THESIS

CHARACTERIZATION OF LIPID COMPOSITION OF SHEET RUBBER FROM *HEVEA BRASILIENSIS* AND RELATIONS WITH ITS STRUCTURE AND PROPERTIES

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Graduate School, Kasetsart University
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THESIS

CHARACTERIZATION OF LIPID COMPOSITION OF SHEET RUBBER FROM *HEVEA BRASILIENSIS* AND RELATIONS WITH ITS STRUCTURE AND PROPERTIES

SIRILUCK LIENGPRAYOON

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Natural rubber produced from *Hevea brasiliensis* latex possesses superior mechanical properties over its synthetic counterpart but lacks consistency in its quality due to its natural origin. This variation has been partly ascribed to non-isoprene components, especially lipids which are the main non-isoprene compounds retained in dry rubber. The aim of this work was to characterize the lipid composition of *H. brasiliensis* latex and derived dry rubber and to study its relationships with natural rubber structure and properties. The study was conducted with four *Hevea* clones: RRIM600, GT1, PB235 and BPM24. Beside latex, unsmoked sheet rubber (USS) prepared using a controlled and repeatable process was chosen as dry rubber sample. Lipid extraction was performed with an optimized method developed for both fresh latex and dry rubber. Lipid content of both sample types was found to be clonal dependent. Samples from PB235, BPM24 and RRIM600 (young) clones contained more lipids (3.4-3.7% w/w dry rubber for latex vs. 2.3-3.3% for USS) than GT1 and RRIM600 (old) clones (2.5-2.8% and 2.0-2.2% for latex and USS, respectively). Polar lipids, namely glycolipids and phospholipids, were found in lower amounts in sheet rubber than in the latex used for its preparation. Lipid composition was further analyzed with various chromatographic techniques. High amounts of a furan fatty acid were found in lipids from PB235 clone while linoleic acid was the main fatty acid in samples from the other clones. Gas chromatography coupled with mass spectrometry permitted to identify the unsaponifiable composition of the samples. The fatty acid compositions of glycolipids as well as phospholipids were elucidated through HPLC-ESI/MS analysis.

In parallel, USS rubber samples were studied for their structure and properties. The studied parameters were mesostructure (gel and molar mass distribution), macrostructure (measured with standardized specification methods), breakdown behavior and vulcanization behavior. Statistical analysis (PCA) of all data showed that samples from PB235 were clearly distinguished from those from the three other clones. A predominant plasticizing effect of esterified fatty acids and especially of furan fatty acids was observed in RRIM600, GT1 and BPM24. Nevertheless, PB235 rubber, that contained high amounts of furan fatty acid, did not exhibit such a behavior as it showed the highest initial plasticity and Mooney viscosity value. Unsaponifiable components seemed to exhibit an antioxidant activity that resulted in a higher plasticity retention index. Vulcanization characteristics of rubber from each clone were found to relate to the properties of rubber in its unvulcanized state. Lipids, especially free fatty acids, act as activators as observed from the shorter scorch time (t_{s2}) of rubber from RRIM600, GT1 and BPM24. The present study permitted a characterization of lipids composition, structure and properties of fully identified natural rubber samples from various *Hevea* clones, collected in a database. This allowed, through statistical analysis, to provide an overview of the relationships between lipid composition and rubber properties.

Siriluck Liengprayoon. 13, May, 2008

Student's signature Thesis Advisor's signature

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Siriluck Liengprayoon

May 2008

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CHARACTERIZATION OF LIPID COMPOSITION OF SHEET RUBBER FROM *HEVEA BRASILIENSIS* AND RELATIONS WITH ITS STRUCTURE AND PROPERTIES

INTRODUCTION

Among the two thousand plant species producing rubberlike material, *Hevea brasiliensis* is the most important source of natural rubber. In the world elastomer market, natural rubber demand has been continuously increasing despite competition by synthetic rubber. This is due to the superior mechanical properties of natural rubber such as high green strength and low internal heat build-up over its synthetic counterpart. Though chemically synthesized polyisoprene is structurally similar to the natural one, synthetic rubber cannot comply with certain technological requirements, especially in the big tyres industry (trucks, planes). Nevertheless, a major drawback of natural rubber is its lack of consistency in quality, which can lead to process accidents during manufacturing.

Apart from *cis*-polyisoprene, natural rubber from *Hevea brasiliensis* contains 3-5% non-isoprene components, absent in synthetic rubber. This non-rubber fraction (proteins, carbohydrates, inorganic constituents and lipids) has been suspected to be responsible for both superior properties and non-consistency of natural rubber. During the coagulation and drying processes, some of these components are lost in the aqueous serum while others, especially lipids, are retained in dry rubber. Lipids have been often reported to affect the properties of natural rubber both in raw and vulcanized states. However, data from literature are hardly comparable as most works dedicated to the effects of lipids on rubber properties have been performed using different rubber sources, extraction and analytical techniques, under various conditions. Moreover, most of these works focused on specific properties or specific classes of lipids.

The purpose of this study was to ascertain the effects of lipids on natural rubber by a qualitative and quantitative analysis of lipids extracted from well characterized natural rubber samples obtained from trees of different clonal origin and

during a long period of time (3 years). Sample characterization concerned agronomical history, mesostructure (gel and molar mass distribution) and physical properties (standardized specification, breakdown behavior and vulcanization behavior).

The first part of this thesis consists in a bibliographic review on natural rubber general processing from the *Hevea* fields to final products, biochemical composition of natural rubber and non-isoprene components, and the current knowledge on relationships between composition and properties. This review provides a general idea of the involvement of all studied parameters concerning lipids and natural rubber structure and properties.

The experimental part of the present work is organized in four chapters. The first one presents our research strategy and the methodological developments realized for this study. The next chapter concerns the lipid composition of both fresh latex and sheet rubber. For this, the development of appropriate and consistent lipid extraction methods from both kinds of samples was a very important step. Structure and properties of sheet rubber samples, determined in parallel through standardized methods, are presented and discussed in the third chapter. The last part deals with the relationships between lipid composition of the various samples and the structure and properties of rubber, through the statistical analysis of all the collected information.

LITERATURE REVIEW

Natural rubber production

This part of our bibliographic study will focus on general information about *Hevea brasiliensis* and the processing commodity chain. A second part entitled "biochemistry of NR" will give details on the biosynthesis and the biochemical composition of NR. The last part is dedicated to the effect of non-isoprene component on natural rubber structure and properties.

1. History and present situation of natural rubber

Natural rubber (NR) is one of the cell constituents of several plant species. Of the several thousands of laticiferous species in the plant kingdom, only about 2000 contain rubber in their latex. Most of the 500 of them that have been tested contain too little rubber to be considered as a potential source of NR. *Hevea brasiliensis*, a forest tree that is indigenous to the tropical rain forests of Central and South America, is presently the most important source of NR and the other rubber bearing plants are of minor importance (George and Panikkar, 2000).

The history of the rubber discovery and the development of the technological processes for converting it into many useful products have involved many countries and people. Rubber came on the world scene with the discovery of the New World. Christopher Columbus is said to have brought back some rubber balls on his return to Europe in the 16th century. The first comprehensive written account of natural rubber was probably the one presented to French Academy in 1751 by Charles Marie de la Condamine reporting on the exploration carried out by Francois Fresneau. This report described the methods of tapping and the preparation of crude rubber from *H. brasiliensis* latex by Brazilian natives.

During the 1850's, most of the rubber for the world industry came from wild *Hevea brasiliensis*, *Ficus elastica* and *Castilla elastica* trees growing in the forests of

Central and South America, India and Africa. In 1876, South and Central America were the main contributors to rubber production with a 71% share. With the growing demand due to rapid economic growth, rubber manufacturing in Europe and America had to widen the source of supply of their raw material. Rubber cultivation in South East Asia was thus initiated in order to feed the industries located in Europe. In 1876, Henry A. Wickham collected 70,000 *Hevea* seeds from Brazil and brought them to the London's Royal Botanic Garden, of which only 2,700 seemed to have germinated, and 1,919 seedlings were sent to Ceylon. During the early days of rubber, Ceylon became the center of activity for rubber seeds and seedlings supply. The growth of plantations in South East Asia was favored by rapid developments in the transportation sector and the opening of the Suez Canal. By the end of the 19th century, NR became one of the major plantation crops under colonial patronage with an export-oriented estate system of production. South East Asia has remained the predominant natural rubber producing region since then (Subramaniam, 1987 ; Thomas and Panikkar, 2000).

Today, *Hevea* trees have lost their wild characters and the production from the Amazonian represents only 1% of the world production (International Rubber Study Group [IRSG], 2007). The total surface area consecrated to *Hevea* production in the year 2006 stood at about 8.8 million hectares, producing about 9.6 million tons of natural rubber. Presently, around 93% of the world production comes from Asia, the major producers being Thailand (32.5%), Indonesia (27.3%), Malaysia (13.3%) and India (8.8%) as presented in figure 1.

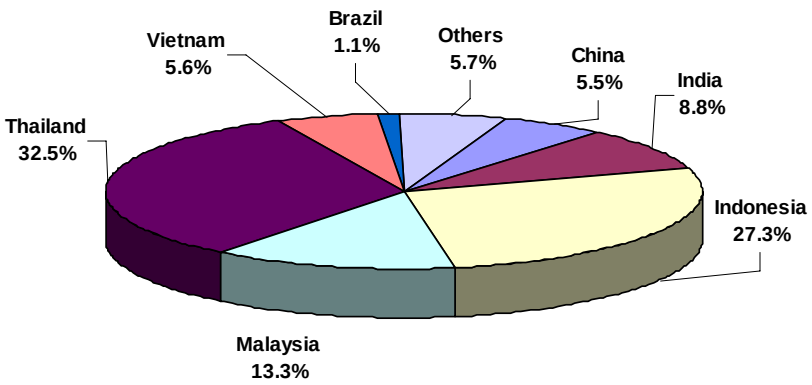


Figure 1 World natural rubber production shares

Source: IRSG (2007)

About three-quarters of the world's natural rubber production is consumed in the tyre manufacturing industry, characterized by a highly automated system and a rigorous quality demand. However, NR is a product of biological origin with a particular complex structure that varies with agronomic parameters associated with the cultivation. Therefore, the properties of raw NR display an important variability compare to its synthetic counterparts (Ehabe *et. al*, 2006).

2. Natural rubber processing

The production of usable rubber includes growing the trees, tapping them for their latex followed by a two steps processing: a first transformation to obtain raw natural rubber and a second one to obtain end products.

2.1 From plantation to raw rubber

2.1.1 Planting and cultivation

Hevea brasiliensis is a tropical tree. It grows best at the temperature of 20 -28 ^{o}C with a well-distributed annual rainfall of 1,800 – 2,000 mm. It grows satisfactorily on most soils provided drainage is adequate. Young seedlings are allowed to grow in a nursery for their first year. They are grafted with clonal bud (obtained from wood garden) after which they are planted out in their final positions. The rubber tree will not be ready to yield rubber until about six years after planting. Before that, the girth is too small and exploitation would be then harmful. During this immature period, maintenance is needed as it is for the whole economic life of the tree which is typically 25-30 years. Once a year, each tree undergoes a "wintering" during which it sheds its leaves, refoliates and flowers. Wintering of rubber tree is fast, lasting only about 1 month. Trees from a given clone tend to winter at the same time in a certain area.

2.1.2 Tapping

The tappers harvest latex from the trees by the process called tapping. A spiral cut is made downwards from left to right almost through the barks. This is to cut the laticeferous vessels in the cortex or inner bark without damaging the tree's cambium layer. This cambium is of particular importance as a lateral meristem; it generates wood and xylem vessels to the inside and the soft bark containing the phloem and the latex vessels to the outside. The first tapping opens up the tapping panel which is then developed by the continued tapping at regular intervals, working down the tree. Bark grows slowly behind the descending cuts. After a panel of a convenient size has been completed, in about 5 years, a new panel is started, leaving the first panel to renew itself. About 10 years after the first tapping, the renewed bark on the first panel may be opened up again and so on. About 3 to 4 hours after tapping, latex is collected, treated to prevent premature coagulation and brought to a factory or a smallholder processing center. Ammonia is the most common stabilizer used. The latex exuded after collection will be gathered later by the tappers as a coagulated form so called cuplump. Cup coagula or field coagula collection may also be performed by leaving the whole harvested latex in the cup (no latex collection) without any stabilization.

2.1.3 Raw rubber production

Hevea latex and natural rubber are distinct products. The former is a stable colloidal dispersion of rubber particles in an aqueous medium while the latter is a solid product obtained following coagulation of the latex, processing and drying of the coagulum (Ehabe *et. al*, 2006). On arrival at the factory, the latex is sieved and blended. Field latex is either centrifuged to give concentrated latex or coagulated and processed into solid dry rubber. A mild acid such as formic or acetic acid is generally used for latex coagulation. Coagulation of latex by the addition of acid is due to the neutralization of negative charges on the membrane that surrounds rubber particles. Because the proteins of the membrane have an isoelectric point close to 4.7, pH has to be brought to this level for a smooth coagulation (Kuriakose and Thomas, 2000).

Field coagulum, *i.e.* coagulated rubber obtained after natural coagulation in the cup (in the field), is a result of acid production by microorganisms at the expense of non-rubber constituents in latex. Low quality field rubber includes the rubber removed from the collection cup (shell scrap), the tapping cut (tree lace, panel scrap), the trunk of the tree (dried rubber from the latex overflow from the tapping cuts) and from the ground beneath the collection cup (earth scrap).

If not concentrated, latex can be processed into ribbed-smoked sheets, air-dried sheets, pale latex crepes, technically specified rubber (TSR), and special quality rubbers. Field coagulum is processed into crepe rubbers or TSR. The scheme of raw rubber processing is presented in figure 2.

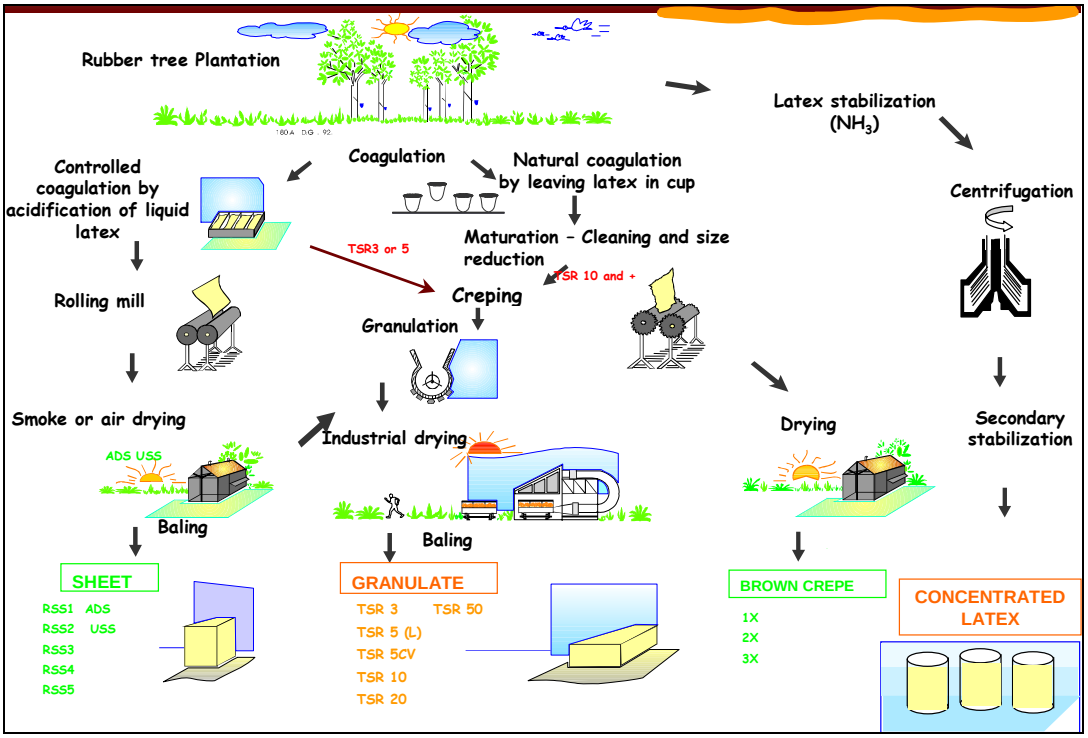


Figure 2 Schematic processes of rubber production

Within each group, there are several grades based on the quality of the product. In the 50's, the conventional forms of natural rubber (sheets, crepes) were visually graded based on the cleanness, uniformity and intensity of color, mold and rust spots. In this way, natural rubber is classified into 8 types and made up of a total

of 35 different grades. However, most visual criteria have no technological basis. TSR, first introduced into market by Malaysia in 1965, has necessitated revolutionary changes in processing methods. Technically Specified Rubber (TSR) is graded according to the source of rubber (latex or field coagulum) and on its properties. Rubber is prepared in granulated or crumb form rather than as sheets or crepes; this allows easier cleaning, especially for the lower grades (Subramaniam, 1987). For Thailand, the exported NR products are block rubber (37%) rubber sheets (33%) and concentrated latex (30%). Exported rubber sheets mostly consist (95%) in ribbed smoked sheets (RSS) of grades 3 and 4 (figure 3).

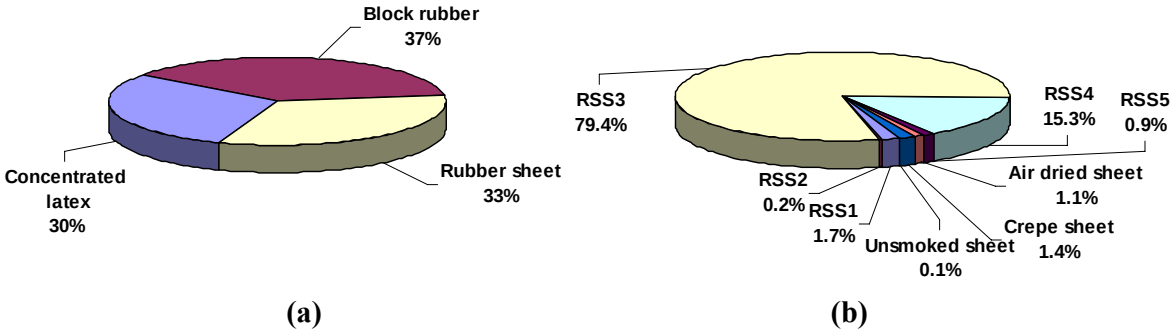


Figure 3 Exportation of NR products of Thailand of year 2006 (a) and proportion of various grades of exported rubber sheets (b).
Source: Thai ministry of commerce (2007)

2.2 From raw dry rubber to end products

In order to obtain usable rubber products, several processing operations are involved. The four main operations are mastication of raw rubber to obtain desirable viscosity, mixing or incorporation of other formulation ingredients, shaping of rubber compound and vulcanization. Mastication and vulcanization are briefly introduced below and are described in details in parts 4 and 5 of this chapter.

2.2.1 Mastication

Mastication is the process whereby the average molar mass of a polymer is reduced by mechanical work. The resulting lower viscosity of the polymer facilitates the incorporation of compounding ingredients and can improve their dispersion (Moneypenny *et. al*, 2004). This also leads to easier down-line processing such as calendaring and extrusion. Shorter processing time and lower power consumption are generally obtained. Mastication is usually limited to NR, since synthetic rubbers are available in processable lower viscosity through choices in polymerization techniques (Fries and Pandit, 1982).

2.2.2 Mixing

Rubber has to be mixed with other ingredients to get the required specific properties. A typical compound contains specific ingredients that in combination with proper processing conditions lead to a product that meets the requirements set beforehand (Van Baarle, 2005). Basically, a rubber compound contains various groups of ingredients such as vulcanizing agents (sulfur, accelerators, and activators), fillers, protecting agents and processing aids. Mixing is carried out either in an internal mixer or a two-roll mill, the choice of a mixer depending upon technical as well as economic requirements.

2.2.3 Shaping

After rubber compounds have been properly mixed to homogeneity, three main ways of shaping are chosen. Moulding is the method where shaping and vulcanization occur in the same place, as the piece is heated under pressure in the mould. Calendering is widely used for the production of a range of finished and semi-finished products such as tire tread blanks and conveyor belts. Extruding is the way used to produce a diverse range of products including sheets, tubing profiles and cables.

2.2.4 Vulcanization

The shaped rubber compound must be vulcanized to get its final properties. During vulcanization, the long chains of rubber molecules become crosslinked by reaction with the vulcanizing agent to form three-dimensional structures. This reaction transforms the soft weak, plastic-like, material into a strong elastic product. After this process, rubber loses its tackiness and becomes insoluble in solvents and more resistant to the deterioration normally caused by heat, light and ageing.

In many industrial goods manufacturing, NR uniformity is required for final compound consistency, which in turn yields consistent processing characteristics. Indeed, the homogeneity of rubber and its flow after mastication condition the efficiency of incorporation and dispersion of other ingredients as well as the consistency of the final compound. The only physical measurements on raw NR that are used to quantify the processing characteristics are Wallace plasticity (P_0) and Mooney viscosity (ML). These tests should predict the effect of variation in raw rubber properties on the processing behavior of the rubber after mastication. However, they were found to be insufficient since the behavior of NR in the standard test occurs under different conditions from those that apply during mastication.

2.3 Concentrated latex process

The field latex is normally not utilized in its original form due to its high water content and susceptibility to bacterial attack. Therefore, it is necessary both to preserve and concentrate it. All the important processes of fabrication of latex articles begin with latex that contains at least 60% rubber. Three methods of latex concentration are employed: centrifugation, evaporation and creaming. Evaporation involves the removal of water only. Hence the ratio of non-rubber constituents to rubber and particle size distribution remain unaffected. In addition, evaporation yields higher rubber content than centrifugation and the resulting latex has somewhat different properties that are preferred for certain products. Creamed latex concentrate

has the smallest share of the market and the process requires several weeks. The main use of creamed latex is the manufacture of latex thread (Allen, 1972). Nevertheless, centrifugation is the preferred method and accounts for 95% of total production (Gazeley *et. al*, 1988).

Latex concentrate is used to make a variety of tubings and dipped goods, and also foam for upholstery and bedding. Latex concentrate is mixed with various compounding chemicals before being processed into gloves, condoms, balloons, catheters and baby soothers for instance. The manufacturing processes have many proprietary features depending on the products (Yip and Cacioli, 2002). Generally, for dipped products, molds of the desired shapes are dipped into the compound latex to enable the deposition of a thin film of latex. The product is washed to remove the excess of chemicals before it is vulcanized in a oven at 100 ^{o}C to 200 ^{o}C. Before the product is stripped from the mold, the gloves are dipped in a powder slurry tank.

For foam products, the compound latex is incorporated with air using a foamer and mixed with gelling agent before introducing to mold. After curing in a mold at 100 ^{o}C, it is washed and dried. In case of latex threads, continuous extrusion of compounded latex into a bath of latex coagulant is performed. Then extruded threads are washed cured, dried and lubricated.

3. Natural rubber structure

Natural rubber consists of long linear chains of isoprene (C_5H_8) units and a variety of non-isoprene components such as proteins, lipids and carbohydrates. Usually, NR and also other types of rubber exhibit the following behaviors: (i) allowing a large deformation without break after several times of stretching (ii) the deformation recovers almost 100% instantly (Nakajima, 2000). The characteristic of reversible extension and retraction is what distinguishes rubber or elastomer from the other types of polymers.

Generally, the properties of materials will depend on its structure at different levels (molecular, macromolecular and supramolecular). Bonfils and Vaysse (2003) divided the structure of natural rubber into three main levels: microstructure, mesostructure and macrostructure.

3.1 Microstructure

Microstructure focuses on molecular level, or chemical structure, inside the rubber such as isoprene units (configuration for example) and lipids, proteins and carbohydrates (non-isoprenes). During the process, especially dry rubber preparation, most water-soluble substances are lost but most of the lipids are retained together with some of non-water soluble proteins, small quantities of inorganic salts and other components. It is to be noted that some of the non-isoprene components greatly influence the properties of NR in both raw and vulcanized states (Subramaniam, 1987).

3.2 Mesostructure

This level focuses on macromolecular structure and gel. The macromolecular structure of natural rubber can be assessed by number-average molar mass (M_n), weight-average molar masse (M_w), molar masses distribution (MMD) and branching.

3.2.1 Molar mass and molar masses distribution

The molar mass and molar masses distribution in polymer systems play an important role in determining their bulk properties. The technique commonly known as gel permeation chromatography (GPC) or size exclusion chromatography (SEC) which allow separation of polymer molecules by their sizes have been used to study macromolecular structure. Subramaniam (1972) was the first who studied MMD of fresh NR latex samples with SEC, expressed as MMD_0. MMD_0 of NR has been described to be either unimodal or bimodal. The unimodal distribution has a high

peak in the high molar masses region and a shoulder in the low molar masses region described as type 3 while the bimodal distribution may be of two types (figure 4): one of type 1 the lower and higher molar mass peaks have nearly the same height, and the other for type 2 the higher molar mass peak height is larger than that of the lower molar mass peak.

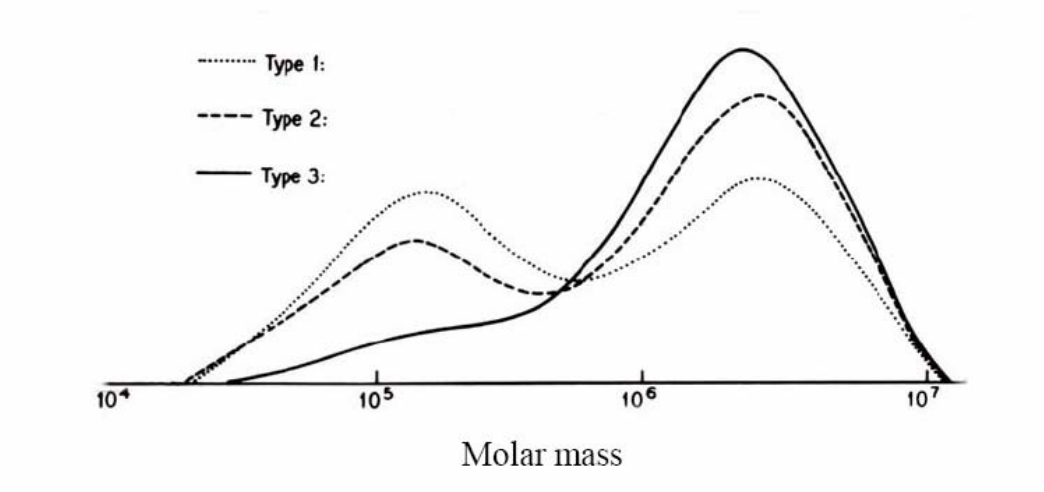


Figure 4 Types of molar mass distribution curves of natural rubber

Type 1: Distinctly bimodal distribution with the peak height in the low molar mass region is nearly equal or slightly less than the peak height at the high molar mass region.

Type 2: Distinctly bimodal distribution with the height of the low molar mass peak is only half or less than the height of the high molar mass peak.

Type 3: Unimodal distribution

Source: Subramaniam (1993)

In young trees, an original situation may also be found where the lower molar mass peak is larger, which was claimed to be due to incomplete biosynthesis of rubber chain (Subramaniam, 1993). This explanation was in accordance with the work of Tangpakdee *et al*. (1996) who studied the number-average molar mass (M_n) of *H. brasiliensis* seedling of different ages (1, 3, 7, 36 and 84 months) by GPC and osmometry. The results lead to the conclusion that M_n increased with the age of the tree. Parth *et al*. (2002) observed that the peak area in the low molar mass region of the RRIM600 *Hevea* clone decreased with age, thus

favoring the peak area in the high molar mass region, while no distinct shift of the MMD curves was observed. The branching index also increased with both the molar mass and age of the trees. Clonal rubber also showed a wide distribution expressed with the polydispersity index (Ip = M_w / M_n) ranging from about 4 to 10 (Subramaniam, 1993). Although the shapes of distribution curves are different, the range of molar mass is approximately the same in rubber from all clones, normally in the region of 10^5 - 10^7 g.mol^{-1} (Eng and Tanaka, 1993).

Furthermore, the type of harvesting and processing also influence on MMD as mentioned in the work of Bonfils *et al*. (2000) which compared the MMD of TSR10 rubber prepared from an unimodal rubber clone (PB217) and bimodal rubber clone (PR107) by SEC. It was found that after processing the inherent MMD (MMD_0) was no longer maintained.

3.2.2 Gel

The gel is defined as a network of polymers formed as a result of extensive branching or crosslinking, which is usually swollen by solvents but does not dissolve in non-destructive solvents (Lee, 1993 ; Eng *et. al*, 1997). Two types of gel exist in NR: macrogel and microgel. Macrogel (macroaggregates) is the part of NR that is visible and insoluble in a conventional polyisoprene solvent and that can be eliminated by centrifugation. Regarding gel quantification method from ASTM, swelling index is used to define type of macrogel. Low and high swelling index refer to hard gel and loose gel, respectively. Hard gel usually indicates the presence of a material that does not break down readily by milling while the latter gel type is opposite (ASTM, 2000). Microgel (microaggregates), contained in the soluble part, cannot be eliminated by centrifugation but can be retained by filtration (porosity $\leq$ 1 μm) (Bonfils *et. al*, 2005).

Latex from virgin trees and from the tree which is not tapped for long period were found to contain extraordinary high gel content; the amounts of macrogel can be as high as 70%. However, the macrogel content decreases on every

tapping and reaches a value of about 5% on regular tapping (Sekhar, 1962). Storage of latex after tapping may also influence gel quantity. Freshly prepared NR has low gel content, of about 5 to 10%. During storage, the gel content increases and may reach 50% or even higher after a long storage (Subramaniam, 1987). Gel content can also be increased by other factors such as clonal characteristics and rubber process (Dogadkin and Kuleznev, 1960 ; Ngolemasango *et. al*, 2003).

Many mechanisms were proposed to explain the formation of a gel phase in NR. They could be attributed to several interactions between abnormal groups on the polyisoprene molecules and non-isoprene components in the latex (Ehabe *et. al*, 2006). Proteins is presumed to relate with gel formation as high nitrogen and mineral content are found in gel phase (Grechanovskii, 1975 ; Eng and Tanaka, 1993) and also deproteinization of NR could lead to a significant decrease of gel content (Bengtsson and Stenberg, 1996). Lipids have also been described to be involved (Tangpakdee and Tanaka, 1997). Increases in gel content are associated with a rise in natural rubber viscosity. Under dry storage, this phenomenon is called “storage hardening” (Rodriguez and De Paoli, 1985 ; Subramaniam, 1993 ; Gan, 1996).

3.3 Macrostructure

This supramolecular level of NR integrates the interactions occurring in the whole product. The global performances of rubber that are influenced by the long chain character of polyisoprene are generally described by some rheological terms such as visoelasticity and plasticity.

4. Natural rubber properties

The behavior of natural rubber mentioned above are generally defined for ambient condition, however, natural rubber or other rubber types will lose those behaviors according to the condition change.

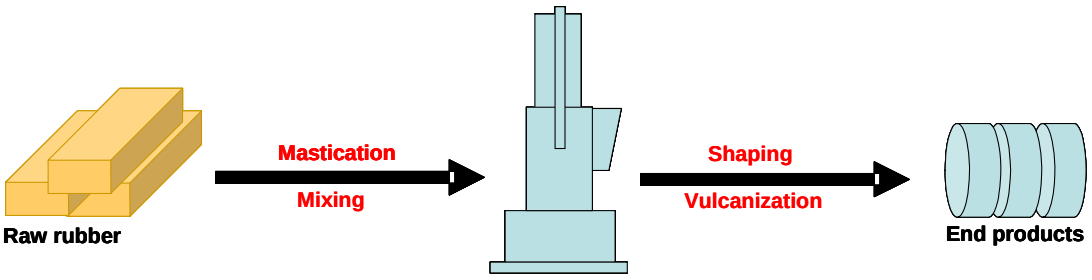


Figure 5 General rubber processing scheme

In rubber processing (figure 5), rheological behavior of raw rubber has to be preliminary determined in order to aid rubber behavior prediction during the process. Mastication is the preparation step especially needed for natural rubber in order to bring its molar mass down to a more easily processable range. To obtain desirable quality of end used products, vulcanization which is the process of crosslinking the essentially linear rubber chains into a three-dimensional network has to be performed by mixing with various needed ingredient. The detail of mastication and vulcanization and characterization of natural rubber properties during each step will be described.

4.1 Rheological properties

The processability of natural rubber was related to its rheological behavior at large deformation and fracture which may be characterized by its viscoelasticity properties (Nakajima, 2000).

4.1.1 Viscoelasticity

This property is an intermediate characteristic between viscous liquid and elastomeric solid and generally rubbers become more liquid-like (viscous) or higher flow properties with elevated temperature or more applied forces (Mathew, 1992 ; Nakajima, 2000). This property of natural rubber affects on the initial mixing of rubber with other compounding ingredients and the subsequent processing of the compounded materials to form the final manufactured products.

4.1.2 Characterization of viscoelasticity

In the rubber manufacturing, Wallace or initial plasticity (P_0) and Mooney viscosity (ML) are the standardized methods used to characterize natural rubber properties. P_0 provides the indication about flow produced during a simple compression at 100 ^oC. The high P_0 for a given natural rubber sample indicates a high resistance of NR to flow under pressure. P_0 of rubber is sensitive to various factors such as clone, storage or rubber processing (Yip, 1990).

Mooney viscosity (ML) gives an indication of the amount of mechanical work required on raw rubber to obtain a mix with consistent rheological properties. A rubber with high Mooney viscosity requires a long mastication time or needs peptizers to obtain a product with a workable and consistent viscosity (Yip, 1990). Mooney viscosity of freshly prepared rubber ranges from 50 to 90 units (ML 1+4 at 100^{o}C) depending on the mixture of clonal latices used in the preparation (Subramaniam, 1987). There is a broad correlation between the results of P_0 and ML (Fuller, 1988). However, both methods measure a flow behavior of rubber at certain shear rates and temperature. It has been reported that same or different grade of natural rubber with similar Mooney viscosities exhibited significantly different flow behavior at high shear rates (Bristow and Sears, 1988). Furthermore, some authors have recently emitted some doubts about the discrimination properties of ML performed in current standardized conditions and suggested to perform this at a much lower rotor speed (Cantaloube and Cocard, 2004). Although technological property measurements are universally standardized to divide rubber into grades, these properties can not readily be combined to forecast rubber behavior during the process (Bristow and Sears, 1984 ; Lim and Lim, 1985 ; Dick *et. al*, 1999).

4.2 Breakdown behavior

Many physical properties of NR are dependent on the mean molar mass of the rubber molecules, their chain length distribution and the extent of their branching. NR is difficult to process without prior breakdown because of its tough

and nervy character. This breakdown is not only a feature of raw rubber but is also evident throughout the stages of compounding and the various down stream processing operations. Some information about mastication and characterization of breakdown will be described in this part.

4.2.1 Mastication of natural rubber

Mastication is effected by applying a shearing force to the rubber in a machine of the same kind as that used for subsequent mixing. This process may be carried out on an open mill, but is usually carried out in an internal mixer. Normal extents of mastication of NR lead to a ten-fold decrease from the initial molar mass of about 10^6 g/mol. (Lim and Ong, 1993).

4.2.2 Mechanism involved during mastication

The two major mechanisms that occur during NR mastication are mechanical and thermo-oxidative breakdowns. The addition of the two phenomena gives a U-shaped breakdown efficiency *vs* temperature curve (figure 6) with a minimum around 100-130 ^{o}C. The thermal mastication efficiency was reported to reach a minimum at around 100 ^{o}C and the mastication mechanical energy decreased with increasing temperature (Lim and Ong, 1985).

At low temperature, the breakages of polymer chains occur exclusively by mechanical shear forces. Long chain molecules are broken by the action of stress. Breakdown of molecular chains under shear or mechanical force is called mechanochemical degradation. Shorter molecules are formed and the ruptured chains have free radicals at their ends. If they are not stabilized by the presence of oxygen, they can recombine into long chain molecules (figure 7). As a result of the reaction with other chains, branching occurs leading to an increase of viscosity and gelation (Dogadkin and Kuleznev, 1960). If they are stabilized, short chain molecules remain free and viscosity thereby is decreased.

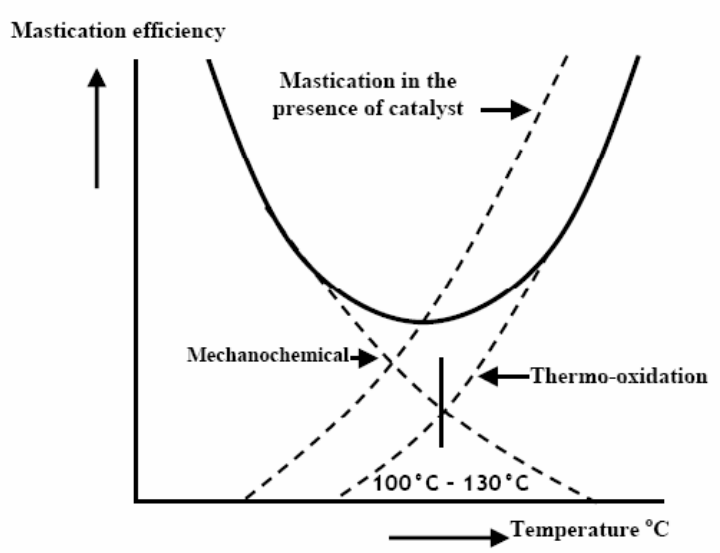


Figure 6 The effect of temperature on the mastication behavior of NR
Source: Dimier *et al.* (2004)

At higher temperature, thermo-oxidative breakdown which is the chemical oxidative reaction that increases with temperature is dominant. Radicals ($R^{\bullet}$), formed during mastication as a consequence of chain rupture, can react with oxygen to form peroxy radicals ($ROO^{\bullet}$), which in turn can form cyclic peroxides or hydroperoxide groups (ROOH) (figure 7). As a result of the hydrogen atom abstraction, free radicals are formed along the chains which absorb oxygen to form new peroxy radicals and propagate chain scissions. The energy consumption in this high temperature mastication is relatively low due to the high plasticity of the elastomer. Meanwhile, mechanical degradation at low temperature largely depends on the mechanical characteristics of mixing equipment. In the mastication process commonly used in industry, both reactions generally superimpose.

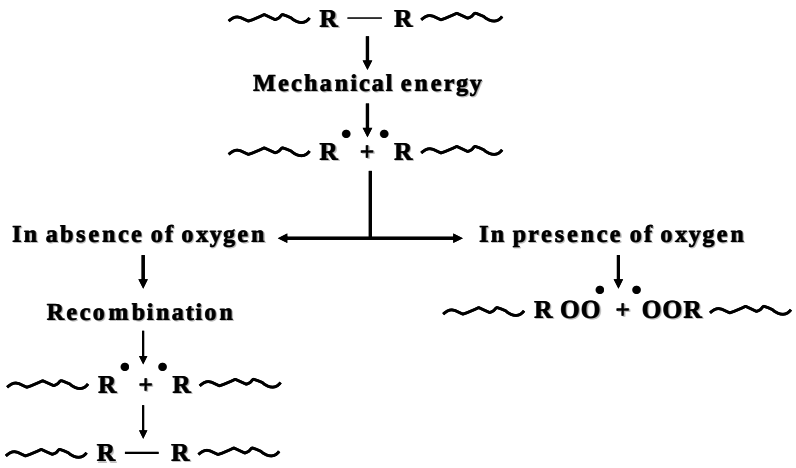


Figure 7 Principles for mastication of rubbers
Source: Fries and Pandit (1982)

Fries and Pandit (1982) said that the important factors for effective mastication of elastomers are the presence of oxygen or radical acceptors, the composition of the elastomer, and mastication conditions. Generally, the oxygen requirement for the breakdown process is too low compared to the amount present in the air during processing in the internal mixer or rubber mill which is usually sufficient. Bristow (1962) and Nakajima (2000) suggested that the fill factor in the brabender head should not be higher than 70 to 75%. The effect of oxygen content on mastication was also studied with another rubber. Arvanitoyannis and Blanshard (1992) found that mastication of Gutta Percha rubber (*trans*-polyisoprene) in oxygen or air results in a sharp increase in modulus. A considerable reduction in elongation at break and a relatively small alteration in strength were also observed whereas its behavior under a flow of nitrogen is much closer to that of initial sample prior to mastication. This behavior is also observed in many polymers.

4.2.3 Characterization of breakdown behavior of natural rubber

The breakdown behavior of raw NR is commonly predicted from the ML and PRI. Even if PRI is dedicated to estimate the resistance of thermo-oxidative breakdown of rubber, some crosslinking which may occur during the test

could lead to difficult interpretation of the result as crosslinking has an opposite effect on P_0 than molecular breakdown. (Rodriguez and De Paoli, 1985 ; Bengtsson and Stenberg, 1996 ; Gan, 1996) Although oxidizability and initial viscosity determined from PRI and ML values are important in determining the rate of breakdown, the role played by other factors such as rubber grade means that they alone do not provide a reliable indicator (Fuller, 1988). Therefore, many researches attempt to find new parameters to forecast this behavior

Bristow and Sears (1984) revealed that the viscosity changes of rubber after being masticated in the Brabender Plasticorder, laboratory scale internal mixer, can be used to predict the breakdown behavior of the same rubber in industrial mixes. Many research works on breakdown behavior of NR have been carried out using this device.

Lim and Ong (1985) have characterized the breakdown behavior of various grades of NR using a Brabender plasticorder. It was reported that the mastication behavior of rubber could be predicted by the ratio of relative drop of Mooney viscosity during mixing versus mechanically consumed energy that is called “breakdown index (BI)”. The work carried out by Lim and Lim (1985) with the calculation of breakdown index found that the drop of viscosity of NR after a fixed cycle of thermal degradation in the heated press gives reasonably good correlations with breakdown and mixing behavior at constant work input in the internal mixer. The method also showed good repeatability.

Other than physical parameters, the determination of MMD before and after NR mastication has been also implemented for the prediction of NR breakdown behavior. Harmon and Jacobs (1966) studied the degradation of NR during mill mastication. The results indicated that as the polymer is milled the distribution is narrowed primarily through the breaking down of large molar mass molecules. The distribution peak shifts to lower molar mass with increasing milling time but a minimal molar mass is achieved after around 76 minutes of milling. Similar results were also found in the study of Subramaniam (1993) who reported that broad

bimodal distribution of NR gradually changes to a narrower distribution in lower molar mass and becomes unimodal after about 10 to 15 minutes of mechanical mastication.

Mastication of RSS 3 in an internal mixer at 80 to 110 ^{o}C shifted the distribution to lower molar masses but the fraction of molecules with low molar masses initially present in the starting material seemed to remain unaffected by the mastication process. In the other words, low molar masses was less degraded during mastication (Bartels *et. al*, 1990). This corresponded to the work of Ehabe *et al.* (2005) that determined the molar mass distribution of TSR 10 and TSR 3CV rubber from PR107 (bimodal) and PB217 (unimodal) clones after mastication in an internal mixer with 100 rpm rotor speed and temperature of 120 ^{o}C. Viscosity stabilized rubber (TSR3CV) of unimodal MMD was progressively degraded by mastication, whereas rubber of the same grade but with a bimodal MMD was degraded more slowly and even stagnated on prolonged mastication. It was described that the higher amount of produced short chains and of which already exist in bimodal rubber could act as plasticizer and facilitate the gliding of the polyisoprene chains. Thereafter, it could prevent the rubber from further degradation.

4.3 Vulcanization characteristics

Vulcanization is the process of treating an elastomer with a chemical to give it useful properties such as elasticity, strength and stability. This process converts a viscous entanglement of long chain molecules into a three-dimensional elastic network by chemically cross-linking of these molecules at various points along the chain.

4.3.1 Vulcanization reaction

Transformation of raw natural rubber into a range of products is accomplished by the addition of a number of ingredients. The objectives of compounding are to facilitate processing and fabrication, to ensure a rapid throughput

with a minimal reject rate and also to achieve the required vulcanizate properties (Fuller, 1988 ; Moneypenny *et. al*, 2004). In general, the materials used can be classified into several major groups namely elastomer, vulcanizing agent, accelerators, activators, fillers, antioxidants and other miscellaneous compounds. A compounding recipe almost always contains some ingredients from each group. Their quantity is usually expressed in parts of the ingredient per hundred parts of rubber (phr).

- Elastomer

The basic component of all rubber compounds, it may be in the form of rubber alone or as a masterbatch of combinations or blends. Elastomers are selected in order to obtain specific physical properties in the final product.

- Vulcanizing agent

Usually, the actual chemical cross-linking is done with sulfur, but there are other technologies, including peroxide-based systems. The combined cure package in a typical rubber compound comprises the vulcanizing agent itself, sulfur or peroxide, together with accelerators and activators.

- Accelerators

In combination with the vulcanizing agent, these materials reduce the vulcanization time (cure time) by increasing the rate of vulcanization. In most cases, the physical properties of the products are also improved. Usually, a reduction in time required for vulcanization is obtained by changes in the amount and/or types of accelerators used. Accelerators are composed of one or two sulfur atoms between a pair of organic end groups. Organic accelerators in vulcanization systems are generally derivatives of 2-mercaptobenzothiazole (MBT). Structurally, all accelerators contain a common functionality, N=C-S.

- Activators

These ingredients form chemical complexes with accelerators, and thus aid in increasing vulcanization rates and improving the final product's properties. Activators can be inorganic compounds such as zinc oxide, hydrated lime, magnesium oxide and hydroxides. Zinc oxide is the most common and it is generally used in combination with a fatty acid to form a rubber-soluble soap in the rubber matrix. Organic acids normally used in combination with metal oxides are stearic, oleic, lauric or palmitic acid.

- Fillers

In principle, reinforcing filler is not needed for NR to improve the tensile properties but it is used to adjust certain mechanical properties such as hardness, abrasion and fatigue. These properties are influenced by the type of filler used. Depending on particle size, fillers can be divided in reinforcing (< 0.03 μm), semi-reinforcing (0.03 to 1 μm) and non-reinforcing fillers (> 1 μm). The type of filler can be divided into two main groups which are carbon black and non-black filler.

a). Carbon black: About 90% of produced carbon black is used in rubber products, predominantly in tyres. Carbon black is also used as a pigment in inks, paints and coatings and in plastics. The physical properties imparted to a given rubber compound by carbon black are dominated by three factors: (1) the loading of carbon black, (2) the specific area of carbon black and (3) the structure of carbon black (Ignatz-Hoover and To, 2004).

b). Non-black fillers: The main non-black fillers are clays, calcium carbonates (both natural and precipitated) and silica (both hydrated and fumed). Although the use of carbon black results in outstandingly high reinforcement, non-black fillers may be also used as they differ not only in their chemical structure but also in their particle size and shape.

- Antioxidants

Most rubber products, particularly those based on unsaturated rubber like NR, are rather susceptible to oxidation during their service life. To improve the resistance against oxidation, antioxidants are used. Rubbers that will be stretched in service and then exposed to outdoor conditions may be susceptible to attack by ozone in the air. To prevent cracking by ozone, antiozonants are used. For black rubbers, the most commonly used antioxidants are of the phenylic type, while phenolic types are employed for non-black rubbers.

- Miscellaneous compounds

Materials that can be used for specific purposes but are not normally required in the majority of rubber compounds can be included in this group. For example, softeners are used to reduce the hardness of the compound and peptizers are used as processing aid to reduce the rubber chain length. It is also useful to use peptizers when NR is blended with synthetic rubber that has lower viscosity.

4.3.2 Vulcanization system

- Sulfur vulcanization

Sulfur remains the major vulcanizing agent, although the system has become far more sophisticated since Goodyear discovered the process in 1839. Sulfur vulcanization can only be applied to rubbers with unsaturations in the backbone or in rubbers with unsaturated side groups. NR is thus commonly vulcanized with sulfur due to its appreciable amount of reactive double bonds. Usually, sulfur alone is seldom used as it requires a very long cure time. Therefore, accelerated sulfur vulcanization, which requires accelerators and activators, is the most common NR crosslinking method. A typical vulcanizing system contains zinc oxide, stearic acid, sulfur or a sulfur donor, and an accelerator.

Sulfur vulcanization occurs by the formation of sulfur linkages or crosslinks between rubber molecules. In conventional sulfur vulcanization (generally formulated as high sulfur / accelerator ratio) the resultant network is rich in polysulfidic sulfur linkages. Sulfur chain linkages can contain six or more sulfur atoms. Lower sulfur/accelerator ratios produce network that are characterized by a greater number of sulfur linkages containing fewer sulfur atoms. Thus, the so-called efficient vulcanizing systems produce higher crosslink densities for the same loading of sulfur. At very low sulfur / accelerator ratio, produced networks are predominantly composed of monosulfidic and disulfidic crosslinks (Ignatz-Hoover and To, 2004).

The chemical reaction pathway involved in the accelerated sulfur vulcanization is presented in figure 8. The reaction starts with the formation of an active accelerator complex that then proceeds to pick up sulfur to form the activated sulfurating species. These polysulfidic species undergoes decomposition and reacts with the allylic carbon in rubber to form a crosslink precursor. This is another polysulfidic species that is terminated with rubber on one end and accelerator on the other. The polysulfide linkage undergoes fragmentation to yield another sulfur containing radical. This radical is then added to a second polymer chain, creating a crosslink. For some properties, the structure of the crosslinks is important. Monosulfide crosslinks are stable thermally up to high vulcanization temperature (upto 200 ^{o}C), but di- or polysulfides are much more reactive (Chapman and Porter, 1988)

- Sulfurless vulcanization

A so-called "sulfur-free" system is a subcategory of accelerated sulfur vulcanization also referred to as sulfur-donor systems. In this system, the sulfur needed for network formation is supplied by the accelerators that function both as accelerators and sulfur-donors. The products from this vulcanization system are more resistant to heat aging since efficient crosslinks containing only 1 or 2 sulfur atoms are found. In addition, the accerelator fragments act as antioxidants.

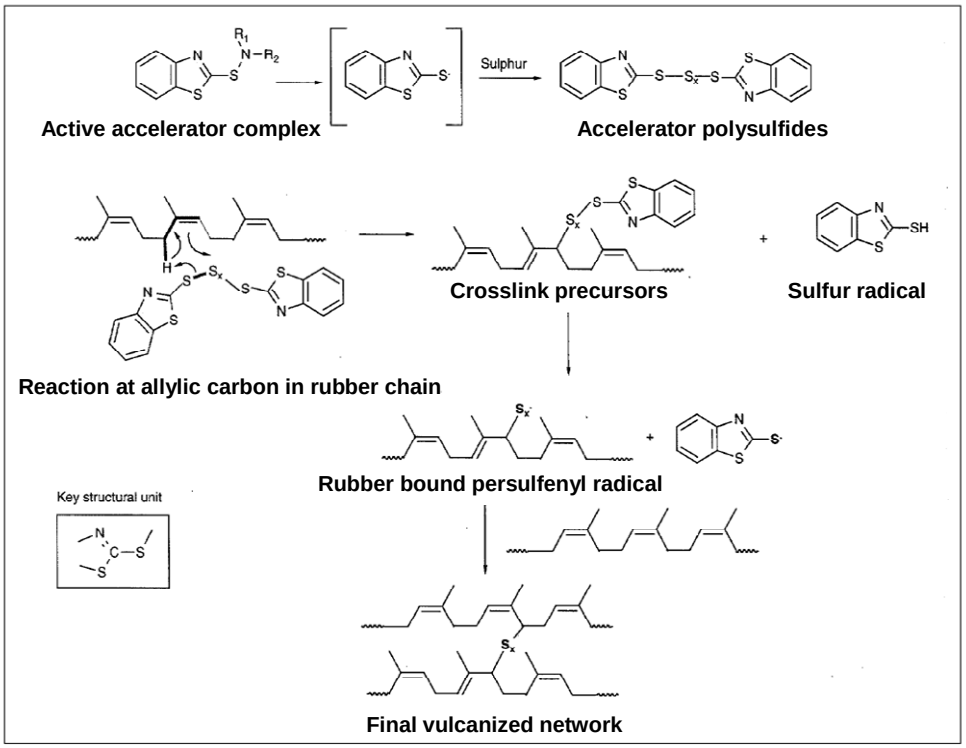


Figure 8 Mechanism of sulfur accelerated vulcanization
Source: Kumar and Nijasure (1997)

- Non-sulfur vulcanization

Though the vast majority of rubber products are cross-linked using sulfur, there are special cases or special elastomers for which non-sulfur crosslinks are desirable. For example, peroxide vulcanization is necessary for saturated rubbers, which cannot be cross-linked by sulfur as it forms direct carbon crosslinks between elastomer molecules and peroxides free radicals formed under vulcanization condition. In general, carbon-carbon bonds from peroxide-initiated crosslinks are more stable than carbon-sulfur-carbon bonds from sulfur vulcanization and therefore provide a cure with good heat resistance. In theory, peroxidic vulcanization, giving simple carbon-carbon crosslinks, should provide a close to ideal crosslink system with good heat resistance. However, in practice the system has a number of disadvantages. One of them is that many antioxidants, particularly p-phenylenediamines, interfere with peroxide vulcanization. Since peroxide

crosslinking occurs by generation of free radicals, it is not surprising that free radical inhibitors, such as antioxidants, reduce its efficiency.

The strength and dynamic mechanical properties of the vulcanizate depend not only on the nature of the polymer chain itself, but also on several variables, such as the type and amount of compound ingredients, the degree and type of cross-linking and compound viscosity.

4.3.3 Characterization of vulcanization

The kinetics of vulcanization is usually studied using a rheometer. The oscillating disc rheometer is the most widely used, in which the specimen is contained in a sealed test cavity under pressure and maintained at controlled temperature. A biconical disc embedded in the specimen oscillates with a small arc (1^o or 3^o). The rheometer records the development of torque as a function of time at a given temperature. The typical cure curve is given in figure 9. The minimum torque (M_L), maximum torque (M_H), scorch time (t_{s2}) and time to 90% cure ($t_{c(90)}$) are indicated.

