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STUDY OF THE EFFECTS OF BIOLOGICAL MATURATION OF COAGULA OF *HEVEA BRASILIENSIS* LATEX ON DRY RUBBER PROPERTIES

by

Jutharat INTAPUN

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Jury

Ms. BANERJEE, R.	Professor, Indian Institute of Technology, Kharagpur,	Reviewer
M. PILARD, J.F.	Professor, Universite du Maine	Reviewer
M. DUBREUCQ, E.	Professor, Montpellier SupAgro	Examiner
Ms. PHONGPAICHIT, S.	Assoc. Professor, Prince of Songkla University	Examiner
Ms. TANRATTANAKUL, V.	Assoc. Professor, Prince of Songkla University	Examiner
M. VAYSSE, L.	Researcher, CIRAD	Examiner
M. SAINTE-BEUVE, J.	Researcher, CIRAD	Invited Examiner

Thesis Directors: Eric DUBREUCQ and Varaporn TANRATTANAKUL

Titre de la thèse	Etude des effets de la maturation biologique des coagula de latex d'*Hevea brasiliensis* sur les propriétés du caoutchouc naturel sec.
Auteur	Melle Jutharat Intapun
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Résumé

L'objectif de ce travail était d'étudier le phénomène de maturation des coagula de latex d'*Hevea brasiliensis*. La stratégie expérimentale a consisté dans un premier temps à caractériser les conditions de maturations régnant sur un site industriel (température, humidité relative, teneur en oxygène de l'air), puis de reproduire ces conditions de maturation à l'échelle du laboratoire dans le but de pouvoir, dans une troisième phase, étudier l'effet de paramètres clés tels que la population microbienne ou l'activité de diverses enzymes sur les propriétés du caoutchouc naturel obtenu à partir de ces coagula.

Les conditions de maturation des coagula de latex empilés sur un site industriel ont été caractérisées. Ceci a nécessité la mise au point d'un matériel expérimental spécifique permettant des mesures fiables et répétables. Nous avons montré que la température et l'humidité relative de l'air au sein du tas augmentent avec la profondeur, alors que la teneur en oxygène diminue jusqu'à des conditions d'anaérobiose. Après 24 jours de maturation dans ces conditions, les propriétés des coagula (pH) et du caoutchouc obtenu (P_0, PRI, masse moléculaire moyenne) dépendent de la position des coagula au sein de la pile.

L'étude de la maturation des coagula dans des conditions de laboratoire contrôlées a nécessité des développements méthodologiques et techniques aboutissant à la mise en place et à l'optimisation d'un dispositif de maturation contrôlée associé à un procédé miniaturisé de production de caoutchouc sec comprenant une étape de crêpage et un séchage au four. Ce dispositif a été confronté au système réel en vérifiant que la réduction d'échelle n'introduisait pas de biais dans les propriétés des

produits étudiés. Le dispositif de maturation contrôlée est constitué de 6 unités pouvant fonctionner en parallèle, chacune d'entre elle pouvant contenir jusqu'à 18 échantillons. Les unités sont contrôlées en humidité relative (10%-90%), température (35°C - 45°C) et teneur en oxygène (0-21%). Sur la base des mesures réalisées sur site industriel, les études on été réalisées à 40°C±1°C et 90%±10% d'humidité relative dans le dispositif de maturation.

Nous avons ensuite démontré le rôle des microorganismes dans la maturation des coagula de latex et leur impact sur les propriétés du caoutchouc naturel sec en comparant les propriétés physiques et structurales, avant et après maturation, d'échantillons de caoutchouc issus de coagula inoculés avec des quantités variables de microorganismes provenant d'un coagulum industriel. Cette étude a mis en évidence un effet-dose des microorganismes sur ces propriétés.

Les microorganismes contenus dans l'inoculum comprenaient des levures et des bactéries gram positives et gram négatives, dont des bactéries lactiques. Quinze des seize souches isolées produisaient des enzymes hydrolytiques, notamment à activité lipase, phospholipase et protéase, qui pourraient participer à la dégradation de lipides et protéines au sein du latex et des coagula.

L'addition de lipase pure à des coagula dans lesquels le développement des microorganismes était inhibé a provoqué la libération d'acides gras sans toutefois modifier les propriétés du caoutchouc après 6 jours de maturation. Par contre, l'addition de papaïne a provoqué une amélioration de la valeur de la viscosité initiale (P_0) des échantillons directement liée à la quantité d'enzyme ajoutée. L'activité de cette protéase a également entraîné une augmentation de la résistance à la thermooxydation (PRI) du caoutchouc.

Les méthodologies et outils développés au cours de se travail devraient permettre le développement de futures recherches visant à comprendre le rôle des microorganismes et des enzymes dans la variabilité de la qualité du caoutchouc naturel.

ชื่อวิทยานิพนธ์	การศึกษาผลทางชีววิทยาในกระบวนการบ่มยางก้อนถ้วยจากน้ำยาง *Hevea brasiliensis* ต่อสมบัติของยางแห้ง
ผู้เขียน	นางสาวจุฑารัตน์ อินทปัน
สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีพอลิเมอร์ (PSU) เทคโนโลยีชีวภาพ-จุลชีววิทยา (Montpellier SupAgro)
ปีการศึกษา	2552

บทคัดย่อ

วัตถุประสงค์ของงานวิจัยนี้ เพื่อศึกษาปรากฏการณ์ที่เกิดขึ้นจากการบ่มยางก้อนถ้วยที่ผลิตจากน้ำยางธรรมชาติ *Hevea brasiliensis* โดยในขั้นแรกได้ทำการศึกษาถึงสภาวะแวดล้อม (อุณหภูมิ ปริมาณความชื้นสัมพัทธ์ และปริมาณออกซิเจน) ในระบบการบ่มกองยางก้อนถ้วยในระดับอุตสาหกรรม และจากนั้นได้จำลองชุดการบ่มในห้องปฏิบัติการ เพื่อใช้ในการศึกษาผลของปัจจัยที่เกี่ยวข้องในการบ่ม เช่น สภาวะการบ่ม จุลินทรีย์ และ เอนไซม์ ที่มีผลต่อสมบัติของยาง

ในการศึกษาผลของสภาวะการบ่มต่อยางก้อนถ้วยในระดับอุตสาหกรรม ชุดอุปกรณ์การเก็บข้อมูลสภาวะการบ่มยางในระดับอุตสาหกรรมได้ถูกสร้างขึ้นและมีการพัฒนาเพื่อให้สามารถเก็บข้อมูลสภาวะการบ่มได้อย่างแม่นยำและสามารถทำซ้ำได้ จากการเก็บข้อมูลพบว่า อุณหภูมิและปริมาณความชื้นสัมพัทธ์ของอากาศภายในกองยางก้อนถ้วยเพิ่มสูงขึ้นตามระดับความลึกของกองยาง ในทางตรงกันข้ามปริมาณออกซิเจนภายในกองกลับลดลง นอกจากนี้เมื่อทำการวัดสมบัติของยางก้อนถ้วย(ค่าความเป็นกรด-ด่าง (pH) ค่าความยืดหยุ่นเริ่มต้น (P_0) ค่าดัชนีความยืดหยุ่นของยาง (PRI) ปริมาณเจล และน้ำหนักโมเลกุลเฉลี่ย) เมื่อผ่านการบ่มเป็นเวลา 24 วัน พบว่าสมบัติเหล่านี้มีการเปลี่ยนแปลงไปจากวันแรกโดยขึ้นอยู่กับตำแหน่งการบ่มภายในกองยาง (ความลึกจากผิวบนสุดของกองยางในระดับ 0, 50, 100, 150 และ 200 เซนติเมตร)

ได้ทำการพัฒนาชุดเครื่องมือและวิธีการควบคุมสภาวะการบ่มในห้องปฏิบัติการ จากการทดสอบพบว่าสามารถควบคุมสภาวะการบ่มต่างๆได้ เช่น การควบคุมปริมาณออกซิเจน อุณหภูมิและปริมาณความชื้นสัมพัทธ์ ได้ทำการทดสอบสภาวะที่ต้องใช้ในการแปรรูป (การบดและการอบยาง) ของยางก้อนถ้วยในระดับห้องปฏิบัติการที่มีขนาด 45 มิลลิลิตร นอกจากนี้พบว่าการลดขนาดยางก้อนถ้วยจากขนาดปกติประมาณ 450 มิลลิลิตร เป็นขนาดเล็กดังกล่าวไม่มีผลกระทบต่อสมบัติของยางก้อนถ้วย ได้สร้างชุดบ่มในห้องปฏิบัติการทั้งหมด 6 หน่วย ซึ่งแต่ละหน่วยสามารถทำการบ่มยางก้อนถ้วยได้จำนวน 18 ก้อน จากการทดสอบการปรับสภาวะการบ่มในชุดบ่ม โดยผสมอากาศแห้งเข้ากับอากาศอิ่มตัว ภายในอุณหภูมิต่างๆ พบว่าสามารถปรับค่าได้ในช่วงอุณหภูมิ 35-45°C ปริมาณความชื้นสัมพัทธ์ 10-90% และออกซิเจน อยู่ในช่วง

0-21% โดยสภาวะที่เลือกใช้ในการศึกษาในห้องปฏิบัติการคือ อุณหภูมิ 40±1°C และปริมาณความชื้นสัมพัทธ์ 90%±10% เนื่องจากพบว่าเป็นสภาวะที่ส่งผลให้ยางก้อนถ้วยเกิดการเปลี่ยนแปลงสมบัติอย่างชัดเจนเมื่อทำการบ่มในระดับอุตสาหกรรม นอกจากนี้พบว่าการแปรรูปยางก้อนถ้วยในระดับห้องปฏิบัติการ โดยใช้ชุดบดขนาดเล็กที่ได้สร้างขึ้นในห้องปฏิบัติการร่วมกับการอบแห้งในตู้อบลมร้อน สามารถควบคุมให้สมบัติของยางไม่แตกต่างจากการแปรรูปในระดับอุตสาหกรรม โดยใช้จำนวนรอบบดเท่ากับ 16 รอบและทำการอบด้วยอุณหภูมิ 125°C เป็นเวลา 2 ชั่วโมง

เพื่อประเมินผลของเชื้อจุลินทรีย์ที่เกี่ยวข้องในขั้นต้นของการบ่มยางก้อนถ้วย ได้ทำการศึกษาผลของการเติมหัวเชื้อจุลินทรีย์ (Inoculum) ที่ปริมาณต่าง ๆกัน ต่อการเปลี่ยนแปลงสมบัติของยางธรรมชาติ พบว่าปริมาณเชื้อจุลินทรีย์ส่งผลอย่างชัดเจนต่อการเปลี่ยนแปลงสมบัติทางกายภาพและสมบัติทางโมเลกุลของยางธรรมชาติ โดยพบว่าปริมาณเชื้อจุลินทรีย์ที่สูงขึ้นนอกจากส่งผลต่อการเกิดออกซิเดชันของยางแล้วยังส่งผลต่อการเกิดการเชื่อมโยงสายโซ่โมเลกุลของยางเช่นกัน ภายใต้การบ่มในสภาวะที่ไม่มีออกซิเจนพบว่ายางธรรมชาติที่เตรียมจากการใช้กรดฟอร์มิกและจากการจับตัวแบบธรรมชาติ มีการเปลี่ยนแปลงสมบัติตามระยะเวลาการบ่มในลักษณะเดียวกัน อย่างไรก็ตามภายใต้สภาวะการบ่มที่มีออกซิเจนระบบการจับตัวทั้งสองแบบส่งผลให้ยางมีสมบัติแตกต่างกัน ซึ่งเห็นผลได้ชัดเจนต่อค่าความยืดหยุ่นเริ่มต้นของยาง ในขณะที่ค่าดัชนีความยืดหยุ่นเกิดการลดลงในช่วงต้นของการบ่มทั้งในสภาวะที่มีและไม่มีออกซิเจน

จากการวิเคราะห์หัวเชื้อจุลินทรีย์ที่ใช้ในการผสมลงในน้ำยาง พบว่าประกอบด้วยเชื้อจุลินทรีย์ทั้งหมดจำนวน $1x10^9 \pm 0.33 \times 10^9$ CFU/mL ซึ่งประกอบด้วย ยีสต์ แบคทีเรียแกรมบวก แบคทีเรียแกรมลบ และ แลคติกเอสิคแบคทีเรีย เมื่อทดสอบการผลิตเอนไซม์ไฮโดรเลสในอาหารเลี้ยงเชื้อเฉพาะ พบว่าแบคทีเรียจำนวน 15 สายพันธุ์ จาก 16 สายพันธุ์สามารถผลิตเอนไซม์ไฮโดรเลส เช่น ไลเปส ฟอสโฟไลเปส และ โปรติเอส ดังนั้นเป็นไปได้ว่าการเปลี่ยนแปลงสมบัติของยางธรรมชาติ ในช่วงเวลาของการบ่มมีความเกี่ยวข้องกับการย่อยกลุ่มโปรตีนและกลุ่มไขมันในยางก้อนถ้วย เมื่อทำการเติมเอนไซม์ไลเปสบริสุทธิ์ที่ความเข้มข้นต่าง ๆ ลงในน้ำยาง พบว่าปริมาณกรดไขมันอิสระที่เกิดจากการย่อยของไลเปสเพิ่มสูงขึ้น แต่ไม่ส่งผลอย่างมีนัยสำคัญต่อค่าดัชนีความยืดหยุ่นของยางธรรมชาติ เมื่อทำการบ่มเป็นระยะเวลา 6 วัน ผลแสดงชัดเจนมากขึ้นเมื่อเติมเอนไซม์โปรติเอสที่เติมลงไปในยาง พบว่าส่งผลให้ค่าความยืดหยุ่นเริ่มต้นและดัชนีความยืดหยุ่นเพิ่มสูงขึ้น

จากการพัฒนาชุดเครื่องมือและวิธีการทดลองในงานวิจัยนี้สามารถนำไปใช้เป็นชุดเครื่องมือพื้นฐาน สำหรับการศึกษาในอนาคต เพื่อให้เข้าใจบทบาทของเชื้อจุลินทรีย์และเอนไซม์ต่อสมบัติของยางธรรมชาติได้ดียิ่งขึ้น

Thesis Title	Study of the effect of biological maturation of coagula of *Hevea brasiliensis* latex on dry rubber properties.
Author	Miss Jutharat Intapun
Major programs	Biotechnology-Microbiology (Montpellier-SupAgro) Polymer Science and Technology (PSU)
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Abstract

The aim of this work was to study the maturation phenomena undergone by cup coagula of *Hevea brasiliensis* latex. The experimental strategy was to first characterize the maturation industrial conditions (temperature, relative humidity and oxygen content of the air), secondly to reproduce this maturation at laboratory scale in order to, thirdly, be able to study the effect of key determinants such as microbial population or enzymatic activity on processed natural rubber properties.

