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**Study of the role of lipids from matured
coagula from *Hevea brasiliensis* latex on
natural rubber behavior in oxidative
conditions**

Presented by

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Defended on 13 MARCH 2015

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ABSTRACTS (English, French, Thai)

Study of the role of lipids from matured coagula from *Hevea brasiliensis* latex on natural rubber behavior in oxidative conditions

Abstract

Natural rubber (NR), a derived product from *H. brasiliensis* latex, is known for its high mechanical properties that are, for some, superior to those of its synthetic counterparts. However, the high degree of unsaturation of poly(*cis*-1,4-isoprene) makes it susceptible to thermo-oxidation. Fortunately, NR is endowed with non-isoprene components of which some have antioxidant properties. Especially, lipids, the main non-isoprene component retained in NR, have been reported to contain antioxidant substances, especially tocotrienols. It is well known that during the maturation of latex coagula, both NR physical properties and chemical composition are altered, but the complex mechanisms of this alteration are still to be elucidated. In the present work, the evolution of some native antioxidant molecules during maturation was followed in relation with some physical properties. Two experimental conditions of maturation were chosen. The first experiment involved uncontrolled conditions based on traditional unsmoked (USS) or ribbed smoked sheet (RSS) processing, while the second was performed in a dedicated maturation device with full control of environmental factors (relative humidity, temperature and oxygen content) followed by a processing based on that of Technically Specified Rubber (TSR). The evolution of samples during maturation was studied at different scales: bulk properties (P_0 , P_{30} and PRI), mesostructure (% gel content, M_w and M_n) and biochemical composition (lipids components). In parallel, *in vitro* antioxidant activity of NR lipid extracts was also investigated using an optimized DPPH method. Lipid quantity and quality evolved during maturation, especially under aerobic conditions. The total amount of lipid extract decreased, with a release of free fatty acids at early stage of maturation followed by a later decrease, unsaturated fatty acids being the first to disappear. The amount of extractable free γ -tocotrienol did not change much during maturation, while its esterified form was enriched in saturated fatty acids. The antioxidant activity measured *in vitro* correlated well with free γ -tocotrienol concentration but not with the resistance of rubber to thermo-oxidation assessed by P_{30} or PRI. Indeed, the *in vitro* conditions of measurement were far from those occurring inside rubber material. The localization of antioxidants in rubber and especially their physical possibility to interact with the double bonds of poly(*cis*-1,4-isoprene) or with oxidant species should be further investigated to understand what drives the drop of P_{30} along maturation time. Non-extractable lipids or more polar non-isoprene molecular species (proteins, polyphenols, etc...) could also influence the resistance to thermo-oxidation.

Keywords Natural rubber, *Hevea brasiliensis*, lipids, thermo-oxidation, antioxidants.

Etude du rôle des lipides de coagula maturés de latex d'*Hevea brasiliensis* sur le comportement du caoutchouc en conditions oxydantes

Résumé

Le caoutchouc naturel (CN), un produit dérivé du latex d'*Hevea brasiliensis*, est connu pour ses propriétés mécaniques supérieures pour certaines à celles de ses concurrents synthétiques. Néanmoins, le haut degré d'insaturation du poly(*cis*-1,4-isoprène) le rend susceptible à la thermo-oxydation. Heureusement, le CN est doté de composés non-isoprènes dont certains ont des propriétés antioxydantes. Les lipides sont les plus importants non-isoprènes retenus dans le caoutchouc et contiennent des molécules à activité antioxydante en particulier les tocotriénols. Il est connu que durant la maturation de coagula de latex, la composition chimique et les propriétés du caoutchouc obtenu sont altérées, mais les mécanismes complexes de cette altération ne sont pas encore complètement élucidés. Dans cette étude, l'évolution de certaines molécules antioxydantes natives pendant la maturation a été suivie en relation avec certaines propriétés physiques du caoutchouc. Deux expérimentations de maturation ont été mises en œuvre. La première mettait en jeu des conditions non contrôlées de maturation suivies d'un procédé de confection du caoutchouc basé sur celui des feuilles fumées (RSS) ou non (USS). La seconde a été conduite dans un dispositif expérimental dédié permettant le contrôle des facteurs de l'environnement tels que l'humidité relative, la température et la concentration en oxygène. Le procédé de confection du caoutchouc était dans ce cas basé sur celui des caoutchoucs spécifiés techniquement (TSR). L'évolution des échantillons pendant la maturation a été étudiée à différentes échelles : propriétés en masse (P_0 , P_{30} et PRI), mésostructure (% gel, M_w and M_n) et composition biochimique (lipides). En parallèle, l'activité antioxydante *in vitro* des extraits lipidiques correspondants a été mesurée en utilisant une méthode DPPH optimisée. La quantité et la qualité des lipides extraits évoluent pendant la maturation, en particulier en aérobiose. La quantité totale de lipides décroît, avec, en début de maturation, une libération d'acides gras dont la quantité diminue ensuite, avec une disparition des espèces insaturées en premier. La quantité de γ -tocotrienol libres extraits change peu au cours de la maturation alors que sa forme estérifiée montre un enrichissement en acides gras saturés. L'activité antioxydante de l'extrait lipidique mesurée *in vitro* est corrélée avec la concentration de γ -tocotrienol libre mais pas avec les valeurs de P_{30} et PRI qui estiment la résistance du caoutchouc à la thermo-oxydation. Cette absence de corrélation pourrait être due à la différence des conditions de mesure *in-vitro* de celles existantes au sein du matériau caoutchouc. La localisation des antioxydants dans le caoutchouc et en particulier leur possibilité physique d'interagir avec les doubles liaisons du poly(*cis*-1,4-isoprène) ou avec des espèces oxydantes reste à étudier afin de comprendre ce qui régit la chute de P_{30} au cours de la maturation. Des lipides non-extractibles ou des molécules non-isoprènes plus polaires (protéines, polyphénols, ...) pourraient également influencer la résistance du caoutchouc à la thermo-oxydation.

Mot clés Caoutchouc naturel, *Hevea brasiliensis*, lipides, thermo-oxydation, antioxydants

การศึกษานาฬิกาขององค์ประกอบไขมันในยางกันถ้วยที่เตรียมจากน้ำยางธรรมชาติต่อการเปลี่ยนแปลงสมบัติภายใต้สภาวะการเกิดออกซิเดชัน

บทคัดย่อ

ยางธรรมชาติที่ได้จากการแปรรูปน้ำยางพารา *Hevea brasiliensis* มีคุณสมบัติทางกลที่ดีกว่ายางสังเคราะห์ อย่างไรก็ตามโครงสร้างของ poly (*cis*-1,4-isoprene) ที่ประกอบด้วยพันธะคู่จำนวนมากทำให้อ่อนแอต่อกระบวนการออกซิเดชัน แต่ยางพาราก็มีกลุ่มของสารตามธรรมชาติที่มีคุณสมบัติในการต้านอนุมูลอิสระที่ทำให้เกิดกระบวนการออกซิเดชัน โดยเฉพาะสารกลุ่มไขมันซึ่งถือเป็นสารกลุ่มที่ไม่ใช่ของหลักที่คงอยู่ในยางธรรมชาติที่มีรายงานว่ามีความเป็นสารต้านอนุมูลอิสระ โดยเฉพาะ tocotrienols เป็นที่ทราบดีว่าในระหว่างการบ่มยางกันถ้วยมีการเปลี่ยนแปลงทั้งคุณสมบัติทางกายภาพและทางเคมีของยางธรรมชาติ ซึ่งกลไกที่ทำให้เกิดการเปลี่ยนแปลงนี้ยังคงไม่เป็นที่ทราบแน่ชัด วัตถุประสงค์ของงานวิจัยนี้เพื่อติดตามการเปลี่ยนแปลงของสารต้านอนุมูลอิสระตามธรรมชาติที่สัมพันธ์กับการเปลี่ยนแปลงของสมบัติทางกายภาพ โดยทดลองทำการบ่มตัวอย่างยางธรรมชาติในสองสภาวะคือ สภาวะที่ไม่มีภาชนะคลุมป้องกันแสงโดยใช้หลักการเดียวกันกับการทำยางแผ่นดิบหรือยางแผ่นรมควัน ในขณะที่การทดลองที่สองจะทำการบ่มตัวอย่างในเครื่องบ่มที่มีออกแบบให้สามารถควบคุมปัจจัยแวดล้อมได้ (ความชื้นสัมพัทธ์, อุณหภูมิ และปริมาณออกซิเจน) โดยใช้กระบวนการในการเตรียมตัวอย่างเช่นเดียวกับการผลิตยางแท่ง TSR ทำการศึกษาการเปลี่ยนแปลงของตัวอย่างในระหว่างการบ่มที่แต่ละระดับโครงสร้าง ได้แก่ คุณสมบัติทางกายภาพ (P_0 , P_{30} and PRI), โครงสร้างระดับ mesostructure (%gel content, M_w และ M_n) และองค์ประกอบทางชีวเคมี (องค์ประกอบของไขมัน) รวมถึงสมบัติในการต้านอนุมูลอิสระของไขมันด้วยวิธี DPPH โดยผลพบว่าทั้งชนิดและปริมาณไขมันมีการเปลี่ยนแปลงในระหว่างการบ่มโดยเฉพาะในสภาวะที่มีออกซิเจน ในช่วงแรกของการบ่มปริมาณของไขมันทั้งหมดลดลงพร้อมกับการปลดปล่อยกรดไขมันอิสระ หลังจากนั้น มีแนวโน้มลดลงโดยกรดไขมันไม่อิ่มตัวเป็นกลุ่มแรกที่สลายตัว ปริมาณของ γ -tocotrienol ที่สกัดได้ในรูปอิสระไม่มีการเปลี่ยนแปลงที่ชัดเจนในระหว่างการบ่ม ในขณะที่รูป esterified มีกรดไขมันไม่อิ่มตัวเป็นองค์ประกอบโดยส่วนใหญ่ ผลการทดสอบสมบัติในการต้านอนุมูลอิสระแบบ *in vitro* ด้วยเทคนิค DPPH พบว่ามีความสัมพันธ์กับความเข้มข้นของ γ -tocotrienol ในรูปอิสระ แต่ไม่สัมพันธ์กับระดับความต้านทานต่อการเกิดออกซิเดชันเมื่อตรวจสอบจากค่า P_{30} หรือ PRI โดยแท้จริงแล้วการทดสอบแบบ *in vitro* มีความแตกต่างจากสภาวะที่เกิดขึ้นจริงในยางธรรมชาติเป็นอย่างมาก ดังนั้นควรมีการศึกษาเพิ่มเติมทั้งตำแหน่งของสารต้านอนุมูลอิสระในยาง โดยเฉพาะความเป็นไปได้ในการเข้าถึงตำแหน่งพันธะคู่ในโครงสร้างของยางธรรมชาติรวมถึงอนุมูลอิสระเพื่อทำความเข้าใจถึงกลไกที่ขับเคลื่อนให้เกิดการลดลงของค่า P_{30} ในระหว่างการบ่ม นอกจากนี้ไขมันที่ไม่ถูกสกัดออกมา หรือสารกลุ่มที่ไม่ใช่ของชนิดอื่นที่มีความเป็นขี้ผึ้งมากกว่า (โพรตีน, โพลีฟีนอล และอื่น ๆ) อาจมีอิทธิพลต่อระดับความต้านทานต่อกระบวนการออกซิเดชันเช่นเดียวกัน

คำสำคัญ ยางธรรมชาติ, *Hevea brasiliensis*, ไขมัน, กระบวนการออกซิเดชัน, สารต้านอนุมูลอิสระ

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To this end, I take full responsibility for any mistake that may be found in this thesis.

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GENERAL INTRODUCTION

Hevea brasiliensis Müll. Arg., commonly called para rubber tree or rubber tree is the most economically important latex source for natural rubber (NR) industry. Today, the world's three largest producing countries are Thailand, Indonesia and Vietnam, which together accounted in 2013 for approximately 68% of total NR production (IRSG, 2014).

NR from *H. brasiliensis* is composed mainly of poly(*cis*-1,4-isoprene) associated with a small portion of some biological elements that give NR highly specific properties. These non-isoprene components comprise proteins, carbohydrates, lipids and inorganic constituents and represent the main difference between NR and its synthetic counterpart (Vaysse *et al.*, 2012). Non-isoprene components have been the subject of numerous studies and are claimed to be involved in many superior mechanical properties of rubber, for instance high green strength, tack and low internal heat build-up, which are highly required for vehicle tire manufacturers. Meanwhile, non-isoprene components have also been mentioned to relate with quality inconsistency of NR compared to synthetic rubber.

NR in raw state is also known to be susceptible to oxidative degradation due to its structure containing appreciable amounts of double bonds. This results in deterioration of its physical properties such as initial plasticity (P_0) and Plasticity Retention Index (PRI). The degree of NR resistance to oxidation could vary depending on conditions of rubber processing. Generally, antioxidants are added during second transformation processing to improve rubber resistance to oxidation. These antioxidants prevent oxidative deterioration of the polyisoprene chain by eliminating free radicals. Beside the synthetic antioxidants commonly used in processing, NR naturally contains its own antioxidant molecules as well (Nadarajah *et al.*, 1971 ; Hasma, 1984 ; Hasma and Othman, 1990).

Previous comprehensive studies on the influence of lipids, a major non-isoprene class of molecules, on NR properties reported that some of the non-isoprene substances present in *Hevea* latex behaved as antioxidants. Tocotrienols, which are vitamin E derivatives, and plant sterols, seem to exhibit an antioxidant activity according to a positive correlation observed with higher PRI of NR (Hasma, 1984 ; Liengprayoon, 2008). Nevertheless, the antioxidant

capacity of these components is not yet fully understood. The present work focuses on native antioxidants from NR samples with a wide range of physical properties. Therefore, NR samples were prepared under various maturation conditions since it is known to have an impact on P_0 and PRI, two industrial indicators used to determine physical properties and resistance of NR to thermo-oxidation. To better understand the impact of native antioxidants against oxidation, NR properties and antioxidant activity of lipids extracts obtained from the different conditions were determined.

The first part of this thesis, reviews general information concerning the production, biochemical composition and non-isoprene components of NR, and the current knowledge on native antioxidants and their influence on rubber properties. The experimental part of this work is divided into three chapters. The first one presents the methodological developments realized for this work; the second and third part concerns the evolution of lipid composition of NR obtained from uncontrolled and controlled maturation, respectively. In these two experimental setups, structure and properties of matured rubber samples and antioxidant activity of NR lipids will be described. The kinetic of evolution of lipid components during maturation will be presented in details.

LITERATURE REVIEW

1. Present situation of natural rubber

Over the past decade, the share of the global consumption of NR in the elastomer market stayed steady around 42%. In 2013, the world production of NR was about 12 million tons (IRSG, 2014); around 93% of the NR world production comes from Asian countries as presented in Figure 1.

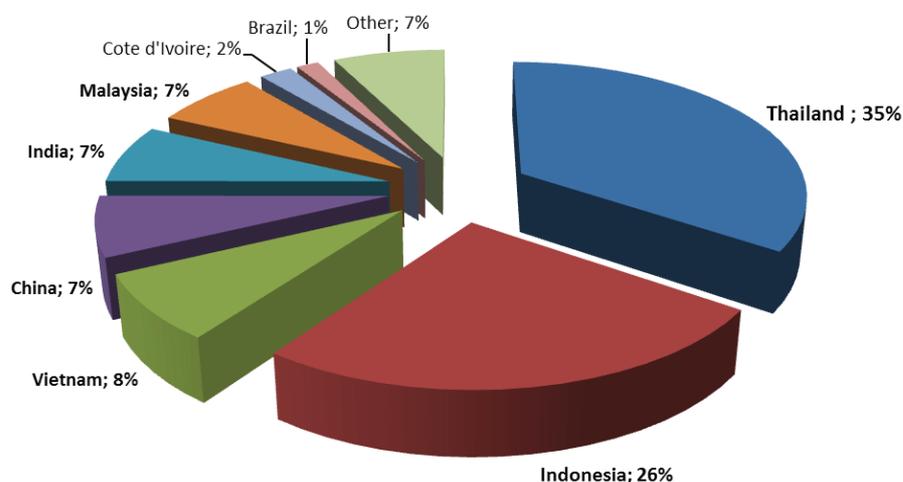


Figure 1 World natural rubber production shares (2013).

Data Source: (IRSG, 2014)

For Thailand, around 4.1 million tons of the NR produced in year 2013 held more than one third of total world NR production (IRSG, 2014). Within this quantity, various NR grades are produced and exported including concentrated latex (19%), block rubber (38%), sheet rubber (22%), compound rubber (19%) and others (2%) as shown in Figure 2. The raw NR produced in the world as well as in Thailand is marketed more in dry form and available in different grades. Sheet and crepe rubbers were the two marketable forms of dry NR before the introduction of technically specified rubber (TSR). TSR is a modern form of marketable rubber and can be produced from latex, field coagulum or a blend of both. Advantages of TSR are assurance of quality with respect to important technical parameters, clean and easy

handling and packing, minimum storage space and reduction in processing time (George and Kuruvilla Jacob, 2000).

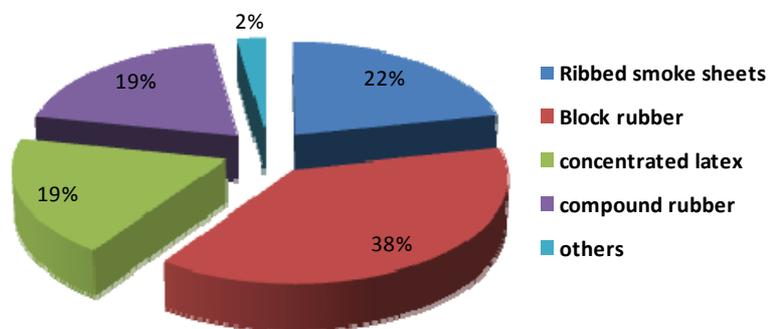


Figure 2 Exportation of NR products of Thailand in year 2013.

Data source: RRIT (2014)

Among the various grades of TSR, TSR20 is the most commonly exported grade for the top exporting countries. In TSR20 rubber processing, the raw material is cup coagula (or cuplump) blended or not with low grade rubber sheets. Cup coagula are coagulated rubber. They present variable qualities related to their origins and collection methods between tapping and processing. The primary processing of latex to raw rubbers as well as production of various forms of dry NR are described afterwards.

2. Natural rubber latex and raw rubber production

The production of usable rubber includes several steps which are tapping, latex collection and processing to transform fresh *Hevea* latex into different marketable forms.

2.1 Tapping and latex collection

The tapping operation consists of cutting bark around the stem without injuring the cambium growth area. About 2-3 mm thick of bark is shaved off to cut open the specialized cells known as laticifers (latex vessels) in the soft bark. Tapping is repeated at a certain frequency by making thin shavings just below the previous cut. The best time for tapping is late at night or early in the morning (low daily temperature). The latex is collected

by allowing it to flow into a small cup attached to the trunk. The collection of latex can be done 3 to 4 h after tapping (Premakumari and Panikka, 1992 ; Kush, 1994).

Once the tree is tapped, latex composition is susceptible to bacterial growth and rapid acidification is observed. This degradation of proteins and the formation of organic acids neutralize the negative charges on rubber particles and the latex gradually coagulates. Therefore, fresh latex cannot be kept for long without a preservative treatment. Ammonia is the most common preservative used to prevent premature coagulation during transfer to a factory or a smallholder processing center. The latex that exudes after collection will be gathered later by the tappers in a coagulated form called cuplump. The development of field coagulum can be induced by adding acid or by leaving the whole harvested latex in the cup without any stabilizers (Intapun *et al.*, 2009).

2.2 Raw natural rubber production

Hevea latex and NR are distinct products. The former is a stable colloidal dispersion of rubber particles in an aqueous medium while the latter is a visco-elastic 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 dry NR. Productions of the three main NR products are described below.

2.2.1 Concentrated latex

Concentrated latex and its by-product, skim rubber are made by separating field latex preserved with ammonia through centrifugation. The degree of ammoniation of latex prior to centrifuging depends upon the period since collection and ranges from 0.25% if centrifuging is immediate, to 1% if the period is two days or more. Only the minimum required quantity of ammonia shall be added (Mathew and Claramma, 2000). Upon arrival at the factory, ammoniated field latex is drawn from each delivered batch and tested using a hydrometer or latexometer to estimate dry rubber content (DRC). Concentration of ammonia, volatile fatty acid (VFA) are also measured. Then, the field latex is filtered through 40 or

60 mesh stainless-steel gauze continuing into a bulking tank. The latex is transferred by gravity into a header tank where another sieve is fitted to remove any coagulum that may have formed during the preliminary treatment in the reception or bulking tanks. From here, a series of chutes distribute the product to each of the centrifuges, where it is separated into concentrated latex and skim latex. Latex concentrate is used to create various tubings, a myriad of dipped goods, and 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 and need (Yip and Cacioli, 2002).

2.2.2 Crepe rubber

Crepe rubbers are processed from fresh latex coagulum, field coagulum or cutting or rubber sheet. After necessary primary treatment of these materials, they are passed through a set of crepe making machines then crinkle and lace-like rubber are obtained. Among different types of starting materials, the latex crepe is classified as the best quality crepe. Pale latex crepe is produced from latex selected specially under strictly controlled conditions and hence is of premium quality. This kind of crepe is used for high quality products such as pharmaceutical articles, light colored and transparent good, adhesives, tapes and tubings (Kuriakose and George, 2000). Since the color and cleanliness are the main concern for crepe quality grading as prescribed in the Green Book, latex to be used for the production shall be free from yellow pigments as far as possible. Therefore, latex has to be collected from clones which are identified on the extent of yellow pigments *i.e* carotenoid in latex. RRIM600 is one of *Hevea* clones that is selected for pale crepe production due to its latex containing minimum β -carotene and is less susceptible to enzymatic coloration (De and White, 2001).

2.2.3 Sheet rubber

Conversion of fresh latex into rubber sheets is the oldest method of processing. This is widely adopted by rubber farmers due to its simplicity, lower cost of processing and the viability even when the quantity of latex is small. Raw rubber sheets are

of various types like ribbed smoked sheets (RSS), air-dried sheets (ADS) and unsmoked sheets (USS) depending on the drying methods.

The bulked latex is usually diluted to a rubber content of 15-16% and placed in coagulation tanks with formic acid or acetic acid. Dilution helps in faster settling of impurities and in obtaining a softer coagulum which can easily be sheeted. It also helps in improving the clarity of sheets. The coagulum from undiluted latex is harder and it contains more proteins and other non-isoprene components which may appear as rust when the sheet is dried (Kuriakose and Thomas, 2000). After complete coagulation, the coagulum is washed and sheeted through the plane and grooved rollers to have around 2.5 to 3.0 mm final thickness. Freshly machined and dripped sheets contain about 20% moisture. For the preparation of RSS, these sheets are dried in smoke houses, while for the production of ADS, smokeless drying chambers are used. Concerning drying methods of sheets, drying in a smoke house has definite advantages. It is quicker than sun drying and does not cause oxidation by UV-radiation. In addition, the burnt rubber trees, coconut husks and other organic materials create a smoke that preserves and gives the rubber its particular odor. For exportation, RSS is the main form exported among different types of sheet rubbers. These RSS are sorted visually into different grades on the basis of color, translucency, presence of mold and other foreign matter. For its marketable form, the sheets are pressed into bales of 113.5 kg and given a protective wrap. The surfaces of the bales are finally coated with a solution of talcum to protect from oxidation. Classification of rubber sheets are based on visual criteria and have no technological basis.

2.2.4 Block rubber

There are many grades of block rubber *i.e.*, TSR XL, TSR 5L, TSR 5, TSR 5CV, TSR 10CV produced from latex, field coagulum or a blend of both. The raw material used for TSR production is processed through a traditional crumb-rubber factory, where it is cleaned by passing the material through a slab-cutter and/or pre-breakers. The resultant crumb is washed in wash tanks and then fed to a series of creper machines which build the rubber into a blanket. The blanket is then crumbed through a shredder or extruder.

The crumb is dried in a forced-air dryer at temperatures between 105°C and 120°C and the final, dried crumb is pressed into blocks.

The production of TSR started in the 1960s, prior to which raw NR was marketed in conventional forms like sheet and crepe. In 1964, The International Standards Organization (ISO) first came out with the draft Technical Specifications for NR. Based on these specifications, Malaysia introduced their Standard Malaysian Rubber (SMR) scheme the following year. Since then all NR manufacturing countries started marketing NR as Technically Specified Rubber, or TSR. TSR is mostly a block type made adopting new methods of processing. The blocks are generally 35 kg bales available on the international market. All the block rubbers are also guaranteed to conform to certain technical specifications as laid out by national schemes or to ISO 2000 standard.

Table 1 The specifications of different grades of TSR rubber.

Parameters	TSR-CV	TSR-L	TSR-5	TSR-10CV	TSR-10	TSR-20CV	TSR-20
Dirt retained (max % wt)	0.05	0.50	0.50	0.10	0.10	0.20	0.20
Ash content (max % wt)	0.60	0.60	0.60	0.75	0.75	1.00	1.00
Nitrogen content (max % wt)	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Volatile matter (max % wt)	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Initial plasticity, P ₀ (≥)	-	30	30	30	30	30	30
Plastic Retention Index, PRI (≥)	60	60	60	50	50	40	40
Color (max) Lovibond units	-	6	-	-	-	-	-
Mooney Viscosity (ML 1+4, 100 °C)	60 ± 5*	-	-	60 + 7,-5*	-	60 + 7,-5*	-

*Not specification status, but are controlled at the producer end.

The specification of TSR are given in ISO 2000:2003; Rubber, raw natural – Guidelines for the specification of technically specified rubber (TSR).

TSR comprises the majority of dry rubber produced from the major NR exporting countries of the world. In Thailand, Malaysia and Indonesia, TSR is designated as Standard Thai Rubber (STR), Standard Malaysian Rubber (SMR) and Standard Indonesian Rubber (SIR), respectively. The main advantages claimed for TSR compared to the conventional sheet and crepe grades of rubbers are: First, its quality can be easily assessed; e.g. P₀, PRI and volatile matter, helping to avoid mistakes in valuation of the material. The specifications in force at present are given in the following Table 1. Secondly, as compact,

polyethylene wrapped bales, handling and transportation costs are reduced through mechanized handling and containerization.

3. Natural rubber structure and properties

3.1 Latex composition

The composition of latex derived from *Hevea* tree is a mixture of many different chemicals, both organic and inorganic. The major component of latex is rubber hydrocarbon poly(*cis*-1,4-isoprene) which forms 30 to 45% w/v latex. Besides rubber and water (55-60% w/v latex), fresh latex has been reported to contain 5-6% of non-isoprene components consisting of carbohydrates, proteins, lipids, amines, and other inorganic constituents. The quantity of these non-isoprene components varies by several factors such as age of the trees, weather, clones, soil and tapping system utilized (Eng and Tanaka, 1993 ; Nair, 2000). Early researchers reported that the tree sap of *Hevea* has three types of particles dispersed in the liquid phase. They are rubber hydrocarbon, Frey-Wyssling particles (Frey-Wyssling, 1929) and the globular particles named lutoids. Cook and Sekhar (1954) were the first to attempt separating the latex particles into fractions by means of ultracentrifugation. Their work was continued later by Moir (1959) under controlled conditions resulting in the identification of several distinct fractions after centrifugation as presented in Figure 3.

3.1.1 Lutoids

Next in abundance is lutoids accounting for 10 to 20% of the volume. Lutoids are a sub-cellular membrane-bound body ranging in size from 2 to 5 μm . This membrane encloses a fluid serum known as lutoids serum or B serum (as located in the bottom fraction of ultra-centrifuged latex) which was mentioned to destabilize rubber hydrocarbon (Nair, 2000). In addition, lutoids contain a wide range of hydrolytic enzymes and some of their enzymatic properties are analogous to the lysosomes of animal cells (Dupont *et al.*, 1976). Approximately 20% of the dry matter in lutoids is made of water-soluble proteins, of which about 70% is hevein. This substance is anionic and shown to contain no less than 5% sulfur, all as cysteine (Webster and Baulkwil, 1989).

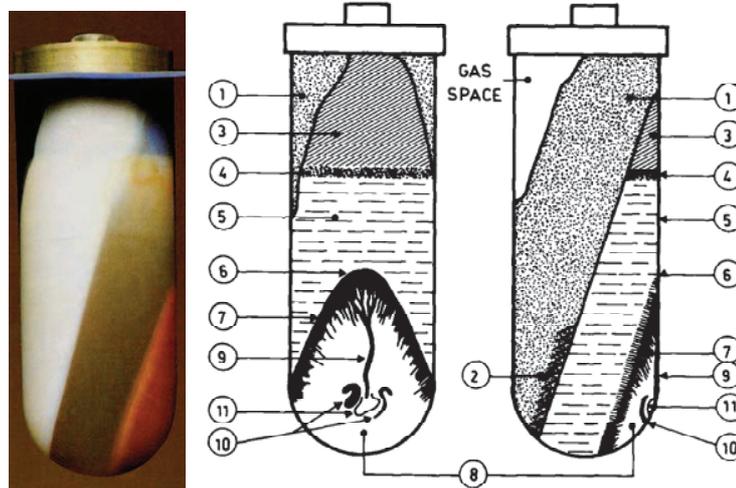


Figure 3 Various fractions of centrifuged latex. Fractions 1-3 correspond to white rubber phase. Fraction 4 is a yellow orange layer constituted by Frey-Wyssling particles. Fraction 5 is an almost clear serum (C-serum) corresponding to the latex cytosol. Fractions 6-11 constitute the bottom fraction where lutoids are highest in quantity.

Source: Moir (1959)

3.1.2 Frey-Wyssling particles

Frey-Wyssling particles are spherical and yellow or orange colored, larger in size and constitute 1% to 3% of latex volume. This particle's diameter ranges from 0.5 to 2 μm . The membrane encloses a fluid serum known as lutoid serum or B serum which is a destabilizer of NR hydrocarbon (D'Auzac and Jacob, 1989).

3.1.3 C-serum

Both rubber particles and the membrane-bound organelles compose most of the bottom fraction to form a colloidal mixture in an aqueous suspension called cytosol. Cytosol represents 40 to 50% of latex volume and has the highest rubber synthesis activity and general metabolism. The latex cytosol 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).

3.1.4 Non-isoprene components

Around 10% of the dry matter of *H. brasiliensis* comprises non-isoprene components. They comprise different biochemical compounds such as proteins, carbohydrates, lipids, and inorganic constituents. The nature and quantity of non-isoprene components in *Hevea* latex can vary greatly depending on the clones, the exploitation system and the environmental conditions (Vaysse *et al.*, 2012).

3.1.4.1 Proteins

Fresh *Hevea* latex contains about 1-2% proteins (w/w) of which about 20% is adsorbed on the surface of rubber particle; an equal quantity is found in bottom fraction (luteoids) and the remainder in the serum phase (Yeang *et al.*, 2002). Adsorbed proteins and phospholipids impart a net negative charge to rubber particles, thereby contributing to the colloidal stability of latex. Moreover, the protein content was also reported to show clonal variations (Wititsuwannakul and Wititsuwannakul, 2001). While over 200 species of proteins can be found in NR latex, most of them are removed during processing. Only a small fraction remains in the products as residual extractable proteins. Though some proteins have been identified to be potential allergens in latex, information about whether all proteins could survive the stringent manufacturing process is presently incomplete (Yip and Cacioli, 2002).

3.1.4.2 Lipids

Lipids in *Hevea* latex occur mainly in the rubber particles and bottom fractions. Ho *et al.* (1975) indicated that lipids associated with the rubber particles and the latex fractions were important in the stability of rubber particles. Depending on the clone, lipids amount to 2.5–3.8% versus dry rubber in latex. The three main families of NR lipids were classified as neutral lipids, glycolipids and phospholipids (Hasma, 1984 ; Hasma and Subramaniam, 1986 ; Liengprayoon *et al.*, 2013).

▪ ***Neutral lipids***

A typical composition in latex lipid consists of more than 50% neutral lipids (% of total lipids) while the other components are mainly phospholipids and glycolipids. Neutral lipids are composed of more than 14 components including sterols, sterol esters, free fatty acids, fatty acid esters, wax esters, monoglycerides, diglycerides, triglycerides, tocotrienols and phenolic substances. As in most plant lipids, the fatty acid composition of *Hevea* latex is mainly linoleic acid (C18:2), stearic acid (C18:0), oleic acid (C18:1) and palmitic acid (C16:0). These fatty acids were detected mainly as esterified forms in latex (Hasma, 1984 ; Liengprayoon, 2008).

▪ ***Phospholipids***

Phospholipids are the major lipid components of biological membranes. 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 (Voet and Voet, 1995). In the latex state, phospholipid represents around 18–25% (w/w total lipids) depending on clones. Phospholipids is of great importance to the physiology of the latex flow since it is closely related to latex stability (Dupont *et al.*, 1976). The stability of rubber particles suspended in latex is dependent on the negatively charged film of proteins and phospholipids. The main phospholipids detected from latex lipids were phosphatidylcholine (PC), phosphatidylinositol (PI), and to a lesser extent, phosphatidylethanolamine (PE) (Hasma, 1984 ; Liengprayoon *et al.*, 2013). Besides its importance in latex stability, phospholipids were claimed to participate in the branching formation in dry state (Rojruthai *et al.*, 2009).

▪ ***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). Glycolipids of fresh latex consisted of four glycolipid families: steryl glucoside (SG), esterified steryl glucosides (ESG), monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG). In fresh latex, glycolipids were found in the range of 0.8-1% (w/w dry rubber) or 21-36% of total lipids. Several works dealing with lipid composition of latex from different clones reported that the relative amount of glycolipids was found to be clone independent (Hasma, 1984 ; Nair *et al.*, 1993 ; Liengprayoon *et al.*, 2013). In addition, clonal variation was also observed in the relative proportions of glycolipid classes with 43–51% DGDG, 30–34% SG, and 7–19% ESG but not MGDG (8%) (Liengprayoon *et al.*, 2011)

3.1.4.3 Carbohydrates and cyclitols

Quebrachitol (monomethyl-1-inositol), sucrose and glucose are the major low molecular weight organic solutes in latex. Quebrachitol, a cyclitol, is the most concentrated single component in the serum phase (75-95% of the total carbohydrates and cyclitols present in latex) (Ohya and Koyama, 2001). Its concentration is found to vary with clones and amounts for around 1% of whole latex. It is a major contributor to the osmotic pressure of the cytosol (D'Auzac and Jacob, 1989). Sucrose is the main sugar in latex as it is the initial molecule in isoprene synthesis and the main element in the laticiferous metabolism. Sucrose concentration in latex is one of the key measurements of latex diagnosis that used to express the physiological state of the exploited trees (Le Roux *et al.*, 2000 ; Silpi *et al.*, 2006). The concentration of sucrose in latex varies with clones and is influenced by exploitation techniques (Nair, 2000).

Non-isoprene components could be dissolved or suspended in the aqueous medium of the latex or absorbed on the surface on rubber particles including contained in other organelles such as lutioids or Frey-Wyssling particles. However, around 50% of these non-isoprene components are leached or degraded during dry rubber processing and their location could not be precisely indicated. In dry matter of latex, 10% is accounted for non-isoprene components and only 5% of those components remained with NR after primary processing (Vaysse *et al.*, 2012).

3.2 Natural rubber mesostructure

Industrial utilization of dry NR in various products originated from its special structure and properties. Due to its natural origin as other biopolymers, NR has a complex structure. Mesostructure is a term used to describe dry NR macromolecular structure and gel. Macromolecular structure concerns the average-molar masses, molar mass distribution (MMD), branching, etc. For polymer characterization, average molar masses and MMD are important features because they could significantly impact on physical properties.

The most common averaged-molar masses used in establishing molar mass-property relationships are the number-averaged molar mass (M_n), the weight-average molar mass (M_w), and the z-average molar mass (M_z). The M_w is inevitably higher than the M_n and a measure of the spread of molar masses within a sample is provided by the ratio M_w/M_n which is defined as polydispersity index (I_p). Any polymer defined as monodisperse indicated that all the molecules are of the same sizes. NR from different clones was reported to have a wide range of I_p from 4 to 10 (Subramaniam, 1993). Currently, size exclusion chromatography (SEC) multi-angle light scattering (MALS) is a useful technique applied for polymer average molar masses and MMD determination. It importantly aids in the establishment of structure-property relationships for polymers. A new innovative and alternative technique is under study to assess the mesostructure of natural rubber such as asymmetrical flow field flow fractionation-multi-angle light scattering (A4F-MALS) (Dubascoux *et al.*, 2012).

3.2.1 Molar masses and molar mass distribution

The molar mass and molar mass distribution in polymer systems play an important role in determining their bulk properties. Subramaniam (1972) was the first who studied MMD of fresh NR latex samples with SEC, expressed as MMD_0 . The MMD_0 of NR is an important criterion to estimate certain NR properties that will be obtained after processing (Bonfils *et al.*, 2000). Depending on the clone as well as ages of rubber trees, MMD_0 expresses two types of distribution: bimodal or unimodal with a shoulder (sometimes also called 'quasi-unimodal') (Figure 4). In young trees, a situation where the lower molar mass peak is larger may be found 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 found the increase of number-average molar mass (M_n) with an increase of *H. brasiliensis* seedling (1, 3, 7, 36 and 84 months) by GPC and osmometry. In addition, though 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 influenced 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.

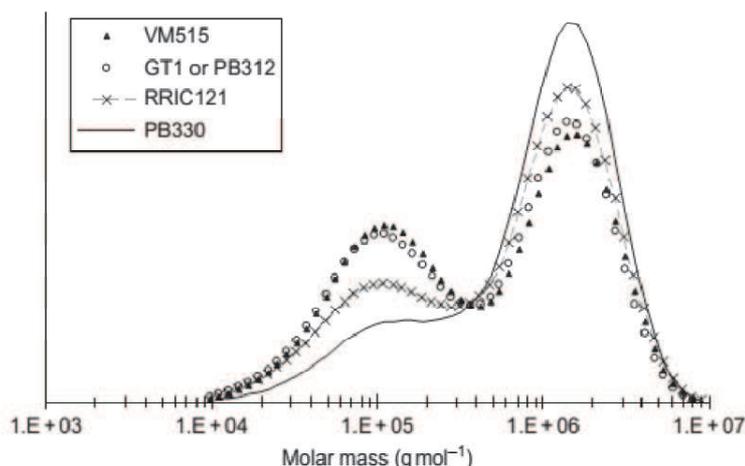


Figure 4 Examples of inherent molar mass distribution for several natural rubber clones (SEC in cyclohexane according to Bonfils and Char (2005)).

3.2.2 Gel

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. Microgel (microaggregates), contained in the soluble part, cannot be eliminated by centrifugation but

can be retained by filtration (porosity $\leq 1 \mu\text{m}$) (Bonfils and Char, 2005). Campbell and Fuller (1984) also used the term microgel to interpret the cloudiness of the solutions obtained when dissolving a sample of NR in dichloromethane.

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). Recently, it was proposed to quantify indirectly NR gel content in terms of “total gel” that was the sum of macrogel and the microgel calculated from the difference between the initial concentration on rubber put in solution and the injected concentration of the rubber solution measured by SEC-MALS (Bonfils and Char, 2005 ; Wisunthorn *et al.*, 2012).

NR gel content was reported to vary with many conditions. Latex from virgin trees and from the tree which is not tapped for long period were found to contain very high gel content; the amount 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). Under dry storage, this phenomenon is called “storage hardening” (Rodriguez and De Paoli, 1985 ; Subramaniam, 1993 ; Gan, 1996). Gel content can also be increased by other factors such as clonal characteristics and rubber process (Dogadkin and Kuleznev, 1960 ; Ngolemasango *et al.*, 2003).

3.3 Natural rubber properties

The properties of NR are generally described by rheological terms such as viscoelasticity and plasticity. In rubber processing, rheological behavior of NR must be preliminarily determined in order to aid rubber property prediction during the process. Initial Wallace plasticity (P_0) and Mooney viscosity (ML) are important quality indicators that are used in rubber manufacturing to describe those properties. P_0 is an indicator showing the NR

flow produced during a simple compression at 100°C. The high P_0 value indicates a high resistance of NR to flow under pressure. Factors such as clones, storages, drying conditions or rubber processing are fundamental and can affect rubber P_0 value (Yip, 1990 ; Moreno *et al.*, 2005). Mooney viscosity (ML) gives an indication of the amount of mechanical force required on raw rubber to obtain a mix with consistent rheological properties. ML and P_0 show, in general, a good linear correlation (Moreno *et al.*, 2005). ML of freshly prepared rubber ranges from 50 to 90 units (ML(1+4) at 100 °C) depending on the mixture of clonal latex used in the preparation (Subramaniam, 1987).

Plasticity retention index or PRI is a measure of the resistance of rubber to molecular breakdown at evaluated temperature. It is the percentage of retention of initial Wallace plasticity value after heating the test pellet at 140 °C for 30 min. This index is used to predict rubber's breakdown during the mixing process. PRI is conversed with P_0 as rubbers with a lower plasticity value have a higher PRI (Yip, 1990). Even if the PRI is dedicated to estimate the amount of rubber molecular breakdown, some crosslinking may occur during this test making interpretation of the results difficult. This is due to an opposite effect the crosslinking on P_0 compared to oxidative breakdown. As well as other property indicators, PRI variations in NR were mentioned to be influenced by genetic differences among clones and by the disparity of climatic conditions during tapping. Fri *et al.* (2007) mentioned that even the effects of some agronomic traits of the *Hevea* plants on the behavior of its rubber have been postulated, indeed NR postharvest treatments such as coagulation, coagula maturation, drying and storage also significantly affected on structure and consequently on NR properties.

4. Natural rubber maturation

Hevea latex is subjected to several treatments before the dry marketable products are obtained. Postharvest treatments of latex influences properties of raw NR material. For the most produced and exported commercial TSR grades of Thailand, TSR10 and TSR20, their main raw materials are the “cup coagula” obtained by natural coagulation of latex. It is well known that these rubber coagula have to be stored for a certain period of time before processing. This period is called “maturation” which is crucial for the processors as it affects

the quality of the processed rubber (Ehabe *et al.*, 2002 ; Fri *et al.*, 2007 ; Intapun *et al.*, 2010). Several works indicated an impact of microbial activity on the change of structure and properties during maturation. The richness in organic compounds of latex and NR coagula allows microbial growth. Studies of NR coagula maturation showed an evolution of microbial growth and activity during maturation. It has been proposed that the biochemical modifications of the coagula caused by microorganisms during maturation have an effect on NR characteristics (Salomez *et al.*, 2014). Indeed, not only polyisoprene but also non-isoprene components are involved in NR structure and properties.

4.1 Modification of latex biochemical composition during maturation

NR is obtained after latex coagulation through two main methods either natural or acid. Natural coagulation could be achieved by neutralizing the negative charges on the rubber particles. This process may result from enzyme activities from *Hevea* latex and from contaminating microorganisms or microbial metabolism in latex. For acid coagulation, excellent separation with almost no rubber left in serum can be achieved simply by a proper adjustment of the pH of latex from neutral to 5.4. The choice of coagulation is usually made according to the primary processing type of dry rubber.

For natural coagulation, initial non-isoprene components in fresh latex including protein, carbohydrates, lipids, organic and inorganic compounds are modified by microbial activity. In this stage, microbial degradation of non-isoprene components results in the change of pH from 6.5 to around 5.2 (first 24 h). This pH drop is due to the acids produced during the acidification phase. These acids, formic, acetic, lactic, butyric, and propionic acids are volatile fatty acids (VFA) which are assumed to be originated from sugar degradation. Nevertheless, the contribution of the major latex carbohydrates, quebrachitol, in this microbial degradation of sugar is not conclusive since in the study of Lowe (1959) ammonia-preserved latex quebrachitol, myoinositol and L-inositol were not degraded during 11 days of storage and it was supposed that quebrachitol was not a substrate for VFA formation. Afterwards in the work of John (1966), 12 strains of microorganisms in latex were found to be able to metabolize carbohydrates and quebrachitol via an oxidative pathway to acids, which implied that it was possible substrate for acid production.

In a later stage of natural coagulation, pH increases more or less fast up to 8 or 9 possibly due to a release of ammonia by protein degradation. Indeed, the evolution of pH seemed to be the result of a balance between acidification by carbohydrate fermentation and an alkalization by organic acid consumption and protein degradation (Intapun *et al.*, 2010 ; Salomez *et al.*, 2014). In fact, the modifications of hydrophilic compounds (carbohydrates and proteins) were described to be involved in natural coagulation more than lipids. These could possibly be due to the fact that an initial phase of microbial growth requires preliminarily carbon source for cell structure and carbohydrates and proteins could be more readily used than lipids. Incidentally, roles of lipids in latex as native antioxidant molecules were more discussed especially in the dry NR since lipids remain as major non-isoprene components in dry rubber due to their hydrophobic properties. In addition, these changes of biochemical composition generate not only an impact on NR quality but also an environmental issue. Apart from fatty acids originating from carbohydrate material, other sulphur and nitrogen containing proteins and amino acids present in the serum could also be degraded by microbial fermentation. In addition to the VFA, these substances cause strong odors in the field and around the rubber coagulation piles.

4.2 Evolution of NR structure and properties during maturation

NR structures and bulk properties are closely related, therefore, mesostructure of matured coagula such as molar mass distribution, weight-averaged molar mass and gel content are regularly determined in order to explain NR property evolution. During the first days of maturation, P_0 could increase or decrease depending on microorganism concentration. Intapun *et al.* (2010) has clearly shown that NR prepared from sodium azide – a biocide latex provide constant P_0 value during maturation, while a significant variation up to 38% increase or 20% decrease was observed with various inoculum concentration applied. Varghese *et al.* (2005) mentioned that microorganism could promote both crosslinking which resulted in an increase of P_0 and scission of polyisoprene macromolecules that brought a decrease of P_0 . In case of PRI that reflects the susceptibility of NR to thermo-oxidation, it was found to decrease proportionally to the microorganism initial concentration in latex; the more inoculated the latex, the more sensitive to thermo-oxidation (decrease of PRI). No change of PRI during maturation was observed when microbial activity was prevented.

Intapun *et al.* (2009) also performed the study on NR coagulation under two different conditions (presence and absence of oxygen) over 45 days of maturation. The result showed that P_0 and PRI parameters were higher in anaerobic than in aerobic condition, which suggests that the decrease of NR property values might be due to oxidative microbial mechanisms.

Intapun *et al.* (2010) also described the mesostructure evolution of NR during maturation. It was observed that averaged-molar mass weight (M_w) decreased slightly after 6 days of maturation (from 1500 kg.mol⁻¹ to 1400 kg.mol⁻¹) when the latex was treated with an biocide (sodium azide). Meanwhile M_w values with a faster drop (from 1500 down to 1150 kg.mol⁻¹) after 6 maturation days were obtained from latex with the presence of active microorganisms. It was also found that the drop rate of M_w increased with the quantity of microorganisms. This showed that microorganisms led to polyisoprene chain scission. A different trend was observed from the average-molar mass (M_n) evolution. M_n of coagula without biocide treatment demonstrated a stable value around 700 kg.mol⁻¹ whereas samples treated with biocide showed an increase of M_n over time, up to 900 kg.mol⁻¹. Gel quantities increased with maturation time and reached a plateau after two days of maturation for both biocide treated or non-biocide treated rubbers meaning that gel formation may occur without microbial activity. High gel containing rubber samples (non-biocide) had a stable M_n over maturation. This could be due to a balance between scissions and the crosslinking illustrated by gel content. Rubber treated with antibiotic seemed to be protected from scission (slight decrease of M_w), while the increase of M_n may be due to gel formations by short chain polyisoprene releases through oxidative reaction.

Beside, significant effects of maturation on mesostructure and the bulk properties of processed NR previously mentioned, *Hevea* clone specific behaviors on property were also studied (Fri *et al.*, 2007). The physical properties (P_0 and ML) of rubber from clones PB217 (slow metabolisms, unimodal MMD), GT1 (intermediate metabolism, bimodal MMD) and PB235 (high metabolism, unimodal MMD) were determined during maturation of 18 to 45 days. PB217 and GT1 clone rubber properties decreased with an increase of maturation whereas PB235 rubber was less sensitive to maturation. Generally, decreasing of bulk properties due to maturation was explained by the degradation of long polyisoprene chains.

However, by observing the PRI evolution, it did not appear that the chains were susceptible to thermal-oxidation. PB235 (high metabolism) and PB217 (low metabolism) clone rubber had PRI remaining constant throughout, while those from clone GT1 and another intermediate metabolism clone, RRIM600 (intermediate metabolism, bimodal MMD), decreased significantly during maturation (Fri *et al.*, 2007).

Contrary results were observed by Ekpini *et al.* (2001) and Zhong *et al.* (2009). P_0 and ML of PR107 and PB217 which are both slow metabolism rubber clones (unimodal MMD) increased during maturation. Unlike the previous work, PRI values of PR107 and PB217 clone matured cup coagula significantly decreased (37% and 19%, respectively). The disparities observed from the various clones implied that the different NR properties could not be directly related only to level of rubber tree metabolism but some inherent dissimilarity in the non-isoprene constituents and/or variations in maturation environments such as temperature and air humidity must be considered.

5. NR oxidation and antioxidants

Most of polymer particularly those based on unsaturated monomer like NR, are rather susceptible to oxidative degradation which results in a loss of physical properties (Abad *et al.*, 2002). Indeed, oxidative degradation could possibly occur at different steps throughout NR production process until in a form of finished product. Thus antioxidants which are considered as a substance capable of preventing or slowing the oxidation are expected to prevent NR from oxidative degradation. They can be either native oxidants naturally present or synthetic antioxidants added in manufacturing process.

5.1 NR oxidation

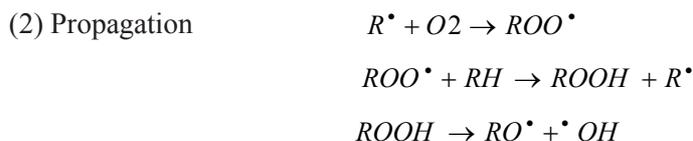
All polymers, natural and synthetic, organic or inorganic will eventually degrade in an oxygen atmosphere at elevated temperatures. Some kind of commercially important polymers could be degraded by thermal oxidation at relatively low temperatures. Oxidation occurring in the temperature ranges from ambient to approximately 200 °C is usually referred to as autoxidation. Reactions which result in oxidative degradation are not identical for all

polymers, but vary with the chemical structure of polymer and with the conditions at which oxidation occurs (Hawkins, 1964).

Oxidative degradation of NR takes place as a result of the reaction between the unsaturation and oxygen in the presence of heat as well as hydrogen abstraction which ultimately leads to chain scission (Nwanorh, 1998). The oxidation of NR in the solid state is complex since two competitive processes; scission and crosslinking, are taking place almost simultaneously. These two mechanisms change the physical properties of the material. Chain scission will result in the loss of stiffness and elasticity, whereas crosslinking will result in increased stiffness and a consequent increase in brittleness. As a general rule, the more predominant effect of the oxidation process is chain scission (Ngolemasango *et al.*, 2006). For polyisoprene, whether a scission or crosslinking mechanism predominates depends strongly on the quantity of oxygen that diffuses into the material. A brief outline of the oxidation process is given below (Tobolsky and Mercurio, 1959).



The formation of free radicals (R^\bullet) during polymerization, processing or service of the rubber product is the first stage of polymer degradation and is called as initiation (1) of degradation process.



Propagation (2) is the second stage when atmospheric oxygen reacts with the free radicals (R^\bullet) to form peroxy radicals (ROO^\bullet). Peroxy radical further reacts with labile hydrogen atom of the polymer molecules to form unstable hydro peroxides ($ROOH$). Hydro peroxides immediately decompose via hemolytic cleavage to form alkoxy and hydroxyl radicals and further propagate the degradation mechanism. Propagation stage of degradation process is very rapid compared to the initiation stage. This autocatalytic oxidation reaction progresses until termination (3) takes place by formation of stable products.



Currently, plasticity retention index (PRI) is the only international standard test used to provide a rapid assessment of the susceptibility of raw natural rubber to thermal oxidative degradation given by the ratio of P_{30} (plasticity after aging at 140 °C for 30min) to P_0 (in %).

5.2 Antioxidants

In biological system, the ability to use oxygen allows the metabolization of various organic compounds for energy. However, as an highly reactive atom, oxygen is capable of becoming part of potentially damaging molecule commonly called “free radical”. Free radicals are electrically charged molecules, with an unpaired electron, this structure causes them to seek out and capture electrons from other substances to neutralize themselves. Though the free radicals from the initial attack could be neutralized, another free radical is formed in the process causing a chain reaction. Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen-containing molecules, including free radicals such as hydroxyl radical, hydrogen peroxide, singlet oxygen and various lipid peroxides (Percival, 1998). Reactive oxygen species (ROS) are generated by biotic and abiotic stresses such as drought, salinity, strong light, extreme temperatures, heavy metals, UV radiation, atmospheric contamination, mechanical wounding, nutrient starvation and pathogen attacks (Jaspers and Kangasjärvi, 2010). Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants.

Antioxidants can be defined as compounds or systems that delay oxidation reaction by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms. Those mechanisms include (1) scavenging species that initiate peroxidation, (2) chelating metal ion such that they are unable to generate active species, (3) quenching $\cdot O_2^-$ preventing formation of peroxides, (4) breaking the autoxidative chain reaction, and/or (5) reducing localized O_2 concentration. The most effective antioxidants are those that interrupt the free radical chain reaction (Nawar, 1996 ; Huang *et al.*, 2005 ; Brewer, 2011).

There are two basic categories of antioxidants, namely synthetic and natural. They are usually containing aromatic or phenolic ring, these antioxidants donate H^+ to free radicals formed during oxidation becoming a radical themselves. These radical intermediates are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of quinone structures. In general, both synthetic antioxidants (BHA, BHT and propyl gallate) and natural botanicals contain phenolic function in this manner (Brewer, 2011). Indeed depending on the scientific discipline, antioxidants could be defined and categorized differently based on their structure, occurrence, mode of action, solubility and kinetic (Flora, 2009).

In biochemistry and medicine point of view, antioxidants are grouped by their enzymatic and non-enzymatic radical scavenging system (Figure 5). There are several intrinsic free radical scavenger systems which involve enzymatic and non-enzymatic reactions in biological system. One of the most effective intracellular enzymatic antioxidants is enzymatic antioxidant defense systems which is super oxide dismutase (SOD) that converts super oxide radicals to hydrogen peroxide (H_2O_2). Glutathione peroxidase (GSH-Px) and catalase will then convert H_2O_2 to a water molecule. SOD, GSH-Px and catalase together provide the primary antioxidant defense mechanism. The non-enzymatic antioxidant defense system includes ascorbic acid (vitamin C), α -tocopherol (vitamin E), glutathione (GSH) and β -carotene (Kharrazi *et al.*, 2008 ; Flora, 2009).

In case of *Hevea* tree, native antioxidants are synthesized by its own biosynthesis pathways and their presences varies according to the physiological status of rubber trees as well as other agronomical conditions. Recently, the development of molecular biology in rubber tree had revealed molecular mechanisms that involve antioxidant mechanisms. For instance, HbAPX, an ascorbate peroxidases gene was reported to be involved in antioxidant system. HbCuZnSOD and HbMnSOD encoded antioxidant superoxide dismutase enzymes, and their roles in drought response were confirmed in rubber tree (Leclercq *et al.*, 2012). Besides oxidation occurring in biological systems, it could also occur in dry NR. At this state, the enzymatic processes could probably not be efficient anymore because of the no or low water activity and the most probably denaturation of enzymes during drying processes.

Therefore, when studying the resistance of dry rubber to oxidation, previously cited non-enzymatic antioxidants should be the primary target.

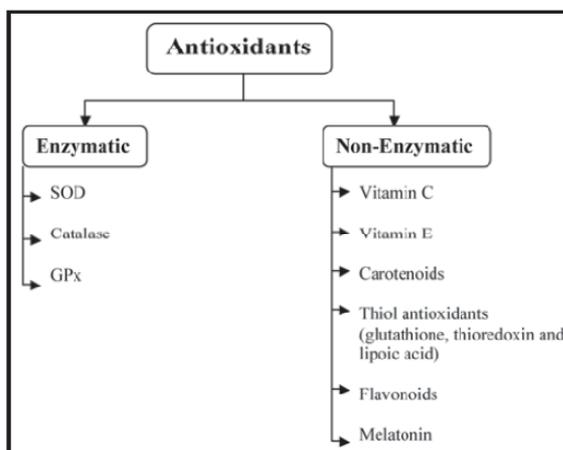


Figure 5 Enzymatic and non-enzymatic classification of antioxidants.

Source: Flora (2009)

5.3 Native antioxidant in natural rubber

NR, being an elastomer containing appreciable amount of unsaturation, is very susceptible to thermal oxidative degradation which generally results in its physical property deterioration both in raw and vulcanized state. Fortunately, due to its natural origin, NR is endowed with native antioxidants that promote its resistance to oxidation. The degree of resistance is however not consistent and depends on the methods of rubber processing. NR especially those prepared from natural coagulation are more susceptible to oxidation than the acid coagulated ones. This variation is attributed to the differences in their non-isoprene content (Hasma and Othman, 1990). Field latex has antioxidants that some of which are predominantly water-soluble and some are predominantly non water-soluble. When latex is converted to rubber by coagulation, most of non-water-soluble antioxidants are retained (Nadarajah *et al.*, 1971).

Various compounds in *Hevea* latex have been described to be native antioxidants such as amino acids, phenols, phospholipids, tocotrienols and betaines (Nadarajah *et al.*, 1971). Their appearance and activity were studied under different conditions. Tan and Audley

(1968) carried out identification of endogenous phenolic substrates in different latex fractions and found three types of betaines i.e. betaines ergothioneine, hercydine and trigonelline mostly accumulated in lutoids or bottom fraction. Indeed, plant betaine is an amphoteric compound that is electrically neutral over a wide range of physiological pH values. Its role was attributed to the stabilization of the structures and activities of enzymes and protein complexes and to the maintaining of the membrane integrity against the damaging effects of abiotic stress (Sakamoto and Murata, 2002). The occurrence of the three betaines compounds in latex was eventually found to be dependent on soil condition and activity of ergothioneine-synthesizing fungi. Though an accumulation of ergothioneine in latex bottom fraction could be confirmed as a phenomenon of vacuolar uptake and storage of materials in plant cell generally, its removal from latex and physiological significance is as yet unknown (Audley and Tan, 1968).

In case of proteins, due to the colloidal nature of latex, proteins are not homogeneously dispersed. Latex proteins are found in the sera and also associated with latex organelles. About 70% of latex proteins are soluble (C-Serum+B-Serum), with the remaining being associated with membranes. A small number of these latex proteins have given rise to the problem of latex allergy. Besides the allergenic issue, proteins present on rubber particles identified as rubber elongation factor (REF) is thought to be involved in rubber biosynthesis, although the exact mechanism is still under investigation (Yeang *et al.*, 2002). Moreover, an antimicrobial activity against invasion of pathogenic fungi of hevein, a small (4.7 kDa) cysteine-rich protein in the lutoids, was also reported (Van Parijs *et al.*, 1991). The presence and/or functions of proteins as antioxidants were less discussed in latex state. In addition, removal of proteins from latex was not found to affect its properties since deproteinized latex films showed good processability and vulcanizate properties compared to ordinary NR (Hayashi *et al.*, 1995 ; Sakaki *et al.*, 1995). Nevertheless, positive effects of NR proteins against thermal oxidation were mentioned in the NR sample prepared from acid coagulation of protein-removed latex. In other works, the reintroduction of proteins or other non-isoprene components in to NR samples was used to determine their effect on NR properties. For example, Boucher and Carlier (1964) reported that phosphoaminolipids, amine and amino acids reduced the oxidisability of raw rubber at 80 °C. In addition, mixing of these nitrogenous substance with other non-isoprene extract could act as antioxidant as effectively

as the whole non-isoprene component. Thus they could be considered as having a synergistic effect. A similar work carried out by Tuampoemsab and Sakdapipanich (2007) also mentioned that addition of the extracted NR protein showed a strong protection on deproteinized rubber toward oxidation by increasing PRI.

Another important antioxidant molecule in NR is tocotrienol, the unsaturated species of vitamin E. Tocotrienol differ structurally from tocopherol only by the presence of trans double bonds at 3',7' and 11' positions in the phytyl tail (Sen *et al.*, 2006) (Figure 6). The discovery of tocotrienol in *Hevea* latex was firstly reported by Whittle *et al.* (1966), They reported that *Hevea* latex is the major non-food source of tocotrienol with the content varying from 0.02% to 0.15% (w/w of dry rubber). In general, the hydrogen donating power of tocotrienol was believed to be in the order of $\alpha > \beta > \gamma > \delta$ form under *in vivo* test and reversed order ($\delta > \gamma > \beta > \alpha$) was obtained under *in vitro* test.