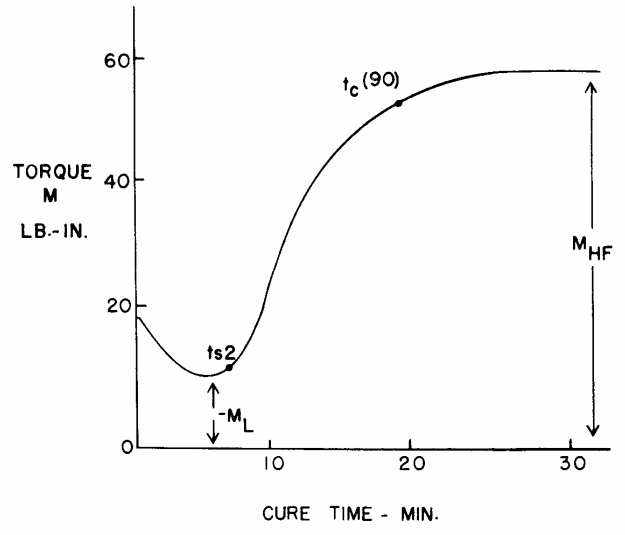


Figure 9 Rheometer cure curve. Torque may be obtained in dN.m as well as in.lb
Source: Conant (1987)

Characteristics related to the vulcanization process are: the time elapsing before crosslink starts or scorch time, the rate of crosslink formation and the final extent of crosslinking. Scorch time generally defines the time of onset vulcanization. There must be a sufficient delay to permit shaping, forming and flowing in the mold before rubber is converted to vulcanized state where further shaping is impossible. The rate of crosslink or cure is the rate at which stiffness (modulus) develops after the scorch point. It determines how long a compound must be cured in order to reach optimum properties. Most of the processes define that the time to reach 90% of maximum cure, namely $t_{c(90)}$, is the state at which most of the physical properties reach optimum values. This parameter will define the total time required, or cure time. Besides that, the formulation of rubber compound, especially the presence of accelerators and activators, will affect the consistency of cure rate, which is of great importance to the manufacturers in modern automated factories with fixed formulation and mixing schedule (Hasma, 1984).

Biochemistry of natural rubber

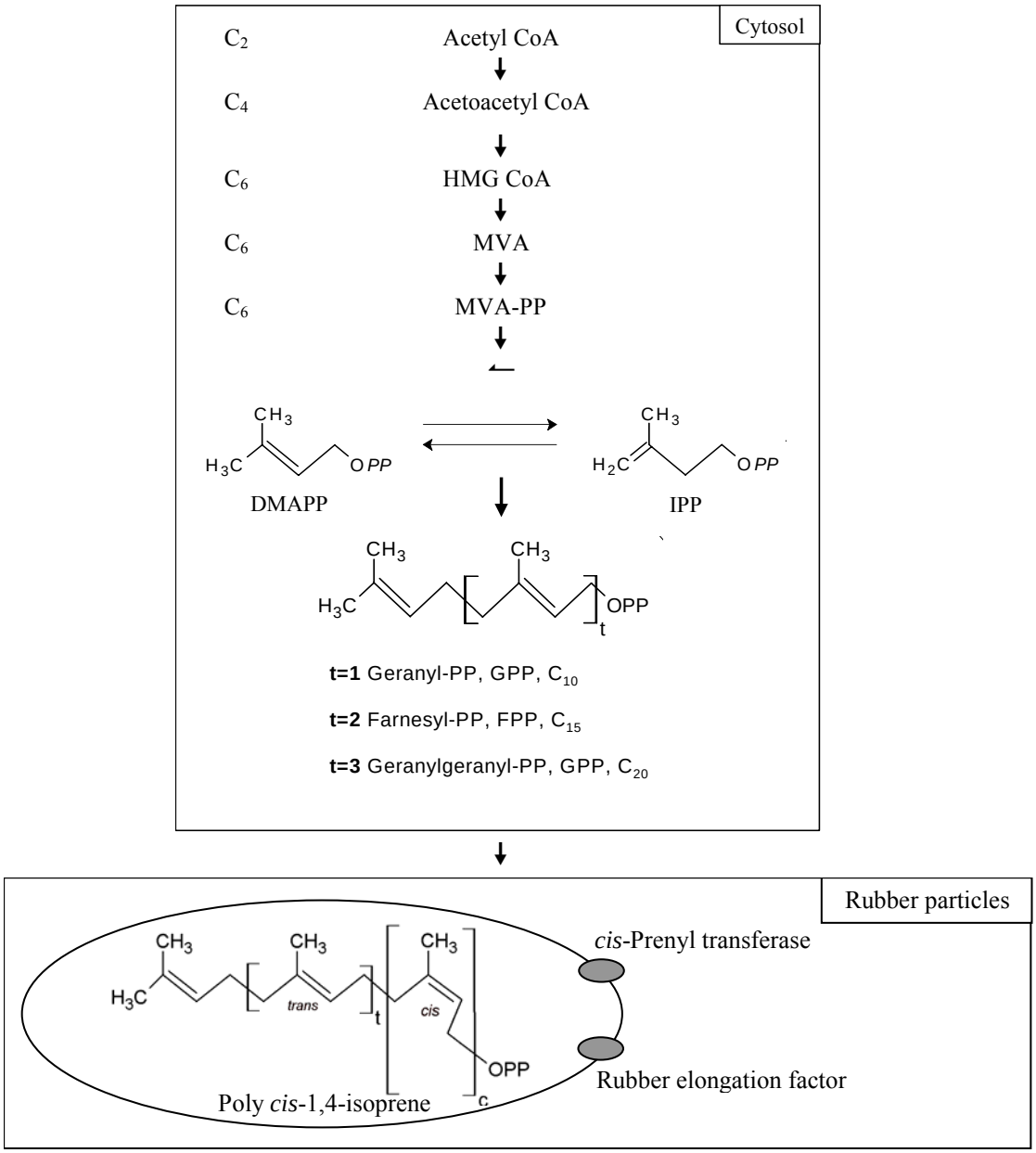
1. Biosynthesis of poly (*cis*-1,4-isoprene)

Hevea brasiliensis is presently the world's sole commercial source of natural rubber (poly *cis*-1,4-isoprene). Rubber is contained in the rubber particles found in the latex. In *Hevea brasiliensis* trees, latex is produced and stored in specialized cells called laticiferous or latex vessels, located in the phloem region in the bark of the tree. These latex vessels are derived from the cambium and are arranged as concentric rings in the bark (Yusof *et. al*, 2000). Latex biosynthesis depends on the number, diameter and anatomical characters of latex vessel system, and on and physiological and biochemical factors. Latex is obtained from the trees by introducing an abnormal physiological function through wounding without damaging the tree during the so-called tapping operation. The capacity of the latex vessels to synthesize and regenerate latex drained during each tapping is critical and is accomplished in the interval between two successive tappings.

NR latex consists of particles of rubber hydrocarbon (poly (*cis*-1,4-isoprene)) and non-isoprene suspended in an aqueous serum phase. Monomers of isoprene units are linked together to form a polymer. NR biosynthesis is a side branch of the ubiquitous isoprenoid pathway. It involves a generation of acetyl-coenzymeA (acetyl-CoA) in successive enzymatically controlled steps where three molecules of acetyl-CoA are converted to isopentenyl pyrophosphate C_5H_8 – PP (IPP), a direct precursor of NR, via mevalonic acid. Subsequently, IPP is polymerized into rubber (Tanaka, 1989). An early scheme proposed by Lynen (1963) for the biosynthesis of polyisoprene, and which still remains the reference, distinctly dissociates the biosynthesis of IPP monomer units from the polymerization as shown in figure 10.

Phase I involves glucose catabolism (glycolysis), during which photosynthe-sized sucrose from the foliar crown is used to produce acetate molecules (polymer precursor). Energy is also generated (in the form of ATP) and the reduction potential is increased by the reduction of nicotinamide adenine dinucleotide phosphate (NAD(P)H) from NAD(P).

Phase II involves polyisoprene biosynthesis, in which energy (ATP) and NADPH are used to elaborate the initial long chain precursor, IPP, from acetyl CoA.



Acetyl CoA: acetyl coenzymeA	DMAPP: dimethylallyl pyrophosphate
Acetoacetyl CoA: acetoacetyl coenzymeA	IPP: isopentenyl pyrophosphate
HMG CoA: β-hydroxy-β-methylglutaryl coenzymeA	GPP: geranyl pyrophosphate
MVA: mevalonic adic	GGPP: geranyl geranyl pyrophosphate
MVA-PP: mevalonic acid 5-pyrophosphate	FPP: farnesyl pyrophosphate

Figure 10 A pathway of polyisoprene biosynthesis

Source: Adapted from (D'Auzac *et. al*, 1989)

The elongation of rubber molecules is catalyzed by two enzymes located on the phospholipoprotein membrane of the rubber particles (figure 10). This "rubber elongation factor" consists in a rubber transferase and a cis-prenyl transferase. Rubber transferase catalyzes the elaboration of polyisoprene by successive additions of IPP molecules into dimethyl allyl pyrophosphate (DMAPP). Although this enzyme may play a role in the initiation rubber molecule synthesis, it does not polymerize rubber. The polymerization of IPP into *cis*-configuration is accomplished by *cis*-prenyl transferase that condenses DMAPP with IPP to give a molecule each of pyrophosphate and geranyl pyrophosphate (C10). This C10 molecule has allylic structure and repeats the condensation, with another molecule of IPP. The propagation, repeated several times, results in the formation of natural rubber with high molar masses (Cornish *et. al*, 1993). Nevertheless, Wititsuwaannakul *et al.* (2004) claimed that the rubber biosynthesis could effectively occur at the bottom fraction membranes (mainly lutoids containing obtained from latex centrifugation) with no requirement of rubber particle preexisted site. Measurements of ^{14}C-IPP incorporation into rubber molecules showed that the rubber biosynthesis activity of washed bottom fraction membranes isolated from freshly tapped latex was up to 6.5 to 7.8 fold higher than the one obtained from the isolated fresh rubber particles.

Tanaka (2001) concludes from ^{13}C and ^{1}H-NMR studies that in natural rubber, the molecular chains are built up from an initiating species ω followed by 2 or 3 *trans*-isoprene units, a sequence of thousands of *cis*-isoprene units and terminated by a terminal α group. The proposed structure is given in figure 11.

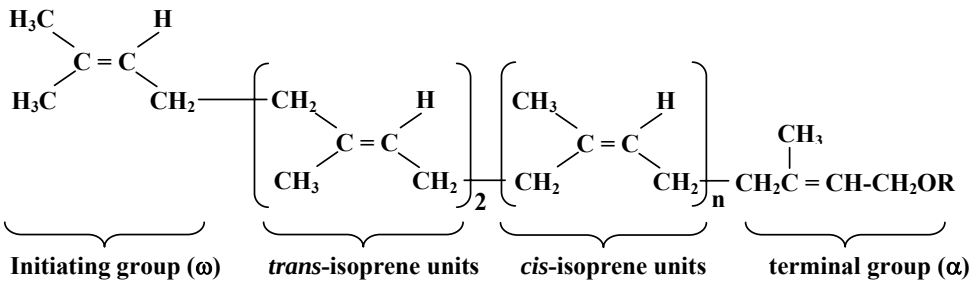


Figure 11 Proposed chemical structure of *cis*-1,4-polyisoprene in *Hevea* rubber
Source: Tanaka (2001)

2. Latex composition

Latex, the milky fluid obtained from tapping, is the cytoplasm coming from laticiferous cells of rubber tree. As described before, the most obvious component in latex is rubber hydrocarbon (poly (*cis*-1,4-isoprene)) which forms 25 to 45% of latex weight. Besides rubber and water, fresh latex contains carbohydrates, proteins and lipids known as non-isoprene components. Numerous other substances are also present in small quantities. Fresh latex can be separated into various fractions by ultracentrifugation at 53,620 g for 40 minutes at 0 to 5 ^{o}C (figure 12).

These latex components can be separated into (1) a white upper layer of rubber particles, or rubber cream, (2) an orange or yellow layer containing Frey-Wyssling particles, (3) an aqueous serum named C serum and (4) a bottom fraction containing greyish yellow gelatinous sediments after ultracentrifugation. The serum contains most of the soluble substances including free amino acids, proteins, carbohydrates, inositol, organic acids, inorganic salts and nucleotidic materials. The bottom fraction consists mainly of lutoid particles and also includes varying amounts of Frey-Wyssling particles, mitochondria and other particulate components of plant cells having a density greater than that of serum (Ancher *et. al*, 1969 ; D'Auzac and Jacob, 1989 ; Jacob *et. al*, 1993 ; Nair, 2000).

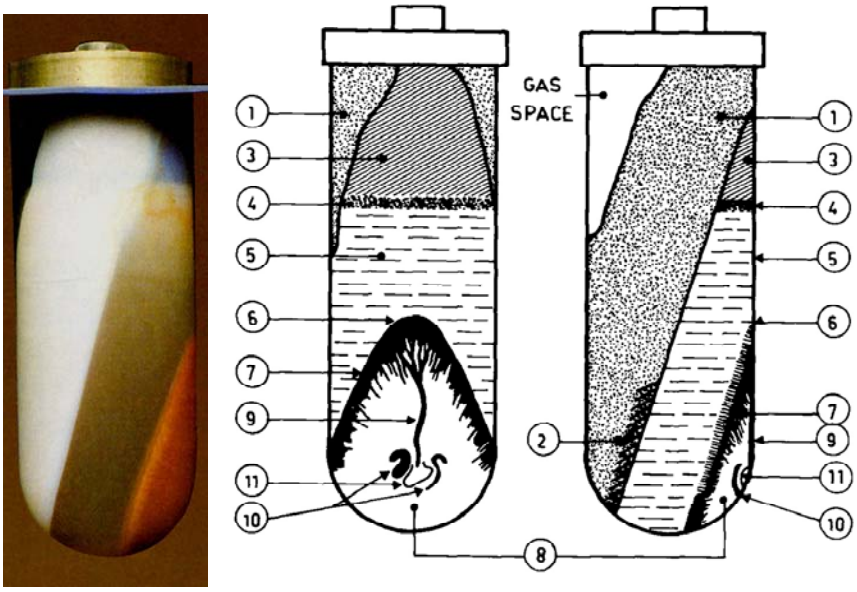


Figure 12 Various fractions of centrifuged latex.

Fractions 1-3 correspond to the white rubber phase. Fraction 4 is a yellow- orange layer constituted by Frey-Wyssling particles. Fraction 5 is an almost clear serum (C- serum) corresponding to the latex cytosol. Fractions 6 to 11 constitute the "bottom fraction" in which fraction 8, quantitatively the most important, is the lutoid fraction intensely pink colored after neutral red absorption.

Source: Moir (1959)

2.1 Rubber particles

Rubber particles (25 to 45% of fresh weight) vary in diameter from 60 nm to 6 μm. The particles are generally spherical, although medium-size and large ones are sometimes pear-shaped. They are strongly protected in suspension by a film of adsorbed proteins and phospholipids. This phospholipoproteic layer imparts a net negative charge to the rubber particle, thus contributing to colloidal stability (Yip and Gomez, 1980 ; Ho *et. al*, 1996). Rubber particles of 0.1 μm diameter probably contain several hundred poly (*cis*-1,4-isoprene) molecules (D'Auzac and Jacob, 1989 ; Tanaka, 1989). The size of rubber particles is believed to be an important parameter related to the biosynthesis mechanism controlling molar mass in NR. Tangpakdee *et al*. (1999) have characterized the structure of rubber particles from the cream phase and the one remaining in the serum phase. The results indicated that the size of rubber

particles of latex obtained from serum is smaller than the one of the cream phase (0.13 μm and 1.03 μm, respectively). The low ester content in small rubber particles (SRP) membrane of latex from serum phase implied that the amount of membrane bound phospholipids was insufficient to terminate the rubber polymerization so that the formation of higher molar mass rubber chains continued in SRP. It is to be noted that the average molar mass and rubber particle size in latex from seedlings were found to increase with the age of trees.

2.2 Lutoids

Next in abundance are lutoid particles, amounting 10 to 20 percent of latex volume. Lutoids are subcellular membrane bound bodies ranging in size from 2 to 5 μm. The membrane encloses a fluid serum known as lutoid serum or B serum. They are directly involved in cellular homeostasis of the laticiferous system and play a major role in the process leading to latex coagulation. The lutoid membrane is a tonoplast with a strong negative charge. It contains a remarkably high content in phospholipids (more than 80%), in which unsaturated and saturated fatty acids are present in equal proportion (Jacob *et. al*, 1993 ; Nair, 2000).

2.3 Frey-Wyssling particles

The Frey-Wyssling particles form 1 to 3% of latex by volume. They are enclosed in a typical double membrane. These particles, the diameter of which is 0.5 to 2 μm, were reported to be the bearers of the yellow color of latex by accumulating lipid globules, isoprenic compounds, carotenoids, plastochromanols and plastoquinones (D'Auzac and Jacob, 1989).

2.4 Latex cytosol and other constituents

Both rubber particles and the membrane-bound organelles composing most of the bottom fraction form a colloidal mixture in an aqueous suspension so-called cytosol. Cytosol represents 40 to 50% of latex volume and is the site of the highest

rubber synthesis activity. The cytosolic part of latex is not fundamentally different from the cytosols of more undifferentiated cells. It contains minerals, organic acids, amino acids, nitrogenous bases, reducing agents and high molecular weight compounds such as proteins, lipids and nucleotides (Jacob *et. al*, 1993). Nucleotides contained in latex are important co-factors and intermediates in biosynthetic processes. Most of the classical amino acids have been reported in latex. Total concentration of inorganic ions in fresh latex is about 0.5% (w/v), the major ions being potassium, magnesium, copper, iron, sodium, calcium and phosphate.

Proteins, carbohydrates and lipid compositions of NR will be presented in the following paragraph.

3. Non-isoprene components

As mentioned earlier, fresh NR latex does not contain only polyisoprene. The other components such as lipids, proteins, amino acids, inositol, carbohydrate and trace elements are called non-isoprene components. They represent around 3-5% of NR (Hasma, 1984). The nature and composition of these non-isoprene components can vary greatly between *Hevea* clones and depend on the exploitation system and the environmental conditions used (Sylla *et. al*, 1996). Some of these components are suspended in the aqueous phase of the latex while the others are the part of rubber particle structure. Some of them could also contribute to specific properties of rubber.

3.1 Protein

The total protein content of fresh latex is approximately 1% of whole latex. About 20% of these proteins are adsorbed on rubber particles; an equal quantity is found in bottom fraction (lutoids) and the remainder in the serum phase (Eng and Tanaka, 1993). Adsorbed proteins and phospholipids impart a net negative charge to rubber particles, thereby contributing to the colloidal stability of latex.

In C serum, nearly half of the enzymes of the glycolytic pathway as well as enzymes for rubber biosythesis are found. Hevein is the major protein found in B serum and accounts for 70% of water soluble protein in the bottom fraction (D'Auzac and Jacob, 1989 ; Nair, 2000). Hevein has been reported to be involved in one of the mechanisms that cause latex coagulation by agglutination with the 23 kDa protein in rubber particles (Jacob *et. al*, 1993 ; Gidrol *et. al*, 1994).

Of the more than 200 different proteins or polypeptides found in NR latex, only about a quarter is allergenic. Most of the protein content is removed when latex is processed into its products. Only a small fraction remains in the products as the residual extractable proteins. Though some proteins have been identified to be potential allergens in latex, the information about whether all of them could survive the stringent manufacturing process is presently incomplete (Yip and Cacioli, 2002).

3.2 Carbohydrates

Quebrachitol (monomethyl 1-inositol) is the most plentiful and the earliest known polyol in *Hevea brasiliensis* latex. Its concentration is found to vary with clones and ranges between 1 to 3% of whole latex. It is a major contributor to the osmotic pressure of the cytosol (D'Auzac and Jacob, 1989). Sucrose is also an important glucid in latex as it is the initial molecule in isoprene synthesis and the main element in the laticiferous metabolism. Sucrose quantity is influenced by seasonal variations, the anabolism increasing with the number of sunshine hours (Le Roux *et. al*, 2000 ; Silpi, 2006). It is also accompanied by smaller amount of glucose, fructose and raffinose. Sucrose concentration in latex is one of the key measurements of latex diagnosis developed to express the physiological state of the exploited trees (Le Roux *et. al*, 2000 ; Silpi, 2006)

In addition to the water soluble polyols and glucids mentioned above, latex also contains galactose derivatives associated with the lipid fraction of whole latex, such as mono- and digalactosyl diglycerides (MGDG and DGDG) (Hasma and Subramaniam, 1986 ; Nair *et. al*, 1993 ; Liengprayoon *et. al*, 2007).

3.3 Lipids

Lipids are hydrophobic substances of biological origin that are soluble in organic solvents such as chloroform and methanol but are only sparingly soluble in water. They contain long-chain hydrocarbon groups in their molecules and are present in or derived from living organisms (Kates, 1972). Many works carried out on lipid analysis from NR reported different lipid contents in *H. brasiliensis* latex. Hasma (1984) reported total lipids extracted from fresh latex of seven *Hevea* clones. The total extract ranged from 1.37% to 3.49% (w/w dry weight of rubber). Of these, about half are neutral lipids, one-third is glycolipids and one-seventh is phospholipids. From many studies, even in works carried out with the same extraction method, considerable variations in lipid content were found in *H. brasiliensis* latex and dry rubber (Hasma and Subramaniam, 1986 ; Nair *et. al*, 1993). These variations were due to different parameters such as the studied clone, the method used to obtain dry rubber sample and extraction procedures.

3.3.1 Neutral lipids

Neutral lipids cover a wide range of compounds including long chain hydrocarbons, esters, tri-, di- and monoacylglycerol, fatty acids, long chain alcohols and sterols.

Altman (1946) isolated the ether-soluble non-isoprene components from alcoholic extracts of five different latex samples. This extract, representing 0.8 to 1.4% of fresh latex volume, comprised 53 to 63% fatty acids, 11 to 15% sterols, 7 to 8% wax alcohols and traces of resin acids.

Smith (1954) reported that fresh latex consisted of 0.59 to 0.87% lipid extract using by methylated spirit (ethyl alcohol/methyl alcohol, 95:5; v/v). It was found that with the alcoholic extraction method, the coagulation of amounts of latex greater that about 40 ml may lead to inefficient extraction owing to the lipid being trapped within the bulky coagulum.

Ho *et al.*(1975) were the first to attempt assessing the quantity of lipid components isolated from rubber particles and non-rubber particles contained in *Hevea* latex. The total extractable lipids from both fractions ranged from 1.7% to 3.7% (w/w dry weight of rubber). The bottom fraction, consisting mainly of lutoid particles, contained less total lipid than rubber cream (0.3-0.5% / dry weight of rubber cream). Around 30 – 45% of neutral lipids in rubber cream consisted of triglycerides whereas free fatty acid and sterol were the main constituents in the bottom fraction. The analysis performed with seven clones showed an apparent clonal variation in the total amount of neutral lipids from both fractions.

Hasma and Subramaniam (1986) carried out further quantitative and qualitative analysis of lipids in fresh latex. Figure 13 shows the identification of neutral lipids by thin layer chromatography (TLC). Among them, are found carotenoid pigments (A), esterified and free sterols (B and K) consisting of β-sitosterol (main sterol), fucosterol and stigmasterol (Nishimura *et. al*, 1977 ; Hasma, 1984). Substance C was shown to be probably an ester of tocotrienol. Substances D and J are esterified and free fatty alcohol, respectively. Substance E is triacylglycerols and a small quantity of tocotrienol ester (E1 on lane1). Substances F, G and H are phenolic compounds, where H is a mixture of two tocotrienol isomers confirmed to be α-tocotrienol and γ-tocotrienol by gas chromatography/ mass spectrometry (GC-MS) analysis. Substance J is a long-chain alcohol, and substances M and L are mixtures of mono- and diacyglycerols, respectively.

As in most plant lipids, the fatty acid composition of *Hevea* latex is mainly palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). A long-chain fatty acid containing a furan ring in the alkyl chain was found in high content for clone RRIM701 especially in triglycerides (Hasma, 1984). Such furan fatty acids occur widely in different plants, vegetable oils, seed oil, seafood and mammals (Morris *et. al*, 1966 ; Spiteller, 2005). However, the presence of high amounts of this acid in *Hevea* lipids has been shown to be a clonal characteristic and may vary with season (Hasma, 1984 ; Visitnonthachai, 2005). This C18 furan fatty

acid was identified by gas liquid chromatography and nuclear magnetic resonance (NMR) as 10,13-epoxy-11-methyl-octadecan-10,12-dienoic acid (Hasma and Subramaniam, 1978 ; Marcel *et. al*, 1981).

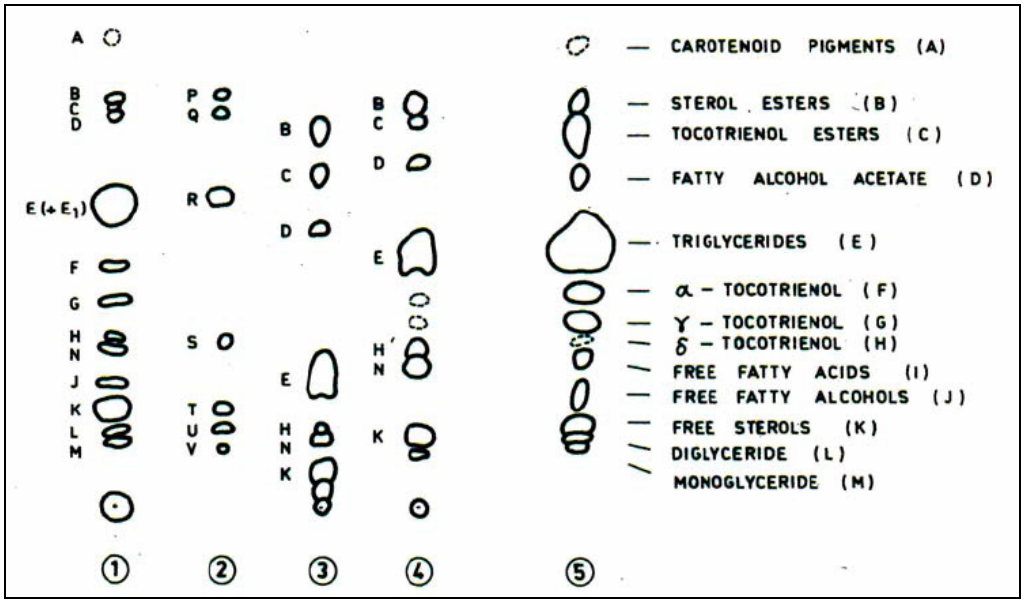


Figure 13 Thin layer chromatography of RRIM501 latex rubber phase neutral lipids. **1**, **2**, and **5**: Developed in n-hexane/benzene (85:15; v/v) followed by hexane/ diethyl ether/acetic acid (69:29:2; v/v/v). **3**: Developed in n-hexane/diethyl ether/acetic acid (90:10:1; v/v/v) **4**: Developed in n-hexane/diethyl ether/acetic acid (80:20:1; v/v/v) Visualization was performed by heating at 180°C after spraying with cupric acetate. **Reference lipids**: Free fatty acids plus traces of tocotrienol (N); sterols (K); diglycerides, monoglycerides, and alcohols (L, M, J); Cholesteryl oleate (P); methyl oleate (Q); trioleine (R); oleic acid (S); β-sitosterol (T); diolein (U); monopalmitin (V).

Source: adapted from Ho *et al*. (1975) and Hasma and Subramaniam (1986)

3.3.2 Phospholipids

Phospholipids are the major lipid components of biological membranes. They consist of *sn*-glycerol-3-phosphate esterified to fatty acids at its C1 (*sn*-1) and C2 (*sn*-2) positions and to a polar group (X) at its phosphoryl group, to

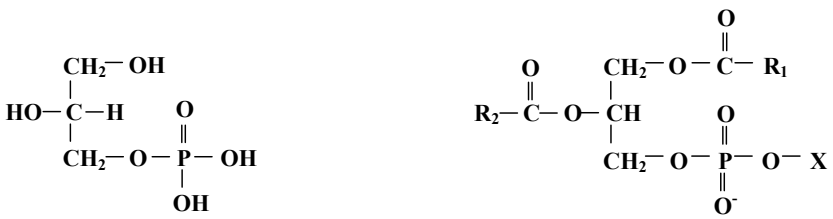
form a class of substances diagrammed in figure 14. Saturated C16 and C18 fatty acids usually occur at *sn*-1 position while *sn*-2 position is often occupied by an unsaturated C16 to C20 fatty acid. The most common phospholipid classes are described in table 1.

Table 1 The common classes of phospholipids. X is a polar molecule esterified to the phosphate group.

Name of X-OH	Formula of -X	Name of phospholipid
Water	-H	Phosphatidic acid
Ethanolamine	$-CH_2CH_2NH_3^+$	Phosphatidyl ethanolamine
Choline	$-CH_2CH_2N(CH_3)_3^+$	Phosphatidyl choline
Serine	$-CH_2CH(NH_3^+)COO^-$	Phosphatidyl serine
Glycerol	$-CH_2CH(OH)CH_2OH$	Phosphatidyl glycerol

Source : Voet and Voet (1995)

The occurrence of phospholipids as essential membrane components is attributable to their tendency to form a variety of structures that will allow them to guard their hydrophobic tails from water. One of such structure is the micelle where the heads all face out and tails all face in, covered from interaction with water by heads (figure 15b). In case of cell membranes, they pack tight together as a double layer as illustrated by figure 15a. These combinations of hydrophilic and hydrophobic areas make it hard for large molecules to pass through the membranes of cells.



sn-glycerol-3-phosphate phospholipids

Figure 14 *sn*-glycerol-3-phosphates and general structure of phospholipids

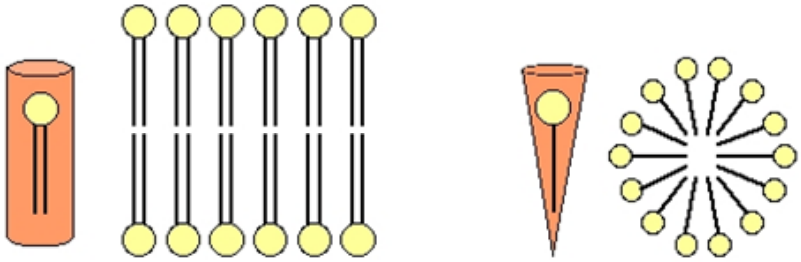


Figure 15 States of aggregation of phospholipids in water (a) and bilayer (b) micelle

The strong negative charge of lutoid membranes was reported to result in the colloidal stability of latex. Dupont *et al.*(1976) carried out a research on the chemical composition of the membrane of lutoids separated from latex by sucrose density gradient. TLC analysis of lutoid membrane phospholipids showed that phosphatidic acid represented 82% of total phospholipids fraction, while the remaining 18% consisted in two unidentified components. TLC result also showed that phosphatidyl choline and phosphatidyl ethanolamine, which are the constituents of most biological membranes, seemed to be absent from the lutoid membrane. However, no phosphatidic acids were detected in rubber particle membranes from the analysis carried out under the same circumstances with full precautions to prevent the effect of phospholipase D. Phosphatidyl choline appeared to be the major component of rubber particle membranes and phosphatidyl ethanolamine also occurred in small amounts together with phosphatidyl glycerol. The exceptionally high phosphatidic acid content in lutoids was proposed by the authors as an explanation of the high electronegative charge observed and the absence of phospholipids containing nitrogenous compound appears to be original. Nevertheless, most of lipids analyses during the same period were carried out with whole latex without specifying their intracellular locations.

Smith (1954) studied phosphatides in *Hevea brasiliensis* latex. Total lipid analysis after latex extraction with boiling methylated spirit showed that the total lipid extract contained approximately 51% phosphatidyl choline (lecithin), 10.5% phosphatidyl inositol, 3% phosphatidyl ethanolamine, 20% triglycerides and

15.5% unsaponifiables. However, the intact structure of phospholipids could not be achieved from this analysis method.

Ho *et al*. (1975) later fractionated phospholipids from rubber cream and bottom fraction of 7 rubber clones by acetone precipitation. TLC analysis allowed identifying phosphatidyl choline, phosphatidyl ethanolamine and two other unknown substances. Phospholipids were mainly located in rubber particles and no apparent clonal variation was observed within them (from 1.07 to 1.18% w/w dry rubber). A later study using the same analysis methods with six different rubber clones showed a constant amount (about 1%) of extractable phospholipids whatever the clone (Hasma and Subramaniam, 1986).

Fatty acids released by the hydrolysis of latex phospholipid are myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) while arachidic (C20:0) acid and furan fatty acid are not detected. Linoleic acid appears to be the main fatty acid in phospholipids of *Hevea brasiliensis*. Linolenic acid (C18:3) was found to be absent from phospholipids of lutoids (Dupont *et. al*, 1976 ; Hasma, 1984 ; Hasma and Subramaniam, 1986)

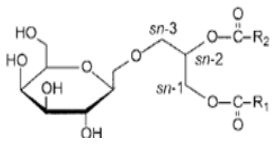
Ehabe *et al.* (2006) noticed the influence of clonal origin on total lipid content and lipid classes in previous works. This information was integrated to classify rubber clones based on their neutral and polar lipid content.

It is to be noted that the study of rubber particles bound lipids is difficult due to the solubility of polyisoprene in most extracting solvents and the rather delicate nature of polar lipids. Bonfils *et al*. (2007) proposed an enhanced solvent extraction method for polar lipids from *H. brasiliensis* solid rubber which appears more suitable that the previous methods for extracting total lipids with optimal phospholipid and glycolipid contents.

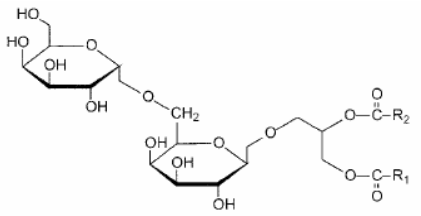
3.3.3 Glycolipids

Different forms of glycolipids, such as glycosylated glycerolipids, esterified sterols and glycosylceramides are present in virtually all biological membranes. Among them, galactolipids are the most abundant plant membrane lipids, especially in green tissues where they represent about 75% of total membrane lipids (Dörmann and Benning, 2002).

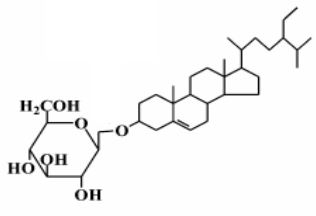
Hasma (1984) reported 0.2-0.5 % (w/w dry rubber) of glycolipid in six different clones and also proved for the first time that fresh *Hevea* latex contains four main glycolipids, namely free and esterified steryl glucosides (SG and ESG), and mono- and digalactosyl diglycerides (MGDG and DGDG). However, only ESG and SG were found to be present in dry rubber. The analysis of their carbohydrate components as TMS ether derivatives by gas liquid chromatography (GLC) revealed that glucose was attached to ESG and SG while MGDG and DGDG were attached to galactose. Their chemical structures are given in figure 16. The sterol components of free and esterified steryl glucosides were described to be fucosterol, stigmasterol and β-sitosterol which was the main one (about 89%).



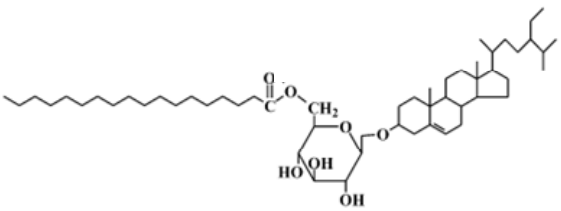
Monogalactosyl diglycerides (MGDG)



Digalactosyl diglycerides (DGDG)



Steryl glucoside (SG)



Esterified steryl glucoside (ESG)

Figure 16 Structure of glycolipids

The fatty acids released by the hydrolysis of glycolipids from *Hevea* latex mainly consist of linoleic acid, oleic acid and stearic acid. Furan fatty acid is also present but in a lower amount than in neutral lipids. In other higher plants, galactolipids contain a higher portion of polyunsaturated fatty acids, up to 95% of which can be linolenic acid (C18:3). Another fatty acid, hexadecatrienoic acid (C16:3), is also found in appreciable amounts especially in MGDG (Christie, 2003). The relative amount of glycolipids in latex from six studied clones was found to be clone independent (Nair *et. al*, 1993).

Effects of non-isoprene components on natural rubber structure and properties

Natural rubber must be technically specified and graded for its wide industrial applications. The quality of NR products is amongst others a function of the quality of raw rubber, which explained the persistent demand by manufacturers for more consistent raw materials. The quality of processed raw NR and its variability depend in part on the quantities and qualities of the non-isoprene components it contains. Non-isoprene components have thus been studied and certain effects on NR structure and properties for both latex and dry rubber have been stated.

1. Concentrated latex

Most non-isoprene components still remain in concentrated latex and have been reported to act on latex properties. During the storage, various reactions occur that can change these interactions.

Carbohydrates become microbiologically oxidized to volatile fatty acids such as formic, acetic and propionic acids. The amount of these fatty acids gives an indication of microbial activity and consequently latex quality as they also have a significant influence on the mechanical stability time (MST) of latex (Galli *et. al*, 2002). MST provides the measure of storage time of preserved latex before it

coagulate. Claramma (1995) suggested that the increase of the MST of latex with storage time was mainly due to the fact that the free fatty acids released by phospholipid hydrolysis provide extra negative charges at the aqueous interface. Hasma (1991) found that MST continues raising even after free fatty acid accumulation ends. This indicates that other factors than free fatty acids are involved in latex stability. One of the non-isoprene component reported to influence latex stability is phosphatidic acid: the negative charges provided by the high amounts of phosphatidic acid in lutoid membranes could results in their colloidal stability (Dupont *et. al*, 1976 ; D'Auzac and Jacob, 1989).

Beside the existing information about latex stability, the morphology of rubber particles obtained after the centrifugation process has been investigated in relation with adsorbed species. Rippel *et al.* (2005) reported that calcium salt crystallites formed around the particles affect film formation and its topology when latex is dried and aged. These crystallites are surprisingly compatible with the hydrocarbon matrix of the rubber particles and are closely associated with their membrane materials. They have been found to prevent the complete coalescence of rubber particles. Divalent cations such as Mg^{2+} and Ca^{2+} present in latex could also form ionic crosslinks between amino acids or carboxylic acids and polyisoprene molecules, thus leading to a higher gel content (Bengtsson and Stenberg, 1996).

Proteins are a part of the non-isoprene component in latex. Allergy caused by the proteins contained in end-used products such as gloves and condoms is of high concern (Yeang *et. al*, 2002). Removal of proteins from latex was attempted using proteolytic enzymes and their allergenic activity and physical properties were tested. The nitrogen content of deproteinized latex was less than 0.02% versus 0.30% in untreated latex. The allergenic potential of the treated latex was lower according to immunologic tests (ELISA). Moreover, deproteinization of latex was not found to affect its properties since deproteinized latex films showed good processability and vulcanizate properties comparable to ordinary NR (Hayashi *et. al*, 1995 ; Sakaki *et. al*, 1995). During the leaching step of dipped latex articles, allergenic proteins and other water soluble components are normally reduced. However, the remaining

hydrophilic non-isoprene components were found to a have negative effect on the tensile strength properties (Amir *et. al*, 1997). In addition, the adsorption of water on proteins can affect the cure characteristics (Metherell, 1980). However, there is so far no structural evidence proving the relationship between proteins and cure characteristics (Sakdapipanich, 2007).

Most latex products are cured in hot air ovens. They are thus open to attack by oxygen. As vulcanization can be inhibited by oxidation, the presence of antioxidants is required. Natural antioxidants are known to be present in NR latex in the form of phospholipids, amino acids, phenols, tocotrienols and betaines. They have been shown to protect NR from deterioration during the coagulation and drying processes.

2. Dry rubber

When latex is converted into dry rubber, most of the water soluble non-isoprene substances are lost by various processes involving water washing. The more hydrophobic components, i.e lipids, are retained in the rubber phase. Lipid content in dry rubber was found to vary with many parameters as mentioned previously, one of them being the rubber tree clone. As found by Visitnonthachai (2005), the variations of physical properties among different rubber clones are correlated to differences in lipid contents. For example, monoclonal USS from RRIM600 clone, with lower lipid content, showed a lower viscosity value than USS from PB235 clone. These clonal characteristics of RRIM600 and PB235 clone were also previously reported by Yip (1990).

In order to investigate the individual effects of the various groups of lipids present on NR properties, experiments have been conducted where selected lipids were added or removed from dry rubber. David *et al*. (2000) studied the effect of non-isoprene constituents on the tack property of NR, which is the ability of two materials to resist to separation after bringing their surfaces into contact for a short time under light pressure. They found that NR submitted to lipid extraction in a Soxhlet apparatus

with boiling acetone for 24 hours and dried at 50 ^{o}C for 12 hours exhibited the highest tack values. In other words, lipids seemed to decrease the tack properties of NR.

Nadarajah *et al.* (1971) extracted and isolated tocopherols and tocotrienols from fresh field latex stabilized with ammonia from various rubber clones. It was found that γ-tocotrienol is the main tocotrienol found in latex, with slight difference among clones. The clones containing the highest amount of δ- and γ-tocotrienol were found to have highest PRI value in their coagulated rubber. It was also noted that coagulation conditions (natural or controlled by acid) had no significant effect on the tocotrienol content of rubber obtained, which is the same as in latex.

Hasma (1984) showed that PRI was improved by the addition to acetone-extracted RSS1 of various neutral lipids fractions from NR. A 0.1 phr amount of tocotrienols was shown to yield an optimal antioxidant activity. However, a similar extra addition of this component did not bring back the PRI value of the acetone extracted RSS1 to the value of PRI of its unextracted rubber. Therefore, PRI of rubber might also be controlled by other factors. Na-Ranong *et. al.* (1995) mentioned that the efficiency of natural antioxidants depended on its localization in membrane of rubber particle .

Generally, thermal oxidative degradation of raw NR is accelerated by numerous factors such as the presence of transition metal ions, sunlight, humidity and heat. The carbonyl group of free fatty acids such as stearic, oleic and linoleic acids and their methyl esters were found to be of prime importance in increasing the rate of oxidation and enhancing chain scission of polyisoprene. Oleic and linoleic acids exhibited a synergistic pro-oxidation activity as a result of their co-oxidation (Arnold and Evans, 1991). Nevertheless, the antioxidant activity of the tocotrienols was found to remain even when the rubber is exposed to a high drying temperature (110 ^{o}C) or to bacterial activity and it was found to increase heat resistance of NR in the vulcanized state (Morimoto, 1985 ; Hasma and Othman, 1990).

Abnormal groups such as fatty acids, lactone, amine, aldehyde and epoxide have been reported to bond to the main-chain molecules. Aldehydes and epoxides are considered to be partly responsible for storage hardening (Bengtsson and Stenberg, 1996 ; Gan, 1996 ; Eng *et. al*, 1997) which is the progressive hardening that occurs in NR when stored under dry conditions.

Fatty acids have been found to have an effect on the crystallization and plasticizing of the rubber. This effect is important as stress-induced crystallization is crucial in enhancing the performance properties of rubber (Burfield and Tanaka, 1987). Saturated fatty acids linked by an ester bond to polyisoprene molecules at the α-terminal end have been shown to play an important role in the crystallization behaviour of NR as nucleating agents (Tanaka *et. al*, 1995 ; Kawahara *et. al*, 1996 ; Kawahara *et. al*, 2000b), while unsaturated fatty acids act as plasticizer (Kawahara and Tanaka, 1995 ; Kakubo *et. al*, 1997). The nucleating effect of saturated fatty acids results in the rapid crystallization of NR, which influences mechanical properties such as tack and green strength in unvulcanized state (Kawahara *et. al*, 2000a), and tear and tensile strengths in the vulcanized state (Ismail *et. al*, 2001). The plasticizing effect of unsaturated fatty acids results in rubber deformation.

Fatty acids are also involved in vulcanization characteristics of NR and are usually used as an activator that increases the vulcanization rate. Indeed, activators are generally high molecular weight monobasic acids or mixtures of stearic, oleic, lauric and palmitic acid (Ismail *et. al*, 1997). Hasma (1984) found that the progressive addition of stearic acid increases torque modulus (ΔT) but that scorch time is unaffected. However, the neutral lipids isolated from fresh latex did not show any activating effect. This may be due to the fact that neutral lipids contain few amounts of free fatty acids as most of them are combined in glycerides.

Proteins and nitrogenous substances were also found to influence the cure characteristics of NR. Othman and Hasma (1988) studied the effects of proteins and amino acids on vulcanization of natural rubber and on vulcanizate properties. They found that neutral and basic amino acids gave a marked increase in cure rate, torque

modulus and a shorter scorch time compared to deproteinised natural rubber. Proteins isolated from B-serum and C-serum and proteolipids were not found to have considerable effects. As well as the study of rheological and processing properties of purified natural rubber (PNR), of which most of non-isoprene constituents are removed by repeated centrifugation, performed by Rattanasom and Suchiva (2005). The results showed that the cure time of PNR is significantly longer than that of normal NR. This may be attributed to the nitrogenous substances naturally present in NR, and that can act as activators for sulfur vulcanization.

Non-isoprene components are present from the early state of latex tapping until the end-use product. The colloidal stability of field latex is mostly influenced by non-isoprene components as mentioned earlier. When they are removed by centrifugation and dry rubber processing, the retained components still express predominant effects. However, the precise role of those various components has not been clearly demonstrated so far.

Since dry rubber is the major derived product from *Hevea brasiliensis* latex, the study of the role of lipids, that constitute the main non-isoprene component, is of great interest. The work presented here aims at studying the evolution of lipids between freshly tapped latex and processed dry rubber state. The information obtained from lipid analyses will be related to their properties.

MATERIALS AND METHODS

Materials

1. Natural rubber (NR) samples

1.1 Latex origin

Four *Hevea brasiliensis* clones: RRIM600, GT1, PB235 and BPM24 were chosen from the plantation of Visahakit Thai Rubber Co., Ltd. located in Chantaburi Thailand. For each clone, the tapping system, sampling number and periods as well as year of planting are listed in table 2.

Table 2 Sample information and sampling periods

Clones	Year of planting	Tapping system	Number of samples	Sampling periods
RRIM600 (old)	1988	1/2s, 2d3	10	April 2004 – June 2004 and December 2005
RRIM600 (young)	1995	1/2s, 2d3	15	April 2004 – December 2006
GT1	1991	1/2s, 2d3	10	April 2004 – June 2004 and December 2005
PB235	1994	1/2s, 2d3	15	April 2004 – December 2006
BPM24	1997	1/2s, d2	15	April 2004 – December 2006

1/2s, 2d3: the trees are tapped on a third of circumference spiral 2 days out of 3
1/2s, d2: the trees are tapped on a half of circumference spiral 1 day out of 2

1.2 Fresh latex collection

The selected trees were tapped at midnight. Latices from around 100 trees of each clone were collected around 5 hours afterwards and filtered through a stainless steel sieve (2 mm pore size). For each clone, the same fresh latex was used for direct lipid extraction and unsmoked rubber sheet preparation.

1.3 Unsmoked rubber sheet preparation

The RRIT technical recommendations for rubber sheet making process were followed. Three liters of filtered fresh latex were diluted with 2 liters of water. The diluted latex was poured into a coagulation tank (35x20x9 cm) and added with 300 mL of 0.42 M formic acid. The pH of latex was lowered to around 4.6. Bubbles

were removed by skimming the acidified latex surface with a metallic plate. The coagulation tank was covered to avoid contamination by dust. After coagulation, the coagulum was removed from the coagulation tank and passed once into a specific crusher to reduce the thickness to around 1 cm. The rubber sheet was then passed through a flat hand mangle for 2-3 times and through a rough hand mangle 2 times to imprint cross lines on the rubber sheet. The rubber sheet was hung and dried under the shade in a ventilated area for 7 to 10 days. The obtained monoclonal unsmoked sheet was used for lipid extraction and study of dry natural rubber properties.

2. Chemicals

Organic solvents, lipid standards and chemicals used for lipid extraction and lipid analysis listed in table 3 were of analytical grade unless otherwise specified.

Methods

1. Lipid extraction

1.1 Lipid extraction from fresh latex

A volume of 25 mL of fresh latex diluted with water (1:1; v/v) was extracted directly in the rubber plantation around 8 hours after tapping using 250 mL of chloroform/methanol 2:1 (v/v). Extraction was performed by adding the sample dropwise into continuously stirred solvent at such a rate that total duration of latex sample addition was 4 minutes. The extract kept at ambient temperature (~30°C) was brought back to the laboratory. The coagulum was then removed and the extract was filtered through Whatman n°1 filter paper (Whatman, England). The filtrate was washed with 1/5 of its volume by a 9 $g.L^{-1}$ NaCl solution as described by Folch *et al.*(1957). After a clear separation between aqueous and organic phases was obtained, the lipid containing organic phase (bottom layer) was collected and evaporated. The obtained dry lipid extract was weighed. The extraction yield was expressed versus dry rubber weight. Three repetitions were performed for each sample.

Table 3 List of solvents, chemicals and standards

Name	Supplier	Country	Ref.
Reagent and organic solvents			
Acetone	Carlo Erba	Italy	
Chloroform	Labscan	Thailand	
Diethyl ether	Carlo Erba	Italy	
Formic acid (94%)	Sakdikamol Lohakij LTD., Part.	Thailand	
n-Hexane	BDH	England	
Methanol	Merck	Germany	
N-methyl-N-(trimethylsilyl)-trifluoro acetamide (MSTFA)	Sigma	Germany	M7891
Orthophosphoric acid (85%)	Merck	Germany	
Pyridine	Labscan	Thailand	
Toluene	Carlo Erba	Italy	
Salts			
Copper (II) acetate	Univar	Australia	
Fast blue BB salt	Fluka	Germany	44670
Molybdenum blue spray reagent 1.3%	Sigma	Germany	M1942
β-naphthyl caproate	Sigma	Germany	N8625
β-naphthol	Sigma	Germany	N1250
Orcinol monohydrate from Lichens	Sigma	Germany	O1875
Rhodamine 6G	Fluka	Germany	83698
Sodium chloride	Univar	Australia	
Sodium hydroxide	Carlo Erba	Italy	
Di-sodium hydrogen phosphate	Univar	Australia	
Trizma Base	Sigma	Germany	T1503
Neutral lipid standards			
Fatty acid methyl esters (FAME): C12:0, C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3 and C20:0	Larodan	Sweden	
Octadecanol	Larodan	Sweden	40-1800-13
Fucosterol	Sigma	Germany	F5379
Plant sterol mixture (β-sitosterol 53%, stigmasterol 7%, campesterol 23% and brassicasterol 13%) (w/w)	Larodan	Sweden	60-1003
α-Tocotrienol	Cayman Chemicals	USA	10008377
γ-Tocotrienol	Cayman Chemicals	USA	10008494
β-Tocotrienol	Cayman Chemicals	USA	10008513
Mix34 mixture for HPLC (oleic acid, methyl oleate, monoolein, diolein and triolein)	Larodan	Sweden	90-3034
Polar lipid standards			
Monogalactosyl diglycerides (MGDG) from plant leaf	Larodan	Sweden	59-1200
Digalactosyl diglycerides (DGDG) from plant leaf	Larodan	Sweden	59-1210
Steryl glucoside (SG) from soybean	Larodan	Sweden	60-1020
Esterified steryl glucoside (ESG) from soybean	Larodan	Sweden	60-1021
Phospholipid mixture for HPLC from Soybean (phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol and lysophosphatidyl choline)	Sigma	Germany	P3817
Lysophosphatidyl inositol sodium salt from soybean	Fluka	USA	62966
3-*sn*-Phosphatidic acid sodium salt from egg yolk	Sigma	Germany	P9511
Mix 40 mixture for HPLC (oleic acid, triolein, phosphatidyl choline from egg yolk, cholesterol and cholesteryl oleate)	Larodan	Sweden	90-4040
Synthetic polyisoprene standards	Polymer Standards Service	Germany	

1.2 Lipid extraction from rubber sheet

A 2.5 grams piece was cut from the rubber sheet. This rubber piece was cut into small pieces (2x1x1 mm). The obtained pieces were then stored at -20°C overnight before grinding using a cryogrinder model Pulverisette 0 (Fritsch, Germany) under liquid nitrogen with 2 mm amplitude for 4 minutes. After grinding, the obtained ground rubber pieces (around 0.2 mm diameter) were transferred immediately into a bottle containing 50 mL of chloroform/methanol (2:1; v/v). After agitation at 160 rpm on a rotary agitator for 6 hours at room temperature (~30°C), rubber was removed by filtration and the total extract was concentrated using a rotary evaporator. Total extract was redissolved with 4 mL of chloroform/methanol (2:1; v/v) and water soluble components were removed with 1 mL of 9 g/l NaCl solution according to Folch *et al.* (1957). After the mixture clearly separated in two phases, the lipid containing bottom layer was taken and evaporated to obtain the lipid extract. Extraction yield was calculated on dry rubber basis and all operations were repeated 3 times for each sample.

2. Lipid analysis

Obtained lipid extracts from both latex and rubber sheet were subjected to various analyses.

2.1 Liquid column chromatography (LC)

The lipid extract was fractionated into neutral lipids, glycolipids and phospholipids by silica gel column liquid chromatography (LC). The procedure described by Rouser *et al.* (1967) was followed, except that the lipid/silica ratio was 1:30 (w/w) instead of 1:100. Silica gel 60 (0.040-0.063 mm, Merck, Germany) was reactivated in an oven at 100 ^{o}C for at least 12 hours. A weight of 1.8 g of silica gel was well mixed with 18 mL of chloroform. The gel was gently transferred into a column (1.6 cm diameter x 10 cm length). Chloroform in excess was drained and

silica gel was washed by 2 volumes of chloroform. The height of silica gel bed was approximately 2.1 cm.

Lipid extract (1 mL of 60 mg.mL^{-1} solution in chloroform) was loaded onto the silica gel. Neutral lipids, glycolipids and phospholipids were successively eluted with 60 mL of chloroform, 90 mL of a mixture of acetone/methanol (9:1; v/v) and 60 mL of methanol, respectively. The flow rate was approximately 1 mL/minute. Each solvent was collected as a separate fraction that was evaporated and weighed.