The maturation conditions of cup rubber coagula stored as a pile in an industrial plant have been characterized. This requested an optimization of the experimental material used to measure conditions in order to get repeatable and consistent data. It was found that temperature and relative humidity of the air increased with depth within the pile. In contrast, oxygen content of the air within the pile decreased as depth increased. Effects of 24-day maturation in these conditions on rubber properties (pH, P_0, PRI and Weight-average molar mass) were found to vary with the position of cup coagula within the pile.

In order to be able to study maturation of coagula in laboratory controlled condition, methodological and technical developments were performed and concerned the design and optimization of a maturation device as well as of a dry rubber process (creping + drying). Before the development of devices, it was checked that the reduction of cup coagula size for practical experimental reasons would not induce any artifact. The developed maturation device in laboratory was set up with 6 maturation units, each of them being able to contain up to 18 mini-cup coagula. An assessment of the regulation of the maturation unit atmosphere showed that the developed device allowed the control of conditions in the following ranges: relative humidity

10% - 90%, temperature 35°C - 45°C and oxygen content 0%-21%. Referring to the study of the conditions in cup coagula pile, it was decided to work at 40°C±1°C and 90%±10% RH in the maturation device. The laboratory process parameters were optimized in order to obtain a rubber with the same quality as if it was processed in factory practice.

In order to assess the involvement of microorganisms in the initial stage of maturation of natural rubber cup coagula, the inoculated rubber was characterized for its physical and structural properties for different maturation times. The results showed that the quantity of microorganisms significantly affected the physical properties and molecular structure (Average-molar mass and gel content) of processed rubber. Microorganisms are not only involved in the increase of sensitivity to thermo-oxidation but also in the crosslinking phenomenon between polyisoprene chains. The total number of microorganisms in the inoculum was around $1x10^9$ CFU/mL, among which were yeasts, gram positive, gram negative and lactic acid bacteria. Fifteen of the 16 bacterial strains isolated from the inoculum used in latex treatments produced hydrolytic enzymes such as lipase, phospholipase and protease when inoculated in specific media, thus potentially participating to lipid and protein degradation in latex and coagula. The addition of lipase in mini-cup coagula resulted in an increased release of free fatty acids but had no significant effect on PRI evolution during the first 6 days of maturation. The main effect was observed in the presence of a protease, papain, which showed a clear positive dose-effect on enzyme concentration on P_0. This enzyme also had an improving effect on PRI.

The methodologies and devices developed during this study should serve as a basis for further studies aiming at a better understanding of the role of microorganisms and enzymes in the variability of quality of natural rubber.

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GENERAL INTRODUCTION

Thailand is the major exporter of raw natural rubber in the world. Recently, 3.02 millions metric tons natural rubber was produced in Thailand, while 2.56 millions metric tons were exported (RRIT, 2008). In fact, natural rubber (NR) currently accounts for more than 40% of the world's elastomer consumption, due to its incomparable properties over synthetic elastomers such as elasticity, crystallization capacity under stress, low internal heat build-up and tackiness. Natural rubber is the raw material used for some vehicle parts, such as tyres and shock absorbers, the construction sector (joints, shock absorber, waterproof joint) and health products (gloves, condoms, catheter) (Burger and Smith, 2002; Budiman, 2002).

About 88 % of the raw NR produced in the world is marketed in dry form; the remaining 12 % is marketed as concentrated latex. In the international market, the dry forms are available in over 40 grades, consisting of technically specified rubber (TSR) in block form, non-TSR grades such as crepes, which include latex-based and field coagulum-based crepes and sheet rubber (Cecil and Mitchell, 2005). NR must be technically specified and graded for its wide range of industrial applications. The overall quality of the raw material determines the grade of the block rubber and its suitability for manufacturing different products. Of the various grades of TSR, TSR 20 is the most commonly exported grade for the top exporting countries. In TSR 20 rubber processing, the raw material is cup coagula (or cuplump) blended or not with low grade rubber sheets. Cup coagula are freshly coagulated rubber. They present variation the qualities related to their origin and collection methods between tapping and processing.

The variation in the qualities of cup coagula causes problems with processing and grading for TSR factories. In industrial practice, cup coagula are stored for a variable period of time before processing i.e. 2-3 weeks. This storage time is called “maturation period”. Currently, TSR 20 factories have also identified empirically a positive effect of a maturation of cup coagula to improve qualities such as Plasticity

Retention Index (PRI) of cup coagula before their final processing. PRI is a parameter defining the processing and grading of raw natural rubber. TSR 20 standard requires a minimum PRI value of 40. The PRI is an indicator of raw rubber susceptibility to thermo-oxidative breakdown. It is determined by Wallace method which evaluates the plasticity of rubber samples before and after ageing in a controlled atmosphere. Low PRI of cup coagula during storage has been reported to be caused by bacterial decomposition of proteins and other non-rubber constituents which can be linked with release of free copper, an active pro-oxidant (Bateman and Sekher, 1966; Hasma and Othman, 1990). Various earlier studies showed the effects of maturation and storage of naturally coagulated latex on some bulk rheological properties and on parameters characterizing the macromolecular chain length. Previous results highlighted the effect of bulk processing parameters at a macromolecular level (Ekpini *et al.*, 2001; Ehabe *et al*., 2002).

The coagulation and maturation of cup coagula are very complex phenomena. The following of endogeneous or heterogeneous metabolic activities inside the serum and the rubber itself are interesting. Latex is an extremely favorable medium for the development of bacteria. Microorganisms use enzymes in the biochemical pathways for growth, and some of these enzymes may participate to the initiation of coagulation. Hanower and coworkers (1977) studied enzymes involved in the coagulation of latex: tyrosinase (polyphenoloxydase), phospholipase C and D. Tyrosinase was found in *Hevea* latex lutoid fraction (Wititsuwannakul *et al*., 2002). Generally, bacteria can enter latex at an early state of tapping including by colonizing laticifers. Taysum (1969) reported the regular occurrence in NR latex of microaerophilic bacteria species usually producing acid. He isolated both *Streptococcus* and *Lactobac*illus in NR latex.

Microbial development in latex and cup coagula has been the subject of various works that were mainly descriptive. However, current scientific knowledge regarding the role of microorganisms and enzymes in this maturation process still is very limited, and these biological activities are not controlled at all. Moreover, latex and cup coagula are complex substances, from both biochemical and structural point

of views (heterogenous solid/liquid media) and the biological and physico-chemical phenomena and changes in structure of NR involved in maturation time are mostly unknown.

The purpose of this research was to investigate the effect of physico-chemical parameters which are involved during the maturation of cup coagula, focusing especially on microorganisms and enzymatic activities and their relationships with processed rubbers properties (P_0, PRI, molar mass and gel content). The study started with laboratory controlled maturation experiments to identify the effect of main physico-chemical parameters (time, temperature, relative humidity and oxygen content) on cup coagula properties. Then, the effect of microorganism and selected enzyme activities on cup coagula properties was studied. This knowledge will then be used for the conception of a protocol permitting the control of microbiological and enzyme activities in the industrial process of TSR 20.

The first part of this thesis consists in a bibliographic review on general natural rubber, TSR processing, post harvested of natural rubber, biological activity involving harvested natural rubber latex, field coagula maturation and their consequence on the properties. This review provides a general idea of the involvement of physical-chemical and biological parameters that concern in cup coagula maturation and their effects on properties.

REVIEW OF LITERATURE

The first parts of our bibliographic study will focus on general information about natural rubber from *Hevea brasiliensis.* The second part concerns primary rubber processing including raw rubber material and Technically Standard Rubber (TSR) processing. In the third part, biological activity of harvested latex is reviewed. The last part concerns field coagula maturation and the factors affecting the evolution of cup coagula properties.

1. Natural rubber

Although approximately 2,000 plants are known to synthesize poly (*cis*-1, 4-isoprene), only natural rubber of *Hevea brasiliensis* (99% of the world market) and guayule rubber of *Parthenium argentatum* (1% of the world market) are produced commercially (Tanaka and Sakdapipanich, 2001). Natural rubber (NR) primarily comprises polyisoprene and is obtained from the milky white latex of a number of species of plants that flourish in the tropics and all plants are from the Spurge family (*Euphorbiaceae*).

1.1 History and present natural rubber production

Natural rubber originally derived from the rubber tree *(Hevea brasiliensis)* in South America, *Hevea Brasiliensis* originated in Brazil, where it was viewed as a very significant crop by the Brazilian government. In 1876, the British Government sent Sir Henry Wickham to investigate the native plants and trees of the Amazonian forests. Wickham brought back some *Hevea* seeds and a few were subsequently made to germinate at the botanical research centre in Kew Gardens, London. This was the beginning of the end for the rubber barons. Soon after the *Hevea* plants were shown to be viable in the UK, the British exported some live plants to Sri Lanka (Ceylon as it was then known) and started some plantations. By the 1890s *Hevea* trees were being planted throughout Sri Lanka and all over the Malayan peninsula.

The rubber tree has achieved considerable commercial importance. Although the production of natural rubber began in the Americas, the introduction of *Hevea brasiliensis* seedling by the British into other countries has led to predominance of the industry in Southeast Asia. Production of rubber from plantations expanded during World War II, and plantation rubber is still produced in Latin America but the production is overshadowed by that from Southeast Asia (Subramaniam, 1987; Thomas and Panikkar, 2000).

Rubber tree is an important industrial crop for natural rubber production. Latex comes from the *Hevea brasiliensis* tree, which grow in tropical regions. They typically reach 20-30 meters height in rubber plantations, and are able to produce commercial quantities of latex at about 7 years of age, depending on climate and location. *Hevea* latex was collected through tapping of trees in the natural forest.

Global rubber yearly production fell from 22.7 million tonnes at the end of 2008 to 21.8 million tonnes in March 2009. The sharp reduction is mainly due to a decline in SR output, which fell to 12.2 million tonnes in March 2009 compared to over 12.8 million tonnes in December 2008. Global yearly NR production is estimated to have fallen from 9.9 million tonnes at the end of 2008 to 9.6 million tonnes in March 2009 (IRSG, 2009).

Presently, around 93% of the world production comes from Asia, Thailand, Malaysia and Indonesia are the largest producers of natural rubber in the world. The major producers being Thailand (31.0%), Indonesia (28.3%), Malaysia (12.1%) and India (8.2%) as presented in Figure 1.

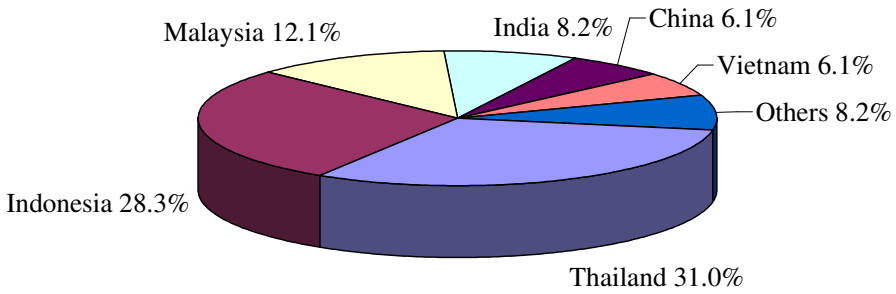


Figure 1 World natural rubber production shares in 2008 (IRSG, 2009).

Global natural rubber consumption declined by over 0.3 million tonnes between September and Decembe in 2008, with consumption growth reversing from 2.1% to -3.4% over the period. The top 10 consuming countries in the world are China, USA, Japan, India, Malaysia, Korea, Indonesia, Brazil, Thailand and Germany. Among the 10 countries 7 countries are in Asia. The world supply of natural rubber is barely keeping up with a global demand for 12 million tons of natural rubber in 2020 (IRSG, 2009).

1.2 Natural rubber latex

Latex is the protective fluid contained in tissue of the bark of the rubber tree. Natural rubber latex (NRL) is a cloudy white liquid collected by tapping a thin strip of bark from the tree and allowing the latex flow into a collection cup over a period of several hours.

1.2.1 Tapping and latex collection

Rubber trees are usually tapped by cutting a spiral groove in the bark halfway or more around the stem, as deep as possible but without injuring the tree's cambium growth. The tapping corresponds to a wound in the tree bark. Only a thin slice of bark, 2-3 mm thick is shaved off to cut open the laticiferous cells in the so called soft bark.

Latex which is the cytoplasm of the specialized cells known as laticifers (latex vessels), gushes out of the tree when laticiferous cells are severed during tapping. This flow is due to the very high pressure inside the laticiferous cells compared to the outside. The latex is collected by allowing it to run into a small cup attached to the trunk. After some time the coagulation of latex plugs this wound and the flow stops. To restart flow from a tapping cut in a subsequent tapping, all that is needed is to cut a thin shaving of the bark along with which the plugs of coagulated latex are also removed and release the latex upward into the new cut (Premakumari and Panikka, 1992; John, 1992; Kush, 1994).

1.2.2 Latex stability

The colloidal stability of latex is attributed to the presence of surface charges on the rubber particles. According to the definition of the stability of latex given by Cockbain and Philpott (1963), latex is stable when there is no aggregation or coalescence of rubber particles. The film or membranes surrounding the particles provide them with a negative charge, as shown by surface potential or zeta potential (Southorn and Yip, 1968). The particle membrane has been described to be composed of proteins, phospholipids, and other substances (Wititsuwannakul and Wititsuwannakul, 2001), and shown to be of a negatively charged nature (Ho *et al*., 1976). The reduction of phospholipids content of latex from a clone of *H. brasiliensis* known for its instability has also been noted, and this observation was subsequently extended to show that the lipid content of rubber particles correlated positively with colloidal stability of the latex (Sherief and Sethuraj, 1978). The colloidal stability was reduced by magnesium released from the damaged or ruptured lutoids (Philpott and Wesgarth, 1953) as the surface charges were neutralized. The effect of inorganic cations was investigated in relation to flocculation of the rubber particles and to plugging of the latex vessels, with a negative effect on latex flow (Yip and Gomez, 1984).

Once the tree is tapped, latex composition is susceptible to modifications by bacteria and rapid acidification is observed. This degradation of proteins and the formation of organic acids neutralize the negative charges on rubber particles and latex gradually coagulated. Therefore, fresh latex cannot be kept for long without a preservative treatment. The field latex must, therefore, be preserved and fortified against bacterial attack, which is accomplished by immediately adding ammonia to the latex, since ammonia creates an environment, disfavourable to bacterial growth and preserving negative charges on rubber particles.

1.2.3 Latex coagulation

The objective of coagulation is to separate the serum (liquid phase in latex) and the coagulum as completely as possible. The development of coagulum can be induced by the addition of acid. Natural rubber latex coagulation is achieved by neutralizing the negative charges on the rubber particles so that they coalesce. In the coagulation procedure the solid rubber is separated from the serum fractions. Coagulation is the process by which a liquid is changed to a thickened, cordlike, insoluble state by chemical reaction. The natural coagulation may also result from enzyme activities, from *Hevea* latex and from contaminating microorganisms or microbial metabolism in latex. The normal latex coagulation methods are acid coagulation and assisted biological coagulation.