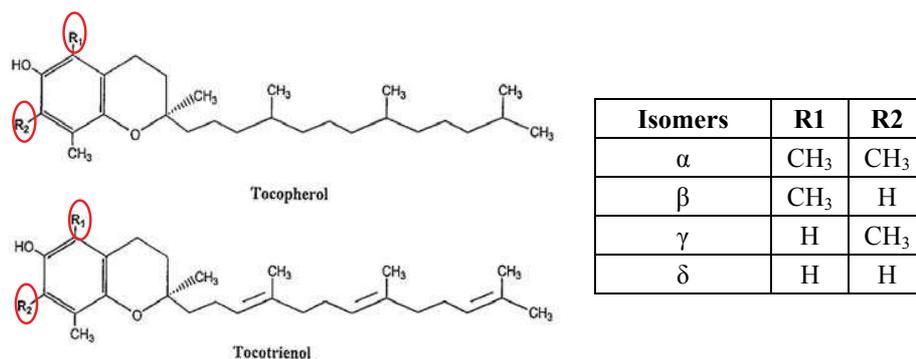


Figure 6 Molecular and chemical structures of tocopherol and tocotrienol.

Sources: Tan (2005)

The different forms of tocotrienols, α -, γ - and δ - form, were found depending on the clones and rubber processing (Nadarajah *et al.*, 1971 ; Hasma and Othman, 1990 ; Horvath *et al.*, 2006). The work of Liengprayoon (2008) reported the quantity of tocotrienol family in unsmoked sheet (USS) prepared from 4 different *Hevea* clones. A positive correlation between tocotrienol obtained after saponification reaction and PRI was observed. Among tocotrienol families, α -tocotrienol seemed to exhibit more antioxidant activity in every studied clone.

Incidentally, Nadarajah *et al.* (1971) studied the content of tocotrienol of *Hevea* latex from six clones that coagulated under different conditions (acid, bacterial and natural coagulations). No significant difference of tocotrienol content from these coagulated samples was observed. However the amount of esterified tocotrienol was found highest in natural coagulated rubber. The drying temperature at 140 °C was found to decrease tocotrienol content whereas the amount of tocotrienol of unheated and heated rubber at 100 °C was similar. Other authors stated that tocotrienol molecules are probably sensitive to oxidation under high temperature (Kamal-Eldin and Appelqvist, 1996).

Besides, studying the content of tocotrienols related to NR properties, the effect of addition the isolated tocotrienol from NR to PRI was also examined. Hasma and Othman (1990) found that isolated tocotrienol had a maximum protection on NR against thermal oxidation at a level of 0.1% (% dry rubber weight) and γ -tocotrienol gave a better protection than the α -form. PRI was increased from 20 to 86 and 81 when γ - and α -tocotrienol were added, respectively. The tocotrienol extract from NR reshown a sufficient protection level even the rubber was subjected to high drying temperature (60 and 110 °C) or to microorganism activity (maturated and/or degraded rubber). Therefore the low resistance of NR toward oxidation might be due to other factors not only the decomposition of this native antioxidant molecule. One of the causes could be the increase of pro-oxidant such as free copper release from copper complex substances by microorganism activity.

In vulcanized rubber, Morimoto (1985) demonstrated a positive correlation between the presence of tocotrienol and the tensile strength of the unspecified clone in the vulcanized state. He reported that the vulcanized heat-aged sample (at 100 °C for 24 h), which was prepared from hot acetone extracted RSS (lipid-removed rubber), had the lowest tensile strength. Meanwhile, the tensile strength value of vulcanized and heat-aged vulcanized of non-extracted rubber did not show any difference. To confirm the influence of tocotrienol, an acetone extracts which was identified to contain tocotrienol as a major component was returned to extracted rubber by roll milling. It was found that the tensile strength of the extracted rubber was increased and finally reached to the original level.

From previous works, activities against thermo-oxidation of native antioxidants were mainly claimed based on the extraction and reintroduction of these isolated compounds into extracted NR and/or normal NR then correlated with certain properties. However the antioxidant activity of isolated compounds from NR itself was not much studied. Since various *in vitro* antioxidant activity assays are available, it could be considered as a complementary experiment to better explain the function of native antioxidants against NR oxidation.

5.4 Antioxidant activity assay

Due to an increase demand in identifying new biomolecules that could be beneficial for health and other applications, several methods for rapid screening of antioxidant activity of molecules were developed. Now, many *in vitro* test methods are available but they are often performed in specific conditions that address different type of antioxidant activity (Laguerre *et al.*, 2009). Therefore, in most of the work carried out on identifying antioxidant activity of plant extract require the positive results from at least two methods to confirm their antioxidant properties. In terms of methods, chemical properties such as structure and solubility as well as mode of action have to be taken into consideration.

Two strategies based the two different measurements are summarized by Laguerre *et al.* (2009). Non-competitive, also called indirect method, are mentioned to be the easiest one. This strategy consists in measuring in the absence of any oxidizable substrate, the ability of an antioxidant to reduce a stable artificial free radical. An only disadvantage of this strategy is that a competitive reaction scheme in which antioxidant and substrate compete for oxidizing species could not be provided. The examples of the most widely used non-competitive methods that measure the antiradical of various molecules are DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid). Therefore, in contrast to a non-competitive strategy, the competitive one involves an oxidizable substrate and consists in measuring the inhibitory effect of an antioxidant on the oxidation of a substrate. As the competition between antioxidant and pro-oxidant occur under the same environment, the approaches of measurement could be performed either (i)

measuring the formation of oxidation products resulting from oxidation of a substrate, or (ii) measuring the substrate loss.

The methods based on the assessment of oxidation product formation are considered to be sensitive and suitable for most cases. However, the disadvantage is that the oxidation reaction that leads to primary oxidative product formation could be further oxidized to secondary products. Due to the lack of any universal oxidation marker it could result in the limited applicability. concerning the second approach, the method named CAT (conjugated autoxidizable triene) developed also by Laguerre *et al.* (2008) is an example of competitive method that measured a substrate loss. The degradation of conjugated triene in emulsion is accompanied by an absorbance decrease at 273 nm and the addition of antioxidant molecules results in a delay of oxidative process and enables the quantification of the antioxidant capacity.

Various works that determined the antioxidant properties of biomolecules have often used the indirect method due to its simplicity and rapid. Most of the work carried out an extraction of bioactive compound from plants using polar organic solvents such as ethanol and methanol as well as water hence most of developed methods are based on aqueous system. In contrast, the antioxidants possibly retained in dry rubber would be lipid-soluble as most of hydrophilic compound are leached out during processing and lipids remain as a major non-isoprene component. In addition, only some of lipid molecules such as tocotrienol could be solubilized in polar organic solvent but to test their synergistic effect as total lipids solubilization in polar organic solvent is impossible. Therefore, the work presented here aims at studying the evolution and activity of native antioxidants with suitable method. Therefore, the information obtained could be correlated with structure and physical properties of NR during maturation analyzed in parallel.

MATERIALS AND METHODS

1. Standards and chemicals

Standards, solvents and chemicals used for lipid extraction and lipid analyses are listed in Table 2.

Table 2 List of standards, solvents and chemicals.

Name	Suppliers and country	Reference
Organic solvents and chemicals		
Acetic acid	Merck, Germany	1000632500
Acetone	Carlo Erba, Italy	400974
Acetonitrile	RCI LabScan, Thailand	75058
Alumina oxide	Sigma, USA	A6139
Chloroform	RCI Labscan, Thailand	67663
Copper (II) acetate	Univar, Australia	860
Diethyl ether	Merck, Germany	1009211000
2,2-Diphenyl-1-picrylhydrazine, DPPH	Sigma, Germany	D9132
Ethanol	Merck, Germany	1009832500
Hydrochloric acid	Merck, Germany	1003172500
Propan-2-ol (HPLC grade)	Fisher, England	67630
Methanol	Merck, Germany	1060092500
Methanolic HCl	Sigma, Germany	33050-U
Molybdenum blue spray reagent 1.3%	Sigma, Germany	M1942
n-hexane	Merck, Germany	1043672500
N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)	Sigma, Germany	M7891
Orthophosphoric acid (85%)	Merck, Germany	1005731000
Pyridine	BDH, England	2038099
Primuline	Sigma, Germany	206865
Toluene	UNIVAR, USA	552
Silica gel60	Merck, Germany	1093851000
Sodium azide	Ajak Finechem	1222
Sodium chloride	Univar, Australia	465
Sodium hydroxide	J.T Baker, Italy	3722
Sodium methoxide solution	Sigma, Germany	403067
Standards		
Cholesteryl stearate	Fluka, Switzerland	K2846
Methyl tetradecanoate, C14:0	Larodan, Sweden	20-1400-13
Methyl hexadecanoate, C16:0	Larodan, Sweden	20-1600-13
Methyl palmitoleate, C16:1	Larodan, Sweden	20-1601-9
Methyl octadecanoate, C18:0	Larodan, Sweden	20-1800-13
Methyl oleate, C18:1	Larodan, Sweden	20-1700-13
Methyl linoleate, C18:2	Larodan, Sweden	20-1802-13
Methyl linolenate, C18:3	Larodan, Sweden	20-1803-9
Methyl eicosanoate, C20:0	Larodan, Sweden	20-2000-13
Glyceryl trioleate	Sigma, Germany	T7140

Table 2 (Continued)

Name	Suppliers and country	Reference
Methyl linoleate	Sigma, Germany	L1876
Mix 34 for TLC (monoolein, diolein, triolein, methyl oleate, oleic acid)	Larodan, Sweden	90-3034
Mix 40 for TLC (cholesterol, cholesteryl oleate, triolein, oleic acid, PC (egg))	Larodan, Sweden	90-3040
Oleic acid	Sigma, Germany	70750
Plant sterol mixture (β -sitosterol 53%, stigmasterol 7%, campesterol 23% and brassicasterol 13% w/w)	Larodan, Sweden	60-1003-7
Stigmasterol	Sigma, Germany	S2424
α -tocotrienol	Cayman Chemicals, USA	10008377
γ -tocotrienol	Cayman Chemicals, USA	10008494
δ -tocotrienol	Cayman Chemicals, USA	10008513
α -tocopherol	Sigma, Germany	T3251
Synthetic polyisoprene standards	Polymer Standards Service, Germany	

2. Latex sources

Two *Hevea* clones, RRIM600 and PB235 were used for all experiments. The rubber trees were tapped early in the morning. After tapping, latex was collected and filtered through a stainless steel sieve. The filtered latex was used for lipid extraction and sample preparation.

2.1 Latex source for uncontrolled maturation conditions experiment

The experiments involving maturation under uncontrolled conditions were carried out in the plantation of Visahakit Thai Rubber Co., Ltd. located in Chantaburi province, Thailand. The field of RRIM600 clone was planted in 1995 and firstly tapping in 2002. The applied tapping system was 1/2S, 2d3 which the trees were tapped on a 1/2 spiral cut downward for 2 out of 3 days while PB235 plantation was planted in 1994 and the tapping was started in 2001 with a 1/2S, d2 system.

2.2 Latex source for controlled maturation conditions experiment

The experiments involving maturation under controlled conditions using a maturation device were performed at Faculty of Sciences and Industrial Technology, Prince of Songkla University, Surat Thani campus in Surat Thani province, Thailand. Latex of

RRIM600 clone was obtained from Prince of Songkla University rubber plantation in Surat Thani campus. This plantation was planted in 1990 and tapped since 1997. The tapping system used was 1/2S, 3d4 which the trees were tapped on a half spiral cut downward for 3 out of 4 days and no stimulation was applied. In the case of PB235 clone, the latex was collected from the plantation of Mrs.Preyawan Sudchamnong located in Khun Talay district, Surat Thani province, Thailand. This plantation was planted in 1988 and tapped since 1994 with a 1/3S, 3d4 tapping system without stimulation.

3. Maturated sample preparation

3.1 Unsmoked rubber sheet (USS) preparation

The RRIT technical recommendations for rubber sheet making process were followed as shown in Figure 7. Three liters of filtered fresh latex were diluted with 2 L 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. During the sheeting process, rubber sheet was cleaned by rinsing with water. The rubber sheet was hung and dried under the shade in a ventilated area for 7 to 10 days.

3.2 NR sample prepared under uncontrolled maturation conditions (mini-sheets)

Unsmoke sheet preparation was used as a model for the experiments where the maturation conditions were not controlled (natural conditions prevailing in the plantation). In order to utilize a reasonable volume of latex, a specific procedure for mini-sheet preparation was developed to reduce the size of the rubber sheet but keeping similar properties as those of USS sheet prepared from RRIT recommendation (3 L of latex). The development of this procedure is detailed in Chapter 1.

Around 10.5 L of freshly tapped latex was filtered and pooled into a 20 L bucket through a metal sieve (2 mm pore size). The pH of filtered latex was measured. For each mini sheet, five hundred milliliters of latex were poured into coagulation box (18x28x7 cm) and gently mixed with 333 mL of water. For acid coagulation sample (0 day of maturation), 50 mL of 0.42 M formic acid solution was added. For matured coagula, pH of fresh coagulum and serum were measured before the coagulated rubber was removed from the coagulation box. The coagulated rubber was passed 1-2 times into a 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. The number of flat hand mangle passes was adjusted in order to obtain similar thickness as that of USS (final thickness of around 3 mm). The obtained mini-sheets were cut into two halves. The first half was dried under the shade in a ventilated area until complete transparent sheet was obtained. The second half was dried in a smoked house (Grand Rubber Company, Kaeng Hang Meao district, Chantaburi province) at 60 °C until no white spot appears on the sheets. The drying time, the weight and thickness of fresh and dry half sheets from both drying methods were recorded. Mini-sheets made from naturally coagulated rubber that were let mature in coagulation box for 1, 2, 3, 7, 9, and 14 days were prepared following a sheeting process similar as that used for acid coagulated sheets. The coagulation boxes were stored in an aerated room in rubber plantation. Three replications were performed for each maturation time. Each sample was used for P_0 and PRI measurement as well as structural and lipid composition analyses. The flow chart of mini-sheet preparation is shown in Figure 7.

3.3 Natural rubber sample prepared under controlled maturation conditions

The cup coagula were used as a model for controlled maturation conditions experiment. The samples were prepared using the procedure described by (Intapun, 2009) with minor modifications. For each clone, fresh latex was filtered through a 1 mm pore size metal sieve and pooled into a bucket. The initial pH of the latex was measured. This filtered latex was used for mini-cup coagula preparation with the conditions summarized in Table 3. In order to follow the evolution of physical properties, structures and lipid composition of NR obtained from matured cup coagula, 3 mini-cup coagula from each maturation condition were randomly sampled after 0, 3, 5, 7, 14, 21 and 28 days.

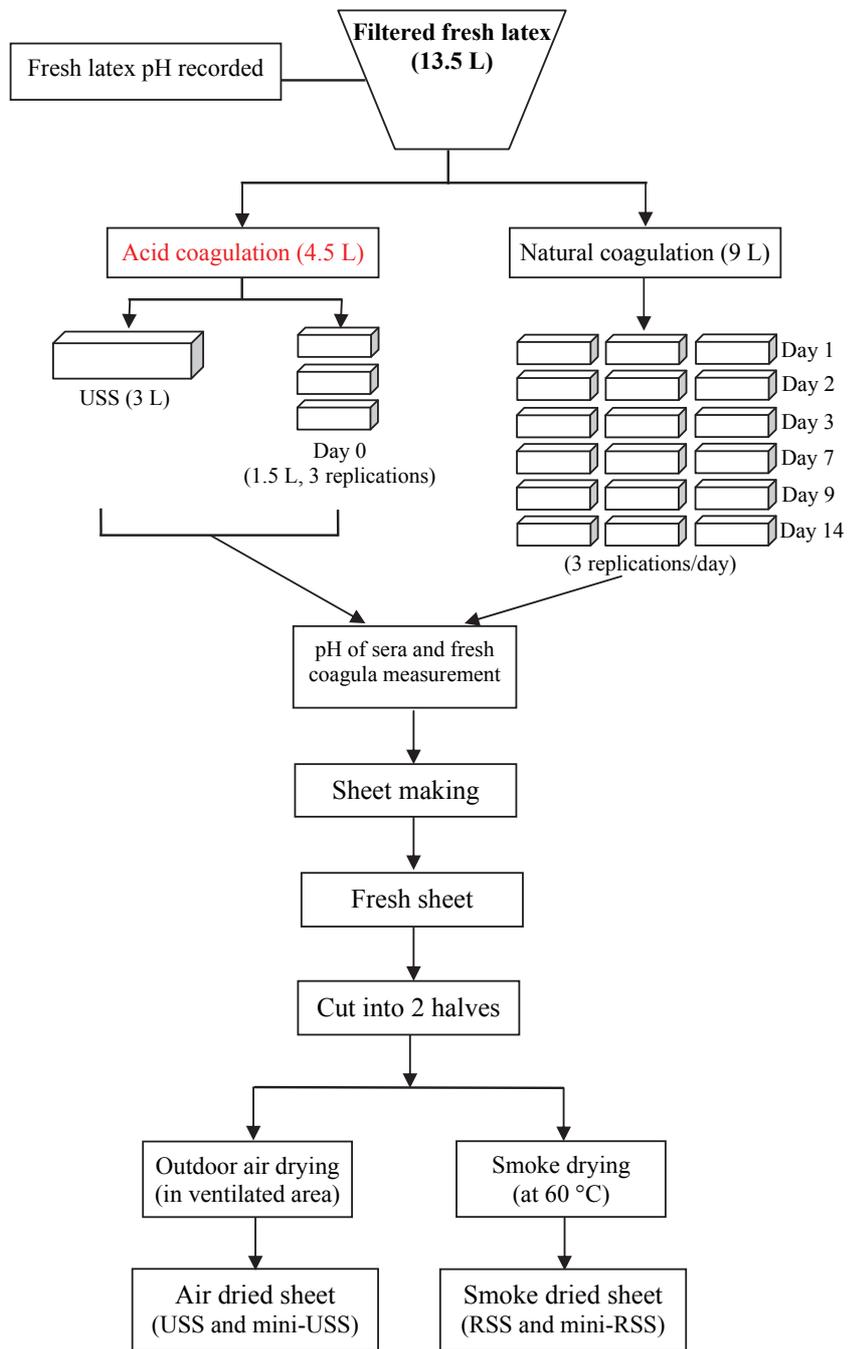


Figure 7 NR sample preparation for uncontrolled maturation condition experiment.

Table 3 The sample treatments and maturation conditions of mini-cup coagula.

Treatments	Maturation conditions	Coagulations	Maturation times (day)	Code
Sodium azide added latex (10% of NaN ₃ solution added)	Anaerobic	Acid	0, 3, 5, 7, 14, 21 and 28	Na+O-
Field latex (no NaN ₃ addition)	Anaerobic	Acid	0	Na-O-
		Natural	3, 5, 7, 14, 21 and 28	
	Aerobic	Acid	0	Na-O+
		Natural	3, 5, 7, 14, 21 and 28	

Forty five milliliters of latex were poured into a 4.7x5.5x6.8 cm mini-glass cup (Figure 8a) that were placed on stainless steel trays in maturation device for 3 days to allow latex to coagulate. After that, the mini-cup coagula were removed from the glass cup and placed in plastic cups (upper diameter 6.5 cm diameter, Figure 8b) until the sampling day. For day 0 sample, 5% of formic acid solution was added into the latex to decrease the pH of latex to 5.2. For sodium azide treated latex, since the coagulation was prevented, therefore, at day 3 the sodium azide added latex containing glass cups were taken out temporarily from the device and 200 μ L of 5% formic acid solution was added into the latex in order to induce coagulation. Then the obtained mini cup coagula were put back in a maturation device until the targeted maturation day. The pH of serum and fresh rubber coagulum were measured by inserting a pH probe in the coagula samples through a precut hole before they were passed through a mini-creper. This mini-creper is a scaled-down version of the creper used in the TSR factory. It has the following specification: roll diameter 10 cm; roll length 30 cm, diamond type groove (space between grooves 6 mm, groove width 2 mm), gap between rolls: 0.04 mm, speed of front roll: 190 rpm.min⁻¹, friction ratio: 1.3:1 (front:rear), engine power: 2.2 kW. The creping procedure consisted in 15 passes with folded crepe and 1 time of pass with unfolded crepe under water shower placed over the nip of rolls (width 30 cm, water flow 6.2 L.min⁻¹). The mini-crepe samples were dried in a hot air oven (UE700, Memmert GmbH & Co. KG., Germany) at 125 °C for 2 h without air renewal. The fresh and dry weights of the obtained mini-crepes were recorded. These dried crepes were used for further analysis (structure, physical properties and biochemical analysis). The flow chart of the mini-coagula sample preparation and maturation conditions is showed in Figure 9.

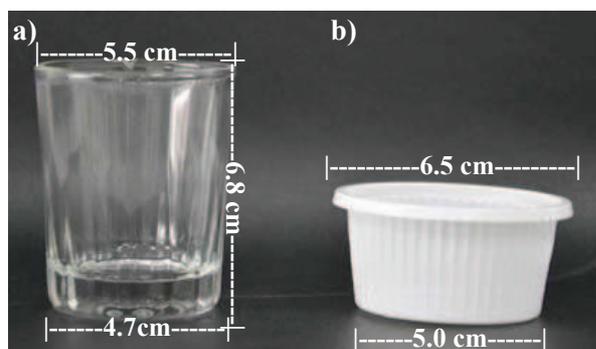


Figure 8 Mini-glass cup (a) and (b) plastic cup for the mini-cup coagula preparation.

3.4 Maturation box device

A maturation box device was used in order to control the environmental conditions during rubber maturation. The device consisted of a water tank, two insulated boxes and air humidifying column. Each insulated box contained 6 independent maturation units which could host 18 mini-cup coagula (Figure 10). The maturation conditions controlled in this experiment were air temperature, air relative humidity and oxygen content.

3.4.1 Air temperature control

The temperature of maturation was controlled through the circulation of water around the maturation units. The temperature of water was regulated by the immersion circulator Polystat model (Cole-Parmer, USA) located in the water tank and the water was circulated by a water pump ($900\text{L}\cdot\text{h}^{-1}$, Power Head AP 1600, Sonic, China) from this tank to the insulated box 1 and 2 as well as to the air humidifying column. Output water flowing from insulated box 1 was controlled by water valves in order to get proper water levels in each compartment (water tank, insulated box 1 and 2, and air humidifying column). The maturation units were immersed in water so that the surrounding water level reached $\frac{3}{4}$ of the height of the maturation units. The air temperature inside each maturation unit was recorded every 30 min by EL-USB-1-LCD temperature data logger (DATAQ Instruments, USA).

3.4.2 Oxygen content and air relative humidity control

The oxygen gas flow in the system was supplied by air compressor (XM 2540 3 HP, Puma). The nitrogen gas from nitrogen tank (9 m³, 99% N₂, Linde PLC td, Thailand) was used for the experiment without oxygen. Both gases were flown in polyurethane tubes (external diameter = 8 mm, internal diameter = 5 mm) equipped with a pressure regulator to delivered a 1.4×10^5 Pa (1378 millibar) pressure. Gas oxygen content was measured with a dedicated probe (Conox3, VWR GmbH, Germany) connect to a multimeter (Multi350 model, VWR GmbH, Germany). The oxygen tube and nitrogen tube were connected to the gas humidifying column separately prior to the maturation system. The air humidifying column consisted of a polycarbonate tube (thickness 2 mm, external diameter 7.5 cm, height 100 cm) equipped with PVC screw cap (diameter 5 cm) at the top. This column was filled with temperature-controlled water coming from the water tank, up to 80 cm in height. The water level in humidifying column was controlled by the relative position of this column to the water tank and by the inlet water flow. The gas inlet tube was plunged into water to a depth of 90 cm from the top of the column. The end of the tube was equipped by an air stone diffuser (ball form, diameter 4.5 cm). The gas bubbles went up through the water, reached the water and the outlet water saturated gas left the humidifying column through the outlet tube which was insulated by an EPDM rubber foam tube (Aeroflex-SSH, Aeroflex International Co., Ltd., Thailand). This was in order to keep the constant temperature and avoid further condensation of water. The gases were distributed to the maturation units by a system of “Y” connectors. Inlet flow of each maturation unit was adjusted by a speed flow controller. Gas relative humidity in each maturation box was recorded every 30 min by EL-USB-1-LCD temperature data logger (DATAQ Instruments, USA) (Figure 10).

4. Lipid extraction from dry rubber

The method of lipid extraction from latex and dry rubber were based on that of Liengprayoon (2008). A piece of dried dry rubber sample (2.8 ± 0.1 g) was cut into small pieces about 2x1x1 mm. The obtained pieces were stored at -20°C overnight before grinding. The grinding procedure was performed by using a cryogrinder model Pulverisette 0 (Fritsch,

Germany) under liquid nitrogen with 2 mm amplitude for 4 min. Then the ground rubber was immediately transferred to a bottle containing 50 mL of a mix of chloroform/methanol (2:1, v/v). The mixture was agitated at 160 rpm on a STUART SO1 orbital shaker (Bibby Scientific, England) for 6 h at 25 °C. The extracted rubber was re-separated from the solvent by filtration with Whatman n°1 filter paper and the total extract was evaporated using a rotary evaporator. The total extract was redissolved with 4 mL of chloroform/methanol.

5. Lipid composition analysis

Lipid extracts obtained from dry rubber samples were subjected to various analyses.

5.1 Qualitative analysis of lipids by thin layer chromatography (TLC)

Five microliters of diluted lipid extract at 10 mg.mL⁻¹ in chloroform from each sample was applied on silica gel 60G TLC plates (Merck, Germany). The plate was developed in a chamber containing the mobile phase. 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 °C for 10 min in a redLine model oven (Binder, Germany). Standard for TLC (mix 40 (Larodan, Sweden)) composed of oleic acid, triolein, phosphatidyl choline from egg yolk, cholesterol and cholesteryl oleate and γ -tocotrienol (Cayman chemical, USA) were used as reference.

5.2 Quantitative analysis of lipids by high performance thin layer chromatography (HPTLC)

5.2.1 Standard preparation and calibration curve

Six commercial lipid standards which were γ -tocotrienol, α -tocotrienol, free fatty acid (oleic acid (C18:1)), free sterol (stigmasterol), esterified sterol (cholesteryl stearate) and glyceryl trioleate (to represent triglycerides of furan fatty acid (TFG) as no commercial standard available) were used. Two of γ -tocotrienol esters and wax standards were purified

from RRIM600 lipid extract. All standard stock solutions were prepared in chloroform. Six concentration levels were used for calibration as detailed in Table 4. The three dimension image of six chromatograms obtained by densitometry analysis of the HPTLC track corresponding to the six concentration levels is shown in Figure 11. The obtained peak areas were plotted against the corresponding concentrations to obtain the calibration curves. Similar calibrations are done for each TLC plate.

5.2.2 Sample preparation

The lipid samples were diluted to 1 mg.mL^{-1} in chloroform and $10 \text{ }\mu\text{L}$ of samples solution were spotted on TLC plate (quantity on plate $10 \text{ }\mu\text{g}$).

5.2.3 Instrumentation and chromatographic conditions

HPTLC was performed on silica gel 60F₂₅₄, $20 \times 10 \text{ cm}$ TLC plates (Merck, Germany). Samples were applied on TLC plates as 4 mm band width at 15 mm apart from the bottom edge of the plate by using an ATS4 (CAMAG, Switzerland) sample applicator under a flow of nitrogen gas. The plates were developed in an automatic developing chamber ADC2 (CAMAG, Switzerland) previously saturated with mobile phase at room temperature for 3 min . The migration distance was 85 mm for general components separation with a mix of n-hexane: diethyl ether: acetic acid $80:20:1 \text{ (v/v/v)}$ as mobile phase. A 90 mm of migration distance and mix of n-hexane: diethyl ether: acetic acid $90:10:1 \text{ (v/v/v)}$ were used specifically for γ -tocotrienol esters and esterified sterols analyses. After elution, the plate was dried with a hair dryer and dipped 2 sec in a mixture of 40% orthophosphoric acid and saturated copper acetate aqueous solution ($1:1; \text{v/v}$) using an CAMAG chromatogram immersion device III (CAMAG, Switzerland). The plate was then heated at $180 \text{ }^\circ\text{C}$ for 10 min in an oven. The photographs of developed plates were taken by using CAMAG Reprostar3 (CAMAG, Switzerland) equipped with a Canon Power Short G2 camera using white light. Densitometric scanning was performed on CAMAG TLC scanner3 (CAMAG, Switzerland) at a wavelength of 325 nm . The operation of all automatic steps and quantitative analysis was performed with Win CATS planar chromatography manager software version 1.4.4.6337.

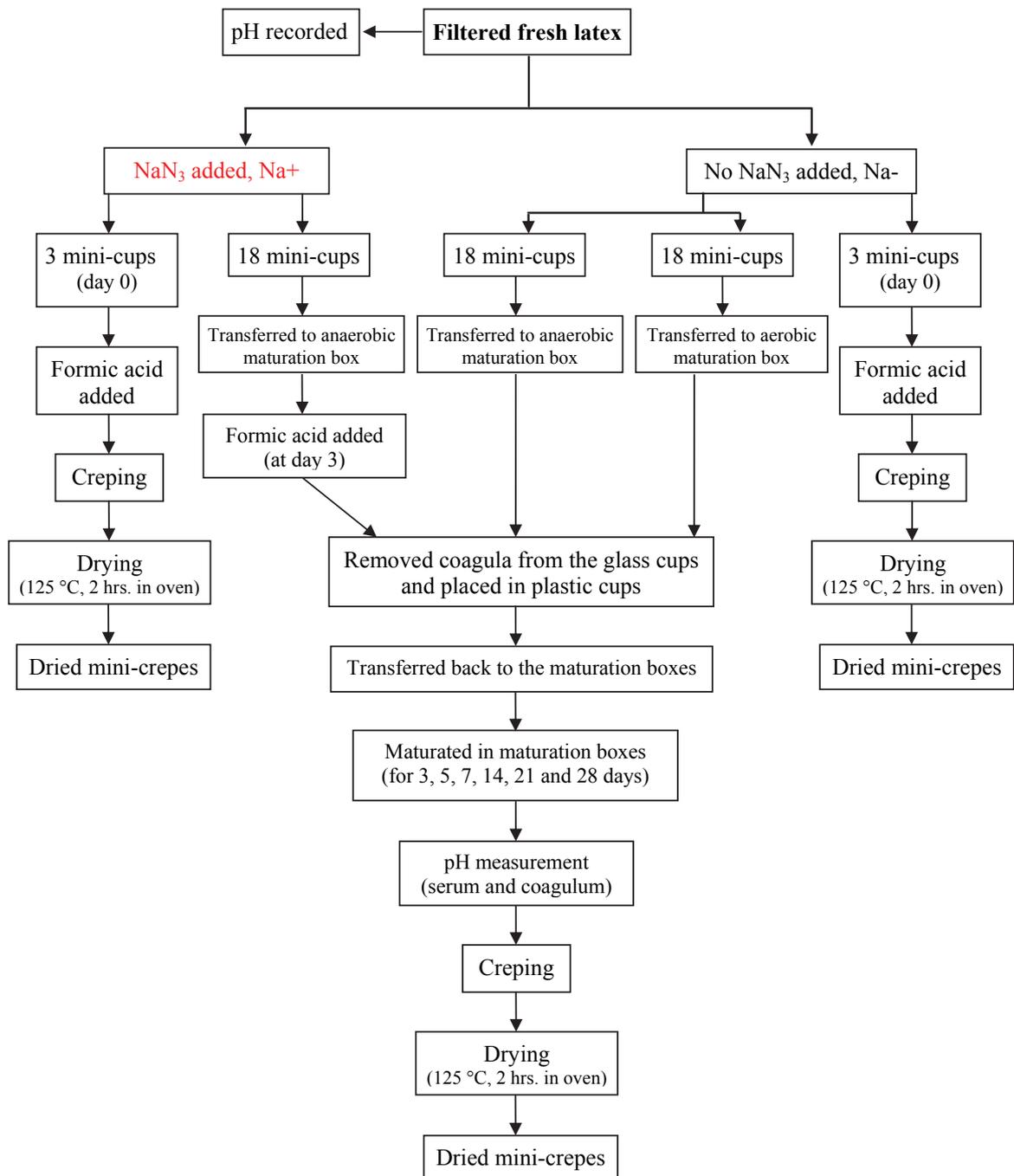


Figure 9 Flow chart of NR sample prepared under controlled maturation conditions.

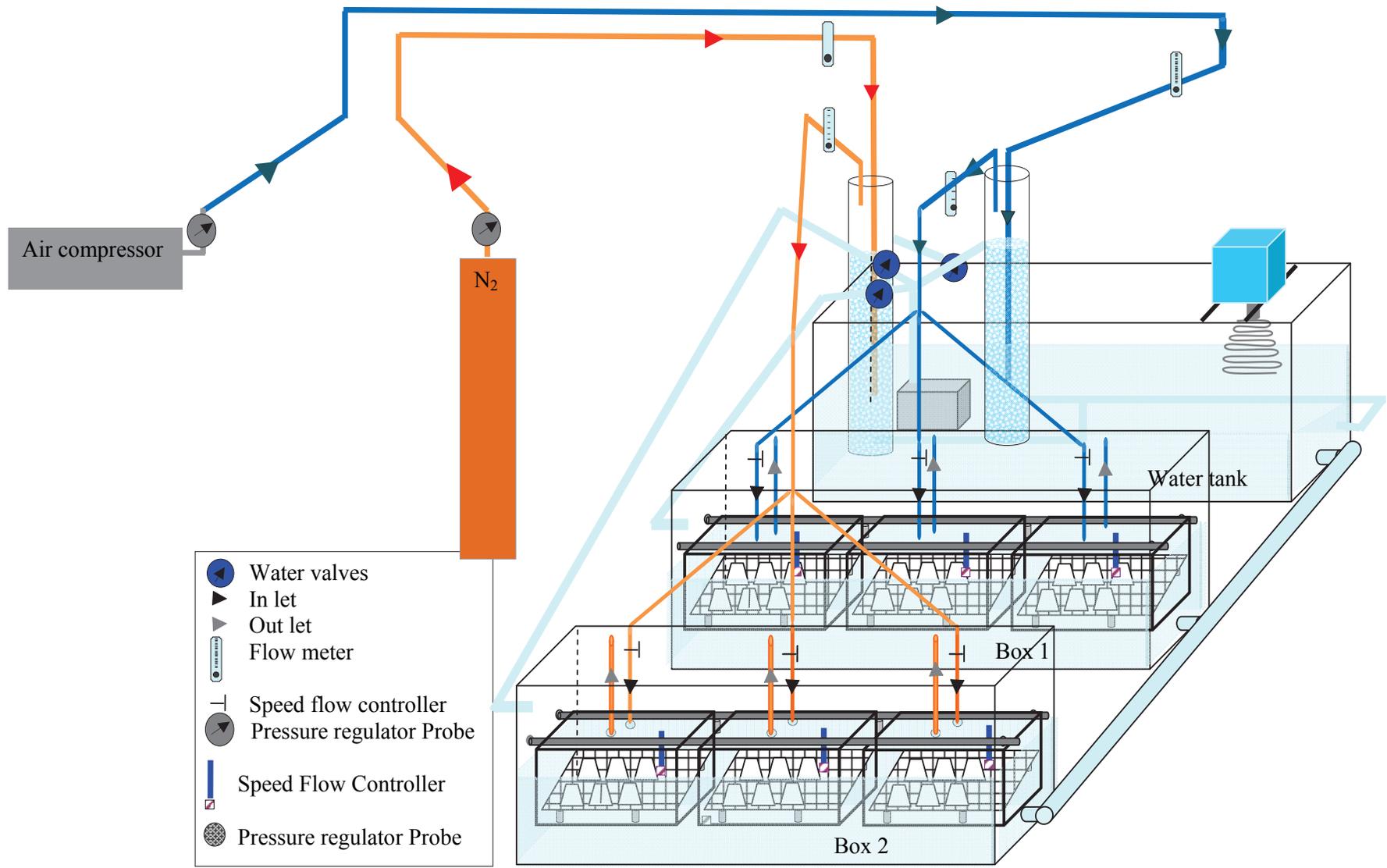


Figure 10 The maturation device system.

Table 4 Lipid standards for HPTLC calibration curves.

Lipid standards	Standard levels (μg on plate)					
	1	2	3	4	5	6
γ -tocotrienol	0.05	0.1	0.2	0.4	0.8	1.0
α -tocotrienol	0.05	0.1	0.2	0.4	0.8	1.0
Oleic acid (free fatty acids)	0.25	0.5	1.0	2.0	4.0	8.0
Stigmasterol (Free sterols)	0.1	0.25	0.5	1.5	3.0	6.0
Cholesteryl stearate (sterol esters)	0.1	0.5	0.75	1.5	2.0	3.0
Glycerol trioleate (triacyl glycerol)	0.1	0.25	0.5	1.5	3.0	6.0
γ -tocotrienol esters (I and II)	0.2	0.4	0.8	1.6	2.4	3.2
Wax	0.2	0.4	0.8	1.6	2.4	3.2

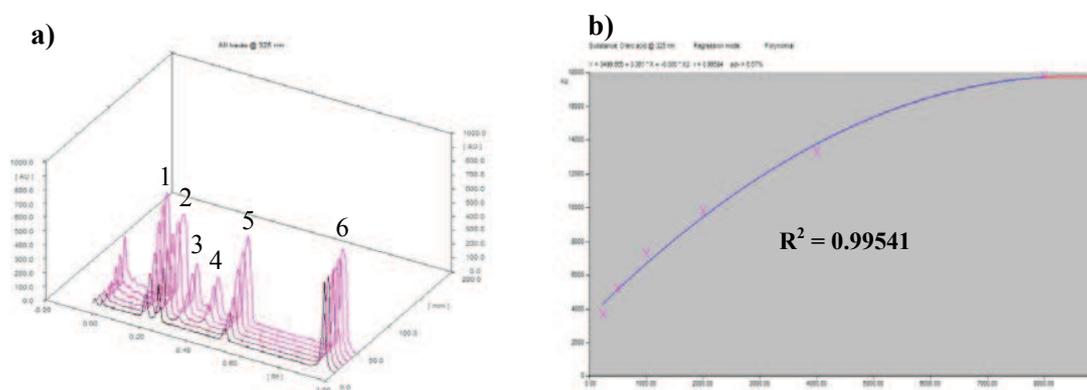


Figure 11 HPTLC three-dimensional image of the calibration curve of six standards (a) and (b) an example of calibration curve obtained for free fatty acids standard.

1: Stigmasterol (free sterols), 2: Oleic acid (free fatty acids), 3: γ -tocotrienol, 4: α -tocotrienol, 5: Glycerol trioleate (triacyl glycerol) and 6: Cholesteryl stearate (sterol esters)

5.2.4 Measurement repeatability and reproducibility

The measurement repeatability was determined from the data of three applications of same concentration of standards within the same day while the measurement reproducibility was determined from the analysis of three applications of same concentration of standards on different days. Each component from the sample was identified by comparing their R_f and the peak area with those of standard at 325 nm.

5.3 Total fatty acid and unsaponifiable compositions analysis by GC-FID/MS

Gas chromatography analysis of total fatty acids of lipid samples obtained from transesterification reaction was performed by converting them to their more volatile derivatives namely fatty acid methyl esters (FAME). The unsaponifiable composition identification by GC-MS was performed after silylation.

5.3.1 Preparation of fatty acid methyl esters (FAME) by transesterification

A 0.05 M sodium methoxide solution was prepared by mixing 62 mL of 0.5 M sodium methoxide solution with 560 mL of methanol (HPLC grade) and a few drops of phenolphthalein. Thirty milligrams of lipids were transferred to a 100 mL flat bottom flask and 3 mL of sodium methoxide solution containing phenolphthalein was added into the flask. The mixture was refluxed at 80 °C for 10 min. Then 3 mL of methanol hydrochloride solution were added and the mixture was refluxed again for 10 min. After cooling down at room temperature, 10 mL of water was added fatty acid methyl esters (FAME) were extracted with 10 mL of hexane for three times. The extracted solution was evaporated to dryness, weighed and re-dissolved in hexane in order to obtain a 1 mg.mL⁻¹ solution ready to be injected in GC-FID and a 2.8 mg.mL⁻¹ solution for GC-MS injection.

5.3.2 Analysis of total 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.s⁻¹. 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 programmed as follows: 160 °C for 0.5 min, increased to 200 °C at 10°C.min⁻¹ then kept constant for 4.6 min. The total run time of the analysis was 9.1 min. 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 of the fatty acid at different known

concentrations as mentioned in the Table 5 and Figure 12. This proportion between FAME was chosen regarding the one generally present in lipid from rubber.

Table 5 Concentrations of standard solution injected in GC ($\text{mg}\cdot\text{mL}^{-1}$).

Fatty acids	Retention times (min)	Level of standard concentrations ($\text{mg}\cdot\text{mL}^{-1}$)							
		1	2	3	4	5	6	7	8
C14:0	2.88	0.2857	0.5714	0.8571	1.4286	2.0000	2.2857	2.5140	2.8571
C16:0	3.76	0.0714	0.1429	0.2143	0.3571	0.5000	0.5714	0.6429	0.7143
C16:1	4.00	0.0500	0.1000	0.1500	0.2500	0.3500	0.4000	0.4500	0.5000
C18:0	4.85	0.0357	0.0714	0.1071	0.1786	0.2500	0.2857	0.3214	0.3571
C18:1	5.09	0.2143	0.4286	0.6429	1.0714	1.5000	1.7143	1.9286	2.1429
C18:2	5.55	0.4929	0.9857	1.4786	2.4643	3.4500	3.9429	4.4357	4.9286
C18:3	6.17	0.1214	0.2429	0.3643	0.6071	0.8500	0.9714	1.0929	1.2143
C20:0	6.29	0.0214	0.0429	0.0643	0.1071	0.1500	0.1714	0.1929	0.2143
Furan fatty acid	7.14	0.2857	0.5714	0.8571	1.4286	2.000	2.2857	2.5714	2.8571
Total		1.58	3.16	4.74	7.89	11.05	12.63	14.15	15.79

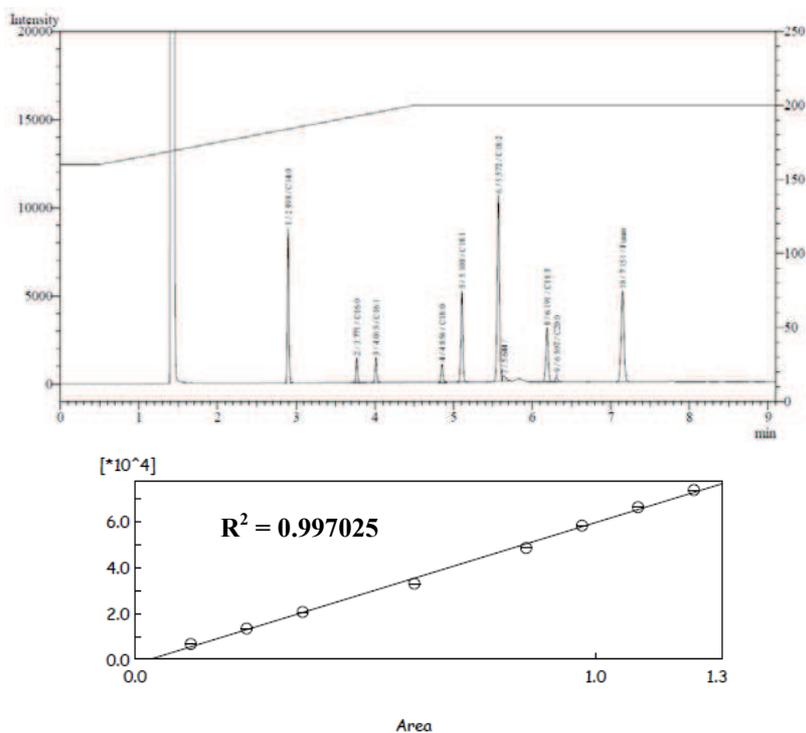


Figure 12 Chromatogram of fatty acid methyl ester (FAME) standards (a) and (b) calibration curve of linoleic acid.

5.3.3 Analysis of unsaponifiable composition by GC-MS

Unsaponifiable compounds were identified by GC-MS after silylation. A 250 μL of lipid sample after transesterified ($2.8 \text{ mg}\cdot\text{mL}^{-1}$ in hexane) was added with 50 μL of MSTFA and 50 μL of anhydrous pyridine. The mixture was incubated at $50 \text{ }^\circ\text{C}$ in a water bath for 30 min before injected to GC-MS. The injected concentration was $2 \text{ mg}\cdot\text{mL}^{-1}$. The gas chromatograph system used was Shimadzu QP2010S GC-MS equipped with a 5% phenyl polysilphenylene-siloxane BPX5 capillary column (30 m, i.d. 0.25 mm, film thickness 0.25 μm , SGE Analytical Science, Australia) and a Shimadzu AOC20i automatic injector (injected volume 1 μL). Linear velocity of helium in column was kept at $32 \text{ cm}\cdot\text{s}^{-1}$. Temperature of the split injector (split ratio 1:20) was $250 \text{ }^\circ\text{C}$. Oven temperature was set at $230 \text{ }^\circ\text{C}$ for 2 min, increased to $300 \text{ }^\circ\text{C}$ at $4 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$ then kept constant for 3.5 min. The mass spectrometer was operated in the electron impact (EI) mode, with electron energy of 70 eV. Mass spectrometric detection was conducted in scan mode from m/z 100 to 800 at 2.2 scans per sec; ion source temperature was $200 \text{ }^\circ\text{C}$. 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 curves were performed using 9 commercial standards at different known concentrations as shown in Table 6 and Figure 13.

Table 6 Concentrations of standard solution injected in GC-MS ($\text{mg}\cdot\text{mL}^{-1}$).

Components	Retention times (min)	Level of standard concentration ($\text{mg}\cdot\text{mL}^{-1}$)							
		1	2	3	4	5	6	7	8
C18 Fatty alcohol	5.43	0.013	0.025	0.050	0.075	0.100	0.150	0.200	0.250
C20 Fatty alcohol	7.67	0.013	0.025	0.050	0.075	0.100	0.150	0.200	0.250
δ -tocotrienol	17.63	0.010	0.020	0.040	0.060	0.080	0.120	0.160	0.200
γ -tocotrienol	19.15	0.010	0.020	0.040	0.060	0.080	0.120	0.160	0.200
Brassicasterol	20.47	0.007	0.013	0.026	0.039	0.052	0.078	0.104	0.130
Campesterol	21.54	0.013	0.026	0.052	0.078	0.104	0.156	0.208	0.260
α -tocotrienol	21.58	0.010	0.020	0.040	0.060	0.080	0.120	0.160	0.200
Stigmasterol	21.99	0.004	0.007	0.014	0.021	0.028	0.042	0.056	0.070
β -sitosterol	23.21	0.027	0.053	0.106	0.159	0.212	0.318	0.424	0.530
Total		0.11	0.21	0.42	0.63	0.84	1.25	1.67	2.09

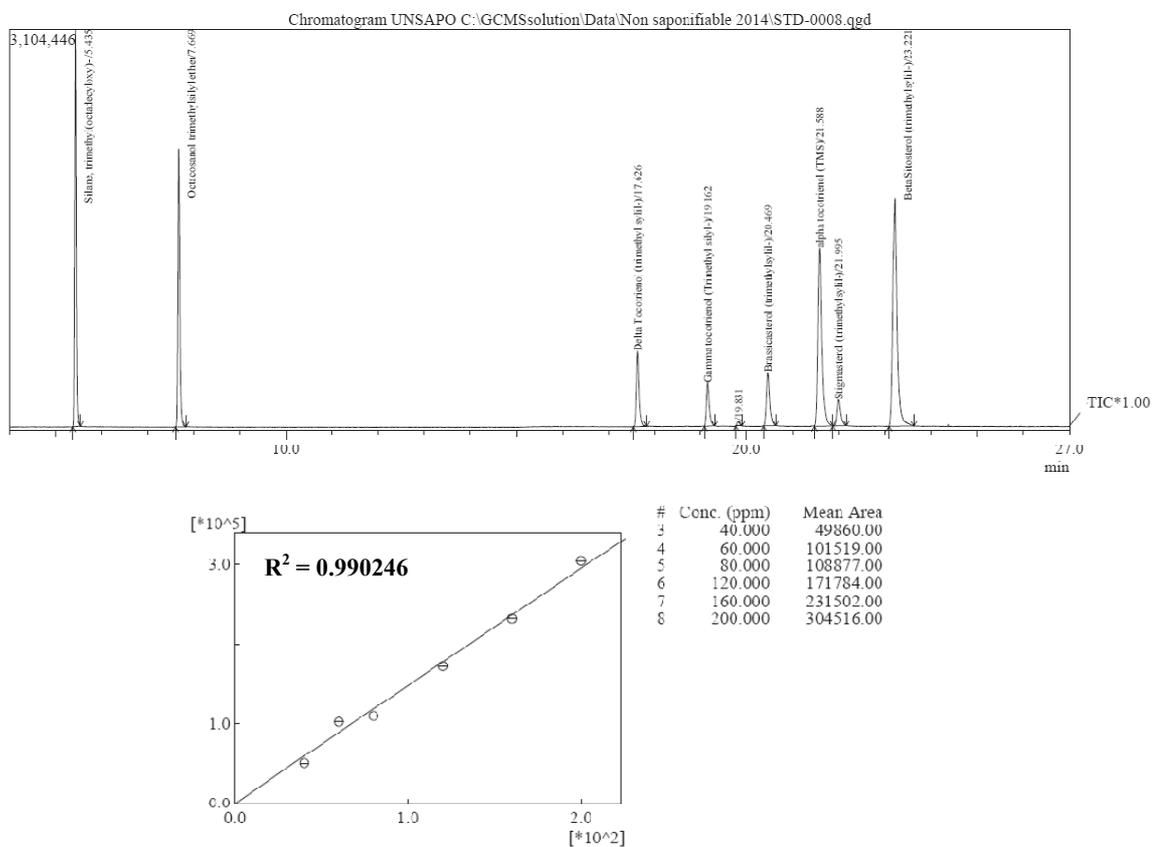


Figure 13 Chromatogram of unsaponifiable standard mixture (a) and calibration curve of γ -tocotrienol (b).

5.4 γ -tocotrienol derivatives identification

5.4.1 γ -tocotrienol dimers identification

The chemical structural identification of γ -tocotrienol dimers obtained from γ -tocotrienol commercial standard was identified by reverse phase HPLC-MS. The analysis was carried out using an Agilent Series 1100 HPLC system consisting of vacuum degasser, auto-sampler, a binary pump and a 1290 Infinity Diode Array Detector, Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was achieved using a Pursuit C18 column (5 μ m particle size, 200 Å pore size, 4.6x250 mm, Varian). Column temperature was maintained at 25 °C. Ten microliters of sample dissolved in acetonitrile/2-propanol (50:50, v/v) at 1 mg.mL⁻¹ was injected. The sample was eluted using an isocratic

solvent system containing acetonitrile/2-propanol/formic acid (50:50:1, v/v/v) at a flow rate of 1 mL.min⁻¹. This HPLC system was connected to an ion-trap mass spectrometer, Agilent LC-MSD-Trap-SL (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray interface operating in positive ion atmospheric pressure chemical ionization (APCI). Ions were detected in ion charged control (ICC) mode (target: 30,000 ions) with an accumulation time of 300 ms, using the following operation parameters: scanning range 200-1000 *m/z*; capillary exit voltage (fragmentor) 158.5 V; skimmer voltage: 40 V; octapole RF 150 V; nebulizer pressure 60 psi; drying gas flow 5 L.min⁻¹; gas temperature 325 °C. A full scan mass spectrum was acquired over the mass range between *m/z* 200 and 1000. Data handling was performed with ChemStation Family Software Products (Rev.A.10.02).

5.4.2 γ -tocotrienol esters purification and identification

5.4.2.1 γ -tocotrienol esters purification by preparative thin layer chromatography scrapping

γ -tocotrienol esters used in experiment were purified from NR lipid extract obtained from RRIM600 clone using thin layer chromatography scrapping technique as there is no commercial standard available. To conduct this purification, 0.8 mL of lipid solution (20 mg.mL⁻¹) from RRIM600 *Hevea* clone was applied on the preparative TLC (preparative silica 60G 20x20 cm, Merck, Germany) for 16 cm length (1 mg.cm⁻¹). The plate was developed in the chamber containing 100 mL of n-hexane/diethyl ether/acetic acid (90:10:1, v/v/v). The dried plate were cut from both edges for 2 cm and sprayed with a mixture of 40% orthophosphoric acid and saturated copper acetate aqueous solution (1:1; v/v) and then heated at 180 °C for 10 min in an oven. This cut plate was used as reference for target compound scrapping. Two bands which corresponded to R_f of γ -tocotrienol esters were then scraped off separately and transferred to a 25 mL brown glass vial. Ten milliliters of diethyl ether were added into the brown glass vial and shaken well for 10 min. The extraction was repeated 3 times with diethyl ether and the extract was filtered through 0.2 μ m syringe filter (Santorius, USA) to remove the silica gel. These purified γ -tocotrienol esters were used for chemical structural analysis by using HPLC-MS and lipid component quantitative analysis by HPTLC.

5.4.2.2 γ -tocotrienol esters chemical structural identification by high pressure liquid column chromatography-mass spectrometry (HPLC-MS)

The chemical structural identification of γ -tocotrienol esters was identified by reverse phase HPLC-MS. The analysis was performed using a Waters Alliance e2695 Separations Module equipped with a 2998 PDA detector (Waters, USA) and a microcolumn CSH130 C18 column (3.5 μm particle size, 2.1x50 mm, Waters, USA). The samples were eluted using an isocratic solvent system containing acetonitrile/Propan-2-ol/formic acid (75:25:0.1, v/v/v) that passed through the column at a flow rate of 0.1 mL.min⁻¹. Ten microliters of sample with a concentration of 1 mg.mL⁻¹ was injected. Components identified by PDA detection were monitored from 200 to 350 nm. Mass spectrum analyses were carried out with a SQ Detector2 single quadrupole mass detector (Waters, USA) equipped with a Z spray introduction source. Nitrogen was used both as a nebulizing gas and drying gas at flow rates of 400 L.h⁻¹ and 20 L.h⁻¹, respectively. The capillary voltage was 3 kV (in the electrospray positive ion mode) with a source temperature of 120 °C and a desolvation temperature of 580 °C. A voltage applied to the orifice of the instrument, also known as the cone voltage was set to a soft value of 20 V to detect protonated molecular ion [M-H]⁺ of the separated molecular species. A full scan mass spectrum was acquired over the mass range between m/z 200 and 1000. Data handling was performed with MassLynx 4.1 software.

6. Natural rubber properties measurement

6.1 Determination of dry rubber properties, Initial plasticity (P_0) and Plasticity retention index (PRI)

The initial Wallace plasticity number (P_0) and the plasticity retention index (PRI) were determined according to SMR bulletin 7 (1992) part. B.8. This P_0 and PRI determination requests a specific equipment: Wallace Rapid Plastimeter.

6.1.1 Homogenization of dry rubber

The dry rubber sample was homogenized following SMR bulletin 7 (1992) part B.2, standard. A 100 g piece of rubber was passed six times through the gap between the 150x300 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 ± 0.16 mm. After each pass, the rubber was rolled into a cylindrical shape 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 the test pieces were cut out for the various tests.

6.1.2 Determination of Initial plasticity (P_0)

The homogenized rubber was blended three times (doubling the sheet between each pass) through the rolls of a roller mill with adjusted nip in order to obtain the final sheet thickness of 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 thickness and 13 mm diameter were punched out from the homogenous dry rubber with a Wallace Punch. The test pellets were divided into two sets (Figure 14); the first three pellets from each homogenous sheet were for plasticity measurement before ageing and the other three pellets were for the measurement after oven ageing.

The test pellet sandwiched between two pieces of cigarette paper (RedStar Treadmark, China), was compressed between the two parallel platens of the Wallace Plastimeter (Wallace & Co. Ltd., UK) to a fixed thickness of 1 ± 0.01 mm and held for 15 ± 0.2 sec to reach approximate thermal equilibrium with the platen temperature (100 ± 1 °C). It was subjected to a constant compressive force of 100 ± 1 N for 15 sec at 100 ± 1 °C. The median value of the thickness after 15 sec compression of three pellets was taken as the measure of initial plasticity.

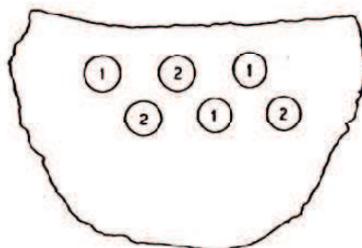


Figure 14 Doubled rubber sheet with punched test pieces

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

6.1.3 Determination of Plasticity retention index (PRI)

The three test pieces were heated in an air Wallace oven at 140 ± 1 °C for 30 ± 0.25 min. After the aged samples were cooled at room temperature, the plasticity of the aged pellets (P_{30}) was taken according the same method used for unaged samples (P_0). The median values of the three unaged and three aged test samples were used to calculate PRI as follows:

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

6.2 Mesostructure analysis by size exclusion chromatography coupled with a multi-angle scattering detector, SEC-MALS

6.2.1 Sample preparation

An amount of 30 mg of each dry rubber sample was weighed and placed in a weighed 50 mL Duran bottle. Filtered tetrahydrofuran (THF) stabilized with 250 mg.L⁻¹ of 2,6-ditert-butyl-4-methylphenol (BHT) was used for sample solubilization and also as mobile phase. Thirty milliliters of this solvent was transferred into the sample containing bottle. The samples were incubated in the water bath at 30 °C for 1 week and agitated daily for 1 h at 30 rpm using a Reax2 rotary agitator (Heildolph, Germany). After that, samples were filtered through 1 μm glass membrane into 1 mL vial before injection.

6.2.2 SEC-MALS analysis

The equipment consisted of a refractive index detector (Optilab rEX, Wyatt Technology) operated at 658 nm and 30 °C and a multi-angle light scattering detector (Dawn DSP, Wyatt technology Corp.) operated at 633 nm. The columns were two PL gel Varian mixed ALS columns (20 μm, 7.5x300 mm ID) with a guard column. The temperature of the column was maintained at 45°C. The mobile phase was THF stabilized with 250 mg.L⁻¹ BHT at a flow rate of 0.65 mL.min⁻¹ and the injected volume was 150 μL. The solvent used for sample solubilization and for mobile phase was prepared at the same time in order to avoid any interference due to the change of solvent lot. The number-average molar mass (M_n) and weight-average molar mass (M_w) were calculated by ASTRA software version 5.3.4.16 (Wyatt Technology). The values of gel content and average molar masses are calculated from the mean of the three injections for every sample (independent solution).

ASTRA software performed the calculation of the mass of rubber sample (m₁) injected after filtration by integrating the whole rubber peak on the chromatogram. Thus the exact concentration of the solution and the injected volume were known before and after filtration. The exact initial mass of sample before filtration, in 0.15 mL was known (m₀). The total of gel content could be calculated as following:

$$\text{Total gel content (\%)} = \left\{ \frac{m_0 - m_1}{m_0} \right\} \times 100$$

m₀ = mass of sample before filtration (in 0.15 mL)

m₁ = mass of injected sample (calculated by ATSR software)

7. Antioxidant activity test of total lipid using 2,2-diphenyl-1-picrylhydrazyl, DPPH method

7.1 γ-tocotrienol and NR lipid extracts samples antioxidant measurement

The antioxidant activity was measured using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) according to the method of Fagali and Catalá (2008) with modification of solvent from ethanol to chloroform:ethanol mixture (1:1; v/v) and the use of microassay device. The

2.5 mM DPPH stock solution was prepared by dissolving 98.5 mg of DPPH with 100 mL in chloroform:ethanol 1:1, v/v and then stored at -20 °C until needed. The 0.25 mM DPPH working solution was prepared freshly before use by diluting 10 mL of DPPH stock solution in 90 mL chloroform:ethanol solution. Tested concentration of lipid extract was 1 mg.mL⁻¹ dissolved in a mixture of chloroform:ethanol. The antioxidant activity of tested samples was compared with the known antioxidant, γ -tocotrienol (Cayman Chemical, USA). Gamma-tocotrienol concentrations used were 0, 0.01, 0.02 and 0.05 mg.mL⁻¹ dissolved in same solvent as sample. A volume of 125 μ L of 0.25 mM DPPH solution was added into the 96-wells plate which contain 125 μ L of the chloroform:ethanol solution containing either samples or γ -tocotrienol. Each sample was analyzed in triplicate.