2.2 Thin layer chromatography (TLC)

Lipid extract was diluted to 10 mg/mL in chloroform. A volume of 5 μl of diluted lipid extract from each sample was applied on silica gel 60G TLC plates. The plates were developed in a chromatography tank containing about 100 mL of suitable solvent systems before being visualized by general or specific detection reagents. The conditions of TLC analyses listed below were used:

2.2.1 Neutral lipids

n-Hexane/diethyl ether/acetic acid (80:20:1; v/v/v) was used as mobile phase. The TLC plate was sprayed with a mixture of 40% orthophosphoric acid and aqueous solution saturated with copper acetate (1:1; v/v) and then heated at 180 ^{o}C for 10 minutes in an oven. The lipid spots appeared brownish on a white background. This technique is particularly effective for the determination of traces of lipids.

2.2.2 Glycolipids

Chloroform/methanol/water (95:20:2.5; v/v/v) was used as mobile phase. Sulphuric orcinol dyeing agent was used for glycolipid detection (2 mg of orcinol per mL of 70% sulphuric acid). This sugar specific reagent is very sensitive (minimum detectable quantity is 0.5 nmol of sugars). The TLC plate sprayed with

orcinol sulphuric was heated at 100 ^{o}C for 10 minutes. The sugars containing spots appear as orange-purple spots on a white background.

2.2.3 Phospholipids

Chloroform/methanol/30% ammonia (65:25:5; v/v/v) was used as mobile phase. The TLC plate was sprayed with molybdenum reagent (molybdenum oxide 1.3% in 4.2 M sulfuric acid) which reacts specifically with the phospholipids within 10 minutes to give blue spots on white background at room temperature. After the plate was heated at 120 ^{o}C for 2 minutes, the phospholipids containing spots appear brownish on a white background and non-phospholipid spots could then be visualized.

2.3 Free fatty acid quantification

Free fatty acids in lipid sample were quantified by the adaptation of the method described by Van Autryve *et al.* (1991) which was used for lipase activity assay. The principle of this method is based on the specific complexation of free fatty acid with rhodamine 6G. The absorbance of the complex was measured a wavelength of 513 nm.

2.3.1 Reactant preparation

The reactant was prepared by dissolving 100 mg of Rhodamine 6G in 100 mL of 0.2 M sodium phosphate adjusted to pH10 with 0.2 M sodium hydroxide. Rhodamine 6G was extracted 2 times with 200 mL of toluene in a separating funnel. After decantation, the obtained organic phase was dried by pouring through sodium hydroxide pellets placed in a funnel. Rhodamine 6G solution was conserved at 4 ^{o}C in the sodium hydroxide containing bottle and protected from light exposure. The calibration was done with linoleic acid in a range from 0 to 10 μg of fatty acid per mL of n-hexane. Calibration was performed each day of measurement.

2.3.2 Quantification of free fatty acids

Lipid extract from rubber sheet and latex were diluted to 60 μg.mL^{-1} and 20 μg.mL^{-1} respectively with n-hexane. Under a fume hood, 0.5 mL of Rhodamine 6G was added to 3.5 mL of diluted sample in a test tube. Before absorbance measurement at 513 nm using a Hitachi model U-2001 spectro photometer (Tokyo, Japan), the solution was left for 5 minutes under a fume hood (25 $\pm$ 2^{o}C) for color stabilization. Free fatty acid concentration was calculated from the response factor obtained from the standard curve.

2.4 Lipase activity assay

2.4.1 Reactant preparation

For the preparation of the Tris-HCl 200 mM, pH 7.0 buffer solution used for substrate preparation and latex dilution, 6.05 g of Trizma-base was dissolved in a small volume of deionized water, pH was adjusted to 7.0 with 6 N HCl then deionized water was added to a final volume of 250 mL. β-naphthyl caproate was used as substrate for lipase activity assay. It was prepared by dissolving 8 mg of β-naphthyl caproate in 10 mL of 5% acetone containing Tris-HCl buffer. β-naphthol, the product of β-naphthyl caproate hydrolysis, was used as the standard. It was prepared by dissolving 3 mg of β-naphthol in 10 mL of Tris HCl buffer,pH 7, added with 5% (v/v) acetone. Fast blue BB salt (1mg.mL^{-1} in ethanol) was used as dyeing indicator and reaction stopper. This indicator has to be well agitated to avoid any precipitate and prepared just before use due to its light sensitive.

2.4.2 Lipase activity assay

Lipase activity in fresh latex was measured directly in the field. In a microplate, 50 μl of fresh latex diluted 5 times with Tris HCl buffer pH7 were added into 50 μl of β-naphthyl caproate solution. After 2 hours of incubation, the product from lipase activity in latex was detected by adding 100 μl of fast blue BB salt. The

color intensity from lipase activity in latex was compared with the color scale obtained with various concentration of β-naphthol and the activity of lipase was estimated and reported in units of lipase activity per L of latex. One unit of lipase activity is the number of μmol of free fatty acid released per minute.

2.5 Gas chromatography (GC)

Gas chromatography analysis of total fatty acid and unsaponifiable compositions of lipids obtained from saponification reaction was performed by converting them to their more volatile derivatives such as fatty acid methyl esters (FAME) and trimethylsilyl (TMS) ether derivatives of sterols.

2.5.1 Preparation of total fatty acids and unsaponifiable by saponification

The saponification method of Kates (1972) was followed. Appropriate volume of lipid extract in chloroform was poured in a flat bottom flask in order to obtain approximately 30 mg of lipid after evaporation by nitrogen stream. Then, 5 mL of a mixture of methanol/3 M NaOH (9:1, v/v) was added to the lipid sample. The mixture was refluxed at 80 ^{o}C for 1.5 hours. After cooling, the unsaponifiable was extracted with 3 x 5 mL of n-hexane. The aqueous bottom phase was acidified with 0.3 mL of 6 M HCl and the free fatty acids were extracted with 3 x 5 mL of n-hexane. Solvents in unsaponifiable and fatty acid extracts were both evaporated to dryness the the samples were weighed. The yield of unsaponifiables and fatty acids were then determined as follows.

$$\% \text{ Fatty acids} = \frac{weight\ of\ \ fatty\ acids}{weight\ of\ \ lipid\ extract} \times 100 \qquad (1)$$

$$\% \text{ Unsaponifiables} = \frac{weight\ of\ \ unsaponifiables}{weight\ of\ \ lipid\ extract} \times 100 \qquad (2)$$

2.5.2 Preparation of fatty acid methyl ester derivatives

A volume of 4.5 mL of 2% of concentrated H_2SO_4 in methanol was added into fatty acid extract and the mixture was refluxed at 80 ^oC for 1.5 hours. After cooling, 0.5 mL of water was added and the methyl esters were extracted with 3 x 5 mL of n-hexane. The methyl ester extract was evaporated to dryness under vacuum, weighed and redissolved in 1 mL of n-hexane.

2.5.3 Preparation of trimethylsilyl ether derivative (TMS) of unsaponifiables

Unsaponifiable fraction was submitted to silylation according to Vaysse *et al*. (2002). The sample dried under a stream of nitrogen was added with 50 µl of MSTFA and 50 µl of anhydrous pyridine. After incubation of the reaction mixture at 50^oC in a water bath for 30 minutes, 400 µl n-hexane was added and the TMS derivative was ready for GC analysis.

2.5.4 Analysis of fatty acid composition by GC/FID

A Shimadzu GC17A gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a fused silica capillary column BPX70 (30 m, i.d. 0.25 mm, film thickness 0.25 µm, SGE, Victoria, Australia) and a Shimadzu AOC20i automatic injector (injected volume 1 µL) were used. Initial linear velocity of helium in column was 34 cm/second. Temperature of the split injector (split ratio 1:15) and of the flame ionization detector were 250 °C and 280 °C, respectively. Oven temperature was 160 °C for 0.5 minutes, increased to 200 °C at 10 °C/minute then kept constant for 4 minutes. Data acquisition was performed using the GC solution v 2.10 software (Shimadzu Co., Kyoto, Japan). Calibration curves were performed using fatty acid methyl ester standard mixtures containing each fatty acid at different known concentrations as mentioned in table 4 and figure 17. This proportion between FAME was chosen regarding the one present in total fatty acid composition of lipid extract from rubber.

2.5.5 Analysis of the unsaponifiables by GC/MS

Unsaponifiable compounds were separated and identified by GC-MS after silylation. The system used was Shimadzu QP2010S GC-MS equipped with a fused silica capillary column SLB-5 MS (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 32 $cm.s^{-1}$. Temperature of the split injector (split ratio 1:20) was 250 °C. Oven temperature was set to 230 °C for 2 min, increased to 300 °C at 4 $°C.min^{-1}$ then kept constant for 3.5 min. The mass spectrometer was operated in the electron impact (EI) mode, with electron energy of 70eV. Mass spectrometric detection was conducted in scan mode from *m/z* 100 to 800 at 2.2 scans per second; ion source temperature was 200 °C. Control and data acquisition and processing were performed using the GCMS solution v 2.40 software and the NIST 05 (Shimadzu Co., Kyoto, Japan) and our own spectrum database.

Calibration of furan fatty acid FAME was performed separately following purification from PB235 lipid extract.

Table 4 Concentration of standard solution injected in GC (mg/mL)

Fatty acids	Retention time (minute)	standard concentration (mg/mL)							
		STD1	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD8**
C14:0	2.95	0.18	0.14	0.11	0.07	0.04	0.02	0.01	0.00
C16:0	3.86	0.53	0.42	0.32	0.21	0.11	0.05	0.03	0.01
C16:1	4.09	0.25	0.20	0.15	0.10	0.05	0.02	0.01	0.01
C18:0	5.97	0.98	0.78	0.59	0.39	0.20	0.10	0.05	0.02
C18:1	5.24	1.22	0.97	0.73	0.49	0.24	0.12	0.06	0.03
C18:2	5.73	2.09	1.67	1.25	0.83	0.42	0.21	0.10	0.05
C18:3	6.38	0.23	0.18	0.14	0.09	0.05	0.02	0.01	0.01
C20:0	6.38	0.20	0.16	0.12	0.08	0.04	0.02	0.01	0.01
Total		**5.66**	**4.53**	**3.40**	**2.26**	**1.13**	**0.57**	**0.28**	**0.14**

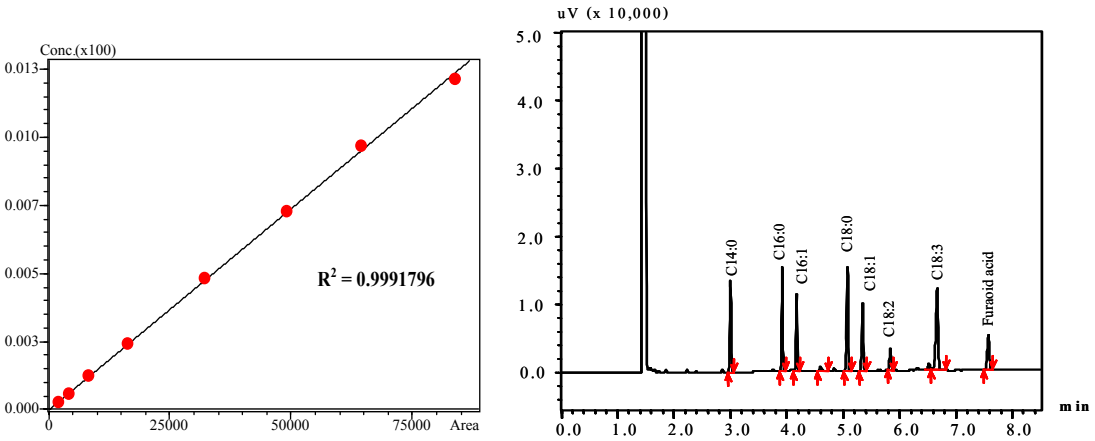


Figure 17 Calibration curve of linoleic acid (C18:2) and chromatogram of fatty acid methyl ester standards.

2.6 High performance liquid chromatography/electrospray ionization -mass spectrometry (HPLC/ESI-MS)

The separation, identification and quantification of glycolipid and phospholipid fractions obtained from LC were carried out with normal-phase liquid chromatography using a Waters Alliance 2695 separation module (Waters Corp., Massachusetts, USA). This module was equipped with an Alltima HP MS silica column (150 mm x 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. A volume of 5 µl of samples were injected using the Waters Alliance 2695 autosampler. The conditions for glycolipids and phospholipids analyses are described below.

2.6.1 Glycolipid analysis conditions

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^{-1}. The total chromatographic run time was 15 min. The mass spectrometer was operated in ESI positive mode (capillary voltage 5 kV; cone voltage 70 V; source temperature 150^{o}C; desolvation temperature 250 ^{o}C; desolvation

nitrogen flow 260 $L.h^{-1}$; cone nitrogen flow 30 $L.h^{-1}$). The full scan mass spectrum was acquired in the *m/z* 300 to 1100 range. Calibration was performed with four glycolipid standards: digalactosyl diglycerides (DGDG), monogalactosyl diglycerides (MGDG), steryl glucoside (SG) and esterified steryl glucoside (ESG).

2.6.2 Phospholipid analysis conditions

The separation was performed with a ternary mixture mobile phase consisting of (A) methanol with 0.1% (w/v) ammonium hydroxide, (B) chloroform with 0.1% (w/v) ammonium hydroxide and (C) deionized water with 0.1% (w/v) ammonium hydroxide. The flow rate was 0.25 $mL.min^{-1}$. Separation was obtained by using gradient starting at 24.5% of A, 74.5% of B and 0.5% of C. Then the ratios were linearly changed to 50% of A, 44.5% of B and 5.5% of C within 10 min and held isocratically for 20 min. The total chromatographic run time was 40 min.

The mass spectrometer was operated in ESI positive (capillary voltage 5 kV; cone voltage 70 V; source temperature 150^{o}C; desolvation temperature 250 ^{o}C; desolvation nitrogen flow 260 $L.h^{-1}$; cone nitrogen flow 30 $L.h^{-1}$) and ESI negative mode (40 and 90 V of cone voltage with same other parameters). The full scan mass spectrum was acquired in the mass range between *m/z* 190 and 1090. Calibration was performed with phospholipid standard mixtures consisting of phosphatidyl choline (PC), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE), phosphatidic acid (PA), lysophosphatidyl choline (LPC) and lysophosphatidyl inositol (LPI).

3. Structural characterization of natural rubber

3.1 Analysis of mesostructure

3.1.1. Determination of weight average molar mass, number average molar mass and molar mass distribution by size exclusion chromatography (SEC)

Sixty milligrams of unsmoked sheet were solubilized in 30 mL of cyclohexane for 2 weeks, and the solution was daily agitated with a rotary stirrer (Model Reax 2, Heildolph, Germany) for 1 hour at 24 rpm the second week. The solution was then centrifuged at 16500 rpm for 1 hour (Avanti JE centrifuge with JA20 rotor, Beckman Coulter Inc., CA, USA) to separate macrogel from the soluble fraction (see 3.1.2). An appropriate dilution of the supernatant was performed to obtain 0.2 mg/mL solution. The diluted solution was filtrated through a disposable filter of 25 mm (glass membrane, pore diameter 1 μm) before injection (Bonfils and Char, 2005).

Calibration was carried out with synthetic polyisoprene with molar masses of 1310, 3030, 8000, 32400, 71950, 97200, 295000, 452000 and 1.2 million (expressed in $g.mol^{-1}$). A series of natural rubber samples (100 μL) and standards were rotatively injected to a SEC system. The system consisted of an ERMA ERC-3112 solvent degasser, a Waters 510 pump (Water Corp., Milford, MA, USA), a Waters 717 plus autosampler, a Waters 486UV detector set to 220 nm. The two styrene-divinylbenzene mixed 30 cm x 7.8 mm columns with a porosity of 20 μm (Styragel, Water corp., Milford, MA, USA) were thermostated at 65 ^{o}C and the flow rate of cyclohexane was 0.8 $mL.min^{-1}$. Three rubber solutions from each sample were prepared and injected.

Number average molar mass (M_n) and weigh average molar mass (M_w) were calculated according to equations (3) and (4), respectively

$$M_n = \frac{\sum N_i M_i}{\sum N_i} \quad \text{computed as} \quad M_n = \frac{\sum H_i}{\sum (H_i / M_i)} \qquad (3)$$

$$M_w = \frac{\sum N_i (M_i)^2}{\sum N_i} \quad \text{computed as} \quad M_w = \frac{\sum H_i M_i}{\sum N_i} \qquad (4)$$

when M_i = molar mass

N_i = number of molecules having molar mass M_i

H_i = height of slice (volts.min) corresponding to molecular weight M_i

3.1.2. Determination of macrogel

After the centrifugation of solution in 3.1.1 was completed and the supernatant was transferred, the centrifuge tubes containing the non-soluble part called macrogel were placed overnight under a fume hood, then dried in a vacuum oven (1 atm) for 4 hours at 45 ^{o}C, cooled at room temperature in desiccator until the weight was constant and weighed for macrogel calculation according to eq. 5.

$$\% \text{ Macrogel} = \frac{macrogel\ weight}{initial\ rubber\ weight} \times 100 \qquad (5)$$

3.1.3. Determination of microgel

For a given sample injected into the SEC apparatus (according to 3.1.1), the integration of chromatographic peak corresponding to rubber polyisoprene after SEC gave the concentration of the solution after filtration. As the concentration of the solution before and after filtration was known (about 0.2 mg.mL^{-1}), the fraction eliminated by filtration, *i.e.* the percentage of microgel, could be determined as follows.

$$\% \text{ microgel} = \frac{[PI_0] - [PI_{SEC}]}{[PI_0]} - (1 - \% macrogel) \qquad (6)$$

when (PI_0) = PI concentration before filtration in mg.mL^{-1}

(PI_{SEC}) = PI concentration estimated from SEC in mg.mL^{-1}

3.2 Analysis of macrostructure

3.2.1 Determination of total solid content of latex (ISO 124; 1992)

A glass Petri dish with cover (diameter approximately 90 mm) was weighed. A weight of 2.0 $\pm$ 0.5 g of latex was poured into the dish and gently spread over the bottom surface of the dish. The dish with latex was dried in oven at 105 $\pm$ 5 ^{o}C for 2 hours. Then, the dish was cooled down to ambient temperature in desiccators and weighed until constant weight. The total solids content was calculated as follows:

$$\% \text{ total solids content (TSC)} = \frac{m_1}{m_0} \times 100 \qquad (7)$$

where m_0 = mass of the test portion

m_1 = mass of the dried material

Three replicates were done. The maximum difference between replicates should not be larger than 0.2 % (w/w).

3.2.2 Determination of dry rubber content of latex (ISO 126; 1995)

The ISO 126 procedure was followed with a five folds reduction of the amounts of latex and reactants. A weight of 2.0 $\pm$ 0.2 g of latex was poured into a glass container. Two milliliters of distilled water were added and gently mixed with 15 $\pm$ 1 mL of 2 % acetic acid. The container was put in boiling water for 15-30 min to clarify the serum; if necessary, 1 mL of 95% ethanol was added to ease the separation. The coagulum was rinsed with plenty of water and then pressed to obtain 2 mm thick sheet. The sheet was dried at 70 $\pm$ 2 ^{o}C until it turned from white to transparent. The sheet was then put to ambient temperature in a desiccator and weighed until constant weight. The dry rubber content was calculated as follows:

$$\% \text{ Dry rubber content (DRC)} = \frac{m_1}{m_0} \times 100 \qquad (8)$$

where m_0 = mass of initial latex

m_1 = mass of the dried sheet

Three replications were done. Maximum difference between replicates should not be larger than 0.1% (w/w).

3.2.3 Analysis of dry rubber

a. Homogenization of dry rubber (SMR bulletin No.7 part B.2; 1992)

A 100 g piece of rubber was passed six times through the gap between the 150 x 300 mm rolls of a laboratory mill rotating at uneven speeds. The rolls were cooled with running water at room temperature and the nip was set at 1.65 $\pm$ 0.16 mm. After each pass, the rubber was rolled into a cylinder and introduced endwise for the next pass. The rubber was not rolled after the fifth pass but folded and passed lengthwise through the rolls. After the sixth pass, the obtained sheet was folded before test pieces were cut out for the various tests.

b. Determination of initial Wallace plasticity (P_0) (ISO 2007; 1991)

A piece of 20 $\pm$ 5 g of homogenized rubber was blended twice through the rolls of a mill at the temperature of 27$\pm$ 3 ^{o}C with nip setting adjusted so that the final sheet thickness was 1.6 - 1.8 mm. The sheet was immediately doubled and the two halves were pressed slightly together by hand. Six test pellets, approximately 3 mm thick and with a 13 mm diameter, were punched out from the doubled sheet with a Wallace Punch. The test pellets were divided into two sets of three (figure 18). The first set was used for initial plasticity (P_0) measurement while the second one was used for plasticity retention index (PRI) determination.

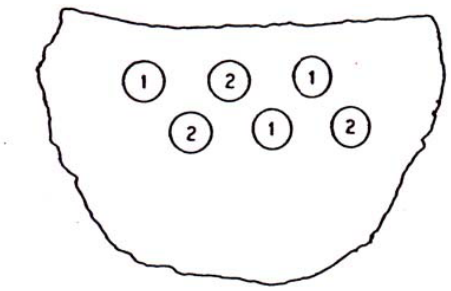


Figure 18 Doubled rubber sheet with punched test pieces

1. Punched pellets for initial Wallace plasticity (P_0) determination
2. Punched pellets for aged plasticity (PRI) determination

The test piece, sandwiched between two pieces of cigarette paper (TST orange), was compressed between the two parallel platens of the Wallace plastimeter to a fixed thickness of 1.00 ± 0.01 mm and held for 15 ± 0.2 s to reach thermal equilibrium with the platen temperature (100°C ± 1°C). It was then subjected to a constant compressive force of 100 ± 1 N for 15 s. The median value of thickness from three tests at the end of this period was taken as the measure of the initial Wallace plasticity.

c. Determination of plasticity retention index (PRI) (ISO 2930; 1995)

The second set of three test pieces prepared according to §3.2.3.a was heated in an air Wallace oven at 140 ± 0.2 °C for 30 ± 0.25 min. After the aged samples cooled down to room temperature, the plasticity of the aged pellets (P_{30}) was taken according the method used for unaged samples (P_0). The median values of the three unaged and the three aged test pieces were used to calculate PRI as follows (eq. 9).

$$PRI = \frac{Aged\ median\ plasticity\ value\ (P_{30})}{Unaged\ median\ plasticity\ value\ (P_0)} \times 100 \qquad (9)$$

Maximum coefficient of variation (CV) should be 3%

d. Determination of Mooney Viscosity (ISO 289-1; 1994)

This measurement was carried out on a Mooney viscometer model Mk III (H.W. Wallace). Test temperature was 100 °C. Twenty five grams of the homogenized rubber were divided into two equal portions. One was put below the preheated rotor (large model) stem through the centre of the portion. Then this whole combination was put in the lower die cavity and the second portion was put on top of the rotor. The dies were closed immediately and the motor was started after 1 minute equilibration time. The maximum (V_{max}) and minimum (V_{min}) viscosities were recorded and the viscosity of the rubber (V_R) was taken at the end of the fourth minute from the instant when the motor had started as shown in figure 19.

A typical test on NR with the normal rotor speed with viscosity number 60 should be reported as follows (eq. 10):

60 ML (1+4) 100^{o}C (10)

60 M	=	the viscosity, in Mooney units
L	=	a large rotor
1	=	the pre-heating time (minute) before starting the rotor
4	=	the running time (minute), after starting the rotor at which the final reading was taken
100 ^{o}C	=	the temperature of the test

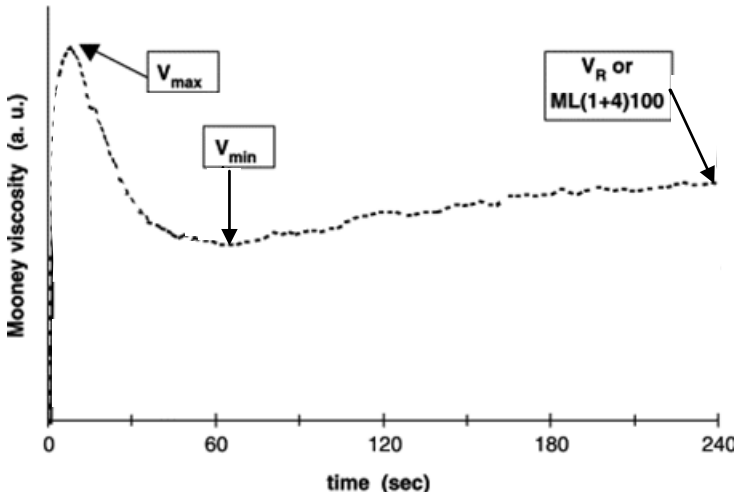


Figure 19 Typical Mooney curve

4. Study of breakdown and vulcanization properties

4.1 Breakdown

The Breakdown Index (BI) is a degradability index that takes into account the effects of thermal and mechanical degradation undergone by NR during mastication. This index was calculated from the ratio of the drop in Mooney viscosity through mastication to the amount of mechanical energy required (Lim and Ong, 1985).

Mastication of NR was carried out using an internal mixer Rheomix 600p (Haake Polylab system) equipped with 2 rotors (roller type 557 – 1302) as shown in figure 20. The Haake mixer is a torque recording rheometer (Rheocord RC300p). Basically, the mixer head consists of a pair of rotors rotating in a closed 65 mL chamber. A weight of 48 $\pm$ 1 g of rubber sheet was fed into the mixing chamber in strips and the instrument recorded the torque needed to turn the rotors. A thermocouple protruding into the chamber enables the rubber temperature to be continuously monitored. The duration of mixing was fixed at 4 minutes.

Figure 20 Internal mixer Rheomix 600p (Haake Polylab system)

Two temperature/rotor speed conditions were tested:

Condition 1, where thermal degradation is promoted

Rotor speed: 50 RPM

Starting temperature: 140 ^{o}C

Condition 2, where mechanical degradation is promoted

Rotor speed: 100 RPM

Starting temperature: 50 ^{o}C

Torque, rubber temperature, and rotor speed were recorded every 0.2 min as the example shown in figure 21. After analysis, total mechanical energy input was provided by the software by integrating the area under the torque-time curve. Volumetric energy input was obtained by dividing total energy input E after 4 minutes of work by the volume of sample as follows (eq. 11):

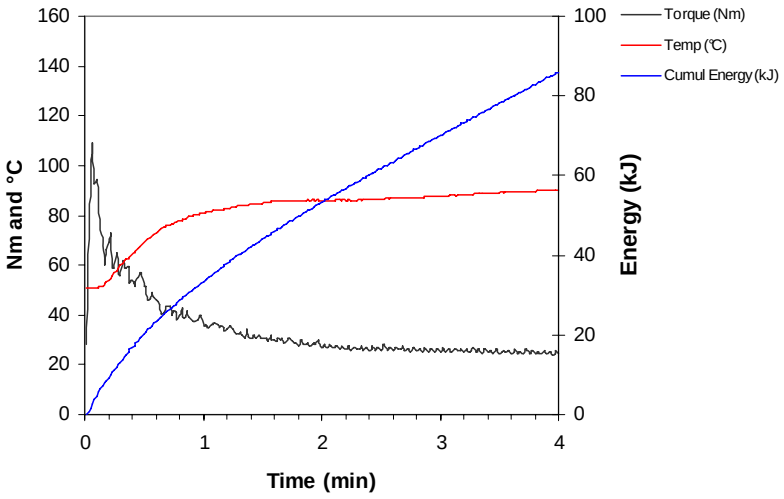


Figure 21 Torque, temperature and cumulated mechanical energy during 4 minutes of mastication under mechanical condition

$$W_u\,(MJ/m^3) = \frac{E\,(J)\,/\,10^6}{rubber\ volume\,(cm^3)\,/\,10^6} = \frac{E\,(J)}{rubber\ weight\,(g)\,/\,0.911} \qquad (11)$$

0.911: average density of raw natural rubber

As defined by Lim and Ong (1985), the breakdown index was calculated from the relative drop of Mooney viscosity (ΔV %) and the volumetric energy input (W_u). The breakdown index, BIv, was calculated as follows (eq. 12):

$$BI_v = \frac{\Delta V\%}{W_u} \times 100$$

$$= \frac{V_1 - V_2}{V_1} \times \frac{1}{W_u} \times 10^4 \quad (12)$$

V_1 = Initial Mooney Viscosity of the raw rubber (ISO 289-1)

V_2 = Mooney Viscosity after mastication (ISO 289-1)

W_u = Energy input ($MJ.m^{-3}$)

The same calculations were performed by replacing the drop of Mooney viscosity, by the drop of initial plasticity (ΔP %). This new index, called BI_P, was calculated according to eq. 13:

$$BI_P = \frac{\Delta P\%}{W_u} \times 100$$

$$= \frac{P_1 - P_2}{P_1} \times \frac{1}{W_u} \times 10^4 \quad (13)$$

P_1 = Initial Wallace plasticity of the raw rubber (ISO 2007)

P_2 = Plasticity after mastication (ISO 2007)

W_u = Energy input ($MJ.m^{-3}$)

4.2 Vulcanization

4.2.1 Vulcanization

Test sample preparation

The cure characteristic measurement was performed following RRIM SMR Bulletin No.7 part C3 (1992). The formulation of pure gum which contain minimum ingredient for vulcanization reaction following recommendation of Rubber Research Institute of Thailand (RRIT) was used as described below.

Homogenized USS	100	
ZnO	5	phr
Stearic Acid	2	phr
Wingstay-L	1	phr
CBS	0.8	phr
Sulfur	2.4	phr

CBS (N-cyclohexylbenzothiazole-2-sulphenamide) was used as an accelerator for sulphur vulcanization and Wingstay-L as an antioxidant.

A weight of 100 g of rubber was first masticated for 1 min with two roll mills (Model R11-3, Kobaira Seisakusho Co.Ltd, Tokyo Japan) preheated to $70 \pm 5\ ^{o}C$ to obtain a smooth band. ZnO, stearic acid, Wingstay-L and CBS were successively added and mixed with rubber for 4 min. The mix was then added with sulphur and homogenized for 2 minutes more. During mixing, each side of the rubber band was alternately cut at 3/4 of the distance across the roll after the addition of all the ingredients for better homogenization. The rubber sample was finally sheeted out and stored in a dark and dry atmosphere at room temperature for a period of time not exceeding 24 hours. A test specimen measuring approximately 30 x 12.5 mm and weighing about 11-20 g was later cut to get a test sample.

Vulcanization condition

The rheometer (Monsanto model MDR2000, Ohio, USA) consists of pair of dies with the biconical disc embedded in the test compound located between them (figure 22). The frequency of the rotary oscillation of the disc was 100 cycles/minute. The arc of rotor oscillation was adjusted to 3^{o} around its center position which corresponds to a total arc swing of 6^{o}. The force required to maintain this oscillation at a constant rate was measured by a torque transducer. The torque (in.lb) was plotted against time. Vulcanization was conducted for 30 min at 150 ^{o}C.

Figure 22 Oscillating disc rheometer (Monsanto model MDR2000)

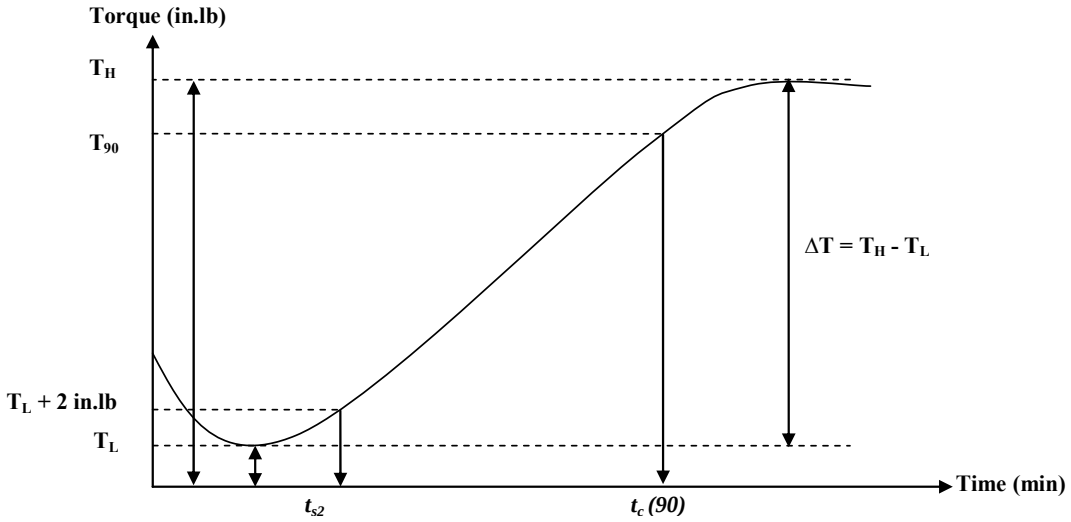


Figure 23 Rheometer curve

The following parameters were determined from the rheometer curve (figure 23).

1. Maximum torque (T_H) corresponds to the maximum modulus of the fully vulcanized specimen at the test temperature.

2. Minimum torque (T_L) corresponds to minimum modulus of the uncured compound.

3. Optimum torque (T_{90}) = T_L + 90% (T_H-T_L) is torque required to reach 90% of cure.

4. Optimum cure time ($t_{c(90)}$) is time in minutes for torque to reach optimum torque.

5. Scorch time (t_{s2}), corresponds to the period from the closure of the die cavity to the time when the torque has risen by 2 in.lb above the minimum torque.

6. Cure rate ($t_{c(90)} - t_{s2}$) is the time required for a given compound to cure to its optimum state.

RESULTS AND DISCUSSION

Chapter 1 Methodology

Lipids have been described to play roles on some rubber properties. Nevertheless, there has been no comprehensive work dealing with the eventual relationships between lipid composition and the lack of consistency of natural rubber properties. Furthermore, the results of the previous studies on the roles of lipid composition in rubber properties are hardly comparable because they have been realized on rubbers from various clones and using different lipid analysis methods.

Our research question was thus:

Is lipid composition related to the lack of consistency of rubber properties?

In order to try to answer this question, the purpose of the present work was the comprehensive characterization of latex and rubber lipids and the analysis of their relations with properties from well defined samples.

1. Research strategy and experimental design

The strategy was to generate a database from a large number of fully characterized samples presenting variability in properties, then to analyze correlations between lipid composition and these properties. This database gathers, for each sample, data on sample origin, lipid composition of latex and dry rubber, rubber structure, raw rubber and vulcanization properties.

Three potential external factors of variation were studied: clone, season and age of trees. Unsmoked sheet (USS) rubber was chosen as dry rubber sample. The criterions that led us to these choices are described below.

1.1 Selection of relevant data

1.1.1 Measurable factors of variation

- ***Clones***

Clonal origin of rubber trees is known to be an important factor affecting natural rubber characteristics in both latex and dry rubber. It has been reported that some physiological parameters were practically the same for a given clone wherever the trees were located (Jacob *et. al*, 1989 ; Gohet *et. al*, 2003). A clonal effect was also found in both biochemical composition and physical properties of derived dry rubber products (Thomas *et. al*, 1990 ; Yip, 1990 ; Nair *et. al*, 1993).

- ***Season***

Season has been frequently mentioned to affect physiological parameters of latex and natural rubber in parallel with clonal origin (Le Roux *et. al*, 2000 ; Moreno *et. al*, 2005). In order to generate a large number of samples, samplings have to be performed over a long period of time. In these conditions, a seasonal effect is unavoidable but measurable, rain value being the most important factor.

- ***Age of trees***

As observed in other plant species, age of rubber trees influences metabolism and latex production and indirectly affects the properties of the produced dry rubber (Bricard and Nicolas, 1989 ; Tangpakdee *et. al*, 1996 ; Nair, 2000). In our work, this factor was studied only for one clone, using trees of two different ages.

1.1.2 Dry rubber type selection

Dry rubber can be obtained from latex through different processes. It was necessary for our study to collect latex from selected trees and to use a process yielding a representative type of dry rubber, chosen on the basis of the following constraints:

(a) Rubber samples should be of monoclonal origin with known agronomical information (clonal conformity, year of planting, tapping system).

(b) Several clones should be available in a short distance area. This is to be able to compare the samples from various clones in a same pedoclimatic environment and to avoid the problem of sample handling, such as uncontrolled coagulation.

(c) The process to obtain dry rubber should be perfectly controllable and repeatable in order to generate comparable samples. Generally, other sources of variation than those identified previously should be avoided.

According to (a) and (b) constraints, experimental plantations such as research institutes or well managed industrial plantations are more suitable than small holder plantations. Concerning (a) and (c), sheet rubber was preferred for the simplicity of its preparation. For example, processing of technically specified rubber (TSR) is difficult to scale down while repeatability is not easy to maintain.

Among the various rubber sheet types namely unsmoked sheet (USS), air dried sheet (ADS) and ribbed smoked sheet (RSS), USS is the simplest one because it does not require a dedicated dryer. Using the simplest process was required to maintain a high level of reproducibility. To validate the choice of USS instead of RSS, which is the common exported form of sheet rubber, a preliminary study was performed to assess potential differences between USS and RSS in terms of physical properties. The results from classical specification of rubber, namely initial plasticity (P_0), plasticity retention index (PRI) and Mooney viscosity (ML) did not indicate any significant difference between these rubber sheet types (Rodphukdeekul ***et. al***, 2008). It was thus decided to use USS as a model for dry rubber.

1.2 Selection of number of samples and sampling period

Clone: The four most planted rubber clones in Thailand were chosen: RRIM600 (most important clone in Thailand), BPM24, PB235 and GT1. PB235 rubber is known for its specific unimodal molar mass distribution (MMD) of polyisoprene chains (Visitnonthachai, 2005) while the 3 others have a bimodal MMD.

Season: Samplings were performed from April 2004 to December 2006. The samplings cover several cycles of rubber tree life (defoliation, tapping interruption) and the three seasons in East Thailand (winter, summer and rainy season) for three years. Rain amount was the most important factor. It was coded into three levels: dry (accumulated rainfall <100 mm), intermediate (100 – 300 mm) and rainy (>300 mm) according to the level of accumulated rainfall during the thirty days before sampling.

Age: This parameter was studied for RRIM600 clone only. All trees were from the same plantation. Trees planted in year 1988 are identified as RRIM600 (old) while RRIM600 (young) were planted in 1995.

1.3 Selection of analyzed data

The studied data can be divided into 3 families. The objective of this study was to obtain as much as possible information from each of the data family mentioned below. The general scheme of analysis is presented in figure 24.

1.3.1 Lipids (in latex and sheet rubber)

- Total lipid extract
- Free fatty acids
- Glycolipids
- Phospholipids
- Neutral lipids
- Unsaponifiable

- Fatty acid composition

1.3.2 Mesostructure

- Gel content
- Molar mass distribution (MMD)
- Number average molar mass (M_n)
- Weight average molar mass (M_w)

1.3.3 Properties

- Specification: dry rubber content (DRC), total solid content (TSC), initial plasticity (P_0), plasticity retention index (PRI), Mooney viscosity (ML) and nitrogen content (N)
- Breakdown behavior during mastication: breakdown index (BI)
- Vulcanization characteristics: scorch time (t_{s2}), optimum cure time ($t_{c(90)}$), minimum torque (M_L) and maximum torque (M_H).

Each data family will be presented in chapter 2 (lipid composition) and chapter 3 (mesostructure and properties), respectively.

1.4 Repetitions

Rubber sheet making process: In order to get enough quantity of dry rubber for analysis, two rubber sheets with the same quality are needed. The repeatability of the process was checked by comparing lipid contents and properties of two sheets prepared under the same conditions. This operation was performed for three samplings dates. Comparison of means from each sampling indicated that the two series of sheets did not show any significant difference in the studied parameters (table 5). This finding was similarly observed for the other studied clones (data not shown).

Table 5 Lipid extract (%w/w dry rubber) and properties of rubber from two series of sheets prepared from BPM24 clones

sampling	Sheet number	%Lipid extract (w/w dry rubber)		P_0		PRI		ML	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
1 (19/01/2006)	1	2.88[a]	0.09	30.1[a]	0.33	84.1[a]	1.09	61.0[a]	0.57
	2	2.97[a]	0.04	30.0[a]	0.58	85.8[a]	0.52	59.8[a]	0.42
2 (28/03/2006)	1	2.52[a]	0.02	30.6[a]	0.33	98.7[a]	0.88	58.1[a]	0.59
	2	2.50[a]	0.01	31.3[a]	0.17	94.6[a]	0.83	58.3[a]	0.64
3 (07/07/2006)	1	2.42[a]	0.04	36.5[a]	0.58	103.4[a]	0.35	64.7[a]	0.33
	2	2.41[a]	0.10	36.7[a]	0.17	103.7[a]	0.58	65.7[a]	0.58

Mean from 3 repetitions of each sampling
For each column and sampling date, letters design groups of means that are not significantly different (student *t* test) SE: standard error

Lipid analysis: Three independent repetitions were performed for each sample, starting by three independent lipid extractions.

Mesostructure: Three repetitions of analysis were performed using one rubber sheet sample for three independent sample preparations

Properties: Three repetitions were performed for each property except breakdown index for which one single measurement was performed by sample. For this analysis, the repeatability of the measurement was verified by comparing mastication parameters recorded from three repetitions of mastication performed for each condition. The results in table 6 show that the coefficient of variation (CV or relative standard deviation RSD%(r)) obtained for each parameter was below 1% under thermal conditions and below 4% under mechanical conditions. This indicated the good repeatability of the measurement.

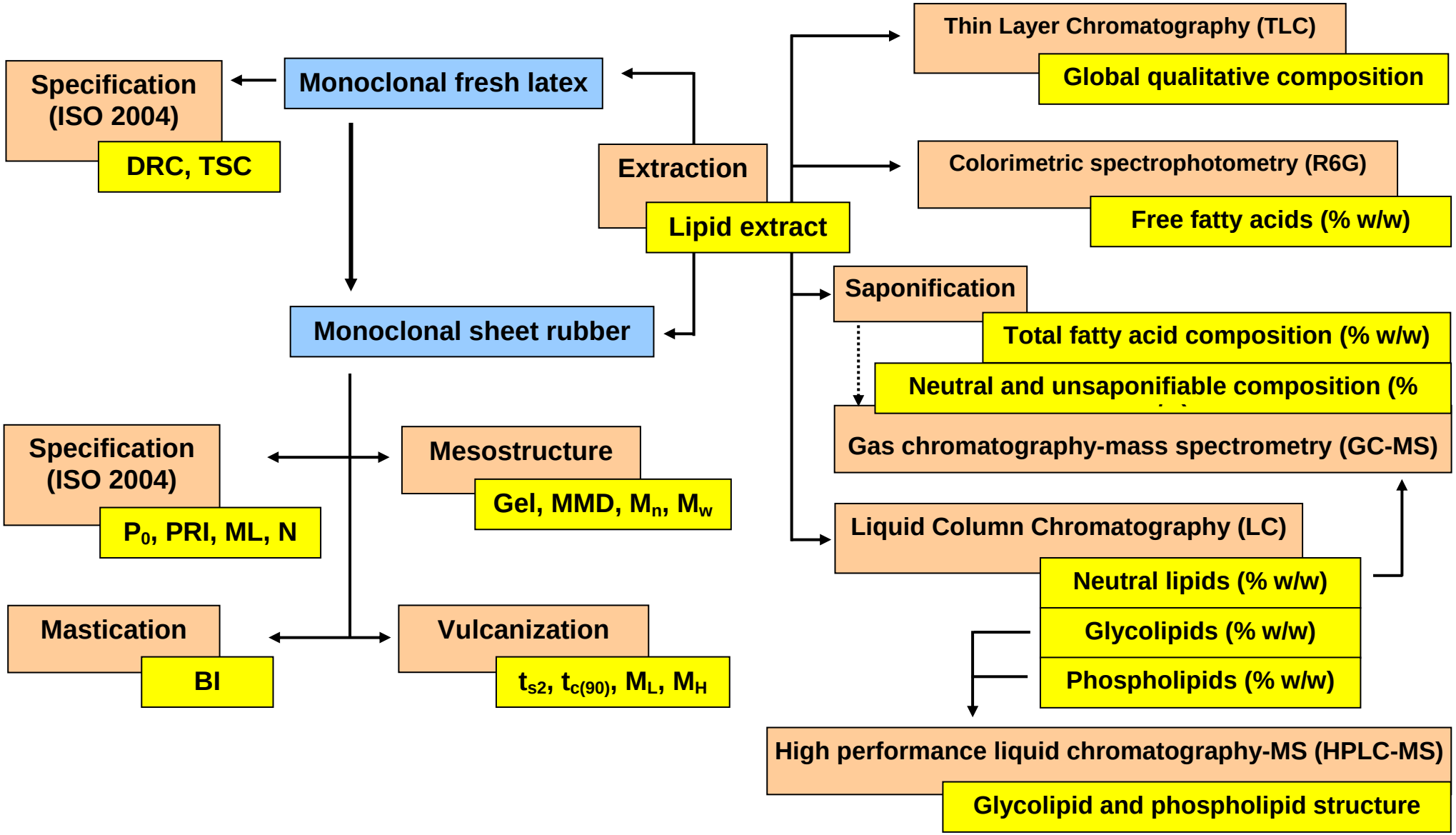


Figure 24 General analysis scheme of structure, properties and lipid composition of natural rubber

Table 6 Values of Breakdown Index characteristics measurement from each repetition of USS mastication under thermal and mechanical conditions.

conditions	repetition	cumulated energy (J)	average torque (kJ)	final torque (kJ)	average temperature (^{o}C)	final temperature (^{o}C)
Thermal conditions	1	32200	25.73	23.41	143.18	157
	2	32000	25.53	23.46	143.10	157
	3	32300	25.76	23.61	144.68	158
	Average	**32165**	**25.70**	**23.50**	**143.70**	**157**
	CV	**0.47%**	**0.49%**	**0.44%**	**0.62%**	**0.37%**
Mechanical conditions	1	87700	34.96	26.23	84.37	105
	2	91000	36.03	26.81	86.46	106
	3	84500	33.95	26.41	85.29	106
	Average	**87733**	**35.00**	**26.50**	**85.40**	**106**
	CV	**3.70%**	**2.97%**	**1.12%**	**1.23%**	**0.55%**

2 Methodology Development: new extraction procedure

This work involves a large number of analysis tools. Most of them have been developed previously and are described in standardized procedures. Concerning lipid extraction, it was necessary to develop further appropriate methods either for latex or sheet rubber as lipid extraction is the key step of quantitative and qualitative lipid analyses. Indeed, previous works have mentioned that the complex nature of natural rubber make the full extraction of non-isoprene difficult because of the highly obstructive nature of rubber. For instance, Hasma (1984) observed that lipids extracted from fresh latex contained polyisoprene while neutral lipids were partly trapped in rubber coagulum. A recent study of cut dry rubber (TSR 3CV) extraction was carried out under high temperature (Soxhlet) and solvent immersion at room temperature. The results showed that lipid extraction at room temperature with chloroform/methanol (1:2 v/v) gives higher amount of glycolipids and phospholipids compared to Soxhlet (Bonfils *et. al*, 2007). In the case of our study, extraction methods were improved in order to optimize the extraction of all classes of lipids from dry sheet rubber as well as fresh latex.

2.1 Optimization of lipid extraction from dry rubber

2.1.1 Study on the effect of extracting solvent and grinding method

The procedure used was that described in materials and methods section, except that the first extracting solvent was either chloroform/methanol (1:2 v/v or 2:1 v/v) or n-hexane/isopropanol (1:2 v/v or 2:1 v/v). The extraction yields obtained for dry rubber are presented in table 7. Among the solvents used, chloroform/methanol 2:1 (v/v) gave the highest lipid extract yield (1.67% vs dry rubber).

Although hexane-containing solvents were the second best in terms of yield, they were found by SEC analysis to extract a high portion of polyisoprene (~15% w/w lipid extract), which interferes with the isolation of lipids. The low value of standard deviation in each case indicated the good repeatability of the extraction procedure. (All relative standard deviations (r.s.d.%(r)) were below 5% but for choloroform/methanol 2:1 (r.s.d.%(r)=9%)). The results show that the higher the ratio of alcohol in the solvent mixture, the less the extraction yield. It was probably because the high polarity of alcohol may lead to a low extraction of neutral lipids.

Table 7 Yield of lipid extraction from non-ground rubber using 4 extracting solvent mixtures

Solvent system	Extraction yield (% /dry rubber)	SD
n-Hexane/Isopropanol (1:2, v/v)	1.42[b]	0.05
n-Hexane/Isopropanol (2:1, v/v)	1.61[a]	0.06
Chloroform/Methanol (1:2, v/v)	1.11[c]	0.04
Chloroform/Methanol (2:1, v/v)	1.67[a]	0.15

Values in column with a common letter are not significantly different
SD: Standard deviation

A comparison between the extraction yield obtained from ground and non-ground rubber extracted in chloroform/methanol (1:2 and 2:1 v/v) and hexane/isopropanol (1:2 and 2:1 v/v) is presented in figure 25. Extraction yield from ground rubber was significantly higher (by 8 to 17 %) than from non-ground rubber in every solvent system. The increase of exchange surface between rubber and solvent obtained by grinding may explain this difference. As mentioned before, hexane-

containing solvent also dissolved polyisoprene. Therefore, chloroform/methanol (1:2 and 2:1 v/v) mixtures were chosen for the following kinetic study.

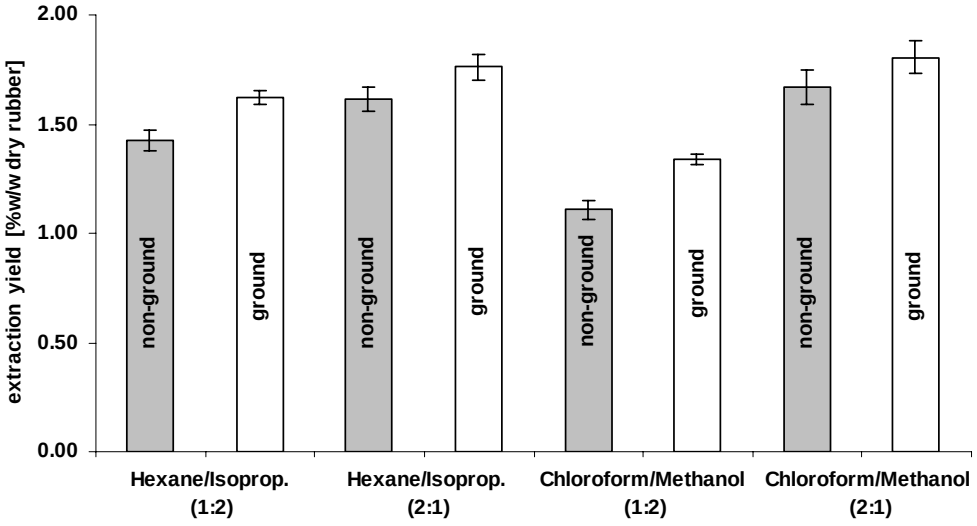


Figure 25 Lipid extraction yield from ground (□) and non-ground (■) RRIM600 unsmoked rubber sheet using 4 extracting solvents.

2.1.2 Study on the effect of extraction duration

For the optimization of the extraction procedure, the evolution of the lipid extraction yield was studied after various incubation times of ground and non-ground rubber in the solvent mixture (chloroform/methanol 1:2 or 2:1, v/v). Figure 26 shows that the lipid extraction yield increased with time from both ground and non-ground rubber samples until reaching a plateau after 6h. Ground rubber was extracted faster than non-ground rubber especially with chloroform/methanol (2:1; v/v). Optimal extraction yield was 1.8 % (w/w vs dry rubber).

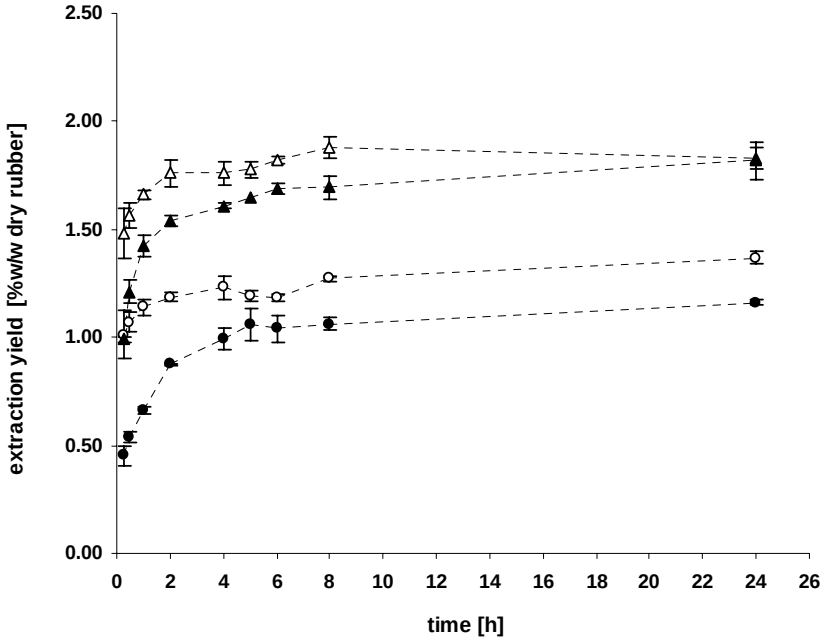


Figure 26 Comparison between lipid extract obtained from non ground rubber extracted with chloroform/methanol 1:2 (●) and 2:1 (▲) and ground rubber extracted with chloroform/methanol 1:2 (○) and 2:1 (△).

2.1.3 Optimal dry rubber extraction conditions

Considering these results, optimal conditions for lipid extraction from dry rubber consist in the use of ground rubber in chloroform/methanol (2:1; v/v) at room temperature for 6 h. The full procedure is described in the materials and methods section.

2.2 Optimization of lipid extraction from fresh latex

2.2.1 Study on effect of dilution

Fresh latex was extracted with chloroform/methanol (2:1, v/v), following the method previously described by Hasma (1984). Extraction yield was approximately 2% after 15 min and did not significantly change during the next 24 h (figure 27). Extraction thus seems to be fast and ends quickly after latex is dropped into solvent. A short extraction time could then be used for lipid extraction from latex.