Normal coagulation is carried out by acidifying latex from approximately neutral (pH of about 7), to pH 5.4. Inorganic acids are used for coagulating skim rubber, but they tend to be too aggressive for coagulating field latex and they leave residues that quickly corrode processing machinery. In practice, formic acid is preferred for normal coagulation but acetic acid is also used, together with formic acid. Excellent separation with virtually no rubber left in the serum can be achieved simply by properly adjusting the pH.

Assisted biological coagulation is a process that can be carried out by auto-coagulation without the addition of acid. Auto-coagulation is not widely used,

however, for various reasons: (i) the rubber produced by auto-coagulation is inferior in certain characteristics and often has an offensive smell. (ii) coagulation takes about 48 hours, much longer than with acid coagulation, and (iii) rubber recovery is often incomplete. Some of these disadvantages have been overcome by Assisted Biological Coagulation, (ABC). ABC is a process developed by RRIM, in which the microbiological production of acid is accelerated by adding sugar (John, 1965). The best ABC results are obtained at 0.2 percent sugar on DRC (molasses at 0.4 percent on DRC, pineapple juice at 2 percent on DRC), giving nearly complete coagulation in 16 hours. The coagulum produced in the ABC process contains bubbles and is therefore unsuitable for the production of RSS sheet. It can, however, be converted into block rubber. In addition when the ABC process is used, it is usually for coagulating skim latex (Cecil and Mitchell, 2005).

1.2.4 Major constituents of latex

Latex is a white milky fluid that is produced by cells of various plants. Many other species exude latex which contains rubber in non-commercial quantities, while *Hevea brasiliensis* is the source of almost all commercial rubber. Very small amounts of other rubbers such as gutta-percha are produced for special applications, but *Hevea brasiliensis* has remained the leading source of natural rubber, and natural rubber can, for all practical purposes, be defined as the result of processing the latex that exudes from cuts in the trunk of *Hevea brasiliensis*.

The composition of latex derived from matured rubber trees is a mixture of many different chemicals, some inorganic but mostly organic. Fresh *Hevea* latex is a polydisperse system in which negatively charged particles of various types are suspended in an ambient serum (C-serum). The two main particulate phases contained in *Hevea* latex are rubber particles constituting 20-45% by volume of fresh latex and lutoid particles (10-20%). The third type, on a quantum basis, is the Frey-Wyssling complexes (13%) (d'Auzac and Jacob, 1989; Premakumari and Panikka, 1992).

Fresh latex can be separated into 4 main fractions by ultracentrifugation (Figure 2). The top fraction consists almost entirely of rubbers particles which lie above the orange-colored layer containing Frey-Wyssling particles, the middle zone is colorless which made up of the aqueous phase of the latex (C-serum), and yellowish bottom fraction consists mainly of membrane organelles called lutoid particles. Details of these fractions are described in Figure 2. Fractions 1-3 correspond to the white rubber phase. Fraction 4 is a yellow- orange layer constituted by Frey-Wyssling particles. Fraction 5 is an almost clear serum (C- serum) corresponding to the latex cytosol. Fractions 6 to 11 constitute the "bottom fraction" in which fraction 8, quantitatively the most important, is the lutoid fraction intensely pink colored after neutral red absorption (Moir, 1959).

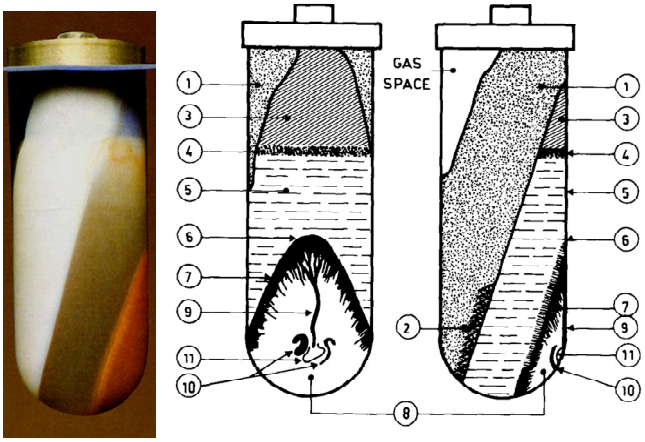


Figure 2 Various fractions of centrifuged latex, (Moir, 1959).

Rubber Particles

Rubber hydrocarbon is the major component of *Hevea* latex. The dry rubber content of latex may vary from 25 to 45%. The rubber molecules are found as particles in the latex. The particles consist mainly of rubber (90%) associated with lipophilic molecules, mainly lipids and proteins, forming the film enclosing rubber particles (Ho *et al*., 1976). This film carries negative charges and is responsible for the stability of rubber particles suspended in aqueous serum. Particles size ranges from 5 nm to 3 μm with a spherical shape. They also show plasticity with a polygonal shape in mature laticifers, where the particles are numerous. The size distribution as

determined by the electron microscopy showed maximum a for 0.1 μm particles (Gomez and Moir, 1979). The other main components of rubber particles are the enclosing membrane consisting of lipids, proteins, and enzymes. These components contribute to the colloidal charge of the rubber particles and their stability in the latex phase (Wititsuwannakul and Wititsuwannakul, 2001).

(a) Rubber particles membrane

The rubber particles are commonly found in association with lipids which are thought to be membrane nature. Microscopically, the particles appear to have a uniform structure, with the rubber molecules enclosed by a thin film (Southorn, 1961). Analyses of the nature of the film enclosing the rubber particles show the presence of phospholipids, proteins and neutral lipids (d'Auzac and Jacob, 1989).

(b) Rubber particles lipids

Rubber particles purified by ultracentrifugation consist up to 3.2% total lipids, of which approximately 2.1% are neutral lipids on a dry rubber weight (Ho *et al*., 1976). Separation of neutral lipid showed it is composed of at least 14 different substances. Triglycerides were the most abundant, accounting for almost 45% of the neutral lipids, while sterols, sterol esters and fatty acid esters constituted about 40%. Other neutral lipids found in trace amounts were diglycerides, monoglycerides, and free fatty acids. In addition, tocotrienols and some phenolic substances were also found to be associated with rubber particles (Ho *et al*., 1976).

Phospholipids are important components of the rubber particles. These components were identified as a considerable quantity of phosphatidylcholine and smaller quantities of phosphatidylethanolamine and phosphatidylglycerol. Phosphatidic acid was found to be predominant in the membrane of lutoids, but was not detected on the rubber particles (Dupont *et al*., 1976). The stability of rubber particles suspension in latex is dependent on the negative charges of proteins and phospholipids (Philpott and Wesgarth, 1953).

(c) Rubber particles proteins

Proteins are found as indigenous components of the film enclosing the rubber particle. Together with lipids, these proteins form the membrane of particles which contribute to their stability. The isoelectric point (pI) of these proteins ranges from 3.0 to 5.0 which is characteristic for surface proteins (Verhaar, 1959).

One of the major proteins in latex is α-globulin with pI of 4.5. It was found both in the cytosol and adsorbed onto the particles surface, and might contribute to their colloidal stability in latex (Archer *et al*., 1963a). A protein group of hydrophobic nature was also found in rubber particles, and proteolipids have been isolated and characterized (Hasma, 1987). This protein was suggested to be a component of the polar lipid backbone that forms part of the membrane of rubber particles.

Lutoid particles and B-serum

The lutoid particle is the vacuolar tonoplast of the latex and is found in the sediment or bottom fraction obtained by centrifuging latex. Lutoids have a liquid content called B-serum. The B-serum has a pH of about 5.5 which consists of an acid serum enriched with divalent cations ($Mg^{2+,}$ Ca^{2+}) and positively charged proteins, these compounds which may neutralize the negative charges of proteins surrounding rubber particles and result in a destabilization of the colloidal solution of latex. Release of protein components from lutoid particles of exuded latex lyses as a result of contact with rainwater can cause cessation of latex flow, due to coagulation, during tapping of rubber trees (Webster and Baulkwil, 1989).

In addition, lutoids contain a wide range of hydrolytic enzymes and some of their enzymatic properties are analogous to those of lysosomes of animal cells (Dupont *et al*., 1976). Approximately 20% of the dry matter in the lutoids is water-soluble protein, of which about 70% is hevein. This substance is anionic and shown to contain no less than 5% sulfur, all as cystine (Webster and Baulkwil, 1989).

Frey-Wyssling particles

On ultracentrifugation of fresh latex the Frey-Wyssling particles may sediment centrifugally or rise centripetally to form a zone beneath the rubber particles. These

particles are mainly composed of lipid materials and are yellow or orange in color due to the presence of carotenoids. Yellow globules, in clusters in tapped latex, were first noted by Frey-Wyssling (Frey Wyssling, 1929). Electron microscopy revealed the structural complexity of these organelles, which consists of one or more inclusions containing the lipid-carotenoid complex. (d' Auzac and Jacob, 1989).

The Frey-Wyssling complexes are considered to have a vital role in metabolic activities. Though Dickenson (1969) proposed that these structures may be possible sites of rubber biosynthesis. The double membrane and presence of carotene and polyphenol oxidase in the FreyWyssling complexes led to a tentative suggestion that it is a type of plastid (Premakumari and Panikka, 1992). Polyphenol oxidase and β-carotene are the classic markers of the Frey-Wyssling complexes.

C-serum

The aqueous phase of the laticiferous cytoplasm contains most of soluble compounds normally found in plant cells such as inositols, carbohydrates, amino acids, proteins, inorganic anions and metal ions, together with the enzymes and intermediates of various biochemical process, including rubber biosynthesis. C-serum is the most non rubber part in the latex and contains proteins approximately 60% of total proteins in latex. A great number of proteins have low isoelectric point and are anionic at the normal pH (6.9) of the serum (Webster and Baulkwil, 1989).

Dehydrated natural rubber latex contains approximately 6% non-polyisoprene constituents depending on the clone, season, and the state of the soil. Natural rubber latex consists of approximately: 34% rubber cis-1,4-polyisoprene, 1% proteins, 0.1-0.5% sterol glycosides, 1.5-3.5% resins, 0.5-1% ash, 1-2% sugars and 55-65% water (Cacioli, 1997). Approximately 20% of the protein part is adsorbed on rubber particles, 20% associated with the sedimentable fraction and thus essentially with the lutoids, andthe remaining 60% is reported as being cytosolic or the serum fraction (d'Auzac and Jacob, 1989).

1.3 Structure characterization of natural rubber from *Hevea brasiliensis*

Sakdapipanich (2007) revealed the structural characterization of natural rubber and showed that natural rubber contains functional groups at the initiating- and terminating-ends of rubber chain, i.e., ω- and α-terminals, bonding with peptides and phospholipids, respectively. Both terminal ends have been presumed to originate branch-points and soft-gel in NR. The formation of branch-points at the α-terminal in NR chain was presumed based on the presence of about two long-chain fatty acid groups and one phosphorus atom accompanied with rubber chain independent of molecular weight even after deproteinisation followed by acetone extraction of NR.

The molecular structure of α-terminal group was elucidated by selective decomposition of ester linkages with enzymatic reactions using lipase and phospholipases A_2, B, C and D. The reaction sites in phospholipids that can be hydrolyzed by phospholipases are shown in Figure 3.

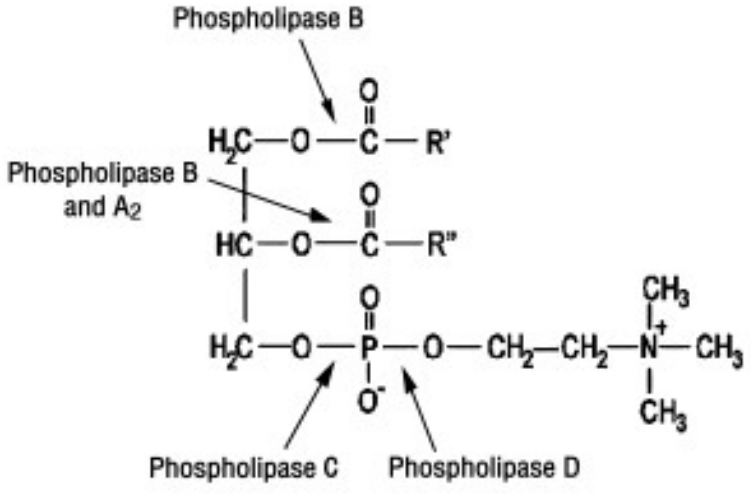


Figure 3 Chemical structure of l-α-phosphatidylcholine and reaction site for phospholipase decomposition (Scherphof, 1993).

The presence of monophosphate, diphosphate and phospholipid linked to rubber molecule was confirmed by treatment of DPNR with lipase, phosphatase,

phospholipases A_2, B, C followed by combination with ^{1}H-, ^{13}C- and ^{31}P-NMR analyses and molecular weight analysis by GPC and dilute solution viscometry (Tarachiwin, *et al*, 2005).

The α-terminal of rubber molecule was postulated to be monophosphate and diphosphate groups linking to phospholipids by Mg^{2+} linkage and hydrogen bonding. Quantitative analysis by solid state ^{31}P-NMR indicates the presence of about two to three phosphorus atoms per α-terminal group in rubber chain. At present, it can be postulated that NR molecule is terminated with monophosphate and diphosphate groups at α-terminal (Figure 4).

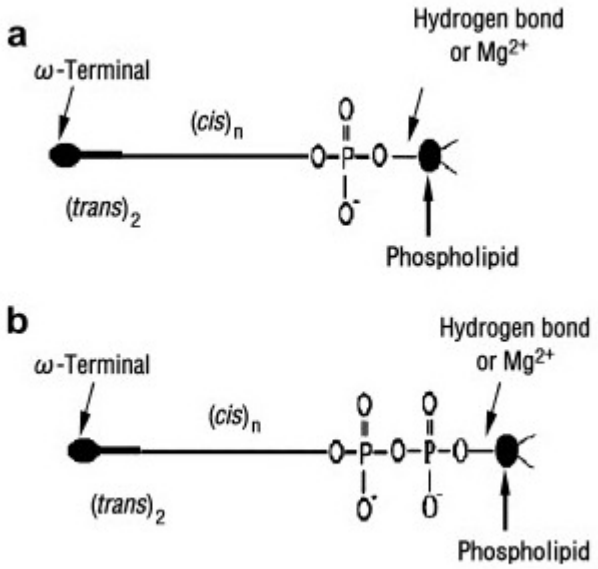


Figure 4 Proposed structures of α-terminal group of NR with terminal monophosphate (a) and diphosphate (b) (Sakdapipanich, 2007).

1.4 Relationships between latex composition and properties

Natural rubber has some specific properties that make of it an irreplaceable product. Natural rubber differs from its synthetic counterpart by a more complex structure that can be described using three levels (Vaysse, *et al.*, 2003).