In the DPPH test, the scavenging of DPPH radicals is followed by monitoring the decrease in absorbance at 517 nm from the initial purple color to yellow which occurs due to reduction by the antioxidant or reaction with a radical species. To determine the reaction kinetics, the assays were continuously monitored at 517 nm for 60 min period at 25 °C by using a Spectra Max190 microplate reader (Molecular Devices, USA). The 96-wells plate was covered with a lid to prevent solvent evaporation during measurement. The plate was shaken for 5 sec before each absorbance measurement. The absorbance value was recorded every 1 min until 60 min, against a blank of chloroform:ethanol solution. % DPPH remaining is reported as A_t/A_0 where A_t is absorbance at each measurement time and A_0 is absorbance at the beginning.

$$\% \text{ DPPH remaining} = \left(\frac{A_t}{A_0} \right) \times 100$$

A_t = Absorbance at each measurement time

A_0 = Absorbance at the beginning

7.2 Method repeatability and reproducibility

To study the method repeatability and reproducibility, both intra-day and inter-day absorbance measurement were performed. Method repeatability was determined by taking different concentrations of γ -tocotrienol, δ -tocotrienol, α -tocopherol and γ -tocopherol standard as shown in Table 7. The given concentrations were applied 3 times to see the

variation of their absorbance measurement within the same working day. For method reproducibility, the same concentrations were applied but their absorbance measurement was analyzed from two different working days.

Table 7 Concentration ranges of tocotrienol and tocopherol standards used for DPPH antioxidant activity method repeatability and reproducibility.

Standards	Level of concentrations (mg.mL⁻¹)
γ -tocotrienol and α -tocopherol	0.01, 0.02, 0.05, 0.1 and 0.2
δ -tocotrienol and γ -tocopherol	0.01, 0.02 and 0.05

CHAPTER 1

Methodology development

1. Introduction

A previous study conducted on Unsmoked Sheet (USS) type of rubber has reported the influence of lipid components on NR physical properties (initial plasticity (P_0), plasticity after ageing at 140 °C for 30 min (P_{30}) and plasticity retention index (PRI)) but the obtained range of properties was too narrow to draw robust conclusions on the quantitative involvement of non-isoprene components especially on PRI which is linked with the resistance of rubber towards thermal oxidation (Liengprayoon, 2008). The purpose of the present work was to continue this approach on a set of samples that displays a wider range of properties, especially PRI. It is well known that maturation of coagula induces a rapid and huge change of PRI index (Intapun *et al.*, 2009). In order to understand the evolution of the non-isoprene components and its effect on physical properties (such as P_0 and PRI) and structure, we have chosen to prepare and study rubber samples with various levels of degradation obtained under controlled conditions. The first approach was to obtain “degraded” USS rubber by inserting coagula maturation in the USS making process. This involved a methodology development step where different parameters were tested (dilution rate, drying conditions, maturation durations and clones). The structure, physical properties and non-isoprene components of matured NR were analyzed along maturation time.

Following the results obtained on the degraded mini-USS model, a second approach was conducted following the protocol previously developed in Prince of Songkla University (PSU, Surat Thani, Thailand) that involves the maturation of mini-coagula under strictly controlled conditions in order to simulate the TSR10 dry rubber processing.

Finally, the optimization of 2 biochemical analyses protocols will be presented. The use of HPTLC (High Performance Thin Layer Chromatography) to assess the evolution of lipid extract composition during the maturation was optimized. Moreover, as the present work intends to focus on resistance of NR towards oxidation through its native antioxidant

content, methodologies to assess antioxidant *in-vitro* activity of lipids extract obtained from matured NR were also investigated.

2. Matured mini-sheet preparation

The previous study of Liengprayoon (2008) has shown that the quality of prepared USS samples was high, characterized by PRI values ranging from 95 to more than 100. This narrow range of variation in physical properties did not allow to correlate the nature and quantity of lipid components with NR superior properties. NR samples with a wider range of physical properties are thus needed.

In a first approach, the USS obtained from laminar coagula preparation was selected as a model for matured rubber sample preparation. The main reason for this choice was that this processing method was fully optimized either technically (totally controlled processing) or geographically, with a dedicated workshop in an industrial rubber plantation (with clone origin controlled rubber trees) located less than 3-h drive from our Kasetsart University laboratory.

For normal size USS preparation, 3 L of latex was used. As a large number of samples is required to allow the study of different maturation times with 3 replications for each condition, it was decided to reduce the sample size, to lower latex consumption and ease the handling of samples. Moreover two drying methods were compared (the classical outdoor air drying and the oven drying). Several constraints were taken into account to develop this scaled down process: latex volume, latex dilution, drying conditions, and coagula maturation times. Two representative clones were used to provide latex (RRIM600 and PB235) for this methodological development.

2.1 Effect of volume reduction

To make a USS, the RRIT (Rubber Research Institute of Thailand) recommends to use 3 L of fresh latex and to dilute it with water at a ratio of 3:2 (latex:water v/v) in order

to obtain a diluted latex with a dry rubber content (DRC) of 18-20%. This level of DRC eases rubber coagulum lamination during sheet making step.

The minimum required quantity for the different analyses of the present study would be approximately 100-150 g of NR (dry weight). To obtain enough dry rubber samples and to minimize latex consumption, the volume of 500 mL of latex was chosen assuming that the averaged DRC was around 30-35%. This volume is 6 times smaller than the traditional one (3 L). All the volumes used in the processing were reduced consequently. The water volume was reduced to 333 mL instead of 2 L, the 0.42 M formic acid solution volume was 50 mL instead of 300 mL. The maturation tank was downscaled as well to 18x8x7 cm instead of 17x42x9.5 cm (Figure 15). The latex of two *Hevea brasiliensis* clones, RRIM600 and PB235, were used to prepare the samples.

To assess the effects of volume reduction, P_0 , P_{30} and PRI of rubber from normal USS and mini-USS prepared with diluted latex were measured.

The result showed that the physical properties of USS and mini-USS from the two studied clones were not different (Table 8). However it was observed that the mini-USS prepared with the latex from both clones tended to have higher P_0 and lower PRI than the normal one. Despite this non significant tendency, the conditions used for reduction of volume were validated.

Table 8 Physical properties of USS (3 L of latex) and mini-USS (500 mL of latex) from RRIM600 and PB235 clones.

Clones	Samples	P_0		P_{30}		PRI	
		Mean	SD	Mean	SD	Mean	SD
RRIM600	USS	41.4 ^a	0.38	35.3 ^a	1.01	85.3 ^a	2.95
	Mini-USS	44.9 ^a	1.76	34.7 ^a	1.45	78.1 ^a	3.51
PB235	USS	51.7 ^a	1.36	43.2 ^a	2.35	83.4 ^a	2.62
	Mini-USS	52.7 ^a	0.49	42.2 ^a	1.92	80.0 ^a	3.11

Data were means from 3 replications. For each column and for each clone, letters design group of means which are not significantly different (student *t* test), $p \leq 0.05$, SD: Standard deviation.

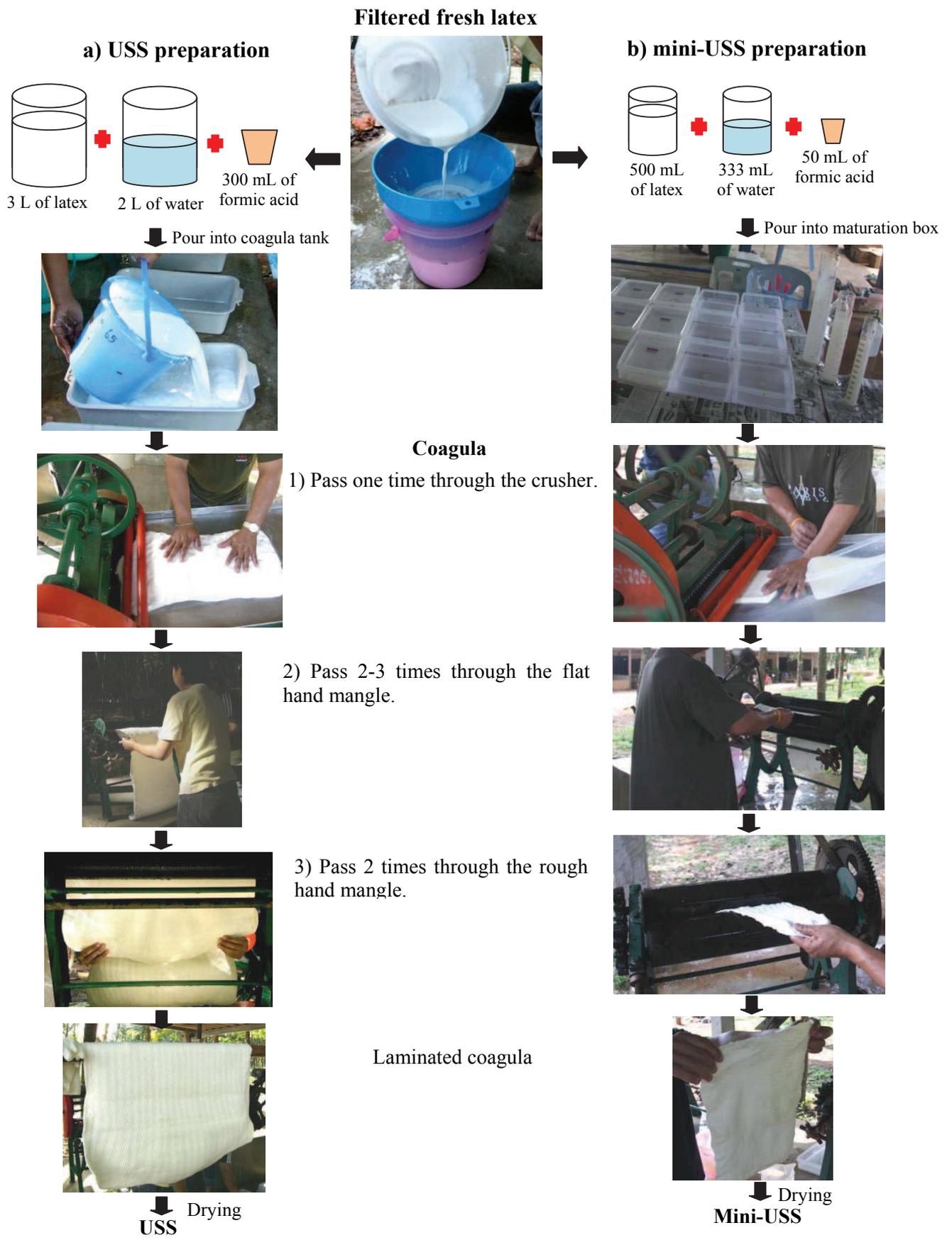


Figure 15 Unsmoked sheet (USS) (a) and (b) mini-USS processing.

2.2 Effect of latex dilution

Secondly, the effect of removing the latex dilution step in the process of making the mini-USS was studied, as the lamination step was easier with small size coagula. The same indicators were used to assess this factor.

When comparing mini-USS prepared from diluted and non-diluted latex, it was found that their physical properties were not different in the rubber from both clones (Table 9). However, it was observed that the samples prepared from the diluted latex were dried within a shorter time compared to the non-diluted samples. It could be explained by the fact that when the coagulum is made from diluted latex, it may increase the gap between each rubber particle. This larger gap would help the rate of water transportation from inside the coagulum to outside faster. Though dilution did not significantly change the properties of the dry rubber, it was decided to keep this step because of drying issue.

Table 9 Physical properties of mini-USS prepared from diluted and non-diluted latex of RRIM600 and PB235 clones.

Clones	Latex	P ₀		P ₃₀		PRI	
		Mean	SD	Mean	SD	Mean	SD
RRIM600	Diluted	45.3 ^a	1.06	33.8 ^a	0.35	74.6 ^a	2.55
	Non-diluted	42.8 ^a	1.89	34.0 ^a	1.41	75.0 ^a	3.18
PB235	Diluted	54.2 ^a	0.76	45.7 ^a	1.26	83.0 ^a	0.35
	Non-diluted	52.3 ^a	1.04	45.3 ^a	1.89	83.9 ^a	0.99

Data were means from 3 replications. For each column and for each clone, letters design group of means which are not significantly different (student t test), $p \leq 0.05$, SD: Standard deviation.

2.3 Effect of the drying method

In the perspective of working with matured samples, it was decided to test other drying methods than the classical outdoor drying. Sheets were hung under shade so that they were protected from direct sun light and rain. Indeed it is known that matured coagula are more difficult to dry than the fresh ones and request stronger drying conditions in terms of duration and temperature. This is the case of TSR10 or TSR20 processing, where

temperatures between 120 to 135 °C are applied for several h to rubber crumbs issued from matured coagula.

Two drying methods (outdoor air drying and oven drying) were tested and compared. Two kinds of laminated coagula were used:

i) Mini-laminated coagula (made from 500 mL latex diluted with 333 mL water and acidified with formic acid as optimized before).

ii) Matured mini-laminated coagula (same volumes of latex and water but no acid, the naturally coagulated coagula being let 1 week in the tank before lamination).

The same physical properties indicators were used (P_0 , P_{30} and PRI) to compare the treatments.

A preliminary trial was performed in our laboratory oven (WTB binder oven, Germany) at the temperature of 120 °C with mini-USS. The formation of numerous bubbles in the rubber led us to decrease temperature to 95 °C for the first comparison trial.

It was found that the non-matured (“day 0”) sample could dry under this temperature in a 3-4 h time. For RRIM600 clone, the P_0 and PRI obtained for non-matured samples were significantly different between outdoor air drying and 95 °C oven drying (Table 10). Indeed, P_0 was lower when the rubber was dried at a higher temperature, and PRI was higher while P_{30} remains the same. These results were not significantly confirmed with PB235 clone though the tendency remained (Table 11).

However, it was observed that at 95 °C, the matured sheet (i.e. 7 days of maturation) tended to melt before complete drying, which prevented the measurement of the physical properties (Table 10 and Table 11 for RRIM600 and PB235 clones, respectively). A lower drying temperature was then tried and it was chosen to use a smoke dryer used to make Ribbed Smoked Sheet (RSS). The smoke dryer, belonging to Grand Rubber Company (Chantaburi province, Thailand), was located not far from the plantation.

Contrary to 95 °C drying, smoke house drying allowed the full drying of matured samples though the drying duration of matured samples was longer than for the non-matured ones (around 7 days instead of 2-3 days). Concerning the properties, smoke drying improved the P₃₀ of RRIM600 rubber and therefore PRI, and same observation can be made for matured sample of PB235 rubber. It is mostly due to the fact that the smoke drying will stop earlier the maturation process in the laminated coagula. It is noted that PRI of matured samples are logically lower than those of non-matured sample.

The smoke drying was therefore selected to conduct further experiments.

Table 10 Physical properties of non-matured mini-USS (day 0) and matured mini-USS (day 7) of RRIM600 clone obtained using 3 drying conditions.

Maturation days	Drying temperatures	P ₀		P ₃₀		PRI	
		Mean	SD	Mean	SD	Mean	SD
0	Outdoor	45.3 ^b	0.75	33.8 ^a	0.25	74.6 ^b	1.80
	95 °C	34.2 ^d	0.17	35.5 ^a	1.00	103.9 ^a	2.74
	60 °C (smoke)	38.2 ^c	0.17	39.0 ^a	0.29	102.2 ^a	0.43
7	Outdoor	58.2 ^a	2.57	11.8 ^b	6.21	20.6 ^c	11.6
	95 °C	N/D	N/D	N/D	N/D	N/D	N/D
	60 °C (smoke)	58.2 ^a	0.58	37.8 ^a	0.35	65.1 ^b	1.41

Data were means from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test), $p \leq 0.05$, SD: Standard deviation. N/D: could not determine (the sample was degraded under test temperature).

Table 11 Physical properties of non-matured mini-USS (day 0) and matured mini-USS (day 7) of PB235 clone obtained from 3 drying conditions.

Maturation days	Drying temperatures	P ₀		P ₃₀		PRI	
		Mean	SD	Mean	SD	Mean	SD
0	Outdoor	54.2 ^a	0.76	45.7 ^a	1.26	83.0 ^{ab}	0.35
	95 °C	49.8 ^a	0.29	44.2 ^a	0.29	88.6 ^a	1.10
	60 °C (smoke)	49.3 ^a	1.53	41.8 ^a	0.76	84.8 ^{ab}	1.31
7	Outdoor	47.0 ^a	8.32	22.3 ^b	6.72	42.8 ^c	11.5
	95 °C	N/D	N/D	N/D	N/D	N/D	N/D
	60 °C (smoke)	54.3 ^a	2.89	39.0 ^a	3.00	71.9 ^b	6.65

Data were means from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test), $p \leq 0.05$, SD: Standard deviation. N/D: could not determine (the sample was degraded under test temperature).

2.4 Maturation duration

The purpose of this methodological development was to obtain significant evolution of P_{30} and PRI indicators in a reasonable period of time. Preliminary experiments were conducted and showed that the 7 days maturation duration was not enough to see very significant changes of physical properties. Therefore it was chosen to extend maturation time up to 14 days. For every maturation duration, 3 independent replications were performed.

Figure 16 presents the evolution of rubber physical properties during 14 maturation days of RRIM600 and PB235 samples dried from 2 drying methods. It has to be noted that after 24 h, RRIM600 latex was not coagulated, which explains that no physical data could be measured for 1-day maturation. It can be observed that PRI and P_{30} evolved significantly during maturation, so 14 days maturation time was extended to 14 days. Chapter 2 will discuss the evolution of these physical properties indicators during maturation and depending on drying condition in details.

However, despite several repetitions of the protocol, the variance of the rubber properties data was not considered enough satisfactory to select this protocol for further studying the lipid composition and the native antioxidant dynamics. This variance could be due to the difference of environmental conditions such as temperature, air relative humidity etc. during rubber maturation and/or drying. Indeed, maturation occurred in plastic boxes that were voluntarily placed in an uncontrolled environment (an open shelter nearby the USS workshop). One key factor that could explain the high variance could be the microbial population. Indeed, the initial number of microorganism in *Hevea* latex and their activity has been reported to affect NR properties (Rose and Steinbuchel, 2005 ; Intapun *et al.*, 2009). In our experiment, latex inoculation was uncontrolled and it is possible that it could vary from one box to another (including between 3 repetitions). Indeed, different contamination levels could be visually observed when the matured coagulum were handled and washed before lamination as presented in Figure 17.

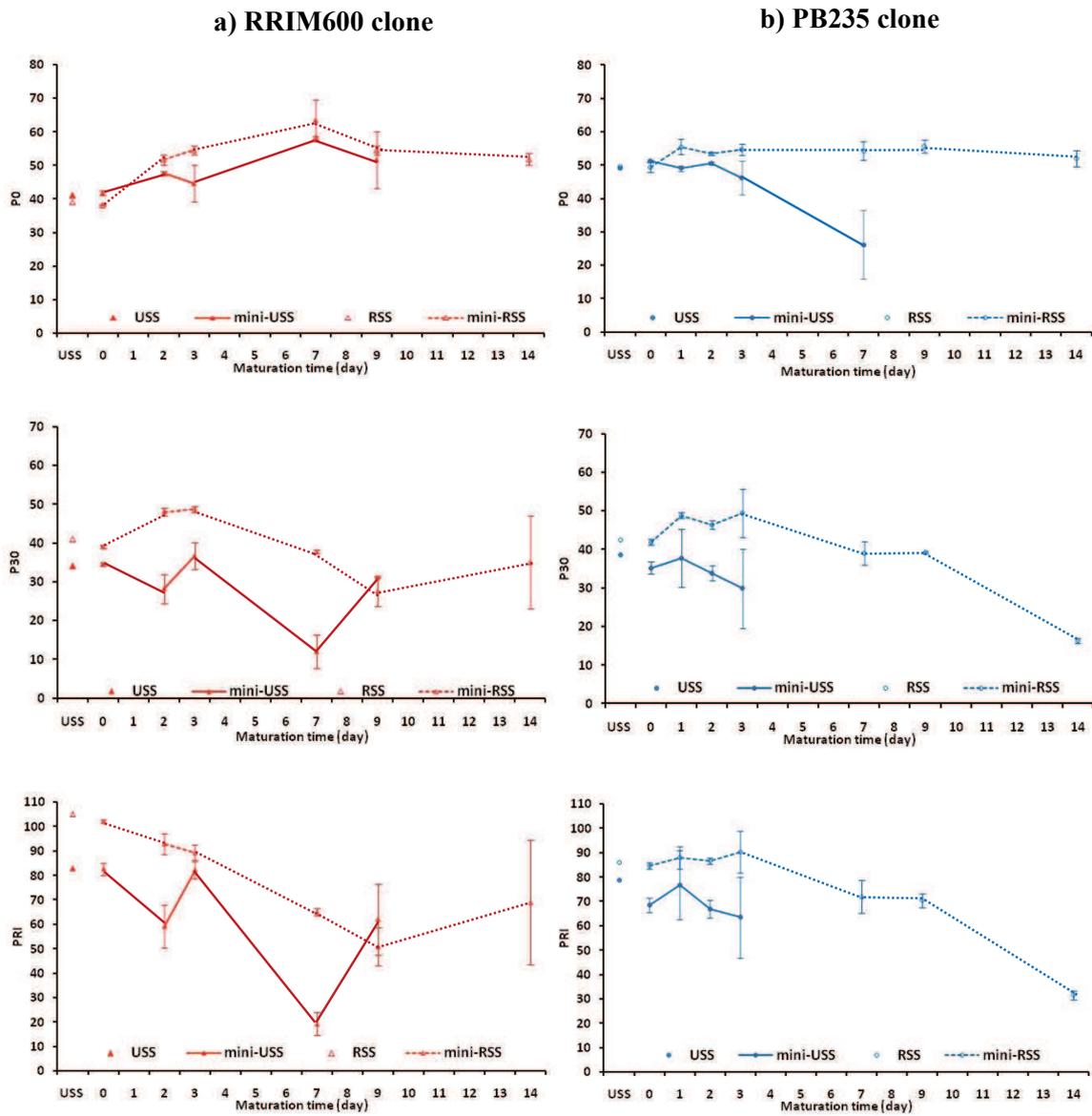


Figure 16 Physical properties (P_0 , P_{30} and PRI) evolution during 14 days of maturation of sheet coagula from (a) RRIM600 and (b) PB235 rubber clones.

Following the not fully satisfactory results obtained from mini-sheet protocol, it was decided to conduct further experiments under strictly controlled environment conditions. The maturation device used was developed during the PhD thesis of Jutharat Intapun in PSU, Surat Thani, Thailand. The detailed description of this device is in Materials and Methods.

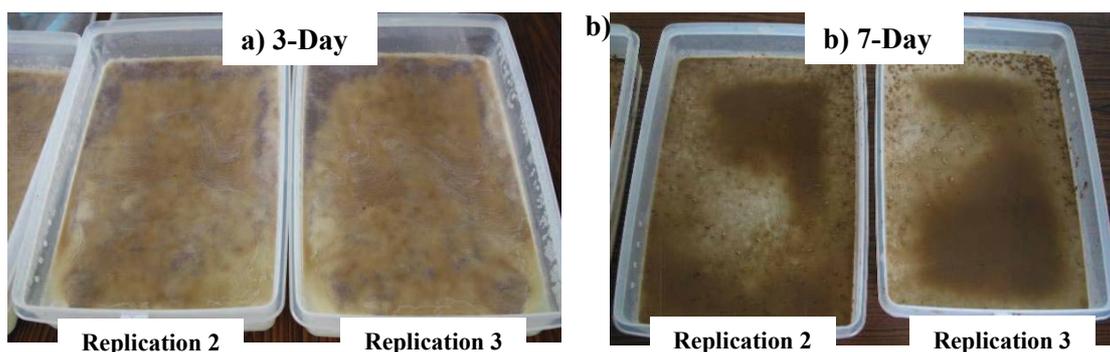


Figure 17 RRIM600 coagula from 2 replications of (a) 3 days and (b) 7 days of maturation.

3. Optimization of High Performance Thin Layer Chromatography (HPTLC) for NR lipid composition analysis

Many modern chromatographic techniques for the analysis of lipid extracted from NR samples, such as Gas Chromatography (GC) and Liquid Chromatography (LC) combined with Mass Spectrometry (MS), have been reported (Liengprayoon, 2008). However, these chromatographic techniques are time consuming and costly.

Among the modern analytical methods, High Performance Thin Layer Chromatography (HPTLC) allows separation with a good resolution and provides qualitative and quantitative results for a wide range of compounds. HPTLC can be applied for the analysis of NR lipid extracts without any pre-treatment, which could avoid the possible structure modification of compounds during sample treatment. Accordingly, the objective of this present methodology development study was to validate a new HPTLC condition for the analysis of lipid compositions obtained from matured NR samples, especially γ -tocotrienol and its esterified forms. The technical details of the HPTLC operations are indicated in Materials and Methods.

3.1 Mobile phase optimization

For optimization, different mobile phases were employed to achieve a good separation of target components which were sterols, free fatty acids (FFA), γ -tocotrienol, triglycerides of furan fatty acid (TGF), two γ -tocotrienol esters (I and II), wax and sterol esters (SE). Initially, a conventional mobile phase consisting of hexane/diethyl ether/acetic acid 80:20:1 (v/v/v) was tried (Figure 18a) with reference standards and PB235 lipid samples from different maturation times. Two other conditions were tested with the same sample:

-Successive elutions with 2 mobile phases consisting of hexane/diethyl ether/acetic acid at ratio of 50:50:1 v/v/v (until 4 cm from the plate bottom, followed by a drying cycle) and 90:10:1 v/v/v (until 8 cm) (Figure 18b)

-Elution of hexane/diethyl ether/acetic acid at ratio of 90:10:1 v/v/v (Figure 18c)

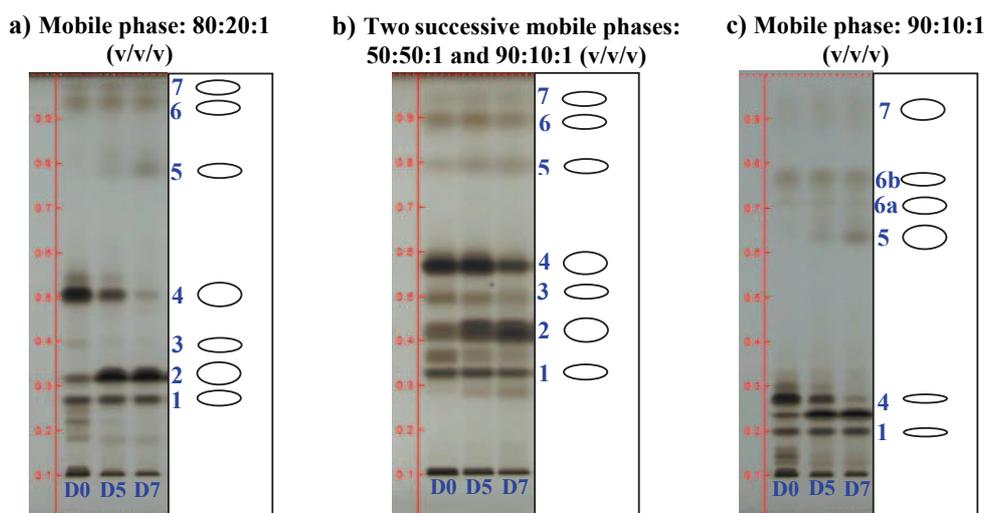


Figure 18 TLC pictures obtained from lipid extracts of PB235 rubber from matured coagula (D0: 0-day, D5: 5-day and D7: 7-day maturation) developed in (a) hexane/diethyl ether/acetic acid 80:20:1 (v/v/v), (b) elution of 2 successive mobile phases, hexane/diethyl ether/acetic acid 50:50:1 (v/v/v) and 90:10:1 (v/v/v), and (c) hexane/diethyl ether/acetic acid 90:10:1 (v/v/v).

1: Sterols, 2: Free fatty acids, 3: γ -tocotrienol, 4: Triacylglycerols of furan fatty acid, 5: Wax, 6: γ -tocotrienol esters (not separated), 6a and 6b: γ -tocotrienol esters (separated), and 7: Sterol esters.

With the classical hexane/diethyl ether/acetic acid 80:20:1 (v/v/v) mobile phase, the two γ -tocotrienol esters could not be separated (Figure 18a, spot no. 6). The successive elutions with of two different mobile phases (Figure 18b) separated well the compounds with a low retention factor (R_f) but did not resolve the γ -tocotrienol esters (Figure 18b, spot no. 6). A single elution with hexane/diethyl ether/acetic acid 90:10:1 (v/v/v) was found to be an appropriate mobile phase to separate the two γ -tocotrienol esters bands, as presented in Figure 18c, spot no. 6a and 6b.

Therefore, it was not possible to analyze all lipid components using a single TLC condition even with a double migration. Lipid components analysis was thus divided into 2 target groups. The group of high R_f components, which are γ -tocotrienol esters (Figure 18c, spot no. 6a and 6b), wax and SE were separated by hexane/diethyl ether/acetic acid 90:10:1, v/v/v. The mix of hexane/diethyl ether/acetic acid 80:20:1, v/v/v was used for separation of low R_f component composed of free sterols, FFA, free γ -tocotrienol and TGF.

Table 12 Lipid standards concentration levels for calibration curves.

Standards	Concentration levels (mg.mL ⁻¹)									
	1	2	3	4	5	6	7	8	9	10
FFA (oleic acid (C18:1))	0.25	0.50	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0
Sterol (stigma sterol)	0.2	0.4	0.8	1.6	2.4	3.2	4.0	4.8	5.4	6.2
γ -tocotrienol	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8
TG	0.1	0.2	0.4	0.8	1.6	2.4	3.2	4.0	4.8	5.4
γ -tocotrienol esters (I and II)	0.2	0.4	0.8	1.6	3.2	4.8	-	-	-	-
Wax	0.2	0.4	0.8	1.6	3.2	4.8	-	-	-	-
SE (cholesteryl stearate)	0.2	0.4	0.8	1.6	2.4	3.2	4.0	4.8	5.4	6.2

3.2 Standard calibration curve

The quantities of the compounds were calculated from the standard calibration curve. In our study, the calibration curves of sterol, FFA, γ -tocotrienol, triacylglycerols (TG) and SE commercial standard were first obtained with 10 different concentrations while only 6 concentration levels were used for two γ -tocotrienol esters and wax purified from RRIM600 lipid as shown in Table 12. The three dimension image of standard chromatograms obtained

by densitometry analysis of the HPTLC tracks corresponding to the concentration levels is shown in Figure 19.

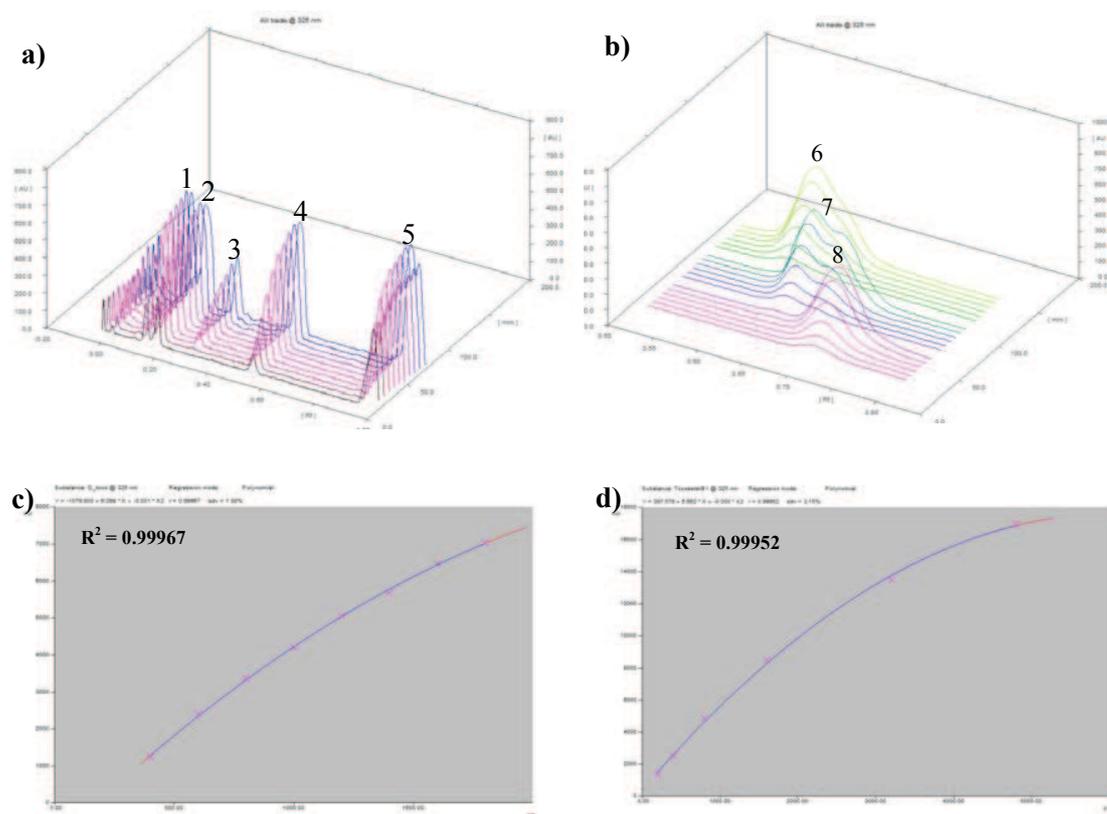


Figure 19 HPTLC three-dimensional image of the calibration curve of 8 standards (a and b), (c) the example of calibration curve of γ -tocotrienol commercial standard and (d) γ -tocotrienol ester (peak no. 6) purified from RRIM600 lipid extract.

1: Sterols, 2: Free fatty acids, 3: γ -tocotrienol, 4: Trioileylglycerols, 5: Sterol esters, 6 and 7: γ -tocotrienol esters and 8: Wax.

The calibration curves were obtained using a 2nd degree polynomial model. The developed HPTLC method allowed the simultaneous quantitative analysis of 21 samples.

3.3 Repeatability and reproducibility of the HPTLC method

The repeatability (3 plates performed within a same working day) and reproducibility (3 plates performed on 3 working days) of the method were verified by the average of peak area obtained from 4 concentration levels of γ -tocotrienol, sterol, TG and FFA standards. After the 4 standards were applied on TLC plate, the plate was developed with hexane/diethyl ether/acetic acid 80:20:1 v/v/v. The calculated coefficients of variation (CV) of the standard peak areas are shown in Table 13 and Table 14. The low value of CV (less than 9%) indicated that this technique can be considered as repeatable and reproducible.

3.4 Conclusion

No single mobile phase was found to separate all compounds so different mobile phases were purposely used to obtain the successful separation of all components. Hexane/diethyl ether/acetic acid 80:20:1, v/v/v was suitable for sterols, FFA, free form of tocotrienols (γ - and α -form) and TGF separation. Meanwhile, using the same solvent with the ratio of 90:10:1, v/v/v was appropriate for the separation of esterified forms of γ -tocotrienol and sterols. The repeatability and reproducibility of the developed method were confirmed by a dedicated study.

Table 13 Measurement repeatability obtained from peak area analysis of γ -tocotrienol, sterol, trioleylglycerols (TG) and free fatty acid (FFA) standards (3 plates on the same day).

Repeatability	Working days	Plates	γ -tocotrienol				Sterol				
			Concentration levels (mg.mL ⁻¹)				Concentration levels (mg.mL ⁻¹)				
			0.2	0.4	0.8	1.0	0.1	0.5	1.5	3.0	
Repeatability	Day 1	a	1404	2703	4911	5658	2761	6935	11002	13608	
		b	1431	2813	5160	6118	2778	7375	11724	14749	
		c	1408	2892	5184	5930	2779	6730	10578	13464	
		Mean	1414	2802	5085	5902	2773	7013	11101	13940	
		SD	14.7	95	151	231	10.25	330	579	704	
		CV	1%	3%	3%	4%	0.4%	5%	5%	5%	
	Day 1			TG				FFA			
				Concentration levels (mg.mL ⁻¹)				Concentration levels (mg.mL ⁻¹)			
				0.25	0.5	1.5	3.0	0.5	1.0	2.0	4.0
		a		2686	4521	8671	12258	4285	5868	8354	11632
b			2654	4196	8004	11144	4256	5937	8126	10859	
c		2821	4639	9309	13079	4707	6510	8896	12269		
		Mean	2720	4452	8661	12160	4416	6105	8458	11587	
		SD	89	230	653	971	252	353	395	706	
		CV	3%	5%	8%	8%	6%	6%	5%	6%	

Table 14 Measurement reproducibility obtained from peak area analysis of γ -tocotrienol, sterol, trioleylglycerols (TG) and free fatty acid (FFA) standards (1 plate/day, 3 working days).

	Working days	Plates	γ -tocotrienol				Sterol				
			Concentration levels (mg.mL ⁻¹)				Concentration levels (mg.mL ⁻¹)				
			0.2	0.4	0.8	1.0	0.1	0.5	1.5	3.0	
Reproducibility	Day 1	a	1404	2703	4911	5658	2761	6935	11002	13608	
	Day 2	d	1392	3015	5140	5984	2604	6855	10894	13877	
	Day 3	e	1424	2811	5371	6228	2663	7649	11475	14014	
		Mean	1406	2843	5141	5957	2676	7146	11124	13833	
		SD	16.07	158.29	230	286	80	437	309	207	
		CV	1%	6%	4%	5%	3%	6%	3%	1%	
				TG				FFA			
		Working days	Plates	Concentration levels (mg.mL ⁻¹)				Concentration levels (mg.mL ⁻¹)			
				0.25	0.5	1.5	3.0	0.5	1.0	2.0	4.0
		Day 1	a	2686	4521	8671	12258	4285	5868	8354	11632
	Day 2	d	2768	4571	8737	12070	4337	6002	8325	11017	
	Day 3	e	2636	4447	8403	11734	4312	5905	7960	10239	
		Mean	2697	4513	8604	12021	4312	5925	8213	10963	
		SD	67	63	177	266	26	69	219	698	
		CV	2%	1%	2%	2%	1%	1%	3%	6%	

4. Optimization of 2,2-di-phenyl-1-picrylhydrazyl (DPPH) antioxidant activity assay for NR lipids

There are many techniques available for antioxidant activity assay. Among those techniques, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is commonly used to determine the antioxidant activity of various plant extracts (Bendini *et al.*, 2006).

The first optimization step was dedicated to the solvent system used for the reaction, as our samples (NR lipid extracts) were not fully soluble in ethanol.

Generally, the measurement of DPPH antioxidant activity is done spectrophotometrically using 1-4 mL cuvettes. This allows the analysis of only a very limited number of samples at the same time and consumes an important amount of reactants and samples. In this present study, DPPH assay using a 96-well microplate spectrophotometer was developed and validated to assess the antioxidant activity of NR lipid extract in a rapid and cost effective manner.

4.1 Solvent modification

The antioxidant activity of NR lipid extracts was measured using DPPH according to the method of Fagali and Catalá (2008) with modification in order to increase the solubility of lipid extract from NR, which is not soluble in polar organic solvent. Initially, the reaction was carried out using 3 solvent systems: pure ethanol and mixtures of chloroform/ethanol 1:1 and 1:2, v/v. It was found that DPPH was soluble in all solvents at the tested concentration of 0.1 mg.mL⁻¹. Concerning lipid samples, at a concentration of 10 mg.mL⁻¹, an unclear solution was observed in ethanol and chloroform/ ethanol 1:2, v/v, while a clear solution was observed with chloroform/ethanol 1:1, v/v. A lower concentration of lipid sample (1 mg.mL⁻¹ in chloroform/ethanol 1:2, v/v) was tested but unclear solution was still observed. Therefore, the ratio of 1:1 v/v of chloroform/ethanol was chosen for further analysis.

4.2 Comparison of a microplate spectrophotometer and cuvette spectrophotometer measurements

The conventional DPPH assay (with chloroform/ethanol 1:1, v/v solvent) method using 4-mL of cuvette volume spectrophotometer was compared with a downscaled method using a 96-well microplate reader. Generally, for the cuvette spectrophotometer method, 1 mL of test sample is mixed with 1 mL of DPPH (0.1 mg.mL⁻¹) using vortex for 5 s before the first absorbance measurement. In our adapted method using microplates, reaction volumes were scaled down from 1 mL to 125 µL for both test sample and 0.1 mg.mL⁻¹ DPPH. The plate was shaken for 5 s between each measurement. Except for the size difference, the analysis sequences were the same in both methods: the absorbance of 2 concentrations of α -tocopherol standard mixture (0.01 and 0.02 mg.mL⁻¹) was measured at 517 nm every minute from 0 to 10 min and every 10 min later on until 60 min.

Table 15 The initial slope and %DPPH_{rem60} of α -tocopherol standard at 0.01 and 0.02 mg.mL⁻¹ concentrations obtained from 2 spectrophotometer measurements.

Concentrations (mg.mL ⁻¹)	Type of spectrophotometer	Initial slopes (%DPPH.min ⁻¹)	SD	%DPPH _{rem60}	SD
0.01	96-well microplate	-4.27 ^a	0.09	83.2 ^a	0.70
	4-mL cuvette	-3.93 ^a	0.22	84.8 ^a	0.98
0.02	96-well microplate	-9.07 ^a	0.21	64.9 ^a	0.80
	4-mL cuvette	-8.03 ^a	0.47	67.5 ^a	0.99

Data were means from 3 replications. For each column and for each concentration, letters design group of means which are not significantly different (student *t* test), $p \leq 0.05$, SD: Standard deviation.

Two indicators were analysed to test whether both methods gave the same results: the initial slope of the curve %DPPH = f(time) and the value of the final plateau at 60 min (%DPPH_{rem60}). The initial slope indicates the rate of reaction and was determined in the time range of 0 to 3 min.

No significant difference of either the initial slope or %DPPH_{rem60} was detected between the 2 methods (Table 15), which validated the developed DPPH microassay measurement.

4.3 Standard concentration range

In the DPPH measurement using microplate reader experiment, the first step was to define the suitable concentration range of standard. Gamma-tocotrienol standard was used as a reference compound for lipid antioxidant activity as it was reported that γ -tocotrienol is the dominant native antioxidant in NR (Nadarajah *et al.*, 1971). A preliminary test was carried out using γ -tocotrienol at the concentrations of 0, 0.01, 0.02, 0.05, 0.1 and 0.2 mg.mL⁻¹. The obtained antioxidant activity of γ -tocotrienol is presented in Figure 20.

At every tested concentration, the decrease of DPPH was fast for the first 3 min and reached a plateau state after 10 min. The DPPH drop rate increased with γ -tocotrienol concentration (Table 16). However it was found that %DPPH_{rem60} of 0.05, 0.1 and 0.2 mg.mL⁻¹ concentrations were not different. Therefore the concentrations of γ -tocotrienol chosen for the experiment were 0.01, 0.02 and 0.05 mg.mL⁻¹.

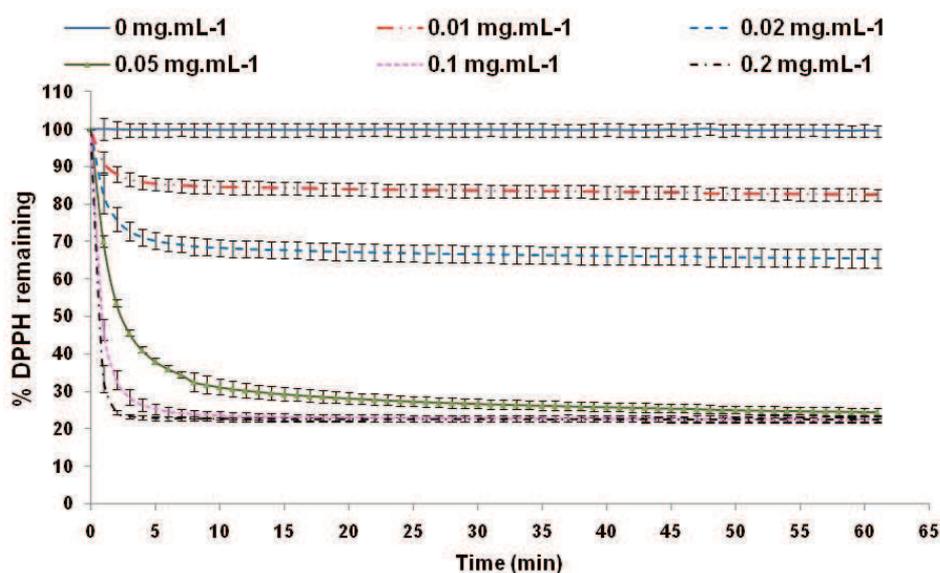


Figure 20 Variation of % DPPH remaining obtained from γ -tocotrienol standard at the concentration of 0, 0.01, 0.02, 0.05, 0.1 and 0.2 mg.mL⁻¹ (3 replications).

Table 16 Initial slope value and %DPPH_{rem60} obtained from γ -tocotrienol standard at the concentration of 0, 0.01, 0.02, 0.05, 0.1 and 0.2 mg.mL⁻¹ (3 replications).

Concentrations (mg.mL ⁻¹)	Initial slopes (%DPPH.min ⁻¹)	SD	%DPPH _{rem60}	SD
0.01	-4.45 ^a	0.23	82.3 ^a	0.52
0.02	-9.11 ^b	0.20	64.4 ^b	0.79
0.05	-17.95 ^c	0.10	24.9 ^c	0.55
0.10	-22.45 ^d	0.11	23.0 ^c	0.55
0.20	-23.58 ^e	0.16	22.6 ^c	0.31

Data were means from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test), $p \leq 0.05$, SD: Standard deviation.

4.4 Measurement repeatability and reproducibility

The repeatability and reproducibility of the measurement using the same indicators: the initial slope of the curve %DPPH = f(time) and %DPPH_{rem60} obtained from 0.01, 0.02, and 0.05 mg.mL⁻¹ concentrations of γ -tocotrienol, δ -tocotrienol, α -tocopherol and γ -tocopherol standards. The DPPH assay was repeated 3 times within the same day for method repeatability. For each assay and each component concentration, 3 wells were used with 3 independently prepared solutions. The reproducibility was estimated by performing 3 assay replications in 3 different days using freshly prepared solutions each day.

As shown in Table 17, the CV values of the 2 indicators obtained during the method repeatability study were less than 5%. A similar observation can be done in the reproducibility study results presented in Table 18 where maximum CV of indicators was 5%. This low CV values indicated the good repeatability and reproducibility of the method for the used standards in the tested concentrations.

4.5 Conclusion

The antioxidant activity of NR lipids could be assessed by a modified DPPH method using chloroform/ethanol (1:1, v/v). The adaptation of the method to the use of 96-well microplates also allowed the simultaneous analysis of a large number of samples, using a low amount of samples and reactants. The measurement repeatability and reproducibility (CV less than 5%) was validated.

Table 17 Measurement repeatability of DPPH assay obtained from initial slope from 0-3 min and % DPPH_{rem60} of γ -tocotrienol, δ -tocotrienol, α -tocopherol and γ -tocopherol standards (Values were expressed as means \pm standard deviations, n=3).

Components	Plate no.	Concentrations (mg.mL ⁻¹)					
		0.01		0.02		0.05	
		%DPPH _{rem60}	Initial slope (%DPPH.min ⁻¹)	%DPPH _{rem60}	Initial slope (%DPPH.min ⁻¹)	%DPPH _{rem60}	Initial slope (%DPPH.min ⁻¹)
γ -tocotrienol	a	81.3	-4.52	64.0	-9.32	25.6	-18.59
	b	82.7	-4.20	63.3	-9.31	25.2	-17.72
	c	82.9	-4.26	65.9	-8.71	23.8	-17.82
	Mean\pmSD	82.3\pm0.89	-4.33\pm0.17	64.4\pm1.36	-9.11\pm0.35	24.87\pm0.95	-18.04\pm0.48
	CV	1%	4%	2%	4%	4%	3%
δ -tocotrienol	a	85.3	-3.01	71.4	-6.41	33.1	-11.58
	b	83.4	-2.97	67.0	-6.25	34.9	-11.92
	c	86.6	-2.86	67.5	-6.24	36.0	-11.94
	Mean\pmSD	85.1\pm1.61	-2.95\pm0.08	68.6\pm2.44	-6.30\pm0.10	34.7\pm1.49	-11.81\pm0.20
	CV	2%	3%	4%	2%	4%	2%
α -tocopherol	a	82.8	-4.40	63.6	-9.50	18.7	-20.98
	b	82.3	-4.31	64.9	-8.81	20.0	-19.79
	c	84.6	-4.18	66.3	-8.88	20.0	-20.33
	Mean\pmSD	83.2\pm1.21	-4.30\pm0.11	64.9\pm1.35	-9.06\pm0.38	19.6\pm0.75	-20.37\pm0.60
	CV	1%	3%	2%	4%	4%	3%
γ -tocopherol	a	89.8	-2.44	75.9	-6.24	36.8	-15.01
	b	86.2	-2.63	73.6	-6.69	38.0	-14.97
	c	90.8	-2.51	77.6	-6.13	38.5	-14.58
	Mean\pmSD	88.9\pm2.42	-2.53\pm0.10	75.7\pm2.01	-6.35\pm0.30	37.8\pm0.88	-14.85\pm0.24
	CV	3%	4%	3%	5%	2%	2%

Table 18 Measurement reproducibility of DPPH assay obtained from initial slope from 0-3 min and %DPPH_{rem60} of γ -tocotrienol, δ -tocotrienol, α -tocopherol and γ -tocopherol standards (Values were expressed as means \pm standard deviations, n=3).

Components	Working days	Concentrations (mg.mL ⁻¹)					
		0.01		0.02		0.05	
		%DPPH _{rem60}	Initial slope (%DPPH.min ⁻¹)	%DPPH _{rem60}	Initial slope (%DPPH.min ⁻¹)	%DPPH _{rem60}	Initial slope (%DPPH.min ⁻¹)
γ -tocotrienol	1	81.3	-4.52	64.0	-9.32	25.2	-18.59
	2	84.8	-4.55	69.6	-8.66	23.1	-17.85
	3	80.6	-4.76	64.9	-8.95	25.6	-18.34
	Mean\pmSD	82.2\pm2.26	-4.61\pm0.13	66.2\pm3.00	-8.98\pm0.33	24.6\pm1.34	-18.26\pm0.38
	CV	3%	3%	5%	4%	5%	2%
δ -tocotrienol	1	85.3	-3.01	71.4	-6.41	33.1	-11.58
	2	88.1	-2.77	70.4	-6.90	32.3	-12.11
	3	89.5	-2.88	71.4	-6.61	34.5	-11.70
	Mean\pmSD	87.6\pm2.14	-2.89\pm0.12	70.0\pm0.52	-6.64\pm0.25	33.3\pm1.14	-11.80\pm0.28
	CV	2%	4%	1%	4%	3%	2%
α -tocopherol	1	82.8	-4.40	63.6	-9.50	18.7	-20.98
	2	84.2	-4.53	65.6	-8.75	19.6	-19.75
	3	85.6	-4.09	67.2	-9.01	20.7	-18.96
	Mean\pmSD	84.2\pm1.40	-4.34\pm0.23	65.5\pm1.80	-9.09\pm0.38	19.7\pm1.00	-19.90\pm1.02
	CV	1.7%	5%	3%	4%	5%	5%
γ -tocopherol	1	89.8	-2.44	75.9	-6.24	36.8	-15.01
	2	90.0	-2.65	77.0	-5.85	37.3	-15.16
	3	84.2	-2.50	78.1	-6.48	35.7	-13.78
	Mean\pmSD	88.0\pm3.29	-2.53\pm0.11	77.0\pm1.10	-6.19\pm0.32	36.6\pm0.82	-14.65\pm0.76
	CV	4%	4%	1%	5%	2%	5%

5. Structural identification of γ -tocotrienol derivatives

The presence of unknown components was observed during NR lipid analysis by TLC as shown in Figure 21. These unknowns had a similar R_f as one of the components observed from γ -tocotrienol commercial standard. It is to be noted that the unknowns were not initially found in standards but possibly appeared following a γ -tocotrienol oxidation during storage after bottle opening.

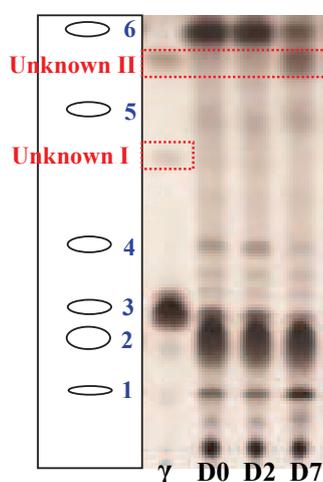


Figure 21 TLC of lipids from RRIM600 lipid samples at 0 day (D0), 2 days (D2) and 7 days (D7) of maturation and γ -tocotrienol standard (γ) developed in the mobile phase of hexane/diethyl ether/acetic acid, 80:20:1 (v/v/v).

1: Sterols, 2: Free fatty acids, 3: γ -tocotrienol, 4: Triacylglycerol of furan fatty acid, 5: Wax, 6: Sterol esters.

5.1 γ -tocotrienol derivatives identification from γ -tocotrienol standard

In order to identify the unknown I and II, an injection to HPLC-MS using γ -tocotrienol standard from a bottle that was kept open from several days was conducted.

The total ion count chromatogram (TIC) and mass spectra obtained after injection of γ -tocotrienol standard kept in an open bottle are presented in Figure 22. Three main peaks were identified at the retention time (R_t) of 4.3 min (peak 1), 8.3 min (peak 2) and 9.1 min

(peak 3), respectively. The detected mass m/z 411 of peak 1 corresponded to the structure of γ -tocotrienol free form $[M+H]^+$. Two same masses, m/z 819, were detected for peak 2 and 3. Indeed m/z 819 is equivalent to twice that of γ -tocotrienol minus 2 hydrogens $[2M-2H+H]^+$. Also, literature reported the presence of γ -tocotrienol dimers from *Hevea* latex (Chow and Draper, 1970) and dimers in tocotrienol oxidation products (Büsing and Ternes, 2011). Moreover, a detailed analysis of fragments supported this assumption (see fragment weights on Figure 22c and Figure 22d). For example, the presence of fragmented mass of m/z 599 corresponded to phytol chain loss of these dimers. The fragmentation pattern was similar for both suspected dimers, except the presence of m/z 425 in peak 3 (9.1 min). This difference allows to attribute the two molecular chemical structures to each peak. This attribution is confirmed by the order of elution. Indeed it is normal that the dimers with the two hydroxyl groups (as called dihydroxy dimer by Büsing and Ternes (2011)) elute before on a reverse HPLC phase due to a higher polarity.

5.2 Tocotrienol derivatives identification from NR lipid extract

5.2.1 Purification and HPLC-MS analysis

Unknown II component (Figure 21) obtained from RRIM600 lipid extract was purified by liquid column chromatography (LC) in order to confirm its chemical structure. The LC experiment was carried out by loading 867 mg of lipids dissolved in 1 mL hexane into a 2.8 cm diameter x16 cm length column using 30 g of Florisil (Activated Magnesium silicate, Restek, U.S.A.) as a packing material. The mobile phase used was hexane/diethyl ether/acetic acid 80:20:1 (v/v/v). The flow rate was approximately $1.4 \text{ mL}\cdot\text{min}^{-1}$. Fifty mL of void volume was collected as a single first fraction then the fraction volume was 9 mL. Twenty fractions were collected and their content checked by TLC. The unknown II component spot appeared mainly in fractions 6 to 9 (Figure 23). However it was observed that sterol esters co-eluted in the same fractions. Those fractions (6-9) were grouped and deposited on a preparative TLC in order to get rid of sterol esters.

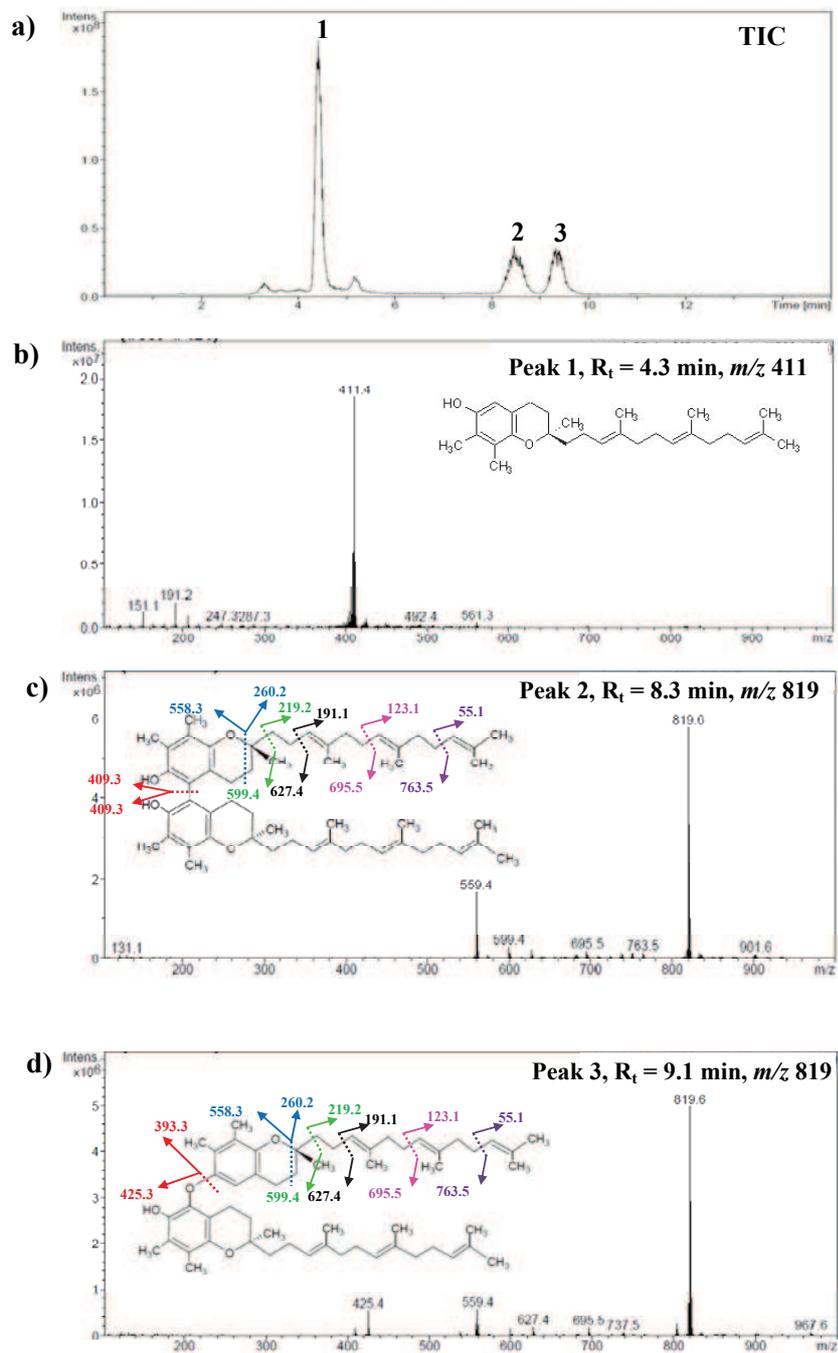


Figure 22 Total ion count chromatogram (TIC) (a) and mass spectra of (b) free γ -tocotrienol (peak 1), γ -tocotrienol dimers at (c) peak 2 and (d) peak 3 obtained from HPLC-MS analysis of γ -tocotrienol standard sample.

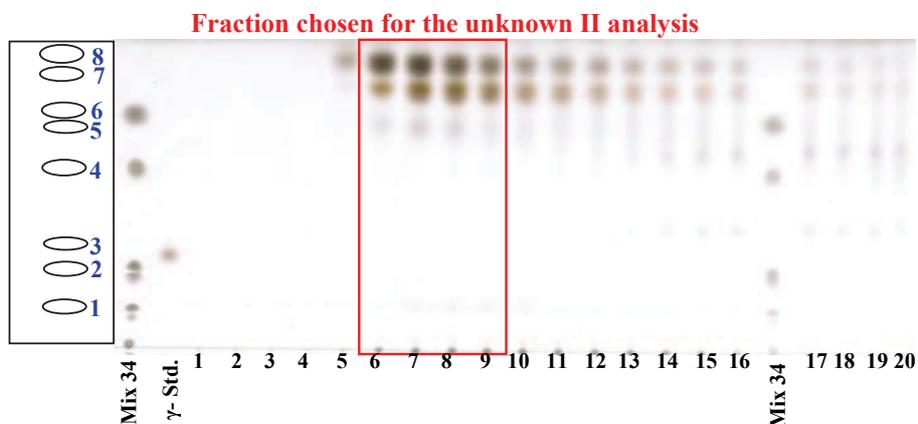


Figure 23 TLC of the first 20 collected fractions from liquid column chromatography on silica gel from RRIM600 lipid developed in the mobile phase of hexane/diethyl ether/acetic acid, 80:20:1 (v/v/v).