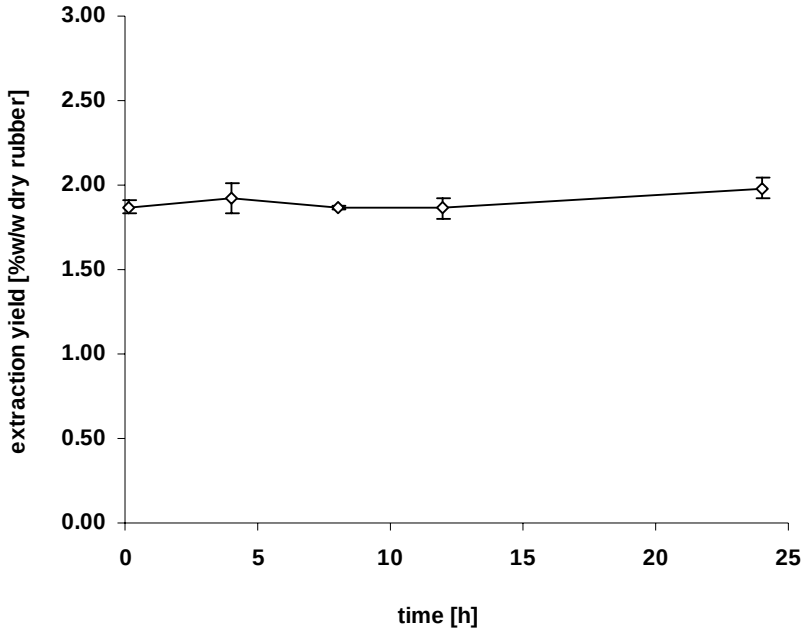


Figure 27 Influence of extraction duration on lipid extraction yield from fresh latex from RRIM600 clone

Nevertheless, lipid extraction from fresh field latex by dropping it in the extracting solvent was found to be incomplete. Indeed, more lipids (w/w dry rubber) were extracted from the dry rubber obtained from fresh latex than from the fresh latex itself. This had also been observed by Hasma (1984). This author assumed that it was due to the early coagulation of latex particles when dropping in the solvent, thus preventing the full extraction by entrapment of lipids in the coagulum.

As dilution is commonly used in polymer extraction, fresh latex was diluted 2, 2.5, 3 and 5 times of its volume with distilled water and gently mixed. Extraction of the diluted latex was then performed in the same conditions as mentioned in materials and methods section.

The extraction yield increased with the dilution factor (straight line in figure 28). Improvement of extraction efficiency by dilution may be due to the increase of the distance between rubber particles that result in a longer particle-

solvent exchange time before coagulation occurs. However, such conditions also facilitate the co-extraction of polyisoprene that became significant for dilutions higher than 2.5x dilution.

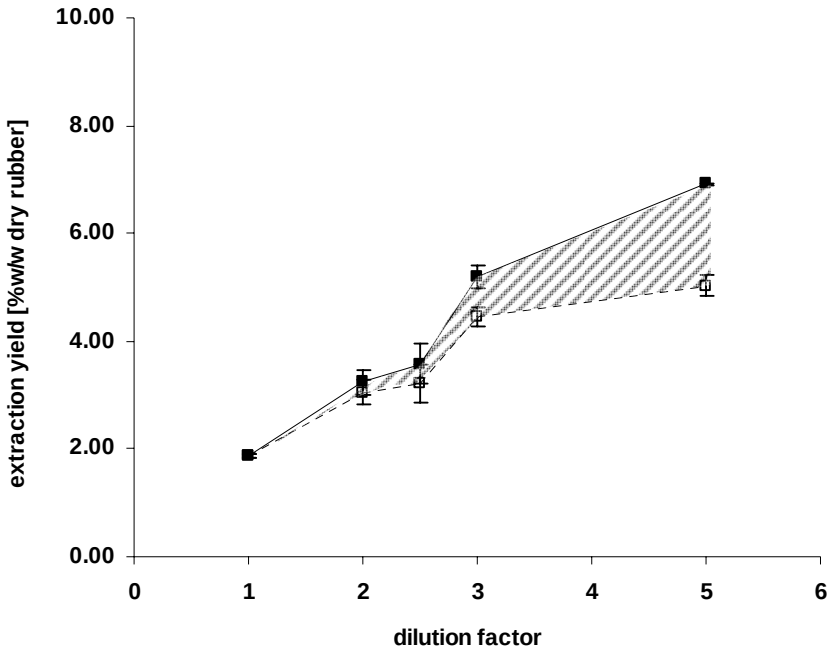


Figure 28 Influence of latex dilution rate on the total lipid extraction yield (–■–) and lipid extraction yield after deduction of contaminating polyisoprene (···□···). Shadow area indicates the level of contaminating polyisoprene. Latex was from RRIM600 *Hevea* clone.

2.2.2 Quantification of contaminating polyisoprene

In order to select the optimum dilution factor to obtain maximum lipid extraction yield with minimum polyisoprene, contaminating polyisoprene in lipid extract was determined by size exclusion chromatography (SEC). SEC chromatogram of lipid extract from 1x, 2x and 3x dilution of RRIM600 clones are presented in figure 29. The results confirmed that contaminating polyisoprene in the extract increases with dilution factor. Area of the peaks corresponding to lipid and polyisoprene can not be compared as these compounds do not have the same extinction coefficient at 220 nm.

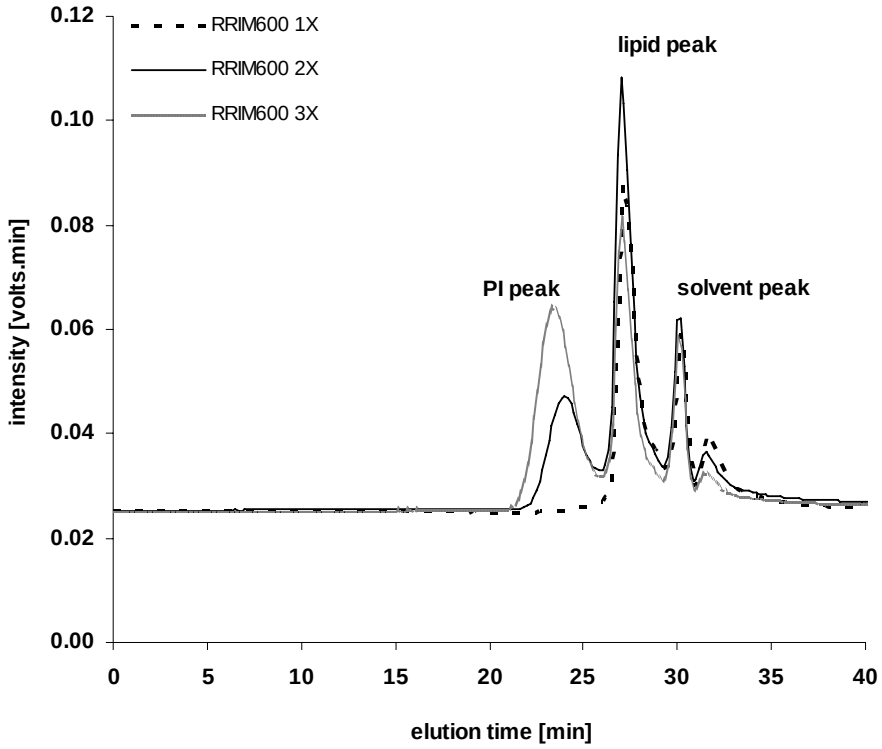


Figure 29 SEC chromatogram of total lipid extract from non-diluted (1x, - - -), 2x (—) and 3x diluted latex (⋯) from RRIM600.

Table 8 presents the lipid extraction yield and amount of contaminating polyisoprene in extracts from RRIM600 clone. The extraction yield from 2x dilution (3.24 %) exhibited around 42 % increase of extraction yield whereas 47 % increase is obtained from 2.5x dilution (3.59 %). However, the yields from 2x and 2.5x dilutions were not significantly different while contaminating polyisoprene double. The higher dilution factor (3x and 5x) also expressed similar phenomenon with even larger increase of polyisoprene level (more than 15% of the extract). Therefore, 2x dilution was the most suitable compromise between high lipid extraction yield and low level of contaminating polyisoprene.

Table 8 Extraction yield and % contaminating polyisoprene for various latex dilution rates

Dilution (times)	Total extract (% /dry rubber)		Polyisoprene in total extract (% /total extract)	
	Mean	SD	Mean	SD
1.0	2.02[d]	0.05	0.56[e]	-
2.0	3.24[c]	0.23	5.98[d]	0.50
2.5	3.59[c]	0.36	10.2[c]	1.17
3.0	5.20[b]	0.22	14.3[b]	0.40
5.0	7.39[a]	0.82	27.2[a]	3.06

Values in column with a common letter are not significantly different

SD: standard deviation

Latex was from RRIM600 *Hevea* clone.

2.2.3 Analysis of lipid classes

Liquid chromatography analysis of lipid classes has been performed to assess if this improvement of lipid extraction equally affects neutral lipids, glycolipids and phospholipids. Results are given in table 9 for each lipid class on a dry rubber basis for the extraction of non-diluted or 2 times diluted latex. The results indicated that latex dilution improves both neutral lipids (by a 2.5 factor) and glycolipids (by 1.4) extraction yield, whereas phospholipids extraction was not significantly changed.

Table 9 Lipid extract by classes after extraction from diluted or non-diluted latex from RRIM600 clone

Sample	Lipid classes	(%/dry rubber)	SD
Non-diluted latex	Neutral lipids	0.59[c]	0.07
	Glycolipids	0.62[c]	0.14
	Phospholipids	0.80[bc]	0.07
Diluted latex (2 times)	Neutral lipids	1.47[a]	0.24
	Glycolipids	0.88[b]	0.18
	Phospholipids	0.89[b]	0.08

Values in column with a common letter are not significantly different

SD: standard deviation

2.2.4 Optimal latex extraction conditions

These results are consistent with the hypothesis of lipid entrapment during non-diluted latex extraction proposed by Hasma (1984). Our results show that by lowering the rate of latex coagulation in the solvent and by changing the texture of the coagulum, dilution seems to favor a better exposure of lipids, especially neutral lipids, to the extracting solvent. Considering the results obtained, optimal conditions for lipid extraction from latex consist in the use of two times diluted latex dropped in chloroform/methanol (2:1; v/v). The full procedure is described in the materials and methods section.

2.3 Conclusion

Lipid extraction from natural rubber was carried out with various combinations of organic solvent in order to obtain both polar and non-polar lipids. Chloroform/methanol (2:1; v/v) was found to be suitable for lipid extraction from unsmoked sheet rubber. Though hexane containing solvents gave higher yield, they also dissolved rubber molecules. The improvement of lipid extraction by increasing the exchange surfaces was performed in dry rubber extraction by grinding under liquid nitrogen. The optimal extraction time was found to be 6 hours at room temperature.

For latex extraction, extracting solvent was chosen according to the result from dry rubber extraction optimization. Latex extraction yield was not found to increase with time. The problem of lipid entrapment in coagulum from immediate coagulation of latex in solvent could be solved by dilution of latex. Increasing the distance between rubber molecules by a two times dilution profited to the extraction efficiency. It gave an improved extraction yield with a minimum quantity of contaminating polyisoprene. Concerning the nature of lipids, we showed that dilution increases mainly neutral lipids extraction, which may suggest that neutral lipids are entrapped by coagulation.

Though this study was carried out using RRIM600 clone, similar studies performed in our laboratory with other clones such as PB235, GT1 or BPM24 confirmed the reported optimal extraction conditions (data not shown).

These optimized extraction methods are proposed in order to approach the genuine quantitative and qualitative data of lipids in natural rubber and be able to conduct further analysis on the effect of these important non-isoprene components to the properties of NR. They have been used for the characterization of samples in the database constituted in our study.

Chapter 2 Lipid composition of natural rubber

1. Lipid extracts

1.1 Lipids from fresh latex

The extraction of lipids from fresh latex of different *H. brasiliensis* clones (RRIM600 old or young, GT1, PB235 and BPM24) using a chloroform/methanol (2:1; v/v) solvent mixture was performed directly in the field following the optimized extraction method previously described. Lipid contents from fresh latex and unsmoked sheet (USS) are presented in table 10. Lipid content of fresh latex varied with clones as well as age of tree in case of RRIM600 clone. PB235 and BPM24 latices contained higher lipid quantity than the other clones. The highest lipid content was found in PB235 clone (3.67% w/w dry rubber) while the lowest lipid content was from RRIM600 (old) clone (2.47% w/w dry rubber).

Table 10 Lipid extraction (%w/w dry rubber) of fresh latex and unsmoked sheet

Clones	lipid extract (% w/w dry rubber)			
	Fresh latex *	SE	Unsmoked sheet**	SE
RRIM600 (old)	2.47^{c}	0.27	1.99^{e}	0.03
RRIM600 (young)	3.36^{ab}	0.13	2.31^{c}	0.03
GT1	2.80^{bc}	0.27	2.15^{d}	0.03
PB235	3.67^{a}	0.11	3.28^{a}	0.03
BPM24	3.36^{a}	0.11	2.67^{b}	0.03

* Mean of 6 samplings; 3 repetitions was performed for each sampling except RRIM600 (old) and GT1 clone (mean from 3 repetitions of 1 sampling)

**Mean of 15 samplings; 3 repetitions was performed for each sampling

For each column, letters design group of mean(s) which are not significantly different (student *t* test)

SE: standard error

1.2 Lipids from unsmoked sheet (USS)

Lipids from unsmoked sheet (USS) prepared from monoclonal latex was in the range of 2.0 - 3.3% (w/w dry rubber) depending on rubber clone and age of tree. Lipid contents of samples were all significantly different from each other. PB235 clone gave the highest lipid content while RRIM600 (old) clone contained the least lipid content.

Lipid content in dry rubber was found to be lower (1.9 – 3.3%) compared to fresh latex but the obtained ranking was the same: PB235, BPM24, RRIM600 (young), GT1 and RRIM600 (old) (from higher to lower). The unequal quantity between latex and USS is due to the loss of lipids during rubber sheet processing, especially of polar lipids that are leached by water and/or hydrolyzed. The *H. brasiliensis* clones studied in this work were categorized into two groups according to their lipid content. PB235, BPM24 and RRIM600 (young) clones were the high lipid group (3.4 – 3.7% of dry rubber content) whereas RRIM600 (old) and GT1 clone were low lipid group (2.5 – 2.8%).

2. Lipid composition

Lipid extracts obtained from both fresh latex and USS samples were further separated by liquid column chromatography (LC) into three main classes: neutral lipids, glycolipids and phospholipids. Each clone was further analyzed by liquid or gas chromatography eventually coupled to mass spectrometer (MS). The results will be presented by lipid fractions as follows.

- Neutral lipids
  - Global neutral lipid composition (TLC)
  - Free fatty acid quantity (Spectrophotometry)
  - Total fatty acid composition (after saponification; GC-FID)
  - Unsaponifiable composition (GC-MS)
- Glycolipids
  - Global glycolipid composition (TLC)
  - Fatty acid composition of glycolipid fraction (GC-FID)

- Sterol composition of glycolipid fraction (GC-MS)
- Study of molecular species of glycolipids (HPLC-MS)

▪ Phospholipids
- Global phospholipid composition
- Fatty acid composition of phospholipids fraction (GC-FID)
- Study of molecular species of phospholipids (HPLC-MS)

2.1 Lipid classes

Lipid classes from fresh latex and USS were characterized by silica gel column liquid chromatography (LC). Lipids were separated into neutral lipids, glycolipids and phospholipids by successive elutions with chloroform, acetone/ methanol (9:1, v/v) and methanol, respectively (Rouser *et. al*, 1967). The amount of each lipid class versus dry rubber weight from fresh latex is presented in table 11 and relative proportion within each lipid extract is presented in figure 30.

Table 11 Lipid classes of lipid extracts from latices (% w/w dry rubber)

Clones	Neutral lipids		Glycolipids		Phospholipids	
	%	SE	%	SE	%	SE
RRIM600 (old)	1.01[c]	0.08	0.82[b]	0.73	0.64[a]	0.12
RRIM600 (young)	1.72[b]	0.11	0.88[b]	0.03	0.90[a]	0.08
GT1	1.10[c]	0.11	1.01[a]	0.04	0.70[a]	0.12
PB235	2.40[a]	0.08	0.84[b]	0.03	0.72[a]	0.08
BPM24	1.88[b]	0.08	0.81[b]	0.03	0.80[a]	0.08

Mean of 6 samplings; 3 repetitions was performed for each sampling except RRIM600 (old) and GT1 (mean from 3 repetitions of 1 sampling)

For each column, letters design group of mean(s) which are not significantly different (student *t* test)

SE: standard error

The results show that neutral lipids were the major lipid class for every studied clone. For RRIM600 (old) and GT1 clones, neutral lipid content was found to be significantly lower than for the other clones (~1% /dry rubber weight and 40% of lipid extract). The neutral lipid content of samples from PB235, BPM24 and RRIM600 (young) clones was 2.40%, 1.88% and 1.72%, respectively, accounting for more than 50% of total lipid weight.

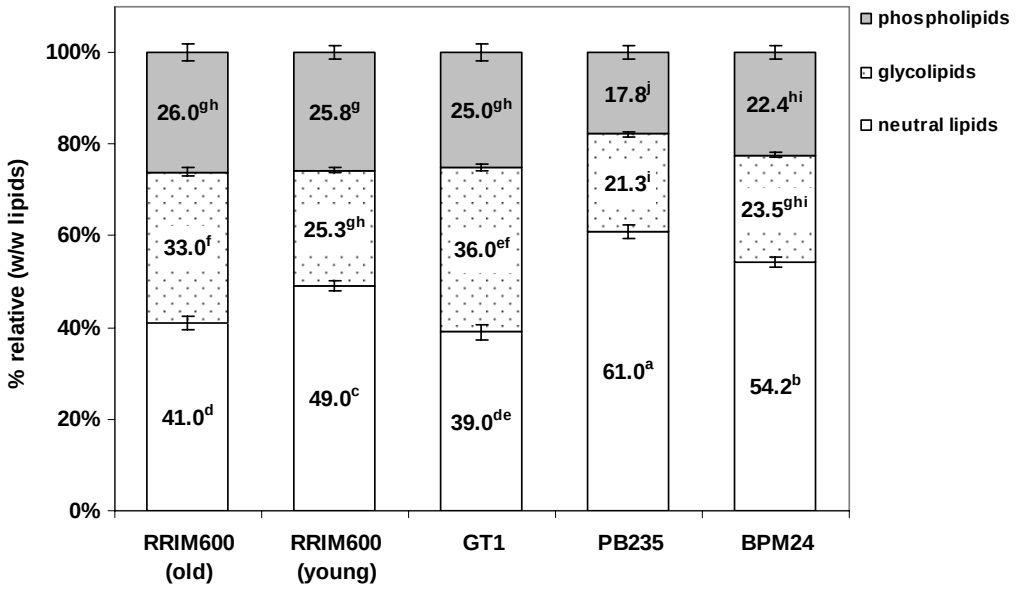


Figure 30 Relative weight, by class, of lipid extracted from latices

Glycolipids were found in the range of 0.8 – 1% (w/w dry rubber) or 21-36% of total lipids. GT1 clone displayed the highest glycolipid content, very close to that of neutral lipids. Phospholipids were present in the range of 0.6 – 0.8%, which accounted for 18% to 29% of total lipids. Phospholipid content was not significantly different among clones.

Lipid classes obtained from rubber sheet extraction are presented in table 12 and their relative proportions are shown in figure 31. A typical rubber sheet contains around 81-86% neutral lipids, 10-14% glycolipids and 4-5% phospholipids. Few differences were observed among clones.

Table 12 Lipid classes of lipid extract from USS (% w/w dry rubber)

Clones	Neutral lipids		Glycolipids		Phospholipids	
	%	SE	%	SE	%	SE
RRIM600 (old)	1.62^{e}	0.08	0.27^{b}	0.73	0.09^{b}	0.01
RRIM600 (young)	1.93^{c}	0.11	0.26^{b}	0.03	0.09^{b}	0.01
GT1	1.82^{d}	0.11	0.26^{b}	0.04	0.09^{b}	0.01
PB235	2.89^{a}	0.08	0.34^{a}	0.03	0.11^{b}	0.01
BPM24	2.18^{b}	0.08	0.34^{a}	0.03	0.15^{a}	0.02

Mean of 10 samplings; 3 repetitions was performed for each sampling

For each column, letters design group of mean(s) which are not significantly different (student *t* test)

SE: standard error

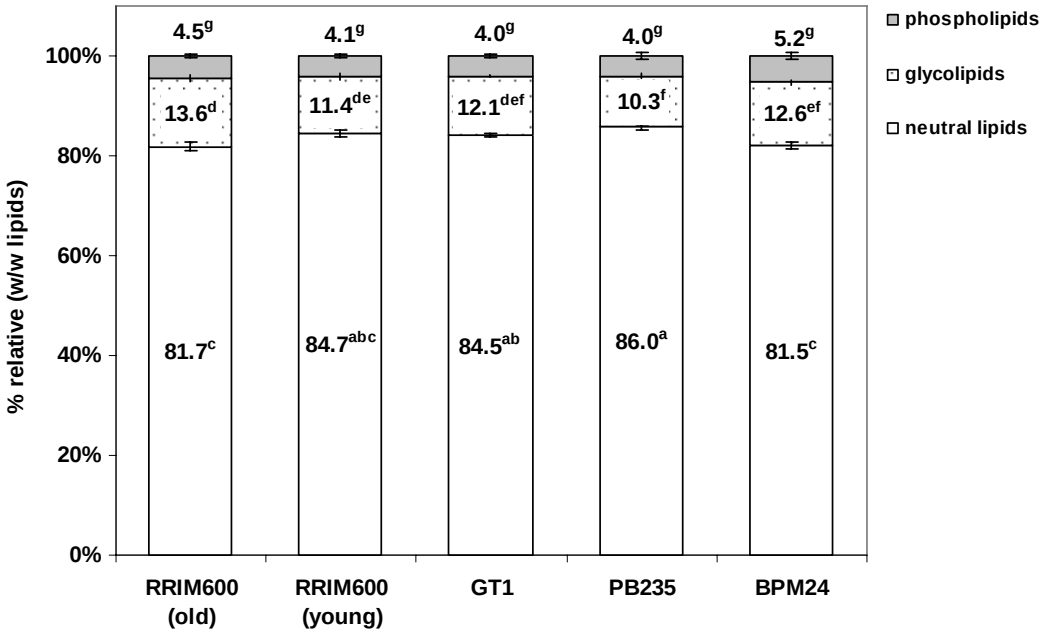


Figure 31 Relative weight, by class, of lipid extracted from USS.

As mentioned earlier, the lower lipid content in USS than in latex (versus dry rubber) was attributed to the loss during rubber sheet processing by water leaching and/or hydrolysis. Figures 30 and 31 confirm that this loss concerns mainly polar lipids, i.e glycolipids and phosopholipid, as neutral lipid constitute more than 80% of the USS lipids. When comparing absolute quantities of each lipid class between fresh latex and USS (table 11 and 12), an increase of neutral lipids and decrease of polar lipids i.e. glycolipids and phospholipids were found. This could indicate that the loss of lipid is more due to hydrolysis phenomena than to only leaching. Indeed, hydrolysis of polar lipids results in the release of diglycerides and free fatty acids, which are mainly found in the neutral lipid fraction. A loss by leaching alone would not have increased the neutral lipid quantity.

2.2 Neutral lipid fraction

2.2.1 Neutral lipid composition

Neutral lipids of lipid extracted from fresh latex and USS were separated using thin layer chromatography (TLC) with a suitable mobile phase.

Figure 32 shows a TLC plate for latex and USS lipids as well as the unsaponifiable fraction from the studied clones compared with commercial standards. The observed spots were identified as sterols, free fatty acids, α-tocotrienol, γ-tocotrienol, triacylglycerols, esterified sterols and fatty acid methyl esters in every clone. The spots of triacylglycerol containing furan fatty acid (10, 13-epoxy-11-methyl-octadecan-10, 12-dienoic acid; FFA) of fresh latex and USS lipids were more important for PB235 compared to the other clones. The structures of the triacylglycerol and free form of this furan fatty acid were confirmed by isolation of furan fatty acid by preparative TLC followed by gas chromatography-mass spectrometry (GC-MS) analysis. The mass spectrum of the isolated FFA is presented in figure 33. Comparison between fresh latex and USS samples showed a clear difference in free fatty acid content, which was more important in USS lipids. The Rf of free furan fatty acid is slightly lower than that of other fatty acids, probably due to the polar nature of the furan ring.

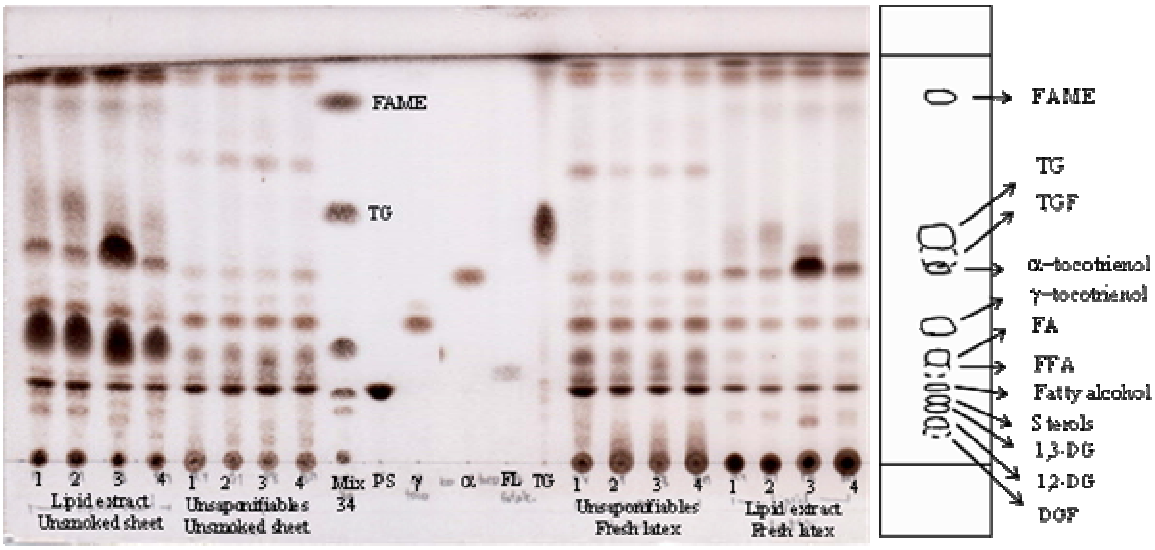


Figure 32 TLC of unsaponifable fraction and total lipid extract from fresh latex and USS. 1: RRIM600 (young); 2: GT1; 3: PB235; 4: BPM24 Developed in hexane/diethyl ether/ acetic acid (80:20:1, v/v/v) and detected by 40% orthophosphoric acid and aqueous solution saturated with copper acetate (1:1, v/v). FA; free fatty acids, FL; free fatty alcohol, DG; -diacylglycerol; TG; triacylglycerol; TGF; triacyl glycerol of furan fatty acid, DGF; diacylglycerol of furan fatty acid, FAME; fatty acid methyl ester, PS; plant sterol, SE; sterol ester, γ; γ-toco trienol and α; α-tocotrienol. All samples are from trip 15 (13 December 2006)

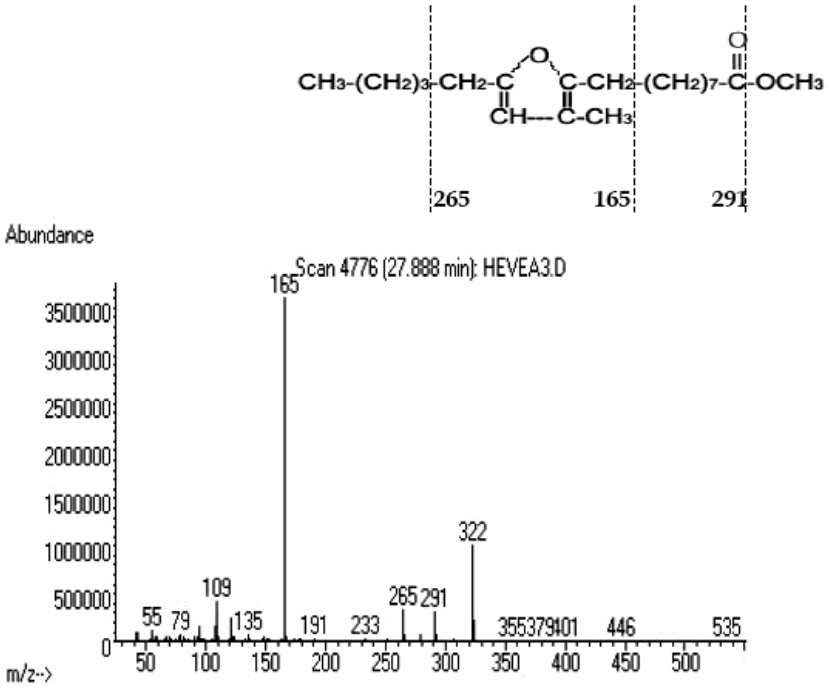


Figure 33 The mass spectrum of furan fatty acid methyl ester from GC-MS analysis

An unknown component (not corresponding to any applied standard) was found with a slightly lower Rf than 1,2-diacylglycerol. The intensity of this spot was more important for PB235. This unknown spot was found in samples from every collection trip. Moreover, it was observed on TLC plate that triacylglycerols of furan fatty acid, and FFA were more polar than that containing other fatty acids. It is therefore possible that this unknown spot is a diacylglycerol of furan fatty acid.

In addition to the main spots already observed on the TLC of total lipids (sterols, tocotrienols), the unsaponifiable fraction from latex and USS displayed a spot with a Rf corresponding to fatty alcohols (Rf between sterols and fatty acids). There was no obvious difference between unsaponifiable fractions from both types of samples. This lipid composition was similar to those of previously reported (Ho *et. al*, 1975 ; Hasma, 1984).

2.2.2 Fatty acids

2.2.2.1 Free fatty acids

Free fatty acids in lipid extracts from latex and rubber sheet were quantified by spectrophotometry using R6G. The results are presented in table 13. Lipid extract from fresh latex contained 1.3 - 2.2 % (w/w lipids) whereas around ten times higher amounts were detected from rubber sheet (18 - 22%). It confirms the assumption made after LC analysis (increase of neutral lipids absolute quantity due to free fatty acid released by hydrolysis) and the results observed from TLC plate (figure 32). The lowest free fatty acid content was from GT1 and BPM24 latex lipids whereas the other clones contained significantly higher amounts. Contrarily, USS lipids from BPM24 clone gave the highest free fatty acid content.

Table 13 Free fatty acids (%w/w lipid) of lipid extracts from latex and rubber sheet

Clones	Free fatty acids in latex*		Free fatty acids in USS	
	%	SE	%	SE
RRIM600 (old)	2.15^{a}	0.20	18.68^{bc}	0.95
RRIM600 (young)	2.08^{a}	0.09	20.63^{ab}	0.59
GT1	1.32^{b}	0.25	20.99^{ab}	0.78
PB235	2.09^{a}	0.11	18.25^{c}	0.79
BPM24	1.49^{b}	0.10	22.04^{a}	0.46

* Mean of 6 samplings; 3 repetitions was performed for each sampling except RRIM600 (old) and GT1 (mean of 3 repetitions from 1 sampling)

For each column, letters design group of mean(s) which are not significantly different (student *t* test)

SE: standard error

- ***Lipase activity in fresh latex***

To confirm that fatty acid release was due to an enzymatic hydrolysis activity, an experiment was set up to assess the presence of lipase/esterase activity in freshly tapped latex using β-napthyl caproate as substrate (see materials and methods 2.4) and following the release of β-naphthol which produces a pink color in the presence of Fast blue B.

A positive result was observed with freshly tapped latex from every clone as shown in figure 34. It was found that after 30 minutes of reaction, some differences in color intensity which represented the level of lipase activity could be observed. BPM24 clone showed slightly less lipase activity whereas in PB235 clone it was slightly higher. The lipase/esterase activities found in RRIM600 (old and young) and GT1 clones were similar. By comparing with known β-naphthol concentrations, the lipase activity in latices could be estimated to a range of 34 to 38 U/L (one U being the quantity of enzyme that catalyzes the release of 1 μmol of fatty acid per min in the conditions applied).

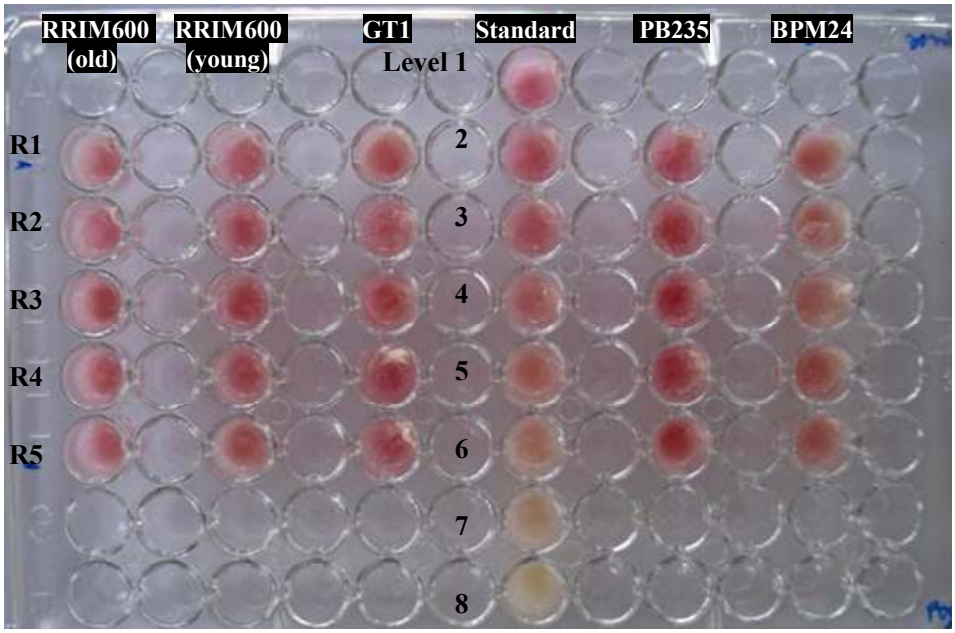


Figure 34 Lipase activity of freshly tapped *H. brasiliensis* latex from different clones after 30 min of reaction. Standard concentration level 1-8 ranged from 0.067, 0.058, 0.048, 0.038, 0.029, 0.019, 0.01 and 0 mg/ml of β-naphthol added to latex. β-napthyl caproate (substrate) concentration was 0.8 mg/ml in 5% acetone containing Tris HCl buffer pH7. Fast blue BB salt 1 mg/ml of ethanol was used as indicator and reaction stopper. R1-5 represented 5 repetitions.

2.2.2.2 Total fatty acid composition

Lipid extracts from fresh latex and sheet rubber were analyzed for their total fatty acid composition using gas chromatography (GC). Total fatty acids obtained after saponification were derivatized as fatty acid methyl esters (FAME) for analysis. Relative fatty acid composition from fresh latex and sheet rubber lipids are presented in table 14 and 15, respectively. For fresh latex, the major fatty acids found in RRIM600, GT1 and BPM24 were linoleic acid (C18:2), stearic acid (C18:0) and oleic acid (C18:1). Depending on clone, those fatty acids ranged from 37-53%, 13-14% and 10-15%, respectively. PB235 showed a different fatty acid profile from the other clones by its higher FFA proportion (74%) and lower linoleic acid (11%) whereas a smaller amount of FFA was found in RRIM600 (7% and 17% for old and young samples, respectively), GT1 (12%) and BPM24 (27%). In plant, it has been proposed that linoleic acid is the precursor of carbon skeleton of furan fatty acid having a pentyl side chain by generation of a hydroperoxide group in position 13. It has been reported that in sugarcane, furan fatty acid constitute about 7% of linoleic acid (Spiteller, 2005). Compared to sugarcane, FFA in *H. brasiliensis* was found to amount to 13-26% of linoleic acid in RRIM600 (old and young) and GT1 clones, and FFA from BPM24 latex was almost 80% of its linoleic acid content. For PB235 clone, FFA was found to contain around 6 times more than linoleic acid. For total fatty acid composition of lipid extract from sheet rubber, similar results were found with slightly higher furan fatty acid content in every clone due to the increased importance of neutral lipid class.

2.2.3 Unsaponifiable composition

2.2.3.1 Qualitative analysis

Analysis of the unsaponifiable fraction obtained after saponification of lipid extracts was performed using gas chromatography-mass spectrometry (GC-MS) after silylation. Identification of compounds was based on the comparison of both their retention time and their mass spectrum with that of commercial standards.

Table 14 Fatty acid composition (%w/w total fatty acids) of lipid extract from fresh latex

clones	C14:0		C16:0		C16:1		C18:0		C18:1		C18:2		C18:3		Furanoic acid		C20:0	
	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE
RRIM600 (old)	0.31^{b}	0.03	7.73^{a}	0.44	0.58^{b}	0.16	14.07^{a}	0.95	13.52^{ab}	1.74	53.21^{a}	2.15	2.34^{b}	0.21	7.44^{c}	4.58	0.81^{b}	0.09
RRIM600 (young)	0.29^{b}	0.02	7.36^{a}	0.19	0.42^{b}	0.09	13.51^{a}	0.41	10.41^{b}	0.75	47.38^{b}	0.93	3.01^{a}	0.09	17.00^{c}	1.98	0.86^{b}	0.04
GT1	0.41^{a}	0.03	7.51^{a}	0.43	0.43^{b}	0.16	13.42^{a}	0.95	15.76^{a}	1.74	46.99^{b}	2.15	1.64^{c}	0.21	12.84^{c}	4.58	1.00^{ab}	0.09
PB235	0.25^{b}	0.02	3.33^{c}	0.18	1.34^{a}	0.07	5.38^{b}	0.40	10.41^{b}	0.73	11.47^{d}	0.90	1.04^{d}	0.09	73.94^{a}	1.92	0.51^{c}	0.04
BPM24	0.42^{a}	0.02	5.91^{b}	0.18	0.44^{b}	0.09	13.16^{a}	0.40	12.17^{ab}	0.73	37.31^{c}	0.90	2.07^{bc}	0.09	27.75^{b}	1.92	1.08^{a}	0.04

Mean of 6 samplings; 3 repetitions was performed for each sampling except RRIM600 (old) and GT1 (mean of 3 repetitions from 1 sampling)

For each column, letters design groups of means that are not significantly different (student *t* test, $P \leq 0.05$)

SE: standard error

Table 15 Fatty acid composition (%w/w total fatty acids) of lipid extract from USS

clones	C14:0		C16:0		C16:1		C18:0		C18:1		C18:2		C18:3		Furanoic acid		C20:0	
	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE
RRIM600 (old)	0.29^{bc}	0.03	6.53^{a}	0.40	0.51^{ab}	0.04	19.56^{a}	3.84	13.00^{ab}	0.82	40.92^{ab}	3.76	1.95^{b}	0.14	15.78^{c}	3.34	1.45^{ab}	0.17
RRIM600 (young)	0.27^{c}	0.01	6.97^{a}	0.16	0.41^{b}	0.01	14.19^{b}	0.49	11.49^{b}	0.55	44.41^{a}	1.20	2.58^{a}	0.11	18.09^{c}	1.45	1.27^{b}	0.10
GT1	0.39^{a}	0.03	6.42^{a}	0.34	0.44^{b}	0.02	15.22^{b}	0.91	14.72^{a}	0.52	37.34^{bc}	2.61	1.45^{c}	0.05	22.89^{c}	3.10	1.58^{ab}	0.16
PB235	0.16^{d}	0.00	2.37^{c}	0.12	0.72^{a}	0.06	4.37^{c}	0.22	2.44^{c}	0.11	7.88^{d}	0.44	0.58^{d}	0.05	81.17^{a}	0.92	0.51^{c}	0.02
BPM24	0.34^{ab}	0.01	5.72^{b}	0.15	0.40^{b}	0.03	14.72^{b}	0.64	11.81^{ab}	0.73	32.97^{c}	1.17	1.69^{bc}	0.06	30.31^{b}	2.09	1.61^{a}	0.13

Mean of 15 samplings; 3 repetitions was performed for each sampling

For each column, letters design groups of means that are not significantly different (student *t* test, $P \leq 0.05$)

SE: standard error

Total ion count (TIC) chromatograms of unsaponifiables from fresh latex and sheet rubber lipid extracts from PB235 clone are presented in figure 35. The same components were found in both extracts. TIC chromatograms from the other clones were qualitatively similar. Components eluted in the following order: stearyl alcohol (octadecanol; RT = 4.98 min), arachidyl alcohol (eicosanol; RT = 7.00 min), γ-tocotrienol (RT = 17.88 min), α-tocotrienol (RT = 20.04 min), stigmasterol (RT = 20.41 min), β-sitosterol (RT = 21.42 min), Δ-5 avenasterol (RT = 21.68 min). Identification of these components is discussed below.

The identification of the two detected fatty alcohols from *H. brasiliensis* was confirmed by the mass spectra of their TMS derivatives, presented in figure 36a and 36b. It is to be reminded that these fatty alcohols could have been esterified to fatty acid (waxes) before saponification. The primary alcohols can usually be identified as TMS ethers by their $(M-15)^+$ fragment (Christie, 2003). The base peak of octadecanol (*m/z* 327) and eicosanol (*m/z* 355) shown in the mass spectra corresponded to those expected.

Mass spectra in figure 36c and 36d also confirmed the presence of γ- and α-tocotrienol in unsaponifiables of fresh latex and USS lipid extracts from every studied clone. Tocotrienol and tocopherol belong to the family group commonly called vitamin E, known for their antioxidant properties (Brigelius-Flohe´ and Traber, 1999). The mass spectrum of γ-tocotrienol displayed a mass fragment *m/z* 223 characteristic of the γ-substituted chromanol ring and the TMS fragment (*m/z* 73). In α-tocotrienol, the *m/z* 227 fragment corresponded to a cleaved α-substituted chroman ring (Birringer *et. al*, 2002).

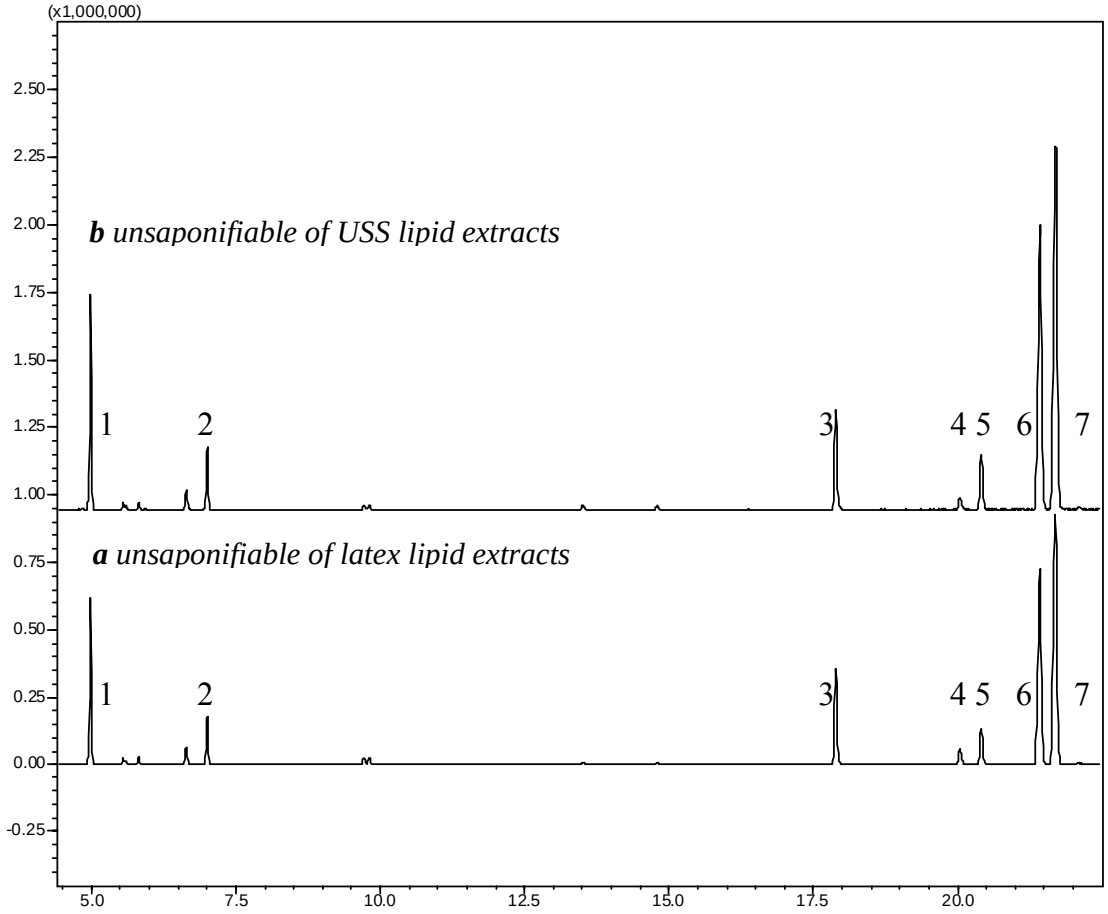


Figure 35 Total ion count (TIC) GC-MS chromatogram of silylated unsaponifiable fractions from total lipids from *H. brasiliensis* PB235 latex (a) and sheet rubber (b). Peaks: 1; octadecanol, 2; eicosanol, 3; γ-tocotrienol, 4; α-tocotrienol, 5; stigmasterol, 6; β-sitosterol and 7; Δ-5 avenasterol.

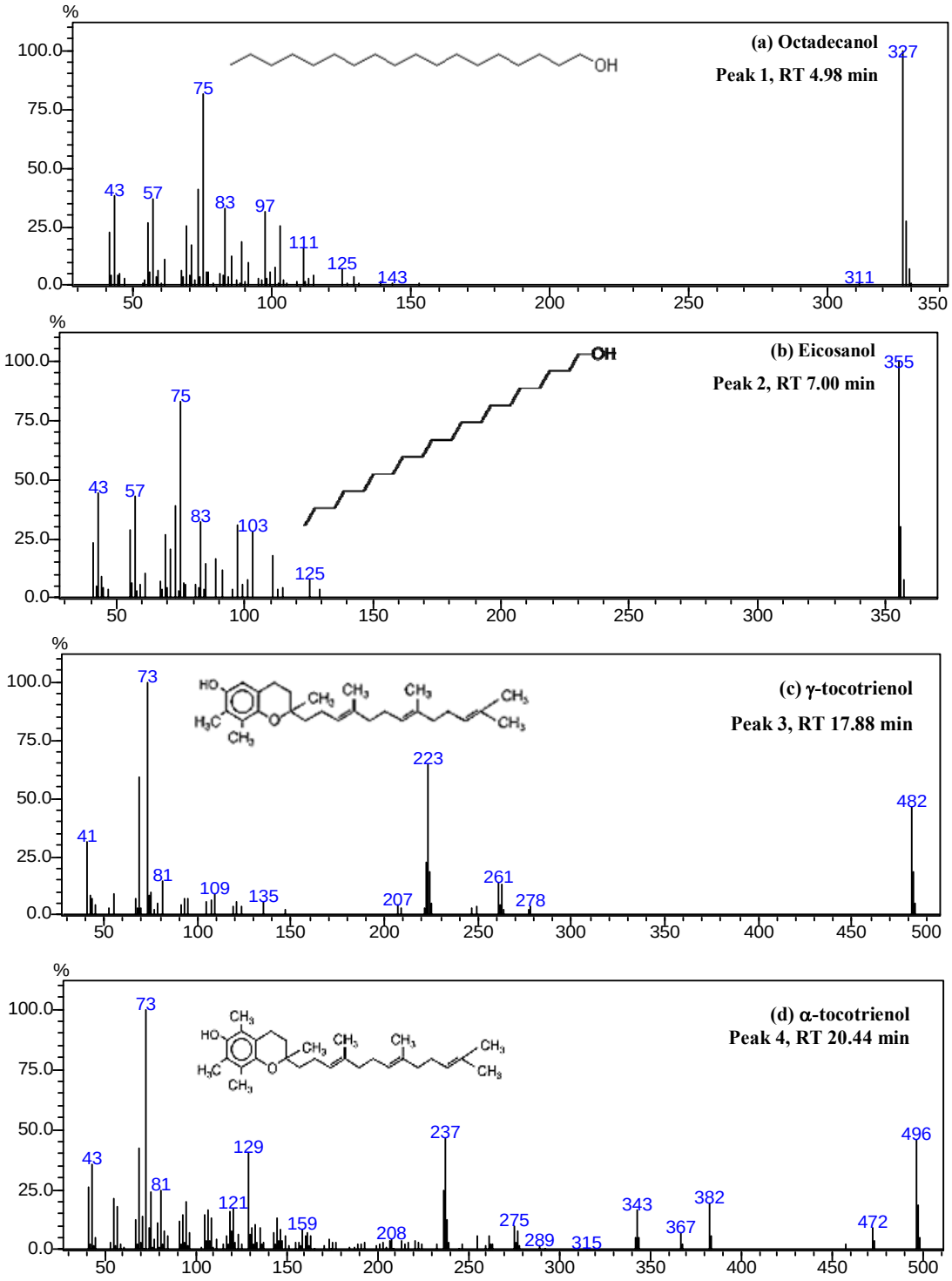


Figure 36 Mass spectra of the TMS ether derivatives of octadecanol (a), eicosanol (b), α-tocotrienol (c), and γ-tocotrienol (d) from *H. brasiliensis* latex. See figure 35 for chromatographic peak position.

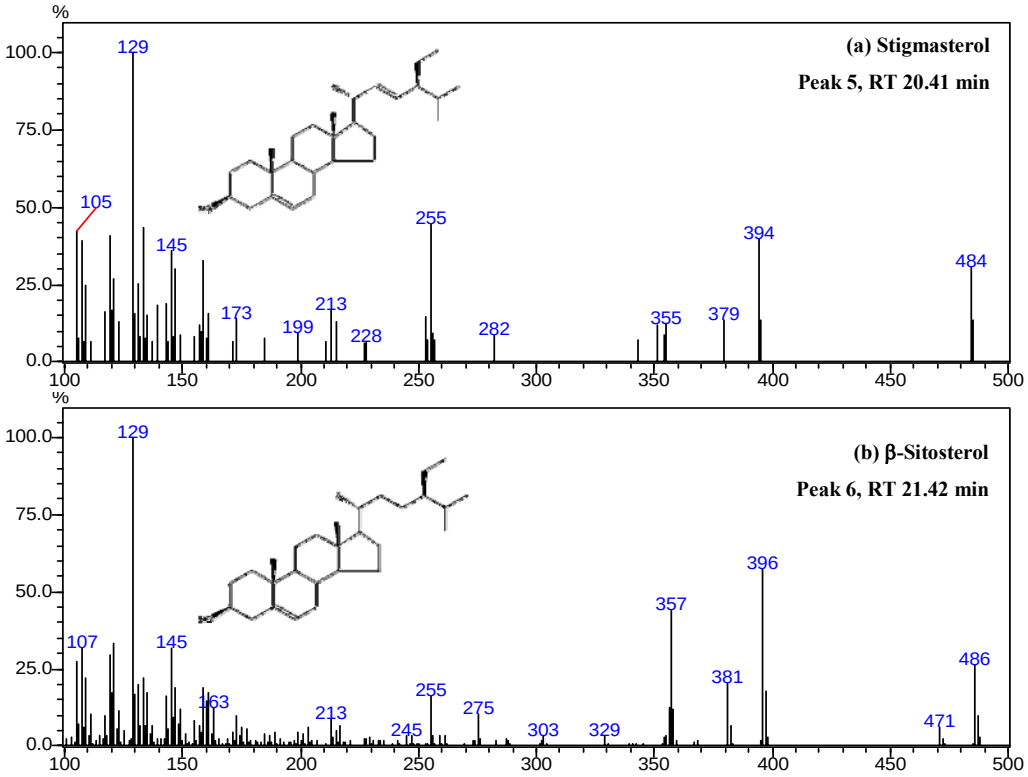


Figure 37 Mass spectra of the TMS ether derivatives of stigmasterol and β-sitosterol from *H. brasiliensis* RRIM600 (young) latex. See figure 35 for peak retention time.

Regarding sterols in unsaponifiable fraction, the presence of stigmasterol and β-sitosterol was confirmed by their mass spectra and retention times as presented in figure 37. The mass spectra are generally characterized by an important pair of ions, *m/z* 129 and its complement $(M-129)^+$, which has been described in several studies (Kamal-Eldin *et. al*, 1998 ; Määttä *et. al*, 1999 ; Pelillo *et. al*, 2003). Stigmasterol, with TMS molecular mass of *m/z* 484, and β-sitosterol with *m/z* 486, showed ion fragment pattern including related peaks such as $(M-15)^+$ and $(M-90)^+$.

The peak eluted after 21.68 min (figure 35) was previously reported by Hasma (1984) to be fucosterol. However, our comparative GC analysis of fucosterol standard and unsaponifiable fraction did not confirm this finding as the retention time of fucosterol (21.43 min) was significantly different (figure 38), while

the retention time of fucosterol was almost identical to that of β-sitosterol. Therefore, the identification of the sterol eluted at 21.68 min was carried out.

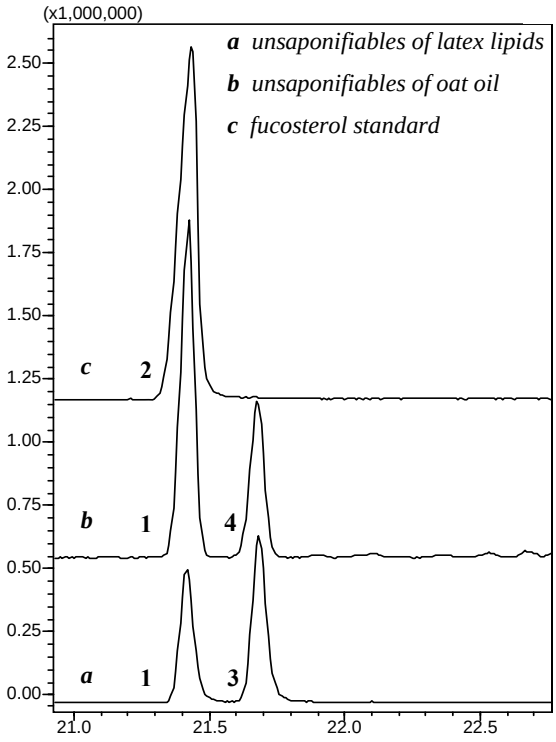


Figure 38 GC chromatogram of unsaponifiable from PB235 latex lipid extract (a), unsaponifiable from oat oil (b) and fucosterol standard (c). Peak 1: β-sitosterol; 2: fucosterol; 3and 4: isofucosterol (Δ-5 avenasterol).