Microstructure or molecular scale focuses on molecular level, or chemical structure, inside the rubber such as isoprene units (configuration for example) and

non-isoprene constituents, lipids, proteins and carbohydrates (non-isoprenes). It is to be noted that some of the non-isoprene components greatly influence the properties of NR in both raw and vulcanized states (Subramaniam, 1987).

Mesostructure level focuses on macromolecular structure and gel. The macromolecular structure of natural rubber can be assessed by number-average molar masse ($\overline{M_n}$), weight-average molar masse ($\overline{M_w}$), molar masses distribution (MMD), branching, etc.

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

Quality and properties of the natural rubber depend, in part, on the quantities of non-rubber elements in the latex (Yip, 1990; Le Roux *et al*., 2000). Non-rubber substances could exhibit considerable influences on some properties of NR. Latex contains proteins, carbohydrates and lipids. After latex exudes into the atmosphere it becomes contaminated with microorganisms present in the tapping cut or in the cup. During biological decomposition, some hydrolysis reactions can lead to the liberation of fatty acid or peptides. These products can influence properties such as fatty acids which are activators for vulcanization (Bengston and Stenberg, 1996).

The total protein content of fresh latex is approximately 1% of which about 20% is adsorbed on rubber particles. The major protein found in the serum phase is α-glubolin (Archer *et al*., 1963a). The proteins and phospholipids adsorbed on the rubber particles impart a net negative charge, thereby contributing to the colloidal stability of latex. The enzymes found to be associated with the rubber surface are isopentenyl pyrophosphase polymerase and rubber transferase (Archer *et al*., 1963a; Lynen, 1967).

Amino acids that can be released from protein hydrolysis among the polyisoprene molecules can cause crosslinking of stored natural rubber. This phenomenon, called storage hardening, affects positively process properties such as Wallace plasticity and Money viscosity (Gan, 1996).

Quebrachitol (methylinositol), sucrose and glucose are the major soluble carbohydrates in latex (Low, 1978). The field latex, which contains carbohydrates, becomes microbiologically oxidised into volatile fatty acids. The quantity of volatile fatty acids is used to determine the quality of latex.

Neutral lipids and phospholipids are present in the lutoid membrane. The fatty acids released by hydrolysis of phospholipids were found to have an effect on crystallization and plasticizing of rubber. Both saturated and unsaturated fatty acids also have an effect on mechanical properties such as tack and green strength of unvulcanized rubber (Kawahara *et al*., 2000a).

In addition, the technological properties of natural rubber vary according to the time of shelf life, type of coagulation, tree age and clonal origin (Le Roux *et al*., 2000; Ferreira *et al*., 2002). Relationships between certain physiological parameters of latex analysed by the micro-latex diagnosis technique and some relevant technological properties of the raw rubber were estimated for some selected clones. These were found to be greatly influenced by eco-climatic conditions. Highly significant clonal variations were also observed in the physiological parameters of latex while these variations were not so consistent for most of the technological properties and physiological parameters notably between the inorganic phosphorus and plasticity retention index and between the total solid content and the Mooney viscosity (Le Roux *et al*., 2000).

The dry rubber content (DRC) is a physiological parameter of the latex, an indicator of the biosynthetic activity in the laticiferous vases. Such behavior is possibly connected to the association of the lesser availability of water in the soil and

the senescence of the leaves which causes a decrease in viscosity of the latex (Van, 1951), favoring its flow and eliciting larger productivity.

Technological properties of latex and natural rubber of clones GT 1, PB 235, IAN 873 and RRIM 600 (*Hevea brasiliensis*) were evaluated by the standard methods of determination of dry rubber content, nitrogen content (%N) and ashes (%ASH) in two consecutive years in the State of Sao Paulo, Brasil. The results showed that properties of latex and natural rubber varied as a function of clone type and among tappings. DRC decreased in the beginning of the dry season (May to June) and %N and %ASH increased in the same period. April to June was a critical period, when %N was above 0.60%. Clone RRIM 600 was less susceptible to climatic variations (Rogerio *et al*., 2005).

2. Primary rubber processing

Raw rubber comes from the field or plantation in two basic forms: field latex and field coagula. Both are primary processed into raw rubber products of different types and grades.

2.1 Raw rubber types

Rubber is the raw material in many products today. It can be divided into two main types, i.e.: natural and synthetic rubber. Here we are looking only at natural rubber. Raw natural rubber involves processing fresh latex or field coagula obtained by cutting the rubber tree, into a raw material ready for use in making rubber products further on. Raw materials in the natural rubber industry consist of fresh (field) latex, obtained by cutting rubber trees, and field coagula, which include cup coagula, cup lump, tree lace, bark scraps, earth scraps and smoked sheet cuttings, etc.

2.2 Raw rubber manufacturers

Raw rubber manufacturers are divided into three types which described as following:

2.2.1 Manufactures of Concentrated latex

Field latex centrifuged products, concentrated latex and its by-product, skim rubber which is made by separating field latex preserved with ammonia in a centrifuge. When the field latex arrives at the factory, a sample is drawn from each delivered batch to determine the amount of ammonia present, the Volatile fatty acid (VFA) and either the Total solid content (TSC) or the Dry rubber content (DRC) (using a hydrometer or latexometer). If the tests show that the DRC is below about 25 percent or the VFA is above acceptable levels (parameters as prescribed by the ISO specifications, VFA No. = 0.05), the latex should not be centrifuged. The field latex is fitted with filters of 40 or 60 mesh stainless-steel gauze, from which the filtered latex runs into the bulking tank. The latex is transferred by gravity into a header tank, in which another sieve is fitted, to remove any coagulum that may have formed during the preliminary treatment of the field latex in the reception tanks or the bulking tanks. From here, a series of latex chutes distribute the latex to each of the centrifuges, where the latex is separated into concentrated latex and skim latex.

2.2.2 Manufacturers of Air Dried Sheets/Smoked Rubber Sheets

Sheet rubbers including unsmoked sheet (USS), ribbed smoked sheet (RSS), air-dried sheet (ADS) and latex grades of crepe rubber. Rubber sheets were processed by diluting the fresh latex to a rubber content of 15 - 16% and coagulated in coagulation tanks using formic acid or acetic acid. Lumps of coagulum are formed after the acid has acted for 3-4 hours. After milling and washing, sheets between 2.5 and 3.5 mm thick, 24 cm wide and 90 or 135 cm in length are produced. The final mill is an embossed mill, which gives the sheets their ribbed structure. Since these rubber sheets are not washed as intensively as crepes, they contain a higher proportion of serum constituents which encourage mold and rotting. For this reason, for RSS, the sheets undergo an additional preservation process in which they are smoked in smokehouses. The sheets are hung in the smokehouses and dried for a week at

temperatures up to 60°C. The smoke resulting from burning *Hevea* (rubber tree) wood and other organic materials such as coconut husks preserves the sheets. The specific smell of these sheets is caused by the materials used to produce the smoke. The sheets are pressed into bales and wrapped in protective sheets. The surface is protected from oxidation by application of a bale coating solution and talcum.

2.2.3 Block rubber industries of different types.

There are many grade of block rubber i.e., TSR XL, TSR 5L, TSR 5, TSR 5CV, TSR 10CV which can be processed from fresh latex or field coagula. These raw coagula is transformed to small granules which are also called wet crumb. The wet crumb is dried in a forced-draught dryer at suitable temperatures depending on grade of rubber block.

2.3 Technically Specified Rubbers (TSR)

Technically specified rubber (TSR) is a model form of marketable raw rubber and can be produced from latex, field coagulum or blend of both. The production of TSR started in 1960s, prior to which raw natural rubber was marketed in conventional forms like sheet and crepe.

The International Standards Organization (ISO) first came out with the draft Technical Specifications for Natural rubber during 1964. Based on these specifications, Malaysia introduced their Standard Malaysian Rubber (SMR) scheme in 1965 and since then all the Natural rubber producing countries started producing and marketing NR as Technically Specified Rubbers.

The TSR are mostly the block types made adopting new methods of processing. The blocks are generally 35 kg bales in the International market. All the block rubbers are also guaranteed to conform to certain technical specifications as laid out by the national schemes or by the ISO 2000 standard.

TSR are the major volume grades of dry rubber in production and exports in the major NR exporting countries of the world. In Malaysia, the TSR is designated as Standard Malaysian Rubber (SMR). In Indonesia, the designation given is Standard Indonesian Rubber (SIR). In Thailand the TSR are called Standard Thai Rubber (STR). In India, the TSR are designated as Indian Standard Natural Rubber (ISNR).

The main advantages claimed for the TSR compare to the conventional sheet and crepe grades of rubbers are that firstly, it can be assessed by different normalized measurement such as P_0, PRI and volatile matter. Secondly, it is marketed as compact, polythene wrapped bales, handling and transportation cost can be saved. Finally, it is in standard size and compact, considerable savings can be made in transport through mechanized handling and containerization.

2.3.1 Generally TSR processing

The raw material such as field coagula used for TSR production is processed through a traditional crumb-rubber factory, where the raw material is cleaned by passing the material through slab-cutter and/or pre-breakers. The resultant crumb is washed in wash tanks, then fed to a series of crepers which build the rubber into a blanket, which is then crumbed through a shredder or extruder. The final crumb is dried in a forced-air dryer at temperatures between 105ºC and 120ºC. Finally, the dried crumb is pressed into blocks.

2.3.2 **TSR processing development** (Sadeesh Babu, *et al.,* 2000)

The processing and marketing of conventional forms of NR like sheet and crepe into TSR were tried in the 1970s. Initial attempts were to develop a processing technique for latex and field coagulum to present them in a form similar to synthetic rubber. Synthetic rubbers were marketed in block form and each block weighted exactly the same. Considerable research has been conducted in the rubber producing countries for the production of NR in block form and several processes are being developed.

Conventional methods are often based on very old techniques, following the relatively simple routine of tapping, collection of latex, coagulation, sheeting and

drying. Under the well-established system of visual grading, the major emphasis in processing was on visual appearance rather than on technical properties.

The new block rubber processes convert raw rubber into a granular form by fast, continuous-flow techniques, carried out in large central factories. Once it is dry, the crumb is compacted into solid blocks of rubber. This is the origin of the name that is now used for the new form. Using the old methods, it takes at least a week to convert field latex into a bale of RSS ready for shipment and up to two weeks for crepe. Using block rubber technology, it takes considerably less than 24 hours to produce blocks.

The most outstanding advantage is that block rubbers are graded to technical specifications, rather than to subjective visual examinations, putting the emphasis where it belongs on inherent physical and chemical properties.

2.3.3 Equipment for TSR grade processing from field coagula

A brief description of the machines and equipment used for TSR grading in large modern factories is as follows:

(1) Conveyors

Two types of conveyors are commonly used. The first type, known as a ‘bucket’, collects crumbed or granulated coagulum from wash/blending tanks for delivery to the next processing machine. The other type of conveyor is the belt conveyor, most commonly used for moving blanketed coagulum from one creper to the next creper in the process line, or to a shredder.

(2) Crepers

A creper is a two-roll mill with an individual motor and gearbox and medium grooves on both rolls.

(3) Extruders

An extruder is similar in design to a pre-breaker. An extruder will be the final machine in a line, for producing a granule of a size suitable for drying.

(4) Granulators

A granulator has a driven rotating shaft holding three or four knives mounted parallel to the axis of the shaft. These rotate past fixed knives that are mounted on the frame, parallel to the axis of the shaft. Granulators are used for pre-cleaning field coagula.

(5) Hammer mills

A hammer mill has a number of metal bars mounted on a cage which is mounted on a driven shaft. Centrifugal force causes the bars, the ends of which are machined with thick ‘teeth’, to stand out from the cage, flailing anything in the cavity in which the hammers rotate. Hammer mills are used both in pre-cleaning lines and in crumbing lines.

(6) Macerators

, Macerators are used for breaking down scrap or coagulum into a thick, rough blanket. The rolls normally have deep diamond grooves and are run with little speed differential. It is in the macerator that most of the acid in the coagulum is removed by squeezing and washing and, as with crushers, the acid soon roughens and forms pits in the surfaces of the rolls.

(7) Pelletizers

A pelletizer was an early form of extruders. Pelletizers have external knives outside the die plate which used to cut the extruded material into pellets.

(8) Pre-breakers

A pre-breaker is very similar in design to an extruder or a pelletizer. A widely-used machine is a twin-scroll pre-breaker, which has twin screws and die

plates. A twin-scroll pre-breaker is used at an early stage of the cleaning process and is fed with coagulum lumps from a slab cutter, hammer mill or granulator. The scrolls knead and mix the rubber, and as the rubber passes through the die plates. The product from a pre-breaker may be fed to extruders or creping machines, depending on the process required. Pelletizers or extruders are essentially single-scroll pre-breakers.

(9) Rotary cutters

A rotary cutter is a granulator, with knives mounted on a drum that rotates rapidly past stationary knives mounted on the machine's housing. A screen on the outlet determines the size of particles leaving the machine. Rotary cutters are used in a cleaning line.

(10) Shredders

A shredder is a high-speed cutting machine, used to produce wet crumb from a fine blanket produced on a creping machine. Shredder was modified as a pair of feed rolls and a cutting roll, working against a fixed plate. Shredder is generally used for final size reduction of crepe blankets from latex coagulum. The crumb is collected in a water-filled tank. A vortex pump conveys the rubber to a dewatering screen, from which the water is returned to the last tank. The dewatered rubber is dropped into a trolley ready for dripping and drying.

(11) Slab cutters

Slab cutter consists of a heavy duty rotor with fixed knives working in tandem with fixed knives on the housing. A slab cutter is used to chop and cut field coagula to be used for blending. With subsequent washing, a slab cutter will remove a large proportion of the dirt and other contaminants from coagula.

(12) Wash tanks

After each piece of equipment, a tank should be installed for washing and blending the material produced by the machine. The wash tank is one of the most important part of the pieces process. A wash tank is usually an open vessel supplied

with a continuous flow of water. Apart from washing cut slab, crumb or whatever is passed through it, a wash tank also serves to blend the material.

2.3.4 Specification for TSR

Technical specified rubber are now graded according to different criteria such as dirt content, ash content, nitrogen content, volatile matter, PRI, Wallace rapid plasticity and color limit. Moreover, TSR are also graded according to their input material such as latex grades, sheet grades, field grades and blended grades including stabilized or Non-stabilized grades. Since 1969, technical specified rubber has been widely accepted worldwide.

The International Standards Organization (ISO) have specified different grades of TSR. The specifications in force at present are given in the following Tables 1.