1: Sterols, 2: Free fatty acids, 3: γ -tocotrienol, 4: Triacylglycerols, 5: Wax, 6: Fatty acid methyl ester, 7: Unknown II and 8: Sterol esters.

The purified spot which has a similar R_f as Unknown II with 80:20:1 of hexane: diethylether: acetic acid (v/v/v) mobile phase was analyzed by HPLC-MS (Ion Trap Mass Spectrometer) with the same method as that used for γ -tocotrienol standard. The obtained TIC chromatogram (Figure 24a) shows that this purified TLC spot contained numerous components. The mass spectra of all peaks were analyzed and the mass spectrum of the identified dimer (m/z 819) was observed only in peak 5 as shown in Figure 24b. This suggested that the unknown II compound observed in lipid extract at the similar R_f of dimer from γ -tocotrienol standard was not actually the same molecule.

To be able to distinguish this group of compounds, different mobile phases were tried in HPTLC analysis on total lipids. The TLC analysis of lipid extract from RRIM600 rubber with a mobile phase of hexane/diethyl ether/acetic acid 90:10:1 (v/v/v) confirmed the chemical difference between unknown II band in NR lipids and dimers of γ -tocotrienol from standard (Figure 25a, showing different R_f). It was also confirmed that the single spot separated with 80:20:1 mobile phase, named unknown II, contained at least two components (spot no. 5 and 6 in Figure 25). To confirm precisely the chemical structure of the components of this 2 spots, a preparative TLC was done again using the 90:10:1 v/v/v

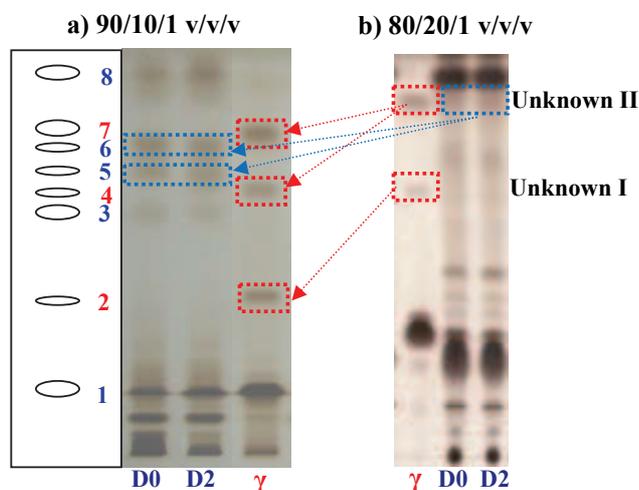


Figure 25 TLC of RRIM600 lipid samples at 0 day (D0), 2 days (D2) of maturation and γ -tocotrienol oxidized standard (γ) developed in (a) mobile phase hexane/diethyl ether/acetic acid, 90:10:1 (v/v/v) compared the same deposits developed with (b) mobile phase hexane/diethyl ether/acetic acid, 80:20:1 (v/v/v).

1: γ -tocotrienol, 2, 4 and 7: γ -tocotrienol dimers, 3: Wax, 5 and 6: Unknown II and 8: Sterol esters.

The TIC chromatogram and mass spectra of corresponding to spot no. 5 and 6 are presented in Figure 26 and Figure 27, respectively. The analysis of the mass spectra led to the identification of several species of γ -tocotrienol esters present as molecular ions $[M+H]^+$, but also as adducts such as $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$.

For spot no.5, two main peaks were identified at 18.1 and 23.3 min. At 18.1 min, the detected species corresponded to the γ -tocotrienol ester of linoleic acid (γ -T3-C18:2; $M = 672.5 \text{ g.mol}^{-1}$; $[M+H]^+$: m/z 674; $[M+NH_4]^+$: m/z 691; $[M+Na]^+$: m/z 696 and $[M+K]^+$: m/z 712). At 23.3 min, the detected species corresponded to the γ -tocotrienol ester of oleic acid (γ -T3-C18:1; $M = 674.54 \text{ g.mol}^{-1}$): $[M+H]^+$: m/z 676; $[M+NH_4]^+$: m/z 693; $[M+Na]^+$: m/z 698 and $[M+K]^+$: m/z 714 (Figure 26).

For spot no. 6, two main peaks were also identified at 24.2 min and 31.5 min. The same analysis led to the identification of γ -tocotrienol esterified respectively with palmitic acid (γ -T3-C16:0, $M = 648.54 \text{ g.mol}^{-1}$; $[M+H]^+$: m/z 650; $[M+NH_4]^+$: m/z 667;

[M+Na]⁺: *m/z* 672 and [M+K]⁺: *m/z* 688) and with stearic acid (γ -T3-C18:0, M = 676.57 g.mol⁻¹; [M+H]⁺: *m/z* 678; [M+NH₄]⁺: *m/z* 695; [M+Na]⁺: *m/z* 700 and [M+K]⁺: *m/z* 716) as shown in Figure 27.

Concerning other fatty acids, trace of γ -tocotrienol esterified with C16:0 were detected in both spots at R_t of 9.5min (detailed data not shown). Meanwhile, γ -T3-C20:0 ester was not detected most probably because the total run duration was not long enough. Moreover, a small peak at 28.69 min in the TIC chromatogram from spot no. 6 gave the mass spectrum of a possible γ -tocotrienol esterified with heptadecanoic acid (C17:0).

The two purified components from PB235 lipid sample were analyzed similarly. The constituents of the two unknown components were not different from those observed in RRIM600 lipid extract (data not shown).

5.2.2 Identification cross-checking by saponification

In order to confirm the previous finding, the scrapped components from spot no. 5 and 6 obtained from PB235 lipid extract were submitted to saponification to check whether the reaction products were made of fatty acids and free γ -tocotrienol.

After saponification, the free fatty acids and unsaponifiable fractions were grouped, submitted to silylation and injected in GC-MS. Figure 28 presents the TIC chromatogram and Figure 29 and Figure 30 present mass spectra of spots no. 5 and 6, respectively. The fatty acid constituent of γ -tocotrienol esters obtained from spot no. 5 was mainly unsaturated fatty acids (C18:1 and C18:2) but contained also palmitic acid (C16:0) (peak 1, Figure 28a), which confirms the HPLC-MS results. It was confirmed also that the fatty acid components of spot no. 6 were saturated fatty acids (C16:0, C18:0 and C20:0) (Figure 28b). It is to be noted that furan fatty acid was not detected from both spot no. 5 and 6, which were isolated from PB235 lipid extract. The fatty acid constituents of the two types of γ -tocotrienol esters from RRIM600 lipid extract were not different from those observed in PB235 lipid extract (data not shown).

The presence of free γ -tocotrienol ($R_t = 19.15$ min) was observed only in the case of spot no. 6. The absence of this component in the saponification products of spot no. 5 could be explained by an artifact (dimerization) that will be discussed in the following paragraph

At this step, the presence of γ -tocotrienol esters in NR lipids could be confirmed. The evolution of γ -tocotrienol esters followed by HPTLC during maturation will be described in Chapter 2.

5.2.3 Effect of saponification on the integrity of the tocotrienols

As mentioned before, no peak of free γ -tocotrienol could be detected in the composition analysis of spot no.5. There was thus an assumption that the saponification reaction used for sample preparation prior to GC-MS analysis could alter the structure of free γ -tocotrienol. Indeed, it was shown previously (§5.1) that γ -tocotrienol standard can be altered by maintaining a bottle open for several days, leading to the formation of dimers. Therefore we could assume that the saponification condition (85 °C, 1.5 h, aeration) could favor this alteration.

To confirm this assumption, the fraction purified by LC, containing sterol esters, γ -tocotrienol esters and wax (Figure 23), was submitted to saponification and the obtained unsaponifiable fraction was spotted on TLC with 2 successive mobile phases in order to optimize the components separation as shown in Figure 31.

After saponification, besides the expected presence of the free γ -tocotrienol, free sterols, and free fatty alcohols, 3 important spots (spot no. 5, 7 and 8 on Figure 31) corresponding to the R_f of γ -tocotrienol dimers were also observed. Those three spots were scrapped off and injected to HPLC-MS for their chemical structural identification. The obtained mass m/z 819 confirmed the partial dimerization of γ -tocotrienol structures due to saponification reaction. Moreover m/z 833 corresponding to heterodimers of γ -tocotrienol and α -tocotrienol were also detected (data not shown).

Spot no. 5

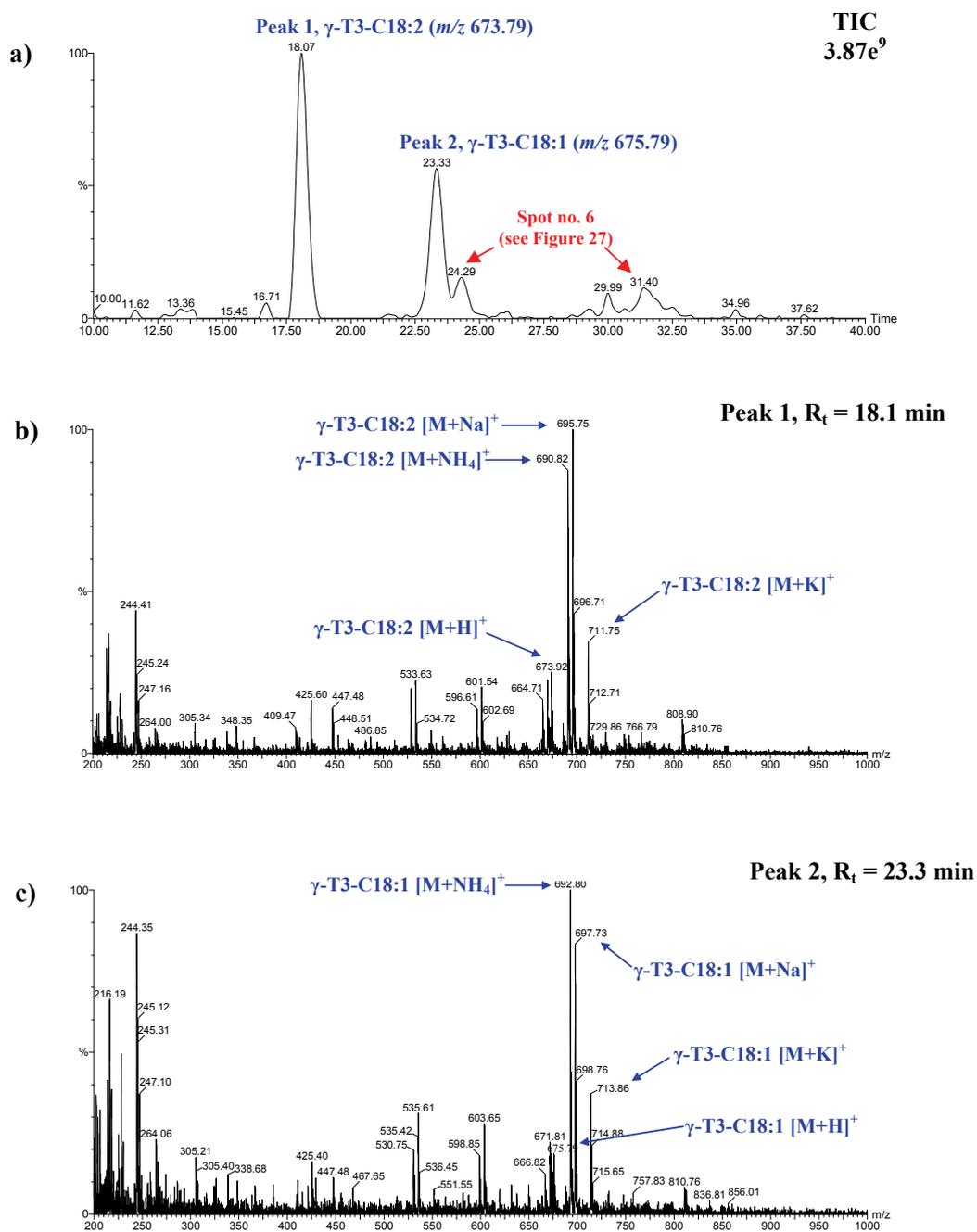


Figure 26 Total ion count chromatogram (a) and mass spectra of spot no.5 purified from RRIM600 lipid extract at (b) peak 1 at R_t of 18.1 min and (c) peak 2 at R_t of 23.3 min.

Spot no. 6

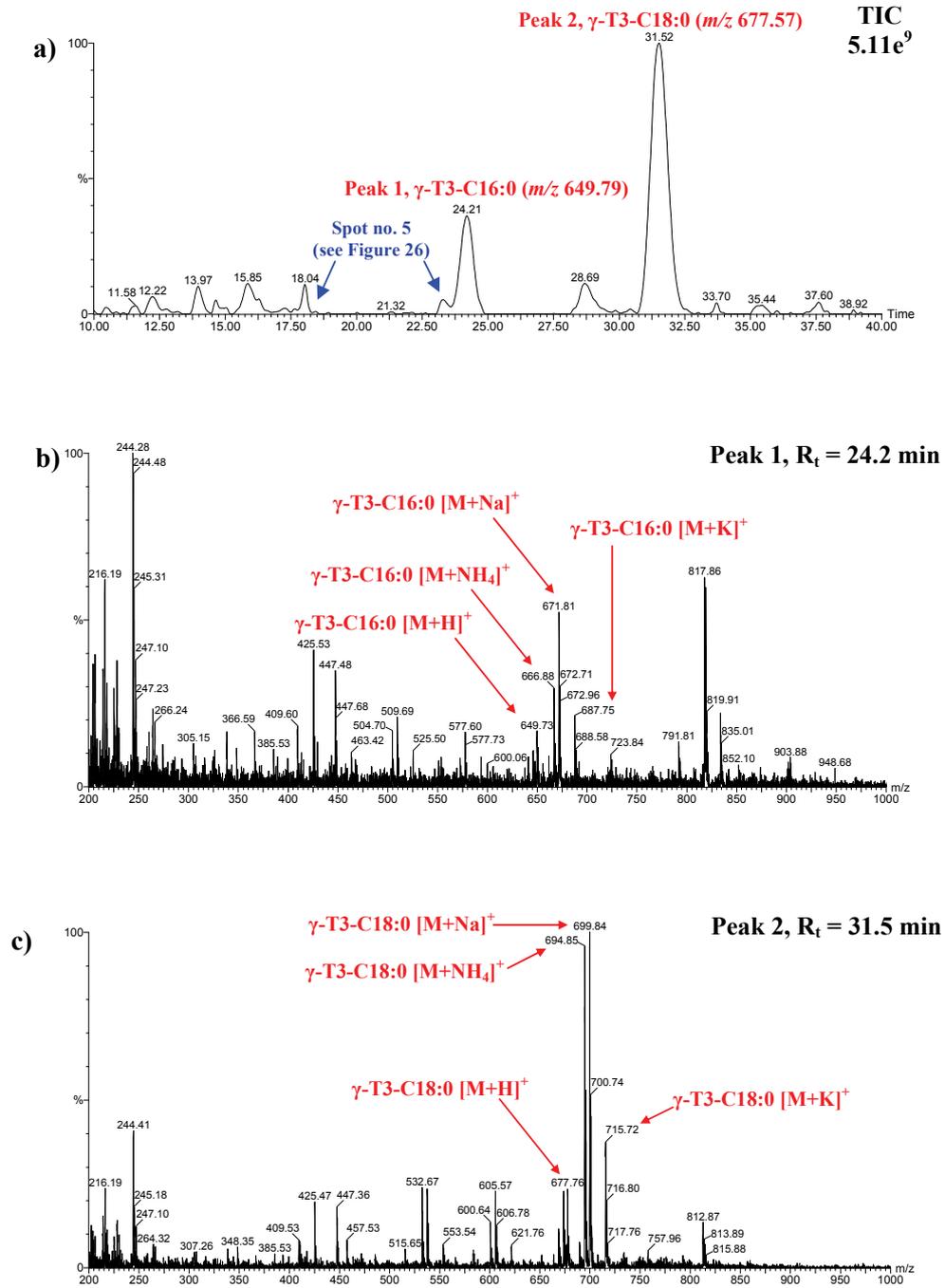


Figure 27 Total ion count chromatogram (a) and mass spectra of spot no. 6 purified from RRIM600 lipid extract at (b) peak 1 at R_t of 24.2 min and (c) peak 2 at R_t of 31.5 min.

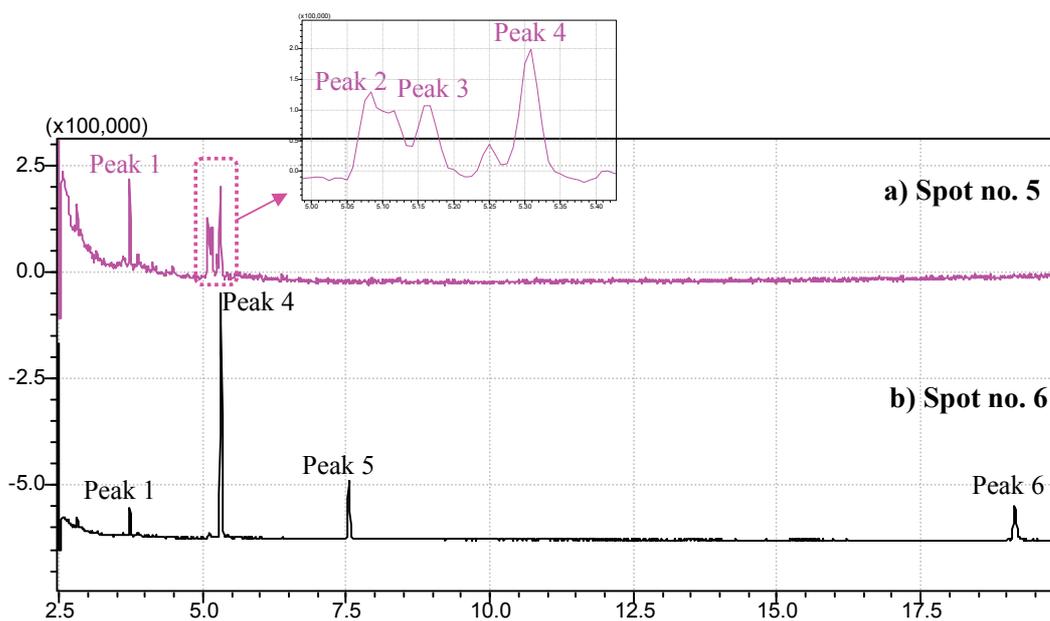


Figure 28 Total ion count GC-MS chromatogram of (a) saponified and silylated spot no. 5 and (b) silylated spot no. 6 from PB235 lipid extract sample.

Peak 1: C16:0, Peak 2: C18:2, Peak 3; C18:1, Peaks 4: C18:0, Peak 5: C20:0 and Peak 6: γ -tocotrienol

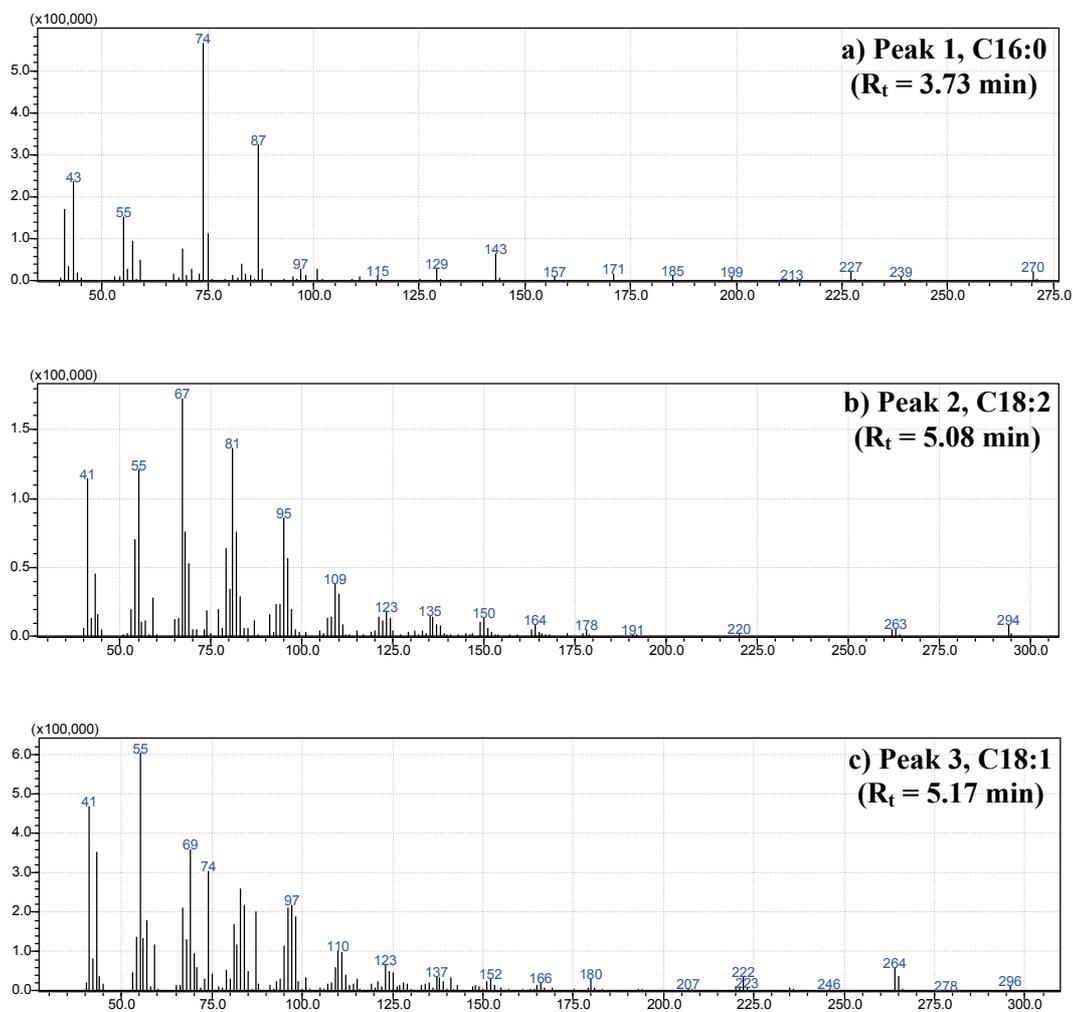


Figure 29 Mass spectra of (a) C16:0, (b) C18:2 and (c) C18:1 from spot no. 5 isolated from PB235 lipid sample.

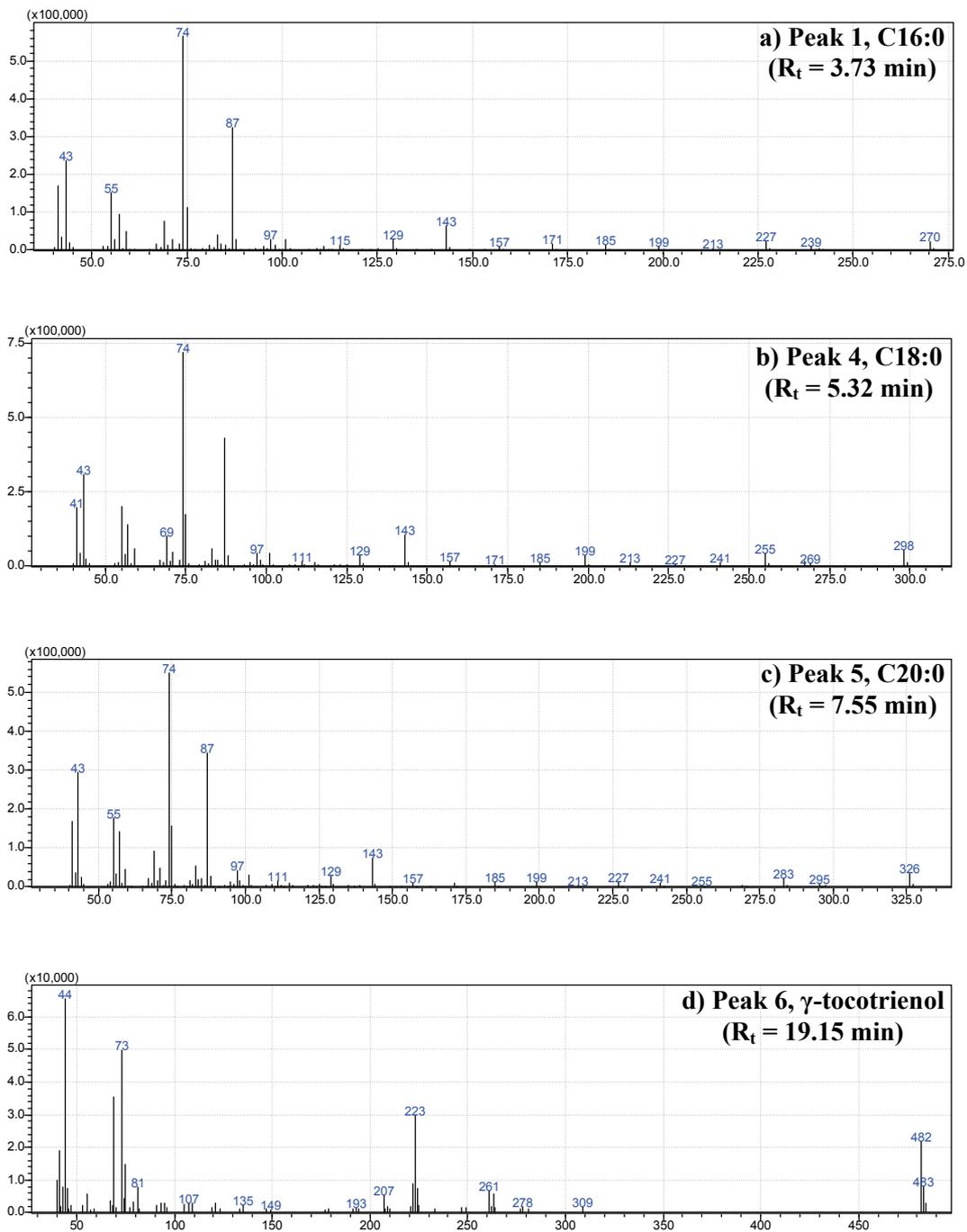


Figure 30 Mass spectra of (a) C16:0, (b) C18:0, (c) C20:0 and (d) γ -tocotrienol from spot no. 6 isolated from PB235 lipid sample.

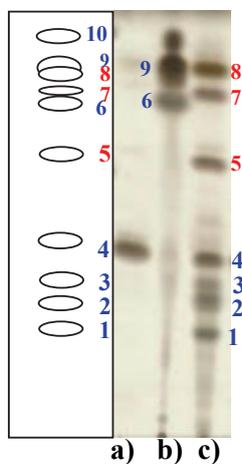


Figure 31 TLC of (a) γ -tocotrienol oxidized standard, (b) γ -tocotrienol esters fraction before saponification and (c) fraction of γ -tocotrienol esters after saponification obtained from liquid column chromatography of PB235 lipid extract. (2 elution times using mobile phase of hexane/diethyl ether/acetic acid, 50:50:1 and 90:10:1, v/v/v).

1: Sterols, 2: Free fatty alcohols, 3: Free fatty acids, 4: γ -tocotrienol, 5, 7 and 8: γ -tocotrienol dimers, 6: Wax, 9: γ -tocotrienol esters and 10: Sterol esters.

Back to Figure 28, it is interesting to observe that this dimerization phenomena during saponification seemed to be total for spot no. 5, which contained mainly unsaturated acids in the initial esters of tocotrienols (free γ -tocotrienol not detected at R_t of 19.15 min), while it seemed only partial for spot no. 6 containing mainly saturated fatty acids (free γ -tocotrienol detected at R_t of 19.15 min but the peak area is low relatively to those of fatty acids). This difference could be either due to chemical composition of the reaction media (presence of saturated versus unsaturated fatty acids, with unsaturated fatty acids favoring the dimerization) or due to limits of detection as the injected quantity for spot no. 5 was lower than that available for spot no. 6.

Therefore, saponification and GC-MS analysis may not be the appropriate techniques to follow the evolution of total tocotrienols during maturation. Indeed, the tocotrienols are altered during saponification, leading to dimers that are not detectable in our GC-MS conditions.

5.3 Conclusion

In our study, it was observed that γ -tocotrienol dimerization could occur during the storage of γ -tocotrienol standard and during the saponification reaction of lipid extract samples. Therefore, the quantity of free tocotrienols assessed from unsaponifiable fraction would most probably be underestimated.

The presence of γ -tocotrienol esters was confirmed in lipid extracts from rubber issued from matured coagulum. As esters of tocotrienols are supposed to have a lower antioxidant activity than their free form (the phenol function being involved in ester bond), it appears very interesting to follow the evolution of both components (free and esterified forms) during maturation. This has been done using the HPTLC protocol optimized for the separation of both groups of γ -tocotrienol esters. In Chapter 2, they will be named γ -tocotrienol ester I (spot no. 6 (Figure 25), containing mainly esters of saturated fatty acids), and γ -tocotrienol ester II (spot no. 5 (Figure 25), containing mainly esters of unsaturated fatty acids).

CHAPTER 2

Characterization of the structure, properties and biochemical composition of natural rubber obtained from coagula matured under uncontrolled condition and treated using a USS or RSS like processing

As presented in Chapter 1, two maturation experimental conditions were studied. The first involved natural conditions while the second was performed in a dedicated device allowing full control of relative humidity, temperature and oxygen content. In the first case, matured coagula were laminated with a hand mangle and outdoor or smoke air dried (USS or RSS like process). In the second case, matured coagula were creped with a mini-creper and dried in an oven (125°C, 2 h) (TSR like process). The sample preparation for two maturation experiments and the environmental conditions set for this device are presented in Materials and Methods (§3.2, §3.3 and §3.4).

The results from USS/RSS like process will be presented in the present Chapter 2 while those obtained after TSR like process will be exposed in the next Chapter 3. In those chapters, figure presentation was preferred to table one. Anyway, tables containing numerical values are provided in Appendices (p. 181).

The indicators that were followed during those experiments can be classified in 3 study levels: bulk properties, mesostructure and biochemical composition. The first study level, bulk properties of NR, illustrates the global performances of rubber and is generally described by rheological measurements assessing viscoelasticity and plasticity. Bulk properties integrate the interactions occurring in the whole product. In this study, the NR property were determined through standardized methods: initial plasticity (P_0), plasticity after ageing at 140°C for 30 min (P_{30}) and plasticity retention index (PRI). The second level, mesostructure of NR concerns the macromolecular structure and is assessed by number-average molar mass (M_n), weight-average molar mass (M_w), molar mass distribution (MMD), and gel content. For biochemical composition, lipid components which is the main non-isoprene component remained in dry rubber was specifically studied. Moreover, the antioxidant activity of lipid extracts was also investigated *in vitro* along maturation time. This

activity was assessed using DPPH test that was developed and reported in Chapter 1. The purpose would be to see if the antioxidant activity of the lipid extract could be linked with the thermo-oxidation resistance properties of rubber.

1. Initial pH of latex, and pHs of coagula and serum during maturation

After the tree is tapped, latex composition is susceptible to modifications by microorganism contamination in the tapping cut or in the collecting cup. Though pH of latex and coagula are not considered as NR properties, it is involved with the level of microorganism activity which was reported to degrade physical properties of rubber during maturation (Salomez *et al.*, 2014). Latex pH at day 0 was recorded, and the evolution of the pH of sera and coagula was followed regularly during 14 days of maturation just before lamination (when the coagula were removed from the coagulation boxes).

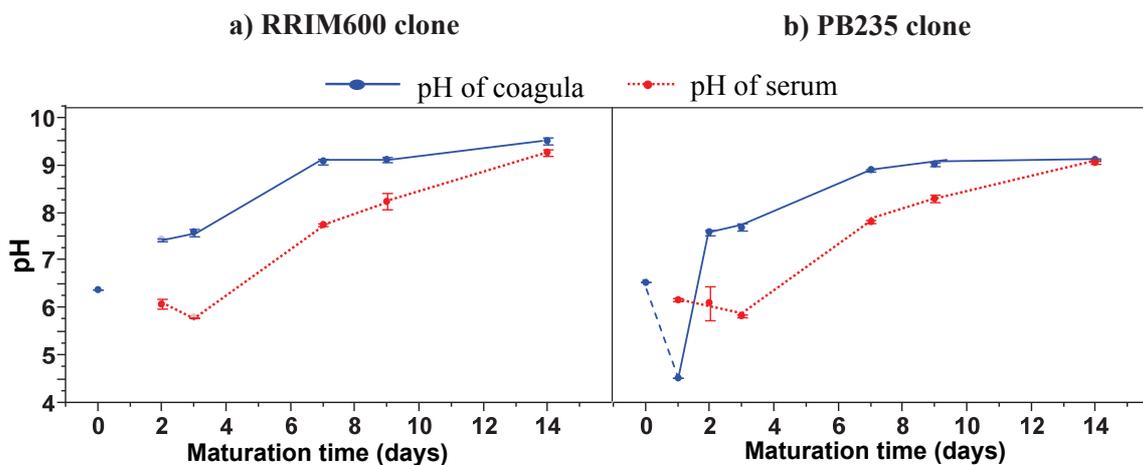


Figure 32 pH evolutions of (a) RRIM600 and (b) PB235 coagula during 14 days of maturation under uncontrolled conditions.

The pH evolutions observed from two clones in this study were similar, as shown in Figure 32. The initial pH of PB235 and RRIM600 fresh latex was at the average value of 6.5 ± 0.1 . The pH of PB235 latex decreased to 4.9 and latex coagulated within 24 h after tapping (Figure 32b) while longer time (between 25-48 h) was needed for RRIM600 latex to coagulate completely (Figure 32a). Unfortunately, the pH of RRIM600 coagula was not

recorded before day 2. From day 2 to day 7, the pH increased up to a plateau at an approximate value of 9. Those results are consistent with literature concerning pH of naturally coagulated latex where a primary acidification followed by a strong alkalization is described. The acidification is reported to be due to the release of volatile fatty acids (VFA) such as formic acid, acetic acid and lactic acid produced during carbohydrate fermentation by microorganism (Mathew and Claramma, 2000). After 1-day maturation, the increase of pH during maturation time may result from both release of ammonia from the proteins degradation and organic acid consumption by microorganism during maturation (Intapun *et al.*, 2010 ; Salomez *et al.*, 2014).

Moreover, the pH of serum that exudated from coagula in the maturation box was also monitored. For PB235 clone, the pH of serum (6.2) just after latex coagulation was higher than the pH inside the coagulum (4.5). From 2 days of maturation and both clones, the pH of sera were lower than the pH of coagula. It could be due to the release of VFA that could solubilize into serum before evaporation. After 14 days of maturation, the same pH was observed in both coagula and sera. Those assumptions should be confirmed by biochemical analysis of serum (VFA, NH₃) that were unfortunately not performed during this study.

2. Fresh and dry matter of laminated mini-sheets

The dry matter of fresh sheet was estimated by comparing the weight of fresh laminated sheets and dry sheets. It can be seen in Figure 33 that whatever the clone, the water content of laminated sheet increased with maturation time. As shown, this increase is supported by that of the fresh weight, while the dry weight remained logically constant. As the weight of the coagula before lamination (unfortunately not recorded) could not increase over maturation time, this increase of fresh weight of laminated sheet could only be explained by a lower amount of water removed by the process of lamination. This is most probably due to a higher resistance of the matured rubber to the force imposed by the mangle rolls. The operators confirmed that this process was indeed more difficult with matured coagula.

A slight difference could be observed between clones: the phenomenon of higher water retention was indeed more important with the clone RRIM600 (water content of freshly

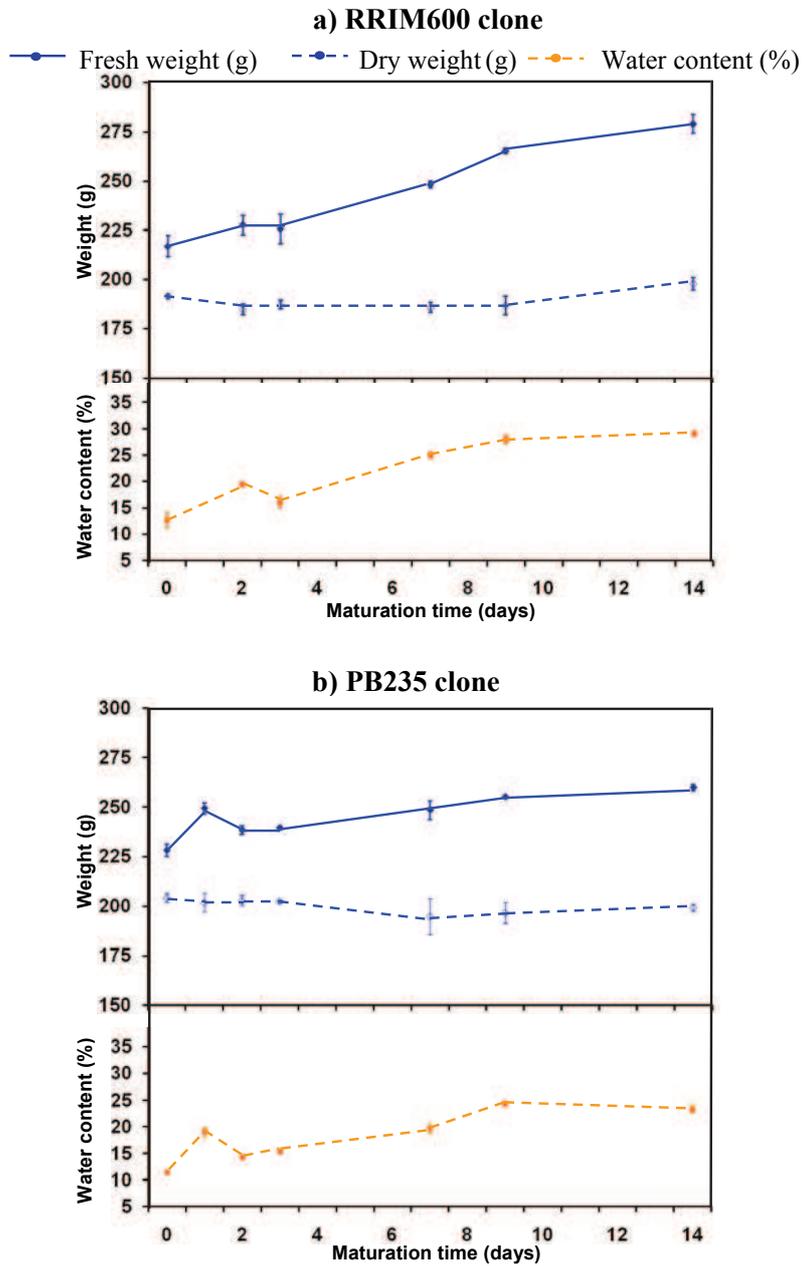


Figure 33 Fresh weight, dry weight and water content of laminated samples obtained from (a) RRIM600 and (b) PB235 rubber clones during 14 days of maturation.

laminated sheet from 15% (day 2) to 30% (day 14)) than with the clone PB235 (water content of freshly laminated sheet from 15% (day 2) to 20% (day 14)).

3. Evolution of bulk properties of NR from matured coagula under uncontrolled maturation conditions (USS/RSS like process)

The physical properties during 14 maturation days obtained from RRIM600 and PB235 samples are shown in Figure 34 and Figure 35. Though smoke drying was selected as an optimized drying method, it appeared interesting to show also results obtained from outdoor air drying. No physical data was obtained from 1-day-old RRIM600 sample as latex was not coagulated.

The values of P_0 of rubber from RRIM600 of outdoor air and smoke dried samples during 9 days maturation ranged from 41.8 to 60.0 and 38.2 to 58.2, respectively. The values of P_0 of matured samples (day 2 and after) are superior to the non-matured and acid coagulated samples (day 0) for both drying conditions (Figure 34). However, P_0 values for 14-day matured samples from outdoor air drying could not be measured (Figure 34a) as the sample were sticky and melted during preparation of the measurement. The condition of maturation (aerated box with serum kept inside) and the long duration of outdoor air drying after lamination (26 days in this case) may have created very degrading conditions. PRI values of RRIM600 smoke dried samples significantly decreased from 102.2 to 50.9 (51.3 units decreased) with maturation duration. This decrease is due to a decrease of P_{30} (Figure 34).

It is known that the P_0 of rubber made from matured coagula (eg TSR10) is often higher than that from non-matured one (eg TSR5). This is most probably due to the increase of gel (crosslinking) within the rubber in the first few days of maturation. However the rubbers issued from matured coagula are also known to be more sensitive to thermo-oxidation. This could explain why 14 days outdoor air dried samples – the most matured ones – melt when submitted to 100 °C conditions of P_0 measurement most probably due to high chain scissions. This lower resistance to thermo-oxidation is illustrated by the decrease of P_{30} which leads to a concomitant decrease of PRI.

Concerning outdoor air dried samples from PB235 clone, P_0 values decrease from 51.3 (day 0) to 20.3 (day 7). P_{30} measurements were impossible on rubber samples made from coagula matured for 7 days or later, while last possible P_0 measurement was done on day 7 (Figure 35a). Meanwhile P_0 of smoke dried sample was constant from 49.3 to 55.7 (Figure 35b) and all measurements could be performed until day 14. PRI of smoke dried samples ranged from 84.8 of day 0 to 32.5 of day 14 as shown in Figure 35. For outdoor air dried samples, PRI was in a range of 84.9 (day 1) to 73.0 (day 3) but after day 3 the sample was completely degraded and PRI could not be measured.

The bulk properties evolution of rubber sample from PB235 showed a similar evolution as that from RRIM600 concerning P_0 and PRI. What differentiated both clones in terms of bulk properties were the initial values of P_0 and their evolution along maturation time. Indeed for PB235 clone, P_0 was around 10 points higher at day 0 ($P_0 = 50$) and did not change significantly during maturation while P_0 from RRIM600 samples started at around 40 and tended to increase along maturation. This is due to the difference of native molar mass distribution (MMD_0) between the two clones. Indeed, PB235 rubber is a unimodal clone the rubber of which contains much less short chains than that of RRIM600 clone. Higher molar masses in PB235 clone rubber explains the higher initial value of P_0 , while the presence of short chains in RRIM600 clone rubber explained the apparent crosslinking phenomenon as described by Ehabe *et al.* (2002). Moreover, our results confirm that outdoor air drying of sheet made from matured coagula provided rubbers very sensitive to thermal-oxidation and high variable results in terms of bulk properties. It is most probably due to the fact that the samples kept maturing under uncontrolled conditions after lamination. Indeed the drying times were longer when the sheets were dried outdoor (around 10 days) than when they were smoke dried (5-7 days).

This higher sensitivity of rubber to thermal oxidation increased in all cases during maturation. It could be due to the decomposition of antioxidants naturally present in NR (Hasma and Othman, 1990) and/or the apparition of pro-oxidant molecules such as free metallic ions released by microorganism activity (Hasma and Othman, 1990 ; Tuampoemsab and Sakdapipanich, 2007).

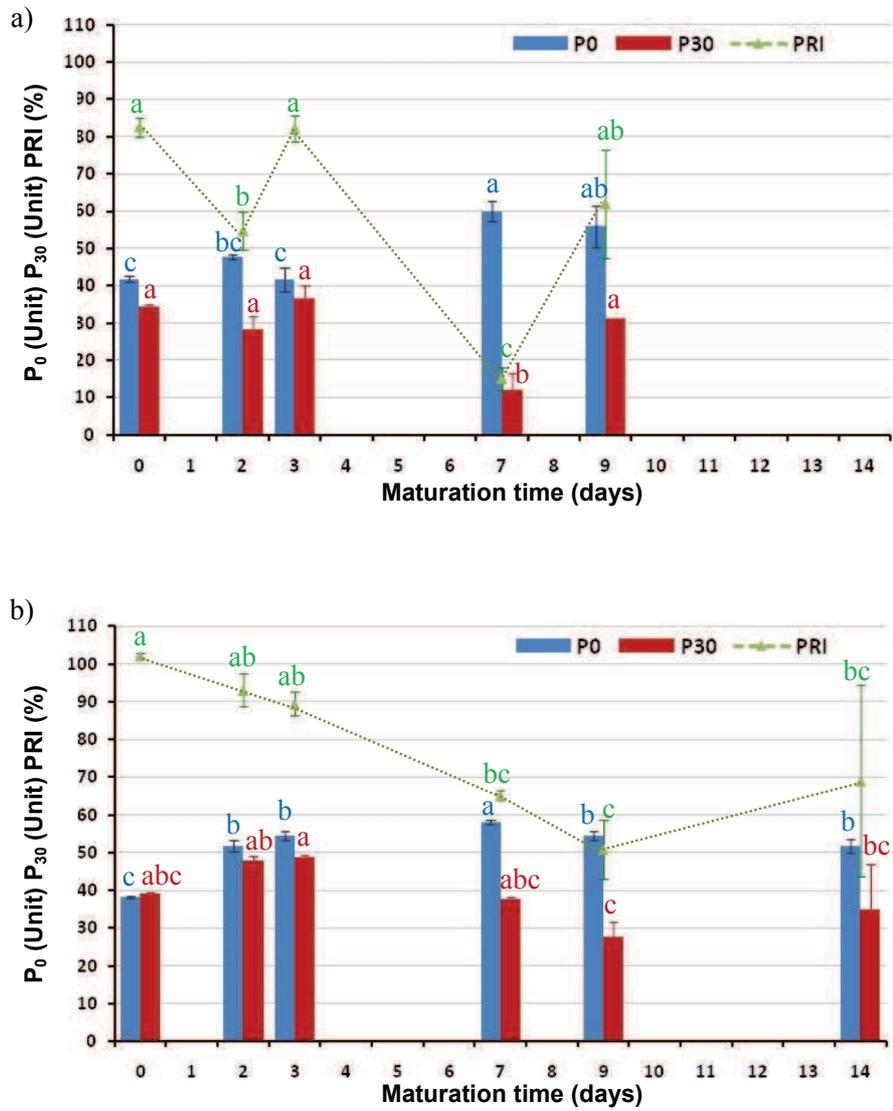


Figure 34 Evolution of physical properties of RRIM600 rubbers obtained from (a) outdoor air drying and (b) smoke drying during 14 days of maturation.

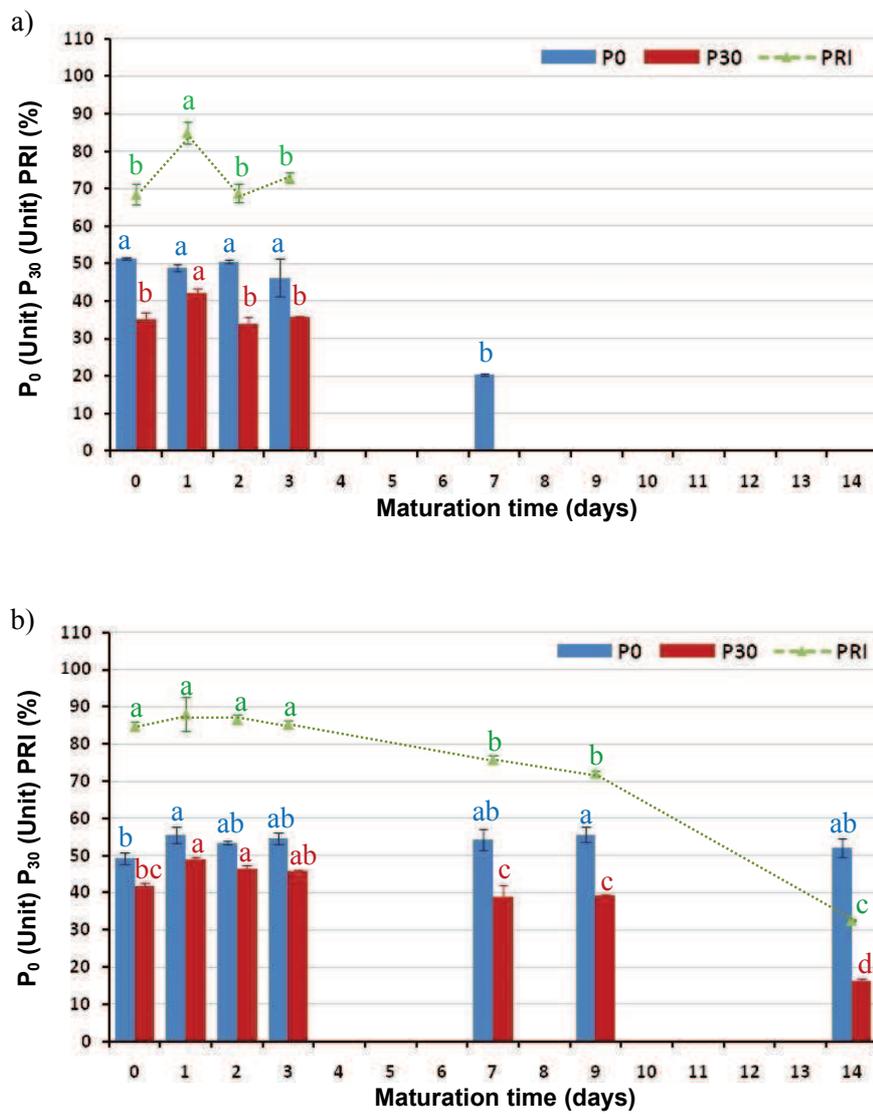


Figure 35 The evolution of physical properties of PB235 rubbers obtained from (a) outdoor air drying and (b) smoke drying during 14 days of maturation.

As few components of lipid extract from rubber were described to have antioxidant properties (Hasma and Othman, 1990), we decided to follow their evolution during maturation.

4. Evolution of lipid extract composition of NR from matured coagula under uncontrolled maturation conditions (USS/RSS like process)

4.1 Lipid quantity

After drying process, lipids of RRIM600 and PB235 samples were extracted with chloroform/methanol mixture (2:1, v/v). The lipid contents (% w/w dry rubber) of sheet rubber from 2 studied clones during maturation are presented in Figure 36.

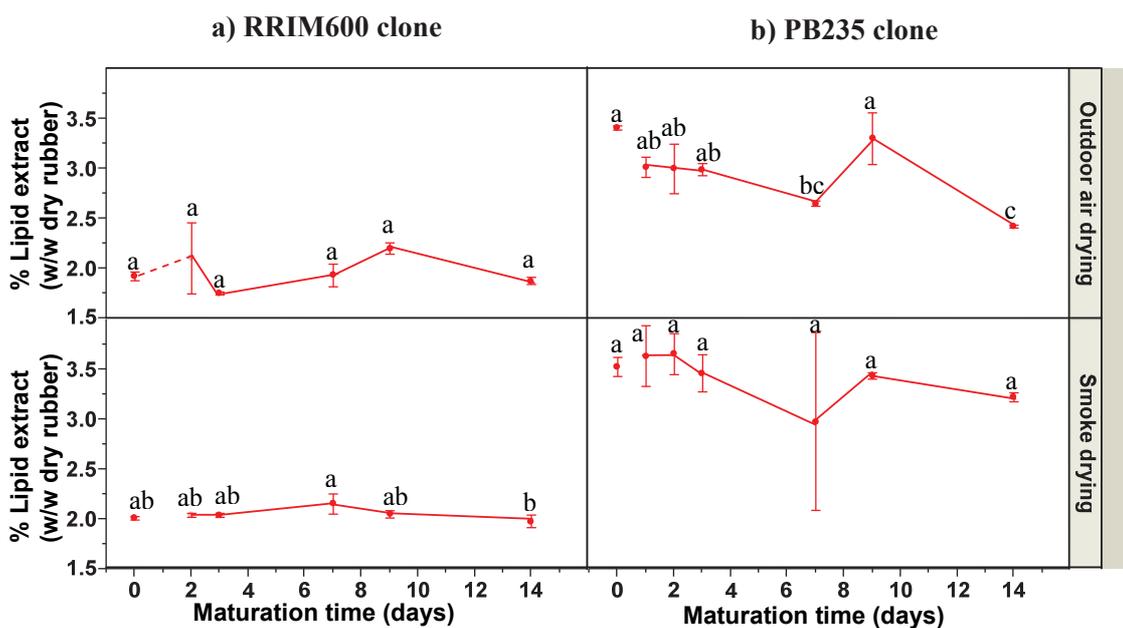


Figure 36 Lipid extracts (%w/w dry rubber) of (a) RRIM600 and (b) PB235 matured sheets from outdoor air and smoke drying during 14 days.

For RRIM600 samples, the obtained lipid content from 2 drying methods did not show significant difference during maturation time. However the smoke dried sample tended to have higher lipid content compared to the outdoor air drying sample (ranged from 1.98-

2.15% and 1.84-2.10%, respectively). This confirmed already published results by Rodphukdeekul *et al.* (2008). The lower lipid content of outdoor air dried sample may be due to lipids degradation caused by microorganism activity, that is inhibited earlier in the case of smoke drying. Indeed, as the drying duration from outdoor air drying was longer than that of smoke drying, the higher water content that remained in the sheet allowed microbial growth. In addition, due to the higher temperature (60°C) used in smoked sheet drying most of microorganism activity was ended sooner than in outdoor air drying.

This difference was also observed in samples obtained from PB235 clone. Moreover, the lipid content from this clone decreased during maturation from 3.41% to 2.42% for outdoor air dried mini-sheets.

Those lipid extract quantities were used further to express all the composition results that will be presented in the following paragraphs versus dry rubber.

4.2 Qualitative analysis of lipid evolution during maturation

Lipid extract of PB235 and RRIM600 clones were analyzed for their global components using TLC. Lipid samples were applied on TLC plates with the suitable mobile phase composed of hexane/diethyl ether/acetic acid (80:20:1, v/v/v).

Figure 37 shows TLC plates of lipids extracts from mini-USS (or mini-RSS) during 14 days of maturation. The common observed spots from both clones were identified as sterols, free fatty acids (FFA), γ -tocotrienol, and esterified lipids such as triacylglycerol of furan fatty acid (TGF), sterol esters (SE), wax and γ -tocotrienol esters (I and II, see Chapter 1 §5.2).

Similar evolutions of lipid components from both drying methods during maturation of the two studied clones were observed and will be described below. However, lipid composition seemed to evolve faster under outdoor air drying than under smoke drying conditions. It is reminded that same lipid extract quantity was deposited for each track

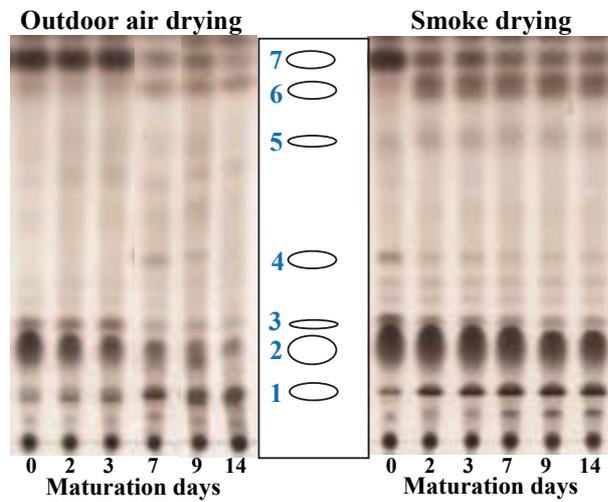
(50 μg) without taking into account the lipid quantity extracted from rubber. Therefore comparison between tracks should be done with caution.

For RRIM600 clone, it can be visually observed that the relative proportions of FFA, γ -tocotrienol and SE decreased while γ -tocotrienol esters and free sterols increased with maturation time. Therefore both esterification (γ -tocotrienol esters) and hydrolysis (sterol esters) seem to occur concomitantly depending of the acyl acceptor. In outdoor drying condition, the FFA proportion seems to be low after 14 days maturation, which could indicate that esterification was more important or that FFA were degraded chemically (oxidation) or biochemically (microbial catabolism). Wax and TGF spots were not obviously seen from outdoor air dried samples but in the case of smoke dried sample, TGF disappeared after 2 days of maturation.

Same observation can be done for lipid extracts from PB235 clone (Figure 37b). Hydrolysis of trifuranoyl glycerol (TGF) seemed faster in outdoor air drying conditions, confirming that biochemical processes continue when the sheets are hung. For smoke dried samples, only TGF and SE spots decreased during maturation. The other components (sterols, FFA, γ -tocotrienol and its esters) seemed not to change with maturation time.

In order to get quantitative data, it was decided to perform HPTLC analysis using the same samples.

a) Lipid extracts from RRIM600 clone



b) Lipid extracts from PB235 clone

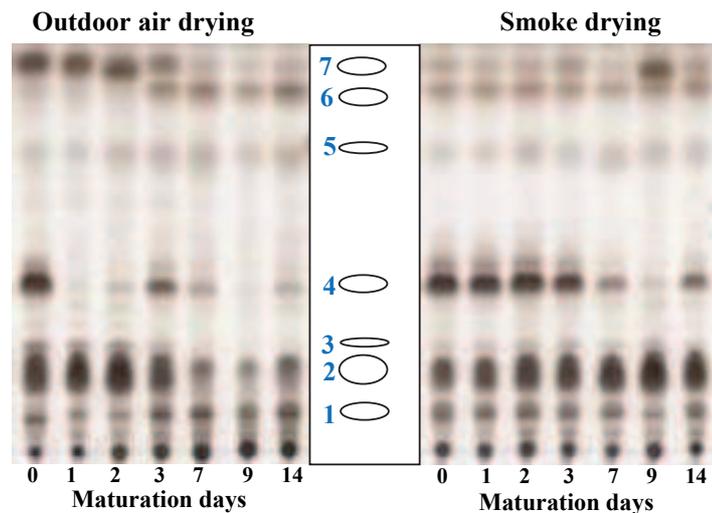


Figure 37 TLC of total lipid extracts from (a) RRIM600 and (b) PB235 matured rubbers during 14 days of maturation, developed in hexane/diethyl ether/acetic acid (80:20:1, v/v/v) and detected by 40% orthophosphoric acid and aqueous solution saturated with copper acetate (1:1, v/v).

1: Sterols, 2: Free fatty acids, 3: γ -tocotrienol, 4: Triacylglycerol of furan fatty acid, 5: Wax, 6: γ -tocotrienol esters (I and II) and 7: Sterol esters.

4.3 Quantitative analysis of lipid evolution during maturation

4.3.1 Neutral lipid composition

Lipid extracts from RRIM600 and PB235 matured rubber were analyzed for the quantity of each lipid component using HPTLC. Lipid components were identified and quantified using the R_f and calibrations of different standards, respectively. Each extract was deposited on two HPTLC plates and run with two appropriate mobile phases as mentioned in Chapter 1.

4.3.1.1 Free fatty acids and TGs

For RRIM600 clone, FFA content of the lipid extract stayed around the value of 1% versus dry rubber whatever the maturation time and the drying condition (Figure 38). As reported before by Liengprayoon *et al.* (2013), these FFA originated from polar lipids (glycolipids and phospholipids) hydrolysis that occurred after coagulation of latex, which originally contains very low amounts of FFA. It is noted that the decrease of FFA with maturation time which was visually observed previously (Figure 37), was not confirmed in HPTLC analysis for RRIM600 lipid extracts.

For PB235 clone, the level of FFA after coagulation was the same than the one measured with RRIM600 (around 1% vs dry rubber) (Figure 38). Under smoke drying condition, FFA kept increasing significantly (from 1% to more than 2%) in the first 7 days. Under outdoor air drying the same increase was observed but seemed to occur faster (within 2 days). Those results confirmed the previous observations on TLC: hydrolysis reaction continued during the sheet hanging. For example, maximum FFA was reached in samples obtained from 2 days maturation in the box followed by 11 days outdoor air drying (13 days in total), while with smoke drying, the maximum was reached when maturation in the box lasted 7 days followed by 7 days of smoke drying (14 days in total). The TGF content, which evolved contrarily to that of FFA, showed that they were the main provider of FFA for this “second increase” of FFA. After this “second increase”, FFA quantity decreased as observed previously. It could be due to disappearance (chemical (oxidation) or biological

catabolic degradation) or esterification with alcohol presents in the latex (sterols, tocotrienols, etc.). Therefore the evolution of these alcohols in their free and esterified forms follows.

4.3.1.2 γ -tocotrienol and its esters

For RRIM600 samples, γ -tocotrienol showed a tendency to decrease with maturation time whatever the drying condition, while its ester form tended to increase (Figure 39). Though it has to be confirmed considering the variance of the measured quantity, this evolution shows that the decrease of tocotrienols during maturation could not be exclusively associated with a degradation of the molecules (oxidation for example) but to an esterification with fatty acids, mostly saturated (see Chapter 1, §5.2).