By comparison with numerous works on sterol analysis (retention time and mass spectra), Δ-5 avenasterol, also called isofucosterol, was identified as a potential candidate due to its closely related structure to fucosterol. It is one of the important phytosterol found in oat (*Avena sativa*) and it is found at a significant level in other plant materials (Moreau *et. al*, 2002).

Unsaponifiable fraction of oat oil, kindly provided from Oat Oil Services, UK was used as standard since not commercial standard was available for Δ-5 avenasterol. Unsaponifiable from oat was analyzed in the same conditions as unsaponifiable from *H. brasiliensis* (figure 38). Peak identification for these

unsaponifiables was performed by comparison with the results of a previous work (Määttä *et. al*, 1999).

The TIC chromatograms of fucosterol standard, *H. brasiliensis* latex and USS unsaponifiable and a mixture of various plant sterols are presented in figure 39. Mass spectra of fucosterol, Δ-5 avenasterol from unsaponifiables of oat oil and the unknown peak from the samples were compared (figure 40).

Mass spectra of fucosterol and Δ-5 avenasterol both displayed the peak at *m/z* 386 (M-98) due to MacLafferty rearrangement and the peak at *m/z* 296 corresponding to (M-98-Trimethylsilanol) (Määttä *et. al*, 1999). These mass spectra of these two sterols were almost identical as their chemical structures differ only by the conformation of a double bond at C24. This mass spectra similarity may explain the erroneous identification by Hasma (1984). Contrarily to fucosterol, the retention time of Δ-5 avenasterol was exactly 21.68 min. Identity of mass spectra as well as of retention time allowed to conclude that peak 7 at RT 21.68 min is Δ-5 avenasterol, also named isofucosterol.

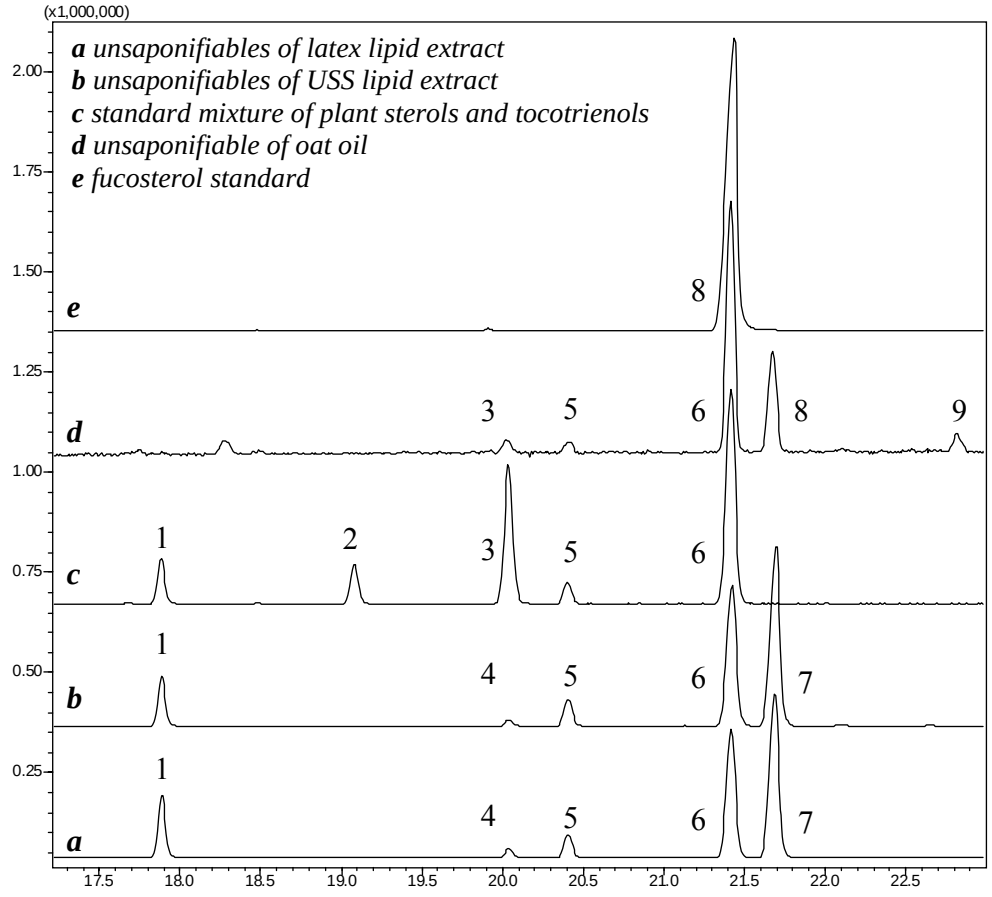


Figure 39 Total ion count GC-MS chromatogram of silylated sterols and tocotrienols from total lipids of *H. brasiliensis* PB235 latex (a) and USS (b) compared to that of standard mixtures (plant sterol mixture and tocotrienols) (c) unsaponifiable fraction from *A. sativa* oil (d) and fucosterol standard (e). Identification of each component was confirmed on the basis of both retention time and mass spectrum using pure standards, except for peak 7 for which no pure standard was available. Peaks: 1, γ-tocotrienol (RT = 17.88 min); 2, brassicasterol (RT = 19.11 min); 3, campesterol + α-tocotrienol (RT = 20.02 min); 4, α-tocotrienol (RT = 20.04 min); 5, stigmasterol (RT = 20.41 min); 6, β-sitosterol (RT = 21.42 min); 7, Δ-5 avenasterol (RT = 21.68 min); 8, fucosterol (RT = 21.43 min); 9, Δ-7 avenasterol (RT = 22.82 min).

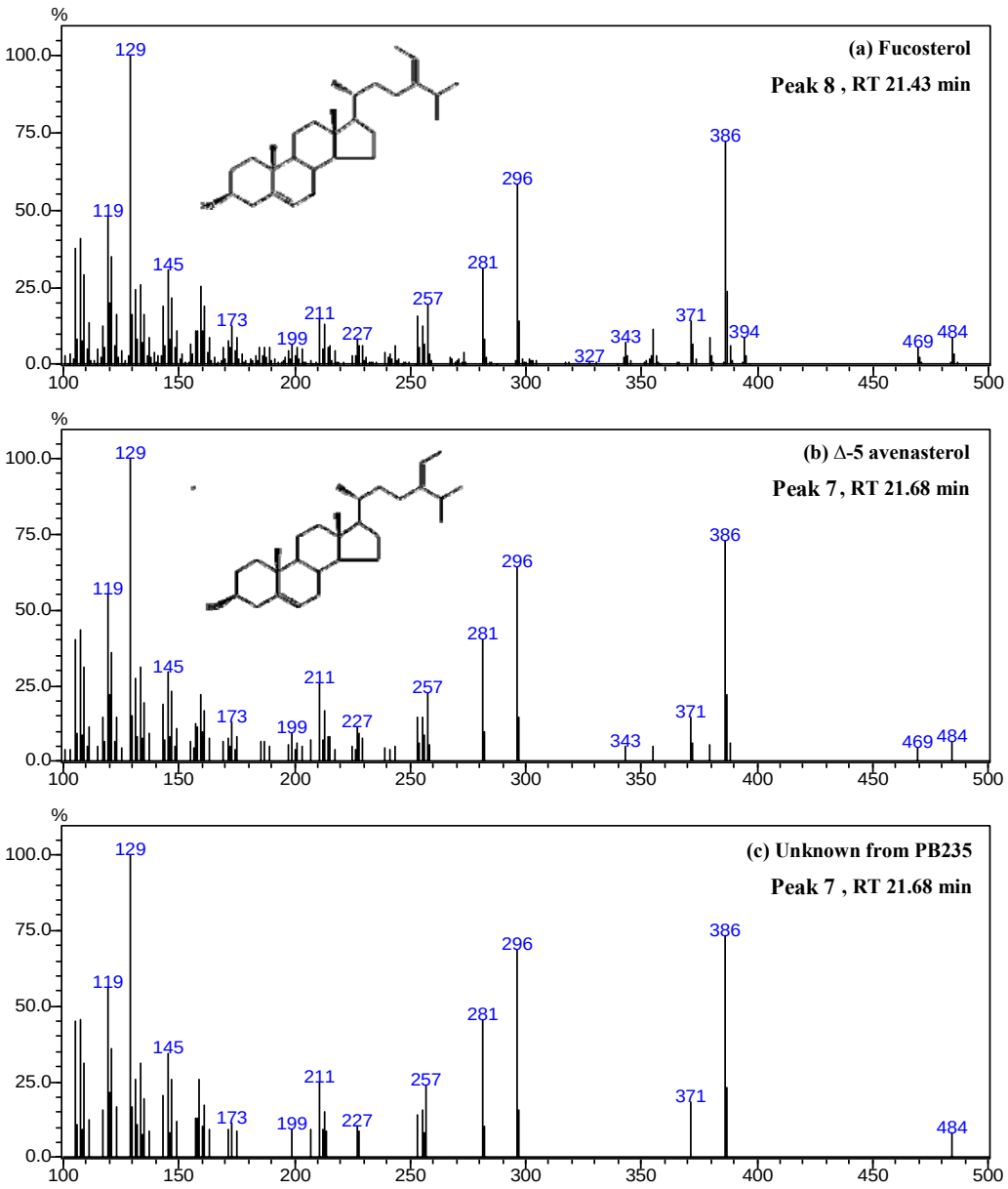


Figure 40 Mass spectra of (a) fucosterol, (b) Δ-5 avenasterol from oat oil unsaponifiable and (c) the peak eluting at 21.68 min (Δ-5 avenasterol) from PB235 latex unsaponifiable.

Table 16 Composition of unsaponifiable from fresh latex (in %w/w of total unsaponifiable)

clones	octadecanol		eicosanol		γ-tocotrienol		α-tocotrienol		stigmasterol		β-sitosterol		Δ-5 avenasterol	
	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE
RRIM600 (old)	3.60[b]	2.83	0.90[b]	0.34	22.20[a]	4.99	4.10[a]	4.06	7.10[a]	1.47	45.88[a]	10.37	16.20[ab]	7.28
RRIM600 (young)	6.63[b]	1.16	1.75[b]	0.84	18.95[a]	2.49	4.30[a]	1.66	8.10[a]	0.60	45.80[a]	4.23	20.68[ab]	2.97
GT1	7.60[ab]	2.83	2.10[ab]	0.84	22.50[a]	4.99	5.70[a]	4.06	6.20[a]	1.47	37.90[a]	10.37	17.90[ab]	7.28
PB235	10.87[a]	1.16	2.17[a]	0.34	17.17[a]	2.49	2.83[a]	1.66	7.15[a]	0.60	34.37[a]	4.23	29.45[a]	2.97
BPM24	9.68[ab]	1.16	2.88[a]	0.34	19.63[a]	2.49	6.55[a]	1.66	6.63[a]	0.60	44.22[a]	4.23	13.06[b]	2.97

Mean of 6 samplings; 3 repetitions was performed for each sampling

For each column, letters design groups of means that are not significantly different (student *t* test, $P \leq 0.05$)

SE: standard error

Table 17 Composition of unsaponifiable from USS (in %w/w of total unsaponifiable)

clones	octadecanol		eicosanol		γ-tocotrienol		α-tocotrienol		stigmasterol		β-sitosterol		Δ-5 avenasterol	
	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE
RRIM600 (old)	5.19^{c}	0.39	0.79^{c}	0.30	10.32^{b}	0.60	4.68^{b}	0.40	8.10^{a}	0.18	58.65^{a}	0.82	11.79^{c}	1.38
RRIM600 (young)	5.52^{c}	0.40	1.30^{bc}	0.17	13.48^{ab}	1.18	5.19^{b}	0.45	7.51^{ab}	0.22	50.59^{b}	2.17	17.12^{b}	1.62
GT1	6.74^{bc}	0.47	1.53^{bc}	0.20	12.17^{ab}	1.61	7.20^{a}	1.02	7.50^{ab}	0.27	57.47^{bc}	1.61	11.44^{c}	1.54
PB235	8.38^{ab}	1.17	1.96^{b}	0.42	13.44^{ab}	0.95	4.57^{b}	0.55	7.27^{bc}	0.21	39.93^{c}	2.33	24.55^{a}	1.86
BPM24	10.36^{a}	0.86	3.16^{a}	0.27	14.33^{a}	1.41	8.05^{a}	0.70	6.71^{c}	0.22	41.74^{c}	2.83	11.44^{c}	1.35

Mean of 15 samplings; 3 repetitions was performed for each sampling

For each column, letters design groups of means that are not significantly different (student *t* test, $P \leq 0.05$)

SE: standard error

2.2.3.2 Quantitative analysis

Quantification of the unsaponifiable fraction was based on standard calibration, except for Δ-5 avenasterol for which the same response factor as for β-sitosterol was applied. Relative proportion of each component in unsaponifiables from fresh latex and USS are given in table 16 and 17, respectively. For fresh latex, octadecanol and eicosanol represented respectively 3 - 11% and 1 - 3% of total unsaponifiable for every clone. PB235 and BPM24 unsaponifiables were found to contain more fatty alcohols than the other clones.

γ-tocotrienol content ranged from 17 to 23% and α-tocotrienol ranged from 3 to 7%. Concerning tocotrienols, no significant difference was observed between clones. Among the three detected plant sterols from *H. brasiliensis*, β-sitosterol was the main sterol found in every clones as well as the main unsaponifiable (> 40%). This sterol is indeed used by some laboratories as a marker of NR presence in unknown vulcanisated rubber (Faridah and Othman, 2007). Though β-sitosterol content was not significantly different between clones, slightly more β-sitosterol was observed in RRIM600 and BPM24 (45% of total unsaponifiable weight). PB235 showed the highest Δ-5 avenasterol content in its unsaponifiable with around 30%, while BPM24 displayed the lowest amount of this sterol (13%).

USS displayed a similar unsaponifiable composition, except a lower relative proportion of γ-tocotrienol (17 - 23% in latex unsaponifiable vs. 10-14% in USS) and a higher proportion of β-sitosterol.

2.3 Glycolipids

2.3.1 Glycolipid composition

2.3.1.1 Glycolipid families

Glycolipid composition of latex and USS lipids was qualitatively analyzed by TLC as presented in figure 41. The four main detected glycolipid families corresponding to glycolipid standards were steryl glucoside (SG), esterified steryl glucoside (ESG), monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG). No obvious clonal difference was found contrarily to neutral lipids. However, a difference in glycolipids was clearly observed between rubber type in the lipid extract from USS, galactosyldiglycerides were almost non-detectable, maybe due to their enzymatic hydrolysis into fatty acids and galactosyl glycerol. These results corroborate the global decrease of the glycolipids content observed in figure 31 and the release of free fatty acid (see 2.2.2.1) during sheet making process. The work carried out by Hasma and Subramaniam (1986) on RRIM501 is also in accordance with this finding.

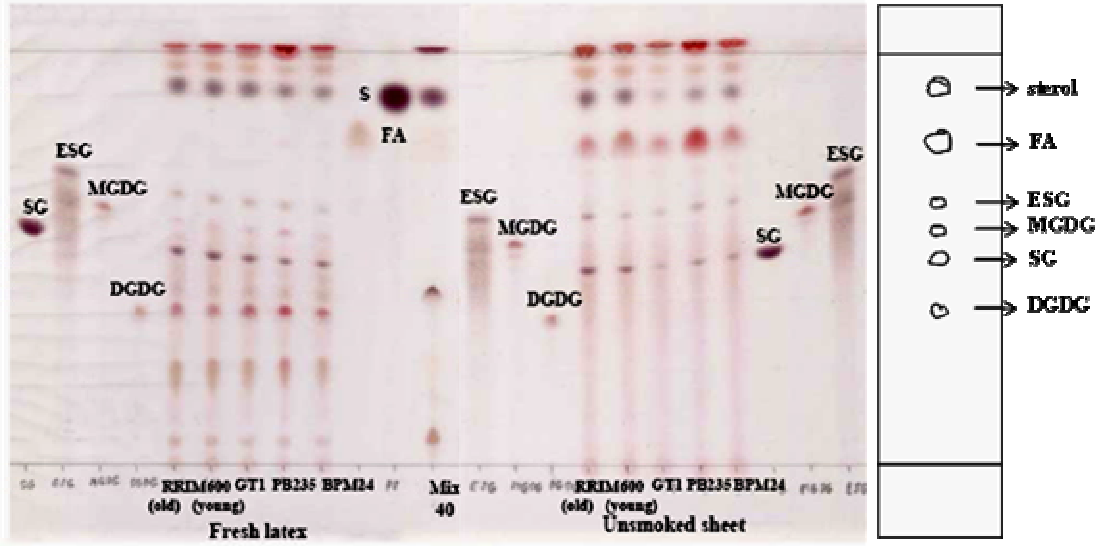


Figure 41 TLC of glycolipids from fresh latices and USS

Clone RRIM600 (old), RRIM600 (young), GT1, PB235 and BPM24 and lipid standards, developed in chloroform/ methanol/ water (95:20:2.5, v/v/v) and detected by orcinol sulphuric reagent. SG; steryl glucoside; ESG; esterified steryl glucoside, MGDG; monogalactosyl diglyceride, DGDG; digalactosyl diglyceride, FA; free fatty acids, S; free sterols. All samples are taken from trip 6 (10 November 2004)

A further identification and quantification by HPLC/ESI-MS of the glycolipids of fresh latex after LC separation will be described below. Indeed, TLC provides only global qualitative results and it remains difficult to obtain reliable quantitative results through this technique. The samples from three clones, namely RRIM600 (young), PB235 and BPM24 were compared. The analysis was performed only on samples from fresh latex due to the low amount of glycolipids in USS lipids (<10 -12% of lipid weight).

2.3.1.2 Fatty acid composition of glycolipids

In order to determine their fatty acid composition, glycolipid fractions obtained from acetone/methanol (9:1, v/v) LC eluent were saponified and derivatized for GC and GC-MS analysis. Fatty acids ranged from C14:0 to C20:0, like in the total lipids extract. The major fatty acid found in glycolipids was linoleic acid, which represented 43% and 40% from all detected fatty acids in RRIM600 and BPM24 glycolipids, respectively (table 18). Meanwhile, PB235 glycolipids distinguished themselves by their higher FFA content (32%) and lower linoleic acid content (25%).

Table 18 Fatty acid composition (w/w total fatty acids) of glycolipid fractions from fresh latex from three *H. brasiliensis* clones, determined by GC

Fraction	**Fatty acid**	***H. brasiliensis* clone**		
		RRIM600 (young)	**PB235**	**BPM24**
Glycolipid fraction	C14:0	0.3	0.5	0.3
	C16:0	9.8	10.9	7.9
	C16:1	0.6	2.0	1.1
	C18:0	19.7	16.7	20.3
	C18:1	16.9	8.0	20.4
	C18:2	43.1	25.4	40.0
	C18:3	3.7	3.4	3.8
	FFA	5.3	32.1	5.2
	C20:0	0.7	1.0	0.9

Data were the mean of 3 repetitions (independent extractions).

Standard deviations were <5% of the means.

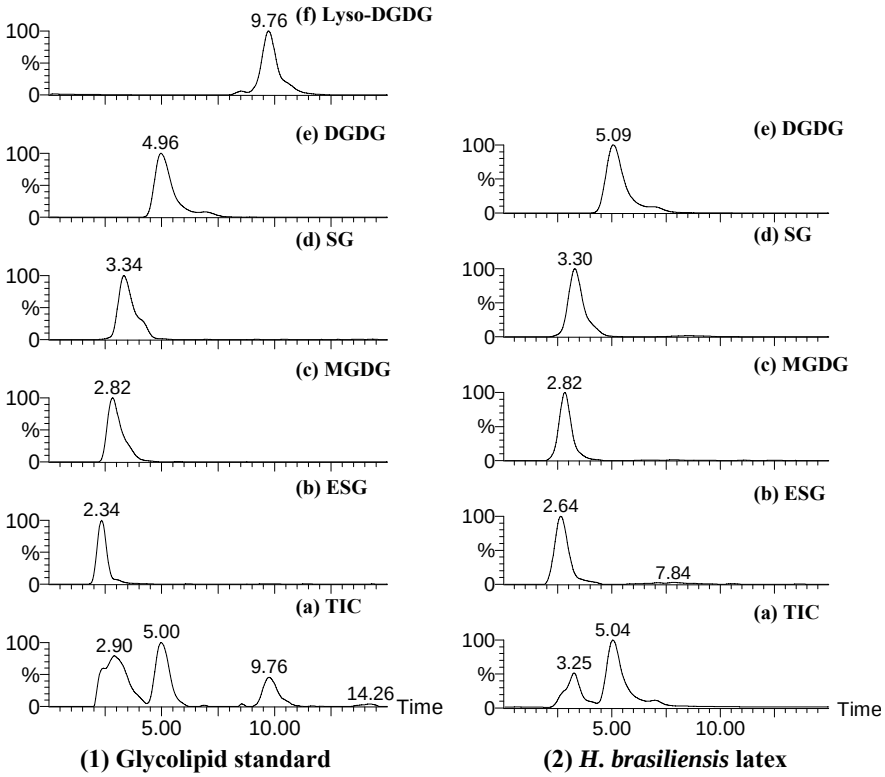


Figure 42 HPLC separation of (1) a mixture of commercial glycolipid (2) the glycolipid fraction from *H. brasiliensis* RRIM600 fresh latex. Chromatograms are given for (a) Total Ion Count and (b to f) sum of (M + Na)+ ions in the m/z range corresponding to each GL family: (b) esterified steryl glucosides (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) digalactosyl monolinolenin (lyso-DGDG, m/z 699).

2.3.1.3 Glycolipid molecular species

Chromatographic separations 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 are presented in figure 42. Discrimination of each glycolipid family under studied HPLC-MS condition was based on their retention times. However, retention time was not sufficient enough to discriminate the four components as the TIC presented only two peaks. The molecular mass of each glycolipid molecular species calculated from their structures with different esterified fatty acids were also utilized for discrimination and for further

quantification. This permitted to distinguish the four specific peaks corresponding to ESG (2.32 min), MGDG (2.82 min), SG (3.32 min) and DGDG (5.04 min). A supplementary compound, eluted after 9.80 min, was present in the glycolipid standard but not in the glycolipid extracted from fresh latex. The mass of the detected ion (*m/z* 699) and the chromatographic behavior of the compound corresponded to that of lyso-DGDG, detected as (digalactosyl monolinolenin + Na)$^+$.

ESI-mass spectra of each glycolipid family peaks permitted to characterize molecular species differing by their fatty acid profile and sterol composition of the total glycolipid fraction from latex. The detected masses and possible molecular species matching masses with fatty acids and sterols are presented in the table 20. The relative molecular composition of each glycolipid family was evaluated by the integration of peaks extracted for each *m/z* from TIC chromatogram. Due to the lack of pure standards for each combination of FA within a glycolipid family, detector's response was assumed to be equal for each molecular species within a family. Four glycolipids molecular species of each family are detailed afterwards.

1) Steryl Glucosides (SG)

Sterol moieties of glycolipids were determined with GC-MS after saponification. Their relative proportions are given in table 19. The major sterol in glycolipid fraction was β-sitosterol as it was observed in total lipid. The clonal difference among clones was observed with higher of Δ-5 avenasterol in PB235. The relative proportion of Δ-5 avenasterol was lower in glycolipids than that observed in total lipids (17% vs 53%). With this analysis, it was not possible to distinguish between the sterol composition from SG and from ESG.

Mass spectra of SG detected by ESI-MS in latex glycolipids are presented in figure 43. The two main ions found corresponded to stigmasteryl or Δ5-avenasteryl glucoside (*m/z* 597) and β-sitosteryl glucoside (*m/z* 599) respectively. (β-sitosteryl glucoside+Na)+ was the most abundent species, which is consistent with the sterol composition of the glycolipid fractions given in table 19. The ion at *m/z* 585

detected in the standard sample corresponded to the structure of (campesteryl glucoside + Na)$^+$, which was not detected in latex GL.

Table 19 Sterol composition of the unsaponifiables from total lipid and glycolipid fractions of fresh latex from three *H. brasiliensis* clones, determined by GC-MS

Fraction	Sterol	*H. brasiliensis* clone		
		RRIM600 (young)	PB235	BPM24
Total lipid unsaponifiable	Stigmaterol	7	6	7
	β-sitosterol	57	41	59
	Δ5-avenasterol	36	53	34
Glycolipid unsaponifiable	Stigmaterol	7	18	13
	β-sitosterol	85	65	78
	Δ5-avenasterol	9	17	9

Total lipids and glycolipid fraction were from trip 15 (December 2006)

Data were the mean of 3 repetitions (independent extractions).

Standard deviations were < 10% of the mean

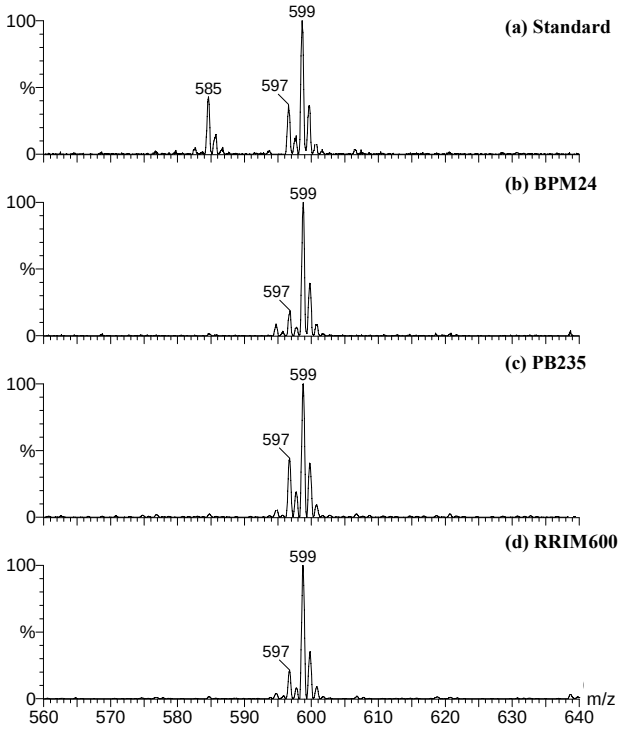


Figure 43 ESI-MS mass spectra of SG from the fresh latex of three *H. brasiliensis* clones (b-d) compared to that of a standard from soybean (a). Details on assigned structures for ions detected in latex glycolipids are given in table 20. The ions at m/z 585 detected in the standard corresponds to the structure of (campesteryl glucoside + Na)$^+$.

2) Esterified Steryl Glucosides (ESG)

The mass spectra of ESG from different *H. brasiliensis* clones are presented in figure 44. Whatever the clone, the most abundant ion of the ESG family peak was *m/z* 859, which 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 20–30% of total detected ESG ions (table 20). The fraction from PB235 clone was characterized by a high amount of FFA esters of β-sitosteryl glucoside (*m/z* 889, 14% of ESG ions).

3) Monogalactosyl diacylglycerols (MGDG)

The mass spectra extracted from the MGDG peak are presented in Figure 45. Different patterns were observed depending on the clone or the plant cell compartment where glycolipids are present. The commercial MGDG standard from plant leaves mainly contained the C18:3 and C16:3 acyl chains reported in the literature (Mongrand *et. al*, 1998). Latex MGDG had a very different composition. In samples from *H. brasiliensis* RRIM600 and BPM24 clones, the main structure, detected with *m/z* 805, corresponded to C18:1/18:1 or C18:0/18:2 MGDG and represented 20-26% of all detected MGDG species, whereas it was 12% only in PB235 clone. In the latter clone, the main peak was at *m/z* 857 and corresponded to the combination of two furan fatty acyl chains (FFA/FFA-MGDG), accounting for 49% of all detected MGDG. Another clonal difference was observed with the unidentified ions at *m/z* 815-819 (table 20), that are highly represented in BPM24 while in lower or very low proportion, respectively, in RRIM600 and PB235 MGDG.

4) Digalactosyl diacylglycerols (DGDG)

In DGDG, FA constituents were found to be similar to those of MGDG (table 20 and Figure 46). FFA/FFA 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 (~32% of total) were C18:1/18:1 or C18:0/18:2. However, the unknown ion (*m/z* 815-819) found in MGDG, mainly from BPM24, had no correspondent in DGDG.

Table 20 Molecular species found in each glycolipid family from latex of three *H. brasiliensis* clones. Proportion of each ion in a family was calculated by 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**
ESG				
837	[β-sitosterol/C16:0+Na]$^+$, [Stigmasterol/C18:2+H]$^+$, [Avenasterol/C18:2+H]$^+$	**21**	**17**	**22**
857	[Stigmasterol/C18:3+Na]$^+$, [Avenasterol/C18:3+Na]$^+$	**4.8**	**3.5**	**7.7**
859	[β-sitosterol/C18:3+Na]$^+$, [Stigmasterol/C18:2+Na]$^+$, [Avenasterol/C18:2+Na]$^+$	**28**	**38**	**34**
861	[β-sitosterol/C18:2+Na]$^+$, [Stigmasterol/C18:1+Na]$^+$, [Avenasterol/C18:1+Na]$^+$	**15**	**8.9**	**15**
863	[β-sitosterol/C18:1+Na]$^+$, [Stigmasterol/C18:0+Na]$^+$, [Avenasterol/C18:0+Na]$^+$	**9.9**	**8.4**	**6.0**
865	[β-sitosterol/C18:0+Na]$^+$, [Stigmasterol/FFA+H]$^+$, [Avenasterol/FFA+H]$^+$	**18**	**9.8**	**15**
889	[β-sitosterol/FFA+Na]$^+$	**3.9**	**14**	**0.7**
MGDG				
777	[C16:0/C18:2+Na]$^+$	**6.3**	**4.6**	**4.5**
779	[C16:0/C18:1+Na]$^+$	**2.8**	**3.0**	**3.9**
797	[C18:3/C18:3+Na]$^+$	**2.4**	**0.9**	**3.2**
801	[C18:2/C18:2+Na]$^+$, [C18:1/C18:3+Na]$^+$	**11**	**2.3**	**7.0**
803	[C18:1/18:2+Na]$^+$, [C18:0/C18:3+Na]$^+$, [FFA/C16:1+Na]$^+$	**14**	**3.9**	**13**
805	[C18:0/C18:2+Na]$^+$, [C18:1/C18:1+Na]$^+$, [FFA/16:0+Na]$^+$, [FFA/C18:3+H]$^+$	**25**	**12**	**18**
807	[C18:0/C18:1+Na]$^+$, [FFA/C18:2+H]$^+$	**12**	**4.3**	**10**
809	[C18:0/C18:0+Na]$^+$, [FFA/C18:1+H]$^+$	**1.6**	**1.2**	**1.0**
819	unknown	**4.9**	**3.0**	**15**
829	[FFA/18:2+Na]$^+$	**1.9**	**2.9**	**1.6**
831	[FFA/C18:1+Na]$^+$, [C18:2/C20:0+H]$^+$	**2.2**	**2.3**	**3.0**
833	[FFA/C18:0+Na]$^+$, [C18:2/C20:0+Na]$^+$	**2.9**	**5.5**	**1.6**
835	[C18:1/C20:0+Na]$^+$, [FFA/FFA+H]$^+$	**1.2**	**3.5**	**2.3**
837	[C18:0/C20:0+Na]$^+$	**4.1**	**2.5**	**6.5**
839	[FFA/C20:0+H]$^+$	**2.2**	**1.6**	**7.0**
857	[FFA/FFA+Na]$^+$	**5.0**	**47**	**2.3**
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]$^+$, [FFA/C16:1+Na]$^+$, [C18:0/C20:0+H]$^+$	**16**	**6.1**	**14**
967	[C16:0/FFA+Na]$^+$, [C18:0/C18:2+Na]$^+$, [C18:1/C18:1+Na]$^+$, [FFA/C18:3+H]$^+$	**31**	**20**	**31**
969	[C18:0/C18:1+Na]$^+$, [C16:1/C20:0+Na]$^+$, [FFA/C18:2+H]$^+$	**15**	**7.9**	**17**
971	[C16:0/C20:0+Na]$^+$, [C18:0/C18:0+Na]$^+$, [C18:3/C20:0+H]$^+$, [FFA/C18:1+H]$^+$	**2.1**	**0.9**	**2.4**
993	[C18:1/FFA+Na]$^+$, [C18:3/C20:0+Na]$^+$	**2.8**	**3.4**	**3.9**
995	[C18:0/FFA+Na]$^+$, [C18:0/C20:0+Na]$^+$	**2.6**	**4.8**	**2.5**
997	[C18:1/C20:0+Na]$^+$, [FFA/FFA+H]$^+$	**1.0**	**1.2**	**1.0**
999	[C18:0/C20:0+Na]$^+$	**0.1**	**0.1**	**0.1**
1019	[FFA/FFA+Na]$^+$	**4.6**	**39**	**2.9**
1023	[FFA/C20:0+Na]$^+$	**0.3**	**0.7**	**0.3**

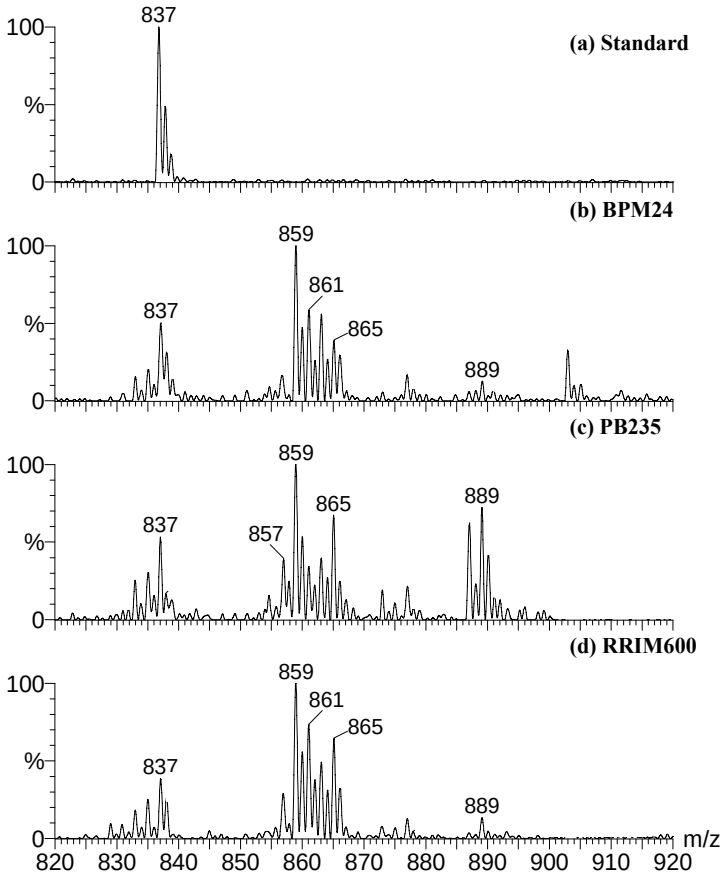


Figure 44 ESI-MS mass spectra of ESG from the fresh latex of three *H. brasiliensis* clones (b-d) compared to a standard from soybean (a). Details on assigned structures for ions detected in latex glycolipids are given in table 20. The ions at *m/z* 837 detected in the standard corresponds to the structure of (β-sitosterol/C16:0 + Na)+ or (Stigmasterol/C18:2 + Na)+ or (Δ-5 Avenasterol/C18:2 + Na)+

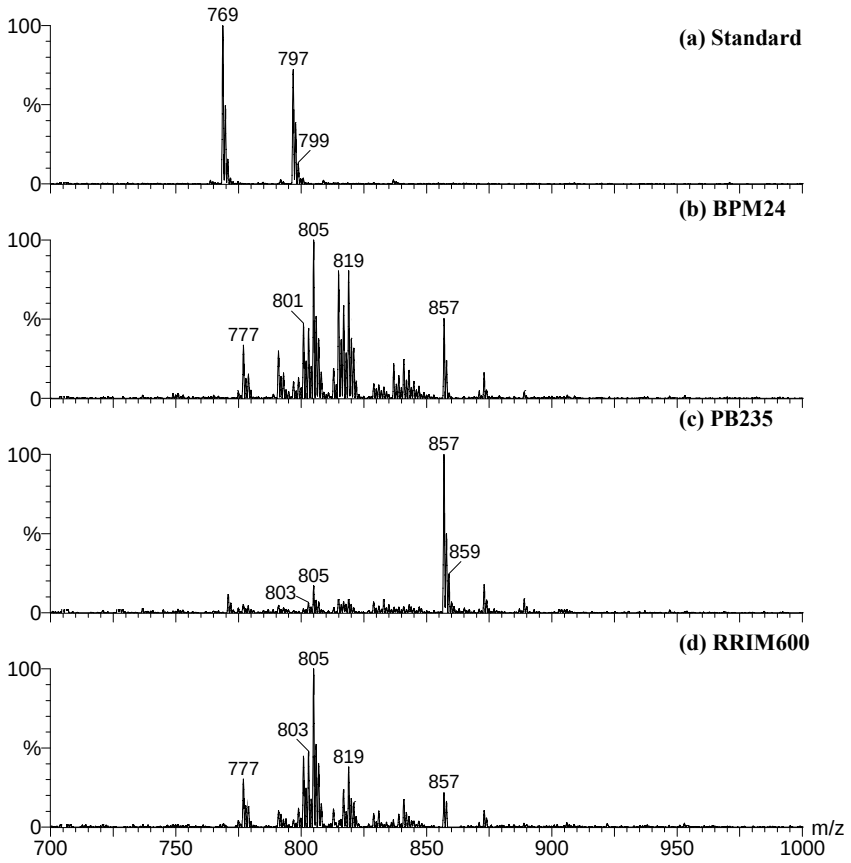


Figure 45 ESI-MS mass spectra of MGDG from the fresh latex of three *H. brasiliensis* clones (b-d) compared to a commercial standard from plant leaves (a).

Details on assigned structures for ions detected in latex glycolipids are given in table 20. The ions at *m/z* 769, 797 and 799 detected 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

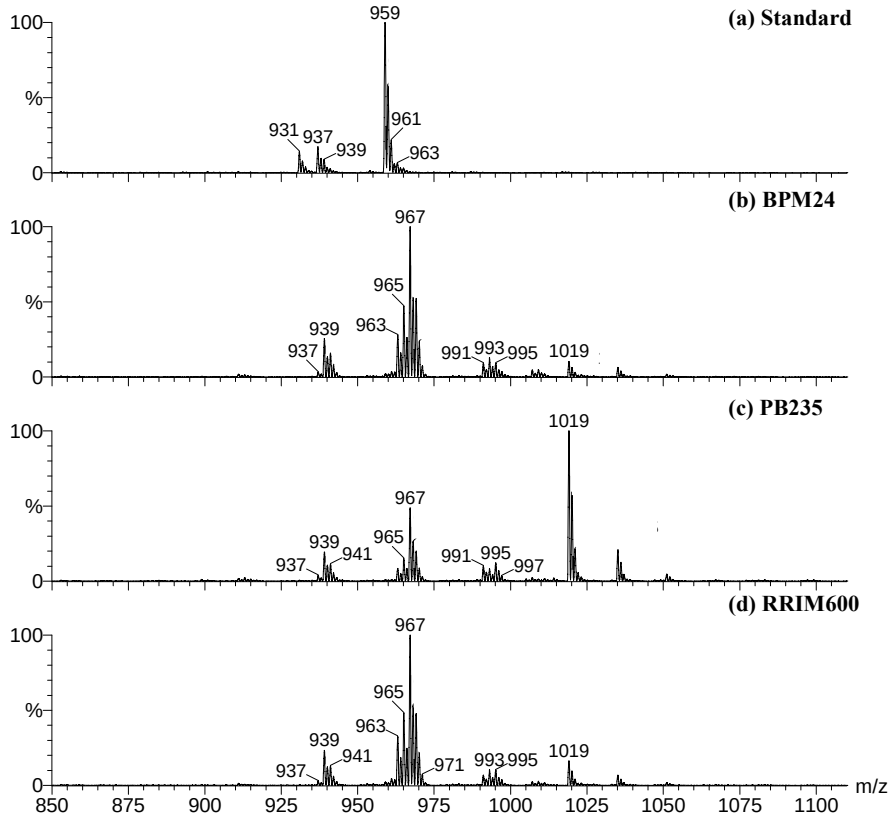


Figure 46 ESI-MS mass spectra of DGDG from fresh latex of three *H. brasiliensis* clones (b-d) compared to a standard from plant leaves (a)**.** Details on assigned structures for ions detected in latex glycolipids are given in table 20. The ions at *m/z* 931 and 959 detected in the standard correspond to the structure of $(C16:3/18:3\text{-DGDG} + Na)^+$ and $(C18:3/18:3\text{-DGDG} + Na)^+$, respectively.

2.3.1.4 Glycolipid quantity

In order to get quantitative results, a calibration was performed with each series of HPLC/ESI-MS injections, using 5 concentrations of standards. 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 figure 41b-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 20). 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 (RT 2.55 min for ESG and 2.95 min

for MGDG) and *m/z* 857 (RT 2.29 min and 2.73 min, respectively). For each family, molar response coefficients were assumed not to depend on the origin (latex, plant leaf, soybean oil) of the glycolipids.

Results given on figure 47 are presented as relative proportions. Around 80% of the injected quantity was recovered. The main glycolipid was DGDG in the three latices (43 – 50% of total glycolipids), followed by SG (30 – 34 %). A higher interclonal difference was observed for ESG, whose content varied from 7 % in the BPM24 samples to 19 % in PB235 clone. These results are similar 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).

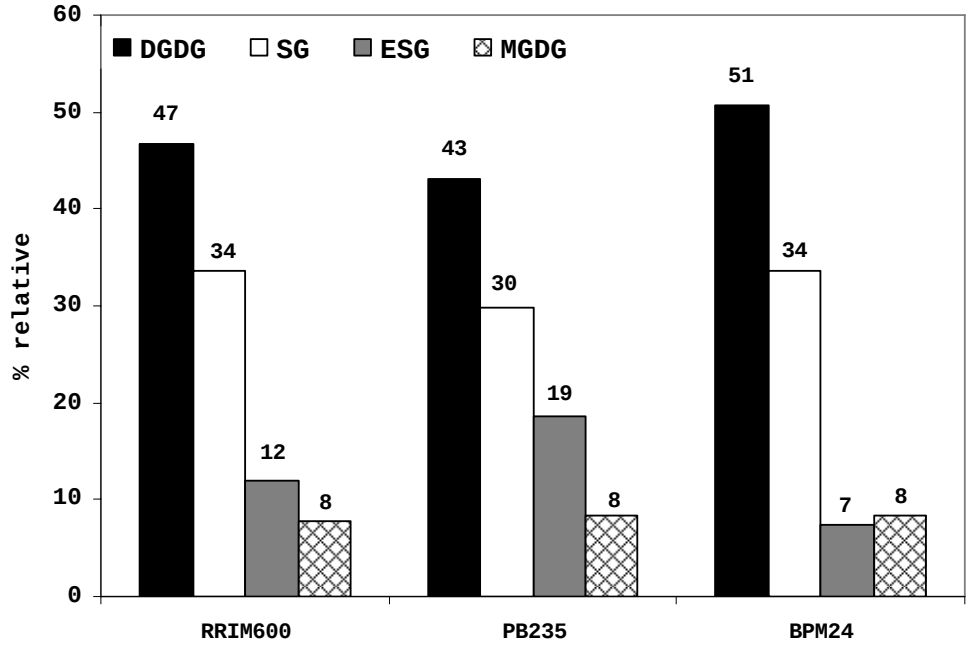


Figure 47 Glycolipid compositions of latices from *H. brasiliensis* RRIM600, PB235 and BPM24, determined by HPLC/ESI-MS.

Data were the mean of 2 repetitions (independent extractions).

MGDG was found in small amount (~8%) in *H. brasiliensis* latex though it has been reported to be a component of lipid bilayers of cell membrane. This could be explained by the fact that galactoglycerolipids, DGDG and

MGDG in plants, especially MGDG, usually accumulate in thylakoid membranes of chloroplast (Benning and Ohta, 2004 ; Kelly and Dormann, 2004). Lee (2000) reported that more than 50% of lipid composition of plant chloroplast membrane is MGDG. Our results suggest that latex MGDG may not originate from leaves.

2.4 Phospholipids

2.4.1 Phospholipid composition

2.4.1.1 Phospholipid families

Phospholipids of fresh latex and USS lipids were separated by TLC as presented in figure 48. The main spots of detected phospholipids in fresh latex corresponded to phosphatidylcholine (PC), phosphatidylinositol (PI) and trace of phosphatidylethanolamine (PE). PC appears to be the most important phospholipids among detected family as seen from the larger blue spot. Clonal difference was not clearly observed. For USS lipids, no phospholipid was visually observed on TLC plates, which confirms the degradation of native phospholipids during rubber sheet processing, similarly to MGDG and DGDG disappearance. Hydrolysis of ester bonds of phospholipids may be partly responsible for the release of free fatty acid previously detected.

In order to access more precisely the chemical composition of phospholipids, fatty acid composition of phospholipids fraction was analyzed (GC-FID) and each molecular species was identified by HPLC/ESI-MS).

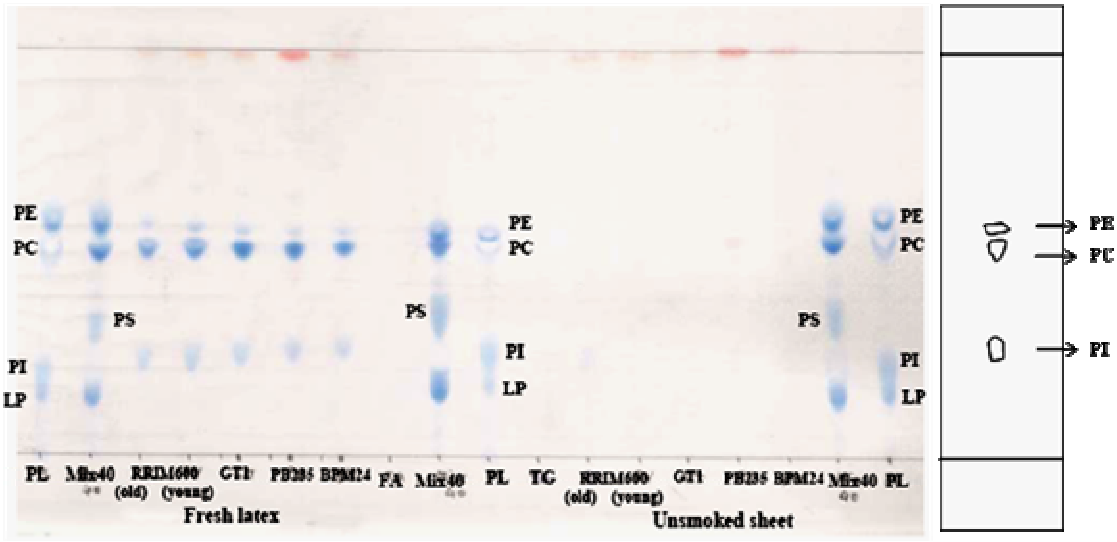


Figure 48 TLC of phospholipids from fresh latices and USS

Clone RRIM600 (old), RRIM600 (young), GT1, PB235 and BPM24 and standard lipid, developed in chloroform/methanol/30% ammonia (65:25:5, v/v/v) and detected by molybdenum reagent. PL; phospholipids mixture, LP; lysophosphatidylcholine, PE; phosphatidylethanolamine, PS; phosphatidyl serine, PC; phosphatidylcholine, PI; phosphatidylinositol, FA; free fatty acids, TG; triacylglycerol.

All samples are taken from trip 6 (10 November 2004)

2.4.1.2 Fatty acid composition of phospholipid family

Global fatty acid composition (table 21) of phospholipid fractions from RRIM600 and PB235 latex was determined by saponification of the phospholipid fraction and GC-FID analysis of methylated fatty acids. The results showed that the major fatty acid in phospholipid fraction from RRIM600 and PB235 was linoleic acid with around 55% and 37% of total fatty acids, respectively. In addition, FFA in phospholipids from PB235 clone was found in lower proportion (15% of phospholipid fatty acids) compared to that found in glycolipids (42% of glycolipid fatty acids) or in total lipid extracts (74%). A previous study, carried out by Hasma (1984) using a phospholipid fraction from RRIM501 latex, also showed that the fatty acyl components of phospholipids obtained from FAME analysis were mostly aliphatic fatty acids (C16:0, C18:0, C18:1 and C18:2).

Table 21 Fatty acid composition (%w/w total fatty acids) of the total phospholipid fraction from fresh latex from two *H. brasiliensis* clones, determined by GC

Fraction	Fatty acid	***H. brasiliensis* clone**	
		RRIM600	PB235
Phospholipid fraction	C14:0	0.0	1.2
	C16:0	9.8	12.9
	C16:1	0.0	4.6
	C18:0	14.3	17.0
	C18:1	16.6	10.8
	C18:2	55.1	38.1
	C18:3	2.6	2.6
	FFA	1.2	13.5
	C20:0	0.3	1.0

Data were the mean of 3 repetitions (independent extractions).

Standard deviations were <5% of the means.

2.4.1.3 Phospholipid molecular species

Phospholipid fractions of fresh latex lipids from RRIM600, PB235 and BPM24 clones were analyzed. Their separation and identification were performed by HPLC-ESI/MS similarly to those of glycolipid fraction. Phospholipids from commercial standard mixtures of phospholipids from soybean (PI, PC, PE and LPC) and egg yolk (PA, LPI) were first compared with the phospholipid fraction of RRIM600 latex. Six phospholipid families were separated by the normal-phase HPLC column, corresponding to PE (6.85 min), PI (7.58 min), LPI (9.56 min) and PA (13.47 min) as shown in figure 49. They were detected under negative-ESI condition while PC (13.51 min) and LPC (19.61 min) were better detected under positive-ESI due to the presence of a quaternary ammonium in their structure. Other peaks, eluted after around 2.4 minutes, were found to be free fatty acids. Though the retention time between standard and samples were not exactly identical, the order of elution and molecular species calculated from the structure and possible fatty acid constituents of each phospholipid family which can be detected with ESI-MS were used to ease peak identification. Pulfer and Murphy (2003) reported that the exact retention time obtained from normal phase HPLC analysis could be difficult to reproduce as the water in the solvent system can alter the affinity of phospholipids for silica in replicate runs.

Phospholipids molecular species of each phospholipid family were determined from ESI mass spectra. Under negative ionization, the mass of the fatty acid constituent released by fragmentation in the ionization probe was observed. The detected *m/z* 255, 277, 279 and 283 corresponded to palmitic acid (C16:0), linolenic acid (C18:3), linoleic acid (C18:2) and stearic acid (C18:0) $(M-H)^-$ ions. These fragmented ions permitted to precise the possible structures as one phospholipid mass can correspond to various combinations of fatty acids. With this information as well as total fatty acid composition, the most probable structures of each molecular species are indicated in table 22.

1) Phosphatidylcholine (PC) and Lysophosphatidyl-choline (LPC)

PC is usually the major phospholipid of plant membranes. The detected molecular masses of PC family from fresh latex lipids are presented in figure 50. The major PC ion of standard was *m/z* 758 (C16:0/C18:2-PC or C18:1/C18:1-PC. Main detected masses in *H. brasiliensis* PC were *m/z* 758, 782, 784, 786 and 788. The most abundant ion was *m/z* 786 (23-26%) for every studied clone, corresponding to C18:0/C18:2-PC or C18:1/C18:1-PC or FFA/C16:0-PC. However, some masses were present in specific proportions depending on the clones. For instance, *m/z* 782 (C18:1/C18:3-PC or C18:2/C18:2-PC) were found in the respective proportion of 29% and 18% in RRIM600 PC and BPM24 PC while it represented only 9% in PB235 PC.

LPC, which contains one fatty acyl, was also detected in *H. brasiliensis* latex. ESI-MS mass spectra of LPC from different *H. brasiliensis* clones are presented in figure 51. The three main fatty acids found in LPC structures were C18:2 (*m/z* 520), C18:1 (*m/z* 522) and C18:0 (*m/z* 524) in the respective proportions of 32-40%, 9-20% and 14-18%. The major detected LPC (*m/z* 520), which contained C18:2, may be a hydrolysate of the major *H. brasiliensis* PC species that gives an ion with *m/z* 786. Indeed, LPC is hardly present in free form in nature. In this case it may be the product of phospholipases activities in fresh latex in the time interval between tapping and extraction.