Table 1 The specifications of difference 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	0.5	0.5	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 (min)	-	30	30	30	30	30	30
Plastic Retention Index (Min)	60	60	60	50	50	40	40
Colour (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).*

The detailed characteristics of the different grades of TSR are as follows:

(1) TSR – CV

TSR-CV is produced from field latex, the CV designating “constant viscosity”, is produced from field latex and is viscosity stabilized to a specified Mooney viscosity. The storage hardening of this grade of rubber is also to be within eight hardness units. The Mooney viscosity of this rubber is stabilized by the addition of hydroxylamine hydrochloride on total rubber in the latex phase, prior to coagulation TSR-CV Rubbers are generally softer than conventional grades. Coupled with its constant viscosity feature, it provides a unique cost advantage in eliminating the pre-mastication process.

(2) TSR-L

It is a light colour rubber produced from latex. The maximum color of this rubber as per the Lovibond scale shall be six units. This natural rubber grade can be used for light colored and transparent products, such as surgical/pressure sensitive tape, textiles, rubber bands, hot water bottles, surgical and pharmaceutical products, large industrial rollers for the paper printing industry, sport wear, bicycle tubes, chewing gum, cable covers, gaskets, and adhesive solutions and tapes.

(3) TSR-5

It is processed using field latex of high quality field coagulum as the main raw materials. TSR-5 has superior strength property together with low mill shrinkage and good tack. It processes better especially in extrusion, calendering, and joining of cycle inner tubes.

(4) TSR-10 and TSR 10CV

TSR - 10 is produced from clean and fresh field coagula or from unsmoked sheets. TSR 10CV is produced in the same way as TSR 10, above, except that during the milling and crumbing process, the raw material is soaked in a hydroxylamine solution. The Mooney viscosity specification is 55–67 MV units.

(6) TSR-20 and TSR 20CV

It is a large volume grade of technically specified natural rubber. It is produced mostly from field coagula, with lower grades of RSS and or unsmoked sheets. TSR-20 has excellent processing characteristics and good physical properties. Its low

viscosity and easier mixing characteristic (compared with the RSS grades) will reduce the mastication and mixing period considerably. TSR 20CV is produced in the same way as TSR 20 with soaking in a hydroxylamine solution. The Mooney viscosity specification is 60-72 MV units.

TSR rubber is presented packing in a modern 1.2 metric tonne pallet which facilitates handling, transportation and store space utilizations. Each pallet consists of 36 bales of 35 kg net. Each bale is wrapped in high quality polythene bag which is dispersible and compatible with rubber at 110°C.

2.3.5 Physical properties of TSR

Physical properties of TSR are general more than adequate to meet the manufacturing and product quality requirement. P_0 and PRI provide a good indication of the physical properties to be expected.

An overall view of the analytical procedures used for the various tests required in the TSR system is given below. These descriptions are indicative of the procedures that are used in the various tests, but are emphatically insufficient to carry out the tests. To carry out the tests properly, the relevant ISO or ASTM test procedure or the RRIM equivalent should be obtained and followed exactly.

(1) Dirt content

For the determination of dirt content, about 10 g of sample should be dissolved in toluene. The solution should be filtered through a previously weighed 44 μm screen. The residue should be washed, dried at 90–100°C for an hour and weighed in the screen. The result is expressed as a percentage of the original sample. For a complete description of the test method, refer to ISO 249:1995; *Rubber, raw natural – Determination of dirt content*, or ASTM D1278-91a (2002); *Standard test methods for rubber from natural sources – chemical analysis.*

(2) Ash content

For the determination of ash content, a 5–10 g portion of the sample should be accurately weighed, wrapped in ash-free filter paper and placed in a weighed crucible. The sample in the crucible should be heated to 550 ± 20°C for 2–4 hours in a gentle flow of air. After cooling, the crucible should again be weighed. The weight difference is ash. The result is expressed as a percentage of the original sample. For a complete description of the test method, refer to ISO 247:1990; *Rubber – Determination of ash*, or ASTM D1278-91a (2002); *Standard test methods for rubber from natural sources – chemical analysis.*

(3) Nitrogen content

For the determination of nitrogen, a semi-micro Kjeldahl procedure should be used: protein is converted into ammonium hydrogen sulphate by heating with a catalyst mixture and concentrated sulphuric acid. The solution is made alkaline and the ammonia is removed by steam distillation. The liberated ammonia is absorbed in boric acid solution and titrated with standard acid. The catalyst can be obtained from any laboratory chemicals supply house. To get an indication of the protein content, the nitrogen content × 6.25 is generally accepted as the weight of protein. For a complete description of the test method, refer to ISO 1656:1996; *Rubber, raw natural, and rubber latex, natural – Determination of nitrogen content or ASTM D3533-90 (2001); Standard method of Nitrogen provides rubber – Nitrogen content*

(4) Volatile matter determination

For the determination of volatile matter, 10–12 g of cooled sample should be accurately weighed and milled and should then be heated to 100 ± 3°C for 4 hours. The sample should then be quickly placed in a sealed bag. The sample should be allowed to cool for half an hour, removed from the bag and weighed again. The difference is expressed as a percentage of the original sample. For a complete description of the test method, refer to ISO 1407:1992; *Rubber – Determination of solvent extract,* or ASTM D1278-91a (2002); *Standard test methods for rubber from natural sources – chemical analysis*.

(5) Wallace Plasticity

The plasticity is measured using a Wallace Rapid Plastimeter. A sample is compressed between two circular platens which are maintained at a temperature of 100°C. The sample is conditioned for 15 seconds at a thickness of 1mm. A compressive force of 100N is then applied for 15 seconds. The final thickness of the test piece expressed in units of 0.01mm, is the plasticity number P_0.

(6) Plasticity Retention Index (PRI)

PRI is a measure of the resistance of raw natural rubber to oxidation. The oxidation effect is assessed by measuring the plasticity before ageing (P_0) and after ageing for 30 min in the ageing oven at 140°C (P_{30}).

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

A high value denotes high resistance to oxidative breakdown. The plasticity is measured using a Wallace Rapid Plastimeter. The value of the index is virtually independent of the initial plasticity of the rubber (P_0). For a complete description of the test method, refer to ISO 2930:1995; *Rubber, raw natural – Determination of plasticity retention index (PRI).*

(7) Accelerated storage hardening test (ASHT)

The ASH analysis measures the change in the Wallace rapid plasticity in pellets stored over phosphorus pentoxide in a closed container at 60°C for 24 hours at atmospheric pressure. The resulting increase in plasticity (ΔP) gives a good measure of storage hardening. For a complete description of the test method, refer to SMR bulletin 7 (1992) part. C.1 standard.

(8) Color analysis

The color of a sample is determined by visual comparison of the sample with graded tinted-glass discs in a color comparator such as the Lovibond colorimeter. For a complete description of the test method, refer to ISO 4660:1999; *Rubber, raw*

natural – Colour index test, or ASTM D 3157-84(2001); The colour of a sample is *Standard test method for rubber from natural sources- Color.*

(9) Mooney viscosity analysis

Mooney viscosity is the shearing torque resisting rotation of a cylindrical metal rotor embedded in the sample within a cylindrical cavity, at 100°C. The dimensions of the shearing disc viscometer, test temperatures and procedures for determining Mooney viscosity are all closely defined. In fact, what is measured is properly called 'stress relaxation'. Mooney viscosity is not a true viscosity: it is a measure of shearing torque averaged over a range of shearing rates. The results are defined by the equipment, the conditions of the test and the sample. A more complete description of the test method is given under ASTM D 1646-03a *Standard test methods for rubber – viscosity, stress relaxation and pre-vulcanization characteristics (Mooney viscometer)* from which the foregoing is condensed, but for a complete description, it is essential to refer to ASTM D 1646-03a itself, or to ISO 289-1:1994; *Rubber, unvulcanized – Determinations using a shearing-disc viscometer – Parts 1, 2, 3 and 4: Determination of Mooney viscosity.*

(10) Cure behavior analysis

The cure behavior of a batch of block rubber is measured using a rheometer – most commonly a Monsanto rheometer. Rubber from the batch under test is mixed with additives in the following proportions to make an 'ACS 1 test' compound; Rubber: 100.0, Zinc oxide: 6.0, Upshur: 3.5, Steric acid: 0.5, Mercaptobenzthiazole: 0.5.

The compound is prepared on a two-roll mill. A small portion is cut into two discs, similar to those used in the Mooney viscosity test, and placed around the rotor of the rheometer. The test is then performed in the prescribed manner. The delta torque, optimum cure time and scorch, derived from the graph produced by the rheometer, can be used to classify the cure behavior of the batch. The graph is sent with the batch to the customer.

In conclusion, fresh latex and fresh coagula can be processed into many types of raw material which were ready for using in marketing rubber products. The raw rubber manufacturing of raw material can be provided into many grades of rubber. TSR are the major volume grades of dry rubber in production and exports in the major NR exporting countries of the world. TSR is a model form of marketable rubber and can be produced from latex, field coagulum or blend of both. The consistencies in the quality of raw material are important to TSR processing.

3. Biological activity of harvested latex

Natural Rubber (NR) is produced from the milky white liquid called latex harvested from rubber trees by the process of tapping. Latex is a colloidal suspension of rubber in aqueous serum. The latex that flows out from the rubber tree on tapping is channeled into an attached container cups. Once the tree is tapped, during latex flowing out the compositions are highly susceptible to attack by bacteria upon exposure to oxygen in the atmosphere. It becomes contaminated with microorganisms present in the tapping cut or in the cup. Some microorganisms can grow and die in latex depending on medium composition and properties, and conditions of maturation.

3.1 Microorganisms in raw rubber

Many types of microorganisms are able to grow in natural rubber by using organic compounds as nutrients. Two types of microorganisms are of particular interest in the degradation of natural rubber latex; these are bacteria and fungi. The importance of fungi as deteriorative agents is a result of the secretion numerous of enzymes which breakdown nonliving substrates in order to supply nutrient materials present in rubber compositions.

Bacteria can be single-cell rods, cocci, or spirilla; others are chain-like or filamentous. They can either be aerobic or anaerobic; in contrast, fungi are necessarily aerobic. Some bacteria are motile; bacteria are predominantly nonchlorophyllous. Their degradative action is also chiefly a result of enzyme production and the

resultant breakdown of the substrates. Bacteria present in soil are important agents for materials degradation.

3.1.1 Microorganisms in latex

Taysum (1957) isolated strains of bacteria from *Hevea* latex system. The bacteria were sampled from the latex of a single tree taken into a sterile container and using a sterile tapping knife. Microbial populations of 12 different families were isolated. Bacteria were also isolated from the latex in the tapper's bucket.

Nearly 100 species of microorganisms were presented in the natural rubber latex samples. It is also evident that many of these species become important under specific ecologic conditions and that symbiosis and antibiosis are frequently encountered. The isolated microorganisms were often characterized from soil and water origins in the families *Pseudomonadaceae and Azotobacteriaceae* (Taysum, 1957) *Micrococcaceae* are extremely common in fresh latex. These bacteria frequently show a preference for high pH conditions and are widespread in ammoniated concentrated latex. *Lactobacteriaceae* are strong acid-producer and also very common.

Taysum (1969) showed results of about 80 samples taken from field latex normal tapping cups. A mean value of $8.02 \pm 0.06 \times 10^6$ CFU/mL was counted for the initial population. It should be emphasized that this count applied to an area where pre-coagulation was rare; more recent experiments suggest that where pre-coagulation is manifest the populations may be considerably higher. Counts on 12 samples of bulk latex arriving at the factory about 5 hours after tapping began gave a mean bacterial content of $4.21 \pm 0.38 \times 10^7$ CFU/mL. The mean count was $1.93 \times l0^8$ CFU/mL, with upper and lower limits of 3.0×10^9 and 4.4×10^4 CFU/mL, respectively. Actually, many of the larger deviations from the mean could be directly attributed to local conditions, principally heavy previous rainfall; this invariably increased the count.

3.1.2 Microorganisms in rubber sheet

Raw natural rubber is processed into different marketable forms of natural rubber such as ribbed sheet, crepe rubber and bock rubber. They can be processed from latex and field coagula. They are higher susceptible to degradation due to bacterial contamination on storage.

The spores of the moulds are present in the air and environmental factors including temperature, humidity and nutrients contained in serum substances are the main factors responsible for mould growth on sheet rubber (Kuriakose and Thomas, 2000). Rubber sheet with more than 0.8 percent humidity favours mould growth. Storing in humid atmosphere or on concrete floor stimulates mould growth on sheet. (Joseph *et al*., 2005). Anandan and Loganathan (1983) identified the genera *Aspergillus* and *Penicillium* in fungi colonizing rubber sheet.

Bubbles in sheet rubber are formed as the result of fermentative changes that take place either in the latex or in the wet coagulum prior to machining. Rubber latex normally contains a large number of bacteria and other microorganisms which are capable of acting on the serum constituents of latex so as to produce changes which results in the formation of quantity of gases and in the appearance of bubbles in the prepared sheet.

3.2 Enzyme in natural rubber latex

3.2.1 Lutoids enzymes involved in latex coagulation

The separation of lutoids from latex by centrifugation makes it possible to show that they contain a range of acid hydrolases in latent form such as cathepsin, acid phosphatase, β-N-acetyl-glucosaminidase, β-galactosidase, ribonuclease, phosphodiesterase, and a β-glucosidase (Pujarniscle, 1968).

A work on the oxidation-reduction enzymes in latex was carried-out by De Haan-Homans (1950), who showed that latex contained catalase, tyrosinase and phenol-oxidase. These enzymes are in most cases accompanied by their inhibitors.

Intralutoidic proteins and enzymes have an important role in latex coagulation. Among these enzymes, all ranges of acidic hydrolases are present and it has been shown that lutoid enzymes can destabilize the negatively charged colloidal suspension of latex particles. The breakdown of lutoids during or after tapping may liberate some hydrolytic enzymes able to attack the phospholipoproteic film that protect the stability of rubber particles. Among the lutoids enzymes, lysozyme is a quantitatively important hydrolytic enzyme (Jacob *et al.*, 2000) but that is unable to provoke the coagulating a suspension of rubber particles.

A lutoid phospholipase has been shown to be activated by small amounts of Ca^{2+} being able to cause the coagulation of latex (Southorn and Edwin, 1968). On the other hand, a study of latex phenoloxidase demonstrated that the enzyme is an o-diphenol oxidase that converts a phenolic substrate, in the presence of Ca^{2+}, H^{+} and dioxygen, into polyphenols or phenones. These products destabilize proteins that normally protect rubber particles (Hannower and Brzozowska, 1977).

Woo (1973) presented evidence for "coagulase" activity originating from C-serum (from cytosol) and not from B-serum (serum of bottom fraction) and able to provoke coagulation in the presence of low concentration of Ca^{+}. In order to obtain an idea of the enzymatic nature of coagulases, they used commercial enzymes for coagulation. They showed that among the lutoids enzymes proposed by Pujarniscle (1968) to be involved in latex coagulation, only a protease may be involved in this process. The role of other lutoid enzymes in latex coagulations thus remains to be elucidated.