For PB235 samples, the free form of γ -tocotrienol was difficult to detect under outdoor air drying condition (Figure 39). Nevertheless, the apparition of γ -tocotrienol ester I was observed. Gamma-tocotrienol ester II was detected in much lower quantity than in RRIM600 lipid extract. It can be explained by the fact that γ -tocotrienol ester II contains mainly C18:2 fatty acid, that are present in much lower quantity in PB235 latex (mainly containing furan fatty acid that was not detected in any of γ -tocotrienol ester species).

4.3.1.3 Sterols and their esters

For RRIM600 clone, the quantities of free forms of sterols increased with maturation time especially under outdoor air drying condition (0.08% at day1-3 to 0.5%) (Figure 40). It seemed partly compensated by a decrease of sterol ester quantities. However, such decrease is not sufficient to explain quantitatively the increase of free form, which means that other sources of free sterols must have been mobilized. The sources could be either indigenous sources such as esterified sterol glucosides (ESG) or sterol glucoside (SG) that are known to be in the lipid extract of rubber, or exogenous, synthesized by microorganism anabolism.

Same observation could be made for PB235 clone (Figure 40). In outdoor air dried samples, the quantities of free sterol increased from 0.05% at day 2 to 0.63% at day 7. As for RRIM600, the decrease of ester form is not sufficient to justify the quantitative increase of free form. Under smoke drying condition, evolutions of free and esterified forms are less important and seemed to compensate each other, which could mean that no other sources of sterols are mobilized.

Though the variance of the data was important and the quantitative calculation approximate (only one sterol used as standard), the obtained value remains consistent with published ones concerning total sterols which is reported to be in the range of 0.6% to 0.7% (w/w dry rubber) for same type of sample (Liengprayoon *et al.*, 2013).

Figure 41 shows the evolution of FFA and total esterified compounds (TGF, γ -tocotrienol esters and SE) during maturation. During the initial maturation period, hydrolysis reaction seemed to dominate (increase of FFA and decrease of total esterified compounds) while after 9 days of maturation esterification seemed to overcome hydrolysis (slight decrease of FFA and increase of esterified compounds).

However, considering the difficulty to get data with variance level that allowed statistical treatment it was decided to perform maturation under strictly controlled condition in order to confirm or not the results obtained during this experiment.

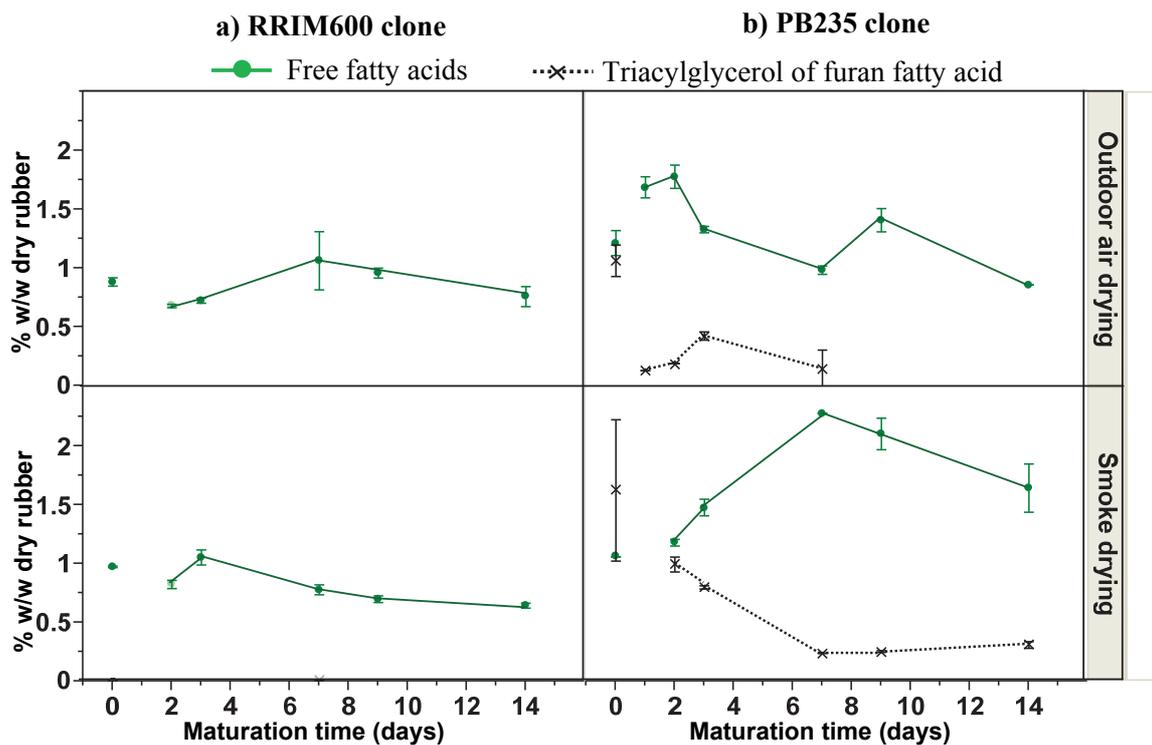


Figure 38 Evolution of free fatty acids and triacylglycerol of furan fatty acid (% w/w dry rubber) of (a) RRIM600 and (b) PB235 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation.

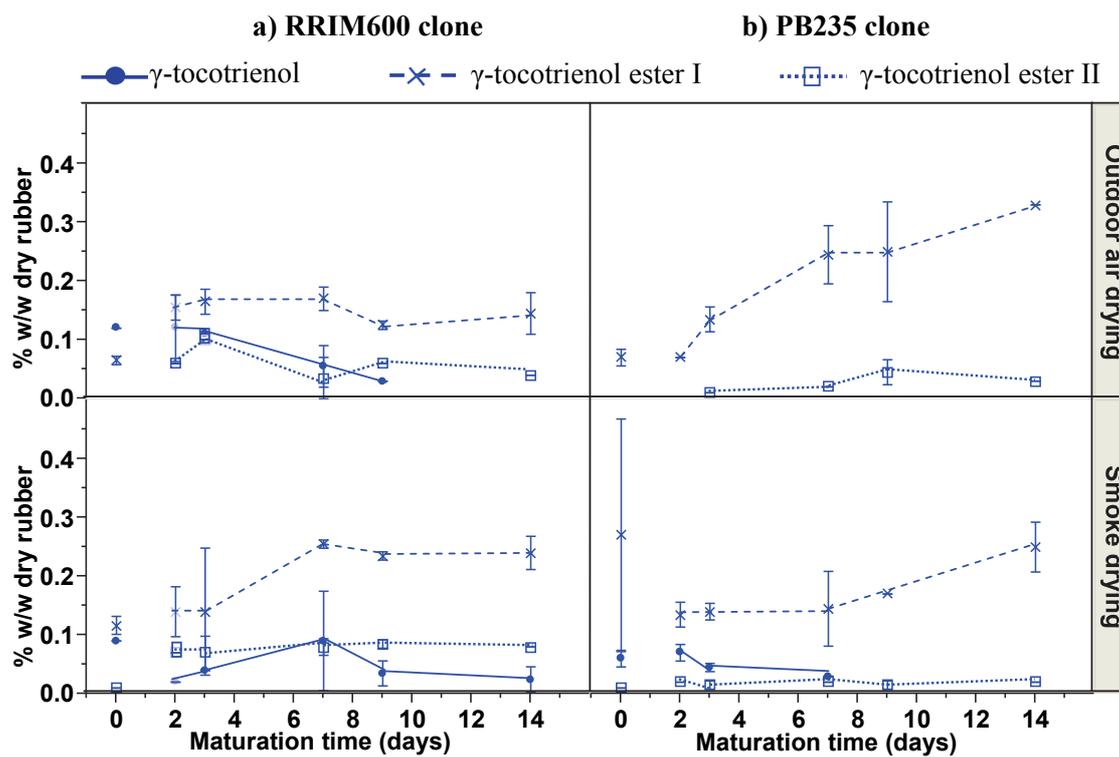


Figure 39 Evolution of γ -tocotrienol and its esters (% w/w dry rubber) of (a) RRIM600 and (b) PB235 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation.

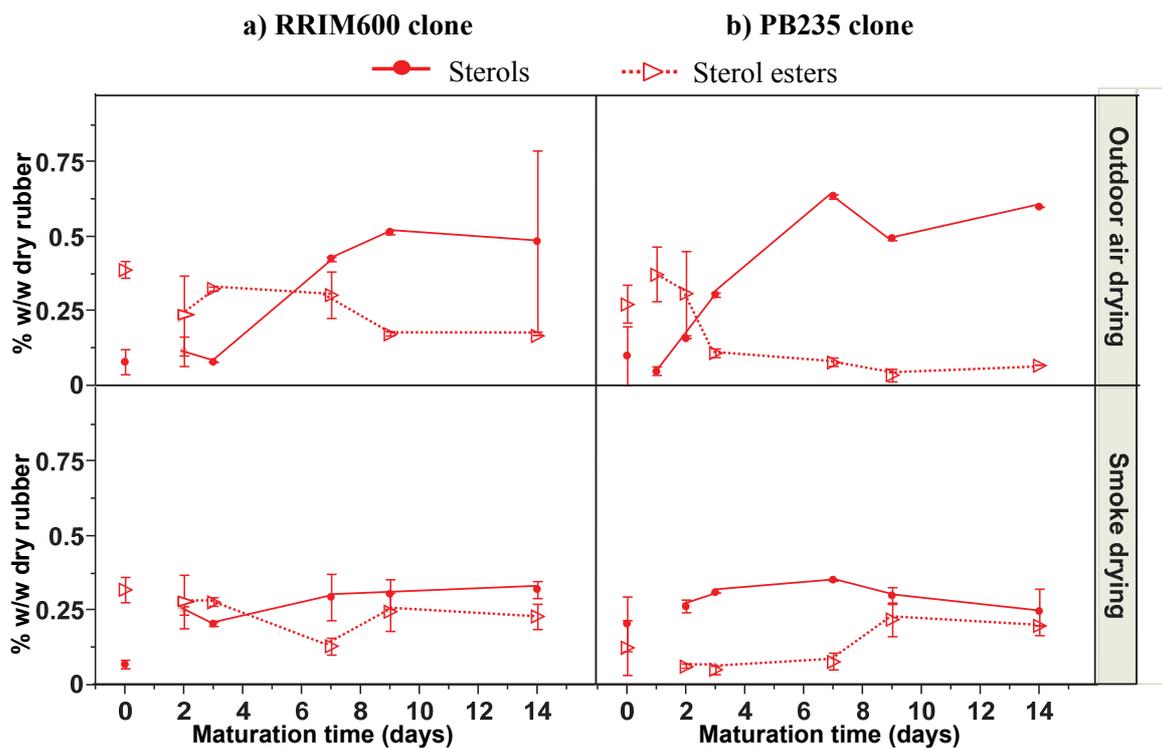


Figure 40 Evolution of sterols and sterol esters (% w/w dry rubber) of (a) RRIM600 and (b) PB235 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation.

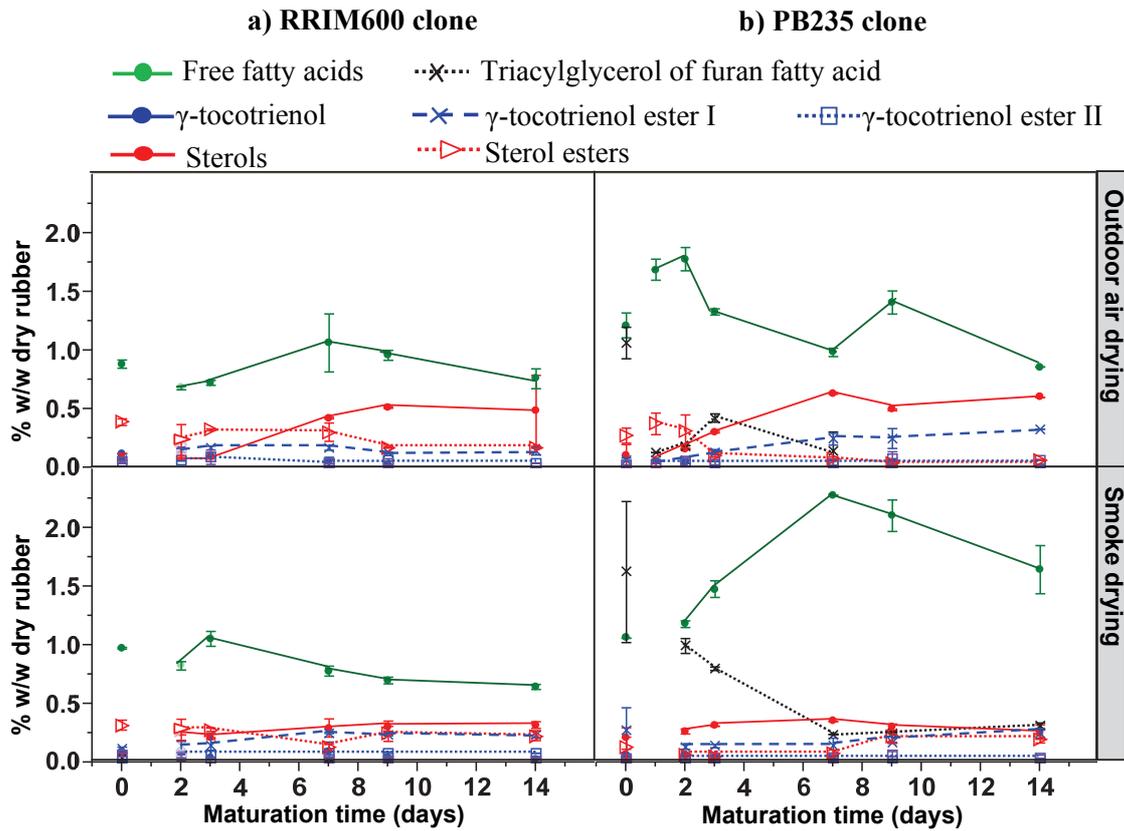


Figure 41 Evolution of the neutral lipid composition evolution (% w/w dry rubber) of (a) RRIM600 and (b) PB235 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation.

4.3.2 Total fatty acid composition

Lipid extract from RRIM600 and PB235 rubber samples obtained from 2 drying methods were analyzed for their total fatty acid composition using gas chromatography (GC-FID) as described in Materials and Methods. The identified fatty acids were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), furan fatty acid and arachidic acid (C20:0). The result would be presented in two ways: as a relative FA composition (versus the TFA weight) and the absolute TFA quantity (versus dry rubber weight). The absolute quantities were calculated taking into account determined concentrations in transesterified lipid, transesterification yields and lipid extracts. Results obtained from RRIM600 and PB235 samples are presented in Figure 42 and Figure 43, respectively. Major fatty acids was linoleic acid (18:2) and furan fatty acid for RRIM600 and PB235 clone lipid extract, respectively.

For RRIM600 clone, the relative content in the 2 main saturated fatty acids palmitic acid and stearic acid increased during 14 days of maturation whatever the drying condition (Figure 42, left). Concomitantly, there was an important decrease of linoleic fatty acid (C18:2) relative proportion (45.1% (day 0) to 5.1% (day14) under outdoor air drying and 45.7% (day 0) to 13.1% (day 14)). Linolenic acid (C18:3; 4.1% at day 0) could be not detected after 3 days of maturation (Figure 42a and Figure 42b). Observation of the absolute calculated quantities shows that the relative enrichment of TFA composition in saturated species is in fact mainly due to the fastest disappearance of the unsaturated ones. Moreover it is observed that the total fatty acid content decreased sharply during maturation followed by outdoor drying (from 0.65% (day 0) to 0.25% (day 14)). This decrease occurs at a lesser extent when mini-sheet were dried under smoke drying ((from 0.71% (day 0) to 0.40% (day 14)). Most probably the fatty acids were submitted to oxidative conditions and it is well known that unsaturated fatty acid are much more sensitive to oxidation that the saturated one, which can explain the observed difference in the kinetic of disappearance (Sousa *et al.*, 2010).

For PB235, the same tendencies were observed (Figure 43). The TFA composition from this clone distinguished itself by the presence of a high amount of furan

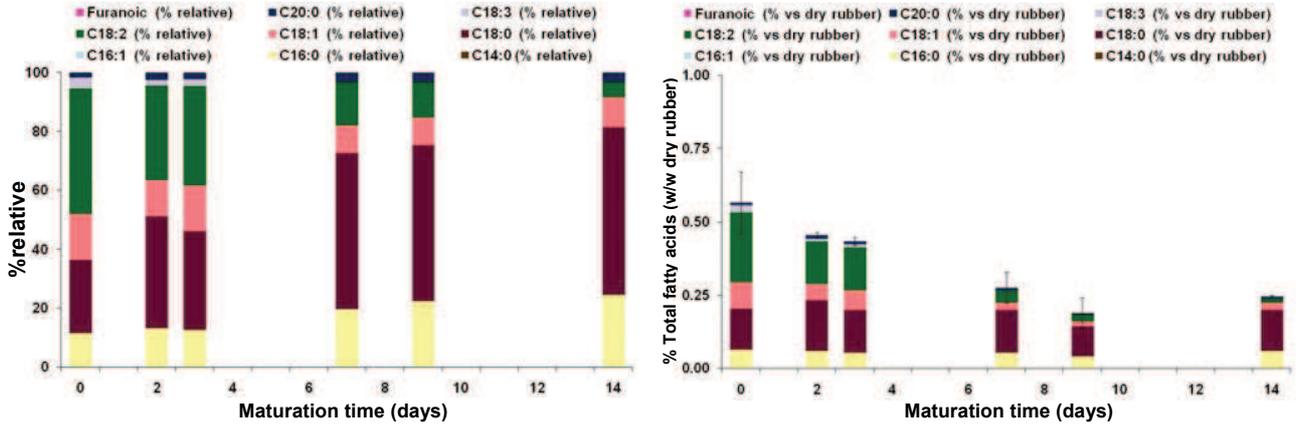
fatty acids and a lower initial amount of C18:2. Alike C18:2 in RRIM600 lipids, this fatty acid quantity decreased faster with maturation time than that of saturated ones (from 1.68% (day 0) to 0.1% (day 7) under outdoor air drying). As for RRIM600 clone, the decrease of TFA quantities during maturation occurred at a lesser extent when the sheets were smoked dried instead of outdoor air dried. This confirmed that the oxidative degradation of molecules keeps going while the sheets are hung. Indeed outdoor air drying was much longer than smoke drying, therefore it provides more “wet” time for the degradation to occur and result in a final quantity of TFA. The difference between the two drying methods in terms of TFA quantities is higher with maturation time. This could be explained by the fact that the laminated sheet obtained from coagula that have been matured very shortly (0 or 1 day) still contained enough native antioxidant to protect the unsaturation of the acyl chains from oxidation during drying time.

4.3.3 Unsaponifiable composition

Lipid extract from RRIM600 and PB235 rubber samples obtained from 2 drying methods were analyzed for their total unsaponifiable composition using Gas Chromatography equipped with a Mass Spectrometer (GC-MS) as described in Materials and Methods. The identified components were octadecanol, eicosanol, γ -tocotrienol, α -tocotrienol, stigmasterol, β -sitosterol and Δ 5-avenasterol. As for TFA, the results would be presented in 2 ways: (i) the relative unsaponifiable composition (versus the total weight of unsaponifiable identified components) and (ii) the absolute quantity of each unsaponifiable component (versus dry rubber weight). The absolute quantities were calculated taking into account determined concentrations in transesterified and silylated lipids, transesterification yields and lipid extracts. Results obtained from RRIM600 and PB235 samples are presented in Figure 44 and Figure 45, respectively.

As it was described before Liengprayoon *et al.* (2013) it is confirmed that unsaponifiable fraction of lipid extracted from rubber (RRIM600 and PB235) is mainly made of phytosterols, β -sitosterol constituting the major one (40-60% of unsaponifiable). In this experiment the RRIM600 lipid extracts seemed richer in sterols than that of PB235.

a) Outdoor air drying



b) Smoke drying

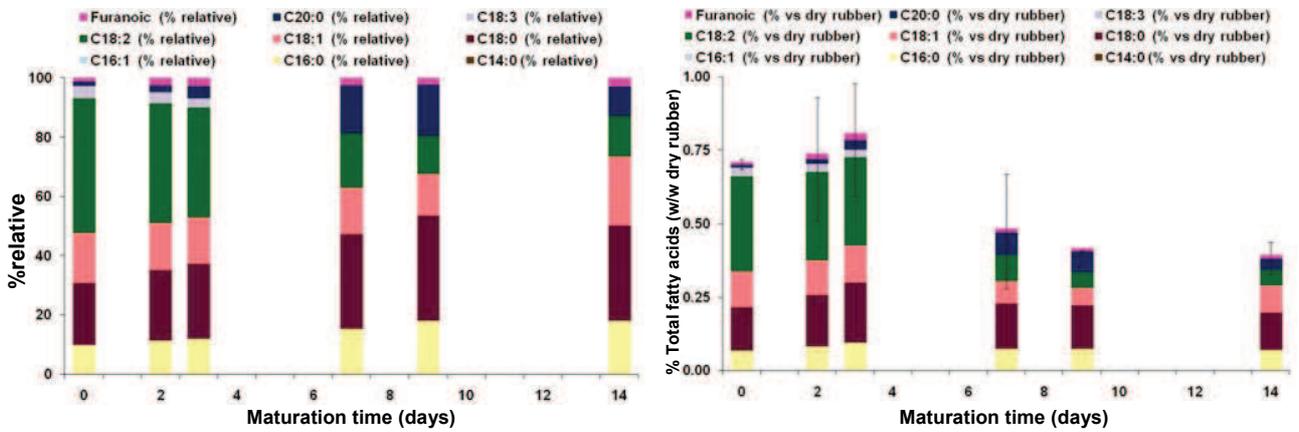
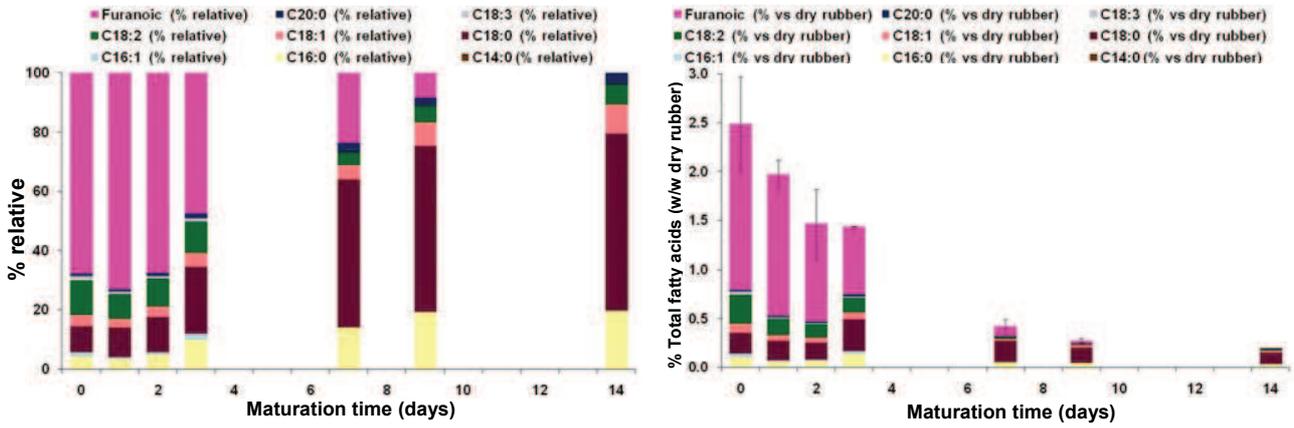


Figure 42 Total fatty acid composition of RRIM600 lipid extracts obtained from (a) outdoor air drying and (b) smoke dried samples during 14 days of maturation.

a) Outdoor air drying



b) Smoke drying

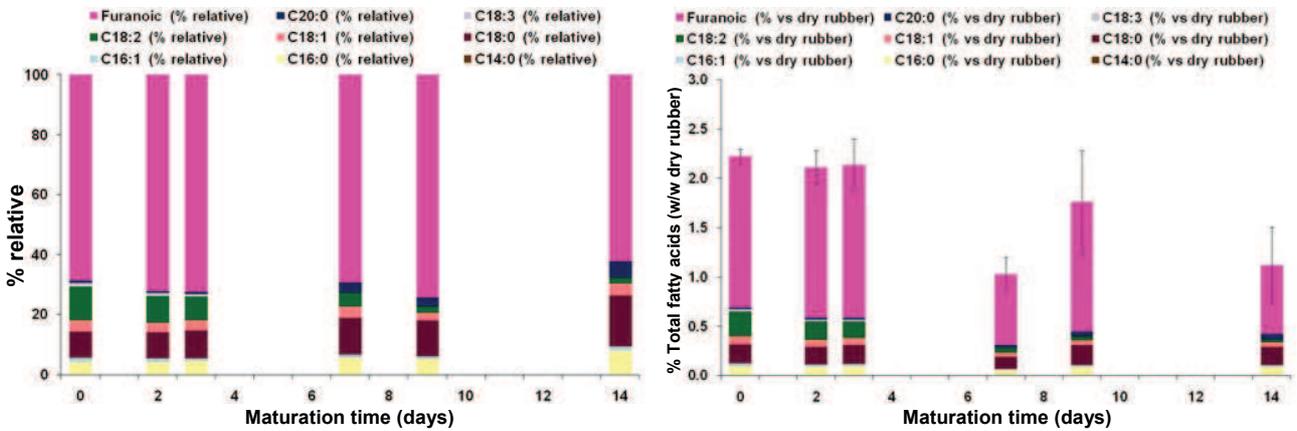


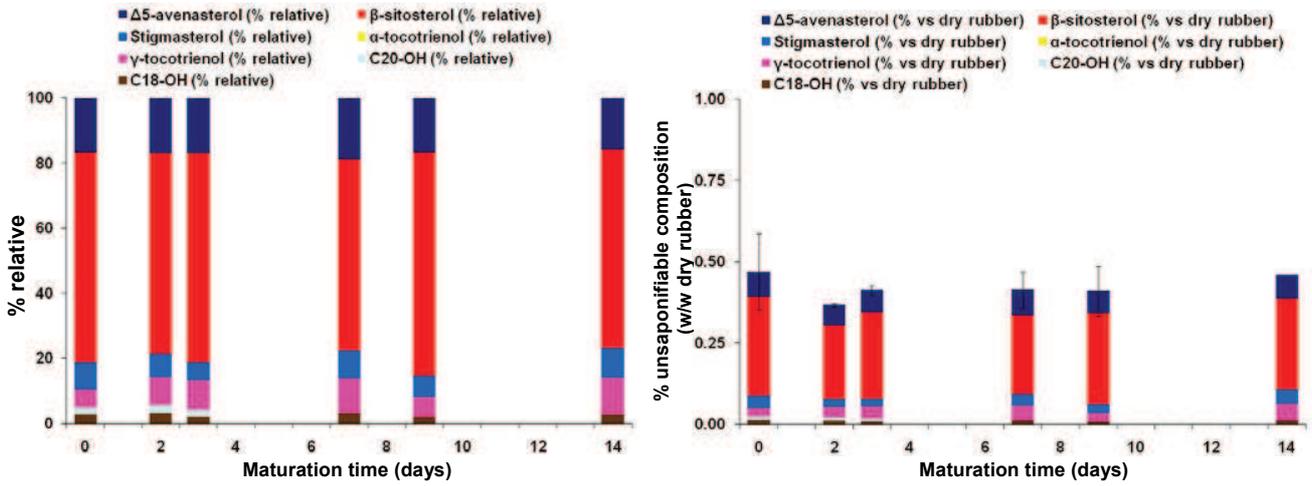
Figure 43 Total fatty acid composition of PB235 lipid extracts obtained from (a) outdoor air drying and (b) smoke dried samples during 14 days of maturation.

It is reminded that samples were saponified, therefore the unsaponifiable components (unsoluble alcohol) could either be free before transesterification or be esterified to a fatty acid. It is also reminded that saponification may lead to alteration of tocotrienol. Moreover, concerning γ -tocotrienol, it is reminded that some of them may have been involved in a dimerization reaction results and it was proved that dimers of γ -tocotrienol are not detected in this GC-MS analysis. Therefore the result concerning total tocotrienols could be underestimated (see Chapter 1, §5.2.2).

For RRIM600 lipids, the composition and absolute quantities seemed not to change significantly with maturation time whatever the drying conditions. Indeed the standard deviation of the total absolute quantities (shown as an error bars on Figure 44) are quite important. However some differences are observed between the two drying conditions: (i) the measured quantities seemed lower with outdoor air drying method (around 0.4% vs NR) than in lipid obtained from smoked dried rubber (around 0.7%); (ii) the tocotrienols (α - and γ -form) content in lipids from smoked dried rubber was higher (around 0.1% w/w dry rubber (smoked dried) versus 0.03% w/w dry rubber (outdoor air drying)). Moreover, no α -tocotrienol was detected from outdoor air dried rubber. Unfortunately, some methodological difficulties (incomplete silylation) were encountered with the samples from RRIM600 lipid from outdoor air-dried samples which may explain partially or not the observed difference between drying methods.

For PB235 lipids extracted from outdoor air dried rubber, the unsaponifiable quantity seemed to decrease with maturation time (0.79% (day 0) and 0.25% (day 14)) with a disappearance of α -tocotrienol after 9 days of maturation. This decrease was not observed when the rubber was smoked dried. Same methodological difficulties and high variability of data prevent from further interpretations. Instead, it was decided to perform the maturation sample under strictly controlled conditions in the hope of getting less variable results.

a) Outdoor air drying



b) Smoke drying

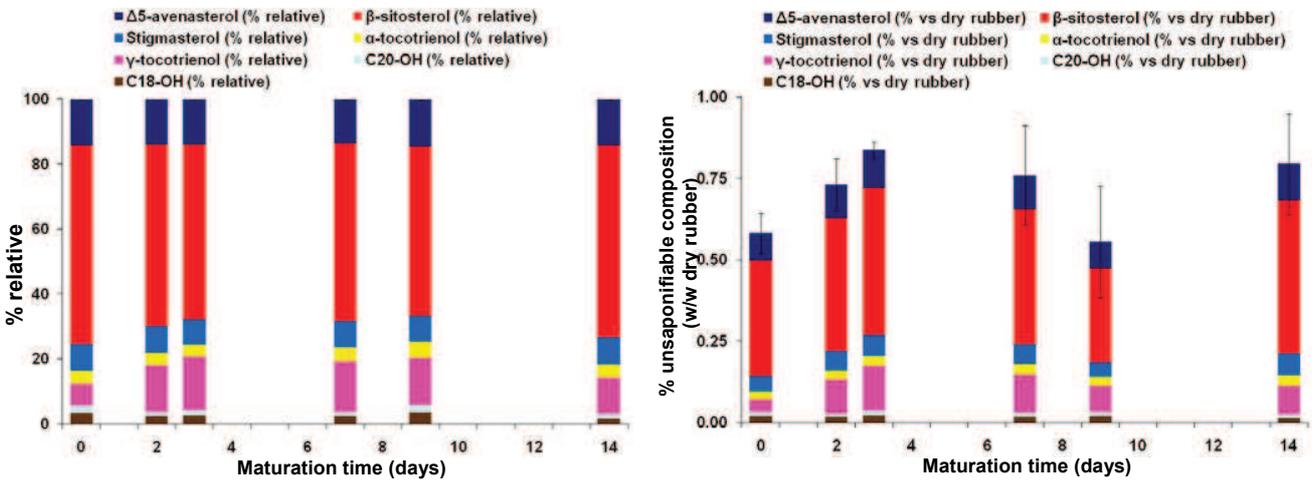
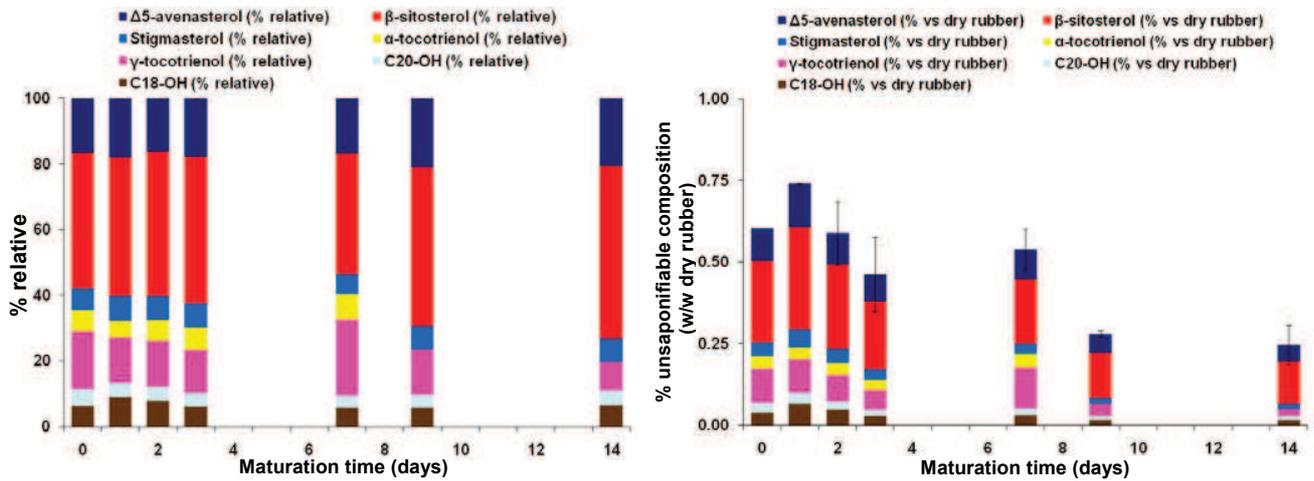


Figure 44 Unsaponifiable composition of RRIM600 lipid extracts obtained from (a) outdoor air drying and (b) smoke dried samples during 14 days of maturation.

a) Outdoor air drying



b) Smoke drying

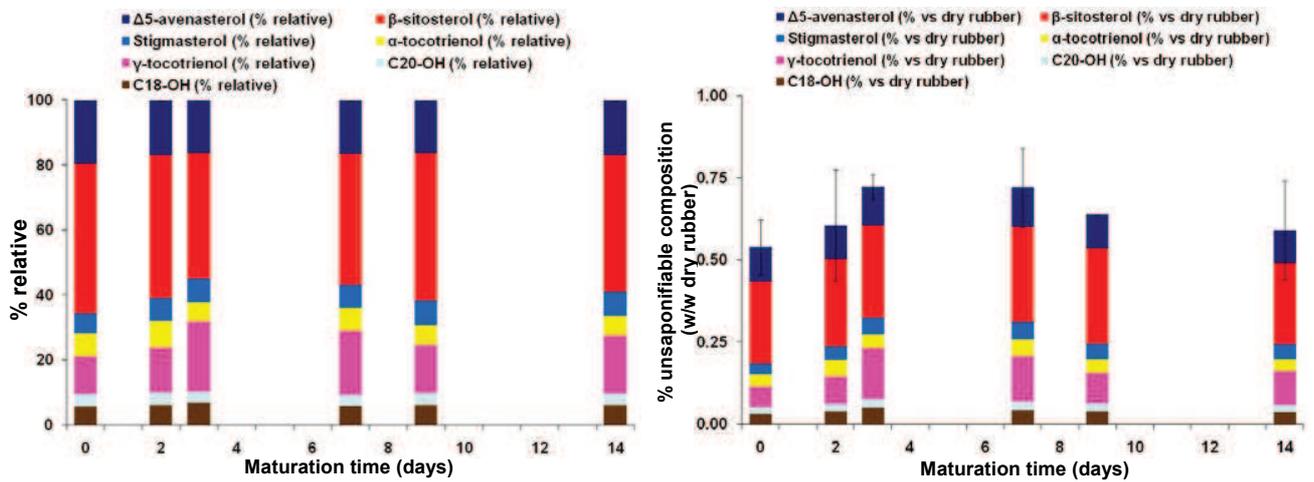


Figure 45 Unsaponifiable composition of PB235 lipid extracts obtained from (a) outdoor air drying and (b) smoke dried samples during 14 days of maturation.

5. *In vitro* antioxidant activity test of NR lipid extract by 2,2-di-phenyl-1-picrylhydrazyl (DPPH) method

5.1 Antioxidant activity of lipid extracts from rubber obtained from matured coagula

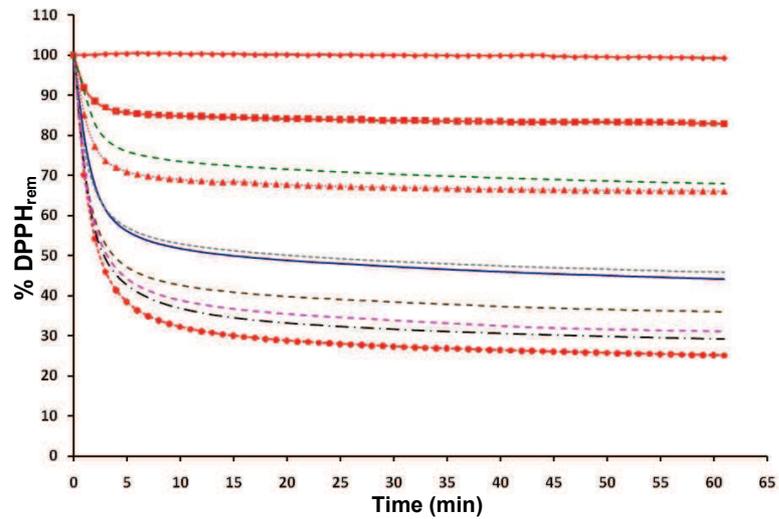
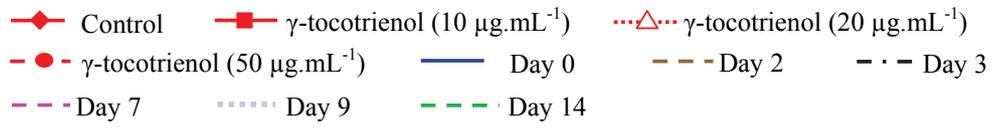
The DPPH assay using a microplate reader spectrophotometer was developed and validated as detailed in Chapter 1 (§4.2). A set of γ -tocotrienol standards with the concentration ranging from 0, 10, 20 and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ were used in parallel for comparison purpose.

Lipid extracts from RRIM600 and PB235 matured samples obtained from outdoor air and smoke drying at the concentration of 1 $\text{mg}\cdot\text{mL}^{-1}$ were tested for their antioxidant activity using the developed method. The result showed that every lipid sample exhibited antioxidant power by lowering the absorbance of DPPH as shown in Figure 46 for RRIM600 lipid samples and Figure 47 for PB235 lipid samples, respectively.

As the interpretation of the graphs is delicate, it was decided to focus on two numerical indicators that are (i) initial slope (0-3 min) of the $\% \text{DPPH}_{\text{rem}} = f(\text{time})$ curve and (ii) value of $\% \text{DPPH}_{\text{rem}}$ at 60 min. During the preliminary experiments described in Chapter 1, it was observed that the absolute values of these two indicators seemed to be inversely correlated. Unfortunately at this stage, not enough data was available to conduct any correlation study and it was subsequently decided to measure both indicators for all tested samples. After 693 measurements (3 measurement replication of 160 samples and 71 tocotrienols standards), the two indicators were plotted versus each other and the correlation was found to be very significant (Figure 48).

The slope value providing the same information as the $\% \text{DPPH}$ remaining after 60 min, using a single indicator is therefore sufficient. For practical reason, the initial slope is the one to be chosen as its measurement can be done in 3 min instead of 1 h. Consequently, only initial slope will be reported in this chapter.

a) Outdoor air drying



b) Smoke drying

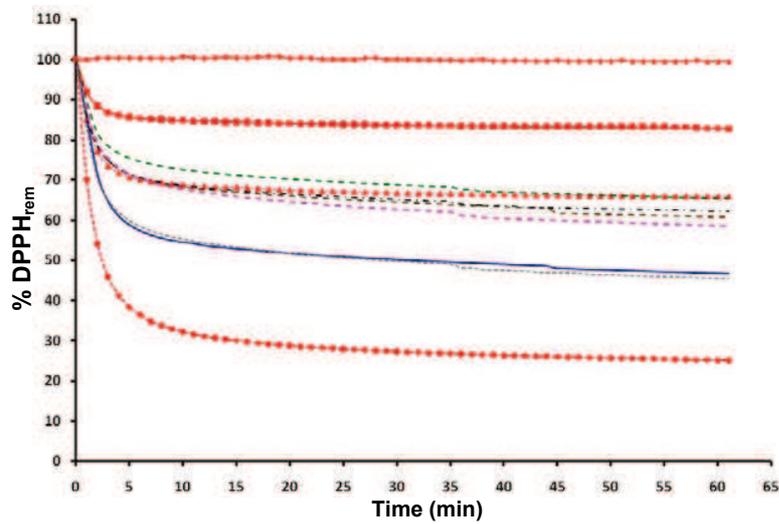


Figure 46 %DPPH_{rem} of RRIM600 lipid extracts obtained from (a) outdoor air drying and (b) smoke drying compared with γ -tocotrienol at 0, 10, 20 and 50 $\mu\text{g.mL}^{-1}$ concentrations (3 replications) from 0 to 60 min of measurement.

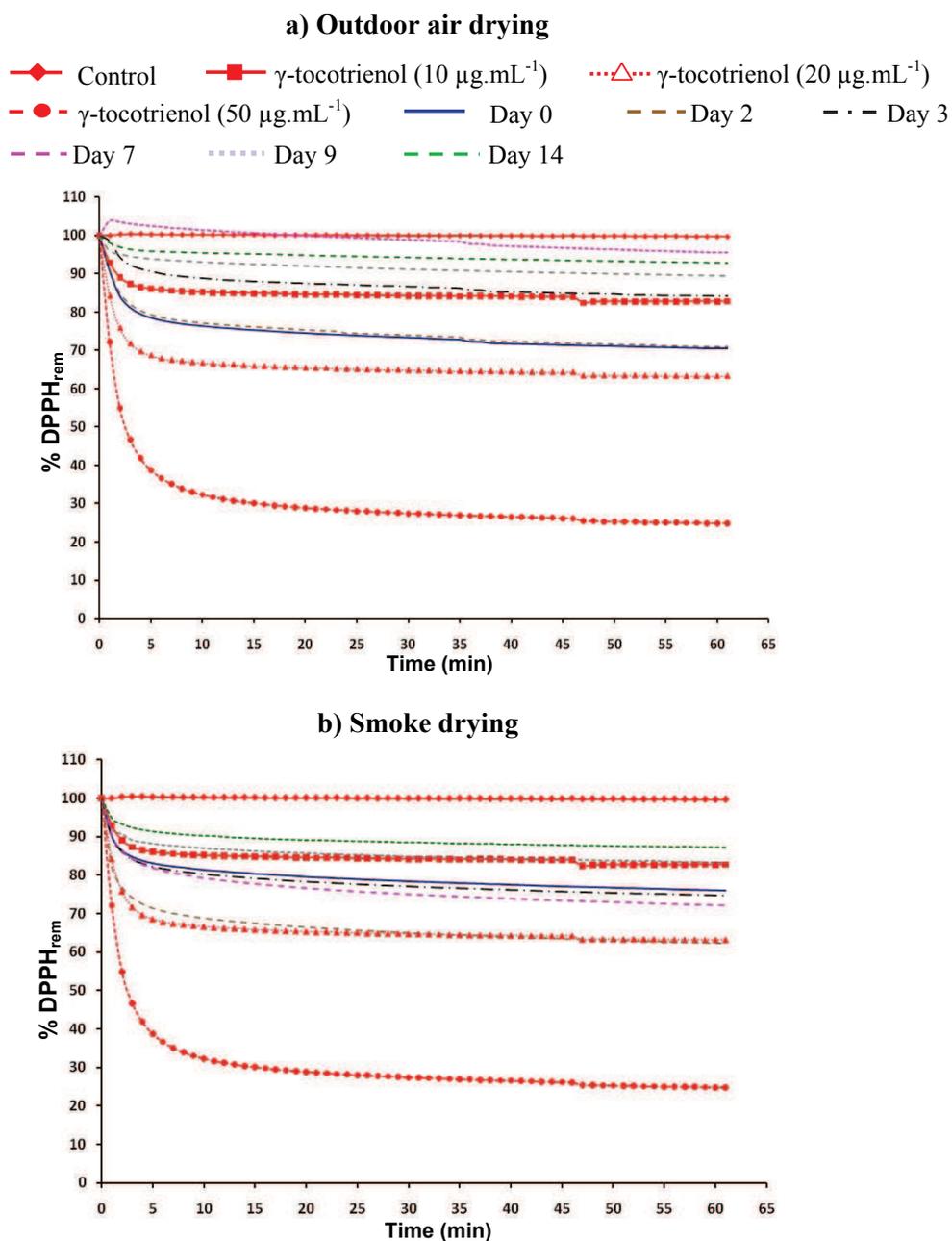


Figure 47 %DPPH_{rem} of PB235 lipid extracts obtained from (a) outdoor air drying and (b) smoke drying compared with γ -tocotrienol at 0, 10, 20 and 50 $\mu\text{g.mL}^{-1}$ concentrations (3 replications) from 0 to 60 min of measurement.

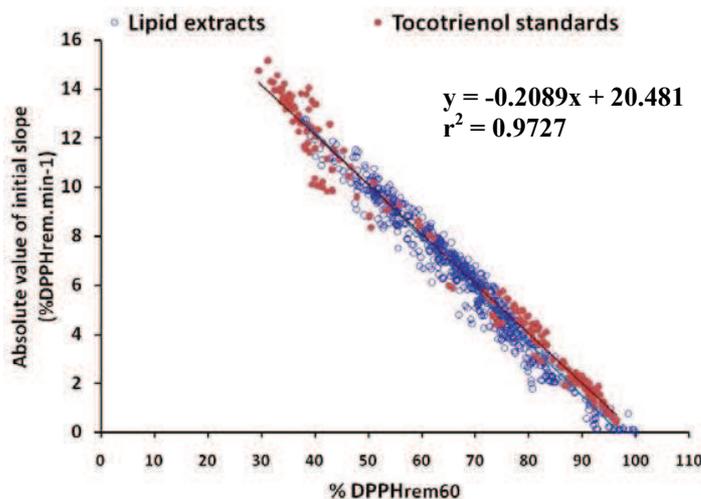


Figure 48 Correlation between absolute initial slope value and %DPPH_{rem60} obtained from lipid extracts of RRIM600 and PB235 rubber clones and tocotrienols standards.

Based on this finding, Figure 49 that shows the absolute value of the slope plotted against time summarized the same information as that contained in Figure 46a and Figure 46b, Figure 47a and Figure 47b. The obtained results are compared with three reference slope absolute values obtained from standard solutions of γ -tocotrienol. Moreover, the green numbers indicate the quantity of native γ -tocotrienol present in the tested lipid extracts as determined by HPTLC analysis.

Antioxidant activity estimated with initial slope indicator was detected for every lipid extracts whatever the clone, the maturation time and drying condition. It means that all obtained lipid extract contain component(s) that can scavenge the DPPH free stable radical. The absolute value of the slope allows the estimation of the effect of the tested parameters (clones, the maturation times and drying conditions) on antioxidant activity.

The antioxidant activity of lipid extracts from RRIM600 rubber obtained from smoke drying was comparable with that of γ -tocotrienol at the concentration of $20 \mu\text{g.mL}^{-1}$ whatever the maturation time. When the RRIM600 sheets were dried outdoor, the antioxidant activity of the lipid extracts evolved between 20 and $50 \mu\text{g.mL}^{-1}$ γ -tocotrienol equivalents. In

that case this indicator seemed to increase for the first 4-6 days of maturation and decreased from day 7 to day 14.

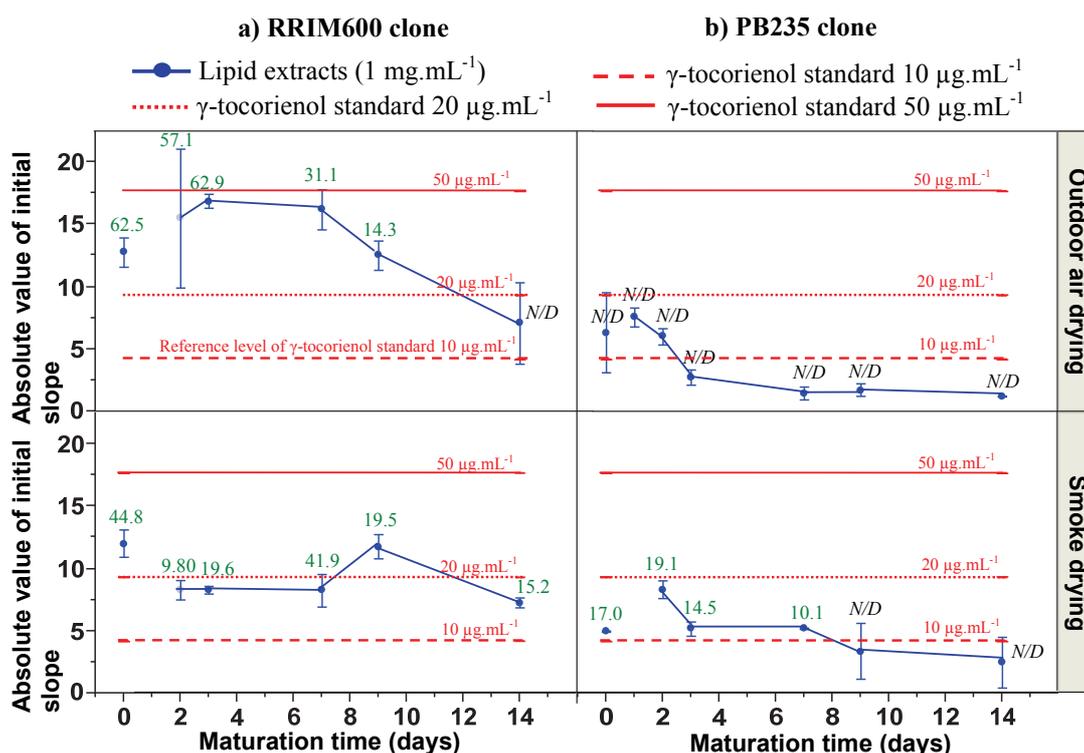


Figure 49 Absolute values of initial slope obtained from (a) RRIM600 and (b) PB235 lipid samples during 14 days of maturation from outdoor air drying and smoke drying. The numbers in green are the free γ -tocotrienol content in the lipid solution ($\mu\text{g.mL}^{-1}$) calculated from HPTLC results. *N/D*: not detected.

The first observation to be made concerning PB235 lipid antioxidant activity is that its level was lower than that of the extracts from RRIM 600 whatever the maturation time or the drying condition. Its level was comparable with that of γ -tocotrienol standard at a concentration of 10 $\mu\text{g.mL}^{-1}$. Under outdoor air drying, the decrease of antioxidant activity is less marked than that observed with RRIM600 clone. This decrease was also observed when the sheets were smoke dried, but at a lesser extent.

As free γ -tocotrienol is often described as a main native antioxidant of *Hevea* rubber and as this molecule was used as a reference standard in this assay, its content measured by HPTLC in lipid extract (w/w) was added to Figure 49. No clear link between its content and antioxidant activity was observed which suggested that this component does not drive alone the antioxidant activity of the lipid extract. Anyway when the antioxidant activity was low (less than $10 \mu\text{g}\cdot\text{mL}^{-1}$ tocotrienol equivalent) no γ -tocotrienol was detected by HPTLC.

In conclusion, it was observed that NR lipids obtained from 2 clones showed an antioxidant activity. During maturation time, the antioxidant activity decreased with maturation time however a clear evolution with time was not obtained. This could be caused from the variation of the lipid components classes containing as observed from the quantitative analysis by HPTLC.

5.2 Antioxidant activity of purified γ -tocotrienol derivatives

Two of γ -tocotrienol derivatives were identified in Chapter 1: γ -tocotrienol esters that are formed during maturation and tocotrienol dimers that are appeared when the free forms are exposed oxidizing conditions (for example during saponification reaction). It was decided to assess the antioxidant activity of these two kinds of derivatives and to compare it with the free form of γ -tocotrienol.

These comparative studies were conducted using the same DPPH test and are presented in Table 19.

No antioxidant activity was observed for γ -tocotrienol esters (I and II) under tested conditions (no slope could be measured). This could be explained by the fact that antioxidant activities of free tocotrienols are attributed to the phenolic hydrogen donation to the free radicals. In the case of esterification, this phenolic hydrogen was transferred to water. The ester bond prevents any free radical scavenging activity which could logically not be detected anymore. This means that this antioxidant “power” has been “silenced” by

esterification but is potentially releasable by hydrolysis. If confirmed, this could lead to have to value added applications.

Concerning dimers, though they were not detected in lipid extract from rubber, the test was conducted to correlate their antioxidant activities with the chemical structures identified in Chapter 1. As shown in Table 19, comparing the antioxidant activity of free and dimer forms of γ -tocotrienol at the same concentration, it was observed that the free form had higher antioxidant activity than the dimer forms at every concentration. Moreover, whatever the tested concentration, γ -tocotrienol dimer III (suspected to contain only one phenolic hydrogen, the other one being involved in the formation of ether bond) had a lower antioxidant activity than the other dimer I and II (suspected to contain the two phenol functions (dihydroxy dimers)). Those results confirm that the availability of phenolic hydrogen is crucial in the antioxidant activity of tocotrienols:

- i) When the phenolic hydrogen is free, the activity is maximum.
- ii) When two phenolic hydrogens are contained in a dimer, the activity is lower (relatively to the concentration) probably due to steric constraints.
- iii) When one of the two phenolic hydrogens has been removed during dimerization (formation of an ether bond), the activity is minimal.

Table 19 Absolute value of initial slope obtained from purified γ -tocotrienol dimers (I, II and III) and esters (I and II) from PB235 lipid extract (Data were means from 3 replications made from the same initial stock solution, *N/D*: not detected).



Concentrations ($\mu\text{g.mL}^{-1}$)	γ -tocotrienol (free form)	γ -tocotrienol derivatives				
		Dimer I	Dimer II	Dimer III	Ester I	Ester II
50	19.8 ± 0.3	6.84 ± 0.3	10.0 ± 0.2	3.74 ± 0.1	<i>N/D</i>	<i>N/D</i>
100	23.6 ± 0.6	12.3 ± 0.2	14.7 ± 0.2	7.19 ± 0.2	<i>N/D</i>	<i>N/D</i>
200	24.3 ± 0.8	18.8 ± 0.2	19.7 ± 0.4	14.6 ± 0.3	<i>N/D</i>	<i>N/D</i>

6. Conclusion

As described in Chapter 1, the methodology used to prepare the matured samples was based on the strategy to modify the protocol of preparation of USS in order to obtain a degraded sample. Indeed, latex was naturally coagulated (instead of formic acid coagulation) and the obtained coagula were matured for a certain period of time (instead of laminating the coagula after coagulation). Though interesting tendencies were observed in terms of physical properties of rubber, of lipid composition, of antioxidant activity of lipid extract, the variance of the obtained data made the interpretation of results very delicate. This variance was most probably due to the environmental parameters that were not controlled during maturation of coagula (temperature, humidity, presence of microorganisms in the atmosphere, etc).

Therefore, it was decided to change the experimental strategy and perform the next trials under strictly controlled maturation conditions (temperature, humidity, oxygen content of the atmosphere) followed by a TSR like processing (creping and drying 125°C, 2 h). This was done in PSU (Surat Thani campus, Surat Thani province, Thailand) where a dedicated maturation device was developed as described in Materials and Methods. The obtained results are shown in the next Chapter.

CHAPTER 3

Characterization of the structure, properties and biochemical composition of natural rubber obtained from coagula matured under controlled condition and treated using a TSR like process

The results obtained when the maturation was not controlled and the coagula were processed using USS/RSS like processing were presented in the previous Chapter 2. The present Chapter 3 will deal with the results obtained from fully controlled maturation conditions (relative humidity, temperature and oxygen content) and followed by a TSR like process (creping and drying 125°C for 2h). The sample preparation methods are presented in Materials and Methods (§3.3).

In this part, the data will be presented always following the same pattern: RRIM600 clone will be on the left while PB235 clone will be on the right. The three treatments will be presented vertically: the first one, on the top, (Na+O-) is a control treatment where the latex was added by a biocide (sodium azide, Na) in order to prevent the growth of most of the microorganisms. The second (Na-O-, maturation atmosphere = nitrogen) and the third, on the bottom, (Na-O+, maturation atmosphere = air) were performed to simulate anaerobic and aerobic maturations, respectively.

1. Initial pH of latex, and pHs of coagula and serum during maturation

The evolutions of pH of coagula and sera obtained from 3 maturation conditions are shown in Figure 50. The initial pH of latex is shown as a blue point at day 0.

1.1 Comparison with uncontrolled conditions

Contrary to uncontrolled condition, a similar evolution was observed for the pH of sera and that of the corresponding coagula. Therefore only pH of coagula will be commented. The absence of value concerning serum pH corresponded to the impossibility of measurement due to complete evaporation of serum.

Moreover, same tendencies of pH evolution between uncontrolled conditions (Figure 32) and the Na-O+ treatment were logically observed though the final high values of pH seemed higher in uncontrolled condition (pH > 9) than in uncontrolled aerobic condition (approx. pH 8).

1.2 pH follow-up of mini-coagula samples under controlled conditions

1.2.1 Control treatment (Na+O-, acid coagulated samples)

It is reminded that all samples in this treatment were acid coagulated. The formic acid was added into latex in each mini-glass cup after 3 days of maturation in order to make the complete coagulation of the latex before removal of all coagula from the mini-glass cups and continuation of maturation in the maturation device as described in Materials and Methods (§3.3)

Despite the biocide activity of sodium azide, it is interesting to see that the initial decrease of pH is still present in the first 3 days for both clones (from 6.6 (day 0, fresh latex) to 5.8 (day3, RRIM600) or 5.2 (day 3, PB235). This could be due to an incomplete inhibition of microbial activity or to a biochemical or chemical reactions that would acidify the latex and would be insensitive to azide. However, it is noted that with PB235 latex this initial pH decrease, which is more important than with RRIM600 latex, is lower when sodium azide is added than when it is not.

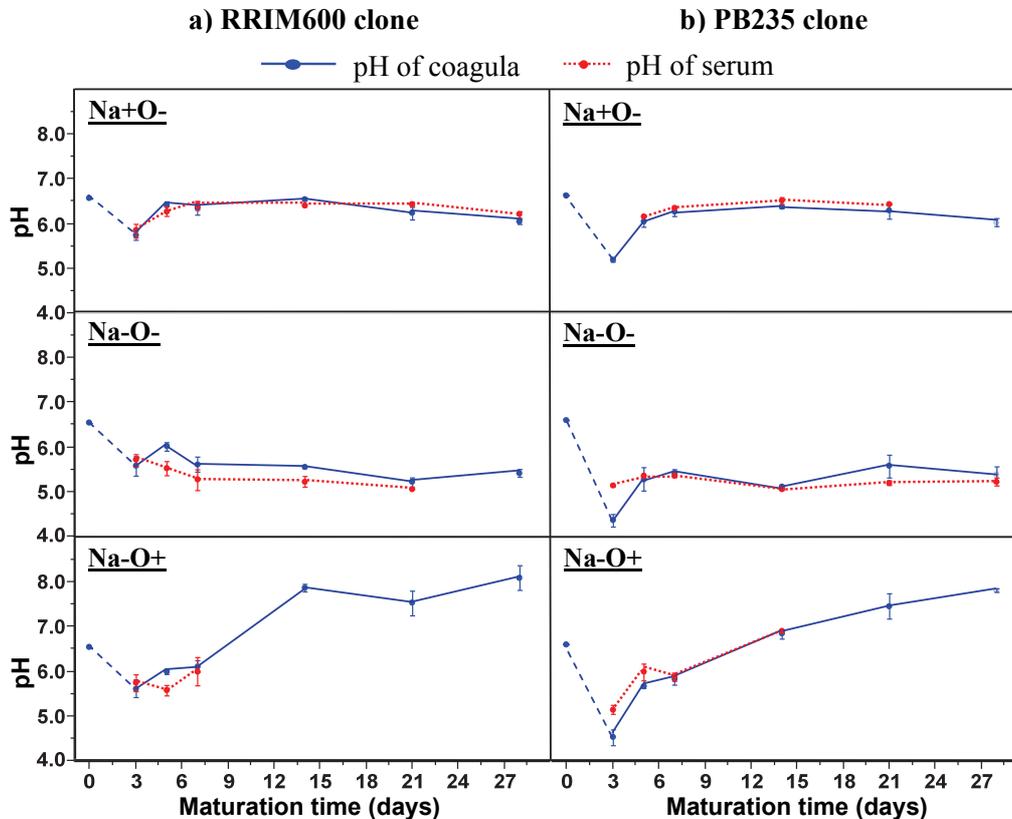
For both clones, after 5 days of maturation, and a forced coagulation by acid addition at day 3, the pH stayed stable between 6 and 6.5 for the total duration of maturation follow-up in the sodium azide added coagula.