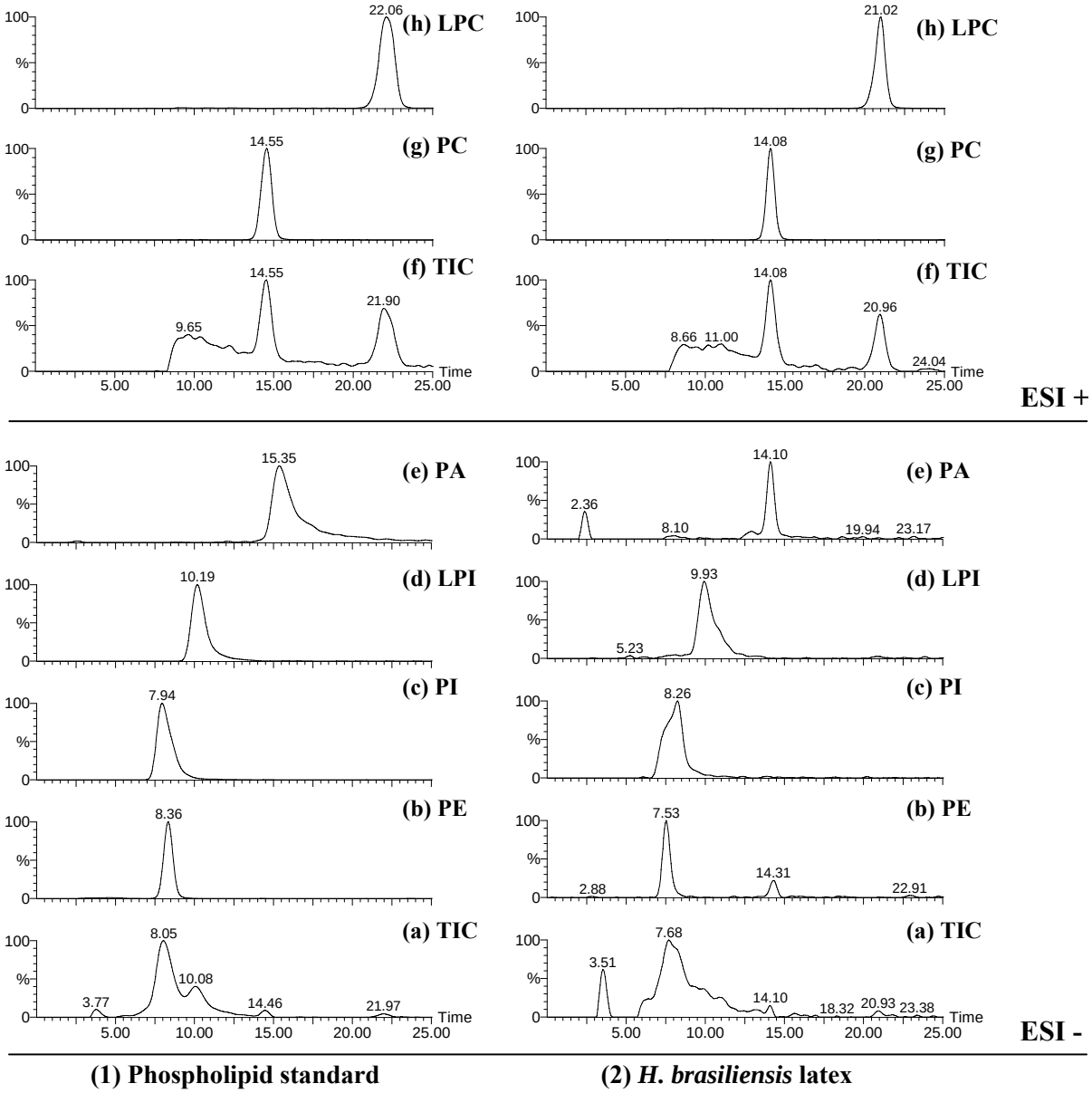


Figure 49 HPLC separation of (1) a mixture of commercial phospholipid and (2) the phospholipid fraction from *H. brasiliensis* RRIM600 fresh latex. Chromatograms are given for (a) Total Ion Count from negative electrospray condition and (b to g) sum of $(M\text{-}H)^-$ and $(M + H)^+$ ions in the *m/z* range corresponding to each PL family: (b) phosphatidylethanolamine (PE, *m/z* 712-772; most abundant species *m/z* 714); (c) phosphatidylinositol (PI, *m/z* 831-863; most abundant species *m/z* 833); (d) lysophosphatidylinositol (LPI, *m/z* 571-863; most abundant species *m/z* 595); (e) phosphatidic acid (PA, *m/z* 671-727; most abundant species *m/z* 725); (f) Total Ion Count from positive electrospray condition; (g) phosphatidylcholine (PC, *m/z* 730-838; most abundant species *m/z* 786) and (h) lysophosphatidylcholine (LPC, *m/z* 494-548; most abundant species *m/z* 520).

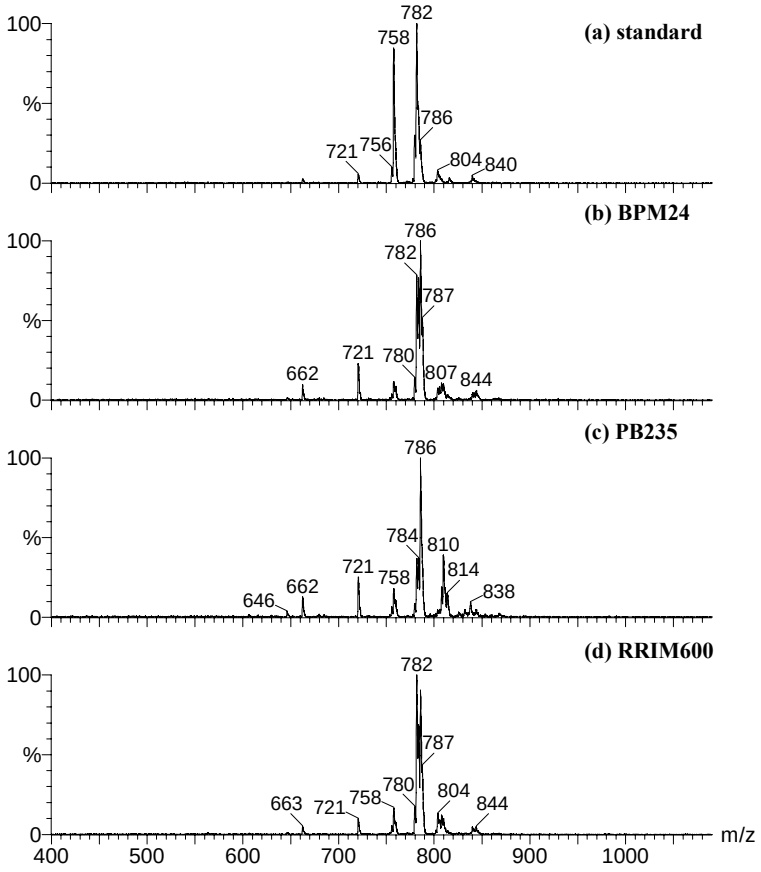


Figure 50 ESI-MS mass spectra of PC from the fresh latex of three *H. brasiliensis* clones (b-d) compared to a standard from egg yolk lecithin (a). Details on assigned structures for ions detected in latex phospholipids are given in table 22.

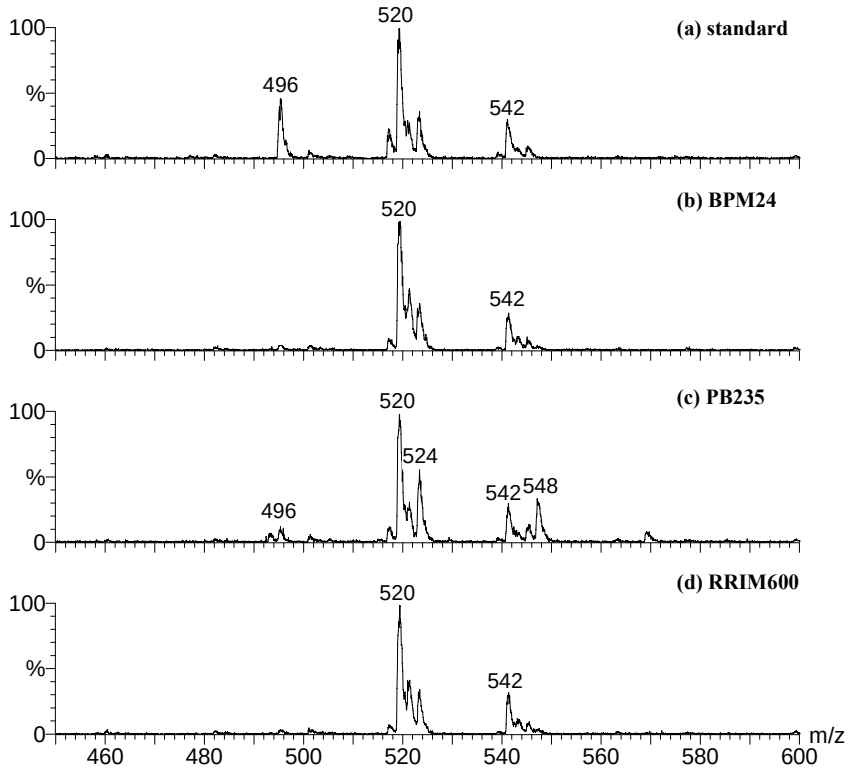


Figure 51 ESI-MS mass spectra of LPC from the fresh latex of three *H. brasiliensis* clones (b-d) compared to a standard from egg yolk lecithin (a). Details on assigned structures for ions detected in latex phospholipids are given in table 22.

2) Phosphatidylethanolamine (PE)

The two major masses detected for PE from *H. brasiliensis* latex were *m/z* 714, corresponding to C16:0/C18:2 or C16:1/C18:1 and *m/z* 742 with C18:0/C18:2, C18:1/C18:1 or FFA/C16:0 as fatty acid constituents (figure 52). These two ions represented together more than 51% of all *m/z* detected in the 712-742 range. These detected masses were similar to those of standard. Other molecular masses such as FFA containing structures with *m/z* 740, 766 and 770 were found in very low quantity even for PB235 clone (5% of all *m/z* detected in 712-772 range).

3) Phosphatidylinositol (PI) and lysophosphatidylinositol (LPI)

PI mass spectra presented in figure 53 showed two main ions with *m/z* 833 and 861 that represented around 44-47% and 27-37% of detected PI, respectively (table 22). They contained the same fatty acid constituents as those found in PE ions. It is to be noted that FFA was not observed as a dominant fatty acid in PI from PB235 phospholipids.

LPI, degraded products from PI, contained C16:0 (*m/z* 571) and C18:2 (*m/z* 595) in their structures (figure 54). The major LPI contained C18:2 (~ 50% of all LPI) whatever the clone.

4) Phosphatidic acid (PA)

ESI-mass spectra of PA standard and of *H. brasiliensis* latex from different clones are presented in figure 55. The molecular species in the PA standard with *m/z* 673 corresponded to C16:0/C18:1-PA while the abundant PA ions from latex samples were of *m/z* 723 to 725. Their structure corresponded to C18:3/C20:0 or FFA/C18:1. These two ions represented 23% and 27% of total detected ions for RRIM600 and PB235 PA, respectively. For BPM24 clone, they accounted for only 10% of total detected masses, *m/z* 723 being the major detected ion (FFA/C18:2-PA).

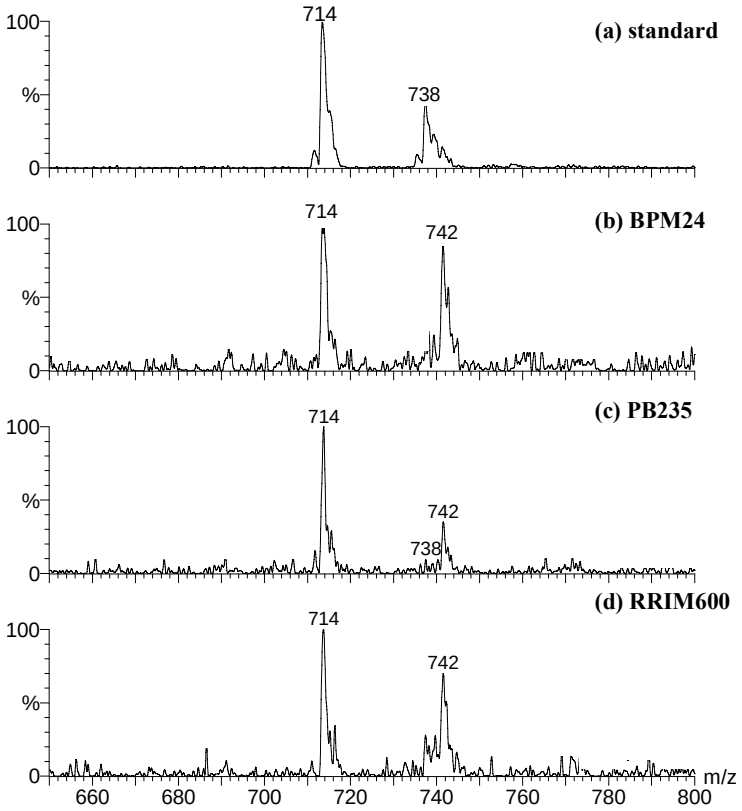


Figure 52 ESI-MS mass spectra of PE from the fresh latex of three *H. brasiliensis* clones (b-d) compared to a standard from soybean (a). Details on assigned structures for ions detected in latex phospholipids are given in table 22.

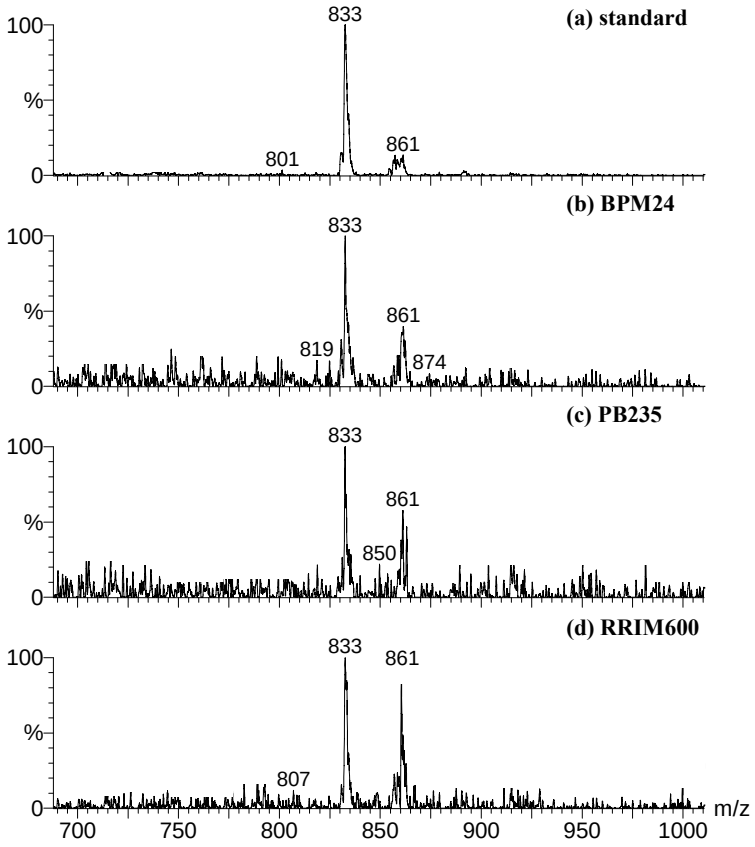


Figure 53 ESI-MS mass spectra of PI from the fresh latex of three *H. brasiliensis* clones (b-d) compared to a standard from soybean (a). Details on assigned structures for ions detected in latex phospholipids are given in table 22.

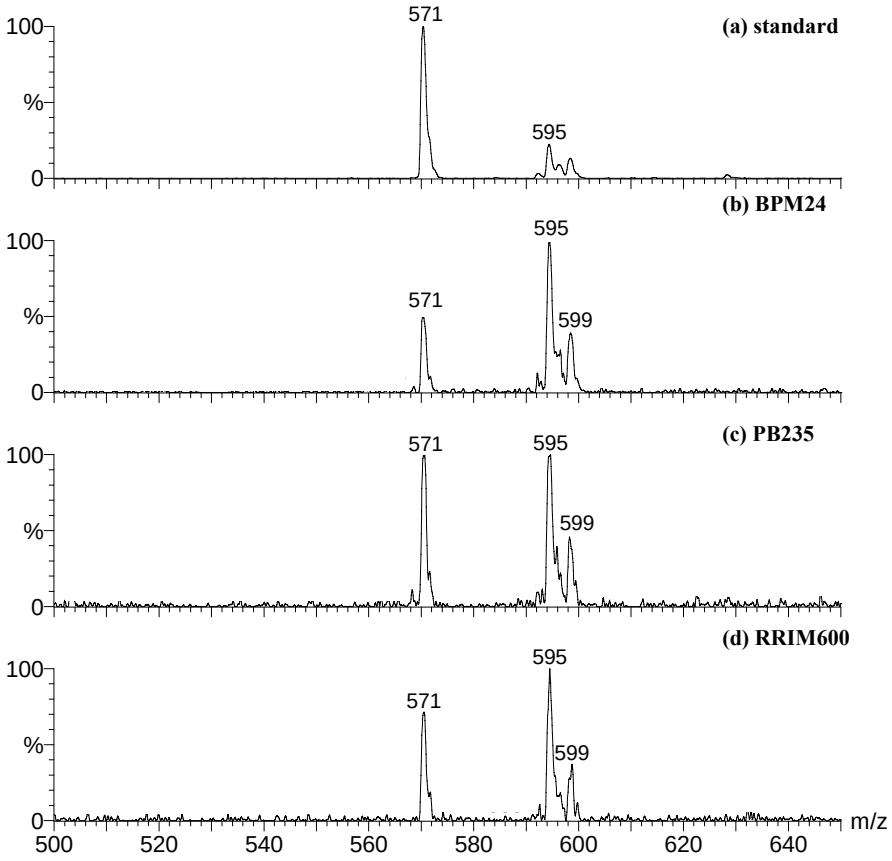


Figure 54 ESI-MS mass spectra of LPI from the fresh latex of three *H. brasiliensis* clones (b-d) compared to a standard from soybean (a). Details on assigned structures for ions detected in latex phospholipids are given in table 22.

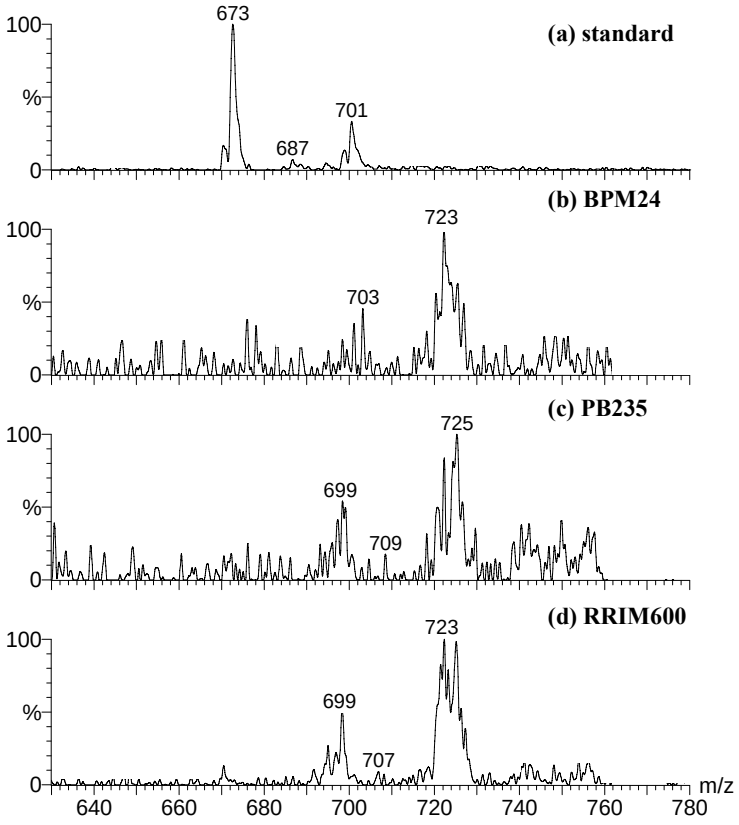


Figure 55 ESI-MS mass spectra of PA from the fresh latex of three *H. brasiliensis* clones (b-d) compared to a standard from egg yolk lecithin (a). Details on assigned structures for ions detected in latex phospholipids are given in table 22.

Table 22 Molecular species found in each phospholipid family from the latex of three *H. brasiliensis* clones. Proportion of each ion in a family was calculated by 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 present in the total phospholipid fraction.

PL mass (*m/z*)	Structure	% relative		
		RRIM600	PB235	BPM24
PC				
730	$[C16:1/C16:1+H]^+$	1.7	2.7	1.9
732	$[C16:0/C16:1+H]^+$	0.9	4.2	1.1
734	$[C16:0/C16:0+H]^+$	0.2	1.1	0.2
756	$[C16:0/C18:3+H]^+$, $[C16:1/C18:2+H]^+$	2.4	3.7	1.6
758	$[C16:0/C18:2+H]^+$, $[C16:1/C18:1+H]^+$	12	13	8.3
760	$[C16:0/C18:1+H]^+$, $[C16:1/C18:0+H]^+$	5.6	8.9	5.6
762	$[C16:0/C18:0+H]^+$	0.6	1.4	0.7
780	$[C16:0/C18:0+NH_4]^+$, $[C18:2/C18:3+H]^+$	5.0	2.5	2.9
782	$[C18:1/C18:3+H]^+$, $[C18:2/C18:2+H]^+$	29	9.3	18
784	$[C18:0/C18:3+H]^+$, $[C18:1/C18:2+H]^+$, $[FFA/C16:1+H]^+$	2.1	9.5	17
786	$[C18:0/C18:2+H]^+$, $[C18:1/C18:1+H]^+$, $[FFA/C16:0+H]^+$	26	23	23
788	$[C18:0/C18:1+H]^+$, $[C16:1/C20:0+H]^+$	10	7.7	12
790	$[C18:0/C18:0+H]^+$, $[C16:0/C20:0+H]^+$	1.1	1.0	1.4
812	$[C18:0/C20:0+H]^+$, $[FFA/C18:1+H]^+$	1.5	3.8	1.3
814	$[C18:2/C20:0+H]^+$, $[FFA/C18:0+H]^+$	1.3	4.1	1.2
838	$[FFA/FFA+H]^+$	0.5	3.4	4.2
LPC				
494	$[C16:1+H]^+$	1.1	6.7	1.2
496	$[C16:0+H]^+$	5.1	9.1	5.7
518	$[C18:3+H]^+$	4.4	5.3	4.1
520	$[C18:2+H]^+$	44	32	40
522	$[C18:1+H]^+$	16	9	20
524	$[C18:0+H]^+$	14	18	17
540	$[C18:1+NH_4]^+$	1.2	0.9	0.8
542	$[C18:0+NH_4]^+$	13	8	10
548	$[FFA+H]^+$	1.1	11	1.4
PE				
712	$[C16:0/C18:3-H]^-$, $[C16:1/C18:2-H]^-$	1.1	3.7	1.1
714	$[C16:0/C18:2-H]^-$, $[C16:1/C18:1-H]^-$	28	31	25
716	$[C16:1/C18:0-H]^-$	7.0	9.5	8.1
738	$[C18:1/C18:3-H]^-$	15	0	11
740	$[C18:0/C18:3-H]^-$, $[FFA/C16:1-H]^-$	9.9	5.3	8.1
742	$[C18:0/C18:2-H]^-$, $[C18:1/C18:1-H]^-$, $[FFA/C16:0-H]^-$	25	27	31
744	$[C18:0/C18:1-H]^-$, $[C16:1/C20:0-H]^-$	6.9	6.1	9.8
766	$[FFA/C18:2-H]^-$	1.9	5.7	1.3
770	$[C18:2/C20:0-H]^-$, $[FFA/C18:0-H]^-$	1.8	7.2	3.5
772	$[C18:1/C20:0-H]^-$	4.0	4.3	1.6
PI				
831	$[C16:0/C18:3-H]^-$, $[C16:1/C18:2-H]^-$	1.6	2.8	8.4
833	$[C16:0/C18:2-H]^-$, $[C16:1/C18:1-H]^-$	44	47	47
835	$[C16:0/C18:1-H]^-$, $[C16:1/C18:0-H]$ -	6.6	12	9.0
861	$[C18:0/C18:2-H]^-$, $[C18:1/C18:1-H]^-$, $[FFA/C16:0-H]^-$	37	33	27
863	$[C18:0/C18:1-H]^-$, $[C16:1/C20:0-H]^-$	11	4.4	8.5
LPI				
571	$[C16:0-H]^-$	24	32	23
595	$[C18:2-H]^-$	52	43	51
597	$[C18:1-H]^-$	9.8	6.9	9.9
599	$[C18:0-H]^-$	14	19	16
PA				
671	$[C16:0/C18:2-H]^-$, $[C16:1/C18:1-H]^-$	4.1	17	4.6
673	$[C16:0/C18:1-H]^-$, $[C16:1/C18:0-H]^-$	9.6	0	10
697	$[C18:0/C18:3-H]^-$, $[FFA/C16:1-H]^-$	11	14	12
699	$[C18:0/C18:2-H]^-$, $[C18:1/C18:1-H]^-$, $[FFA/C16:0-H]^-$	13	20	14
701	$[C18:0/C18:1-H]^-$, $[C16:1/C20:0-H]$ -	6.9	0	3.1
723	$[FFA/C18:2-H]^-$	19	10	25
725	$[C18:3/C20:0-H]^-$, $[FFA/C18:1-H]^-$	23	27	10
727	$[C18:2/C20:0-H]^-$, $[FFA/C18:0-H]^-$	13	12	21

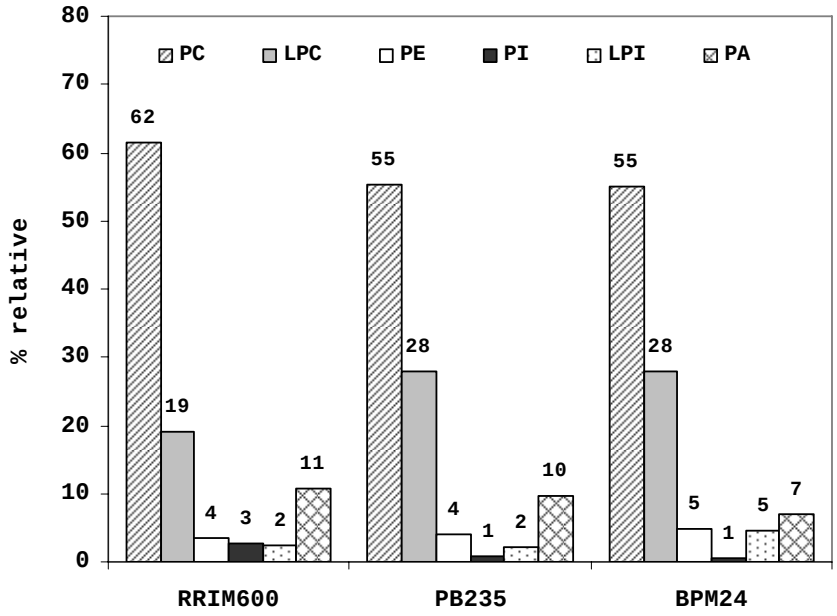


Figure 56 Phospholipid composition of latices from *H. brasiliensis* RRIM600, PB235 and BPM24, determined by HPLC/ESI-MS. Data were the mean of 2 repetitions (independent extractions).

2.4.1.4 Phospholipid quantity

The relative proportion of each phospholipid family was evaluated by the integration of peaks extracted for each *m/z* from the TIC chromatogram (figure 49), following the same calculation principles as for glycolipids. All detected masses and the correspondent structures are presented in table 22. Around 70% recovery was obtained. For each clone, the proportion of each phospholipid family is presented in figure 56. The results showed that PC was the main phospholipid found in natural rubber latex samples from every clone (55 - 62 % of total phospholipids) while LPC amounted to 19 - 28%. The sum of PC and LPC from each clone indicated a similar amount of native PC initially present in latex (~80% of total phospholipids). PA, reported by Dupont *et. al.* (1976) to be the main phospholipid in lutoid membrane, was found to account for 7-11% of total phospholipids. However, PA, LPC and LPI could also be artifacts due to the activity of phospholipases A1, A2 or D. Compared to the results obtained from HPLC-MS, the indirect analysis of phospholipids from fresh latex of RRIM501 clone reported by Hasma (1984) showed a similar PC content (58%) but much more PI and PE (~20%

of each). Trace of LPC and LPI were also observed on TLC and they were also considered as artifacts from enzymatic reactions occurring during latex extraction.

3. Conclusion

Lipids from fresh latex and USS from four *H. brasiliensis* clones, RRIM600 (two different ages), GT1, PB235 and BPM24, were characterized both in quantity and quality. Lipid content in fresh latex was 2.5-3.7% versus dry rubber content depending on clones and tree age. PB235, BPM24 and RRIM600 (young) were classified as high lipid containing clones whereas RRIM600 (old) and GT1 contained a lower amount of lipids. In USS, the amount of lipids was found to be lower than in latex (1.9-3.3% versus dry rubber) but the ranking from high to low lipid content remained the same. Lipids are partly either eliminated by washing or hydrolyzed during USS making process. Indeed lipase activity was detected in fresh latex.

Lipids from fresh latex and USS were separated into 3 main families: neutral lipids, glycolipids and phospholipids. In fresh latex, neutral lipids was the major lipid class with a relative proportion ranging from 39 to 61%. Lipids from PB235 and BPM24 clone latices contained more than 50% neutral lipids. Glycolipid content in GT1 latex and RRIM600 (old) latices was higher compared to the other clones (33-36% against 21-25%). For phospholipids, RRIM600 (old and young) and GT1 clones were found to contain a higher amount of phospholipids (25-28%) compared to PB235 and BPM24 clones (18%).

Lipid families in USS lipids were found to be similar in every clone and significantly different from latex. Relative proportions of 81-86% of neutral lipids, 10-13% of glycolipids and 4-5% phospholipids were found. The higher proportion of neutral lipids found in these samples was consistent with the hypothesis that polar lipids were partly eliminated by the washing of rubber sheets, or hydrolyzed: indeed rubber sheet lipid were extracted after the long drying process of sheets (~7 days) whereas latex lipids were extracted immediately on field, a few hours after tapping.

Neutral lipids of fresh latex and USS lipids were characterized for their composition using TLC and GC-MS. The main difference was found in free fatty acid content, resulting from lipid hydrolysis catalyzed by hydrolases. The free fatty acid content in USS lipids was around 18-22% versus lipid weight, i.e. about ten times higher than that of fresh latex (1.3-2.2%).

Regarding total fatty acid composition, linoleic acid (C18:2) was the major fatty acid found in RRIM600, GT1 and BPM24 clones (37-53% of total fatty acids). PB235 clone contained only 11% of linoleic acid but 74% of furan fatty acids. Total fatty acid composition of fresh latex and USS lipids was found to be similar.

The unsaponifiable fraction contained two fatty alcohols (C18:0 and C20:0), α-tocotrienol and γ-tocotrienol, stigmasterol, β-sitosterol and Δ-5 avenasterol which was previously reported to be fucosterol. β-sitosterol (34-46%) was the main unsaponifiable for every studied clone. The amount of γ-tocotrienol ranged from 17 to 22% and was found to be similar among clones while that of Δ-5 avenasterol differed: RRIM600 (old and young), GT1 and BPM 24 clones contained around 13-20% of Δ-5 avenasterol in its unsaponifiable compared to 29% in PB235 clone. Stigmasterol and α-tocotrienol contents were in the range of 6-8% and 2-7%, respectively. Octadecanol content was found to be higher than eicosanol (3-11% against 2-3%). Both fatty alcohols may come from waxes (esterified form of fatty alcohol).

Glycolipids of fresh latex consisted of four glycolipid families, namely steryl glucoside (SG), esterified steryl glucosides (ESG), monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (MGDG). Same glycolipid families were also found in USS lipids but with fewer amounts of MGDG and DGDG. Analysis of molecular species of each glycolipid family was performed with RRIM600 (old), PB235 and BPM24 latex glycolipids. Glycolipids contained β-sitosterol as the major sterol moiety in their structures for every clone, which is similar to free sterol composition. Total fatty acid composition of glycolipids was similar to that of total lipids, except furan fatty acids that was found in fewer amount in PB235 clone (74%

in total lipids compared to 32% in glycolipids). Linoleic acid was the main fatty acid in glycolipids of every clone. The main detected ions for SG and ESG were *m/z* 599 (70-80% of total detected SG) and *m/z* 859 (20-30% of total detected ESG), which corresponded to the respective structures of β-sitosteryl glucoside and β-sitosteryl (linolenoyl) glucoside. The most abundant ions for MGDG and DGDG were *m/z* 805 (20-26% of total MGDG) and *m/z* 967 (32% of total DGDG). Both molecular masses corresponded to the C18:1/18:1 or C18:0/18:2 diacylglycerol part. For most of detected MGDG and DGDG, the same diacylglycerol part was found in detected ions. Among clones, the glycolipid structures containing furan fatty acid were found in slightly higher proportion in PB235 glycolipids compared to those of other clones. Concerning, glycolipid quantity, the main glycolipid family was DGDG, which comprised around 43-50% of total glycolipids. The content was descending from SG (30-34%) to ESG (7-19%) and MGDG (8%). Clonal difference was observed for ESG with the lowest content in BPM24 glycolipids (7%) whereas 12% and 19% were respectively found in RRIM600 and BPM24 glycolipids.

Phospholipids in *H. brasiliensis* latex consisted of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidyl inositol (PI) and lysophoaphatidylinositol (LPI) and phosphatidic acid (PA). All these phospholipids were detected in every studied clone.

The fatty acid composition of phospholipids was similar to those of total lipids as well as observed for glycolipids except for furan fatty acids in PB235 clone (13% versus 74% in total lipids) while linoleic acid in phospholipids of this clone represented 37%. The most abundant PC ion was *m/z* 786 (C18:0/C18:2, C18:1/C18:1 or FFA/C16:0), representing 23-26% in every clone. LPC, a product of the hydrolysis of PC, contained mainly linoleic acid in its structure (*m/z* 520; 32-40%).

The major molecular species of PE in fresh latex were *m/z* 714 and *m/z* 742. They represented altogether around 51% of total PE. The most probable diacylglycerol parts corresponding to these masses were C16:0/C18:2 or C16:1/C18:1 for *m/z* 714 and C18:0/C18:2, C18:1/C18:1 or FFA/C16:0 for *m/z* 742.

PI also showed two main molecular species. An ion with *m/z* 833 corresponded to the same fatty acid constituent as *m/z* 714 of PE while *m/z* 861 had the same fatty acid constituents as *m/z* 742. The amount of *m/z* 833 and *m/z* 861 were 44-47% and 27-37% of detected PI, respectively. As well as LPC, the main LPI molecular species contained linoleic acid in its structure (*m/z* 595; ~50%).

Major PA molecular species formed ions with *m/z* 723, corresponding to FFA/C18:2 and *m/z* 725 which corresponded to C18:3/C20:0 or FFA/C18:1. A clonal difference was detected as BPM24 PAcontained only 10% of the *m/z* 725 species but 25% of the *m/z* 723 species while the contrary was observed for PB235 clone. RRIM600 PA contained similar proportions of *m/z* 723 (19%) and *m/z* 725 (23%).

PC was the main phospholipid family found in every clone, representing 55 to 62% of total phospholipids. The amount of LPC was three times lower than that of PC in RRIM600 phospholipids (19% LPC against 62% PC), whereas it represented around half of PC quantity in PB235 and BPM24 phospholipids (28% LPC against 55% PC). PA represented around 10% of total phospholipids and only trace amount of PE, PI and LPI (1-5%) were observed. The scheme of global lipid profile is presented in figure 57.

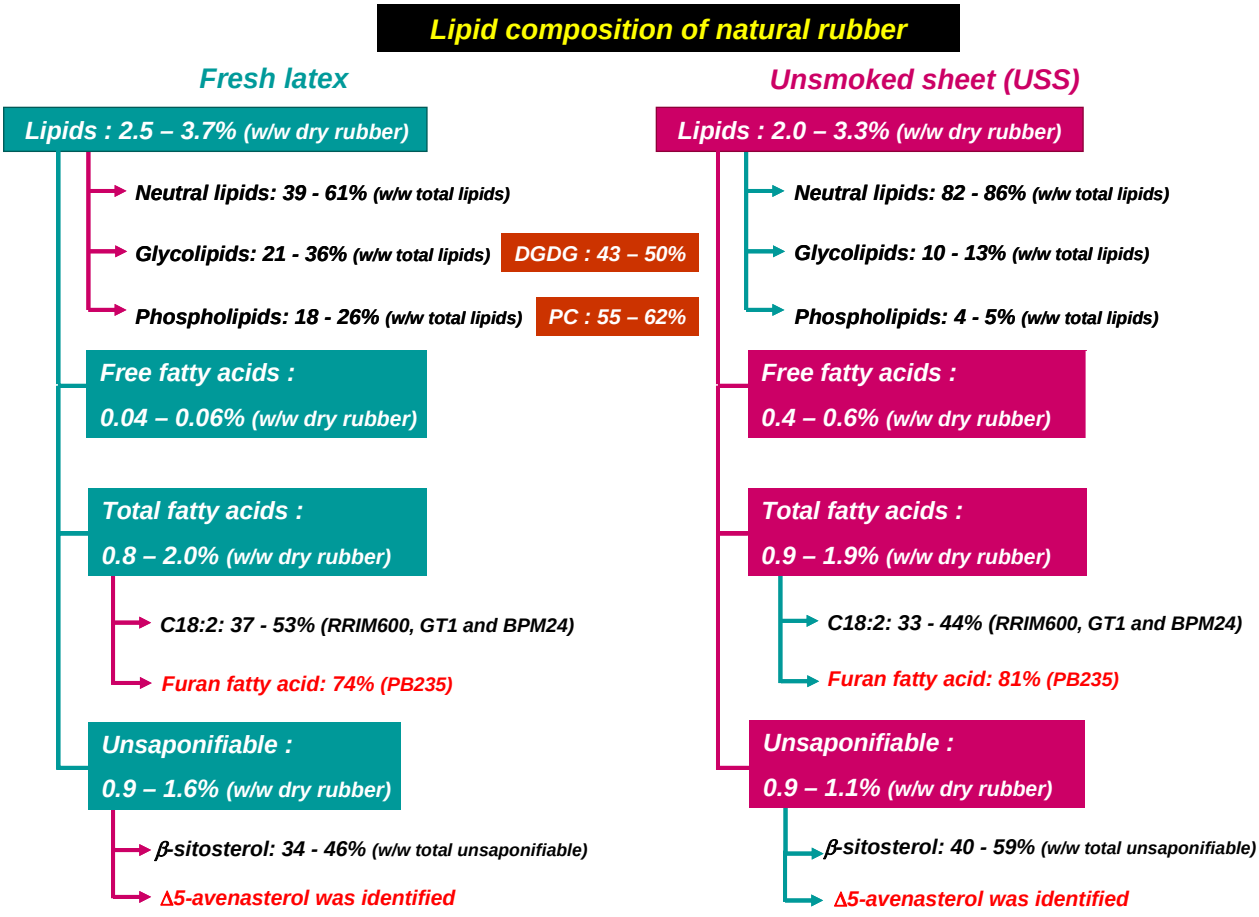


Figure 57 Global lipid profile of fresh latex and unsmoked sheet (USS) samples

Chapter 3 Natural Rubber Structure and Properties

1. Natural rubber structural characterization

1.1 Macrostructure characterization

Macrostructure and mechanical properties of USS prepared from monoclonal latex were determined through standardized methods: initial plasticity (P_0), plasticity retention index (PRI) and Mooney viscosity (ML). Total solid content (TSC) of the latex used for USS preparation was also measured. The mean values of each measurement from 15 samplings are presented in table 23.

Table 23 Means of total solid content (TSC), initial plasticity (P_0), plasticity retention index (PRI), and Mooney viscosity (ML).

Clones	TSC		P_0 (P_0 unit)		PRI (PRI unit)		$ML_{(1+4)\ 100}$	
	mean	SE	mean	SE	mean	SE	mean	SE
RRIM600 (old)	47.90^{a}	1.06	39.10^{b}	0.98	104.42^{a}	1.17	68.97^{b}	1.04
RRIM600 (young)	41.37^{c}	0.82	35.30^{c}	0.45	102.07^{ab}	0.86	61.93^{c}	0.79
GT1	47.07^{a}	1.06	35.69^{c}	0.77	101.23^{ab}	1.60	63.26^{c}	1.28
PB235	44.29^{b}	0.87	47.83^{a}	0.42	91.95^{c}	0.73	78.78^{a}	0.56
BPM24	40.79^{c}	0.75	33.55^{d}	0.45	100.92^{b}	1.02	58.88^{d}	0.51

Mean of 15 samplings; 3 repetitions were performed for each sampling

For each column, letters design group of mean(s) which are not significantly different (*t* test)

SE: standard error

Although TSC of latex is not considered as a quality index of dry rubber, it is a preliminary test that provides information about polyisoprene content of the sampled latices. Indeed, dry rubber content (DRC) constitutes 90% of total solid content. RRIM600 (old-18 years old) and GT1 (15 years old) clones had the highest TSC value while RRIM600 (young) and BPM24 clones with respective ages of 11 and 9 years gave the minimum TSC value. PB235 clone (12 years old) TSC value was in between. TSC of the samples were found to be related with ages of rubber trees. Though age depending character is well known (Jacob *et. al*, 1989), variation of TSC

is also brought about by other factor such as the tapping system and season (Yip, 1990).

Initial plasticity, P_0, of USS samples ranged from 34 to 48 depending on rubber clone. PB235 rubber gave the highest value of P_0 which indicated its higher resistance to flow whereas the USS from BPM24 clone gave the lowest P_0 value. A difference was observed between P_0 of the two USS obtained from RRIM600 clone. Such a difference from the same clone might be influenced by polyisoprene chain length and/or branching which may depend on the age of the tree (Tangpakdee *et. al*, 1996). However, GT1 clone which was also old (15 years), and had same TSC value (47) as RRIM600 (old) clone, gave P_0 value (35) as low as that of RRIM600 (young) clone. Further mesostructure analyses are requested to conclude.

Mooney viscosity, ML, may be used as an indicator of the amount of work required on the raw rubber to give a constant rheological property (Yip, 1990). As for initial plasticity, ML of studied rubbers was found to be clone-dependent. PB235 clone gave the highest ML value (79) while BPM24 gave the lowest one (59). Discrimination of rubber samples obtained from ML value was in accordance with that from P_0 as mentioned by Fuller (1988). Moreover, ML was reported to have a positive correlation with TSC (Le Roux *et. al*, 2000) in the case of STR20 but this claim was not observed in our study as the highest ML value was not obtained either from RRIM600 (old) or GT1 that had the highest TSC (~ 47%).

Plasticity retention index is often described as an assessment of the sensitivity of certain rubber to thermo-oxidation. Thermo-oxidation provokes scissions of poly- isoprene chain under high temperature resulting in a decrease of Wallace plasticity. Therefore, PRI should be always lower than 100, which was not the case for all of our samples (table 23). In addition, during the test, another phenomenon occurred under the influence of high temperature (140^oC, 30 min) and increased the plasticity. This phenomenon is called crosslinking which is the creation of a network between abnormal groups of polyisoprene chains, such as aldehyde, epoxides and lactones (Eng *et. al*, 1997), leading to an increase of Wallace plasticity

of the material (Gan, 1996). Therefore, PRI is in fact the measurement of the balance between scission (thermo-oxidation) and crosslinking which make the interpretation rather difficult. In our case, obtained PRI values were quite high (> 90) which is generally the case of acid coagulated rubber. Value exceeding 100 indicated that the crosslinking phenomenon described before was more important than the chain scission during the heating period of PRI test. PB235 clone distinguishes itself by a lower PRI (92) which may be due to either a higher sensitivity to thermo-oxidation (less antioxidant content) and/or lower ability of its polyisoprene chain to crosslink (less abnormal groups). This crosslinking was reported to involve more the short chain of polyisoprene (Ngolemasango *et. al*, 2003). Further molar mass distributions are then needed to understand the phenomenon.

1.2 Mesostructure characterization

Mesostructure of USS samples were analyzed using size exclusion chromatography (SEC). The studied parameters, weight average molar mass (M_w), number average molar mass (M_n), polydispersity index (I) and gel content are presented in table 24. The molar mass distribution (MMD) of USS for each *H. brasiliensis* clone is presented in figure 58.

It is necessary to state as a preliminary reminder that the molar mass distributions presented here concern only the soluble part of the polymer. The MMD of RRIM600, GT1 and BPM24 clones showed a bimodal shape where the higher molar mass peak height is higher than the lower molar mass peak height. This distribution was qualified by Subramaniam (1993) as type 2. PB235 clone showed a unimodal distribution with shoulder (type 3). PB235 clone seems then to contain less short chains and more long ones. Ratio between M_w/M_n known as polydispersity index (I) was consequently significantly lower for PB235 (5.98) compared with that from the other clones (>7.8). It confirmed that PB235 rubber has a narrow distribution of its molar mass. The presence of fewer short chains in PB235 may reduce the crosslinking ability of polyisoprene chain as suggested by Ngolemasango *et. al.* (2003). This could explain the lower PRI of PB235.

The M_n and M_w values were found to be different as suggested by MMD shape. PB235 clone showed distinct higher values of both molar masses (M_w=1340 kg/mol, M_n=226 kg/mol). No significant difference was observed for the other studied clones. Bonfils (1999) found clear positive correlation between P_0 and M_w but mentioned that this relationship was dependent not only on clonal origin but also on the post harvest treatments (off latex or cuplump). In our study, a global P_0 and M_w correlation could be observed as shown in figure 59 even if this relation is not clear when data are observed separately by clone.

Concerning gel (non soluble part), macrogel was reported to be secondary bonded network incorporating microgel and most of the proteinaceous material (Bengtsson and Stenberg, 1996). Fuller (1988) suggested that nitrogen content of dry rubber which indicated the quantity of present proteins may possibly provide an indication of relative amounts of gel. However, as seen from table 24, observed gel content was not correlated to nitrogen content. Gel could be formed by various mechanisms in which proteins are either involved or not (Ehabe *et. al*, 2006). Moreover, the relationship between gel and certain property was reported only in the case where microgel and macrogel quantity are added together and expressed as "total gel content" (Ehabe *et. al*, 2005). In our study, RRIM600 (old) clone contained the highest amount of macrogel, using cyclohexane as solvent, with about 15% while the other clone rubber contained around 10% of macrogel. The content of microgel ranged from 39% (GT1) to 52% (PB235). High total-gel containing clones were PB235 and RRIM600 (old) with more than 60% of its isoprene being non-soluble. Those two clones are also the one which displayed the highest P_0.

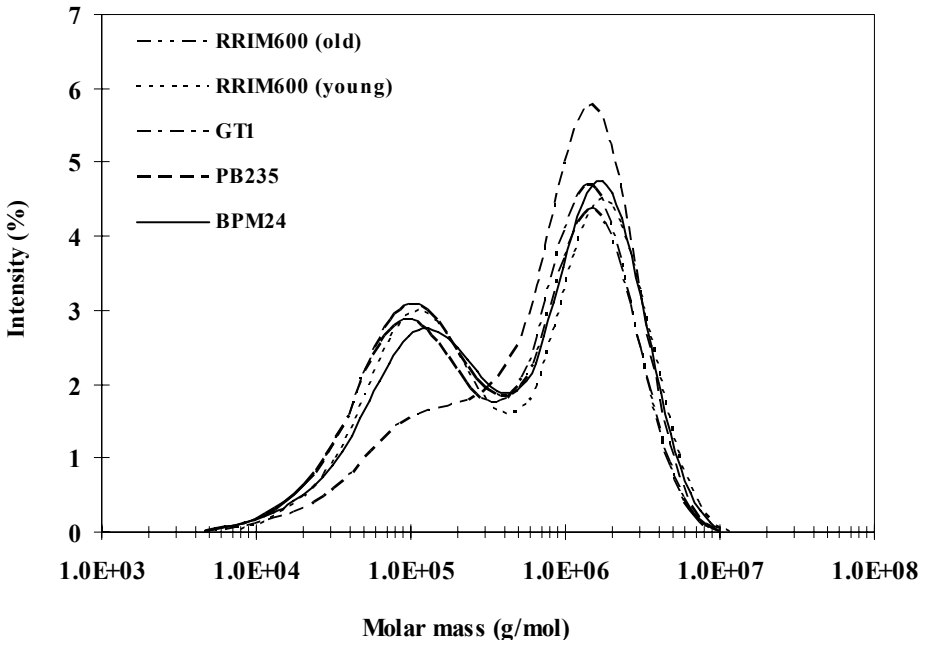
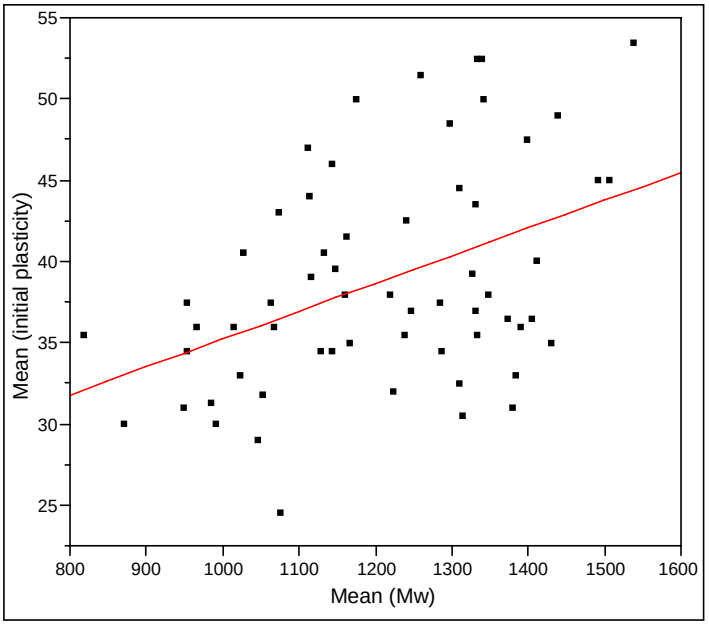


Figure 58 Example of molar mass distributions of USS from studied *H. brasiliensis* clones. (All sample were from 13 December 2005)



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	568.0492	568.049	15.5044
Error	60	2198.2744	36.638	Prob > F
C. Total	61	2766.3236		0.0002

Figure 59 Correlation between weight average molar mass (M_w) and initial plasticity (P_0)

Table 24 Mesostructure parameters (weight average molar mass (M_w), number average molar mass (M_n), polydispersity index (I), gel content and nitrogen content of USS rubber.

Clones	Mw (kg/mol)		Mn (kg/mol)		I		Macrogel (%)		Microgel (%)		Total gel (%)		% Nitrogen	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
RRIM600 (old)	1186.31^{b}	39.28	150.00^{b}	6.55	7.89^{a}	0.26	15.13^{a}	1.69	48.29^{bc}	2.74	62.62^{a}	3.47	0.35^{b}	0.01
RRIM600 (young)	1204.71^{b}	17.79	145.83^{b}	2.56	8.39^{a}	0.19	11.43^{b}	0.97	43.30^{cd}	1.43	55.02^{bc}	1.26	0.38^{a}	0.01
GT1	1172.05^{b}	32.23	150.68^{b}	3.28	7.80^{a}	0.23	10.70^{b}	1.19	39.30^{d}	3.01	49.51^{c}	2.95	0.35^{b}	0.01
PB235	1340.03^{a}	16.95	226.22^{a}	5.37	5.98^{b}	0.16	10.71^{b}	0.93	52.54^{a}	1.09	63.03^{a}	1.30	0.34^{b}	0.01
BPM24	1160.56^{b}	24.86	144.29^{b}	2.84	8.13^{a}	0.15	11.43^{b}	0.97	47.74^{b}	1.65	59.16^{ab}	1.77	0.39^{a}	0.01

Mean of 15 samplings; 3 repetitions were performed for each sampling

For each column, letters design group of mean(s) which are not significantly different (*t* test)

SE: standard error

(%) was expressed versus dry rubber weight (w/w)

2. Study of breakdown and vulcanization behaviors

2.1 Breakdown behavior

2.1.1 Mastication and breakdown index (BI)

Mastication of USS samples from different *H. brasiliensis* clones was performed under two conditions: thermal (140°C starting temperature, 50 rpm rotor speed) and mechanical (50°C starting temperature, 100 rpm rotor speed). Under "thermal" mastication, scissions of chains are mainly due to thermo-oxidation while under "mechanical" conditions, scissions are obtained mainly by mechanical shearing. Three parameters were monitored during mastication (torque, temperature and cumulated mechanical energy). Examples of evolution of these parameters under the two conditions are presented in figure 60.

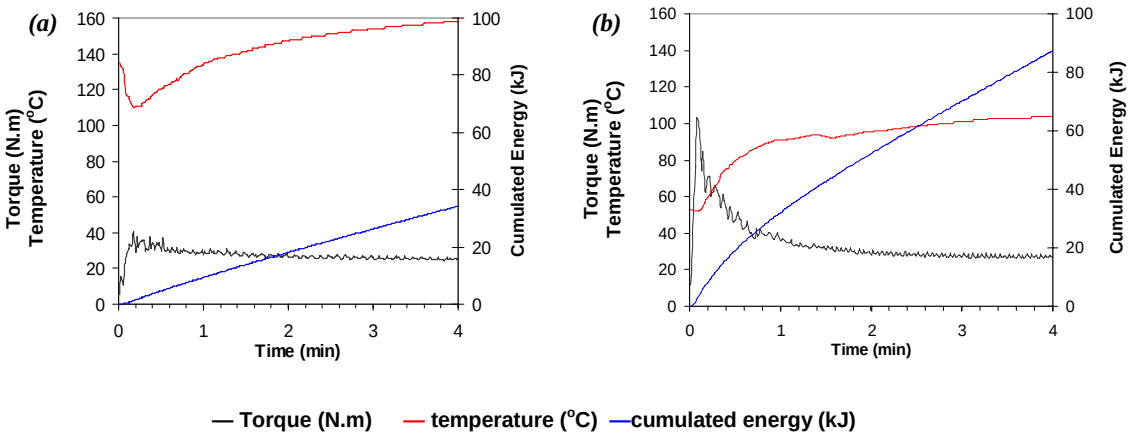


Figure 60 Mastication of RRIM600 rubber sheet samples under thermal conditions (a) (140°C starting temperature, 50 rpm rotor speed for 4 min) and mechanical conditions (b) (50°C starting temperature, 100 rpm rotor speed for 4 min).

In both cases, torque increased after introducing the sample into internal mixer and then decreased once the degradation occurred. Range of variation of torque values was larger under mechanical conditions. Consequently, the cumulated mechanical energy recorded from thermal and mechanical mastication showed obvious difference as mechanical energy consumption observed from

mechanical degradation (> 80 kJ) is much higher than that of thermal conditions (< 40 kJ). Torque value of thermal conditions (figure 60a) seemed to become stable in a shorter time than that observed under mechanical conditions (figure 60b). Temperature of both conditions was found to increase due to heat generation during rubber mastication. After 4 min of mastication, temperature was around 160°C for thermal conditions though initial temperature was set at 140°C while a value of around 100°C was recorded under mechanical mastication (starting temperature set to 50°C).