Hannower and Brzozowska (1977) studied the mechanism of the coagulation of latex by *in vitro* coagulation experiments. They showed that acidic pH, divalent cations (Ca^{2+} and Mg^{2+}), B-serum, oxygen and some phenolic compounds act as coagulating agents. Addition of trypsin or phenol oxidase provoked a much faster coagulation than the addition of other proteins. Some factors resulted in a slower coagulation, such as C-serum, antioxidizing agents, atmosphere devoid of O_2 and stimulation of tree by ethrel.

3.2.2 Microbial degradation enzymes

The degradation of polymers may refer to an attack by microorganisms. The microorganisms must first excrete extracellular enzymes which depolymerize the polymers in their growth medium. As a consequence, if the molar mass of the polymers can be sufficiently reduced to generate water–soluble intermediates, these can be transported into the microorganisms and fed into the appropriate metabolic pathway(s). As a result, the end products of these metabolic processes include water and carbon dioxide together with new biomass.

The lack of microbial enzymes for rubber biodegradation, particularly enzymes catalyzing the cleavage of the rubber backbone, was one of the last obstacles to natural rubber degradation as none had been isolated until recently. However, strains able to use natural rubber as carbon and energy source have been selected. The chemical analysis of degradation products which were transiently formed due to incomplete biodegradation and the analysis of mutants unable to use natural rubber as a carbon source for growth finally led to the identification of the first genes coding for enzymes catalyzing the cleavage of polyisoprene (Braaz *et al*, 2003; Rose and Steinbuchel, 2005).

During rubber degradation by microorganisms, an oxidative cleavage of the double bond in the poly (*cis*-1,4-polyisoprene) backbone must occur as the first step. Furthermore, most of the degradation products detected contained aldehyde and keto groups. A crude enzyme extract prepared from the supernatant of a culture of this *Xanthomonas* strain incubated with natural rubber latex revealed activity with natural rubber. *Xanthomonas* secretes a protein having an apparent molecular mass of 65 kDa during growth on latex, which has been referred to as rubber oxygenase (RoxA). The purified RoxA protein contained about 2 mol heme per mol RoxA, which is characteristic of heme-containing proteins. Incubation of the purified RoxA protein with latex and oligo(*cis*-1,4-isoprene) resulted in the accumulation of a major 236-Da degradation product identified as 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al (Braaz et al., 2003). Such oxygenases could thus be major enzymes in the microbial

degradation of rubber. Enzyme mediator systems yielded *in vitro* degradation products containing aldehyde or keto groups. Enzyme systems such as the lipoxygenase, peroxidase, or laccase system depend on mediators, which are radicalized by these enzymes and which subsequently generate polyisoprene radicals that are cleaved by scission. If a radical mechanism also applies to the *in vivo* cleavage of polyisoprene by bacteria, radicals must be generated (Rose and Steinbuchel, 2005). The mediators involved have not yet been identified.

3.2.3 Effect of latex enzymes on microbial activity

Giordani and team (1999) reported a study of enzyme activities in C-serum and B-serum from *Hevea brasiliensis* latex. They studied the specificity of glycosidases activities at pH 5.0 in C-serum and L-serum from latex using chromogenic substrates (pNP-α-D-galactoside, pNP-β-D–galactoside, pNP-α-D-glucoside, pNP-β-D–glucoside, pNP-N-acetyl-β-D-glucosaminide, pNP-α-D-mannoside, pNP-β-D–fucoside). The determination of specific activities of glycosidases in subcellular fractions showed a strong activity of N-acetyl-β-D-glucosaminidase in L-serum and, at a lesser level, α-D-mannosidase activity. Both enzyme activities are potentially able to degrade the cell wall of *Candida albicans*. Otherwise, the C-serum contains, at a lesser level, N-acetyl-β-D-glucosaminidase activity; no α-D-mannosidase activity was detected. Therefore, in view of the potential cell wall degradation, both the C-serum and L-serum were tested for antifungal properties, that were confirmed.

In conclusion, natural rubber latex is highly susceptible to attack by bacteria during the tapping process and collection. It becomes contaminated with microorganisms. They produce enzymes which depolymerize the polymer in rubber. As a consequence, the molar mass of rubber and some qualities can be significantly reduced.

4. Field coagula maturation and consequences on properties

Field coagula are obtained from field latex, they are collected by cutting a thin strip of bark from the tree and allowing the latex to exude into a collecting vessel over a period of hours. Once it has been collected in a cup or other container, it is called field latex. It continues to be referred to as field latex until it reaches a factory for processing.

Field coagula are mainly raw material for rubber block processing. Field coagula are obtained when latex auto-coagulates, in the cup, on the cut, on the panel, or in the surroundings at the base of the tree. These field coagula are processed into field grades of block rubber and field grades of crepe. In general, cup coagula are produced by smallholders who are characterized by the small size of plantation (many are 2 hectares or less in Thailand) and their relatively low incomes. They are so far away from a factory that the latex they collect would coagulate anyway before it reached the factory.

Microorganisms can enter in the latex in the earlier stage of tapping (Taysum, 1961). Latex contains all the nutrients that microorganisms need, so it is very susceptible to bacterial growth and spoilage. Bacterial action can spontaneously coagulate fresh latex to form a solid (field coagula).

There are four types of field coagula as material for processing of block natural rubber (Cecil and Mitchell, 2005). Typical field coagula are cuplumps, treelaces, smallholders' lumps and earth scraps. Each of these four types has significantly different properties.

1. Cuplump is the coagulated material found in the collection cup when the tapper next visits that tree. It arises from latex clinging to the walls of the cup after the latex was last poured into the bucket, and from late-dripping latex exuded before the latex-carrying vessels of the tree become blocked. It is of higher purity and of greater value than the other three types.

2. Treelace is the coagulum strip that the tapper peels off the previous cut before making a new cut. It usually has higher copper and manganese contents than cuplumps. Both copper and manganese are pro-oxidants and can lower the physical properties of the dry rubber.

3. Smallholders' lump is a lump produced by smallholders who collect rubber from trees a long way away from the nearest factory. Acidic materials and fermented fruit juices are used to coagulate the latex.

4. Earth scrap is the material that gathers around the base of the tree. It arises from latex overflowing from the cut and running down the bark of the tree, from rain flooding a collection cup containing latex, and from spillage from tappers' buckets during collection. It contains earth and other contaminants and has variable rubber content depending on the amount of contaminants mixed with it.

4.1 Cup coagula maturation

Generally, the rubber industry typically uses "maturation" processes to improve some properties of rubber. Maturation is a term used for different processes, and different definitions are found in the literature, such as:

(1) Maturation is the storing time of latex compound before use for production, after mixing the vulcanizable latex with the compounding ingredients (Naus, Collard and te Winkel, 2000). Typical storage time is 12-16 h at 25-30°C.

(2) Maturation allows adequate dispersion of the compounding ingredients and the rising of air bubbles to the surface. Maturation of cuplumps is the storage time between latex coagulation and processing (granulation) (Watson, 1969).

(3) Maturation is the phenomena that occur in coagulated cup lumps before processing (granulation/ washing/drying) (Ehabe *et al*., 2002). Typical maturation times are 18 to 45 days in the shade or exposed to the sun.

In this thesis, maturation was based on the definition of Watson, 1969. Cup coagula maturation is the phenomenon that occurs during time between latex coagulation and processing.

After being tapped, *Hevea* latex is normally subjected to several treatments before it is marketable. For sheet and off-latex grades production, rubber is rapidly coagulated from latex and subjected to operations that eliminate most of the serum and non rubber constituents. The crumb is then dried appropriately and baled, with the product constituting premium technically specified and conventional sheet grades.

A more commonly adopted practice today is to wait for natural coagulation of rubber in cups. Cup coagula are the coagulated material found in the collection cup when field latex was collected in the cup. They constitute raw rubber materials for TSR 10 and 20 processing. The tapper may repeatedly taps the tree for several times until the cup is full. Coagula are then taken out of the cup after latex coagulation. Cup coagula are collected at variable frequencies, and stored for extended periods before factory processing. Coagulation and maturation are known to modify the structure of natural rubber, and this modification could either increase in viscosity due to crosslinking or decrease viscosity due to chain scission.

The variation in the quality of raw natural rubber causes serious problems associated with processing and grading. Indeed, the quality of processed rubber varies with changes in season, clonal origin and especially with the maturation time of the cup coagula material (field coagula). Field coagula are more sensitive to degradation by thermo-oxidation than NR harvested in the form of latex and then coagulated under controlled condition. Post harvest microbiological and enzymatic activities in raw natural rubber could influence the properties of the dry product.

4.2 Environmental factors

Environmental factors do not only influence the rubber to be degraded, they also have a crucial influence on the microbial population and on the activity of different microorganisms themselves. Parameters such as humidity, temperature, pH, salinity, the presence or absence of oxygen and the supply of different nutrients have important effects on the microbial degradation of rubber.

The major environmental factors that affect the long-term maturation are light intensity, relative humidity and temperature. Radiant energies such as UV light can initiate or accelerate damages by chemical reactions, especially in rubber materials. Daylight also contains infrared radiation which can initiate and accelerate damaging chemical reactions by heating.

Relative humidity (RH) refers to the relative amount of moisture in air. It is stated as a percentage, with 100% being air fully saturated with water vapor. Organic materials absorb or give off moisture continuously to achieve equilibrium with the atmosphere. Prolonged exposure of organic materials to RH above 60% - 65% will allow fungal growth.

Temperature is a significant factor because it affects on RH and on biological and chemical activities. When moisture in air is heated, RH decreases; when it is cooled, RH increases. Temperature is also important because deterioration of organic material progresses much more quickly at higher temperatures than at lower ones. Exposure to heat can drastically accelerate the aging of organic materials. Temperature also affects, in a more complex manner, biological activities.

4.3 Effect of maturation on processed cup coagula properties

Natural rubber present in the latex vessels of the tree has a high molecular weight and is well protected by anti-oxidants but, between tapping and processing, certain collection and processing practices and some environmental conditions can impair these desirable characteristics. During maturation of coagulated latex, the types of coagulation and maturation time may modify the structure of natural rubber, and these modifications affect its viscosity. However, the biological environment characterizing natural rubber coagulation and maturation is very complex, a change in the serum activity with maturation should be associated with the selective activation of antioxidants from their less reactive forms.

The Plasticity Retention Index (PRI) test gives a measure of the oxidisability of rubber, which may be affected by the concentration of anti-oxidants and metal-chelating agents and by any partial oxidation it may have already suffered. PRI is the measure of raw rubber susceptibility to thermo-oxidative breakdown by measuring the Wallace plasticity of a rubber sample before and after ageing in an oven at 140^{o}C for 30 minutes in a controlled atmosphere. It is a standardized parameter to determine sensitivity of natural rubber to thermo-oxidation. It has to be mentioned that for natural rubber there is a balance between scission and crosslinking during the thermo-oxidative process. In most of the cases, scissions are prominent. The initial Wallace Plasticity (P_0) is similar to a constraint stress compression relexometer stress. P_0 can provide on idea of the hot-flow behavior of the raw rubber and on the energy required during compound rubber operation.

The factors that lower the PRI of various forms of NR are (Watson, 1969):

- exposure to direct sunlight
- adventitious contamination with copper, iron or manganese
- excessive soaking in water
- excessive creping particularly with fine nip setting
- over-heating during drying or incorrect drying procedures

The importance of these factors varies widely, and may or may not significantly depend on other parameters such as collection time and concentration of added chemicals. Rubber processed from acid-coagulation has higher PRI values than rubber processed from auto coagulation. If very small quantities of unchelated copper are present, PRI is decreased (Hasma and Othman, 1990).

Soaking the coagulum in aqueous solutions (such as oxalic acid, phosphoric acid, sodium oxalate and EDTA or pyrogallol) can increase the PRI values. The oxalic acid treatments of copper-contaminated field latex coagula result in a rise of PRI values but the PRI levels of the processed rubber after treatment are still low compared with those made from uncontaminated and unmatured coagula (Watson,

1969). Chin *et al.* (1971) stated that the maturation of cup coagula and their storage environment have a marked effect on the viscosity and thermo-oxidative degradation of processed dry rubber. Hasma and Othman (1990) attributed a drop in PRI to the deterioration of natural antioxidants in the cup coagula during maturation.

Le Roux *et al.* (2000) found relationships between tree physiological parameters and eco-climatic conditions on technological properties of raw rubber but the maturation step which occurred under different climatic conditions was not suspected as a source of property variability.

Condition of maturation has an influence on PRI values of cuplump. Ekpini and coworker (2001) showed that the PRI value of PB 217 clone dropped from around 80 to 30 after 15 days of maturation in the cup whereas, for matured cuplumps from clone PR 107, the change was slightly downwards, with a loss of 19 % only. More recently Varghese *et al.* (2005) showed the effect of storage on P_0 and PRI of field coagulum. It was observed that there was an increase in P_0 and a decrease in PRI. The increase in P_0 was proposed to be due to the crosslink of the abnormal groups in rubber with the non-rubber constituents. The main reason for low PRI of field coagulum during storage was considered to be the bacterial decomposition of proteins and other non-rubber constituents.

4.4 Effect of maturation on macromolecular structure

Macromolecular theories explain the visco-elastic performance of high molecular weight polymers in relation to their processability. The molecular measurements involved in relaxation phenomena and in the viscoelastic properties of polymers in natural rubber are molecular weight, molecular weight distribution, gel, the degree of branching along the chain and the length of the branches.

During maturation of clone PB 235 rubber, these parameters decreased with maturation and may be related with the antioxidant activity and *Hevea* metabolism (Ehabe *et al*., 2000). It is likely that the chain-scission process is more pronounced in clone PB 235 rubber because of insufficient natural antioxidants. However, the

scission could also result in the formation of unstable terminal groups that crosslink the molecular chains. The reduction in molar mass observed for clone PB 235 rubber was proposed to be the sum of the two antagonistic processes, indicating a predominance of chain-scission reactions on prolonged rubber maturation. The molar mass distribution of clone GT1 was different from that of the other clones, as a significant reduction in the intensity of the very long chains was observed after maturation and storage days increased (Ehabe *et al.*, 2000).

MATERIALS AND METHODS

Materials

1. Origin of natural rubber latex samples

The latex was sampled from 32 *Hevea brasiliensis* trees from RRIM600 clone located in an area of the rubber plantation of Prince of Songkla University, Surat Thani Campus in Surat Thani Province, the southern part of Thailand. The plantation was planted in 1990 and started tapping in 1997.

2. Industrial cup coagula

Industrial cup coagula samples were collected from the factory of Von-Bundit Co., Ltd. (Khun Talay branch, Surat Thani province, Thailand). These cup coagula were naturally coagulated in small- holder rubber plantation and stored for 1-2 weeks by the middle man. Then they were transferred to the factory and stored in a pile of 3-meter in height and 20-meter in width. The industrial cup coagula of the pile (Figure 5) were stored outdoor and were exposed to sunlight during 14 to 30 days before being transferred to the processing line. All experiments were performed during February 2007 to March 2008.