1.2.2 Anaerobic (Na-O-) and aerobic (Na-O+) maturation condition (natural coagulation)

It is reminded that all samples in this treatment were naturally coagulated except for the day 0 sample, which was acid coagulated to allow the rubber sample making from a fresh coagulum (Materials and Methods, §3.3).

For RRIM600 samples, an initial decrease of one point of pH (from 6.6 to 5.6) was observed in both conditions (aerobic and anaerobic maturations) and corresponded to the coagulation. Then, the evolutions of pH were different depending on the maturation condition. Indeed, pH of Na-O+ coagula increased significantly and continuously during maturation and reached a maximum of 8.2 at day 28 while for Na-O-, the pH stayed in the narrow range of 5.4 to 6.0. Therefore, the presence of oxygen seemed to be a major factor of the pH evolution of rubber coagula over maturation time. This higher evolution of pH of coagula matured under aerobic condition could be linked with a higher protein degradation and/or lower organic acid production by aerobic microorganism, leading to higher ammonia and/or lower organic acid concentrations and resulting in a higher increase of pH. The same phenomenon was also observed in the work of Intapun (2009) who reported a higher increase of pH of RRIM600 matured cup coagula under the presence of oxygen than in its absence during 45 days of maturation.

Concerning the matured coagula prepared from PB235 rubber clone latex, the pH decreased from 6.6 (day 0, fresh latex) to 4.4 at day 3 which is 1 point lower than the minimum value reached by RRIM600 latex. This higher decrease could be partially explained with a higher FFA content, as FFA is known to be released by enzymatic hydrolysis in the early stage of maturation. This enzymatic process being most likely not sensitive to sodium azide addition, it can explain only partially this decrease to pH 4.4 (in the case of sodium azide added coagula, the pH decreased to 5.2). After this lowest pH, the evolutions of pH followed the same trend as those observed with RRIM600 (important increase in the presence of oxygen, and plateau at pH 5.2-5.6 range in anaerobic conditions).



Na+O-: Sodium azide added latex and matured under anaerobic condition.
 Na-O+: No sodium azide added latex and matured under aerobic condition.
 Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 50 pH evolutions of mini-cup coagula obtained from (a) RRIM600 and (b) PB235 rubber clones.

2. Fresh and dry matter of mini-crepes

As for uncontrolled conditions, the fresh weight and dry weight of the laminated coagula (called in the present case “mini-crepe” and “mini-laminated sheet” in uncontrolled conditions) were followed during maturation and the water content was calculated as the following ratio: (fresh weight minus dry weight)/fresh weight.

Figure 51 presents the evolutions of those indicators by treatment for RRIM600 and PB235 clones. It is observed that the dry weight (17-18 g) is constant whatever the treatment,

which means that the water content variation depends on those of the fresh weight of the crepe and that the drying seemed complete for all samples.

2.1 Comparison with uncontrolled conditions

As shown in Table 20, in every cases (aerobic controlled or uncontrolled maturation conditions) the water content increased with maturation time, but it is noted that the absolute values are always higher with controlled conditions, especially at the early stages (day 0, acid coagulated or day 3, natural coagulation). This difference is most probably due to the difference of laminating processing; a hand mangle in the case of uncontrolled conditions and a mini-creper in the case of controlled conditions. The difference in shape of coagula could also be involved.

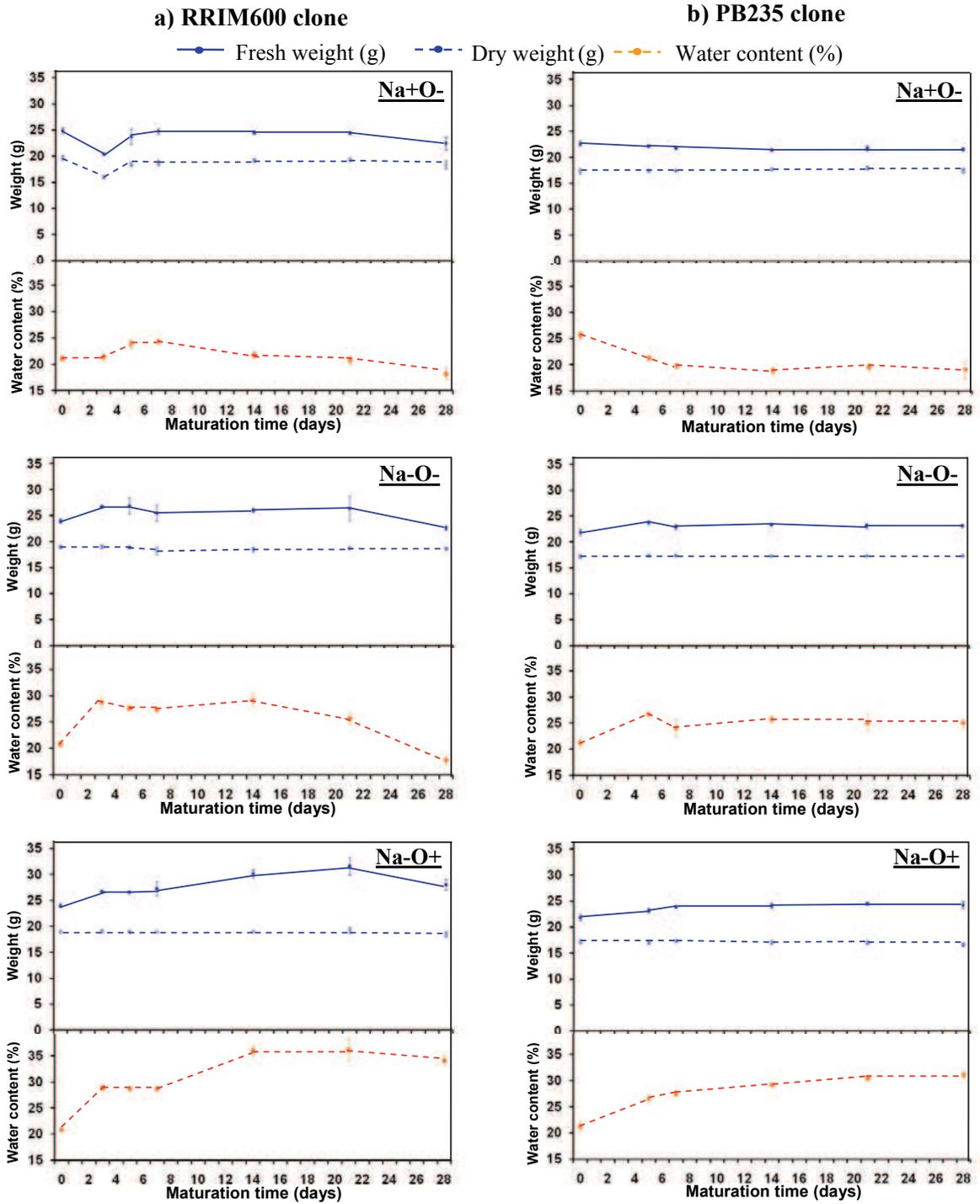
Table 20 The ratio of water contents of fresh laminated crepes and fresh laminated USS sheets obtained from RRIM600 and PB235 coagula.

Clones	Maturation duration (days)	Water content (%) of		Ratio of water contents (controlled /uncontrolled conditions)
		fresh laminated USS sheets (uncontrolled conditions)	fresh laminated crepes (controlled aerobic conditions)	
RRIM600	0	13 ± 2.0	21 ± 0.4	1.6
	3	16 ± 0.9	29 ± 0.7	1.8
	14	29 ± 0.5	37 ± 1.6	1.3
PB235	0	10 ± 1.7	21 ± 0.9	2.1
	3	15 ± 0.2	25 ± 1.1	1.7
	14	23 ± 0.7	29 ± 0.2	1.3

Values were expressed as means ± standard deviations (n=3.). The data of controlled condition were from aerobic maturation condition.

2.1 Evolutions of fresh and dry matter of matured coagula under controlled conditions

When sodium azide was added, the water content of the crepe stayed in the range of 17-25% for both clones. A slight decrease in function of maturation time was observed with PB235 crepes.



Na+O-: Sodium azide added latex and matured under anaerobic condition.
 Na-O+: No sodium azide added latex and matured under aerobic condition.
 Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 51 Fresh weight, dry weight and water content of mini-creped samples obtained from RRIM600 and PB235 clones during 28 days of maturation.

Under aerobic maturation conditions (Na-O+), the water content of the crepe increased significantly with maturation time reaching 35% for RRIM600 crepe obtained from 21 day-maturated coagula and 28% for PB235. This could be linked with microbial activity, that is known to be correlated with water retention in the coagula (Intapun *et al.*, 2010).

Under anaerobic conditions (Na-O-), the water content values stayed around 25% and seemed not to depend on the maturation time, except for that of day 28 of RRIM600 crepe that decreased to 18%.

It is therefore worth noting that the materials that were put in the oven and that were dried for the same time (2 h) at the same temperature (125 °C) were containing different quantities of water to be evaporated by drying. Therefore, the phenomenon occurring during drying could differ from one sample to another. Unfortunately, there was not enough knowledge and appropriate equipment to adapt oven temperature and drying time to the specific initial water content of each sample.

3. Evolution of bulk properties of NR from matured coagula under controlled maturation conditions (TSR like process)

The physical properties evolutions of RRIM600 and PB235 mini-crepes during 28 days of maturation are presented in Figure 52 and Figure 53, respectively.

3.1 Comparison with uncontrolled conditions

As expected, the variance of the obtained values and the number of outlier were found to be much lower when the maturation conditions were controlled. The evolutions of the bulk property indicator values over maturation time seemed easier to interpret especially for RRIM600 clone.

Moreover the maturation was followed on a longer duration (28 days) which provide information on a longer term.

3.2 Evolution of bulk properties of mini-crepes under controlled conditions

3.2.1 Control treatment (Na+O-, acid coagulated samples)

It is reminded that all samples in this treatment were acid coagulated. Concerning RRIM600 clone, though the growth of microorganisms was inhibited (Na+O-), the initial plasticity P_0 increased during the first few days of maturation (from P_0 39 at day 0 to P_0 54 at day 7). Then, this value stayed still until 28 days of maturation. P_{30} value followed the same evolution, which means that this hardening seems resistant to the thermo-oxidation condition imposed in P_{30} measurement (140 °C, 30 min). Therefore PRI did not change much and stayed at a high range of 73-93. Concerning PB235 clone, the hardening described for RRIM600 clone was not observed and the values of P_0 , P_{30} and therefore PRI stayed constant for all maturation durations (P_0 50; P_{30} 37; PRI 72).

3.2.2 Anaerobic maturation condition (Na-O-, natural coagulation day 3-28)

It is reminded that all samples in this treatment were naturally coagulated except the one at day 0. Therefore the evolution of value will be commented from day 3 to day 28 for RRIM600 and from day 5 to day 28 for PB235 clone due to a technical issue.

For RRIM600 clone, P_0 values increased from 51 (day 3) to 60 (day 7) and stayed still until day 28. Concerning P_{30} , a slight decrease was observed from 46 (day 3) to 40 (day 28) which resulted in a decrease of PRI from 90 (day 3) to 64 (day 28).

For PB235 clone, from day 5 to day 28, the values of P_0 stayed constant around the value of 40 except for day 7 (P_0 48). P_{30} values were low and decreased from day 7 (22) to day 21 (5). Therefore PRI decreased from 39 (day 5) to 4 (day 21).

Concerning non-maturated samples (day 0) which were obtained after acid coagulation, the P_0 value was higher for PB235 (P_0 44) than from RRIM600 (P_0 38) and corresponded to the values measured in uncontrolled conditions (mini-sheets).

Under anaerobic maturation, two main differences were observed between 2 clones (i) The hardening of RRIM600 rubber during maturation was not observed for PB235. This difference was already noted for sodium azide added treatment, which means that it could be independent from microorganisms but more linked with the mesostructure of rubber that is known to differ between 2 clones. Mesostructural characterization which will be described later in this chapter would provide more information ; (ii) The rubber obtained from PB235 clone seemed more sensitive to thermo-oxidation than that of RRIM600. This difference was not noticed in sodium azide added treatment which means that it could be linked with microbial activity.

3.2.3 Aerobic maturation condition (Na-O+, natural coagulation (day 3-28))

It is reminded that all samples in this treatment were naturally coagulated except day 0. Therefore the evolution of values will be commented from day 3 to day 28 for RRIM600 and from day 5 to day 28 for PB235 clone due to technical issue.

For RRIM600 clone, P_0 values increased from 51 (day 3) to 55 (day 7) and stayed still until day 28. A decrease of P_{30} values was observed from 44 (day 3) to 26 (day 28) which resulted in a significant decrease of PRI from 87 (day 3) to 46 (day 28). The main difference with anaerobic condition is a faster and higher drop of P_{30} which is due most probably to higher degradation of native antioxidant because of the presence of oxygen. The values of P_0 measured in this experiment are abnormally high for long maturation times. Indeed, it was expected that P_0 value should decrease after the initial hardening due to the scission of polyisoprene chains. Mesostructural analysis will provide more information.

For PB235 clone, from day 5 to day 28, the values of P_0 stayed constant around the value of 36 except for day 7 (P_0 43). P_{30} values were very low and decreased from day 7 (P_{30} =12) to day 28 (P_{30} =5). PRI value was low in stayed in the range from 16 to 26. As for RRIM600, the lack of decrease of P_0 over maturation time is abnormal especially when the P_{30} value are very low at early stage of maturation which should illustrate a lack of native antioxidant power which should lead to the scission of the polymer chain during the drying (125 °C, 2 h) and therefore a decrease of P_0 .

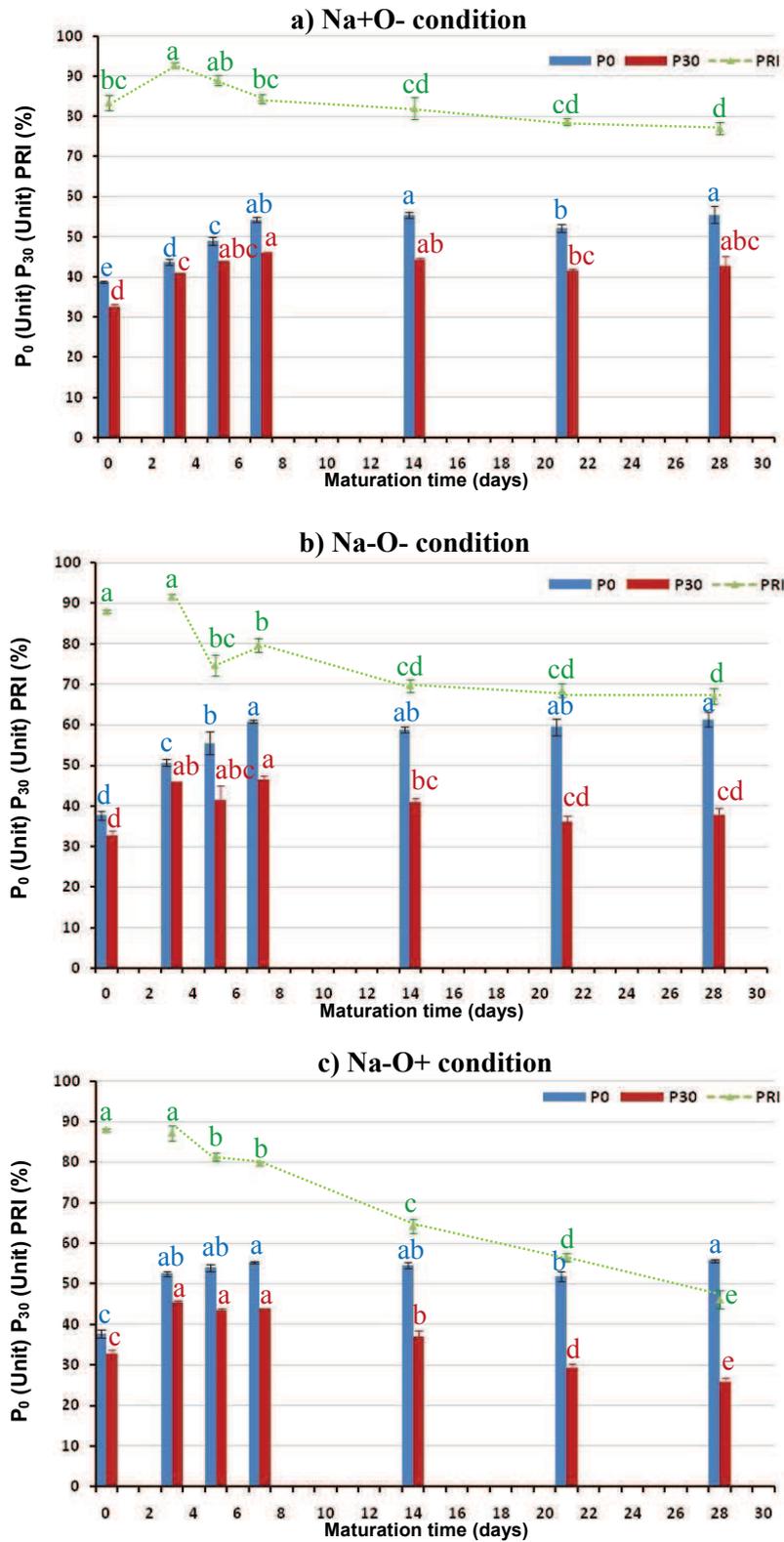


Figure 52 Evolutions of physical properties of (a) Na+O-, (b) Na-O- and (c) Na-O+ coagula obtained from RRIM600 clone during 28 days of maturation.

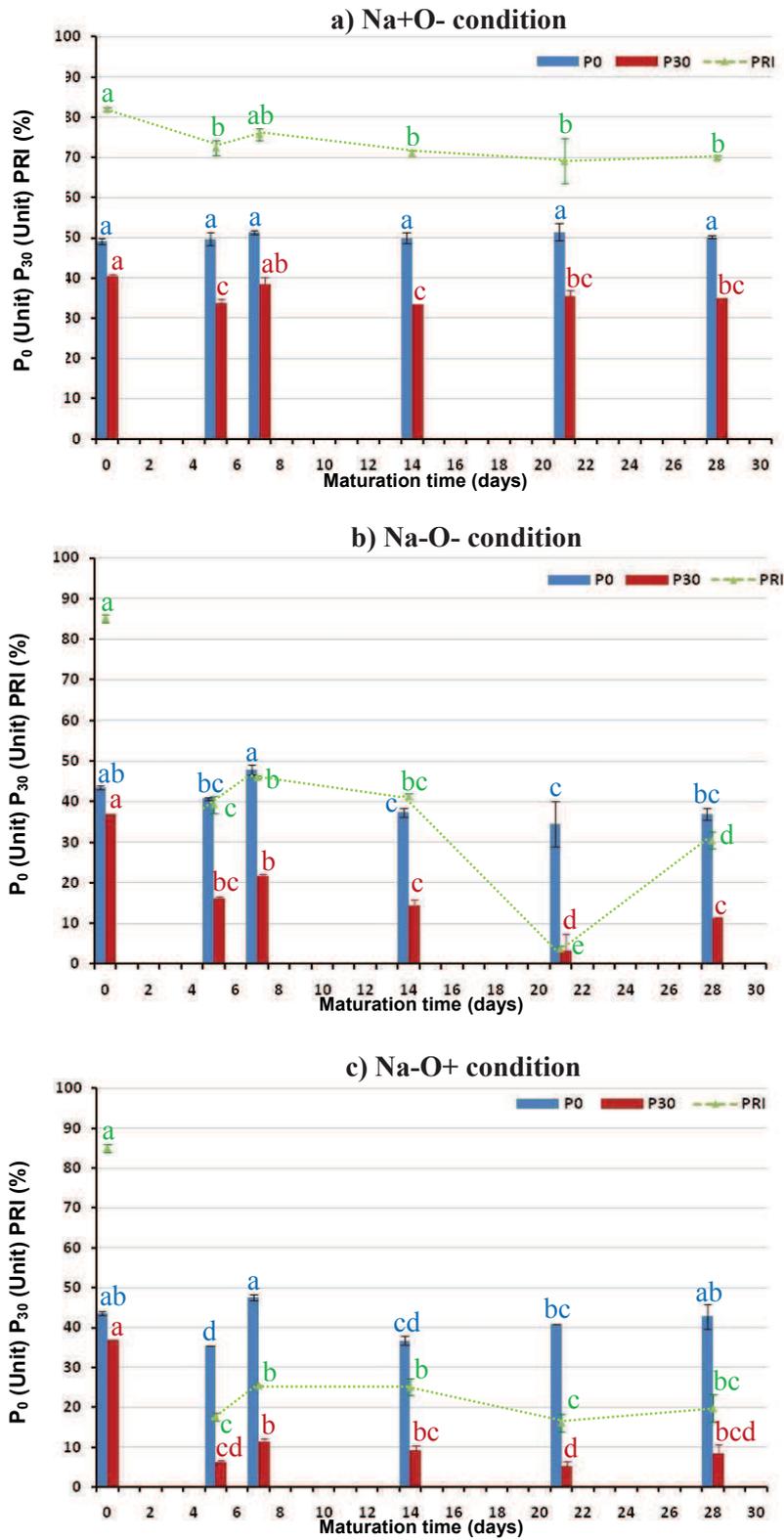


Figure 53 Evolutions of physical properties of (a) Na+O-, (b) Na-O- and (c) Na-O+ coagula obtained from PB235 clone during 28 days of maturation.

4. Mesostructure analysis

Mesostructure of RRIM600 and PB235 matured mini-cup coagula were analyzed by Size Exclusion Chromatography (SEC) equipped with a Multi-Angular Light Scattering (MALS) detector. The evolutions of the studied parameters: total gel content, weight-average molar mass (M_w) and number-average molar mass (M_n) are described below. It is precised that the mesostructural analyses were performed 6 months after the sample making and were stored at room temperature for 4 months in Bangkok laboratory and for 2 months in Montpellier laboratory. Therefore storage hardening may have occurred. Anyway, care was taken to ensure strictly identical storage history for all samples.

Following the analysis of bulk properties, the mesostructural indicator should confirm the main evolution over maturation time (initial hardening of the rubber for RRIM600 clone, etc.).

4.1 Total gel content

Figure 54 presents the evolution of total gel content (macrogel + microgel) during 28 days of maturation obtained from RRIM600 and PB235 mini-cup coagula.

4.1.1 Control treatment (Na+O-, acid coagulated samples)

There was a significant increase of gel within the first few days of maturation. This increase was much more significant for RRIM600 clone (from 34% (day 0) to 52% (day 7)) than for PB235 clone (from 28% (day 0) to 36% (day 5)). This correlates well with the observation of the evolution of P_0 values under this treatment that inhibits microorganism activity. This increase of gel content in presence of sodium azide was reported before by Intapun *et al.* (2010) for RRIM600 clone. Concerning PB235 clone, though the gel content increased also from day 0 (28%) to day 5 (36%), the extent of this increase was lower and the gel contents of PB235 sample were always lower than those of RRIM600 clone whatever maturation time and treatment. This lower variation and lower level of gel during maturation of PB235 coagula fit well with the variation of P_0 .

This clonal difference could be explained by the different molar mass distribution between RRIM600 and PB235 clones, RRIM600 rubber initially containing more short chain polymer that are known to be involved in gel formation (Ehabe *et al.*, 2002).

In addition, it is important to note that the acid coagulation had no significant effect on the increase of the gel content with the maturation time. Indeed, all samples were acid coagulated and the evolution of gel content followed the same trend as the other treatments (Figure 54).

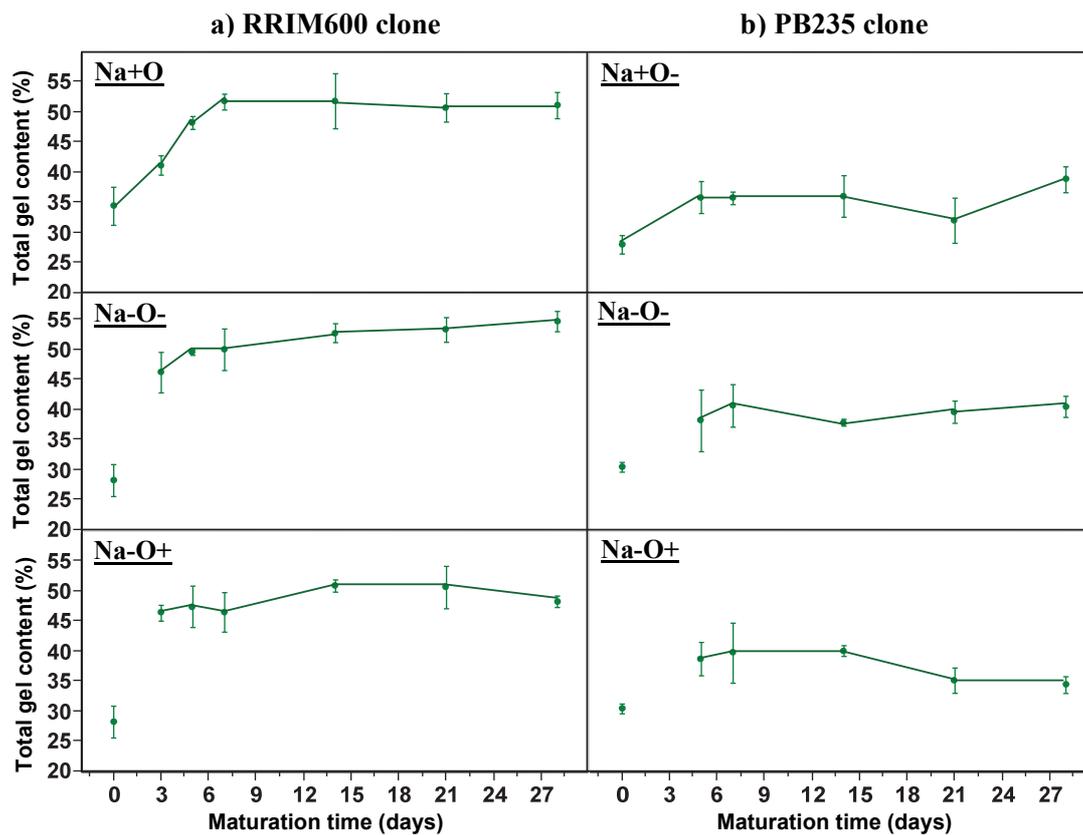
4.1.2 Anaerobic maturation condition (Na-O-, naturally coagulated samples)

For RRIM600 clone, an increase of gel content was observed as well at the beginning of the kinetic (from 28.5% (day 0) to 50% (day 7)). Concerning PB235 clone, the gel content increased also at the beginning of the kinetic (from 31% (day 0) to 41% (day 7)). Again, the plateau for the gel content observed during the kinetic of maturation ($7 < \text{time} < 28$ days) is significantly lower for ADS from PB235 clone.

4.1.3 Aerobic maturation condition (Na-O+, naturally coagulated samples)

The evolutions of gel content for both clones in aerobic condition were very similar to those observed under anaerobic ones.

The value of gel content at day 0 (acid coagulated, non-maturated sample) whatever treatment and clone was significantly lower than the naturally coagulated and maturated sample (for example for RRIM600 in anaerobic condition, the gel content of day 0 sample was 29% while after 3 day of maturation it was 47%). As mentioned before, it appeared that acid added for coagulation had no influence on the evolution of gel content. In addition, in our conditions of experiments there were no significant difference between treatments (Na+O-, Na-O-). At day 0, it can be noted that there is no clonal difference of gel content. However, along maturation a large difference was observed for gel content for the samples from the two clones (gel PB235 < gel RRIM600).



Na+O-: Sodium azide added latex and maturated under anaerobic condition.
 Na-O+: No sodium azide added latex and maturated under aerobic condition.
 Na-O-: No sodium azide added latex and maturated under anaerobic condition.

Figure 54 Evolutions of total gel content (%) obtained from (a) RRIM600 and (b) PB235 rubber clones during 28 days of maturation.

4.2 Weight-average molar mass (M_w) and number-average molar mass (M_n)

4.2.1 Control treatment (Na+O-, acid coagulated samples)

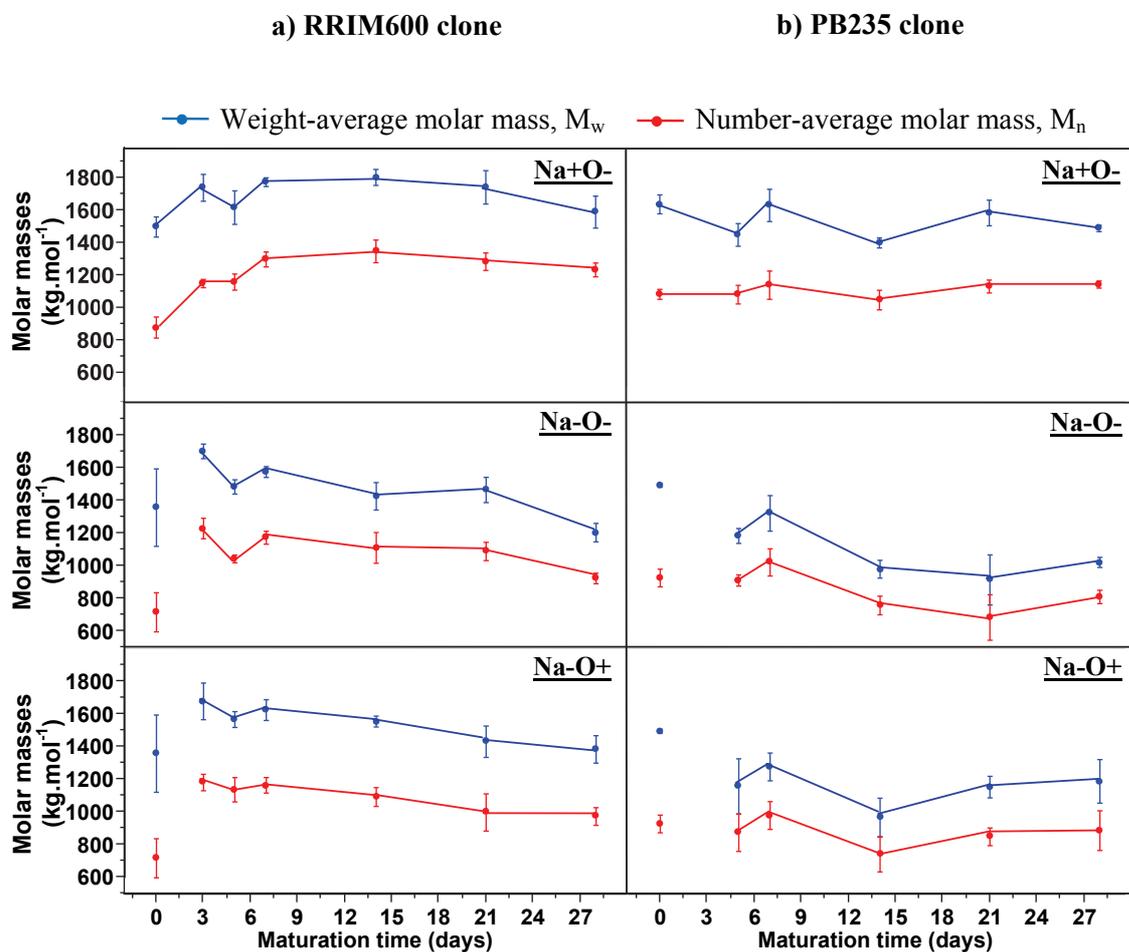
As presented in Figure 55, at day 0, the molar masses of PB235 sample (M_n 1083 kg.mol⁻¹; M_w 1637 kg.mol⁻¹) were significantly higher than those of RRIM600 (M_n 879 kg.mol⁻¹; M_w 1497 kg.mol⁻¹) which was expected from their known molar mass distribution (MMD₀ unimodal for PB235 and bimodal for RRIM600). After maturation (from day 3 to day 28), the molar masses did not change for PB235 sample while those for RRIM600 increased significantly and reached the value of 1347 kg.mol⁻¹ for M_n and 1802 kg.mol⁻¹ for M_w . This significant change is probably correlated with the high increase of gel that is associated with a disappearance of short chain in the soluble fraction that is used to assess molar masses.

4.2.2 Anaerobic maturation condition (Na-O-, natural coagulation day 3-28)

For RRIM600 clone, both molar masses decreased with maturation time from day 3 (M_n 1237 kg.mol⁻¹; M_w 1710 kg.mol⁻¹) to day 28 (M_n 930 kg.mol⁻¹; M_w 1212 kg.mol⁻¹). For PB235 rubber, the same tendency was observed with a higher decrease of average molar masses for longer maturation times.

4.2.3 Aerobic maturation condition (Na-O+, natural coagulation day 3-28)

The evolutions of both M_n and M_w were similar than in anaerobic conditions but the decrease of the values appeared less marked under aerobic condition.



Na+O-: Sodium azide added latex and matured under anaerobic condition.
 Na-O+: No sodium azide added latex and matured under aerobic condition.
 Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 55 Evolutions of weight-average molar mass (M_w) and number-average molar mass (M_n) obtained from (a) RRIM600 and (b) PB235 rubber clones during 28 days of maturation.

5. Evolution of lipid extract composition from matured coagula under controlled maturation conditions (TSR like process)

5.1 Lipid quantity

The lipid extract quantity obtained from RRIM600 and PB235 mini-cup coagula during 28 days of maturation are presented in Figure 56.

5.1.1 Comparison with uncontrolled conditions

The lipid extract from unmaturation samples (day 0) from both clones were found to be similar in both experimental conditions (Chantaburi fields, mini-sheet processing versus Surat Thani fields, mini-crepe processing), with a marked clonal difference (RRIM600 2.01% (Chantaburi mini-RSS day 0) vs 2.04% (Surat Thani mini-crepe day 0) and PB235 3.53% (Chantaburi mini-RSS day 0) vs 3.38% (Surat Thani mini-crepe day 0)). This confirms that lipid extract is a clonal character. Moreover the expected reduction of variance of values was clearly obtained by controlling the maturation conditions (Figure 56 and Figure 36).

Concerning the evolution of this indicator during maturation same tendencies were observed between the two maturation conditions but the decrease of the amount of lipid extract during maturation from PB235 samples was more marked under controlled maturation condition (aerobic treatment): 3.38% (day 0) to 2.51% (day 28).

5.1.2 Evolution of lipid quantity under controlled conditions

5.1.2.1 Control treatment (Na⁺O⁻, acid coagulated samples)

As shown in Figure 56, the lipid extract quantity obtained from both clones did not change much during maturation (for RRIM600 clone, it ranged from 2.17% (day 0) to 1.89% (day 28) and for PB235 clone ranged from 3.47% (day 0) to 3.16% (day 28)). This is most probably due to the absence of catabolic lipid degradation because of an inhibition of microorganism activity.

5.1.2.2 Anaerobic maturation condition (Na-O-, natural coagulation day 3-28)

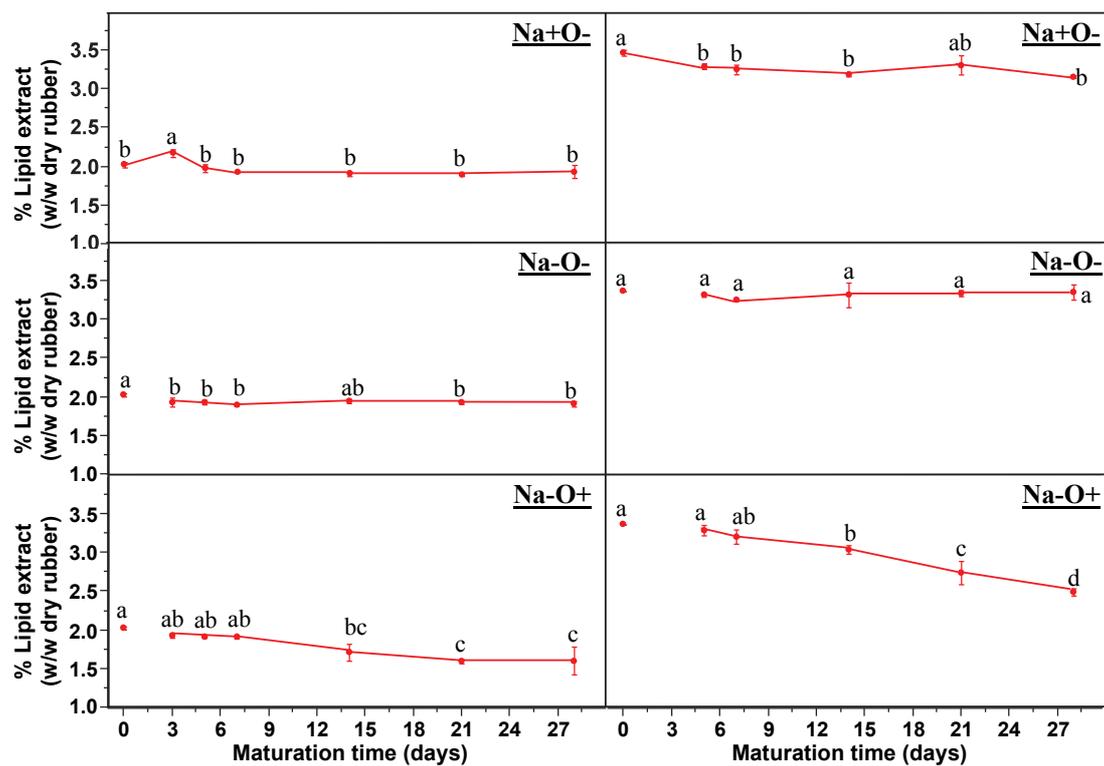
For both clones, under anaerobic maturation condition, the lipid extract did not change and stayed at its initial respective levels (for RRIM600 clone, the value of lipid extract stayed still in the range 1.91%-1.95% and 3.26-3.37% for PB235 clone). Therefore it seems that in absence of oxygen, the lipid extract quantity remained unchanged. It does not mean that there is no biochemical change (such as hydrolysis and release of free fatty acids) but it means that the products of this reaction remained not water soluble. For example the β -oxidation of fatty acids leading to water soluble acetyl-CoA would lead to a decrease of lipid extract if no mechanisms compensate this loss. Indeed, lipid anabolism by anaerobic microorganisms could also occur and compensate a possible catabolic degradation of native lipid extract.

5.1.2.3 Aerobic maturation condition (Na-O+, natural coagulation day 3-28)

Contrary to anaerobic maturation condition, a significant evolution of lipid contents was observed from the samples which matured under aerobic conditions from both clones. For RRIM600 samples, the lipid contents decreased significantly from 1.94% (day 3) to 1.61% (day 28) and for PB235, the lipid contents decreased significantly from 3.31% (day 5) to 2.51% (day 28). The more important decrease for PB235 clone could be linked with the higher fatty acids content of this clone. It could be therefore concluded that the presence of oxygen is an important factor for lipid catabolic degradation caused from microorganism activity during rubber maturation. The evolutions of each lipid component will be described in details in the following pages.

5.2 Qualitative analysis of lipid evolution during maturation

Figure 57 shows the TLC plates of lipids extract from mini-cup coagula of two rubber clones during 28 days of maturation.



Na+O-: Sodium azide added latex and matured under anaerobic condition.
 Na-O+: No sodium azide added latex and matured under aerobic condition.
 Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 56 Lipid extract (% w/w dry rubber) obtained from (a) RRIM600 and (b) PB235 rubber clones during 28 days of maturation.

5.2.1 Comparison with uncontrolled conditions

For both clones, the relative proportion of FFA and TGF obtained from aerobic controlled condition was similar to those of uncontrolled conditions in every drying condition. However the degradation rate of those components seemed visually lower under controlled conditions.

It is interesting to notice that when the sample were oven dried (125 °C, controlled maturation conditions, Figure 57) and at a lesser extent smoked dried (60 °C, uncontrolled maturation conditions, Figure 37), the esterified species (sterol esters and tocotrienol esters) were present for all maturation times, while when the samples were outdoor air dried the sterol esters tended to disappear while the tocotrienol esters appeared with maturation. Therefore there could be an effect of drying method on the esterification state of FA and alcohols in the lipid extract. This will be analyzed in the following quantitative reports.

5.2.2 Evolution of lipid composition under controlled conditions

5.2.2.1 Control treatment (Na+O-, acid coagulated samples)

A similar evolution of lipid components during maturation was observed between lipid extracts from 2 studied clones. The main change over maturation time was FFA spot increase corresponding to a hydrolysis of esterified species, especially polar lipids (Liengprayoon *et al.*, 2013).

5.2.2.2 Anaerobic maturation condition (Na-O-, natural coagulation day 3-28)

The evolutions of lipid components of RRIM600 samples were similar to the control treatment. However it was observed that the intensity of wax spot of natural coagulated sample seemed to be higher than in the sodium azide added and acid coagulated samples. Same observation could be made for lipid components evolution of PB235 samples except for TGF spots. It was found that the hydrolysis of TGF seemed faster

in the case of naturally coagulated samples (Na-) than in that of sodium azide and acid coagulated samples (Na+). Moreover the wax spot for PB235 samples seemed to be doubled. It could be due to the presence of more polar furan fatty acid in wax species. It would be interesting to analyze the fatty acid composition of waxes.

5.2.2.3 Aerobic maturation condition (Na-O+, natural coagulation day 3-28)

For both samples, the hydrolysis of TGF seemed to occur faster under aerobic than anaerobic conditions. Moreover, free fatty acids spot intensity seemed to decrease at long maturation time (day 14-28). HPTLC quantification would help to confirm these visual observations.

5.3 Quantitative analysis of lipid evolution during maturation

5.3.1 Neutral lipid composition

Lipid extracts obtained from RRIM600 and PB235 matured rubbers were analyzed for each lipid component quantity using HPTLC. Compared with the previous HPTLC results obtained with extracts issued from uncontrolled maturation conditions, the variance of result was much lower, and the obtained tendencies easier to interpret.

5.3.1.1 Free fatty acids and TGs

Figure 58 shows the evolution of free fatty acids (FFA) and trifuranoyl-glycerol (TGF) quantities over maturation time for the 3 treatments and 2 clones.

When sodium azide was added, an identical and limited release of free fatty acids (till around 0.7 % rubber) was observed for both clones after 3-5 days of maturation. For PB235 lipid extract which contains a lot of TGs it was observed that they were partially hydrolyzed only by native latex lipases. Another source of fatty acids can also be polar lipids (glyco- and phospholipids) that are known to be hydrolyzed during the process of dry rubber sample making.

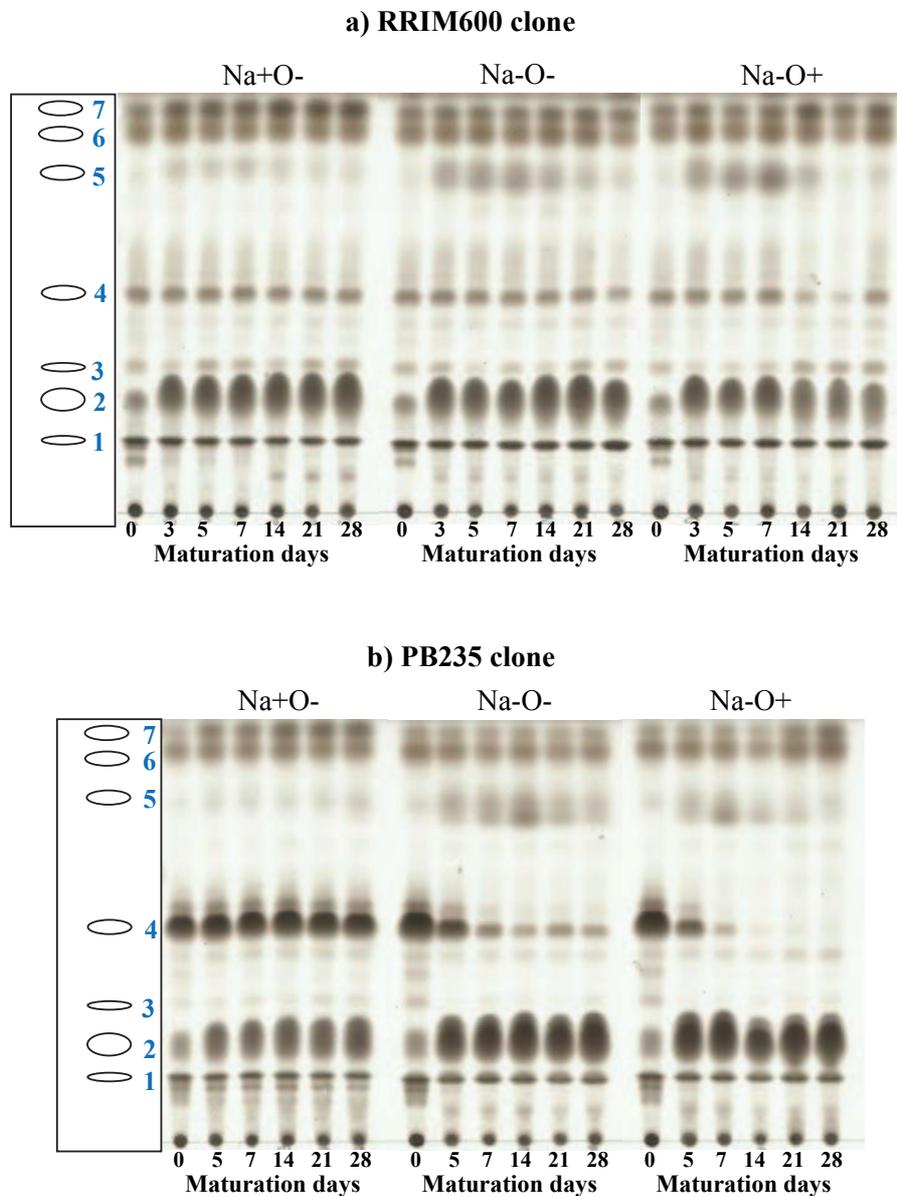


Figure 57 TLC of total lipid extract obtained from (a) RRIM600 and (b) PB235 matured mini-cup coagula during 28 days from 3 maturation conditions. Developed in hexane/diethyl ether/acetic acid (80:20:1, v/v/v) and detected by 40% orthophosphoric acid and aqueous solution saturated with copper acetate (1:1, v/v).

1: Sterols, 2: Free fatty acids, 3: γ -tocotrienol, 4: Triacylglycerol of furan fatty acid, 5: Wax, 6: γ -tocotrienol esters (I and II) and 7: Sterol esters.

Under anaerobic maturation, same observation could be made for RRIM600 clone. The evolution of TGF and FFA for PB235 clone was different compared with sodium azide added treatment: indeed, the hydrolysis of TGF was almost complete after 14 days of maturation, which logically corresponded to a higher free fatty acids (mostly furan fatty acid) release. Therefore the anaerobic microorganisms may have provided supplementary lipase activity to perform this complete hydrolysis of TGF.

Under aerobic conditions, at the early stage of maturation (3-5 days) the quantities of TGF were similar than in anaerobic conditions. But two differences can be noted for the later stage of maturation: (i) the complete hydrolysis of TGF occurred earlier and (ii) a decrease of FFA after 7 days of maturation replaced the plateau state observed before. The decrease could be either due to an esterification with alcohol (sterols, tocotrienols, fatty alcohols) or to a catabolic degradation.

5.3.1.2 γ -tocotrienol and its esters

Figure 59 shows the evolution of free γ -tocotrienol and its esters over maturation time for the 3 treatments and 2 clones. It is reminded that γ -tocotrienol ester I contains mainly saturated fatty acids while γ -tocotrienol ester II contains unsaturated fatty acids. The very low level of ester II (around 0.02%) in PB235 sample compared with those from RRIM600 (0.07-0.15 %) whatever the treatment or the maturation is consistent with the facts that (i) PB235 clone is known to have a much lower quantity of C18:2 compared with RRIM600 and (ii) no furan fatty acid was detected in both γ -tocotrienol esters.

When sodium azide was added in RRIM600 latex, the free form of γ -tocotrienol initially present at 0.1% tended to decrease to 0.05% after day 7 and its quantity remained stable after. At the same time γ -tocotrienol ester I decreased from 0.2% (day 0) to 0.15% (day3) and stayed stable. Concerning γ -tocotrienol ester II, a slight decrease over maturation time was observed. For PB235 sample with the same biocide added treatment, no clear change was observed for γ -tocotrienol free form. It is noted that the initial level (0.06%) is lower than that of RRIM600 sample (0.1%). Contrarily, tocotrienol ester I initial amount in

PB235 sample (0.3%) was higher than that of RRIM600 (0.2%) and decreased in the first 5 days of maturation.

When the microorganism activity was not inhibited and the coagula were naturally coagulated and matured under anaerobic conditions, the evolutions of free and esterified γ -tocotrienol were different. In the case of RRIM600 samples, the evolutions of both esters I and II were symmetrical: the ester species got enriched in saturated fatty acids after 14 days of maturation but their total amount did not change. Concerning PB235 samples, their content in ester I seemed to increase after 14 days of maturation as well, while both free and ester II stayed steady at low levels.

When aerobic conditions were applied, same evolutions of esters species were observed for both clones, but a slight increase of the free forms was also observed. The enrichment of ester species by saturated fatty acids during maturation could be linked to a parallel enrichment of saturated species in total fatty acids.

5.3.1.3 Sterols and their esters

The evolution of sterols and their esters quantities over maturation time obtained from three treatments and two clones are presented in Figure 60.

For sodium azide added samples, an esterification of some of the initially free sterols seems to occur at early stage, most probably catalyzed with endogenous enzyme from latex. Anyway, the increase of esterified species did not balance fully the decrease of free sterols that could be involved in other reactions (glucosylation?). The increase of esterified sterols was not observed clearly when microorganism activity was not inhibited whatever clone and treatment. For both clones, the free sterol content in rubber samples remained stable for naturally coagulated samples (day 3-day 28) and stayed in the range of 0.2-0.3% which is much lower than that of acid coagulated sample at day 0 (>0.5%).

5.3.1.4 Wax

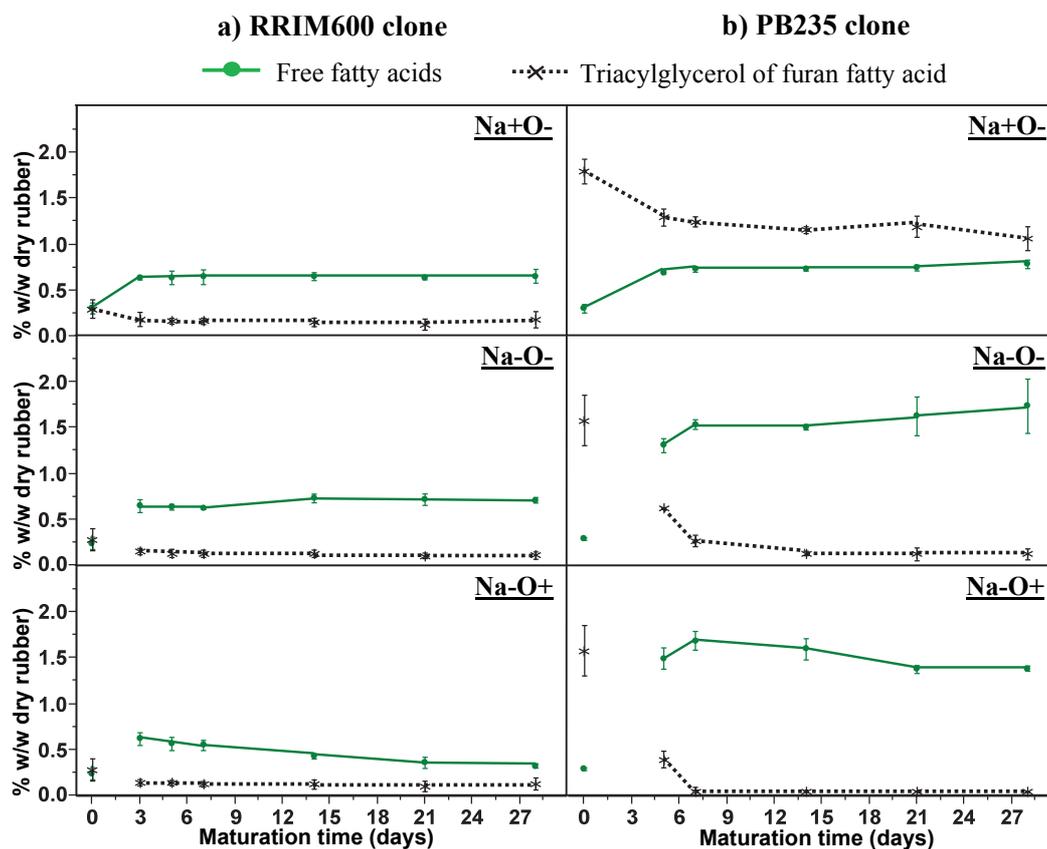
Wax species appeared temporarily during maturation when microorganism activity was not inhibited (Figure 61). The main period of appearance of wax was from 3 to 14 days in RRIM600 samples and was more important under aerobic condition, while this period was broader for PB235 samples. Unfortunately the corresponding fatty alcohols were not quantified in this HPTLC experiment.

The scheme of neutral lipid composition evolutions of RRIM600 and PB235 clones are shown in Figure 62.

5.3.2 Total fatty acid composition

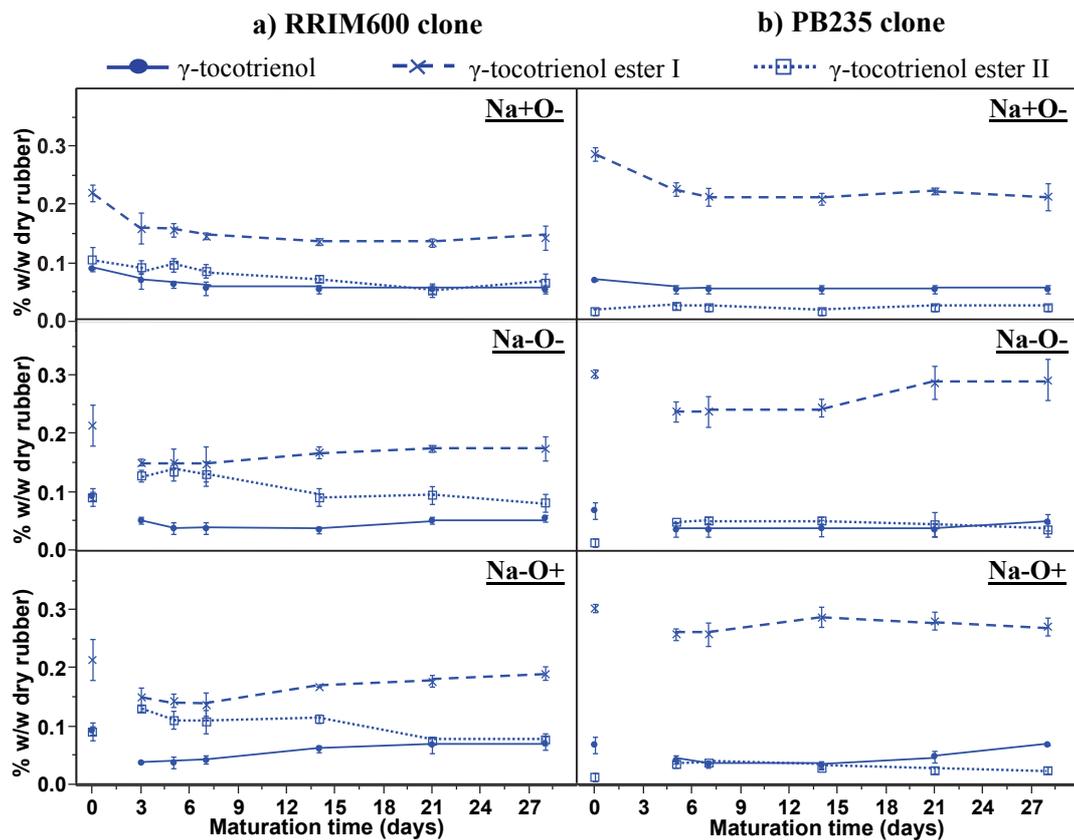
Lipid extracts from RRIM600 and PB235 rubber samples obtained from three maturation conditions were analyzed for their total fatty acids composition using gas chromatography (GC-FID) as described in Materials and Methods. It is to be reminded that the absolute quantities were calculated taking into account determined concentrations in transesterified lipid, transesterification yields and lipid extracts. The results are shown separately for RRIM600 and PB235 clones in Figure 63 and Figure 64, respectively.

For RRIM 600 samples, the initial TFA composition made mainly of C18:2, C18:1 and C18:0 fatty acids did not change much with maturation time when sodium azide was added (Na+O-) (Figure 63a, left), or when the naturally coagulated latex was kept under anaerobic condition (Na-O-) (Figure 63b, left). Contrarily, when the maturation was performed in the presence of oxygen, the TFA relative composition changed dramatically after 7 days of maturation: the unsaturated C18 fatty acid proportion decreased while proportion of saturated C16:0 and C14:0, as well as of furan fatty acid increased. The absolute quantity graph (Figure 63c, right) suggests that the total quantity of TFA decreased significantly from 14 days of maturation and is mainly due to a disappearance of unsaturated FA. This confirms the decrease of intensity of FA spot on TLC (Figure 57).



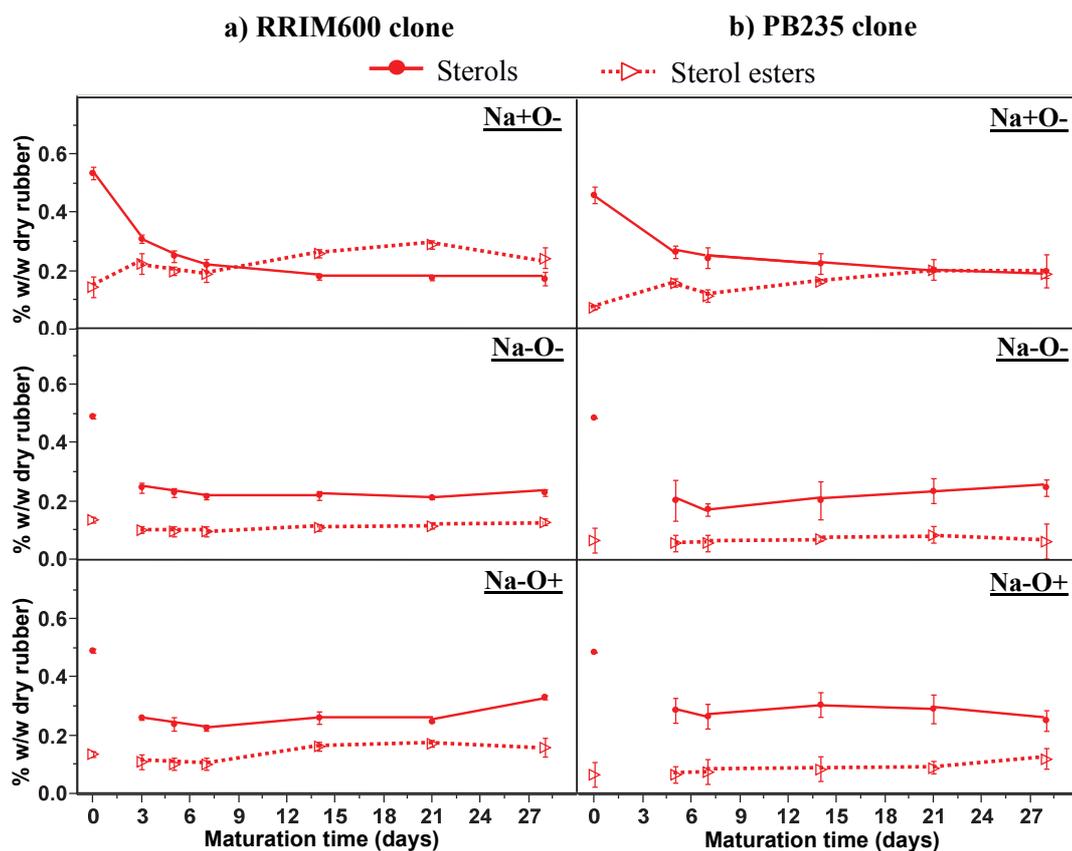
Na+O-: Sodium azide added latex and matured under anaerobic condition.
Na-O+: No sodium azide added latex and matured under aerobic condition.
Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 58 Evolutions of free fatty acids (FFA) and triacylglycerol of furan fatty acid (TGF) (% w/w dry rubber) obtained from (a) RRIM600 and (b) PB235 clones during 28 days of maturation.