From those observed parameters, breakdown behavior of NR samples under each mastication condition was assessed by breakdown index (BI) following method of Lim and Ong (1985). BI has been used to monitor the breakdown behavior of rubber by following the change of ML before and after mastication. Generally, ML provides information about the energy required to masticate the raw rubber until desirable viscosity is obtained. BI is the ratio of relative drop of ML to specific consumed mechanical energy. The breakdown indexes calculated from two studied conditions are presented in table 25. In this study, drop of P_0 was also considered to calculate another breakdown index. The results obtained using drop of P_0 were less discriminating and are therefore not shown here.

Table 25 Breakdown index of USS

clones	Breakdown index (BI)			
	Thermal conditions*		Mechanical conditions**	
	mean	SE	mean	SE
RRIM600 (old)	1.58^{b}	0.11	1.14^{ab}	0.03
RRIM600 (young)	2.12^{a}	0.11	1.24^{a}	0.04
GT1	1.89^{ab}	0.08	1.18^{ab}	0.03
PB235	0.60^{c}	0.06	0.54^{c}	0.03
BPM24	1.86^{b}	0.10	1.06^{b}	0.05

Mean of 15 samplings

For each column, letters design group of mean(s) which are not significantly different (*t* test)

SE: standard error

* Thermal conditions; 140°C starting temperature, 50 rpm rotor speed

** Mechanical conditions; 50°C starting temperature, 100 rpm rotor speed

The BI values were found to vary with clones. Maximum degradation for a given amount of mechanical energy was from USS of RRIM600 (young) with the respective BI values from thermal condition and mechanical condition of 2.12 and 1.24. The lowest BI was obtained from PB235 rubber with the value of 0.60 and 0.54 from thermal and mechanical conditions, respectively. For sheet rubber with bimodal distribution (RRIM600 (old and young), GT1 and BPM24), BI were higher than BI from unimodal distribution rubber (PB235). In the study on clonal rubber of TSR3CV grade, no significant differences were found between unimodal (PB217) and bimodal (PR107) samples (Ehabe *et. al*, 2005). In the other words, under the same mastication condition, BI alone was not enough to characterize breakdown behavior of rubber even they were different in molar mass distribution. In addition, Lim and Ong (1985) who worked on breakdown behavior of commercially available NR grades (SMR 5, 10, 20, L, CV and RSS1) found that sheet rubber (RSS1) was 2 to 4 times less degraded compared to the other grades of natural rubber.

Though BI values in our study could differentiate the breakdown behavior of rubber from different *H. brasiliensis* clones, BI values from two different mastication conditions were hardly comparable. Indeed, thermal energy input which help the breakdown especially when high temperature conditions are applied is not taken into account in the BI calculation. Parameters used to calculate the breakdown index, i.e. relative drop of ML and consumed mechanical energy were therefore considered individually.

2.1.2 Relative drop of ML and mechanical energy input

Table 26 shows the respective values of relative drop of ML and specific energy (calculated by dividing the total energy input used for mastication with volume of the sample). The higher relative drops of ML from mechanical conditions than those from thermal conditions indicated the higher breakdown of rubber from mechanical condition. Logically, the mechanical energy consumption associated with this higher drop of ML was also higher. Roughly, under mechanical mastication condition, the relative drop of ML was less than twice higher than under

thermal conditions while the mechanical specific energy input was 2.5 times higher. This explains why BI was lower under mechanical conditions for every clone but PB235. In the latter case, BI was almost the same in both conditions, meaning that even the drop of ML was more important under mechanical condition, the relative mechanical energetic cost of these drops were the same. Thus PB235 seems quite resistant to thermal oxidation under shearing, meaning that its low PRI value could be more attributed to its low ability to crosslink under PRI heating step rather than its sensitivity to thermo-oxidation.

At low temperature (<100°C), the chain scission is mainly done by mechanical shearing. The mobility of polyisoprene is rather low and their disentanglement is rather difficult. Therefore, the degradation is obtained solely by mechanical shearing as new radical cannot recombine since they rapidly react with oxygen (Fries and Pandit, 1982 ; Dimier *et. al*, 2004 ; Ehabe *et. al*, 2006). Under this so-called ¨mechanical¨ mastication, PB235 distinguishes itself with the highest consumed specific mechanical energy (1713 MJ/m^3) for the lowest drop of ML (-10%).

Table 26 Ratio of chain scission and energy consumption from thermal and mechanical mastication

Clones	Relative drop of ML (%)				Energy input (MJ/m^3)			
	Thermal*		Mechanical**		Thermal*		Mechanical**	
	mean	SE	mean	SE	mean	SE	mean	SE
RRIM600 (old)	10.52^{b}	0.74	19.16^{ab}	0.37	640.93^{ab}	8.99	1687.03^{ab}	20.03
RRIM600 (young)	12.89^{a}	0.67	20.50^{a}	0.75	611.88^{c}	9.92	1651.46^{b}	14.57
GT1	11.88^{ab}	0.57	19.73^{a}	0.68	622.03^{bc}	9.60	1645.73^{b}	7.53
PB235	3.96^{c}	0.40	9.59^{c}	0.64	659.29^{a}	4.85	1712.93^{a}	11.83
BPM24	11.31^{b}	0.59	17.36^{b}	0.75	595.72^{c}	9.59	1596.48^{c}	15.26

Mean of 15 samplings

For each column, letters design group of mean(s) which are not significantly different (*t* test)

SE: standard error

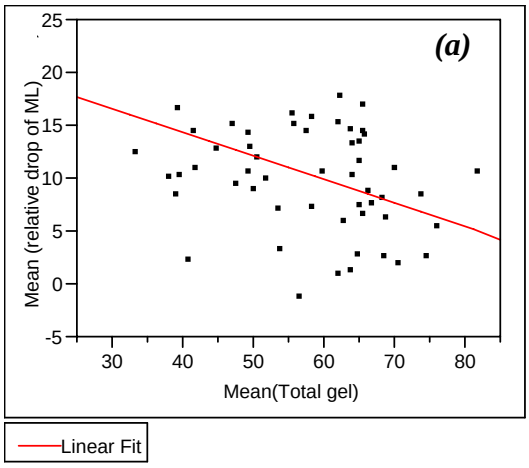
* Thermal condition; 140^{o}C starting temperature, 50 rpm rotor speed

** Mechanical condition; 50^{o}C starting temperature, 100 rpm rotor speed

2.1.3 Relationships with other structural parameters

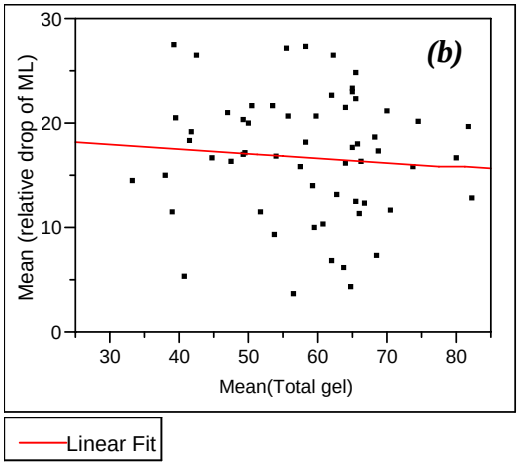
2.1.3.1 Mesostructure: initial total gel content, Mw and Mn

For both conditions, it was noted that the drop of ML obtained from PB235 rubber was much less than the other clones even the same level of mechanical energy was consumed. The correlation between drop of ML during mastication and mesostructure were analyzed. It was found that total gel had a negative correlation with the relative drop of ML obtained from thermal mastication (figure 61a). This correlation confirmed the stiffening effect of gel. Nevertheless, under mechanical conditions no correlation between drop of ML and total gel was found (figure 61b).



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	394.8271	394.827	11.9439
Error	53	1752.0138	33.057	Prob > F
C. Total	54	2146.8408		0.0011



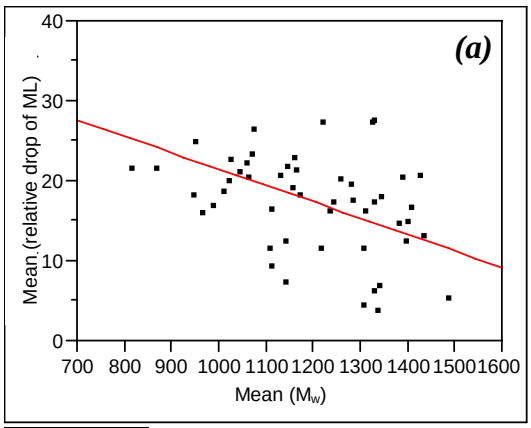
Analysis of Variance

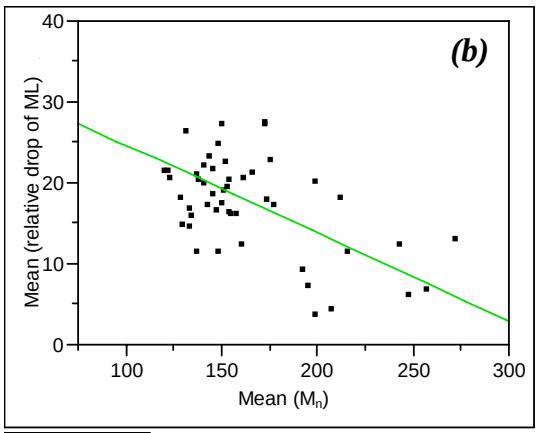
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	13.5541	13.5541	0.3806
Error	57	2030.1561	35.6168	Prob > F
C. Total	58	2043.7102		0.5398

Figure 61 Correlation between initial total gel content (%/ dry rubber weight) and relative drop of ML after thermal mastication (a) and mechanical conditions (b).

M_w and M_n also expressed the same relationship with the drop of ML with both mastication conditions as shown in figure 62 and 63. PB235 which had significantly higher M_w and M_n values than the other clones showed less degradation. These correlations could lead to the conclusion that under mechanical

conditions only chain lengths regulate the drop of ML while at high temperature the gel content becomes a factor affecting on breakdown.





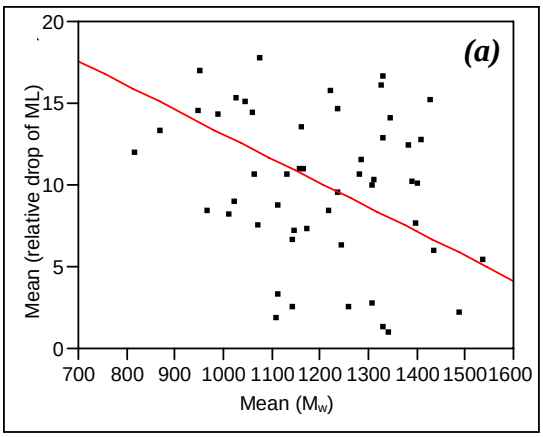
Analysis of Variance

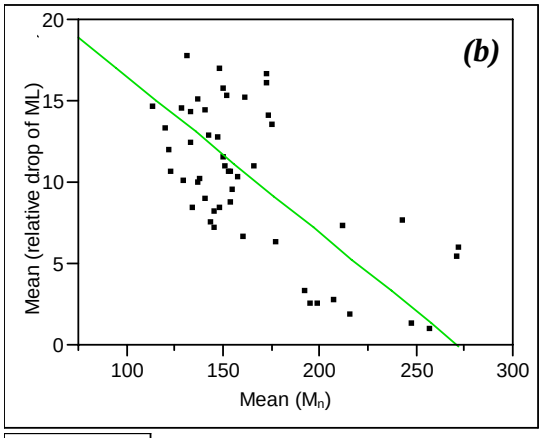
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	631.5847	631.585	16.0955
Error	49	1922.7535	39.240	Prob > F
C. Total	50	2554.3382		0.0002

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	805.7346	805.735	24.3357
Error	48	1589.2365	33.109	Prob > F
C. Total	49	2394.9711		<.0001

Figure 62 Correlation between initial M_w (a) and M_n (b) and relative drop of ML after mechanical mastication.





Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	361.9962	361.996	11.7176
Error	51	1575.5621	30.893	Prob > F
C. Total	52	1937.5583		0.0012

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	758.7465	758.747	34.0818
Error	50	1113.1251	22.263	Prob > F
C. Total	51	1871.8716		<.0001

Figure 63 Correlation between initial M_w (a) and M_n (b) and relative drop of ML after thermal mastication

2.1.3.2 Plasticity retention index (PRI)

PRI from both conditions before and after mastication was measured as presented in table 27. As mentioned before, PRI indicates the balance between thermo-oxidation and crosslinking which occurs under high temperature conditions (140°C, 30 min). Except for GT1, PRI values increased after thermal mastication while those from mechanical conditions were lower.

Table 27 PRI measured before and after thermal and mechanical degradation

clone	PRI before mastication		PRI after thermal mastication		PRI after mechanical mastication	
	mean	SE	mean	SE	mean	SE
RRIM600 (old)	100.44[a]	1.55	101.39[b]	1.57	94.64[b]	1.43
RRIM600 (young)	102.34[a]	1.17	105.96[a]	1.19	99.48[a]	1.08
GT1	101.29[a]	1.55	99.90[b]	1.57	91.60[b]	1.43
PB235	91.47[b]	1.13	94.41[c]	1.15	85.81[c]	1.04
BPM24	100.18[a]	1.17	102.31[b]	1.19	94.00[c]	1.08

Mean of 15 samplings

For all PRI values (3 columns), letters design group of mean(s) which are not significantly different (*t* test)

SE: standard error

Considering PRI results alone, it is likely that after thermal mastication, crosslinking reaction which occurs under PRI measurement is more important than chain scission (PRI > 100) and that this phenomenon is more important than before mastication (PRI after thermal mastication > PRI before thermal mastication).

In order to interpret further this result, P_0 and P_{30} before and after mastication were compared as presented in figure 64. Among two mastication conditions, the decrease of P_0 and P_{30} after mechanical mastication was much more important than that of thermal mastication. Theses figures show clearly that thermal mastication keep or even increase the crosslinking ability of the rubber, while mechanical mastication seems to decrease partially this ability.

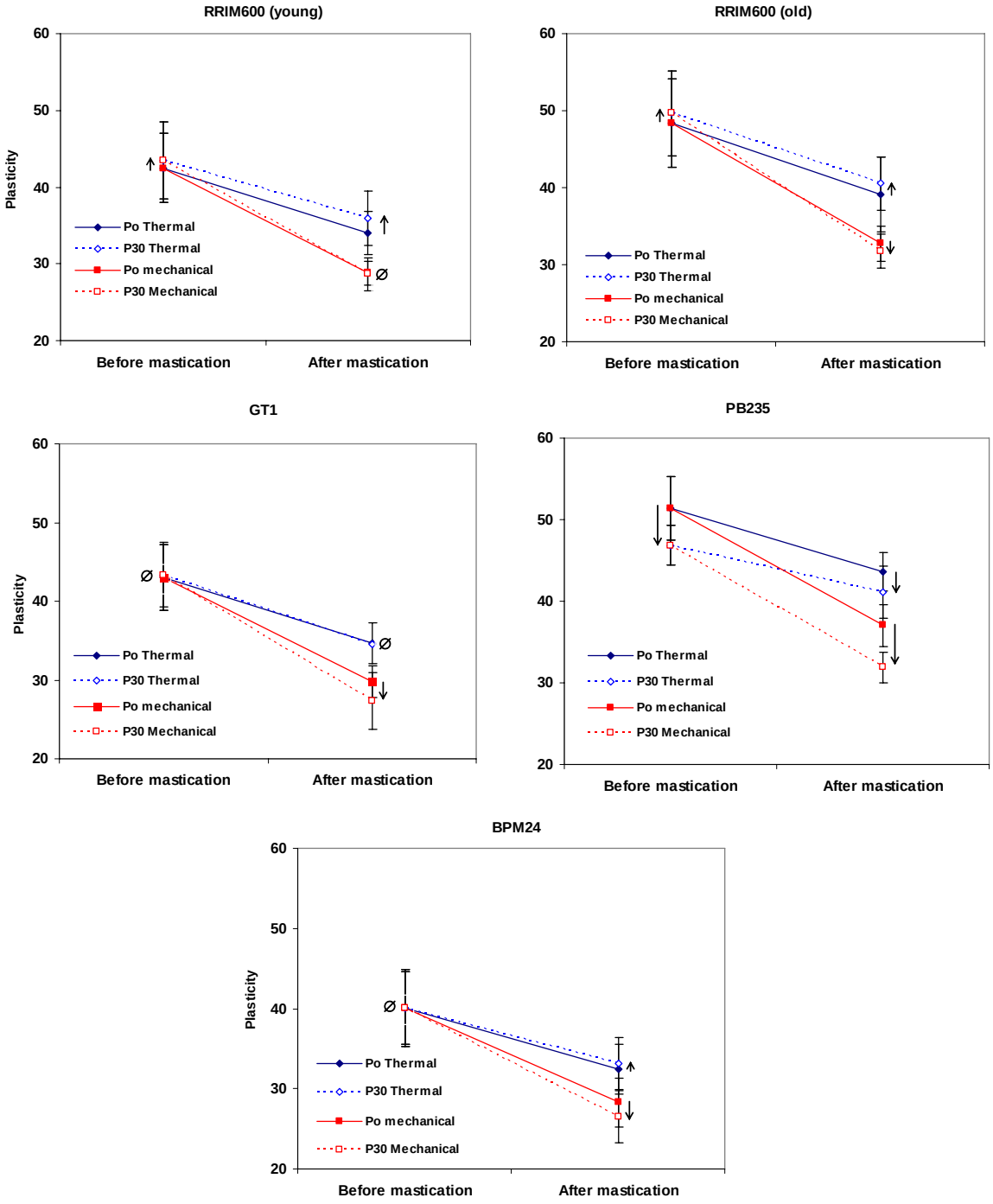


Figure 64 P_0 and P_{30} of USS samples before and after thermal and mechanical mastication. Symbols indicate the reaction which occurred under PRI measurement (∅: balance between crosslinking and chain scission, ↑: crosslinking reaction was paramount and ↓: chain scission reaction was paramount).

In other words, rubber masticated under mechanical conditions got a lower P_0 and a lower ability to crosslink under PRI heating conditions compared to rubber masticated under high temperature conditions. It could be supposed that thermal mastication may have activated more abnormal groups which could help the crosslinking phenomena and that this "activation" requires a minimum amount of thermal energy which is not provided during mechanical mastication.

2.2 Vulcanization

In attempt to study the cure characteristics of different *H. brasiliensis* clones, USS rubber from each sampling was vulcanized. The formulation used in this study contained 0.8 phr of CBS (accelerator), 5 phr of zinc oxide, 2 phr of stearic acid (activators), 2.4 phr of sulfur (sulfur donor) and 1 phr of Wingstay-L (antioxidant). This formulation contained the general ingredient needed for common vulcanization but filler. The parameters derived from cure curves are presented in table 28.

Table 28 Vulcanization parameters from different *H. brasiliensis* clones

clones	t_{s2} (min)		$t_{c(90)}$ (min)		T_L (in.lb)		T_H (in.lb)	
	mean	SE	mean	SE	mean	SE	mean	SE
RRIM600 (old)	6.63^{b}	0.22	11.65^{b}	0.21	12.14^{b}	0.26	67.94^{a}	0.33
RRIM600 (young)	6.15^{bc}	0.20	10.95^{bc}	0.23	11.28^{c}	0.13	66.12^{b}	0.27
GT1	6.19^{bc}	0.31	11.24^{bc}	0.29	11.16^{cd}	0.18	65.35^{bc}	0.64
PB235	7.48^{a}	0.20	13.27^{a}	0.24	12.82^{a}	0.17	64.20^{d}	0.32
BPM24	5.90^{c}	0.20	10.65^{c}	0.22	10.70^{d}	0.10	65.04^{c}	0.24

Mean of 15 samplings; 3 repetitions were performed for each sampling
For each column, letters design group of mean(s) which are not significantly different (*t* test)
SE: standard error

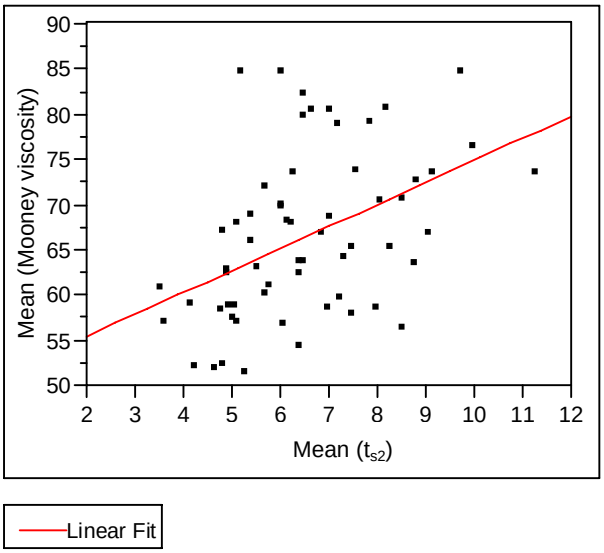
The scorch time (t_{s2}) is the time taken for the minimum torque value to increase by two units. It can be seen that the shortest scorch time was obtained from BPM24 rubber (5.90 min), whereas PB235 gave the longest scorch time (7.48 min). It is known that the main factor of influence of scorch time is the nature of accelerator contained in formula (Krejsa and Koenig, 1992). Since all the rubber was vulcanized with the same formula, the difference in scorch time might be due to the nature of

rubber: initial viscosity and/or natural accelerator content may be of importance in this matter. Concerning initial viscosity, a positive correlation between initial ML of raw rubber and scorch time was observed among our samples (figure 65). PB235 rubber which was more viscous than the other clones as seen from its higher ML value presented a significantly longer scorch delay (7.5 min). Likewise, the rubber with the lowest initial ML, namely BPM24, had the shortest scorch time (5.9 min).

The optimum cure time ($t_{c(90)}$) which is the time when 90% of the increase of torque is accomplished was found to be longest in PB235 rubber compound (13.27 min) which may be due to the longer scorch time. Indeed, BPM24 rubber coumpound had the shortest t_{s2} and also the shortest $t_{c(90)}$ (10.65 min).

Concerning minimum torque (T_L) PB235 and BPM24 compound presented the two extreme values with 12.8 and 10.7 in.lb, respectively. The minimum torque is an indication of the stiffness of unvulcanized test specimen. T_L has been described to often correlate well with ML of a raw rubber (Ignatz-Hoover and To, 2004). This correlation was observed in this study (figure 66).

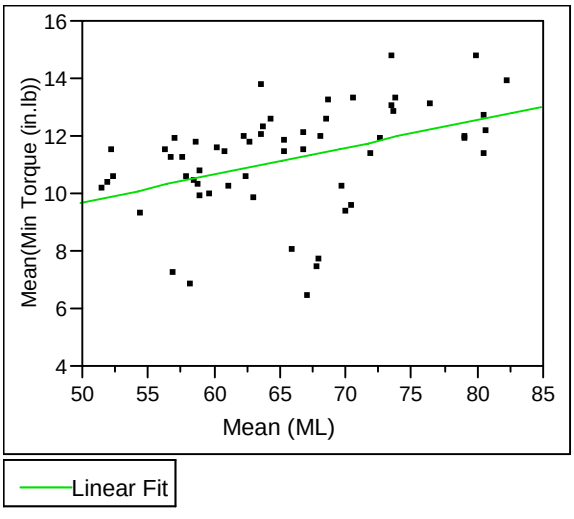
Except for the maximum torque, RRIM600 and GT1 compound showed vulcanization parameters values situated in between those observed from PB235 and BPM24. In case of maximum torque (T_H), which is a measure of the stiffness of the fully vulcanized test specimen at the vulcanization temperature, the situation is different. Indeed PB235 rubber which has the highest ML in raw state presented the lowest T_H. This rubber display a particular vulcanization behavior: the vulcanization starts later (longest t_{s2}), from a compound which has a higher stiffness (highest T_L) but lead to a vulcanizate which has a lower stiffness at vulcanization temperature (lowest T_H). The initial higher stiffness could be explained by the properties of raw PB235 rubber that has the highest weight average molar mass therefore higher ML. Meanwhile, the lowest maximum torque of obtained vulcanizate may be due to its less efficient sulfur crosslinking (Ignatz-Hoover and To, 2004). Moreover, non-isoprene component could also affect either scorch time or vulcanization rate.



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	959.2088	959.209	14.3162
Error	59	3953.1026	67.002	Prob > F
C. Total	60	4912.3114		0.0004

Figure 65 Correlation between Mooney viscosity (ML) and scorch time (t_{s2})



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	35.24452	35.2445	12.6338
Error	56	156.22305	2.7897	Prob > F
C. Total	57	191.46757		0.0008

Figure 66 Correlation between Mooney viscosity (ML) and minimum torque (T_L).

3. Conclusion

Macrostructure and mechanical properties of USS prepared from monoclonal latex were determined through standard measurements. PB235 showed the highest value of Wallace plasticity P_0 (48) while BPM24 and RRIM600 (young) showed the minimum value (40) for this parameter. Similar results were also observed for ML as PB235 showed the maximum ML value (79) while the minimum ML was observed for BPM24 clone (59). This difference could be due not only to the clone but also to age as differences in P_0 and ML values were also observed between RRIM600 (young) and RRIM600 (old). Though PB235 has the highest value of P_0 and ML, it was found to have the lowest PRI (92) value which means that it is either more sensitive to thermo-oxidation or have a lower ability to crosslink under thermal treatment undergone during PRI test.

Concerning mesostructure, RRIM600, GT1 and BPM24 rubbers were found to have a bimodal molar mass distribution (type 2) which contained both long and short chain polyisoprenes while unimodal distribution (type 3) was observed for PB235 rubber. PB235 rubber contained mainly longer chain as seen from its higher average molar mass compared to the other clones. Macrogel content in studied rubber was in the range of 10-15% while that of microgel was from 39 to 52%. RRIM600 (old) rubber had the highest macrogel content (15%) while PB235 had the highest microgel content (52%). Moreover, these two clones were the highest total gel containing clones (~63% w/w dry rubber).

Breakdown index (BI) values were determined in thermal and mechanical mastication conditions by calculating the ratio between the drops of ML to mechanical energy consumed. BI was found to be clone-dependent. PB235 and RRIM600 (young) showed respectively the lowest and highest BI value in both conditions. However, BI from two mastication conditions was hardly comparable due to the fact that thermal energy input which facilitate rubber breakdown especially under thermal mastication was not taken into account. Therefore, other parameters observed during mastication were considered for further breakdown behavior

explanation. The relative drop of ML obtained from PB235 clone was lower than those from other clones while the amount of mechanical energy used was the same. This was found to be related with the peculiar mesostructure of PB235 (highest M_w, M_n and total gel content). The total gel content was negatively correlated with relative drop of ML under thermal mastication while M_w and M_n were negatively correlated with both relative drop of ML from two mastication conditions. It is likely that polyisoprene chain length influences on rubber breakdown under mastication at temperature below 100°C. At higher temperatures, the disentanglement of polyisoprene chain is facilitated and total gel seems to become the predominant factor of rubber breakdown. In addition, rubber obtained after mechanical mastication seems to partially lose the crosslinking ability as observed when measuring PRI after mastication in both conditions. This could be due to the fact that the crosslinking ability could be "activated" under high temperature only.

Vulcanization characteristics of each sample showed an obvious clonal difference which could be linked to their properties in unvulcanized state. USS sample which had a lower P_0 and ML value such as BPM24 rubber (P_0 = 40 and ML = 59) resulted in a lower T_L value (10.7 in.lb). Meanwhile, RRIM600 (old) and PB235 rubber which have higher P_0 and ML showed the highest values of T_L (12.14 and 12.82 in.lb respectively). In addition, the stiffness of the rubber compound due to higher ML of raw rubber may be one of the factors that could delay scorch times as found with PB235 clone.

The differences in the properties of rubber sheet sample among studied clones were only partially explained by the difference of the mesostructure. Nevertheless, certain properties such as PRI which is very high even after mastication at high temperature could not be explained by mesostructural approach only and non-isoprene component may be involved. Moreover, the peculiar characteristics of PB235 may be also linked to its lipid composition which is particular as stated in Chapter 2. Therefore, various possible roles of lipid composition on natural rubber structure and properties will be considered and presented in next chapter.

Chapter 4 Correlations between lipid composition and natural rubber structure and properties

Correlations between lipid composition and natural rubber structure and properties of studied clones namely RRIM600 (two different ages defined as old and young respectively), GT1, PB235 and BPM24 were analyzed using the data obtained from 15 samplings (April 2004 – December 2006). In our study, two measurable parameters, clone and season, were chosen as source of variability as mentioned earlier in Chapter 1. Concerning season, three levels were defined based on accumulated rainfall collected 30 days before sampling day. When accumulated rainfall amount was higher than 300 mm the season was defined as "Rainy". "Dry" season corresponded to an accumulated rainfall below 100mm while intermediate rainfall level (100-300mm) was attributed to "intermediate" season.

The variability of the collected data, i.e. lipid composition, natural rubber structure and properties was represented using principal component analysis (PCA). The representation of every sample in function of the two first PCA axes is shown in figure 67 while figure 68 shows the variables represented in correlation circle.

The first axis of PCA analysis represented 26% of total variation, separated clearly PB235 from the other clones (figure 67). No seasonal effect was observed. The variables (figure 68) which mostly contributed in the first axis were principally the variables in the group of fatty acid composition from rubber sheet (55 – 80) excepted free fatty acids (63 and 72) and C14:0 (55 and 64). Those latter variables were not different between PB235 and the other clones. The importance of furan fatty acid in axis 1 appeared clearly (47 and 48). Other types of variable contributed to the discrimination between PB235 and the other clones: the most noticeable were mastication parameters (17 to 22) and mesostructural ones (83, 85 and 87).

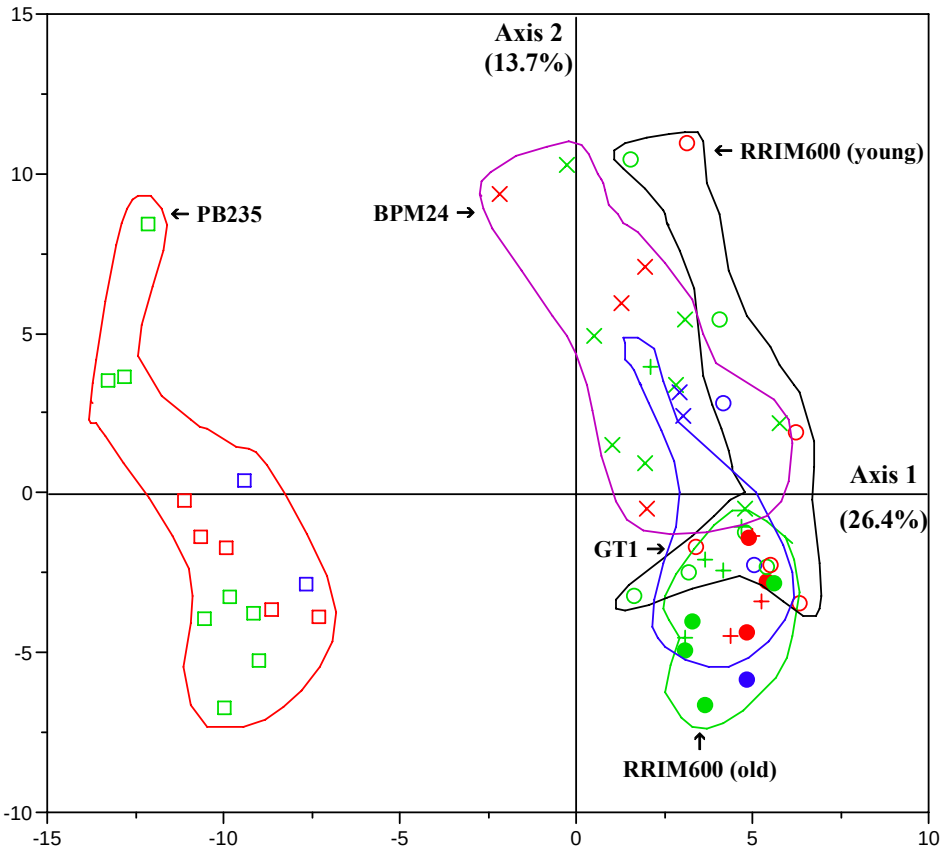


Figure 67 Principle component analysis of mean values of lipid composition and natural rubber structure and properties represented by the two forst axes (clones: ●: RRIM600 (old); ○: RRIM600 (young); ✚: GT1; □: PB235 and ×: BPM24, season: ■ dry season ■ intermediate and ■ rainy season)**.**

The second axis which represented 14% of the total variability showed less evident clonal difference. Nevertheless, BPM 24 seemed different from RRIM600 old and GT1 clones (almost all samples from those 2 latter clones have negative coordinates on this axis while BPM24 coordinates are almost all positives). Seasonal effect was still not predominant. This finding confirmed that the principle factor of variability for measured parameters was clone. The variation that mostly contributed the second axis was unsaponifiable components (especially fatty alcohols 116 and 117), free fatty acid (63), and polar lipids (glycolipid (1) and phospholipid (5)) All these variables varied independently to those identified for the first axis.

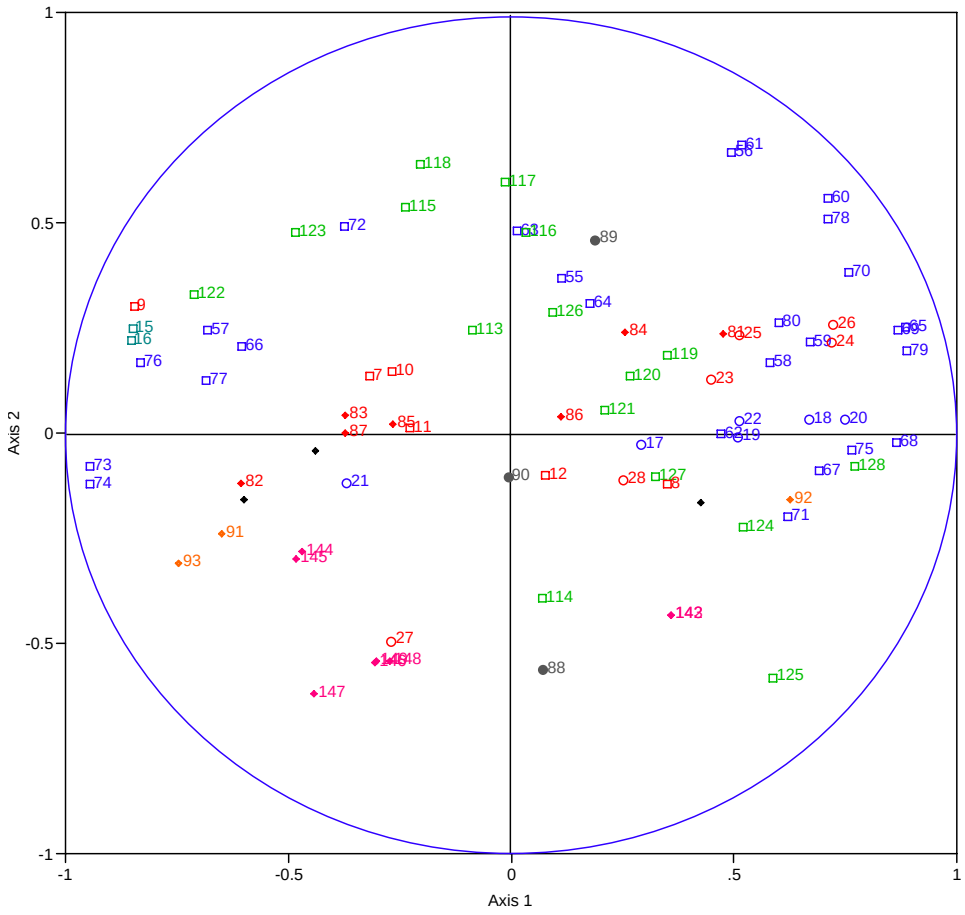


Figure 68 Circle of correlation among lipid composition and structure and property parameter of USS samples. Details of number and symbol of each variable are listed in table 29.

Analysis of the two first axis of PCA (representing together 40% of the total variability) leads the following remarks:

- there is not marked seasonal effect (in this matter it is worth noting that the variable total rain (90) is almost in the center of the correlation circle which means that this variable do not contribute in neither axis 1 nor axis 2).
- Clonal effect appears clearly on axis 1 and to a lesser extent on axis 2. It confirms the adopted presentation by clone as mentioned in Chapter 1.
- PB235 samples are clearly separated by the first axis. Data from PB235 clone will therefore be presented apart in the further analysis of correlations.

The strongest pair correlation between lipid data and the other measured variables (structures and properties) will then be presented in details.

Table 29 Number and symbol representing each variable in circle of correlation

number	symbol	variables	category	number	symbol	variables	category
7	□	glycolipids (%w/w dry rubber)	Lipid classes	78	□	unsaturated FA (%w/w dry rubber)	fatty acid composition
8	□	glycolipids (%w/w lipids)	Lipid classes	79	□	unsaturated FA (%w/w total fatty acids)	fatty acid composition
9	□	neutral lipids (%w/w dry rubber)	Lipid classes	80	□	saturated FA (%w/w dry rubber)	fatty acid composition
10	□	neutral lipids (%w/w lipids)	Lipid classes	81	◇	I	mesostructue
11	□	phospholipids (%w/w dry rubber)	Lipid classes	82	◇	Mn	mesostructue
12	□	phospholipids (%w/w lipids)	Lipid classes	83	◇	Mw	mesostructue
15	□	Lipid extract (%w/w dry rubber)	Lipid extract	84	◇	Mz	mesostructue
16	□	lipid extract weight (mg)	Lipid extract	85	◇	Total gel	mesostructue
17	○	%drop Po mechanical	breakdown (mechanical masticaiotion)	86	◇	macrogel	mesostructue
18	○	%drop ML mechanical	breakdown (mechanical masticaiotion)	87	◇	microgel	mesostructue
19	○	BI Po mechanical	breakdown (mechanical masticaiotion)	88	●	age	other
20	○	BI ML mechanical	breakdown (mechanical masticaiotion)	89	●	Nitrogen content sheet	other
21	○	Cumulated Energy mechanical	breakdown (mechanical masticaiotion)	90	●	total rain month (mm)	other
22	○	PRI mechanical	breakdown (mechanical masticaiotion)	91	◇	ML_RRIT	specification
23	○	%drop Po thermal	breakdown (thermal masticaiotion)	92	◇	PRI_RRIT	specification
24	○	%drop ML thermal	breakdown (thermal masticaiotion)	93	◇	Po_RRIT	specification
25	○	BI Po thermal	breakdown (thermal masticaiotion)	94	◇	ML_Fr	specification_Fr
26	○	BI ML thermal	breakdown (thermal masticaiotion)	95	◇	PRI_Fr	specification_Fr
27	○	Cumulated Energy thermal	breakdown (thermal masticaiotion)	96	◇	Po_Fr	specification_Fr
28	○	PRI thermal	breakdown (thermal masticaiotion)	113	□	γ–tocotrienol (%w/w total unsaponifiable)	unsaponifiable
55	□	C14:0 (%w/w dry rubber)	fatty acid composition	114	□	stigmasterol (%w/w total unsaponifiable)	unsaponifiable
56	□	C16:0 (%w/w dry rubber)	fatty acid composition	115	□	C18-OH (%w/w total unsaponifiable)	unsaponifiable
57	□	C16:1 (%w/w dry rubber)	fatty acid composition	116	□	C18-OH (%w/w dry rubber)	unsaponifiable
58	□	C18:0 (%w/w dry rubber)	fatty acid composition	117	□	C20-OH (%w/w dry rubber)	unsaponifiable
59	□	C18:1 (%w/w dry rubber)	fatty acid composition	118	□	C20-OH (%w/w total unsaponifiable)	unsaponifiable
60	□	C18:2 (%w/w dry rubber)	fatty acid composition	119	□	unsaponifiable (%w/w dry rubber)	unsaponifiable
61	□	C18:3 (%w/w dry rubber)	fatty acid composition	120	□	α-tocotrienol (%w/w dry rubber)	unsaponifiable
62	□	C20:0 (%w/w dry rubber)	fatty acid composition	121	□	α-tocotrienol (%w/w total unsaponifiable)	unsaponifiable
63	□	free fatty acids (%w/w lipids)	fatty acid composition	122	□	Δ5-avenasterol (%w/w total unsaponifiable	unsaponifiable
64	□	C14:0 (%w/w total fatty acids)	fatty acid composition	123	□	Δ5-avenasterol (%w/w dry rubber)	unsaponifiable
65	□	C16:0 (%w/w total fatty acids)	fatty acid composition	124	□	β-sitosterol (%w/w dry rubber)	unsaponifiable
66	□	C16:1 (%w/w total fatty acids)	fatty acid composition	125	□	β-sitosterol (%w/w total unsaponifiable)	unsaponifiable
67	□	C18:0 (%w/w total fatty acids)	fatty acid composition	126	□	γ-tocotrienol (%w/w dry rubber)	unsaponifiable
68	□	C18:1 (%w/w total fatty acids)	fatty acid composition	127	□	stigmasterol (%w/w dry rubber)	unsaponifiable
69	□	C18:2 (%w/w total fatty acids)	fatty acid composition	128	□	unsaponifiable (%w/w lipids)	unsaponifiable
70	□	C18:3(%w/w total fatty acids)	fatty acid composition	142	◇	Max torque (in.lb)	vulcanizaton
71	□	C20:0 (%w/w total fatty acids)	fatty acid composition	143	◇	Max torque (Nm)	vulcanizaton
72	□	free fatty acids (%w/w dry rubber)	fatty acid composition	144	◇	Min torque (in.lb)	vulcanizaton
73	□	furan FA(%w/w dry rubber)	fatty acid composition	145	◇	Min torque (Nm)	vulcanizaton
74	□	furan FA (%w/w total fatty acids)	fatty acid composition	146	◇	tc10 (min)	vulcanizaton
75	□	saturated FA (%w/w total fatty acids)	fatty acid composition	147	◇	tc90 (min)	vulcanizaton
76	□	total fatty acids (%w/w dry rubber)	fatty acid composition	148	◇	ts1 (min)	vulcanizaton
77	□	total fatty acids (%w/w lipids)	fatty acid composition	149	◇	ts2 (min)	vulcanizaton

1. Influence of lipid composition on natural rubber structure

Correlations between lipid composition and structure of USS were determined by analysis of variance with an observed significant probability of 0.05 ($P \leq 0.05$). The results will be presented according to the correlations between each structure and three groups of lipid data: lipid classes (lipid extract, neutral lipids, glycolipids and phospholipids), fatty acid composition (free fatty acids and total fatty acids after saponification) and unsaponifiable composition (total unsaponifiables and individual unsaponifiable component). All lipid data will be expressed relatively to dry rubber weight. Some examples of the correlation will be presented. Samples originating from RRIM600 (old and young), GT1 and BPM24 clones will be referred as from 'three clones'. PB235 data will be presented separately according to the difference shown on axis 1 of PCA.

1.1 Mesostructure

Crosslinking of abnormal groups such as aldehyde (Eng *et. al*, 1997), ester (Eng *et. al*, 1994) or lactones (Gregg and Macey, 1973), that are present on the polyisoprene chain, were proposed as the main cause of the formation of branching in natural rubber (gel formation). Recently, natural rubber latex have been reported to contain two kinds of functional groups at the initiating- and terminating-ends of the rubber chain, i.e., ω - and α-termini, respectively. The ω-terminus was mentioned as being bonded with a protein, while the hydroxyl of α-terminus was supposed to be linked to a phospholipids (consisting of a phosphoric ester and two long-chain fatty acid ester groups). Both terminal ends have been presumed to be the origin of the branch-points that are involved in the gel formation and the increase of M_w (Tanaka *et. al*, 1997 ; Tangpakdee and Tanaka, 1997 ; Tarachiwin *et. al*, 2005).

1.1.1 Weight average molar mass

1.1.1.1 Weight average molar mass (M_w) and lipid classes

In our study, correlation between phospholipids and M_w was not observed. Mild lipid extraction condition used may not be able to extract covalently-bonded phospholipids from isoprene (this extraction requires normally transesterification under strong alkaline conditions). For that reason, phospholipids presented in total lipids are likely from other source such as particle membrane component. This could explain the non-correlated results between phospholipids and M_w.

1.1.1.2 Weight average molar mass (M_w) and fatty acid composition

Negative correlation between total fatty acid (%w/w dry rubber) of three clones and M_w was observed. This result indicates that the increase of total fatty acids resulted in decreasing of M_w (figure 69a). Taken individually, arachidic acid and furan fatty acids also exhibited this correlation. In PB235 this correlation was not observed (Figure 69b).

1.1.2 Total gel

Total gel content is the sum between macrogel and microgel content. Due to the fact that total gel contained 77 – 81% of microgel, the correlation between total gel and lipid composition were found to be similar with those of microgel. The correlation between gel and lipid composition is therefore presented only with total gel.

1.1.2.1 Total gel content and lipid classes

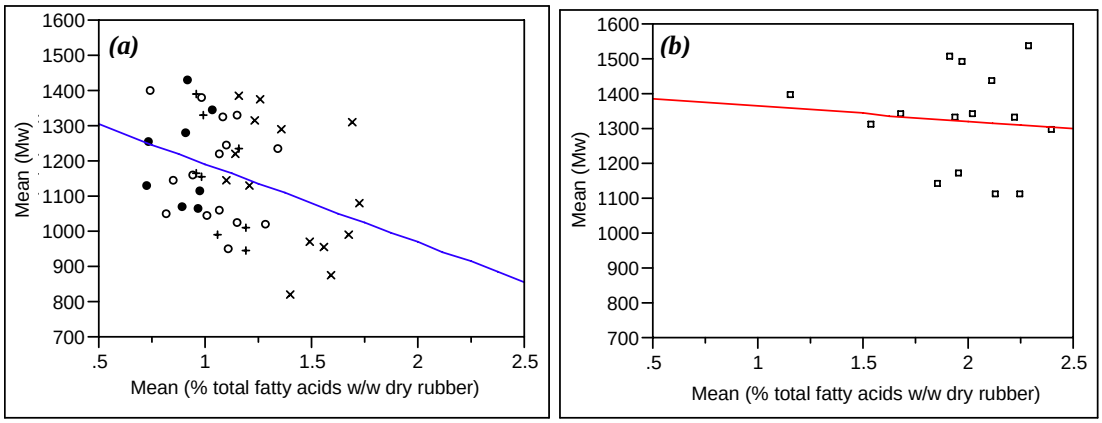
No correlation between each lipid class of dry rubber and total gel was observed which could be explained by the same reason as for M_w – lipid class correlation.

1.1.2.2 Total gel content and fatty acid composition

Total saturated fatty acids which is the addition of myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0) altogether was found to be positively correlated with total gel content (figure 70). This could be possible that saturated fatty acids were involved with gel formation (Tanaka *et. al*, 1997).

1.1.2.3 Total gel content and unsaponifiable composition

No correlation between unsaponifiable composition and total gel content was observed for studied clone.



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	135744.9	135745	5.9684
Error	43	977984.9	22744	Prob > F
C. Total	44	1113729.8		0.0187

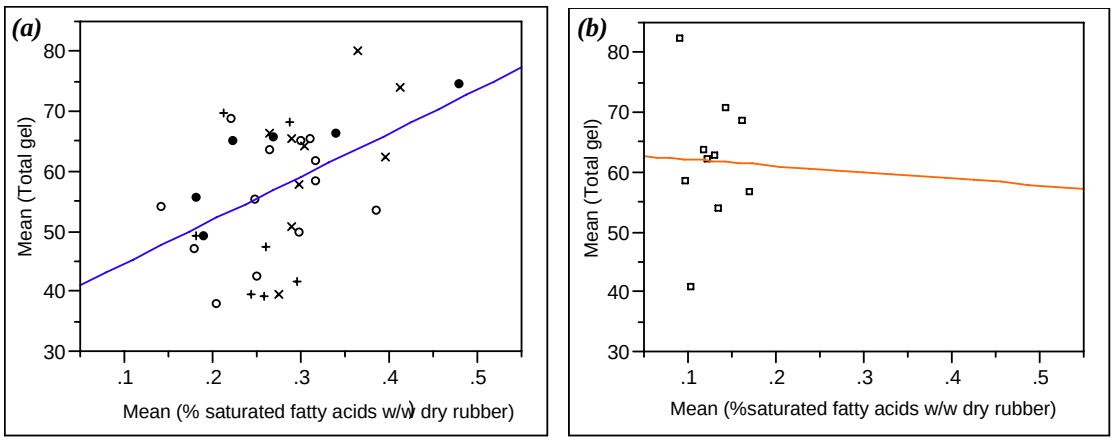
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	2817.78	2817.8	0.1361
Error	13	269168.49	20705.3	Prob > F
C. Total	14	271986.27		0.7181

Figure 69 Correlation between total fatty acid (%w/w dry rubber) and weight average molar mass (M_w) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young); ✚: GT1; ×: BPM24 and □: PB235).

1.2 Macrostructure

Macrostructural characteristics of natural rubber are determined and expressed by standardized methods. Wallace initial plasticity (P_0) and Mooney viscosity (ML) determine the flow behavior of the rubber under low strain rate. Their values are meant to be predictive indicators of energy amount to be applied to obtain requested rheological properties of rubber. Unfortunately, those indicators are not sufficient enough to get a confident prediction of behavior. PRI is generally used to indicate the behavior of natural rubber under conditions where thermal-oxidation is promoted.



Linear Fit
Mean(total gel) = 37.367151 + 72.827412 Mean(% saturated FA)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	935.9868	935.987	8.9958
Error	33	3433.5587	104.047	Prob > F
C. Total	34	4369.5456		0.0051

Linear Fit
Mean(total gel) = 63.391036 - 10.866817 Mean (% saturated FA)

Analysis of Variance

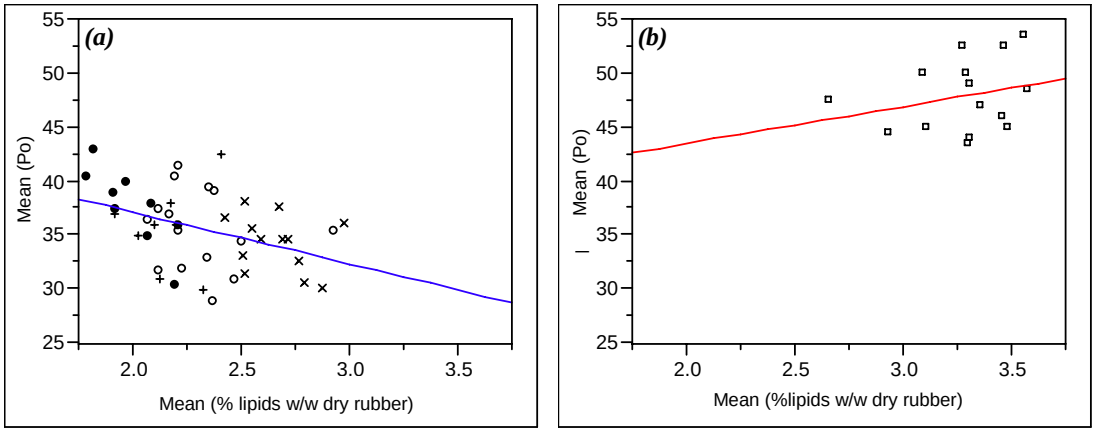
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	0.7666	0.767	0.0056
Error	8	1094.0337	136.754	Prob > F
C. Total	9	1094.8003		0.9422

Figure 70 Correlation between saturated fatty acids (%w/w total fatty acids) and total gel content of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ×: BPM24 and □: PB235)

1.2.1 Initial plasticity (P_0)

1.2.1.1 Initial plasticity (P_0) and lipid classes

The lipid extracts from RRIM600, GT1 and BPM24 clones were found to negatively correlate with P_0 (figure 71a). Neutral lipids which constituted about 80% of lipids weight show logically a similar correlation, while polar lipids did not show any. This negative correlation suggests that lipids, mainly neutral lipids, may act as plasticizer which facilitates the flow property of rubber and results in decreasing of P_0. For PB235 clone, no correlation between its lipids (either total lipids or classes) and P_0 was observed. Lipid extract from PB235 rubber displayed even a positive non significant relation with Po (Figure 71b). It confirms the necessity to analyze separately PB235 data as suggested by PCA preliminary analysis.



Linear Fit
Mean (Po) = 46.786238 - 4.8091438 Mean(% lipids)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	91.27269	91.2727	8.4643
Error	43	463.68008	10.7833	Prob > F
C. Total	44	554.95278		0.0057

Linear Fit
Mean(Po) = 36.634284 + 3.4414313 Mean (% lipids)

Analysis of Variance

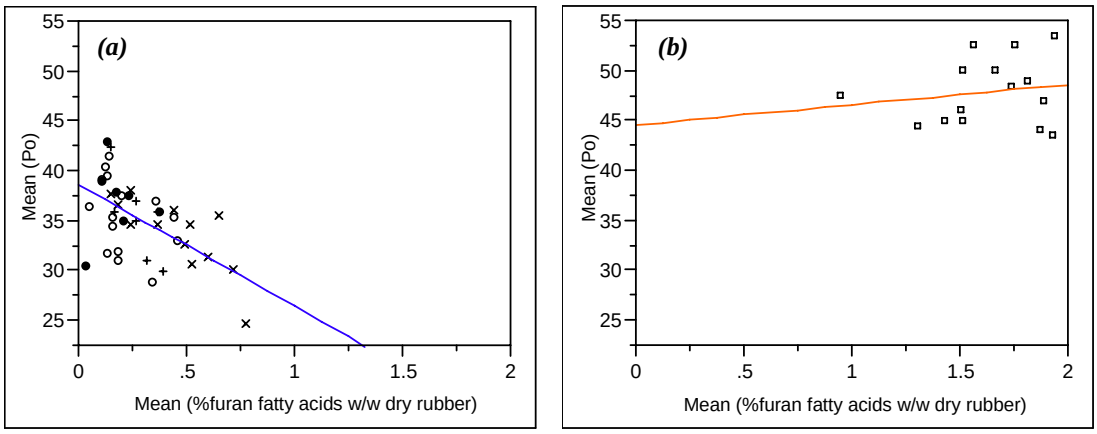
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	10.07594	10.0759	0.9255
Error	13	141.52406	10.8865	Prob > F
C. Total	14	151.60000		0.3536

Figure 71 Correlation between lipid extracts (%w/w dry rubber) and initial plasticity (P_0) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young); ✚: GT1; ×: BPM24 and □: PB235)

1.2.1.2 Initial plasticity (P_0) and fatty acid composition

Total fatty acids content of the three clones shows a negative correlation with P_0. Furan fatty acid was found to have similar correlation (figure 72). This could mean that the previous correlation (lipid extract (mainly neutrals) and P_0)

are mainly due to total fatty acids which include both free and esterified forms. Nevertheless, no correlation was observed when only free fatty acid was analyzed. This finding implies that esterified fatty acids which accounted around 24-29% of lipid extract for the three clones play an important role on plasticization of natural rubber.