Figure 5 Commercial cup coagula pile.

3. Source of microorganism's inocula

During the laboratory maturation operations, controlled inoculation of latex was performed. In order to get a representative inoculum, microorganisms were collected from the serum of the cup coagula after three days of maturation in their cups. The collected inoculum was from the same trees as the ones used for latex sampling (paragraph 1 on page 40). The preparation of inoculum is described in the method part.

4. Chemicals

Reagents, organic solvents and chemicals used for enzyme activity testing and determination of enzyme activity products are presented in Table 2.

Table 2 List of solvents and chemicals.

Name	Supplier	Country
Reagents and solvents		
Acetone	Merck	Germany
Benedict's reagent	Merck	Germany
Benzoic acid	Carlo	Italy
Chloroform	Merck	Germany
Citric acid	Merck	Germany
3,5-Dinitrosalicylic acid	Sigma	Germany
Di-Sodium hydrogen phosphate 12 H_2O	Merck	Germany
Ethanol	Merck	Germany
Folin&Ciocalteu's Phenol reagent	Merck	Germany
Formaldehyde	Carlo	Italy
Formic acid	Commercial	Thailand
Iodine solution	Merck	Germany
p-Nitrophenyl b-D-glucopyranoside	Sigma	Germany
Potassium dihydrogen phosphate	Merck	Germany
Phosphorous pentoxide	Merck	Germany

Safranin solution 1% w/v	Fluka	Germany
Sodium azide	Riedel	Germany
Sodium bicarbonate	Merck	Germany
Sodium carbonate anhydrous	Merck	Germany
Sodium hydroxide	Merck	Germany
Sodium dihydrogen phosphate	Merck	Germany
Thymolphthalein	Merck	Germany
Trichloroacetic acid	Fluka	USA
Tyrosine standard	Sigma	Germany
Enzymes and media substrate		
Beta-Glucosidase from almond	Sigma	Germany
Cane sugar	Commercial	Thailand
Casein from bovine milk	Fluka	USA
Gram Arabic	Fluka	USA
Lipase from *Candida parapsilosis*	**	France
Olive oil	Food	Thailand
Protease	Sigma	Germany
Skim Milk Powder	Merck	Germany
Tributyrine	Fluka	USA
Plate Count Agar (PCA)	Difco	USA
Mannitol Salt Agar (MSA)	Difco	USA
Mac Conkey Agar	Merck	Germany
MRS	Difco	USA
Malt Extract Agar (MEA)	Difco	USA

**provided by laboratory "Biotechnologie microbienne et enzymatique des lipides et des agropolymères, Département des Sciences pour les Agro-Bioprocédés, Montpellier SupAgro, France).

5. Equipments

The equipments used in this work are listed as follows:

➢ Air compressor	XM 2540 3 HP, Puma	Taiwan
➢ Autoclave	SX 700, TOMY Digital Biology, Ltd.	Japan
➢ Bright field Microscope	CX31, Olympus	UK
➢ RH & Tem Data logger	V2.2, Scientific Equipment Co. Ltd.	Thailand
➢ Flow meters	RM SERIES, Dwyer Instruments, Inc.	USA
➢ Humid/Temp meter	HT-765-232, Digicon	Japan
➢ Hot air oven	UE700, Memmert GmbH & Co. KG.	Germany
➢ Digital Humidity Sensor	SHT 15, Sensirion AG, Staefa ZH	Switzerland
➢ Incubator	BD Series 53, Binder gmbh,	Germany
➢ Industrial Crepper	Lihoe Co. Ltd, Selangor	Malaysia
➢ Nitrogen tank	Thai Industrial Gazes PCL	Thailand
➢ Multimeter with	Multi 350 model, VWR GmbH,	Germany
o Oxygen probe	Conox 3, VWR , Weilheim	
o pH probe	Sentix SP S7, Weilheim	
➢ One-Touch connector	JEL PC, DINGAN	China
➢ SEC-MALS	Dawn DSP, Wyatt technology Corp.	USA
➢ Temperature controller	Compatible CCI, huher	USA
➢ Water pump	Power Head AP 1600, Sonic	China

Methods

1. Preparation of natural rubber samples

1.1 Natural rubber latex samples collected by normal tapping

The 32 selected rubber trees were tapped in the early morning. The following tapping system was used: S/2, 3d4 (the trees are tapped on one half spiral cut tapped downward 3 out of 4 days without stimulation). Latex was collected from the clean cup about 3 hours after tapping and filtered through a stainless steel sieve (pore size, 1.0 mm). This fresh latex was used for the cubic coagula (paragraph 8, page) and mini-cup coagula samples (chapter 3). The periods of sampling were from August to November 2008.

1.2 Clean natural rubber latex sample (CL) collected by "clean" tapping

A special tapping procedure was developed for the collection of Clean Latex (as clean as possible) which contains a minimum number of microorganisms. The collecting material was sterilized at 121°C for 15 min. in autoclave before using. Such material included a tapping knife, a spout, a plastic tube connected with a collection plastic bag (15 cm x 23 cm), a plastic sheet (60 cm x 70 cm) and water (1 L).

The bark downward from the cut was slightly scrapped with a sterile metallic blade (5 cm x 12 cm) 3 cm below the cut (Figure 6). This part of the bark, the cut and the scrapped area 3 cm up from the cut were cleaned successively with commercial sterile cotton added with sterile water, ethanol (70 %) and sterile water respectively. The clean panel was covered with a plastic sheet fixed on the tree by a rubber band, 10 cm above the tapping panel. Tapping was performed with a sterile tapping knife and a new sterile spout was fixed on the tree. Latex was turned toward the sterile spout connecting with the sterile plastic tube which was inside the sterile plastic bag. The bag was placed inside an ice containing cup (Figure 6). When the latex flow

stopped (around 3 h after tapping), plastic bag containing latex was transferred to a laminar flow cabinet in the nearby laboratory.

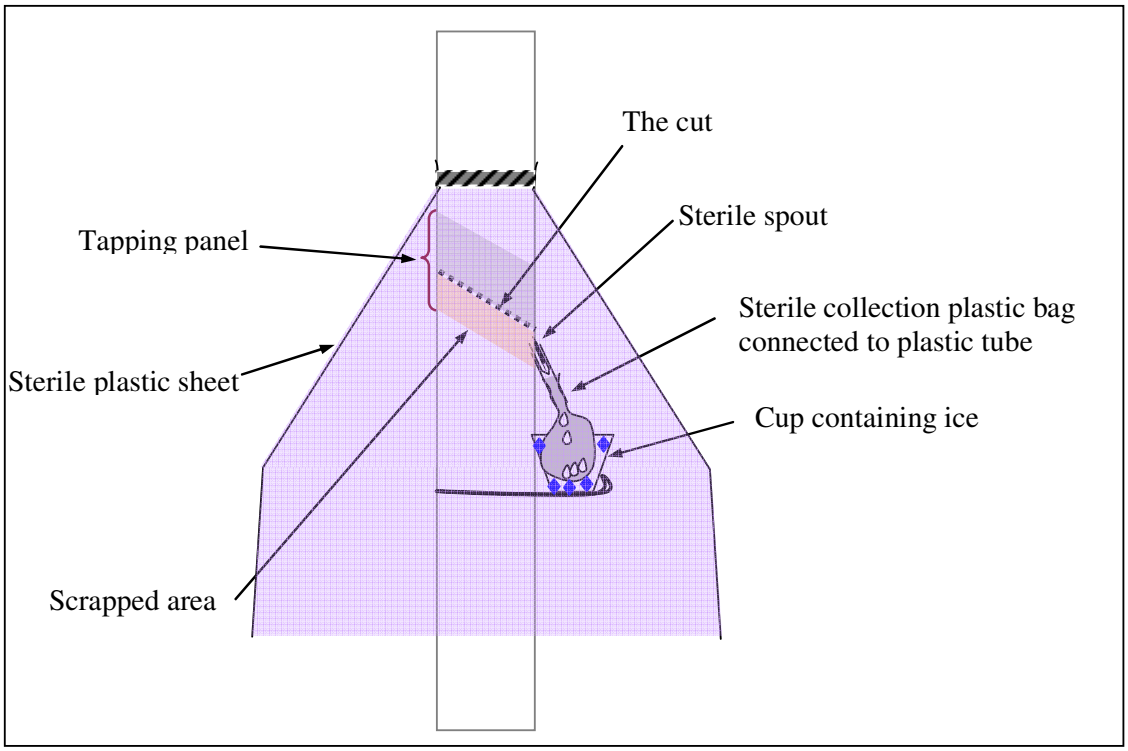


Figure 6 Clean latex tapping system.

2. Mini-cup coagula preparation

Two groups of cup coagula were used in this study. The first was industrial cup coagula obtained from smallholders through their delivery to STR 20 factory Von Bundit Co, LTD. (Materials section paragraph 2). The second group was mini-cup coagula which were prepared at a laboratory scale with origin-controlled latex obtained by the “clean” tapping (Methods section paragraph 1.2).

2.1 Preliminary preparation

Several solutions were prepared before the latex collection.

2.1.1 Sodium azide solution (10%)

Ten percent (w/v) of sodium azide solution was prepared by dissolving 10 g of sodium azide (NaN_3) in a total volume of 100 mL of distilled water measured in a volumetric flask. The solution was agitated with a magnetic stirrer for 20 min.

2.1.2 Sodium chloride solution

Sodium chloride 0.9% solution was prepared by dissolving 9 g of sodium chloride in 1000 mL of distilled water in a volumetric flask. After mixing by using a magnetic stirrer for 20 min, it was autoclaved at 121°C for 15 min.

2.1.3 Formic acid solution

Formic acid 5% solution was prepared by diluting 13.3 mL of formic acid to a total volume of 250 mL in sterile distilled water. The volume adjustment was performed in a volumetric flask.

2.1.4 Inoculum

The microbiological inoculum preparation is described in paragraph "9-Microbiological techniques".

2.1.5 Hydrolase solution preparation

In some experiments, the microbial inoculation was replaced by enzyme addition.

Hydrolase enzyme solutions were prepared by dissolving enzyme powder in 100 mM phosphate buffer pH 5.2. Two hydrolase activities were tested during this study:

➔ **protease** (papain from *Carica papaya* skin) was from Sigma (ref : P3375)

➔ **lipase** from *Candida parapsilosis* CBS 604 was produced in Montpellier SupAgro by fermentation of a recombinant strain of *Pichia pastoris* as described by Brunel, *et al*. (2004).

The activity of each enzyme powder was checked prior to the utilization (enzyme assay is detailed in paragraph 10 page 33). Table 3 indicates the initial activity and the concentration used to obtain the enzyme stock solutions which were prepared before adding in the latex.

Table 3 Enzyme concentration and activity of the stock solutions of enzymes.

Enzyme	Substrate	Measured Product	Standard activity (U/mg)	Measured activity (U/mg)	Weight (mg) of enzyme in 26 mL of phosphate buffer	Activity of the stock solution (U/mL)
Protease (Sigma)	Casein	Tyrosine	0.5-2	1.1	1300	55
Lipase (SupAgro, France)	Olive Oil	Fatty acid	80	113	58	250

For each enzyme, one unit (U) of activity corresponds to the release of 1µmol of product per minute in the testing condition. (Activity testing methods are detailed in paragraph 10)

2.2 Preparation of latex before coagulation

2.2.1 Mini-cup coagula with controlled microbial population

Several kinds of mini-cup coagula were prepared in this study. Coagulation was either natural or induced by addition of formic acid while microorganisms population was controlled with inocula or, on the contrary, antibiotic (sodium azide) addition.

The different treatments are shown in Figure 7 while the details of the volume of each additive are indicated in Table 5. All these operations were performed under sterile laminar flow or in some cases, in an area of 15 cm around a flame. The symbols of each treatment are listed in Table 4:

Table 4 Signification of the symbols of treatments.

Treatments	with	without
sodium azide	N+	N-
microorganisms	M+	M-
formic acid	F+	F-

In every case, Clean Latex (approx. 5 L) was transferred from the sterile plastic bags to a 5 L sterile glass container. The Clean Latex was homogenized by gentle stirring with a sterile glass rod. The initial pH of clean latex was measured. Depending on various treatments indicated in Table 5, this latex was split into different lots and each lot was added with all additives except formic acid and gently agitated using a magnetic stirrer. Then, in the case of controlled coagulation, 5% formic acid solution was added into the latex mixture until pH of latex decreased to 5.2. The pH evolution was monitored by a disinfected pH probe (Sentix SP S7, VWR, Weilheim, Germany) connected to a multi 350 data logger (VWR GmbH, Weilheim, Germany).

As shown in Figure 7, three mini-cup coagula (N+M-F-, N+M+F- and N-M-F) were not prepared because the coagulation time could not be controlled properly in a short period (more than 2 days were indeed required).

Nevertheless, the treatment N-M+F- was considered as a control representing the natural coagulation of the field latex in the cup. Indeed, the addition of microorganisms led to an adjustment of the microorganisms population in the initially clean latex to the level that was present in field latex ($3X10^6$ CFU/mL) harvested by smallholders. M1+, M2+ and M5+ represented different quantities of microorganisms added to clean latex and concentration of inoculum adding are shown in Table 5.

Maturation time had been studied in order to follow the evolution of cup coagula properties during 7 weeks, period of storage in industrial practices. The total volume of latex used for each lot or treatment (approximately 1 L) was for a test of up

to 6 maturation times with 3 repetitions with about 45 mL of each mini-cup coagula (18 mini-cup coagula per treatment).