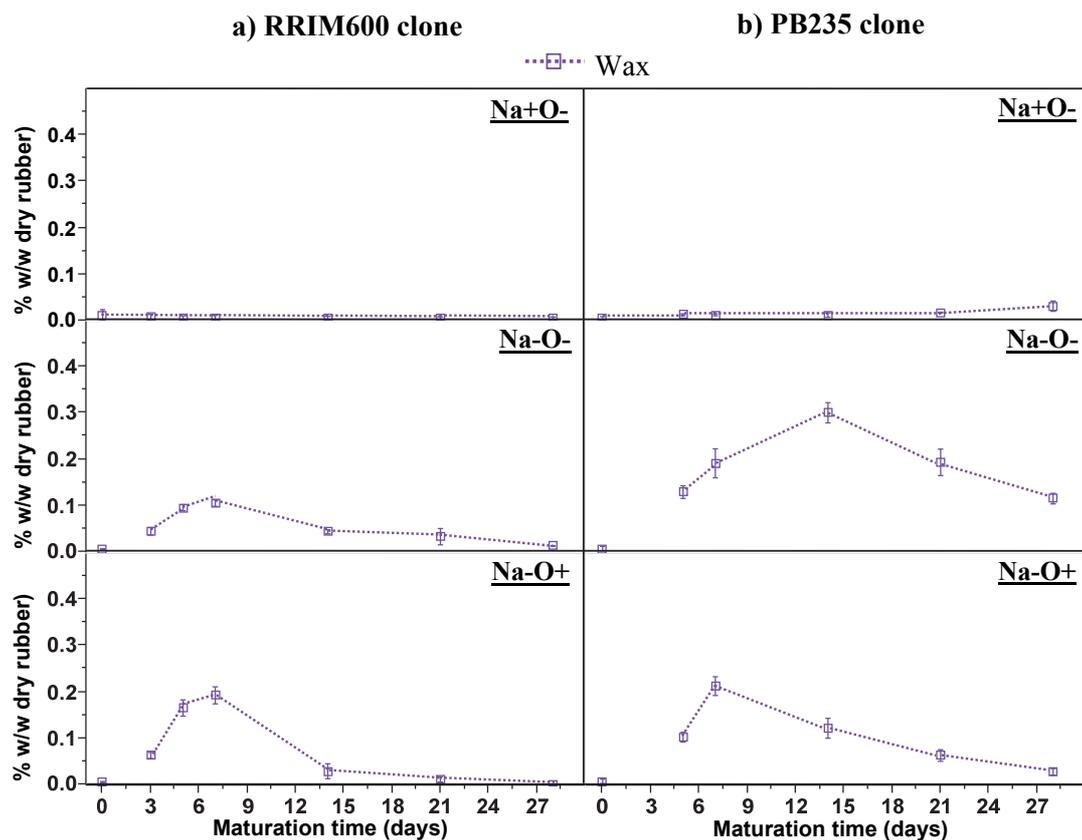
Na+O-: Sodium azide added latex and matured under anaerobic condition.
Na-O+: No sodium azide added latex and matured under aerobic condition.
Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 59 Evolutions of γ -tocotrienol and its esters (% w/w dry rubber) obtained from (a) RRIM600 and (b) PB235 clones during 28 days of maturation.



Na+O-: Sodium azide added latex and matured under anaerobic condition.
 Na-O+: No sodium azide added latex and matured under aerobic condition.
 Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 60 Evolutions of free sterols and sterol esters (SE) (% w/w dry rubber) obtained from (a) RRIM600 and (b) PB235 clones during 28 days of maturation.



Na+O-: Sodium azide added latex and matured under anaerobic condition.
 Na-O+: No sodium azide added latex and matured under aerobic condition.
 Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 61 Evolutions of wax (% w/w dry rubber) obtained from lipid extracts of (a) RRIM600 and (b) PB235 clones during 28 days of maturation.

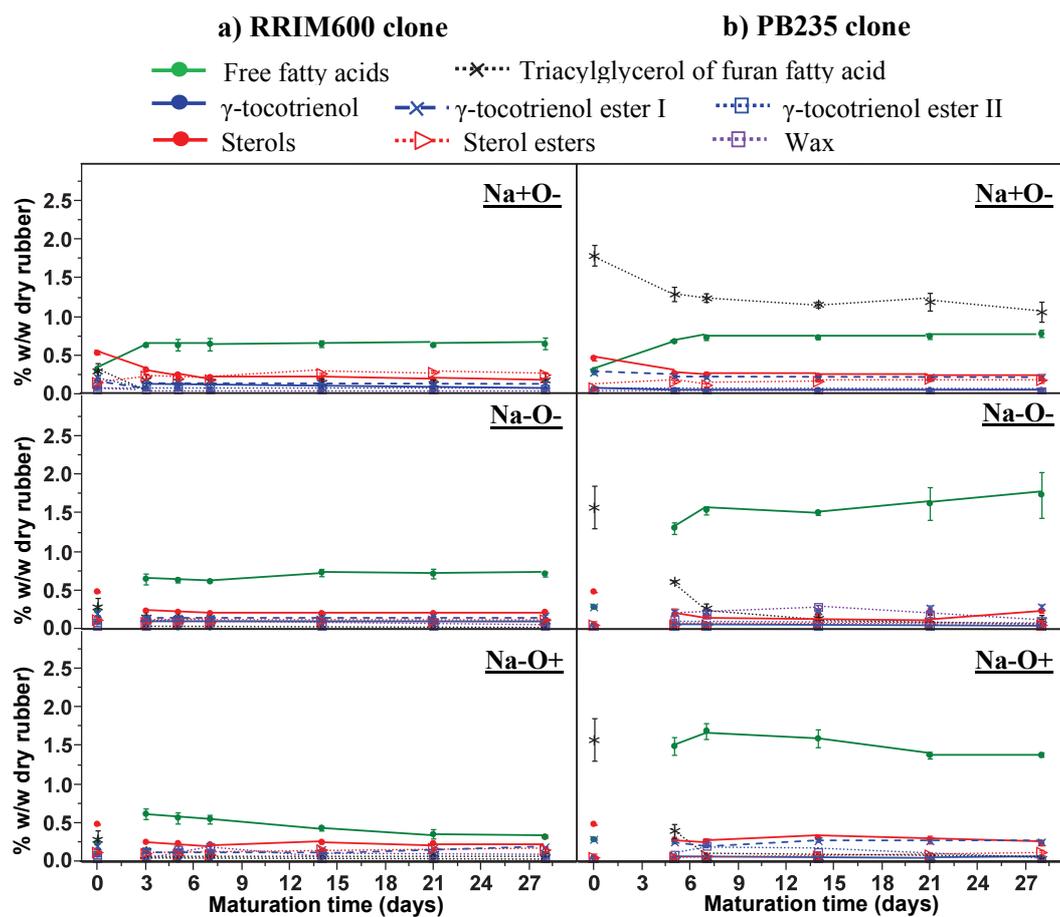


Figure 62 Neutral lipid component evolutions (% w/w dry rubber) obtained from lipid extracts of (a) RRIM600 and (b) PB235 clones during 28 days of maturation.

For PB235 (Figure 64), though the presence of high quantities of furan fatty acid makes the evolution of other species more difficult to observe, same comment as that done for RRIM 600 clone could be made: the composition and quantity of TFA remained constant during maturation time for sodium azide added and anaerobic treatments while change could be observed when the maturation was conducted in presence of oxygen. The unsaturated C18 FA proportion and quantities decreased significantly after 7 days of maturation.

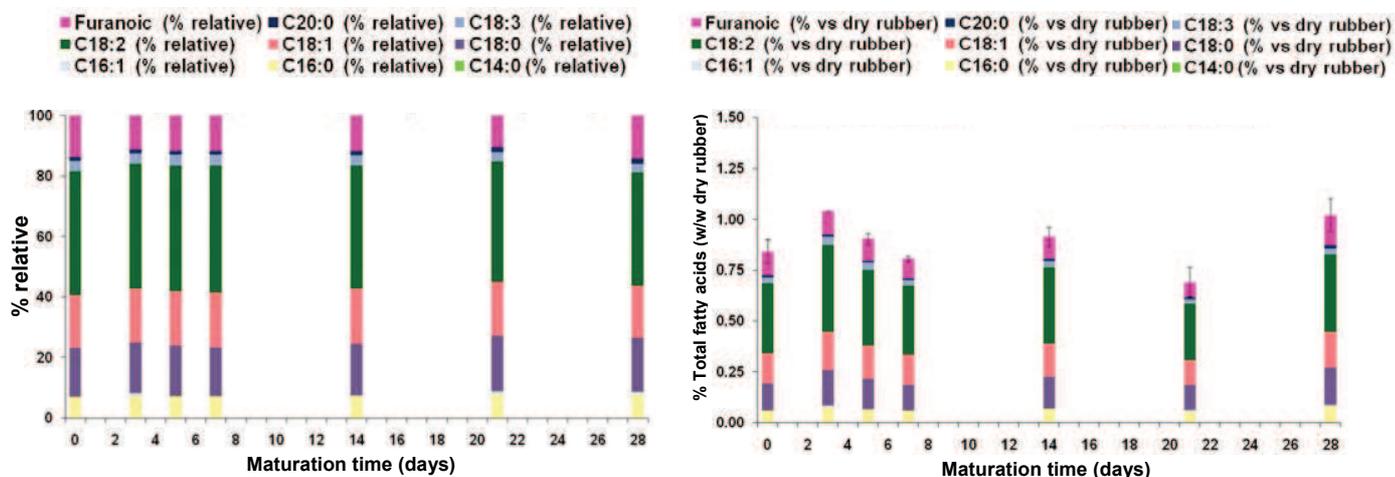
Therefore in the presence of oxygen, it is confirmed that microorganisms are able to perform a catabolic degradation (e.g. FA β -oxidation) that leads to the quantitative disappearance of fatty acids in the lipid extracts. The targeted FA to be degraded are the unsaturated one (i.e. C18:1; C18:2). It is also interesting to note that this disappearance coincide with the elevation of pH to high level (>8) that is only observed in the presence of oxygen. Therefore, fatty acids could participate to the pH of the coagula (together with other organic acids and ammonia). However, care must be taken in the interpretation because this result concerns TFA (total fatty acids) and not FFA (free fatty acids). Finally, this decrease of TFA quantity under aerobic condition fits well with the lipid extract quantity decrease that was discussed previously (§5.1.2).

5.3.3 Unsaponifiable composition

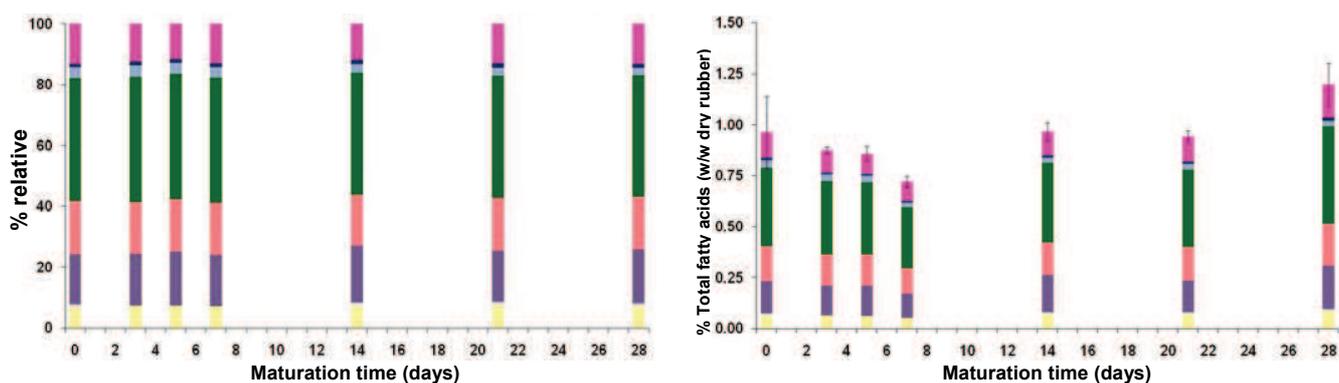
The total unsaponifiable composition of lipid extract from RRIM600 and PB235 rubber samples obtained from 3 maturation conditions were analyzed using gas chromatography equipped with a mass spectrometer (GC-MS). The obtained absolute quantities were calculated in the same way as it was for total fatty acids composition analysis.

The unsaponifiable composition of RRIM600 samples did not change significantly whatever the maturation duration or the treatment as shown in Figure 65. As

a) Na+O-



b) Na-O-



c) Na-O+

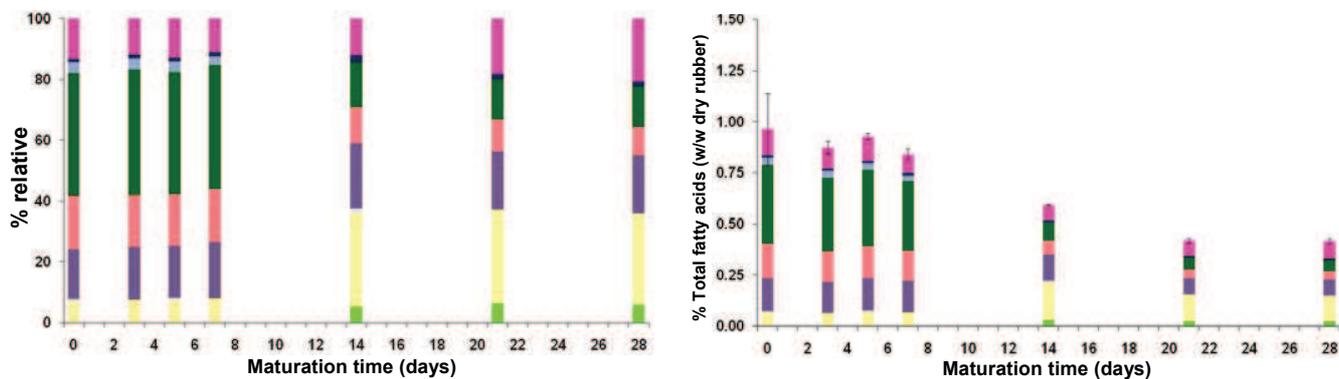
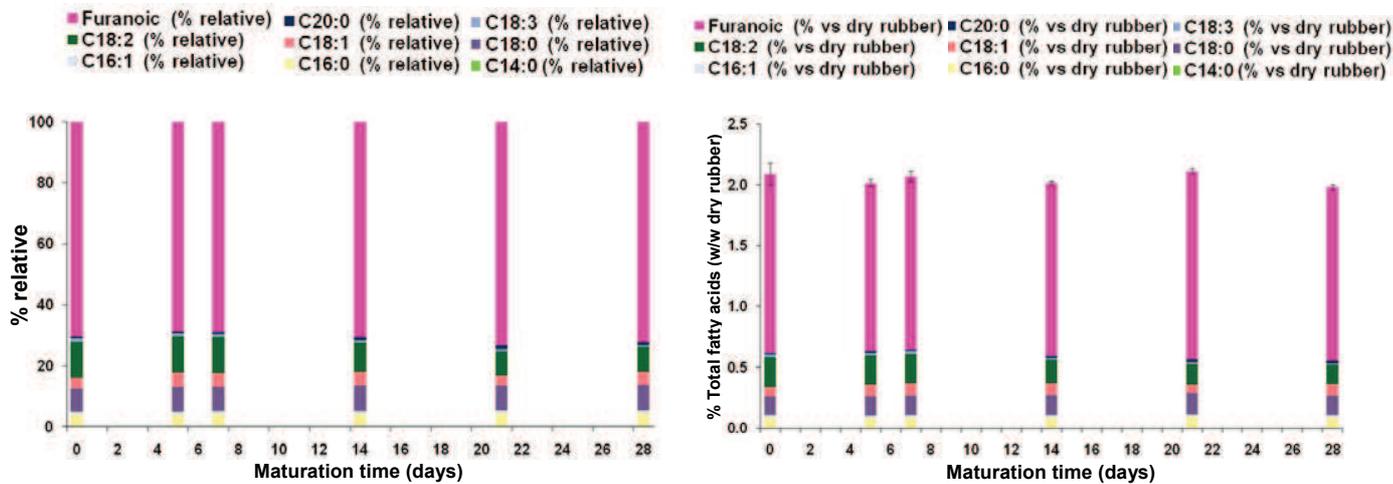
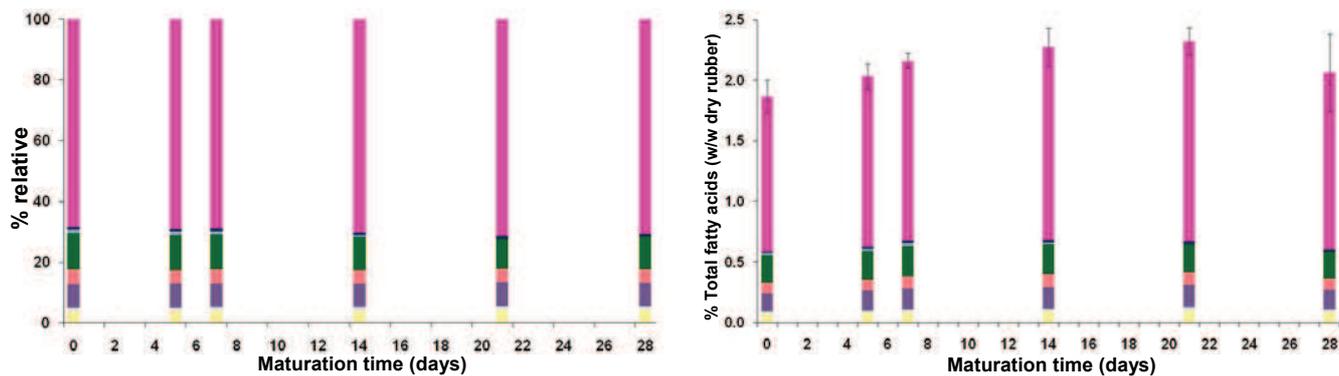


Figure 63 Total fatty acid composition of RRIM600 lipid extracts obtained from (a) Na+O-, (b) Na-O- and (c) Na-O+ maturation conditions during 28 days of maturation.

a) Na+O-



b) Na-O-



c) Na-O+

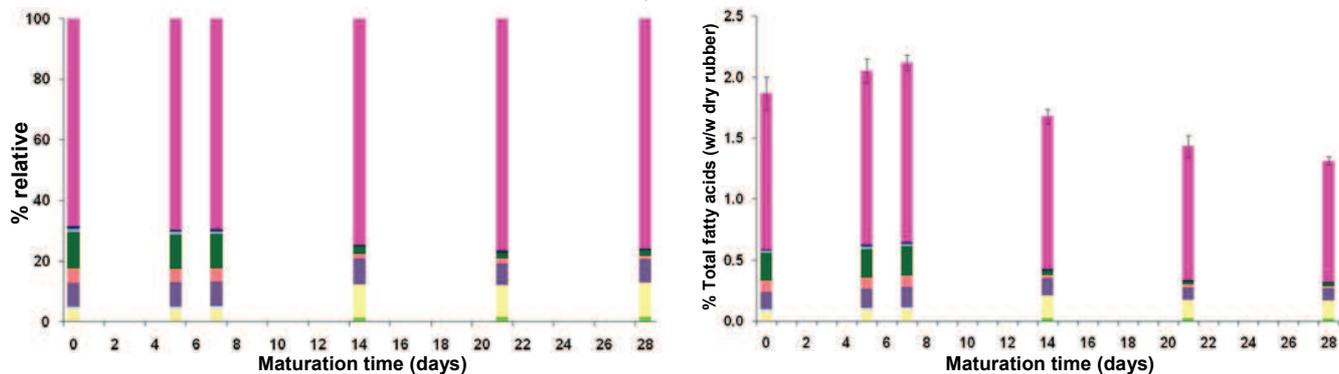


Figure 64 Total fatty acid composition of PB235 lipid extracts obtained from (a) Na+O-, (b) Na-O- and (c) Na-O+ maturation conditions 28 days of maturation.

described previously by Liengprayoon *et al.* (2013) the main unsaponifiable was β -sitosterol (range 0.39-0.65%) followed by γ -tocotrienol (range 0.07-0.18 %). Concerning the absolute quantities, the unsaponifiable amounted for approximately 0.85% of rubber dry weight at day 0 for all treatments. The quantitative evolution during maturation time is difficult to assess with the obtained results as no clear trend can be detected for sodium azide added and anaerobic treatments. However, for aerobic treatment, the total quantity of unsaponifiable seems to decrease after 14 days (down to approximately 0.65%).

Concerning PB235 clone, the results could not be obtained due to methodological difficulties (uncomplete silylation).

6. *In vitro* antioxidant activity test of NR lipid extract by 2,2-di-phenyl-1-picrylhydrazyl (DPPH) method

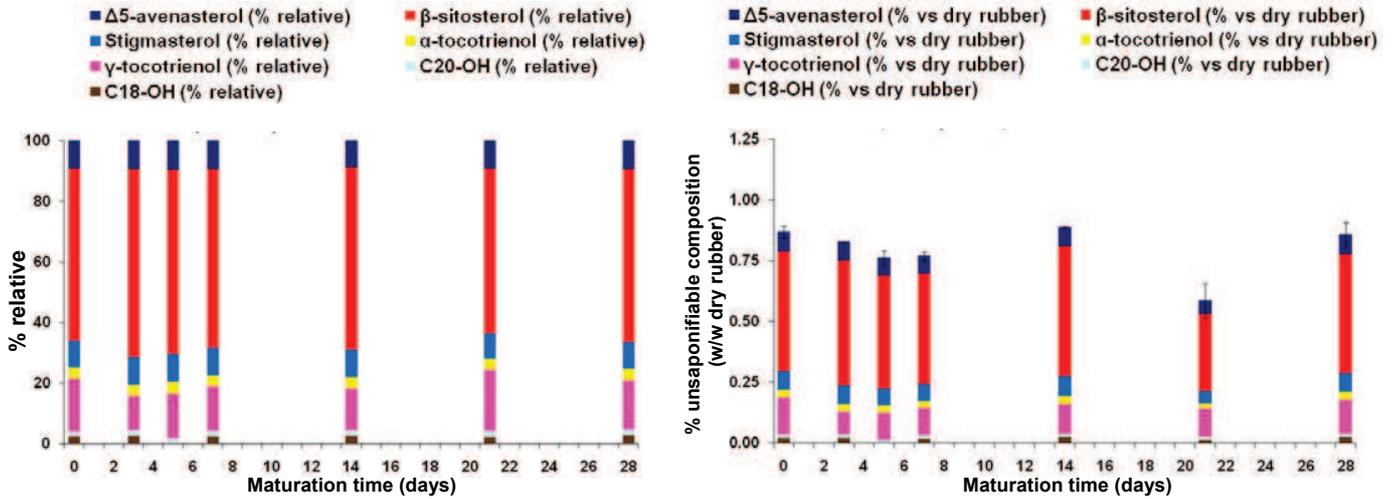
Figure 66 present the antioxidant activity of lipid extract samples obtained from RRIM600 and PB235 clones. As demonstrated previously in Chapter 2 (§5.1), this activity is expressed as the absolute value of the initial slope of the curve %DPPH remaining = f(time). The obtained results are compared with 3 reference slope absolute values obtained from standard solutions of γ -tocotrienol. Moreover, the green number indicates the quantity of native tocotrienol present in the tested lipid extracts as determined by HPTLC analysis.

6.1 Comparison with uncontrolled experiment

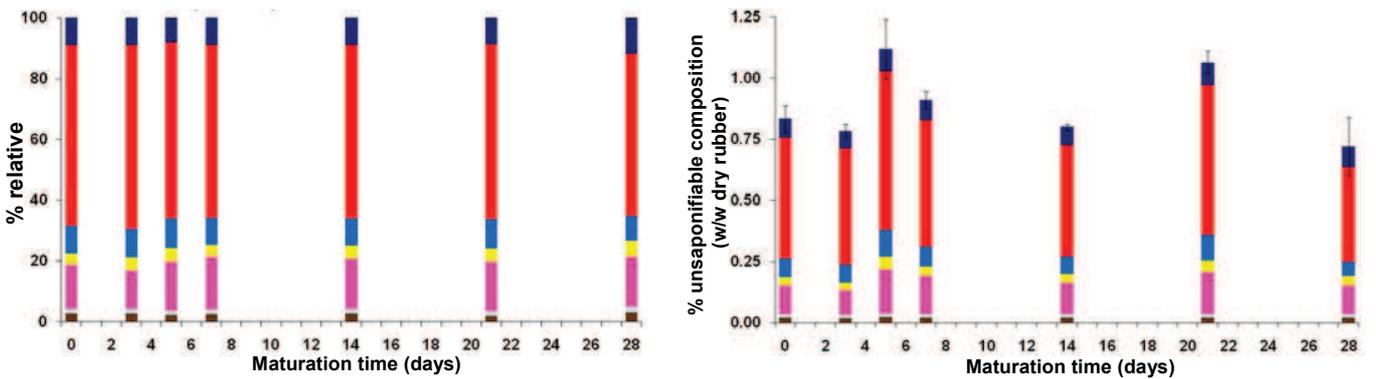
As expected, the variance of the obtained data was much lower when the lipid extracts were obtained from controlled maturation conditions. Whatever the clone and maturation time, the lipid extracts obtained from aerobic controlled condition had higher antioxidant activities than those of lipid extracts obtained from uncontrolled conditions.

As for uncontrolled conditions, it was observed that the RRIM600 clone lipid extract showed a higher antioxidant activity compared with those from PB235 clone.

a) Na+O-



b) Na-O-



c) Na-O+

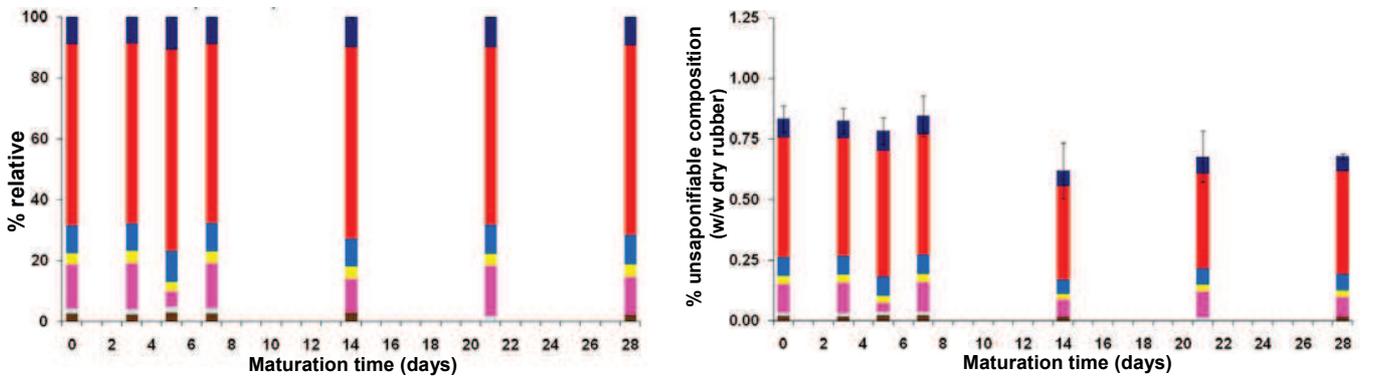


Figure 65 Unsaponifiable composition of RRIM600 lipid extracts obtained from (a) Na+O-, (b) Na-O- and (c) Na-O+ maturation conditions during 28 days of maturation.

6.2 Evolution of lipids extract antioxidant activity under controlled conditions

6.2.1 Control treatment (Na+O-, acid coagulated samples)

During maturation time, the antioxidant activity of lipid extracts of sodium azide added RRIM600 samples increased while that of PB235 lipid extracts seemed not to change. For the latter, it is observed that the tested lipid extracts provided always $20 \mu\text{g.mL}^{-1}$ of native γ -tocotrienol, and that the level of activity remains slightly above the level measured for $20 \mu\text{g.mL}^{-1}$ of γ -tocotrienol standard. It could mean that native γ -tocotrienol makes most of the measured antioxidant activity. However, for RRIM600 the evolution under this treatment is different. Indeed, while the quantity of native γ -tocotrienol provided by the tested lipid extract remained at a level of $30 \mu\text{g.mL}^{-1}$ from day 3 to day 28, the absolute value of the slope was abnormally low compared to standard at day 3 and increased significantly until day 7. In this case, it seems that the antioxidant activity of the extract is not driven by γ -tocotrienol alone. Other components that change in quantity during the initial step of maturation under this sodium azide added treatment may also act as pro-oxidants and antagonists of γ -tocotrienol antioxidant activity. Indeed it is well known that the antioxidant activity of a mixture differs from the activity of the individual components (Choe and Min, 2009 ; Wang *et al.*, 2012). In this case, it could be noted that the quantity of free sterols decreased significantly in the early stage of maturation (see Figure 60). However this decrease was also observed for PB235 lipid extract.

6.2.2 Anaerobic maturation condition (Na-O-, natural coagulation day 3-28)

The lipid extract from RRIM60 sample matured under anaerobic condition tended to show an increasing trend of antioxidant activity during maturation time after an initial decrease until day 5. However those variations are not significant.

Concerning PB235 extract, and except for day 21, the level of antioxidant activity stayed constant at an equivalent of $20 \mu\text{g.mL}^{-1}$ γ -tocotrienol standard while γ -tocotrienol provided by the tested lipid extract did not exceed $10 \mu\text{g.mL}^{-1}$. It means that

another components acted as an additional antioxidant or had a synergetic effect on γ -tocotrienol antioxidant activity.

6.2.3 Aerobic maturation condition (Na-O+, natural coagulation day 3-28)

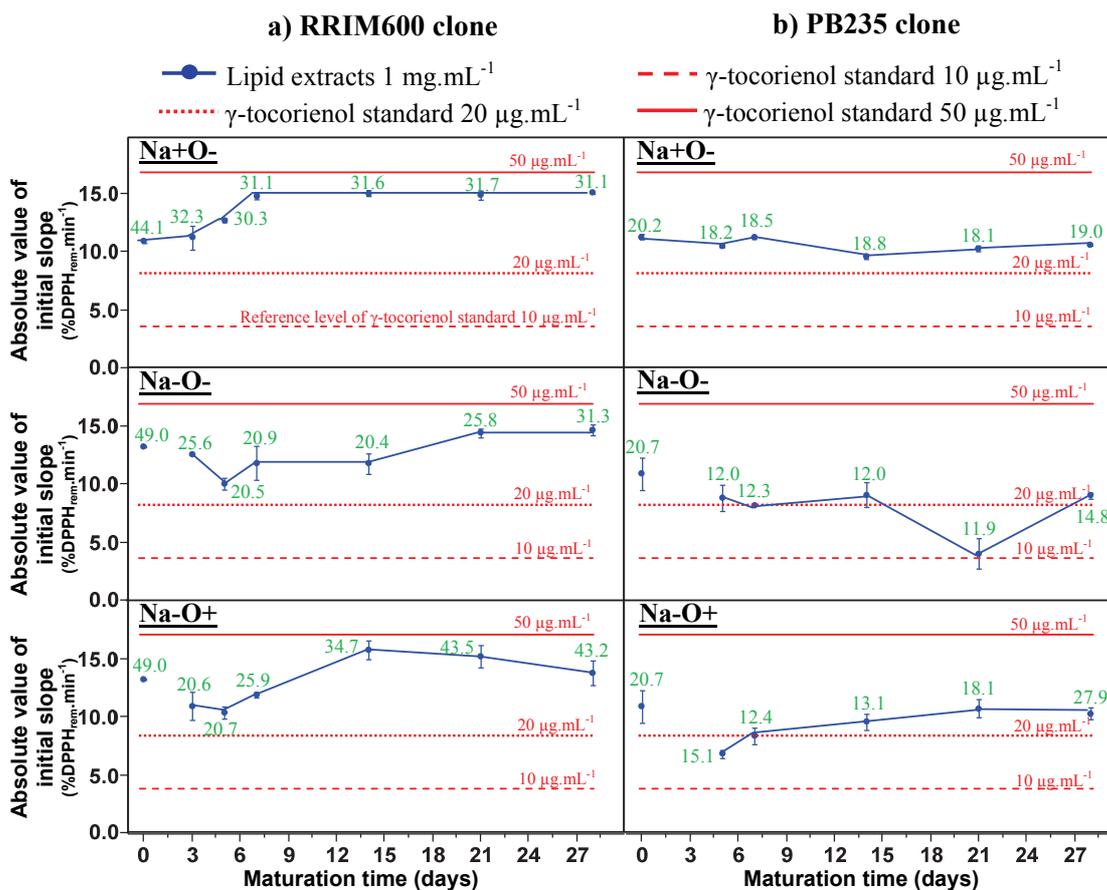
For both clones, and whatever maturation time, the evolution of antioxidant activity of lipid extracts that were observed in absence of oxygen (Na-O-) are similar in the presence of oxygen (Na-O+). However it noted that the real content of free γ -tocotrienol in the tested lipid extracts were higher under aerobic conditions.

7. Conclusion

Under controlled maturation conditions followed by a TSR like process, the evolution of every followed indicators (bulk properties, mesostructure and lipids components) were studied and the variance of obtained data was much lower than that of the data obtained from uncontrolled conditions (USS/RSS like process). The effect of presence of microorganisms on NR structure and properties was studied by comparing control treatment where the growth of most of microorganisms in latex was inhibited by a biocide addition (sodium azide, Na+) and the other two other treatments conducted under anaerobic and aerobic conditions. Generally, higher evolutions of the followed indicators were obtained on non-biocide added treatments especially under aerobic maturation condition.

pH values of coagula increased during maturation, and the maximum increase was under aerobic condition. The water content of the fresh crepe ready to be dried increased with maturation. Initial plasticity (P_0) and gel content increased at the beginning of the maturation especially for RRIM600 clone which contains a higher amount of short chain of polyisoprene. P_{30} values tended to decrease under aerobic maturation condition. This decrease was absent when the microorganism was inhibited. Lipid quantity and quality evolved during maturation. Those evolutions were maximal under aerobic condition. In these conditions, the total quantity of lipid extract decreased, the free FA content increased at early stage of maturation, later on the TFA content decreased (the unsaturated FA being the first to disappear), the unsaponifiable quantity decreased at a lesser extent. Concerning extractable

free γ -tocotrienol, it was found that their quantity did not change much during maturation, while their esterified form get enriched in saturated fatty acid during maturation. The antioxidant activity measured by DPPH test correlated well with free γ -tocotrienol concentration but not with the resistance of rubber to thermo-oxidation assessed by P₃₀ or PRI.



Na+O-: Sodium azide added latex and matured under anaerobic condition.
 Na-O+: No sodium azide added latex and matured under aerobic condition.
 Na-O-: No sodium azide added latex and matured under anaerobic condition.

Figure 66 Absolute value of initial slope obtained from (a) RRIM600 and (b) PB235 lipid extracts. The numbers in green are the free γ -tocotrienol content in the lipid solution (μ g.mL⁻¹) calculated from HPTLC results.

GENERAL CONCLUSION

This present work aimed at better understanding the effects of NR native antioxidants on the physical properties of rubber, especially against oxidative degradation. To do so, the evolution of native antioxidants during natural rubber maturation was followed. Indeed, maturation is known in industrial practice to alter the physical properties of NR. This evolution was followed along two maturation conditions (USS/RSS like and TSR like conditions) using different lipid characterization techniques including HPTLC, GC-FID/MS and HPLC-MS, as well as by *in vitro* antioxidant activity assessment (DPPH test). In parallel, the change of physical properties was followed the determination of standard parameters (P_0 , P_{30} and PRI). Moreover, due to the inter relationships between structure and properties, mesostructure characteristics (MMD, M_w , M_n and gel content) of matured NR samples were also studied.

Firstly, rubber samples were prepared following a USS/RSS-like process. All parameters concerning sample preparation had to be developed in order to obtain degraded NR samples with a wide range of physical properties that could allow the study of correlations with the evolution of antioxidants and/or lipids molecules. Though following industrial USS/RSS like conditions, samples were prepared in a mini-sheet form using 500 mL latex instead of 3 L as recommended by RRIT. Minimizing the latex quantity allowed to study more parameters and to cope with all physical and chemical analyses. The optimum protocol to prepare sheet samples corresponded to a dilution of 500 mL of latex with 333 mL of water and a coagulation either with formic acid (control) or by natural coagulation (maturation). A maturation period of 14 days was found to be sufficient to generate different physical properties. Both control and matured samples were dried under outdoor air and smoke drying conditions. It was found that smoke drying allowed the full sample drying even if a longer time was required for matured samples. Concerning physical properties, smoke drying improved P_{30} and therefore PRI and moreover, less variation of data was obtained compared to outdoor air drying condition. Besides the optimization of sample preparation, a lipid analysis technique to follow the change of composition of matured samples was also developed. HPTLC was chosen as it could provide both qualitative and

quantitative results. With two different mobile phases, hexane/diethyl ether/acetic acid at the ratio of 90:10:1 (v/v/v) and 80:20:1 (v/v/v), both non-polars (wax and sterol esters) and more polar lipids (free sterols, free fatty acids, free γ -tocotrienol and TGF) could be well separated. Indeed, repeatability and reproducibility measurements confirmed the effectiveness of this analysis method including its high throughput. It was thus chosen for rapid lipid qualification and quantification. In addition, an HPLC-MS analysis method was specifically developed to analyze antioxidant molecules, γ -tocotrienol and its derivatives. In the case of antioxidant activity assays, different solvents for DPPH and lipid solubilization were also tested since methanol, the conventional solvent, could not completely solubilize lipid samples. Chloroform/ethanol (1:1, v/v) was found to be the most suitable solvent mixture for both DPPH and lipids. Moreover, to reduce the analysis time and sample size compared to conventional spectrophotometry, instead of using cuvettes the DPPH assay was performed in microplates.

Accordingly, samples from RRIM600 and PB235 clones along 14 days of maturation were prepared and analyzed under these optimized conditions. At the initial state of coagulation, pH of both coagula and sera from two samples followed the general tendency previously reported by displaying a decrease of pH from 6.5 to 4.9 during the first 24 h reported to be due to carbohydrate fermentation, followed by an increase up to 9 which could be due to protein degradation by microbial activity. No obvious clonal difference was observed during the coagulation and laminated sheet preparation steps besides a higher water content in PB235 laminated sheets (30%) than in that of RRIM600 (20%) in the latest days of maturation. Finally, though smoke drying was found to be more effective, it appeared that under outdoor air drying interesting results were evidenced. For bulk properties, P_0 of outdoor air and smoke dried samples from RRIM600 clone decreased similarly during 9 days of maturation. However, P_0 after 14 days of maturation could not be determined because of an excessive degradation of rubber after the long drying time taken for outdoor drying. Meanwhile, when using smoke drying, in which sample was dried in a shorter period, a significant decrease of P_0 and PRI could be observed. This implied that higher degradation occurred in outdoor air drying than in smoke drying. The bulk properties of rubber samples from PB235 clone showed a similar evolution as that from RRIM600 concerning P_0 and PRI.

What differentiated both clones in terms of bulk properties were the initial values of P_0 and their evolution along maturation time. Indeed, P_0 and PRI of PB235 samples during maturation varied less compared to RRIM600 clone. PB235 is a unimodal clone the rubber of which contains much less short polyisoprene chains than that of RRIM600 clone. Higher molar masses in PB235 clone rubber explain the higher initial value of P_0 , while the presence of short chains in RRIM600 clone rubber explained the apparent crosslinking phenomenon. Concerning the evolution of lipid extract composition, lipid content was not found to be dependent on maturation. Nevertheless, smoke dried samples tended to have a higher lipid content compared to outdoor dried samples. This could be due to the shorter drying duration of smoke drying that inhibited further degradation while the longer time as well as the higher water activity in outdoor air drying allowed microbial degradation. For the composition, lipids seemed to evolve faster under outdoor air drying. Esterification and hydrolysis seemed to occur concomitantly as observed from the increase and decrease of certain esterified lipids at different stages of maturation. Globally, the composition of lipids from the two clones changed similarly along maturation, with only differences in the type of lipids according to clonal origin. To relate the changes of bulk properties and lipids with antioxidant activity, lipids extracts from both RRIM600 and PB235 matured samples were measured for their antioxidant activity using the DPPH test. The results indicated that obtained lipid extract contained component(s) that can scavenge the DPPH free stable radical. In addition, at the same concentration, the antioxidant activity of lipids from clone PB235 was lower than that of the extracts from RRIM 600 whatever the maturation time or the drying condition. Though a positive result was observed from DPPH, a clear evolution with time was not obtained, which could possibly be due to the variation of the lipid components. Though interesting tendencies were observed in terms of physical properties of rubber, lipid composition and antioxidant activity of the lipid extracts, the variance of the obtained data made the interpretation of results very delicate. Therefore, it was decided to change the experimental strategy and to perform another trial under strictly controlled maturation conditions followed by a TSR like processing.

In this trial, latex from two *Hevea* clones -RRIM600 and PB235- were coagulated and matured under 3 controlled conditions - control (sodium azide added, no oxygen, acid coagulation), anaerobic (no sodium azide added, no oxygen) and aerobic (no sodium azide

added, oxygen). From the obtained dry NR, the evolutions of indicators from three study levels - bulk properties, mesostructure and lipid composition - were followed along maturation time. For all measured indicators, a lower variance of the value was obtained as expected with the controlled samples compared to those obtained in uncontrolled maturation conditions.

A marked clonal character was observed in the indicator values from every study levels during 28 days of maturation. It confirmed that having contrasted material help to interpret obtained results. Indeed previous maturation studies (Intapun, 2009 ; Intapun *et al.*, 2010) dealt only with RRIM600 clones.

Concerning pH, after a decrease from the initial value (pH of fresh latex) to the lowest one at 3 days of maturation, pH increased with maturation time for both clones. The higher increase of pH was observed when the coagula were matured under aerobic condition whatever the clone. This could be linked with the protein degradation (NH₃ release) and/or organic acids disappearance by aerobic microorganism metabolism. Moreover it was observed that pH values of PB235 coagula after 3 days of maturation were lower than that of RRIM600. This could be explained by a higher free fatty acids content in PB235 clone. Indeed, these free fatty acids (mainly furan fatty acid) are released by native *Hevea* enzymes during early stage of maturation.

The dry matter of laminated coagula was estimated by comparing the weight of fresh and dry laminated coagula. The results showed that under anaerobic conditions and for both Na⁺ and Na⁻ samples, the water content of the crepes seemed not to depend on the maturation time from two clones. Meanwhile, under aerobic maturation conditions (Na-O⁺), the water content increased significantly with time. This could be linked with microbial activity, especially from aerobic microorganisms, which is known to be correlated with water retention in the coagula (Intapun *et al.*, 2010).

Concerning the evolution of physical properties, when the microbial activities were inhibited the properties (P₀, P₃₀ and PRI) did not change much during time whatever the clone. When microorganism activities were not inhibited, especially under aerobic condition,

a higher and faster drop of P_{30} was observed and resulted in a decrease of PRI for both clones. This is most probably due to a higher degradation level of native antioxidants because of the presence of oxygen. A different evolution of physical properties between two clones was observed. Whatever maturation condition, P_0 value of RRIM600 samples increased during the first few days of maturation while for PB235, P_0 remained constant over time. This could be explained by the fact that these two rubber clones have different molar mass distributions (unimodal for PB235 and bimodal for RRIM600 clones) and that the short polyisoprene chains are reported to be involved in rubber reticulation (formation of gel, increase of P_0). Concerning P_{30} and PRI, it was observed that the value of these properties in samples obtained from PB235 were lower than that from RRIM600 rubber whatever the condition and time.

Concerning mesostructural analysis, the total gel content of both clones was found to increase during during first few days when the microorganism activities were inhibited. Therefore gel formation could be due to a phenomenon occurring after coagulation and not directly correlated to microbial activity. When the microbial activities were not inhibited, the evolution of gel remained similar as with the sodium azide added treatment. This evolution of gel was well correlated with the evolution of P_0 values during time.

When microorganism activities were inhibited, M_w and M_n , for RRIM600 clone increased significantly in the first days of maturation while when microorganism activities were not inhibited, M_w and M_n decreased during maturation. Microorganisms could therefore contribute to the chain scission phenomena during maturation. In the case of PB235 clone, M_w and M_n did not change significantly during maturation whatever the treatment though a non significant decreasing trend was observed for anaerobic treatment.

From this study, whatever maturation condition and time the lipid extract quantity of samples from PB235, which was classified as a high lipids containing rubber clone (Liengprayoon *et al.*, 2013) was higher than that issuing from RRIM600 clone. Among maturation conditions, it was observed that the lipids content decreased significantly in the presence of oxygen for samples from both clones. For RRIM600, the lipid content decreased from 1.94% (day3) to 1.61% (day 28) and for PB235 clone, decreased from 3.31% (day 3) to

2.51% (day 28). Therefore a major factor of NR lipids degradation could be linked to aerobic microorganism activity and the presence of oxygen during maturation.

Neutral lipid evolution during 28 days of maturation was characterized both in quality and quantity. Concerning neutral lipids quality, analyzed using TLC, the main observed change during maturation concerned free fatty acids and TGF contents for both clones. The hydrolysis of TGF to furan fatty acids was found to be the fastest under aerobic conditions. HPTLC analyses of neutral lipids showed that the higher evolution of the amount of each component during maturation was observed when the microorganism activities were not inhibited. Gamma-tocotrienol and its derivatives were especially targeted during this study because of their known antioxidant activity. They were found to be present in free or esterified forms in rubber. Two γ -tocotrienol esters were identified: ester I, rich in saturated fatty acids and ester II, rich in unsaturated fatty acid. Samples from PB235 clone had a lower γ -tocotrienol content than that from RRIM600. This could explain the lower P₃₀ value of PB235 rubber. A different evolution of γ -tocotrienol and its esters was observed between three treatments. When microorganism activities were inhibited, in the case of RRIM600 sample, the abundance of γ -tocotrienol and its esters seemed to decrease during the first 3 days and stayed constant after that until 28 days. Meanwhile, for PB235, the concentration of γ -tocotrienol ester I showed a slight decrease during first 5 days. When microorganism activities were not inhibited, a slight increase of free γ -tocotrienol content after 14 days and a symmetrical evolution of γ -tocotrienol esters I and II were observed whatever the clone.

Regarding total fatty acid composition (TFA), linoleic acid (C18:2) was the major fatty acid found in RRIM600 (40% w/w total fatty acids at day 0) while PB235 clone contained mainly furan fatty acids (70% w/w total fatty acids at day 0). During maturation, the composition and quantities of TFA remained constant under anaerobic condition from both clones. Meanwhile, when the maturation was performed in the presence of oxygen, the composition and quantities of TFA decreased significantly due to the disappearance of unsaturated FA (especially C18:2).

For unsaponifiable composition, β -sitosterol was the main unsaponifiable followed by γ -tocotrienol whatever the clone, maturation condition and time. For RRIM600 samples no clear evolution of the quantity of unsaponifiable was observed under anaerobic condition while it seemed to decrease during time under aerobic condition (from 0.85% (day 3) to 0.65% (day 28) w/w dry rubber). Unfortunately, in the case of PB235 clone the results could not be obtained due to methodological difficulties (uncomplete silylation).

Antioxidant activity of lipid extract was assessed by the optimized DPPH method. The obtained results correlated well with free γ -tocotrienol content. The presence of this molecule explains most of the antioxidant activity of the extract but not all. It can be assumed that other molecules in the extract have antagonist or synergetic effect on this activity. RRIM600 lipid extracts showed a higher antioxidant activity than that from PB235 whatever the maturation condition and time. This could be linked to the known lower resistance to thermo-oxidation of the rubber from PB235 clone. However, the evolution of antioxidant activity along maturation time stayed stable (PB235) or even increased (RRIM600) while P_{30} and PRI values decreased. Therefore, no direct link was evidenced between the antioxidant activity of the lipids extracted from rubber and the ability of the material to resist to thermo-oxidation. Indeed, the *in vitro* conditions of measurement were far from those occurring inside solid state rubber. Moreover, the localization of antioxidants in rubber and especially their physical possibility to interact with the double bond of polyisoprene should be investigated to understand more what drives the drop of P_{30} along maturation time. In addition, the lipid analyses were conducted on extractable molecules only. Non extractable lipids or other molecules belonging to more polar non-isoprene species (proteins, polyphenols, etc...) could also influence the resistance to thermo-oxidation.

It is worth mentioning that all the informations from characterized lipids, especially the potential antioxidant molecules as well as structure and properties, allowed us to understand better their relationships. However, the possible synergistic effect of lipids during maturation would be worth studying further. Developing a method to determine the oxidation-antioxidation reactions with other approaches closer to the *in vivo* condition would also be useful.

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APPENDICES

APPENDIX A

UNCONTROLLED CONDITIONS

(USS/RSS Like process)

Table 1 Fresh and dry weights (g) and water content (%) of laminated sheet samples obtained from RRIM600 and PB235 rubber clones during 14 days of maturation

Maturation time (days)	RRIM600 Clone						PB235 Clone					
	Fresh weight (g)		Dry weight (g)		Water content (%)		Fresh weight (g)		Dry weight (g)		Water content (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	216.8 ^d	5.43	191.4 ^{ab}	1.10	12.7 ^c	1.42	228.2 ^d	3.10	204.3 ^a	2.16	11.4 ^d	0.23
1	-	-	-	-	-	-	249.3 ^b	3.02	201.9 ^a	4.61	19.0 ^b	0.89
2	227.8 ^d	5.00	185.0 ^b	2.72	19.4 ^c	0.34	238.4 ^c	2.28	202.9 ^a	2.58	14.3 ^c	0.07
3	225.8 ^d	7.53	187.3 ^b	2.19	16.1 ^d	1.05	239.6 ^c	0.49	202.6 ^a	0.83	15.4 ^c	0.24
7	248.2 ^c	1.66	186.1 ^b	2.59	25.0 ^b	0.56	248.5 ^b	4.71	194.7 ^a	9.19	19.6 ^b	0.95
9	262.3 ^b	1.53	186.9 ^b	4.71	28.0 ^a	0.74	255.3 ^{ab}	0.58	196.6 ^a	5.34	24.3 ^a	0.06
14	279.0 ^a	4.74	197.8 ^a	3.22	29.1 ^a	0.46	259.9 ^a	1.62	199.3 ^a	1.92	23.3 ^a	0.65

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P < 0.05$). SD: standard deviation.

Table 2 Physical properties of RRIM600 matured rubbers obtained from outdoor air and smoke drying during 14 days of maturation

Maturation time (days)	Outdoor air drying						Smoke drying					
	P ₀ (unit)		P ₃₀ (unit)		PRI (%)		P ₀ (unit)		P ₃₀ (unit)		PRI (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	41.8 ^c	0.76	34.5 ^a	0.50	82.5 ^a	2.48	38.2 ^c	0.29	39.0 ^{abc}	0.50	102.2 ^a	0.75
2	47.7 ^{bc}	0.58	28.2 ^a	3.75	54.7 ^b	5.16	51.7 ^b	1.53	48.0 ^{ab}	1.00	93.0 ^{ab}	4.33
3	41.8 ^c	3.18	36.6 ^a	3.45	82.1 ^a	3.43	54.5 ^b	1.32	48.7 ^a	0.76	89.4 ^{ab}	3.21
7	60.0 ^a	2.83	12.0 ^b	4.36	15.1 ^c	3.04	58.2 ^a	0.58	37.8 ^{abc}	0.35	65.1 ^{bc}	1.41
9	56.0 ^{ab}	5.65	31.0 ^a	0.00	61.9 ^{ab}	14.4	54.5 ^b	1.32	27.7 ^c	3.88	50.9 ^c	4.03
14	-	-	-	-	-	-	51.8 ^b	1.76	35.0 ^{bc}	12.2	69.0 ^{bc}	25.5

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P < 0.05$). SD: standard deviation.

Table 3 Physical properties of PB235 matured rubbers obtained from outdoor air and smoke drying during 14 days of maturation

Maturation time (days)	Outdoor air drying						Smoke drying					
	P ₀ (unit)		P ₃₀ (unit)		PRI (%)		P ₀ (unit)		P ₃₀ (unit)		PRI (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	51.3 ^a	0.29	35.2 ^b	1.61	68.5 ^b	2.86	49.3 ^b	1.52	41.8 ^{bc}	0.76	84.8 ^a	1.31
1	49.0 ^a	0.87	42.0 ^a	1.41	84.9 ^a	2.90	55.5 ^a	2.29	48.8 ^a	0.76	88.1 ^a	4.60
2	50.5 ^a	0.50	33.8 ^b	2.02	68.8 ^b	2.55	53.5 ^{ab}	0.50	46.3 ^a	1.04	86.6 ^a	1.22
3	46.2 ^a	5.00	35.8 ^b	0.35	73.0 ^b	1.41	54.7 ^{ab}	1.61	45.8 ^{ab}	0.28	85.3 ^a	1.06
7	20.3 ^b	0.35	-	-	-	-	54.3 ^{ab}	2.89	39.0 ^c	3.00	75.8 ^b	1.06
9	-	-	-	-	-	-	55.7 ^a	2.02	39.2 ^c	0.29	72.1 ^b	0.64
14	-	-	-	-	-	-	52.0 ^{ab}	2.50	16.3 ^d	0.58	32.5 ^c	0.28

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P \leq 0.05$). SD: standard deviation.

Table 4 Lipid extracts (%w/w dry rubber) of RRIM600 and PB235 matured sheets obtained from outdoor air and smoke drying during 14 days of maturation

Maturation days	RRIM600 clone				PB235 clone			
	Outdoor air drying		Smoke drying		Outdoor air drying		Smoke drying	
	Mean	<i>SD</i>	Mean	<i>SD</i>	Mean	<i>SD</i>	Mean	<i>SD</i>
0	1.92 ^a	0.04	2.01 ^b	0.02	3.41 ^a	0.02	3.53 ^a	0.10
1	-	-	-	-	3.01 ^{ab}	0.10	3.64 ^a	0.30
2	2.10 ^a	0.36	2.04 ^b	0.02	3.00 ^{ab}	0.25	3.66 ^a	0.21
3	1.75 ^a	0.01	2.04 ^b	0.02	2.99 ^{ab}	0.06	3.46 ^a	0.19
7	1.93 ^a	0.11	2.15 ^a	0.10	2.65 ^{bc}	0.03	2.98 ^a	0.89
9	2.10 ^a	0.06	2.05 ^b	0.04	3.30 ^a	0.26	3.44 ^a	0.03
14	1.84 ^a	0.04	1.98 ^b	0.06	2.42 ^c	0.01	3.22 ^a	0.05

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P < 0.05$). *SD*: standard deviation.