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	199.10425	199.104	19.2068
Error	40	414.65320	10.366	Prob > F
C. Total	41	613.75744		<.0001

Analysis of Variance

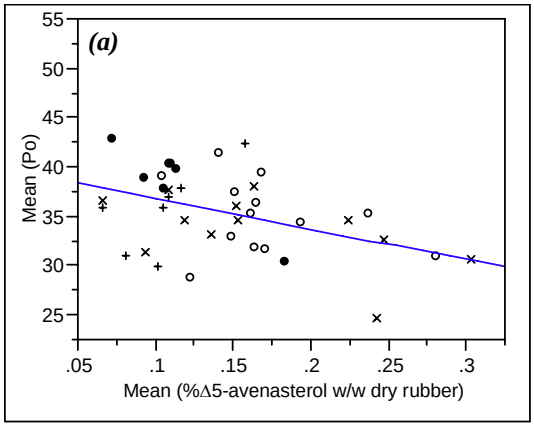
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	4.21516	4.2152	0.3718
Error	13	147.38484	11.3373	Prob > F
C. Total	14	151.60000		0.5525

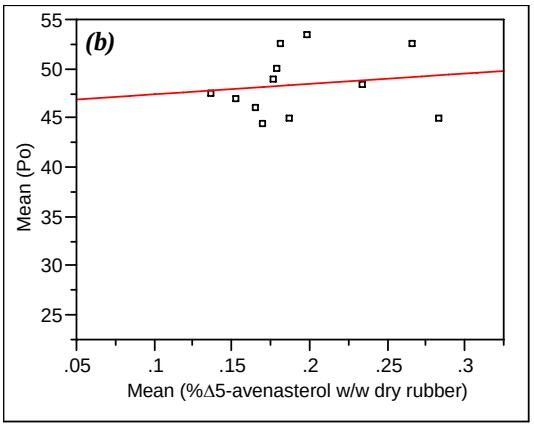
Figure 72 Correlation between furan fatty acid (%w/w dry rubber) and initial plasticity (P_0) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ✕: BPM24 and □: PB235)

Concerning PB235 clone which displayed a significantly higher P_0 than that of the other clones, no significant correlation was observed between individual fatty acids and P_0. Contrarily to the other clones, furan fatty acid, which is present in very important amount in this clonal lipids (~85% of all fatty acids or 40% of total lipids), did not correlate negatively with P_0. Kawahara and Tanaka (1995) have mentioned that unsaturated fatty acids at low concentration act as plasticizer in rubber but at higher concentration they could also improve crystallization rate of natural rubber.

1.2.1.3 Initial plasticity (P_0) and unsaponifiable composition

Total unsaponifiable amount of the three rubber clones was not found to correlate with P_0 but a negative correlation between Δ5-avenasterol and P_0 was observed as presented in figure 73. This relationship suggests that some unsaponifiable could act as plasticizer as the other lipids.





Linear Fit

Linear Fit

Mean (P_0) = 39.938955 - 30.680455 Mean (%Δ5-avenasterol)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	119.84551	119.846	8.5374
Error	37	519.39808	14.038	Prob > F
C. Total	38	639.24359		0.0059

Linear Fit

Linear Fit

Mean (P_0) = 46.400876 + 10.383524 Mean (%Δ5-avenasterol)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	2.35920	2.3592	0.2204
Error	10	107.05746	10.7057	Prob > F
C. Total	11	109.41667		0.6488

Figure 73 Correlation between Δ 5-avenasterol (%w/w dry rubber) and initial plasticity (P_0) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ×: BPM24 and □: PB235)

1.2.2 Mooney viscosity (ML)

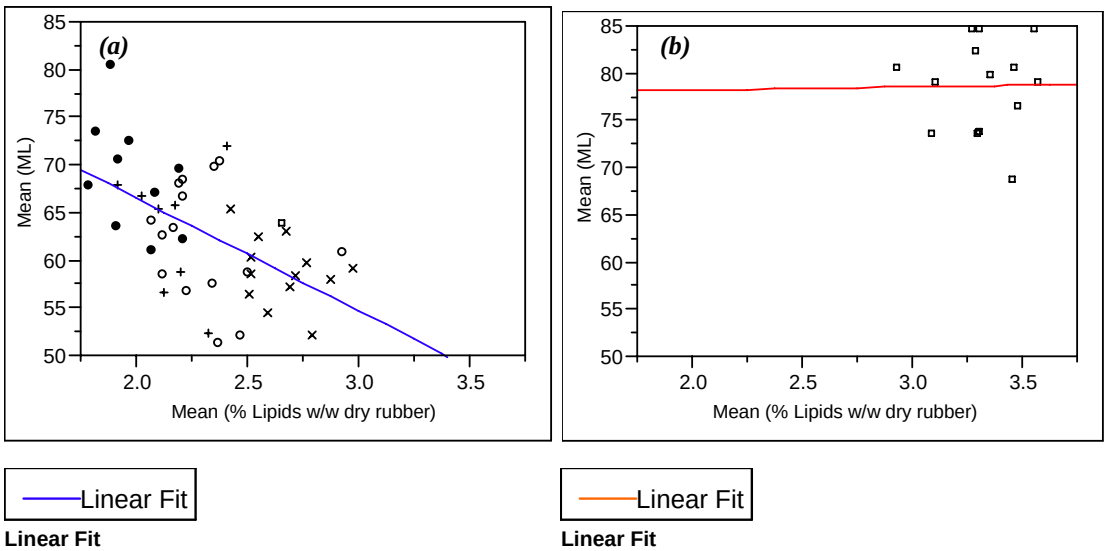
1.2.2.1 Mooney viscosity (ML) and lipid classes

ML has been described to have a broad correlation with P_0, therefore similar tendency of correlation was observed. Lipid extract and neutral lipids of the three clones were all negatively correlated with ML. The results indicated that the global effect of lipids is a plasticizing effect as observed in P_0 analysis. Lipid

extract and also individual lipid classes of PB235 did not show any correlation to ML compared to the other clones (figure 74).

1.2.2.2 Mooney viscosity (ML) and fatty acid composition

Total fatty acid of the three clones was found to correlate with ML as presented in figure 75. The same correlation was observed in furan fatty acid while other individual fatty acid did not show any correlation. Furan fatty acid seems therefore to be prominent in this plasticizing effect. However, this effect may come from a synergistic effect of all fatty acids which is hardly detectable by pair analysis of correlation. For PB235, no correlation between fatty acid composition and ML was found.



Linear Fit
Mean (ML) =90.302141 - 11.830384 Mean (% Lipids)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	593.8334	593.833	20.6457
Error	45	1294.3353	28.763	Prob > F
C. Total	46	1888.1687		<.0001

Linear Fit
Mean (ML) = 77.587754 + 0.3481224 Mean (% Lipids)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	0.05262	0.0526	0.0020
Error	12	309.34667	25.7789	Prob > F
C. Total	13	309.39929		0.9647

Figure 74 Correlation between lipid extract (%w/w dry rubber) and Mooney viscosity (ML) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ✕: BPM24 and □: PB235)

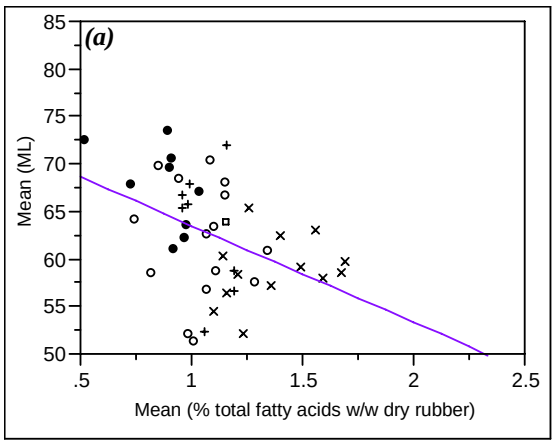
1.2.2.3 Mooney viscosity (ML) and unsaponifiable composition

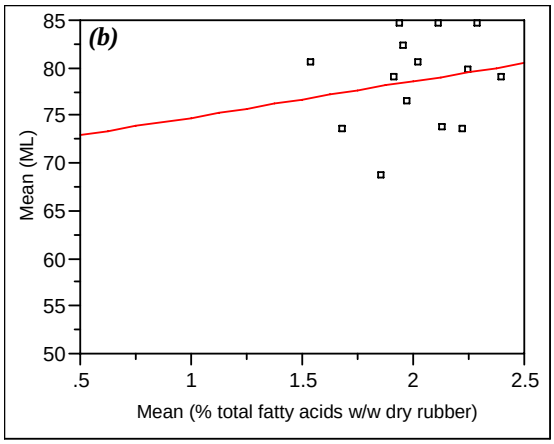
Unsaponifiable composition of the three clones shows a negative correlation with ML (figure 76). Taken individually, octadecanol (C18-OH), eicosanol (C20-OH) and α-tocotrienol exhibited a similar negative correlation. None of those significant correlations were observed in PB235.

1.2.3 Plasticity retention index (PRI)

1.2.3.1 Plasticity retention index (PRI) and lipid classes

Lipids (either lipid extracts or individual classes) were not found to exhibit any correlation with PRI whatever the clone.





Linear Fit

Linear Fit

Mean (ML) = 73.758176 - 10.151396 Mean (% total fatty acids)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	271.2504	271.250	9.2088
Error	44	1296.0476	29.456	Prob > F
C. Total	45	1567.2980		0.0040

Linear Fit

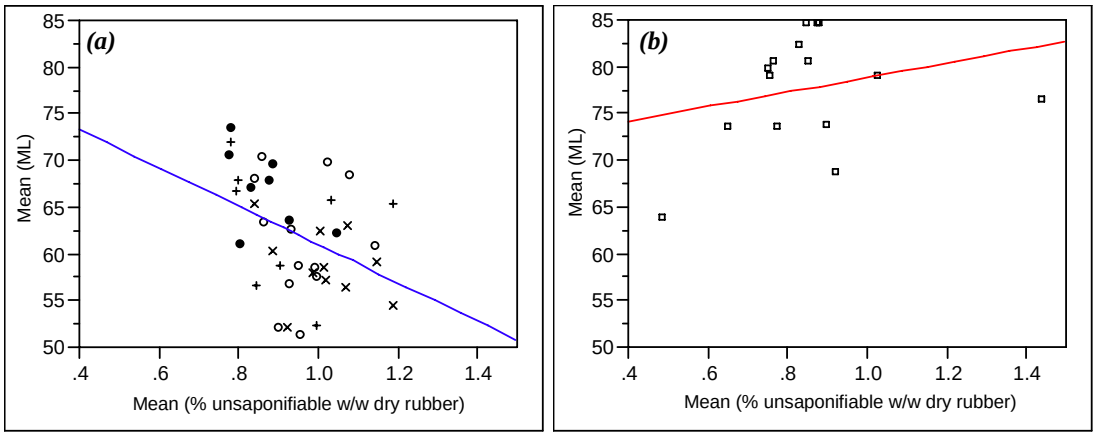
Linear Fit

Mean (ML) = 71.103852 + 3.7833614 Mean (% total fatty acids)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	10.45373	10.4537	0.4196
Error	12	298.94555	24.9121	Prob > F
C. Total	13	309.39929		0.5293

Figure 75 Correlation between total fatty acids (%w/w dry rubber) and Mooney viscosity (ML) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ×: BPM24 and □: PB235)



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	213.4506	213.451	6.7732
Error	38	1197.5242	31.514	Prob > F
C. Total	39	1410.9748		0.0131

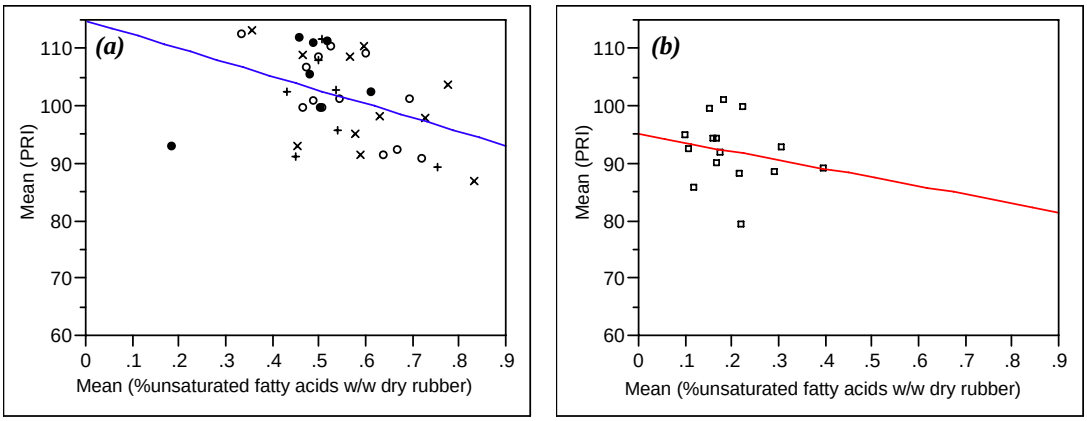
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	35.14385	35.1438	0.9493
Error	13	481.26615	37.0205	Prob > F
C. Total	14	516.41000		0.3477

Figure 76 Correlation between unsaponifiables (%w/w dry rubber) and Mooney viscosity (ML) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ✕: BPM24 and □: PB235)

1.2.3.2 Plasticity retention index (PRI) and fatty acid composition

For the three clones, a negative correlation was found between free fatty acids and PRI while total fatty acids did not show any. However, among total fatty acids, unsaturated fatty acids alone showed a negative correlation with PRI as presented in figure 77. Among unsaturated fatty acids, linoleic acid showed a similar correlation with PRI. Indeed, linoleic acid was the main fatty acid for these three clones (35-41% of total fatty acids).



Linear Fit

Linear Fit

Mean (PRI) = 115.10816 - 24.356458 Mean (%unsaturated FA)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	352.4416	352.442	6.7407
Error	36	1882.2903	52.286	Prob > F
C. Total	37	2234.7319		0.0136

Linear Fit

Linear Fit

Mean (PRI) = 95.15767 - 14.962911 Mean (%unsaturated FA)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	20.38304	20.3830	0.6036
Error	13	438.98929	33.7684	Prob > F
C. Total	14	459.37233		0.4511

Figure 77 Correlation between unsaturated fatty acid (%w/w dry rubber) and plasticity retention index (PRI) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ✕: BPM24 and □: PB235)

It was described that the carbonyl group of free fatty acids such as stearic acid, oleic and linoleic acid in natural rubber were of primary importance in enhancing polyisoprene chain scission. In addition, the unsaturation of free fatty acids was found to increase the rate of auto-oxidation (Arnold and Evans, 1991). This chain scission could consequently result in lower PRI. No correlation between lipid composition and PRI was observed in PB235 clone. PB235 rubber had a significantly lower PRI than that of the other clone. Furan acid could be involved in this difference, even if no effect of this peculiar fatty acid was noted on PRI of the other clones.

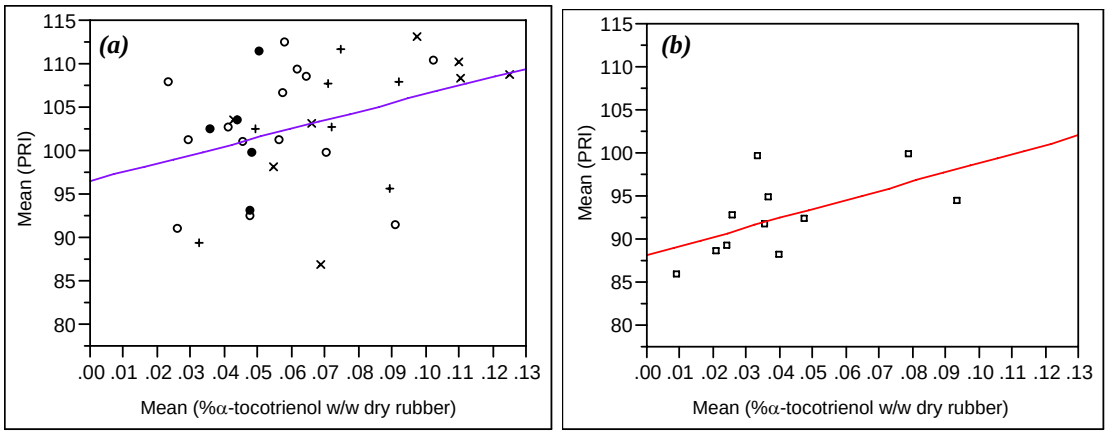
1.2.3.3 Plasticity retention index (PRI) and unsaponifiable composition

Unsaponifiable composition was previously mentioned to have antioxidant activity in natural rubber both in raw and vulcanized state especially

γ-tocotrienol (Nadarajah *et. al*, 1971 ; Hasma and Othman, 1990 ; Na-Ranong *et. al*, 1995). In our study, α-tocotrienol of the three clones (0.025 to 0.125% of rubber) was found to have positive correlation with PRI (figure 78). Its antioxidant activity was more obvious than that of γ-tocotrienol which ranged from 0.05 to 0.25% of rubber and has been reported to be the major antioxidant in natural rubber. Nevertheless, the comparison should be done with caution as those works discussed mainly the effect of addition of tocotrienol on extracted rubber but not the effect of inherent antioxidant content. Stigmasterol also showed the similar correlation. Phytosterols have been already reported as potential antioxidant (Yoshida and Niki, 2003). They could also be involved in the cross linking phenomena occurring during PRI measurement and leading to the increase of its value. Concerning PB235 clone, no significant correlation between unsaponifiable component and PRI was observed but it is likely that the activity of α-tocotrienol as antioxidant is not different between clones.

It is worth noting that the components that illustrated antioxidant activity were those which found in smaller content (average of α-tocotrienol = 0.04 – 0.07% of dry rubber weight and stigmasterol = 0.06 - 0.08%) while β-sitosterol (0.6%) which was predominant sterols did not display any correlation.

Though high PRI of the USS samples have been mentioned to be also due to crosslinking under the PRI test condition, it was difficult to identify lipid involved in this phenomena by observing correlations only.



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	221.8027	221.803	4.7794
Error	32	1485.0711	46.408	Prob > F
C. Total	33	1706.8738		0.0362

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	71.80608	71.8061	4.8244
Error	9	133.95619	14.8840	Prob > F
C. Total	10	205.76227		0.0557

Figure 78 Correlation between α-tocotrienol (%w/w dry rubber) and plasticity retention index (PRI) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ✕: BPM24 and □: PB235)

2. Influence of lipid composition on natural rubber properties

2.1 Breakdown behavior

Breakdown behavior of rubber was assessed by breakdown index (BI) value. However, no correlation between BI value and lipid composition was clearly identified. Therefore, mastication parameters such as cumulated mechanical energy and drop of ML, which were measured during mastication, were considered.

2.1.1 Thermal degradation

2.1.1.1 Cumulated mechanical energy and lipid classes

For the three clones, negative correlation between lipid extract and cumulated mechanical energy consumed during mastication was found as presented in figure 79 while no correlation with individual lipid classes was observed.

Lipids may act as plasticizer during mastication that may ease the flow of rubber leading to a lower requirement of mechanical energy to maintain rotor speed at its set value of 50 rpm. Lubricant property of lipids is well known. Indeed, various types of oils such as palm oil or castor oil, are widely used as processing aids (Moneypenny *et. al*, 2004). Concerning PB235 clone, no significant correlation was observed.

2.1.1.2 Cumulated mechanical energy and fatty acid composition

For the three rubber clones, furan fatty acid was the only fatty acid that correlated negatively with cumulated mechanical energy (figure 80). This correlation suggested that furan fatty acid acts as plasticizer. However, in PB235 rubber a contrary result was observed as furan fatty acid showed a non-significant positive correlation with cumulated mechanical energy. These observations are in full agreement with the previous discussion dealing with P_0 (figure 72).

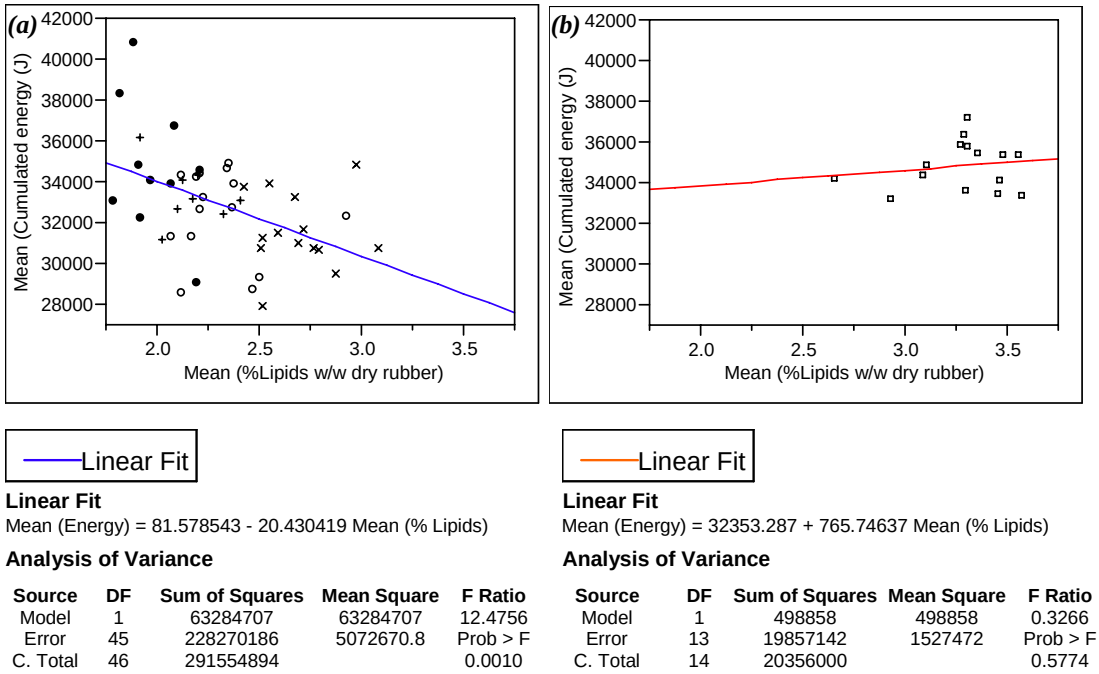
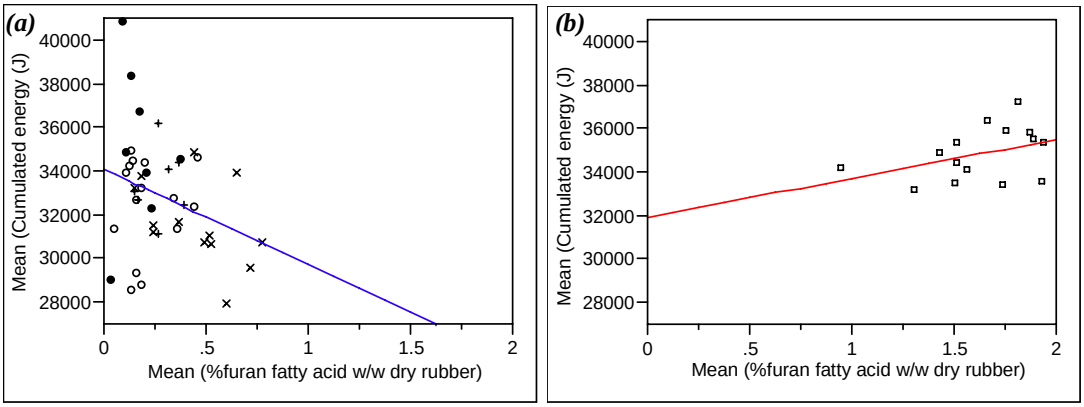


Figure 79 Correlation between lipid extracts (%w/w dry rubber) and cumulated mechanical energy consumed for thermal mastication (140°C initial chamber temperature, 50 rpm rotor speed) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);+: GT1; ×: BPM24 and □: PB235)



Linear Fit

Linear Fit

Mean (Energy) = 34128.505 - 4383.1905 Mean (%furan FA)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	26415608	26415608	4.1855
Error	41	258762066	6311269.9	Prob > F
C. Total	42	285177674		0.0472

Linear Fit

Linear Fit

Mean (Energy) = 31958.737 + 1786.9896Mean (%furan FA)

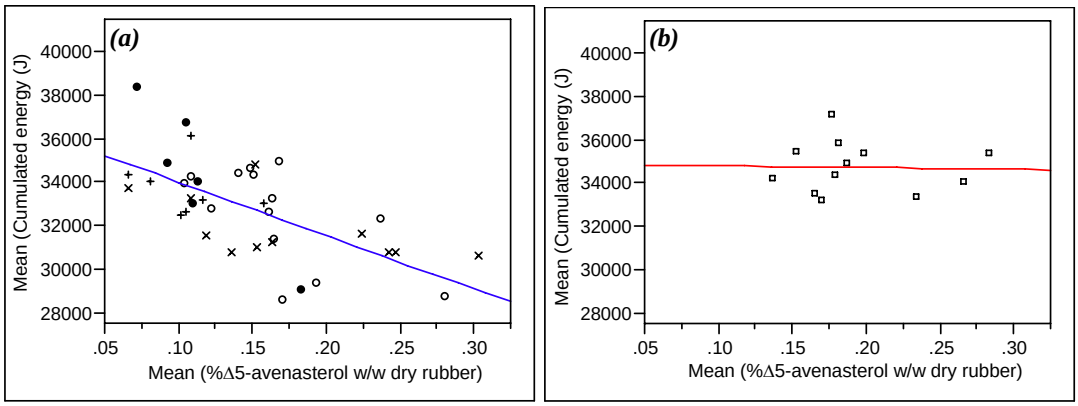
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	3278221	3278221	2.4955
Error	13	17077779	1313675	Prob > F
C. Total	14	20356000		0.1382

Figure 80 Correlation between furan fatty acids (%w/w dry rubber) and cumulated energy consumed for thermal mastication (140°C initial chamber temperature, 50 rpm rotor speed) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);+: GT1; ×: BPM24 and □: PB235)

2.1.1.3 Cumulated mechanical energy and unsaponifiable composition

For the three clones, Δ-5 avenasterol was found to have negative correlation with cumulated mechanical energy as presented in figure 81. This correlation is of the same kind as the one observed between ML or P_0 and unsaponifiable where the latter displayed a plasticizing property.



Linear Fit

Mean(Energy) = 36488.842 - 24466.072 Mean(%Δ5-avenasterol)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	74409514	74409514	25.2704
Error	36	106003117	2944531	Prob > F
C. Total	37	180412632		<.0001

Linear Fit

Mean (Energy) = 34919.817 - 831.8173 Mean (%Δ5-avenasterol)

Analysis of Variance

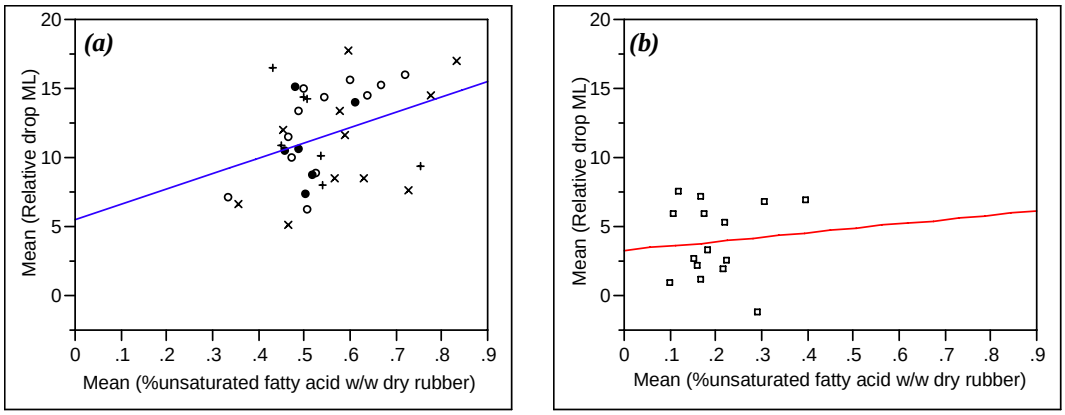
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	15140	15140	0.0098
Error	10	15374026	1537403	Prob > F
C. Total	11	15389167		0.9229

Figure 81 Correlation between Δ-5 avenasterol (%w/w dry rubber) and cumulated mechanical energy consumed for thermal mastication (140°C initial chamber temperature, 50 rpm rotor speed) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ✕: BPM24 and □: PB235)

2.1.1.4 Relative drop of ML and fatty acid composition

For the three clones, relative drop of ML was found to be correlated with total unsaturated fatty acids (figure 82). This correlation indicates that with higher amount of unsaturated fatty acid rubber is more degraded under mastication. At high temperature these fatty acids are susceptible to oxidation and their oxidation products (hydroperoxides) could act as initiators in polyisoprene oxidation (Keller *et. al*, 1981). This is supported by the negative correlation between unsaturated and PRI previously observed in figure 77.

However, this effect of unsaturated fatty acids on the drop of ML was not observed in PB235, possibly due to the fact that unsaturated fatty acids represented only around 13% of total fatty acid for PB235 rubber while around 60% were found in the other clones (furan fatty acid was not considered as unsaturated fatty acids as unsaturations are included in a furan ring).



Linear Fit
Mean (drop ML) = 5.5945854 + 11.107898 Mean(%Unsaturated)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	54.40647	54.4065	4.9648
Error	34	372.58757	10.9585	Prob > F
C. Total	35	426.99404		0.0326

Linear Fit
Mean (drop ML) = 3.3278683 + 3.2058487 Mean (%Unsaturated)

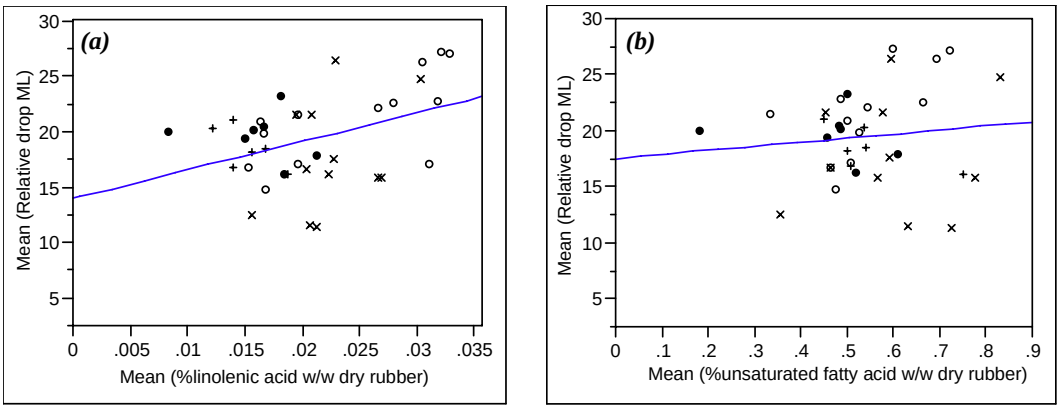
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	0.93567	0.93567	0.1152
Error	13	105.60792	8.12369	Prob > F
C. Total	14	106.54359		0.7397

Figure 82 Correlation between unsaturated fatty acids (%w/w dry rubber) and relative drop of ML from thermal mastication (140°C initial chamber temperature, 50 rpm rotor speed) of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);+: GT1; ×: BPM24 and □: PB235)

2.1.2 Mechanical degradation

For the three clones, same kind of correlation between lipid composition and cumulated mechanical energy were observed with mechanical mastication (50°C initial temperature and 100 rpm rotor speed). Nevertheless, the correlation mentioned above between drop of viscosity and unsaturated fatty acid was not observed (figure 83b) while taken individually linolenic acid showed a correlation with the drop of ML (figure 83a). This may be due to the difference of mastication temperature. At high temperature (around 160°C at the end of the "thermal" mastication) all unsaturated fatty acids may be involved in the generation of pro-oxidant molecules (hydroperoxides) while at lower temperature (around 100°C at the end of "mechanical" mastication), only the most unsaturated fatty acid could be involved (linolenic fatty acid contains 3 unsaturations while the other unsaturated fatty acids contain either 1 or 2 unsaturation(s)). PB235 lipids did not show any correlation with mechanical mastication parameters.



Linear Fit
Mean (drop ML) = 14.034999 + 257.63687 Mean (%C18:3)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	94.87261	94.8726	6.6015
Error	36	517.36640	14.3713	Prob > F
C. Total	37	612.23901		0.0145

Linear Fit
Mean (drop ML) = 17.585131 + 3.6296745 Mean (%Unsaturated)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	7.53205	7.5321	0.4359
Error	34	587.52957	17.2803	Prob > F
C. Total	35	595.06162		0.5136

Figure 83 Correlation between relative drop of ML from mechanical mastication (50°C initial chamber temperature , 100 rpm rotor speed) and linolenic acids (%w/w dry rubber) (a) and unsaturated fatty acids (b) of RRIM600, GT1 and BPM24 (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1 and ×: BPM24)

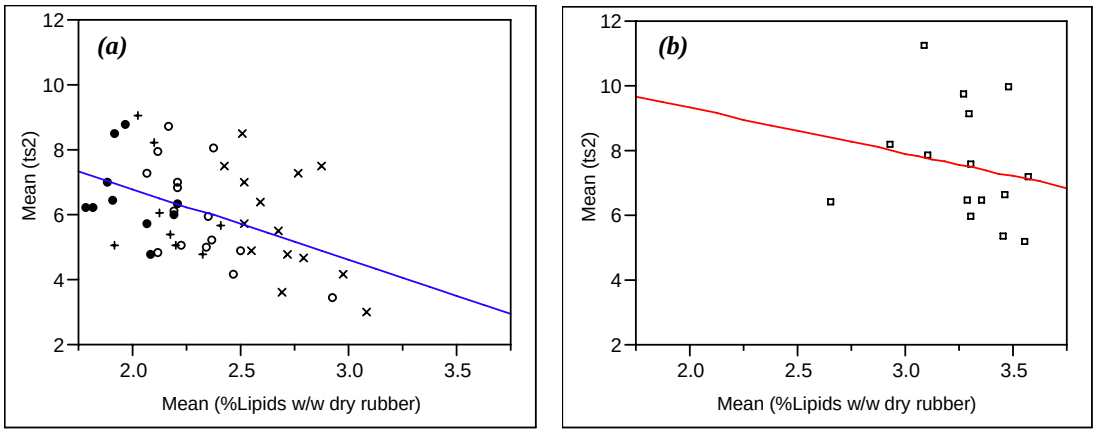
2.2 Vulcanization

In case of latex, various non-isoprene components have been reported to act as natural activator of vulcanization. The most cited are nitrogenous compound such as amines, betaines, choline and amino acids in latex (Altman, 1948 ; Othman *et. al*, 1993 ; Rattanasom and Suchiva, 2005). In case of dry rubber vulcanization, fatty acids especially stearic acid is usually added as activators together with zinc oxide. In our study, we focused on the role of lipid composition as natural activators.

2.2.1 Scorch time (t_{s2}) and lipid class

For the three clones, lipid extract showed a negative correlation with scorch time (t_{s2}) which confirms an activator property as presented in figure 84. Neutral lipids also displayed a similar correlation. These correlations were not

observed in polar lipids which could be due to their small quantities especially phospholipids.



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	22.53231	22.5323	12.3168
Error	45	82.32299	1.8294	Prob > F
C. Total	46	104.85531		0.0010

Analysis of Variance

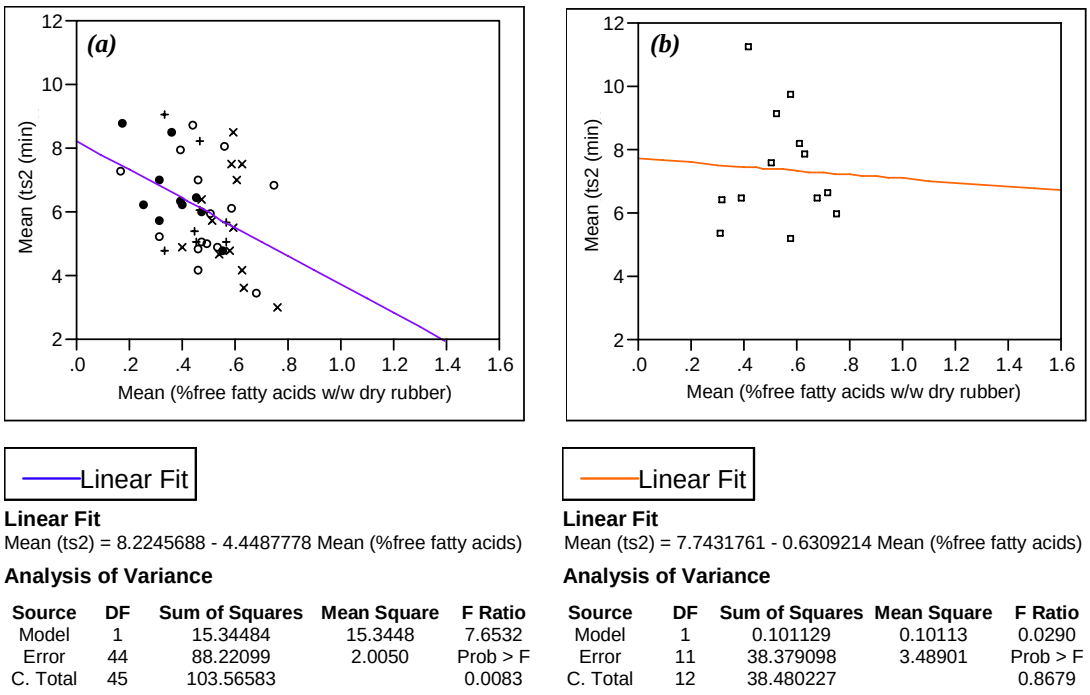
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	1.684848	1.68485	0.5092
Error	13	43.012395	3.30865	Prob > F
C. Total	14	44.697243		0.4881

Figure 84 Correlation between lipid extracts (%w/w dry rubber) and scorch time (t_{s2}) from vulcanization of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ×: BPM24 and □: PB235)

2.2.2 Scorch time (t_{s2}) and fatty acid composition

Free fatty acids from the three clones were found to have negative correlation with scorch time (figure 85). This observation is in agreement with the clonal difference in scorch time observed from the vulcanization of USS samples presented previously in chapter 3. BPM24 clone which has the highest free fatty acid content compared to the other clones gave the shortest scorch time. In addition, individual fatty acid was not found to correlate with scorch time. This may be due to the fact that they are not all in the reactive form therefore, the results may not be obviously observed.

In case of PB235 clone, its free fatty acids was slightly lower than BPM24 and higher than the other clone but their activating activity was not observed in this rubber as it presented the longest scorch time.



Linear Fit
Mean (ts2) = 8.2245688 - 4.4487778 Mean (%free fatty acids)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	15.34484	15.3448	7.6532
Error	44	88.22099	2.0050	Prob > F
C. Total	45	103.56583		0.0083

Linear Fit
Mean (ts2) = 7.7431761 - 0.6309214 Mean (%free fatty acids)

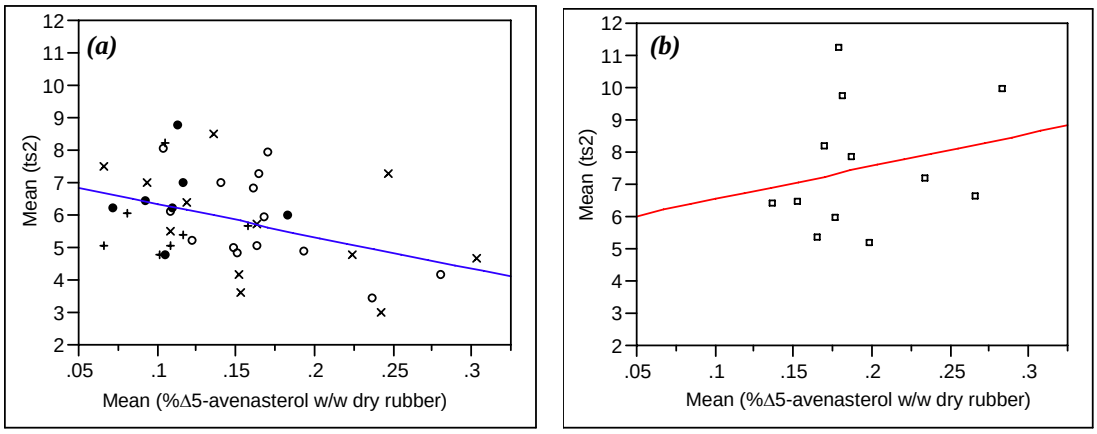
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	0.101129	0.10113	0.0290
Error	11	38.379098	3.48901	Prob > F
C. Total	12	38.480227		0.8679

Figure 85 Correlation between free fatty acids (%w/w dry rubber) and scorch time (t_{s2}) from vulcanization of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young);✚: GT1; ×: BPM24 and □: PB235)

2.2.3 Scorch time (t_{s2}) and unsaponifiable composition

Activity of unsaponifiable content for the three clones as natural activators (i.e. negative correlation with scorch time) was observed for octadecanol, eicosanol, α-tocotrienol and Δ-5 avenasterol (figure 86) while β-sitosterol shows positive correlation with scorch time (figure 87).



Linear Fit
Mean (ts2) = 7.3623215 - 9.9703202 Mean (%Δ5-avenasterol)

Analysis of Variance

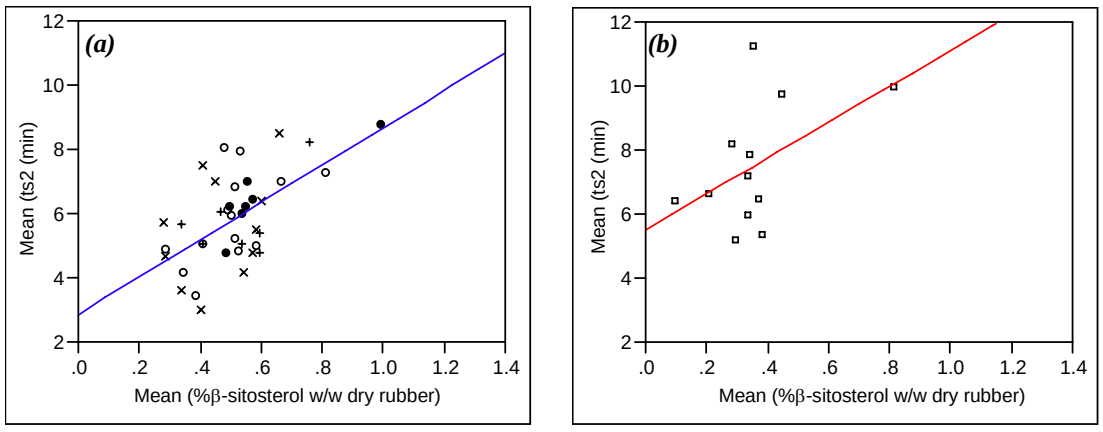
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	12.741636	12.7416	7.4275
Error	38	65.187592	1.7155	Prob > F
C. Total	39	77.929228		0.0097

Linear Fit
Mean (ts2) = 5.544378 + 10.168028 Mean (%Δ5-avenasterol)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	2.262295	2.26230	0.5840
Error	10	38.738821	3.87388	Prob > F
C. Total	11	41.001117		0.4624

Figure 86 Correlation between Δ5-avenasterol (%w/w dry rubber) and scorch time (t_{s2}) from vulcanization of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young); ✚: GT1; ×: BPM24 and □: PB235)



Linear Fit
Mean (ts2) = 2.8847683 + 5.81286727 Mean (%β-sitosterol)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	26.631086	26.6311	19.9079
Error	37	49.495462	1.3377	Prob > F
C. Total	38	76.126547		<.0001

Linear Fit
Mean (ts2) = 5.5344808 + 5.5993276 Mean (%β-sitosterol)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	9.935501	9.93550	3.1982
Error	10	31.065615	3.10656	Prob > F
C. Total	11	41.001117		0.1040

Figure 87 Correlation between β-sitosterol (%w/w dry rubber) and scorch time (t_{s2}) from vulcanization of RRIM600, GT1 and BPM24 (a) and PB235 (b). (●: RRIM600 (old); ○: RRIM600 (young); ✚: GT1; ×: BPM24 and □: PB235)

Furthermore it is to be noted that scorch time may depend not only on chemical action of activators such as lipids identified above, but also on initial rheological properties of raw rubber. As mentioned in chapter 3 (§2.2), an initial higher ML of raw rubber may be responsible by itself of a longer delay before crosslinking starts. That supports the fact that PB235 rubber, even containing a high amount of lipids, displayed the longest scorch time. Synergy between polymer initial rheological properties and the actions of activators remains to be elucidated.

3. Conclusion

Principal component analysis showed clearly that data from PB235 clone play an important role in the global variance of obtained data. Indeed, in most cases, PB235 clone displayed distinguishable characteristics. To get rid of unknown (or not lipid-dependent) PB235 clonal effect, correlations were therefore observed by putting PB235 apart from the others. The correlations found afterwards were mainly the representatives of three rubber clones, namely RRIM600 (old and young), GT1 and BPM24.

Concerning mesostructure, weight average molar mass (M_w) was found to decrease with the increase of total fatty acids. It could be due to the involvement of fatty acid containing molecules in branching of polyisoprene chains which would result in an increase of the M_w.

Macrostructure of the samples was assessed by P_0, PRI and ML. P_0 and ML were found to correlate to lipid composition similarly. For the three clones, total lipids, neutral lipids, and total fatty acids (i.e. esterified and free forms) showed a negative correlation with either P_0 or ML. This correlation was not observed for free fatty acid alone, which indicated the predominant involvement of esterified fatty acid. When the fatty acids were taken individually from the total fatty acid content, furan fatty acid was found to play an important role. We could then suspect esterified furan fatty acid and especially triacylglycerols of furan fatty acid to have an important influence on macrostructure of sheet rubber. However, this finding was not evident in

PB235 unimodal rubber that displayed concomitantly highest amount of furan fatty acids and higher values of P_0 and ML.

PRI was found to be affected by free fatty acids in a negative way as an increase of fatty acids could lead to the auto-oxidation of rubber and resulted in lower PRI. Regarding total fatty acid composition, unsaturated fatty acids especially linoleic acid seemed to be involved in this negative effect on PRI. Moreover, unsaponifiable showed its antioxidant activity with PRI. Moreover, some unsaponifiable component showed antioxidant activity by increasing PRI. Among them, α-tocotrienol and stigmasterol were the most active in every clone. It has to be noted also that the range of PRI in sheet rubber samples was very high and narrow, therefore the correlation between lipids and PRI has to be validated with the wider range of PRI (40-100).

Breakdown behavior of USS samples, lipid composition and mastication parameters were found to be interrelated with each other. Lipids class was found to ease the mastication by acting as plasticizer leading to lower mechanical energy consumption to maintain rotor speed. Nevertheless, fatty acids especially furan fatty acid showed a different effect for the two groups of sample (as mentioned for P_0). High amount of furan fatty acid in PB235 leaded to high P_0 and therefore more energy was needed for mastication which was contrarily to the other clones. Therefore, in case of PB235 even total lipids eases mastication process meanwhile it creates the resistance of rubber to mastication also. This may be one of the reasons, apart of its high Mw, that mastication of PB235 consumed the most energy but it was found to be the least degraded rubber compared to the other clones. This assumption was obvious in both thermal and mechanical mastication.

The relationship between lipid composition and vulcanization characteristics was mainly focused on the activity of natural activator. It was found that lipids especially free fatty acid could act as natural activator. Indeed, high free fatty acid containing rubber gave shortest scorch time for the three clones. Concerning PB235 clone which contained high free fatty acids, the previous relation was not verified as the scorch time was the longest among all clones.

Lipids were found to have various correlations with natural rubber structures and properties depending on rubber clones. Many correlations were not significant for PB235 clone due to the small variance of data but tendencies could be observed.

CONCLUSION

The aim of the present work was to characterize the lipid composition of *H. brasiliensis* latex and derived dry rubber and to study its relationship with natural rubber structure and properties. Samples were selected from the four most planted *H. brasiliensis* clones in Thailand with known agronomical background: RRIM600, GT1, PB235 and BPM24. Unsmoked rubber sheets (USS) prepared according to a repeatable process, were used as the source of dry rubber. USS rubber was shown to have physical properties similar to those of ribbed smoked sheet (RSS) rubber, which is the most exported form of sheet rubber in Thailand.

Characterization of lipids from fresh latex and sheet rubber was carried out after the development of optimized extraction methods. A two fold dilution of fresh latex before extraction was found to overcome the trapping of lipids inside the rubber coagulum formed immediately after solvent immersion. With dry rubber, increasing the exchange surface with solvent by ultra-low temperature (-196°C) grinding of rubber pieces prior extraction at room temperature also improved significantly the extraction yield.

Total lipid amount in fresh latices (2.5-3.7% w/w dry rubber) was found to be clonal dependent. Latices from three clones, PB235, BPM24 and RRIM600 (young) contained a higher amount lipids (3.4-3.7% w/w dry rubber) while lipids amounted only to 2.5-2.8% in GT1 and RRIM600 (old). TLC analysis of lipid extracts from fresh latex showed the presence of the same lipid classes described in previous works. A further analysis of lipids in each class evidenced no obvious qualitative difference between clones except for PB235. Indeed, the quantity of furan fatty acid (FFA) in the neutral fraction of PB235 lipids was found to be significantly higher than for the other clones. This fatty acid clearly distinguished PB235 from the other clones as it represented around 74% of its total fatty acids content, while linoleic acid was the main fatty acid in the other clones (37-53%). In this study, the presence of Δ-5 avenasterol, which was previously claimed to be fucosterol, was also evident. The

amount of this sterol was twice higher in PB235 than in the other clones, whereas β-sitosterol was the major sterol in every clone.

The structural analysis of fresh latex polar lipids using HPLC/ESI-MS showed that both FFA and Δ-5 avenasterol were more present in neutral and glycolipid classes than in phospholipids for every clone even in PB235. Four main latex glycolipids were identified and quantified, i.e., by order of importance, digalactosyldiglycerides (DGDG), sterylglucosides (SG), esterified sterylglucoside (ESG) and monogalactosyl diglycerides (MGDG). Six families of phospholipids were identified and quantified, i.e. by decreasing order: phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and lysophosphatidylinositol. Chemical structures, especially fatty acid composition of glycolipids and phospholipids, were elucidated thanks to HPLC-MS analysis.

Processing of latex was found to affect total lipids amount in dry rubber as less lipid extract was found in sheets (2.0-3.3%) than in latex (2.5-3.7%). Beside total quantity, differences were also observed between lipid classes, especially concerning polar lipids. The latter were found to be decreased in sheet rubber by around 50% for glycolipids and 80% for phospholipids from their initial quantity in latex. This change could be mainly due to enzyme-catalyzed hydrolysis, as lipase activity was observed in fresh latex. However, the clonal characteristics of lipid composition observed in fresh latex are still evident in their corresponding sheet rubber.

In parallel with lipid analysis, USS samples were characterized for their structure and properties in order to identify possible correlations. Among various measurable factors, i.e. clone, season and age of rubber trees, principle component analyses have pointed out that the variable that mostly created the variability of the results was the clonal origin of samples. PB235 clone was clearly distinguished from the other three clones. This statistical analysis confirmed what was observed in lipid composition results.

Initial plasticity (P_0) of rubber was in the range of 34 to 48, decreasingly ranked from PB235 (48), RRIM600 (old), GT1, RRIM600 (young) to BPM24 (34). Mooney viscosity (ML) showed a same ranking as observed for P_0, ranging from 59 to 78, as they both measure the flow behavior of rubber under low strain rates. In terms of relationships between lipids and P_0 and ML, a predominant plasticizing effect of esterified fatty acids and furan fatty acids was observed in the three rubber clones. Nevertheless, PB235 rubber, that contained high amounts of furan fatty acid, did not exhibit such a behavior as it showed the highest P_0 and ML values.

High PRI values were found in every studied clone (92 to 106), the lowest value being from PB235 clone. All samples thus expressed a high resistance to thermal degradation after mastication. The lower PRI measured on PB235 rubber samples could be partly explained by the fact that this rubber, characterized by a unimodal distribution of polyisoprene chain lengths, contained less short chains, which could reduce its ability to create crosslinks under high temperature conditions. The involvement of lipids in PRI was found to have both beneficial and deleterious effects. Increase in free fatty acid amounts tended to decrease PRI value while the antioxidant activity of unsaponifiable components such as α-tocotrienol and stigmasterol resulted in an increase of PRI.

Breakdown behavior of natural rubber was estimated through breakdown index (BI) by masticating rubber under thermal and mechanical conditions. This study showed that rubber samples with a low initial P_0 and ML were more degraded than that with higher ones. Such rheological behaviors were also influenced by plasticizing effect of lipids. This effect is widely used in the rubber industry, where lipids are added as processing aids in order to ease rubber mastication by requiring a lower mechanical energy consumption.

Vulcanization characteristics of each rubber clone were found to relate to the properties of rubber in unvulcanized state. Lipids, especially free fatty acids, displayed an activator activity as observed from the shorter scorch time (t_{s2}) of BPM24, RRIM600 and GT1 rubber samples.

From the present study it can be seen that lipids display both advantageous and disadvantageous effects on natural rubber properties. Some involvements with structure of natural rubber have been also mentioned. The purpose of this study was to cover a broad range of lipids as well as properties to get a general overview of the relationships without *a priori*. At this step, further studies focusing on specific points that have been pointed out are required.

Finally, it is worth mentioning that the unsmoked rubber sheet samples in our study cover a high but narrow range of rubber properties as observed, for instance, for PRI. This specific rubber type was chosen for the possibility of fully control and repeating its making process. With the knowledge learned from the present study, and after a proper selection of parameters to be measured, a similar approach could be done on more degraded rubber samples such as dry rubber obtained from maturated cuplumps. Such a study is indeed requested to further understand the involvement of lipid in rubber properties.

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