Table 5 Neutral lipid composition (% w/w dry rubber) of RRIM600 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation

Drying methods	Days	Neutral lipid composition													
		Sterols		Free fatty acids		TGF		γ -tocotrienol		γ -tocotrienol ester I		γ -tocotrienol ester II		Sterol esters	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Outdoor air drying	0	0.08 ^a	0.04	0.89 ^a	0.04	N/D	-	0.12 ^a	-	0.07 ^b	0.01	N/D	-	0.39 ^a	0.03
	2	0.12 ^a	0.05	0.68 ^a	0.01	N/D	-	0.12 ^a	0.06	0.16 ^a	0.02	0.06 ^a	-	0.24 ^a	0.13
	3	0.08 ^a	0.00	0.73 ^a	0.02	N/D	-	0.11 ^a	0.00	0.17 ^a	0.02	0.10 ^a	-	0.33 ^a	0.01
	7	0.43 ^a	0.01	1.07 ^a	0.25	N/D	-	0.06 ^a	0.04	0.17 ^a	0.02	0.04 ^a	0.04	0.31 ^a	0.08
	9	0.52 ^a	0.01	0.96 ^a	0.04	N/D	-	0.03 ^a	-	0.13 ^{ab}	0.01	0.06 ^a	-	0.18 ^a	0.01
	14	0.49 ^a	0.30	0.76 ^a	0.08	N/D	-	N/D	-	0.15 ^a	0.04	0.04 ^a	-	0.17 ^a	-
Smoke drying	0	0.07 ^b	0.01	0.98 ^a	0.01	N/D	-	0.09 ^a	-	0.12 ^a	0.02	0.01 ^a	-	0.32 ^a	0.04
	2	0.25 ^a	0.01	0.83 ^b	0.04	N/D	-	0.02 ^a	-	0.14 ^a	0.04	0.07 ^a	-	0.28 ^a	0.09
	3	0.21 ^{aβ}	0.01	1.06 ^a	0.06	N/D	-	0.04 ^a	-	0.14 ^a	0.11	0.07 ^a	0.03	0.28 ^a	0.01
	7	0.30 ^a	0.08	0.78 ^{βγ}	0.04	N/D	-	0.09 ^a	0.08	0.26 ^a	0.01	0.08 ^a	0.01	0.13 ^a	0.03
	9	0.31 ^a	0.05	0.70 ^{βγ}	0.03	N/D	-	0.04 ^a	0.02	0.24 ^a	0.01	0.09 ^a	0.01	0.25 ^a	0.06
	14	0.32 ^a	0.03	0.65 ^γ	0.02	N/D	-	0.03 ^a	0.02	0.24 ^a	0.03	0.08 ^a	0.00	0.23 ^a	0.04

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 6 Neutral lipid composition (% w/w dry rubber) of PB235 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation

Drying methods	Days	Neutral lipid composition													
		Sterols		Free fatty acids		TGF		γ -tocotrienol		γ -tocotrienol ester I		γ -tocotrienol ester II		Sterol esters	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Outdoor air drying	0	0.10 ^d	0.10	1.22 ^{bc}	0.11	1.07 ^a	0.13	N/D	-	0.07 ^b	0.01	N/D	-	0.28 ^{ab}	0.06
	1	0.05 ^d	0.01	1.69 ^a	0.09	0.13 ^b	0.00	N/D	-	0.07 ^b	0.01	N/D	-	0.38 ^a	0.09
	2	0.16 ^{cd}	-	1.78 ^a	0.10	0.19 ^b	-	N/D	-	0.07 ^b	0.00	N/D	-	0.31 ^{ab}	0.14
	3	0.31 ^{bc}	0.01	1.33 ^b	0.03	0.43 ^b	0.04	N/D	-	0.14 ^{ab}	0.02	0.01 ^a	-	0.11 ^{ab}	0.01
	7	0.64 ^a	0.01	0.99 ^c	0.04	0.15 ^b	0.16	N/D	-	0.25 ^a	0.05	0.02 ^a	0.00	0.08 ^{ab}	0.01
	9	0.50 ^{ab}	0.01	1.41 ^b	0.10	N/D	-	N/D	-	0.25 ^a	0.08	0.05 ^a	0.02	0.04 ^b	0.02
	14	0.60 ^a	-	0.86 ^c	-	N/D	-	N/D	-	0.33 ^a	-	0.03 ^a	-	0.07 ^{ab}	-
Smoke drying	0	0.21 ^a	0.09	1.06 ^b	-	1.63 ^a	0.60	0.06 ^a	0.01	0.27 ^a	0.20	0.01 ^a	0.00	0.13 ^a	0.09
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	0.27 ^a	0.02	1.18 ^b	0.03	1.00 ^{ab}	0.06	0.07 ^a	0.01	0.14 ^a	0.02	0.02 ^a	0.00	0.07 ^a	0.01
	3	0.31 ^a	0.00	1.48 ^b	0.07	0.80 ^{ab}	0.01	0.05 ^a	0.01	0.14 ^a	0.01	0.02 ^a	0.01	0.05 ^a	0.01
	7	0.35 ^a	-	2.28 ^a	-	0.24 ^{ab}	-	0.03 ^a	-	0.15 ^a	0.06	0.02 ^a	-	0.08 ^a	0.03
	9	0.30 ^a	0.03	2.11 ^a	0.13	0.26 ^b	0.01	N/D	-	0.17 ^a	0.00	0.02 ^a	0.01	0.22 ^a	0.06
	14	0.25 ^a	0.08	1.65 ^{ab}	0.21	0.31 ^b	0.03	N/D	-	0.25 ^a	0.04	0.02 ^a	-	0.20 ^a	-

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 7 Total fatty acid composition (% w/w dry rubber) of RRIM600 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation

Drying methods	Days	Fatty acids															
		C16:0		C16:1		C18:0		C18:1		C18:2		C18:3		C20:0		FuFA	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Outdoor air drying	0	0.06 ^a	0.007	N/D	-	0.14 ^{ab}	0.015	0.09 ^a	0.036	0.24 ^a	0.096	0.02 ^a	0.008	0.01 ^a	0.003	N/D	-
	2	0.06 ^a	0.008	N/D	-	0.17 ^a	0.013	0.05 ^{ab}	0.011	0.15 ^{ab}	0.024	0.01 ^b	0.001	0.01 ^a	0.003	N/D	-
	3	0.06 ^a	0.008	N/D	-	0.17 ^a	0.013	0.05 ^{ab}	0.011	0.15 ^{ab}	0.024	0.01 ^b	0.001	0.01 ^a	0.003	N/D	-
	7	0.05 ^a	0.011	N/D	-	0.15 ^{ab}	0.017	0.07 ^{ab}	0.009	0.15 ^{ba}	0.022	0.01 ^b	0.002	0.01 ^a	0.001	N/D	-
	9	0.05 ^a	0.007	N/D	-	0.15 ^{ab}	0.029	0.03 ^b	0.006	0.04 ^b	0.008	N/D	-	0.01 ^a	0.002	N/D	-
	14	0.05 ^a	0.002	N/D	-	0.12 ^b	0.006	0.02 ^b	0.006	0.03 ^b	0.004	N/D	-	0.01 ^a	0.001	N/D	-
Smoke drying	0	0.07 ^a	0.003	N/D	-	0.15 ^a	0.003	0.12 ^a	0.007	0.32 ^a	0.009	0.03 ^a	0.000	0.01 ^a	0.001	0.01 ^a	-
	2	0.08 ^a	0.022	N/D	-	0.18 ^a	0.046	0.12 ^a	0.032	0.30 ^a	0.084	0.03 ^a	0.007	0.02 ^a	0.004	0.02 ^a	-
	3	0.10 ^a	0.025	N/D	-	0.20 ^a	0.051	0.13 ^a	0.031	0.30 ^a	0.074	0.03 ^a	0.006	0.03 ^a	0.008	0.02 ^a	0.005
	7	0.07 ^a	0.046	N/D	-	0.15 ^a	0.090	0.08 ^a	0.060	0.09 ^b	0.068	N/D	-	0.08 ^a	0.067	0.01 ^a	0.010
	9	0.08 ^a	0.013	N/D	-	0.15 ^a	0.019	0.06 ^a	0.014	0.05 ^b	0.024	N/D	-	0.07 ^a	0.021	0.01 ^a	0.001
	14	0.07 ^a	0.006	N/D	-	0.13 ^a	0.008	0.09 ^a	0.008	0.05 ^b	0.038	N/D	-	0.04 ^a	0.013	0.01 ^a	-

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 8 Total fatty acid composition (% w/w dry rubber) of PB235 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation

Drying methods	Days	Fatty acids															
		C16:0		C16:1		C18:0		C18:1		C18:2		C18:3		C20:0		FuFA	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Outdoor air drying	0	0.11 ^{ab}	0.020	0.04 ^a	0.007	0.22 ^{ab}	0.042	0.09 ^a	0.016	0.29 ^a	0.057	0.03 ^a	0.007	0.02 ^{ab}	0.003	1.68 ^a	0.331
	1	0.07 ^{ab}	0.018	0.01 ^a	0.005	0.20 ^{ab}	0.001	0.06 ^{bc}	0.001	0.17 ^b	0.049	0.01 ^b	0.001	0.02 ^{abc}	0.000	1.44 ^a	0.171
	2	0.07 ^a	0.012	0.01 ^a	0.001	0.18 ^{ab}	0.033	0.05 ^{bcd}	0.009	0.14 ^{bc}	0.025	0.01 ^b	0.002	0.02 ^{abc}	0.004	0.99 ^{ab}	0.351
	3	0.14 ^{ab}	0.049	0.03 ^a	0.017	0.32 ^a	0.100	0.07 ^{ab}	0.003	0.15 ^b	0.018	0.02 ^b	0.001	0.02 ^a	0.004	0.68 ^{ab}	0.148
	7	0.06 ^{ab}	0.012	N/D	-	0.21 ^{ab}	0.037	0.02 ^d	0.003	0.02 ^d	0.003	N/D	-	0.01 ^{abc}	0.001	0.10 ^b	0.119
	9	0.05 ^{ab}	0.013	N/D	-	0.16 ^{ab}	0.028	0.02 ^d	0.002	0.02 ^d	0.005	N/D	-	0.01 ^c	0.001	0.02 ^b	-
	14	0.04 ^b	0.015	N/D	-	0.12 ^b	0.042	0.02 ^{cd}	-	0.01 ^{cd}	-	N/D	-	0.01 ^{bc}	-	N/D	-
Smoke drying	0	0.09 ^a	0.001	0.03 ^a	0.003	0.19 ^a	0.003	0.08 ^a	0.001	0.25 ^a	0.003	0.03 ^a	0.001	0.02 ^c	0.001	1.53 ^a	0.062
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	0.09 ^a	0.008	0.02 ^{ab}	0.002	0.18 ^a	0.016	0.07 ^{ab}	0.001	0.19 ^b	0.006	0.02 ^{ab}	0.001	0.02 ^c	0.002	1.52 ^a	0.134
	3	0.10 ^a	0.014	0.02 ^{ab}	0.003	0.20 ^a	0.023	0.07 ^{ab}	0.011	0.17 ^b	0.025	0.02 ^b	0.003	0.02 ^c	0.002	1.54 ^a	0.182
	7	0.06 ^a	0.001	0.01 ^c	0.001	0.12 ^a	0.001	0.04 ^b	0.001	0.05 ^c	0.006	N/D	-	0.04 ^{bc}	0.006	0.72 ^a	0.151
	9	0.09 ^a	0.022	0.02 ^{bc}	0.004	0.21 ^a	0.047	0.05 ^b	0.017	0.04 ^c	0.011	N/D	-	0.06 ^{ab}	0.014	1.30 ^a	0.407
	14	0.09 ^a	0.023	0.02 ^{bc}	0.003	0.19 ^a	0.055	0.05 ^b	0.004	0.02 ^c	0.001	N/D	-	0.06 ^a	0.006	0.70 ^a	0.294

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 9 Unsaponifiable composition (% w/w dry rubber) of RRIM600 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation

Drying methods	Days	Unsaponifiable composition													
		C18-OH		C20-OH		γ -tocotrienol		α -tocotrienol		Stigmasterol		β -sitosterol		Δ 5-avenasterol	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Outdoor air drying	0	0.01 ^a	0.001	0.01 ^a	0.010	0.03 ^a	-	N/D	-	0.04 ^a	0.015	0.30 ^a	0.063	0.08 ^a	0.018
	2	0.01 ^a	0.005	0.01 ^a	0.003	0.03 ^a	-	N/D	-	0.03 ^a	0.001	0.23 ^a	0.010	0.06 ^a	0.011
	3	0.01 ^a	0.001	0.01 ^a	0.000	0.04 ^a	0.014	N/D	-	0.02 ^a	0.002	0.26 ^a	0.004	0.07 ^a	0.004
	7	0.01 ^a	0.006	N/D	-	0.04 ^a	0.026	N/D	-	0.04 ^a	0.004	0.24 ^a	0.003	0.08 ^a	0.020
	9	0.01 ^a	0.001	N/D	-	0.02 ^a	-	N/D	-	0.03 ^a	0.008	0.28 ^a	0.039	0.07 ^a	0.014
	14	0.01 ^a	-	N/D	-	0.05 ^a	-	N/D	-	0.04 ^a	-	0.28 ^a	-	0.07 ^a	-
Smoke drying	0	0.02 ^a	0.002	0.01 ^a	0.002	0.04 ^a	0.003	0.02 ^a	0.002	0.05 ^a	0.004	0.40 ^a	0.050	0.08 ^a	0.005
	2	0.02 ^a	0.002	0.01 ^a	0.002	0.10 ^a	0.022	0.03 ^a	0.002	0.06 ^a	0.004	0.41 ^a	0.034	0.10 ^a	0.013
	3	0.02 ^a	0.001	0.01 ^a	0.000	0.14 ^a	0.081	0.03 ^a	0.005	0.07 ^a	0.001	0.45 ^a	0.026	0.12 ^a	0.004
	7	0.02 ^a	0.000	0.01 ^a	0.001	0.12 ^a	0.076	0.03 ^a	-	0.06 ^a	0.006	0.42 ^a	0.018	0.10 ^a	0.027
	9	0.02 ^a	0.008	0.01 ^a	0.004	0.08 ^a	0.018	0.03 ^a	0.001	0.04 ^a	0.016	0.29 ^a	0.136	0.08 ^a	0.011
	14	0.01 ^a	0.002	0.01 ^a	0.000	0.09 ^a	-	0.03 ^a	-	0.07 ^a	0.008	0.47 ^a	0.050	0.11 ^a	0.010

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 10 Unsaponifiable composition (% w/w dry rubber) of PB235 lipid extracts obtained from outdoor air and smoke dried samples during 14 days of maturation

Drying methods	Days	Unsaponifiable composition													
		C18-OH		C20-OH		γ -tocotrienol		α -tocotrienol		Stigmasterol		β -sitosterol		$\Delta 5$ -avenasterol	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Outdoor air drying	0	0.06 ^a	-	0.03 ^{ab}	-	0.11 ^a	-	0.04 ^a	-	0.06 ^a	-	0.36 ^a	-	0.13 ^a	-
	1	0.06 ^a	0.003	0.03 ^a	0.001	0.10 ^a	0.029	0.04 ^a	0.001	0.05 ^a	0.005	0.31 ^a	0.044	0.13 ^a	0.019
	2	0.05 ^{ab}	0.009	0.03 ^{abc}	0.004	0.08 ^a	0.009	0.04 ^a	0.006	0.04 ^{ab}	0.012	0.26 ^{ab}	0.064	0.10 ^{ab}	0.011
	3	0.03 ^{bc}	0.002	0.02 ^{bcd}	0.002	0.06 ^a	0.039	0.03 ^a	-	0.03 ^{ab}	0.006	0.21 ^{ab}	0.022	0.08 ^{ab}	0.021
	7	0.03 ^{bc}	0.004	0.02 ^{bcd}	0.003	0.13 ^a	0.056	0.04 ^a	0.001	0.03 ^{ab}	0.001	0.20 ^{ab}	0.009	0.09 ^{ab}	0.007
	9	0.02 ^c	0.002	0.01 ^{cd}	-	0.04 ^a	0.016	N/D	-	0.02 ^b	0.002	0.14 ^b	0.004	0.06 ^b	0.001
	14	0.02 ^c	0.004	0.01 ^d	0.002	0.02 ^a	-	N/D	-	0.02 ^b	0.008	0.13 ^b	0.034	0.05 ^b	0.002
Smoke drying	0	0.03 ^a	0.005	0.02 ^a	0.001	0.06 ^a	0.021	0.04 ^a	-	0.03 ^a	0.006	0.25 ^a	0.058	0.11 ^a	0.007
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	0.04 ^a	0.016	0.02 ^a	0.007	0.08 ^a	0.052	0.05 ^a	-	0.04 ^a	0.018	0.27 ^a	0.085	0.10 ^a	0.023
	3	0.05 ^a	0.007	0.03 ^a	0.001	0.16 ^a	0.042	0.04 ^a	0.002	0.05 ^a	0.003	0.28 ^a	0.038	0.12 ^a	0.002
	7	0.04 ^a	0.021	0.03 ^a	0.013	0.14 ^a	-	0.05 ^a	-	0.05 ^a	0.021	0.29 ^a	0.119	0.12 ^a	0.045
	9	0.04 ^a	0.006	0.02 ^a	0.004	0.09 ^a	0.031	0.04 ^a	0.008	0.05 ^a	0.010	0.29 ^a	0.058	0.10 ^a	0.028
	14	0.04 ^a	0.006	0.02 ^a	0.005	0.10 ^a	-	0.04 ^a	-	0.04 ^a	0.005	0.25 ^a	0.024	0.10 ^a	0.011

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 11 Absolute initial slope value (%DPPH_{rem}.min⁻¹) of RRIM600 and PB235 lipid extracts obtained from outdoor air dried and smoke dried samples during 14 days of maturation

Maturation time (days)	RRIM600 clone				PB235 clone			
	Outdoor air drying		Smoke drying		Outdoor air drying		Smoke drying	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	12.8 ^a	1.18	12.0 ^a	1.10	6.32 ^{ab}	3.21	4.97 ^{ab}	0.05
1	-	-	-	-	7.54 ^a	0.76	-	-
2	15.5 ^a	5.57	8.28 ^{bc}	0.78	5.99 ^{abc}	0.66	8.32 ^a	0.71
3	16.9 ^a	0.56	8.28 ^{bc}	0.30	2.72 ^{bcd}	0.61	5.17 ^{ab}	0.57
7	16.2 ^a	1.61	8.24 ^c	1.31	1.45 ^{cd}	0.52	5.19 ^{ab}	0.05
9	12.5 ^a	1.17	11.8 ^{ab}	0.98	1.71 ^{cd}	0.51	3.38 ^{ab}	2.24
14	7.06 ^a	3.27	7.26 ^c	0.40	1.20 ^d	0.04	2.46 ^b	2.04

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, P < 0.05). SD: standard deviation.

APPENDIX B

CONTROLLED CONDITIONS

(TSR Like process)

Table 12 Fresh and dry weight (g) and water content (%) of laminated crepe samples obtained from RRIM600 and PB235 rubber clones from 3 conditions during 28 days of maturation

Conditions	Days	RRIM600 clone						PB235 clone					
		Fresh weight		Dry weight		Water content		Fresh weight		Dry weight		Water content	
		(g)	(g)	(%)	(g)	(g)	(%)	(g)	(g)	(%)	(g)	(g)	(%)
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	24.9 ^a	0.59	19.6 ^a	0.42	21.1 ^c	0.37	22.7 ^a	0.46	17.3 ^a	0.46	25.7 ^a	0.64
	3	20.4 ^c	0.17	16.0 ^b	0.25	21.4 ^c	0.57	20.6 ^c	0.43	16.2 ^b	0.31	21.3 ^b	0.31
	5	23.7 ^{ab}	1.43	18.5 ^a	0.42	23.8 ^{ab}	0.76	22.2 ^{ab}	0.23	17.4 ^a	0.19	21.3 ^b	0.35
	7	24.7 ^a	0.58	18.7 ^a	0.45	24.3 ^a	0.13	21.8 ^{ab}	0.16	17.5 ^a	0.05	19.8 ^{bc}	0.44
	14	24.5 ^{ab}	0.35	19.2 ^a	0.36	21.8 ^{bc}	0.44	21.5 ^{bc}	0.18	17.7 ^a	0.19	18.9 ^c	0.66
	21	24.4 ^{ab}	0.25	19.3 ^a	0.31	20.8 ^c	0.74	21.8 ^{ab}	0.48	18.0 ^a	0.31	19.6 ^{bc}	0.46
	28	22.4 ^{bc}	1.26	18.4 ^a	0.78	18.2 ^d	1.09	21.6 ^{bc}	0.21	17.5 ^a	0.35	19.0 ^c	1.66
Na-O-	0	23.9 ^{αβ}	0.37	19.0 ^α	0.20	20.8 ^γ	0.44	21.8 ^β	0.60	17.2 ^α	0.30	21.3 ^β	0.91
	3	26.7 ^α	0.08	19.1 ^α	0.25	28.8 ^α	0.97	22.8 ^{αβ}	0.29	17.4 ^α	0.04	23.7 ^{αβ}	0.85
	5	26.9 ^α	1.60	18.9 ^α	0.16	27.6 ^{αβ}	0.42	23.7 ^α	0.29	17.4 ^α	0.15	26.6 ^α	0.29
	7	25.5 ^{αβ}	1.54	18.2 ^α	0.74	27.3 ^{αβ}	0.13	22.9 ^{αβ}	0.47	17.4 ^α	0.05	24.1 ^{αβ}	1.68
	14	26.1 ^{αβ}	0.38	18.5 ^α	0.45	29.2 ^α	1.27	23.3 ^α	0.19	17.3 ^α	0.06	25.8 ^α	0.75
	21	26.4 ^α	2.39	18.8 ^α	0.27	25.7 ^β	0.99	23.1 ^α	0.45	17.3 ^α	0.09	25.0 ^α	1.69
	28	22.6 ^β	0.41	18.6 ^α	0.25	17.7 ^δ	0.84	23.1 ^α	0.13	17.3 ^α	0.09	25.1 ^α	0.81
Na-O+	0	23.9 ^{AD}	0.37	19.0 ^A	0.20	20.8 ^C	0.44	21.8 ^C	0.60	17.2 ^A	0.30	21.3 ^F	0.91
	3	26.8 ^C	0.13	19.0 ^A	0.24	29.0 ^B	0.68	23.1 ^{BC}	0.51	17.2 ^A	0.15	25.3 ^E	1.08
	5	26.6 ^{CD}	0.25	19.0 ^A	0.14	28.7 ^B	0.31	23.2 ^{AB}	0.46	17.1 ^A	0.23	26.5 ^{DE}	0.61
	7	27.3 ^C	1.32	18.9 ^A	0.02	28.7 ^B	0.14	23.9 ^{AB}	0.11	17.3 ^A	0.18	27.5 ^{CD}	0.40
	14	30.1 ^{AB}	0.81	19.0 ^A	0.04	36.0 ^A	1.03	24.1 ^{AB}	0.48	17.1 ^A	0.28	29.1 ^{BC}	0.23
	21	31.6 ^A	1.73	19.0 ^A	0.57	36.7 ^A	2.14	24.5 ^A	0.25	17.0 ^A	0.28	30.5 ^{AB}	0.42
	28	28.0 ^{BC}	1.01	18.5 ^A	0.44	34.1 ^A	0.89	24.2 ^{AB}	0.68	16.7 ^A	0.31	31.1 ^A	0.71

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, P < 0.05). SD: standard deviation.

Table 13 Physical properties of RRIM600 and PB235 matured mini-cup coagula obtained from 3 conditions during 28 days of maturation

Conditions	Days	RRIM600 clone						PB235 clone					
		P ₀ (unit)		P ₃₀ (unit)		PRI (%)		P ₀ (unit)		P ₃₀ (unit)		PRI (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	38.8 ^c	0.29	32.5 ^d	0.69	83.4 ^{bc}	1.77	49.2 ^a	0.76	40.3 ^a	0.58	82.0 ^a	0.45
	3	43.8 ^d	0.76	41.0 ^c	0.02	92.7 ^a	0.78	-	-	-	-	-	-
	5	49.0 ^c	1.00	44.0 ^{abc}	0.00	88.9 ^{ab}	1.27	49.7 ^a	1.61	33.8 ^c	1.03	72.5 ^b	1.91
	7	54.3 ^{ab}	0.58	46.0 ^a	0.20	84.4 ^{bc}	1.13	51.5 ^a	0.50	38.3 ^{ab}	1.76	75.9 ^{ab}	1.48
	14	55.5 ^a	0.71	44.3 ^{ab}	0.37	82.0 ^{cd}	2.83	50.0 ^a	1.41	33.5 ^c	0.02	71.1 ^b	0.78
	21	52.2 ^b	1.04	41.5 ^{bc}	0.51	78.7 ^{cd}	0.85	51.5 ^a	2.12	35.5 ^{bc}	1.41	69.1 ^b	5.59
	28	55.5 ^a	2.12	42.8 ^{abc}	2.50	77.0 ^d	1.56	50.3 ^a	0.35	35.0 ^{bc}	0.00	70.0 ^b	0.49
Na-O-	0	37.7 ^δ	1.04	32.8 ^δ	1.06	88.0 ^α	0.35	43.5 ^{αβ}	0.50	37.0 ^α	0.01	85.1 ^α	1.00
	3	50.7 ^γ	0.76	46.0 ^{αβ}	0.00	91.6 ^α	0.64	-	-	-	-	-	-
	5	55.5 ^β	2.83	41.5 ^{αβγ}	3.53	74.7 ^{βγ}	2.55	40.8 ^{βγ}	0.35	16.0 ^{βγ}	0.70	39.3 ^γ	2.05
	7	60.8 ^α	0.35	46.5 ^α	0.99	79.7 ^β	1.70	47.8 ^α	1.26	21.7 ^β	0.35	46.0 ^β	0.28
	14	58.8 ^{αβ}	0.76	41.0 ^{βγ}	0.87	69.7 ^{γδ}	1.55	37.3 ^γ	1.15	14.5 ^γ	1.33	41.3 ^{βγ}	0.64
	21	59.5 ^{αβ}	2.12	36.0 ^{γδ}	1.42	68.5 ^{γδ}	1.70	34.5 ^γ	5.66	3.33 ^δ	4.04	3.75 ^μ	0.64
	28	61.3 ^α	1.76	37.8 ^{γδ}	1.77	67.0 ^δ	1.91	37.0 ^{βγ}	1.41	11.3 ^γ	0.33	30.5 ^δ	2.05
Na-O+	0	37.7 ^C	1.04	32.8 ^C	1.06	88.0 ^A	0.35	43.5 ^{AB}	0.50	37.0 ^A	0.01	85.1 ^A	1.00
	3	52.5 ^{AB}	0.71	45.5 ^A	0.50	87.2 ^A	1.84	-	-	-	-	-	-
	5	54.0 ^{AB}	0.87	43.5 ^A	0.49	81.4 ^B	0.99	35.5 ^D	0.00	6.25 ^{CD}	0.35	17.6 ^C	0.99
	7	55.3 ^A	0.35	44.0 ^A	0.01	79.7 ^B	0.49	47.5 ^A	0.71	11.5 ^B	0.69	25.6 ^B	0.07
	14	54.5 ^{Ab}	0.71	37.0 ^B	1.39	64.3 ^C	1.77	36.8 ^{CD}	1.06	9.25 ^{BC}	1.07	25.2 ^B	2.19
	21	51.8 ^B	1.26	29.3 ^D	1.06	56.6 ^D	0.92	41.0 ^{BC}	0.00	5.33 ^D	1.15	16.2 ^C	2.19
	28	55.8 ^A	0.35	25.8 ^E	1.10	46.2 ^E	2.26	42.8 ^{AB}	3.18	8.50 ^{BCD}	2.11	19.8 ^{BC}	3.46

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, P < 0.05). SD: standard deviation.

Table 14 Total gel content and molar masses (weight-average molar mass, M_w and number-average molar mass, M_n) of RRIM600 and PB235 matured mini-cup coagula obtained from 3 conditions during 28 days of maturation

Conditions	Days	RRIM600 clone						PB235 clone					
		Gel (%)		M_w (kg.mol ⁻¹)		M_n (kg.mol ⁻¹)		Gel (%)		M_w (kg.mol ⁻¹)		M_n (kg.mol ⁻¹)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	34.4 ^c	3.15	1497 ^b	61.9	878.7 ^d	64.9	28.0 ^b	1.53	1637 ^a	58.4	1083 ^a	30.2
	3	41.2 ^{bc}	1.61	1738 ^a	82.8	1149 ^c	25.0	-	-	-	-	-	-
	5	48.2 ^{ab}	1.07	1617 ^{ab}	103	1158 ^{bc}	49.4	35.9 ^a	2.65	1449 ^{bc}	69.5	1081 ^a	57.7
	7	51.7 ^a	1.31	1773 ^a	27.0	1298 ^{ab}	45.8	35.7 ^a	1.04	1630 ^a	99.3	1139 ^a	87.0
	14	51.8 ^a	4.57	1802 ^a	49.0	1347 ^a	69.5	36.1 ^a	3.45	1399 ^c	30.6	1048 ^a	60.3
	21	50.7 ^a	2.35	1741 ^a	103	1284 ^{abc}	54.3	32.0 ^{ab}	3.75	1583 ^{ab}	78.7	1131 ^a	39.3
	28	51.1 ^a	2.17	1589 ^{ab}	98.3	1233 ^{abc}	42.2	38.8 ^a	2.15	1488 ^{abc}	19.9	1143 ^a	22.3
Na-O-	0	28.5 ^γ	2.66	1365 ^{βγ}	237	723.3 ^γ	120	30.7 ^β	0.81	1502 ^α	12.1	933.7 ^{αβ}	54.0
	3	46.4 ^β	3.37	1710 ^α	44.8	1237 ^α	62.7	-	-	-	-	-	-
	5	49.8 ^{αβ}	0.45	1492 ^{αβγ}	43.7	1050 ^{αβ}	23.9	38.4 ^α	5.13	1191 ^{βγ}	45.7	918.0 ^{αβ}	34.1
	7	50.2 ^{αβ}	3.46	1583 ^{αβ}	33.2	1180 ^α	39.3	40.9 ^α	3.54	1330 ^{αβ}	109	1029 ^α	83.2
	14	53.0 ^{αβ}	1.57	1434 ^{αβγ}	84.5	1118 ^{αβ}	94.6	38.1 ^{αβ}	0.56	987.3 ^{γδ}	54.4	764.7 ^{βγ}	57.0
	21	53.5 ^α	2.06	1473 ^{αβγ}	78.0	1096 ^{αβ}	55.8	39.8 ^α	1.85	921.7 ^δ	153	691.3 ^γ	140
	28	54.9 ^α	1.69	1212 ^γ	56.5	930.3 ^β	32.5	40.7 ^α	1.76	1029 ^{γδ}	30.5	817.0 ^{βγ}	40.9
Na-O+	0	28.5 ^B	2.66	1365 ^A	237	723.3 ^B	120	30.7 ^B	0.81	1502 ^A	12.1	933.7 ^A	54.1
	3	46.6 ^A	1.31	1685 ^A	112	1188 ^A	50.5	-	-	-	-	-	-
	5	47.7 ^A	3.45	1574 ^A	48.5	1144 ^A	74.3	39.0 ^A	2.78	1165 ^{BC}	169	879.7 ^A	114
	7	46.7 ^A	3.29	1632 ^A	63.7	1171 ^A	47.8	39.9 ^A	5.00	1283 ^{AB}	85.3	986.0 ^A	85.0
	14	51.1 ^A	1.03	1562 ^A	33.3	1099 ^A	57.6	40.3 ^A	0.89	972.0 ^C	119	748.3 ^A	109
	21	50.9 ^A	3.52	1438 ^A	96.3	1005 ^A	114	35.4 ^{AB}	2.11	1160 ^{BC}	66.1	854.7 ^A	54.4
	28	48.5 ^{AC}	0.95	1391 ^A	84.1	979.3 ^A	53.9	34.6 ^{AB}	1.40	1195 ^{BC}	134	893.0 ^A	122

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student t test, $P < 0.05$). SD: standard deviation.

Table 15 Lipid extract (% w/w dry rubber) of RRIM600 and PB235 mini-cup coagula samples from 3 conditions during 28 days of maturation

Maturation time (days)	RRIM600 Clone						PB235 Clone					
	Conditions						Conditions					
	Na+O-		Na-O+		Na-O-		Na+O-		Na-O+		Na-O-	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	2.04 ^b	0.03	2.04 ^a	0.02	2.04 ^a	0.02	3.47 ^a	0.04	3.38 ^a	0.00	3.38 ^a	0.00
3	2.17 ^a	0.05	1.94 ^{ab}	0.03	1.95 ^b	0.06	-	-	-	-	-	-
5	1.98 ^b	0.05	1.93 ^{ab}	0.02	1.95 ^b	0.03	3.29 ^b	0.04	3.31 ^a	0.06	3.33 ^a	0.03
7	1.93 ^b	0.01	1.93 ^{ab}	0.02	1.91 ^b	0.01	3.25 ^b	0.06	3.22 ^{ab}	0.09	3.26 ^a	0.01
14	1.90 ^b	0.03	1.73 ^{bc}	0.11	1.96 ^{ab}	0.03	3.19 ^b	0.03	3.06 ^b	0.06	3.33 ^a	0.16
21	1.89 ^b	0.01	1.61 ^c	0.03	1.94 ^b	0.02	3.31 ^{ab}	0.12	2.76 ^c	0.15	3.36 ^a	0.04
28	1.93 ^b	0.08	1.62 ^c	0.18	1.92 ^b	0.04	3.16 ^b	0.02	2.51 ^d	0.05	3.37 ^a	0.10

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test, $P \leq 0.05$). SD: standard deviation.

Table 16 Neutral lipid composition (% w/w dry rubber) of RRIM600 lipid extracts obtained from 3 conditions during 28 days of maturation

Conditions	Days	Components															
		Sterols		Free fatty acids		TGF		γ -tocotrienol		Wax		γ -tocotrienol ester I		γ -tocotrienol ester II		Sterol esters	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	0.54 ^a	0.021	0.30 ^a	0.060	0.30 ^a	0.100	0.09 ^a	0.000	0.012 ^a	0.012	0.22 ^a	0.014	0.11 ^{ab}	0.021	0.15 ^b	0.035
	3	0.31 ^b	0.014	0.64 ^b	0.021	0.18 ^a	0.076	0.07 ^{ab}	0.014	0.008 ^a	0.006	0.16 ^b	0.026	0.09 ^{ab}	0.012	0.23 ^{ab}	0.035
	5	0.25 ^c	0.020	0.64 ^b	0.074	0.16 ^a	0.032	0.06 ^{ab}	0.006	0.006 ^a	0.004	0.16 ^b	0.011	0.10 ^{ab}	0.012	0.20 ^{ab}	0.014
	7	0.22 ^{cd}	0.020	0.64 ^b	0.081	0.16 ^a	0.035	0.06 ^b	0.012	0.006 ^a	0.005	0.15 ^b	0.006	0.09 ^{abc}	0.012	0.19 ^{ab}	0.028
	14	0.18 ^d	0.010	0.65 ^b	0.044	0.15 ^a	0.046	0.06 ^b	0.007	0.005 ^a	0.004	0.14 ^b	0.006	0.07 ^{abc}	0.006	0.26 ^a	0.014
	21	0.18 ^d	0.007	0.63 ^b	0.014	0.13 ^a	0.061	0.06 ^b	0.007	0.005 ^a	0.003	0.14 ^b	0.007	0.05 ^c	0.012	0.29 ^a	0.014
	28	0.17 ^d	0.023	0.65 ^b	0.075	0.18 ^a	0.089	0.06 ^b	0.007	0.005 ^a	0.003	0.14 ^b	0.021	0.07 ^{bc}	0.015	0.25 ^{ab}	0.035
Na-O-	0	0.50 ^a	0.007	0.25 ^b	0.067	0.29 ^a	0.121	0.10 ^a	0.007	0.008 ^{γ}	0.001	0.22 ^a	0.035	0.09 ^{ab}	0.015	0.14 ^a	0.010
	3	0.25 ^b	0.017	0.66 ^a	0.070	0.16 ^{ab}	0.035	0.05 ^b	0.006	0.047 ^b	0.008	0.15 ^b	0.006	0.13 ^a	0.010	0.11 ^{ab}	0.012
	5	0.23 ^b	0.015	0.65 ^a	0.031	0.14 ^{ab}	0.045	0.04 ^b	0.010	0.098 ^a	0.007	0.15 ^b	0.023	0.14 ^a	0.015	0.10 ^b	0.017
	7	0.22 ^b	0.010	0.64 ^a	0.015	0.14 ^{ab}	0.040	0.04 ^b	0.010	0.108 ^a	0.007	0.15 ^b	0.030	0.13 ^a	0.021	0.10 ^b	0.017
	14	0.22 ^b	0.015	0.74 ^a	0.047	0.14 ^{ab}	0.040	0.04 ^b	0.006	0.046 ^b	0.004	0.17 ^{ab}	0.010	0.09 ^{ab}	0.015	0.11 ^{ab}	0.012
	21	0.22 ^b	0.006	0.73 ^a	0.062	0.12 ^b	0.031	0.05 ^b	0.006	0.035 ^{bγ}	0.018	0.18 ^{ab}	0.006	0.10 ^{ab}	0.015	0.12 ^{ab}	0.010
	28	0.23 ^b	0.012	0.72 ^a	0.031	0.12 ^b	0.040	0.06 ^b	0.006	0.016 ^{γ}	0.001	0.18 ^{ab}	0.021	0.08 ^b	0.015	0.13 ^{ab}	0.012
Na-O+	0	0.50 ^A	0.007	0.25 ^D	0.067	0.29 ^A	0.121	0.10 ^A	0.007	0.008 ^C	0.001	0.22 ^A	0.035	0.09 ^{BC}	0.015	0.14 ^{ABC}	0.010
	3	0.26 ^C	0.006	0.63 ^A	0.069	0.15 ^{AB}	0.035	0.04 ^C	0.000	0.065 ^B	0.007	0.15 ^{BC}	0.015	0.13 ^A	0.006	0.11 ^{BC}	0.025
	5	0.24 ^C	0.023	0.58 ^{AB}	0.072	0.15 ^{AB}	0.030	0.04 ^C	0.010	0.168 ^A	0.018	0.15 ^{BC}	0.011	0.11 ^{AB}	0.015	0.11 ^C	0.021
	7	0.23 ^C	0.010	0.56 ^{AB}	0.056	0.14 ^{AB}	0.031	0.05 ^{BC}	0.007	0.195 ^A	0.019	0.14 ^C	0.020	0.11 ^{ABC}	0.020	0.11 ^C	0.021
	14	0.27 ^C	0.021	0.44 ^{BC}	0.026	0.13 ^{AB}	0.050	0.06 ^{BC}	0.006	0.031 ^{BC}	0.016	0.17 ^{ABC}	0.000	0.12 ^{ABC}	0.007	0.17 ^{AB}	0.015
	21	0.25 ^C	0.000	0.37 ^{CD}	0.061	0.11 ^B	0.058	0.07 ^{ABC}	0.014	0.014 ^C	0.006	0.18 ^{ABC}	0.010	0.08 ^C	0.006	0.18 ^A	0.012
	28	0.34 ^B	0.007	0.34 ^{CD}	0.023	0.14 ^{AB}	0.066	0.07 ^{ABC}	0.012	0.003 ^C	0.000	0.19 ^{AB}	0.012	0.08 ^{BC}	0.010	0.16 ^{ABC}	0.032

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test, $P \leq 0.05$). SD: standard deviation.

Table 17 Neutral lipid composition (% w/w dry rubber) of PB235 lipid extracts obtained from 3 conditions during 28 days of maturation

Conditions	Days	Components															
		Sterols		Free fatty acids		TGF		γ-tocotrienol		Wax		γ-tocotrienol ester I		γ-tocotrienol ester II		Sterol esters	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	0.46 ^a	0.028	0.30 ^c	0.046	1.80 ^a	0.134	0.07 ^a	0.000	0.005 ^b	0.004	0.29 ^a	0.012	0.02 ^a	0.006	0.08 ^c	0.007
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	0.27 ^b	0.021	0.69 ^b	0.012	1.29 ^b	0.093	0.06 ^a	0.007	0.013 ^b	0.002	0.23 ^b	0.012	0.03 ^a	0.006	0.16 ^{ab}	0.014
	7	0.25 ^b	0.035	0.73 ^{ab}	0.029	1.25 ^b	0.055	0.06 ^a	0.007	0.012 ^b	0.002	0.21 ^b	0.015	0.02 ^a	0.006	0.12 ^{bc}	0.021
	14	0.23 ^b	0.035	0.73 ^{ab}	0.020	1.16 ^b	0.038	0.06 ^a	0.007	0.012 ^b	0.005	0.21 ^b	0.010	0.02 ^a	0.006	0.17 ^{ab}	0.007
	21	0.21 ^b	0.035	0.74 ^{ab}	0.032	1.19 ^b	0.115	0.06 ^a	0.007	0.017 ^{ab}	0.001	0.22 ^b	0.006	0.02 ^a	0.006	0.21 ^a	0.007
28	0.20 ^b	0.057	0.78 ^a	0.046	1.06 ^b	0.130	0.06 ^a	0.007	0.031 ^a	0.006	0.21 ^b	0.023	0.02 ^a	0.006	0.19 ^a	0.014	
Na-O-	0	0.50 ^α	-	0.30 ^β	0.014	1.60 ^α	0.276	0.07 ^α	0.014	0.01 ^δ	0.005	0.31 ^α	0.007	0.02 ^β	0.007	0.07 ^α	0.042
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	0.21 ^β	0.070	1.32 ^α	0.075	0.64 ^β	0.006	0.04 ^α	0.012	0.13 ^γ	0.014	0.24 ^α	0.017	0.05 ^α	0.000	0.06 ^α	0.028
	7	0.18 ^β	0.021	1.55 ^α	0.053	0.28 ^γ	0.062	0.04 ^α	0.012	0.19 ^β	0.031	0.24 ^α	0.026	0.05 ^α	0.006	0.06 ^α	0.028
	14	0.21 ^β	0.065	1.52 ^α	0.028	0.14 ^γ	0.025	0.04 ^α	0.014	0.30 ^α	0.022	0.25 ^α	0.015	0.05 ^α	0.006	0.08 ^α	0.007
	21	0.24 ^{αβ}	0.042	1.64 ^α	0.212	0.13 ^γ	0.070	0.04 ^α	0.012	0.20 ^β	0.029	0.29 ^α	0.028	0.05 ^α	0.021	0.09 ^α	0.028
28	0.25 ^{αβ}	0.028	1.75 ^α	0.297	0.13 ^γ	0.060	0.05 ^α	0.014	0.12 ^γ	0.011	0.30 ^α	0.035	0.04 ^α	0.012	0.07 ^α	0.060	
Na-O+	0	0.50 ^A	-	0.30 ^C	0.014	1.60 ^A	0.276	0.07 ^A	0.006	0.01 ^E	0.005	0.31 ^A	0.007	0.02 ^C	0.007	0.07 ^A	0.042
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	0.29 ^{AB}	0.042	1.51 ^{AB}	0.115	0.41 ^B	0.091	0.05 ^{AB}	0.006	0.11 ^{BC}	0.011	0.26 ^A	0.010	0.04 ^{AB}	0.006	0.07 ^A	0.028
	7	0.27 ^{AB}	0.042	1.70 ^A	0.103	0.06 ^B	0.042	0.04 ^B	0.005	0.22 ^A	0.020	0.26 ^A	0.020	0.04 ^A	0.000	0.08 ^A	0.042
	14	0.31 ^{AB}	0.042	1.61 ^{AB}	0.115	0.06 ^B	-	0.04 ^B	0.006	0.12 ^B	0.022	0.29 ^A	0.017	0.03 ^{AB}	0.000	0.09 ^A	0.042
	21	0.30 ^{AB}	0.049	1.39 ^B	0.044	0.06 ^B	-	0.05 ^{AB}	0.005	0.07 ^{CD}	0.013	0.28 ^A	0.015	0.03 ^{BC}	0.006	0.10 ^A	0.021
28	0.26 ^B	0.035	1.40 ^B	0.028	0.05 ^B	-	0.07 ^{AB}	0.009	0.03 ^{DE}	0.007	0.27 ^A	0.015	0.03 ^{BC}	0.006	0.13 ^A	0.035	

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test, $P \leq 0.05$). SD: standard deviation.

Table 18 Total fatty acids composition (% w/w dry rubber) of RRIM600 lipid extracts obtained from 3 conditions during 28 days of maturation

Conditions	Days	Components																	
		C14:0		C16:0		C16:1		C18:0		C18:1		C18:2		C18:3		C20:0		FuFA	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	N/D	-	0.06 ^a	0.005	N/D	-	0.14 ^a	0.012	0.15 ^a	0.013	0.34 ^a	0.024	0.03 ^a	0.002	0.01 ^b	0.001	0.11 ^{ab}	0.004
	3	N/D	-	0.08 ^a	-	0.01 ^a	-	0.18 ^a	-	0.19 ^a	-	0.43 ^a	-	0.04 ^a	-	0.01 ^{ab}	-	0.12 ^{ab}	-
	5	N/D	-	0.07 ^a	0.002	N/D	-	0.15 ^a	0.004	0.16 ^a	0.005	0.37 ^a	0.011	0.03 ^a	0.002	0.01 ^b	0.000	0.11 ^{ab}	0.005
	7	N/D	-	0.06 ^a	0.006	N/D	-	0.13 ^a	0.013	0.15 ^a	0.016	0.34 ^a	0.038	0.03 ^a	0.003	0.01 ^b	0.001	0.09 ^{ab}	0.013
	14	N/D	-	0.07 ^a	0.015	N/D	-	0.16 ^a	0.033	0.16 ^a	0.038	0.37 ^a	0.101	0.03 ^a	0.010	0.01 ^{ab}	0.001	0.11 ^{ab}	0.032
	21	N/D	-	0.05 ^a	0.028	0.01 ^a	0.001	0.13 ^a	0.065	0.12 ^a	0.064	0.27 ^a	0.143	0.02 ^a	0.012	0.01 ^b	0.005	0.07 ^b	0.039
	28	N/D	-	0.08 ^a	0.008	0.01 ^a	0.001	0.18 ^a	0.014	0.18 ^a	0.017	0.38 ^a	0.024	0.03 ^a	0.002	0.02 ^a	0.005	0.14 ^a	0.015
Na-O-	0	N/D	-	0.07 ^{αβ}	0.013	0.01 ^α	-	0.16 ^{αβγ}	0.028	0.17 ^{αβ}	0.031	0.39 ^{αβ}	0.067	0.03 ^α	0.006	0.01 ^{αβγ}	0.002	0.13 ^{αβ}	0.022
	3	N/D	-	0.07 ^β	0.001	N/D	-	0.15 ^{βγ}	0.003	0.15 ^β	0.003	0.36 ^{αβ}	0.010	0.03 ^α	0.001	0.01 ^{βγ}	0.000	0.11 ^{αβ}	0.002
	5	N/D	-	0.07 ^β	0.004	N/D	-	0.15 ^{βγ}	0.007	0.15 ^β	0.007	0.36 ^{αβ}	0.011	0.03 ^{αβ}	0.001	0.01 ^{βγ}	0.001	0.10 ^β	0.098
	7	N/D	-	0.05 ^β	0.001	N/D	-	0.12 ^γ	0.008	0.12 ^β	0.001	0.30 ^β	0.007	0.02 ^{αβ}	0.001	0.01 ^γ	0.000	0.09 ^β	0.094
	14	N/D	-	0.07 ^{αβ}	0.004	0.01 ^α	0.000	0.18 ^{αβ}	0.018	0.16 ^{αβ}	0.005	0.39 ^{αβ}	0.011	0.03 ^{αβ}	0.001	0.01 ^{αβ}	0.001	0.11 ^{αβ}	0.114
	21	N/D	-	0.07 ^{αβ}	0.006	0.01 ^α	0.001	0.16 ^{βγ}	0.017	0.16 ^{αβ}	0.019	0.38 ^{αβ}	0.050	0.02 ^β	0.004	0.01 ^{αβ}	0.001	0.12 ^{αβ}	0.123
	28	N/D	-	0.09 ^α	0.011	0.01 ^α	0.003	0.21 ^α	0.027	0.21 ^α	0.037	0.48 ^α	0.092	0.03 ^{αβ}	0.006	0.02 ^α	0.002	0.16 ^α	0.157
Na-O+	0	N/D	-	0.07 ^C	0.013	0.01 ^A	-	0.16 ^A	0.028	0.17 ^A	0.031	0.39 ^A	0.067	0.03 ^A	0.006	0.01 ^{AB}	0.002	0.13 ^A	0.022
	3	N/D	-	0.07 ^C	0.003	N/D	-	0.15 ^A	0.006	0.15 ^A	0.006	0.36 ^A	0.015	0.04 ^A	0.002	0.01 ^{AB}	0.001	0.10 ^A	0.001
	5	N/D	-	0.07 ^C	0.009	0.01 ^A	-	0.16 ^A	0.019	0.16 ^A	0.018	0.37 ^A	0.043	0.03 ^A	0.002	0.01 ^{AB}	0.001	0.12 ^A	0.019
	7	N/D	-	0.07 ^C	0.001	0.01 ^A	0.002	0.16 ^A	0.005	0.15 ^A	0.006	0.34 ^A	0.012	0.03 ^A	0.002	0.01 ^{AB}	0.001	0.09 ^A	0.004
	14	0.03 ^A	0.001	0.18 ^A	0.026	N/D	-	0.13 ^A	0.010	0.07 ^B	0.008	0.09 ^B	0.001	0.03 ^A	0.002	0.02 ^A	0.005	0.07 ^A	0.026
	21	0.03 ^A	0.003	0.13 ^B	0.013	N/D	-	0.08 ^B	0.013	0.04 ^B	0.006	0.06 ^B	0.009	N/D	-	0.01 ^B	0.001	0.08 ^A	0.031
	28	0.03 ^A	0.004	0.13 ^B	0.028	N/D	-	0.08 ^B	0.016	0.04 ^B	0.011	0.05 ^B	0.009	N/D	-	0.01 ^B	0.002	0.09 ^A	0.016

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 19 Total fatty acids composition (% w/w dry rubber) of PB235 lipid extracts obtained from 3 conditions during 28 days of maturation.

Conditions	Days	Components																	
		C14:0		C16:0		C16:1		C18:0		C18:1		C18:2		C18:3		C20:0		FuFA	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	N/D	-	0.08 ^a	0.001	0.02 ^a	0.002	0.16 ^a	0.010	0.07 ^a	0.052	0.24 ^a	0.015	0.02 ^a	0.002	0.02 ^a	0.001	1.55 ^a	0.089
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	N/D	-	0.08 ^a	0.004	0.02 ^a	0.001	0.16 ^a	0.009	0.10 ^a	0.005	0.24 ^a	0.012	0.02 ^a	0.002	0.01 ^a	0.001	1.47 ^a	0.111
	7	N/D	-	0.08 ^a	0.002	0.02 ^a	0.001	0.16 ^a	0.002	0.10 ^a	0.002	0.24 ^a	0.005	0.02 ^a	0.001	0.01 ^a	0.001	1.43 ^a	0.044
	14	N/D	-	0.09 ^a	0.006	0.02 ^a	0.002	0.16 ^a	0.011	0.09 ^a	0.007	0.19 ^b	0.018	0.02 ^b	0.002	0.02 ^a	0.000	1.42 ^a	0.111
	21	N/D	-	0.09 ^a	0.006	0.02 ^a	0.001	0.17 ^a	0.013	0.07 ^a	0.050	0.17 ^{bc}	0.008	0.02 ^b	0.001	0.03 ^a	0.011	1.42 ^a	0.097
	28	N/D	-	0.09 ^a	0.002	0.02 ^a	0.001	0.16 ^a	0.002	0.09 ^a	0.002	0.16 ^c	0.006	0.01 ^b	0.002	0.03 ^a	0.003	1.38 ^a	0.096
Na-O-	0	N/D	-	0.07 ^α	0.002	0.02 ^α	0.001	0.15 ^α	0.006	0.09 ^α	0.004	0.22 ^α	0.009	0.02 ^β	0.001	0.01 ^α	0.001	1.28 ^α	0.111
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	N/D	-	0.08 ^α	0.002	0.02 ^α	0.001	0.16 ^α	0.008	0.09 ^α	0.003	0.24 ^α	0.006	0.02 ^β	0.001	0.02 ^α	0.001	1.41 ^α	0.085
	7	N/D	-	0.09 ^α	0.003	0.02 ^α	0.001	0.17 ^α	0.003	0.10 ^α	0.003	0.25 ^α	0.007	0.03 ^{αβ}	0.001	0.02 ^α	0.002	1.50 ^α	0.041
	14	N/D	-	0.10 ^α	0.006	0.02 ^α	0.001	0.18 ^α	0.013	0.10 ^α	0.006	0.25 ^α	0.023	0.02 ^γ	0.001	0.02 ^α	0.002	1.59 ^α	0.099
	21	N/D	-	0.10 ^α	0.015	0.02 ^α	0.003	0.19 ^α	0.026	0.10 ^α	0.015	0.23 ^α	0.029	N/D	-	0.03 ^α	0.010	1.65 ^α	0.260
	28	N/D	-	0.09 ^α	0.026	0.02 ^α	0.007	0.16 ^α	0.047	0.09 ^α	0.026	0.22 ^α	0.083	0.03 ^α	-	0.02 ^α	0.003	1.46 ^α	0.449
Na-O+	0	N/D	-	0.07 ^C	0.002	0.02 ^A	0.001	0.15 ^A	0.006	0.09 ^A	0.004	0.22 ^A	0.009	0.02 ^A	0.001	0.01 ^A	0.001	1.28 ^{AB}	0.111
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	N/D	-	0.08 ^C	0.004	0.02 ^A	0.001	0.17 ^{AB}	0.008	0.09 ^A	0.004	0.23 ^A	0.010	0.02 ^A	0.001	0.01 ^A	0.001	1.42 ^A	0.072
	7	N/D	-	0.09 ^C	0.003	0.02 ^A	0.001	0.17 ^A	0.006	0.09 ^A	0.004	0.24 ^A	0.011	0.02 ^A	0.001	0.02 ^A	0.001	1.47 ^A	0.037
	14	0.03 ^A	0.003	0.18 ^A	0.010	N/D	-	0.14 ^A	0.010	0.03 ^B	0.003	0.04 ^B	0.004	N/D	-	0.01 ^A	0.001	1.25 ^{AB}	0.109
	21	0.03 ^A	0.004	0.15 ^B	0.025	N/D	-	0.10 ^C	0.015	0.02 ^B	0.005	0.03 ^B	0.003	N/D	-	0.01 ^A	0.002	1.10 ^B	0.137
	28	0.02 ^A	0.002	0.15 ^B	0.013	N/D	-	0.10 ^C	0.010	0.02 ^B	0.003	0.02 ^B	0.002	N/D	-	0.01 ^A	0.001	1.00 ^B	0.078

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 20 Unsaponifiable composition (% w/w dry rubber) of RRIM600 lipid extracts obtained from 3 conditions during 28 days of maturation.

Conditions	Days	Components													
		C18-OH		C20-OH		γ -tocotrienol		α -tocotrienol		Stigmasterol		β -sitosterol		$\Delta 5$ -avenasterol	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	0.02 ^a	0.002	0.01 ^a	0.001	0.15 ^a	0.021	0.03 ^a	0.001	0.08 ^a	0.001	0.49 ^a	0.003	0.08 ^a	0.003
	3	0.02 ^a	-	0.01 ^a	-	0.09 ^a	-	0.03 ^a	-	0.08 ^{ab}	-	0.52 ^{ab}	-	0.08 ^a	-
	5	N/D	-	0.01 ^a	0.001	0.11 ^a	0.022	0.03 ^a	0.001	0.07 ^{ab}	0.007	0.46 ^{ab}	0.043	0.07 ^a	0.002
	7	0.02 ^a	0.003	0.01 ^a	0.001	0.11 ^a	0.025	0.03 ^a	0.003	0.07 ^{ab}	0.004	0.45 ^{ab}	0.018	0.07 ^a	0.005
	14	0.02 ^a	0.002	0.01 ^a	0.001	0.12 ^a	0.065	0.03 ^a	0.006	0.08 ^a	0.004	0.53 ^a	0.025	0.08 ^a	0.012
	21	0.01 ^a	0.002	0.01 ^a	0.001	0.12 ^a	0.049	0.02 ^a	0.009	0.05 ^b	0.017	0.32 ^b	0.118	0.05 ^a	0.021
	28	0.02 ^a	0.002	0.01 ^a	0.002	0.14 ^a	0.039	0.03 ^a	0.005	0.08 ^a	0.005	0.49 ^a	0.023	0.08 ^a	0.008
Na-O-	0	0.02 ^{α}	0.001	0.01 ^{α}	0.000	0.12 ^{α}	0.029	0.03 ^{β}	0.002	0.08 ^{$\alpha\beta$}	0.003	0.65 ^{α}	0.016	0.08 ^{α}	0.006
	3	0.02 ^{α}	0.001	0.01 ^{α}	0.001	0.10 ^{α}	0.006	0.03 ^{β}	0.002	0.07 ^{$\alpha\beta$}	0.003	0.61 ^{α}	0.028	0.07 ^{α}	0.005
	5	0.02 ^{α}	0.003	0.01 ^{α}	0.002	0.18 ^{α}	0.066	0.05 ^{α}	0.008	0.11 ^{α}	0.008	0.52 ^{α}	0.037	0.09 ^{α}	0.011
	7	0.02 ^{α}	0.002	0.01 ^{α}	0.001	0.16 ^{α}	0.029	0.04 ^{$\alpha\beta$}	0.004	0.08 ^{$\alpha\beta$}	0.009	0.49 ^{α}	0.064	0.08 ^{α}	0.011
	14	0.02 ^{α}	0.001	0.01 ^{α}	0.001	0.13 ^{α}	0.053	0.04 ^{$\alpha\beta$}	0.009	0.07 ^{$\alpha\beta$}	0.007	0.48 ^{α}	0.034	0.07 ^{α}	0.009
	21	0.02 ^{α}	0.002	0.01 ^{α}	0.000	0.17 ^{α}	0.034	0.05 ^{$\alpha\beta$}	0.003	0.10 ^{$\alpha\beta$}	0.005	0.46 ^{α}	0.024	0.09 ^{α}	0.006
	28	0.02 ^{α}	0.004	0.01 ^{α}	0.003	0.12 ^{α}	0.076	0.04 ^{$\alpha\beta$}	0.005	0.06 ^{β}	0.041	0.38 ^{α}	0.253	0.09 ^{α}	0.008
Na-O+	0	0.02 ^A	0.001	0.02 ^A	0.0001	0.12 ^A	0.029	0.03 ^A	0.002	0.08 ^A	0.003	0.49 ^{AB}	0.016	0.08 ^{AB}	0.006
	3	0.02 ^A	0.001	0.01 ^B	0.0002	0.13 ^A	0.025	0.03 ^A	0.002	0.07 ^{AB}	0.003	0.49 ^{Ab}	0.020	0.07 ^{AB}	0.006
	5	0.02 ^A	0.001	0.01 ^B	0.0002	0.04 ^A	0.015	0.03 ^A	-	0.08 ^A	0.006	0.52 ^A	0.039	0.08 ^A	0.006
	7	0.02 ^A	0.001	0.01 ^b	0.0005	0.13 ^A	0.034	0.03 ^A	0.006	0.08 ^A	0.010	0.49 ^{Ab}	0.061	0.08 ^{AB}	0.009
	14	0.02 ^A	-	N/D	-	0.07 ^A	0.045	0.02 ^A	0.009	0.06 ^B	0.007	0.39 ^B	0.047	0.06 ^B	0.003
	21	N/D	-	0.01 ^B	-	0.11 ^A	0.070	0.03 ^A	0.007	0.07 ^{AB}	0.005	0.39 ^B	0.017	0.07 ^{AB}	0.000
	28	0.02 ^A	0.000	N/D	-	0.08 ^A	0.032	0.03 ^A	0.006	0.07 ^{AB}	0.004	0.42 ^{AB}	0.033	0.06 ^B	0.008

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test, $P \leq 0.05$). SD: standard deviation. N/D: not detected.

Table 21 Unsaponifiable composition (% w/w dry rubber) of PB235 lipid extracts obtained from 3 conditions during 28 days of maturation

Conditions	Days	Components													
		C18-OH		C20-OH		γ -tocotrienol		α -tocotrienol		Stigmasterol		β -sitosterol		$\Delta 5$ -avenasterol	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na+O-	0	0.03 ^a	0.003	0.02 ^a	0.001	0.09 ^a	0.007	0.04 ^a	-	0.03 ^a	0.002	0.23 ^a	0.012	0.07 ^{ab}	0.004
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	0.03 ^a	0.002	0.02 ^a	0.001	0.10 ^a	0.003	0.04 ^a	-	0.02 ^a	0.004	0.21 ^a	0.028	0.07 ^{ab}	0.004
	7	0.01 ^a	0.000	0.02 ^a	0.000	0.07 ^a	0.006	N/D	-	N/D	-	0.10 ^a	0.006	0.06 ^b	0.000
	14	0.02 ^a	0.002	0.02 ^a	0.003	0.04 ^a	0.003	N/D	-	0.02 ^a	0.004	0.22 ^a	0.018	0.07 ^{ab}	0.005
	21	0.04 ^a	0.013	0.02 ^a	0.004	0.12 ^a	0.072	0.06 ^a	0.008	0.04 ^a	0.019	0.32 ^a	0.184	0.09 ^a	0.023
	28	0.03 ^a	0.009	0.02 ^a	0.002	0.11 ^a	0.033	0.04 ^a	0.002	0.04 ^a	0.006	0.28 ^a	0.049	0.07 ^{ab}	0.002
Na-O-	0	0.03 ^{αβ}	0.004	0.02 ^α	0.000	0.11 ^{αβ}	0.003	N/D	-	0.03 ^α	0.001	0.22 ^{αβ}	0.003	0.07 ^{αβ}	0.002
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	0.01 ^β	0.004	0.02 ^α	0.001	0.05 ^β	0.002	N/D	-	N/D	-	0.09 ^β	0.003	0.06 ^β	0.003
	7	0.02 ^{αβ}	0.004	0.02 ^α	0.001	0.05 ^{αβ}	0.015	N/D	-	0.02 ^α	0.002	0.24 ^{αβ}	0.009	0.08 ^{αβ}	0.004
	14	0.04 ^α	0.004	0.02 ^α	0.001	0.09 ^{αβ}	0.045	0.05 ^α	0.006	0.04 ^α	0.009	0.38 ^α	0.102	0.09 ^{αβ}	0.009
	21	0.04 ^α	0.004	0.02 ^α	0.000	0.15 ^α	0.043	0.04 ^α	0.006	0.05 ^α	0.004	0.40 ^α	0.034	0.10 ^α	0.011
	28	0.03 ^{αβ}	0.003	0.02 ^α	0.004	0.09 ^{αβ}	0.039	0.04 ^α	0.005	0.04 ^α	0.008	0.34 ^α	0.102	0.08 ^{αβ}	0.024
Na-O+	0	0.03 ^A	0.000	0.02 ^A	0.001	0.11 ^A	0.003	N/D	-	0.03 ^A	0.001	0.22 ^{AB}	0.003	0.07 ^A	0.002
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	0.02 ^A	0.012	0.02 ^A	-	0.07 ^A	0.036	N/D	-	0.03 ^A	-	0.15 ^B	0.106	0.07 ^A	0.015
	7	0.03 ^A	0.004	0.02 ^A	0.003	0.07 ^A	0.039	0.04 ^A	-	0.03 ^A	0.003	0.20 ^{AB}	0.101	0.07 ^A	0.014
	14	0.02 ^A	0.010	0.02 ^A	0.001	0.06 ^A	0.031	0.04 ^A	-	0.03 ^A	0.015	0.25 ^{AB}	0.085	0.07 ^A	0.005
	21	0.03 ^A	0.003	0.02 ^A	0.002	0.13 ^A	0.078	0.04 ^A	0.012	0.04 ^A	0.005	0.39 ^A	0.021	0.08 ^A	0.018
	28	0.03 ^A	0.001	0.02 ^A	0.001	0.11 ^A	0.042	0.03 ^A	0.002	0.04 ^A	0.001	0.30 ^{AB}	0.024	0.07 ^A	0.008

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test, $P \leq 0.05$). SD: standard deviation. *N/D*:not detected.

Table 22 Absolute initial slope value of RRIM600 and PB235 lipid extracts obtained from 3 conditions during 28 days of maturation.

Maturation time (days)	RRIM600 clone						PB235 clone					
	Conditions						Conditions					
	Na+O-		Na-O-		Na-O+		Na+O-		Na-O-		Na-O+	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	10.9 ^c	0.17	13.4 ^{abc}	0.07	13.4 ^{abc}	0.07	11.3 ^a	0.23	11.0 ^a	1.41	11.0 ^a	1.41
3	11.2 ^{bc}	1.03	12.7 ^{abc}	0.05	11.1 ^{bc}	1.22	-	-	-	-	-	-
5	12.7 ^b	0.16	10.2 ^d	0.51	10.5 ^c	0.52	10.5 ^{bc}	0.16	8.94 ^a	1.13	6.92 ^b	0.35
7	14.8 ^a	0.26	12.0 ^{bcd}	1.46	12.0 ^{bc}	0.23	11.2 ^{ab}	0.12	8.29 ^a	0.03	8.48 ^{ab}	0.71
14	15.1 ^a	0.25	11.9 ^{cd}	0.88	15.9 ^a	0.81	9.60 ^d	0.21	9.23 ^a	1.07	9.70 ^{ab}	0.70
21	14.9 ^a	0.39	14.5 ^{ab}	0.38	15.4 ^a	0.96	10.3 ^{cd}	0.25	4.14 ^b	1.32	10.8 ^a	0.78
28	15.1 ^a	0.09	14.8 ^a	0.47	13.9 ^{ab}	1.06	10.6 ^{abc}	0.09	9.13 ^a	0.26	10.4 ^a	0.52

Data were mean from 3 replications. For each column, letters design group of means which are not significantly different (student *t* test, $P \leq 0.05$). SD: standard deviation.