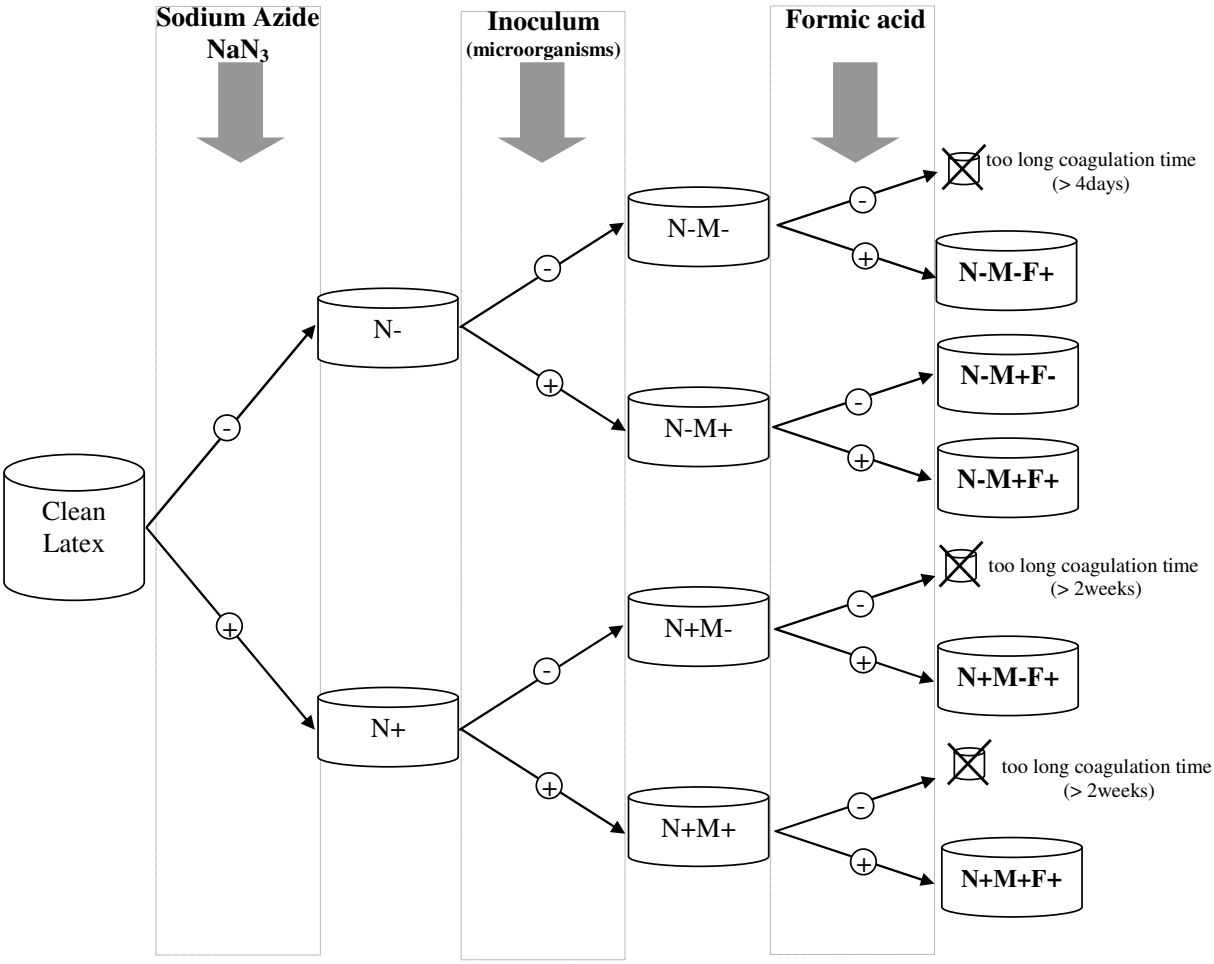


Figure 7 Flow chart of mini-cup coagula preparation with controlled microbial population.

2.2.2 Hydrolase-added mini-cup coagula

An anther set of samples was made to test the effect of selected hydrolases on the maturation of mini-cup coagula with microbial agent. Clean latex was split into different lots and each lot was added with sodium azide solution and formic acid. For both protease and lipase activities, five concentrations of enzyme were tested by adding different volumes of previously prepared stock solutions of enzyme. The mixture was homogenized by a gentle agitation using a magnetic stirrer. Figure 8 illustrates the mini-cup coagula preparation while Table 6 gives the details of the volumes used in the mixture.

2.3 Transferring to mini-cup and coagulation

Sterile 45 mL glass mini-cups (3 cm x 4 cm x 5 cm) were fully filled (around 45 mL) with prepared latex shortly after formic acid addition, if any (Figure 9). Mini-cup coagula in glass cups (with formic acid) were stored in sterile hermetically closed plastic containers (28 cm x 18 cm x 18 cm) in an incubator at 40°C for 3 hours. The mini-cup coagula reference in glass cup (without formic acid) was stored for 2 days at 40°C in other sterile hermetically closed plastic container in the incubator. After latex coagulation, all the plastic containers were moved out from the incubator. The mini-cup coagula were removed from their cups and replaced on a sterile stainless steel tray in the same plastic containers (18 mini-cup coagula in each plastic container, Figure 10). All transfer operations were carried out in a laminar flow cabinet.

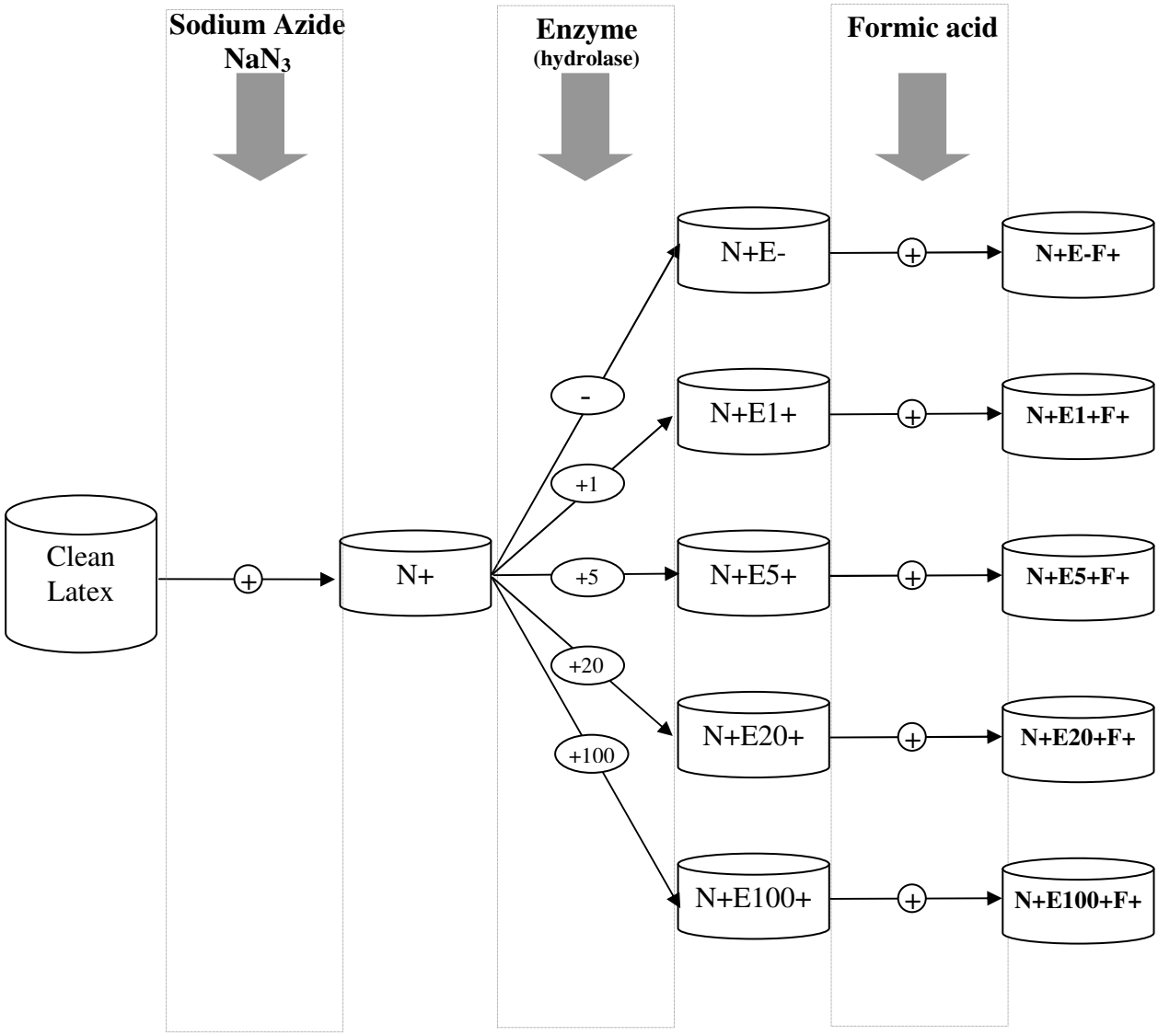


Figure 8 Flow chart of mini-cup coagula preparation added with hydrolytic enzyme.

Table 5 Composition of lattices with controlled microbial population.

Label	N-M-	N-M+F-	N-M+F+	N+M-F+	N+M+F+	N-M1+F+	N-M2+F+	N-M5+F+
Clean Latex (ml)	1000	900	900	900	900	1000	1000	1000
10 % Sodium azide solution	-	-	-	16	16	-	-	-
Sterile water (mL)	-	24	16	-	-	-	-	-
Inoculum (mL)	-	20	20	-	20	14	28	-
Concentrated inoculum 2.5X	-	-	-	-	-	-	-	28
Sterile 0.9% NaCl (mL)	28			20	-	14	-	-
5% Formic acid (mL)	9	-	8	8	8	9	9	9
Total volume (mL)	*1037*	*944*	*944*	*944*	*944*	*1037*	*1037*	*1037*
Approx CFU/mL (before	*3x10^5*	*3x10^6*	*3x10^6*	*3x10^4*	*Nd*	*2x10^6*	*9x10^6*	*2x10^7*

NB: fresh latex harvested in normal tapping (not clean) contains 3x10^6 CFU/mL

Table 6 Composition of lattices added with hydrolytic enzymes.

Label	N+E-F+	N+E1+F+	N+E5+F+	N+E20+F+	N+E100+F+
Clean latex (mL)	960	960	960	960	960
10 % Sodium azide solution	20	20	20	20	20
Hydrolase enzyme stock solution (mL)*	0	0.2	1	4	20
- Protease (U/mL)	0	11	55	220	1100
- Lipase (U/mL)	0	50	250	1000	5000
100mM Phosphate buffer (mL)	20	19.8	19	16	0
5% Formic acid (mL)	15	15	15	15	15
Total volume (mL)	*1015*	*1015*	*1015*	*1015*	*1015*

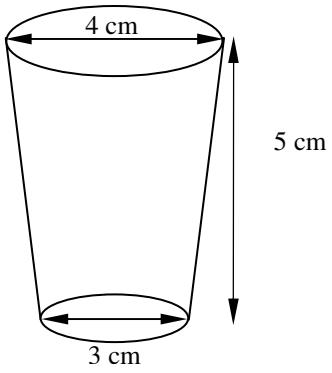


Figure 9 Glass mini-cup for the mini cup coagula preparation.

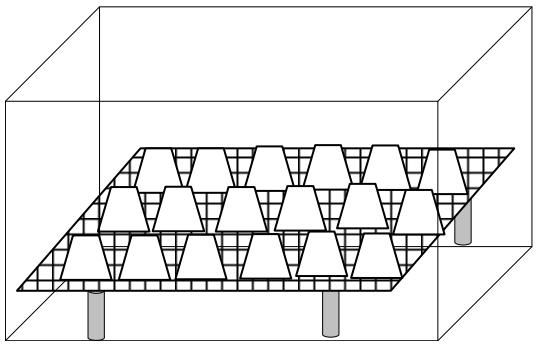


Figure 10 Mini-cup coagula placed on sterile stainless steel trays in hermetically closed plastic container.

3. Maturation in controlled devices

3.1 Short-term maturation in closed container

In this case, the mini-cup coagula were kept in the hermetically closed plastic containers [without air circulation with high relative humidity due to serum exudates (Figure 11)]. The maturation was performed over a period of 6 days under a temperature of 40 °C in an incubator.

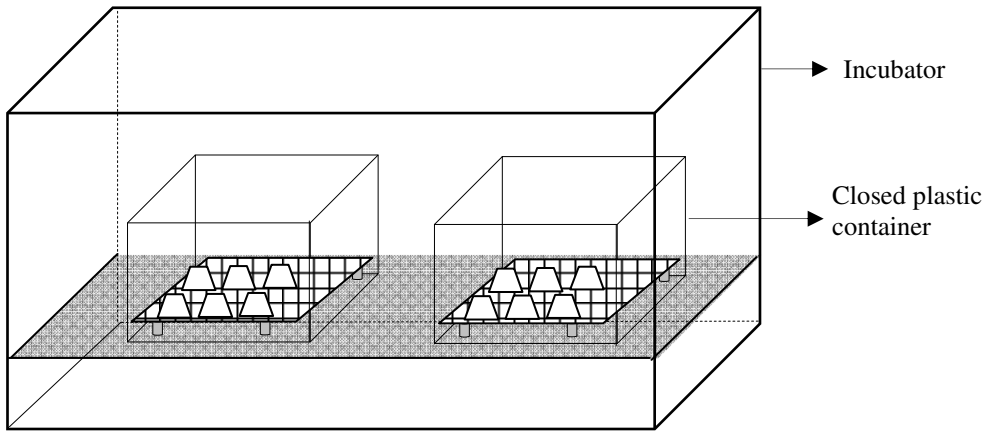


Figure 11 Two hermetically closed plastic containers equipped with stainless steel trays placed in an incubator.

3.2 Long-term maturation in a maturation device

3.2.1 Maturation device

A long-term maturation device was developed in order to be able to conduct maturation experiments under controlled conditions including air sterility, air relative humidity, air temperature and air oxygen content. The development steps of this device are detailed in Chapter 2. This pilot could contain 6 maturation units with 36 mini-cup coagula (Figure 12).

3.2.1.1 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 (compatible CCI, huher, USA) located in the water tank and the water was circulated by a water pump (900L/h, Power Head AP 1600, Sonic, China) from this tank to the insulated boxes 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 boxes 1 and 2, and air humidifying column) (Figure 13). The maturation units were immersed in water so that the surrounding water level reached ¾ of the height of the maturation units. The air temperature inside each maturation unit was measured by a probe (Digital Humidity Sensor SHT 15, Sensirion AG, Staefa ZH, Switzerland) connected to a data logger system (Scientific Equipment Temperature & %RH Data logger V2.2, PSU Hat Yai, Thailand) and linked to a computer. The temperature of each maturation unit was recorded every 30 min.

3.2.1.2 Control of gas flow inside the maturation unit

Three parameters were controlled: the flow rate, the oxygen content and the relative humidity. The gases were flown in polyurethane tubes (external diameter = 8 mm, internal diameter = 5 mm) connected with One-Touch type connector (JEL PC, DINGAN, China). Figure 12 illustrates the gas flow control of the device, and a simplified chart is given in figure 14.

- **Oxygen content:**

Two gases supplier units were used:

- one air compressor (XM 2540 3 HP, Puma)
- one Nitrogen tank (9 m^3, 99% N_2, Thai Industrial Gazes PCL td, Thailand).

Both were equipped with a pressure regulator which delivered a 1.4 X 10^5 Pa (1378 millibar) pressure to the system. Both tubes were joined with a “Y” type one-

touch connector. The balance between compressed air and nitrogen was set by two flow meters (RM SERIES, Dwyer Instruments, Inc., Michigan, USA) indicated as “A” and “N” in the Figures 12 and 14. The two gases were mixed in a plastic box containing plastic tips to enhance the homogenization. Oxygen content of the obtained gas was calculated taking as an assumption that air oxygen content was 21%.

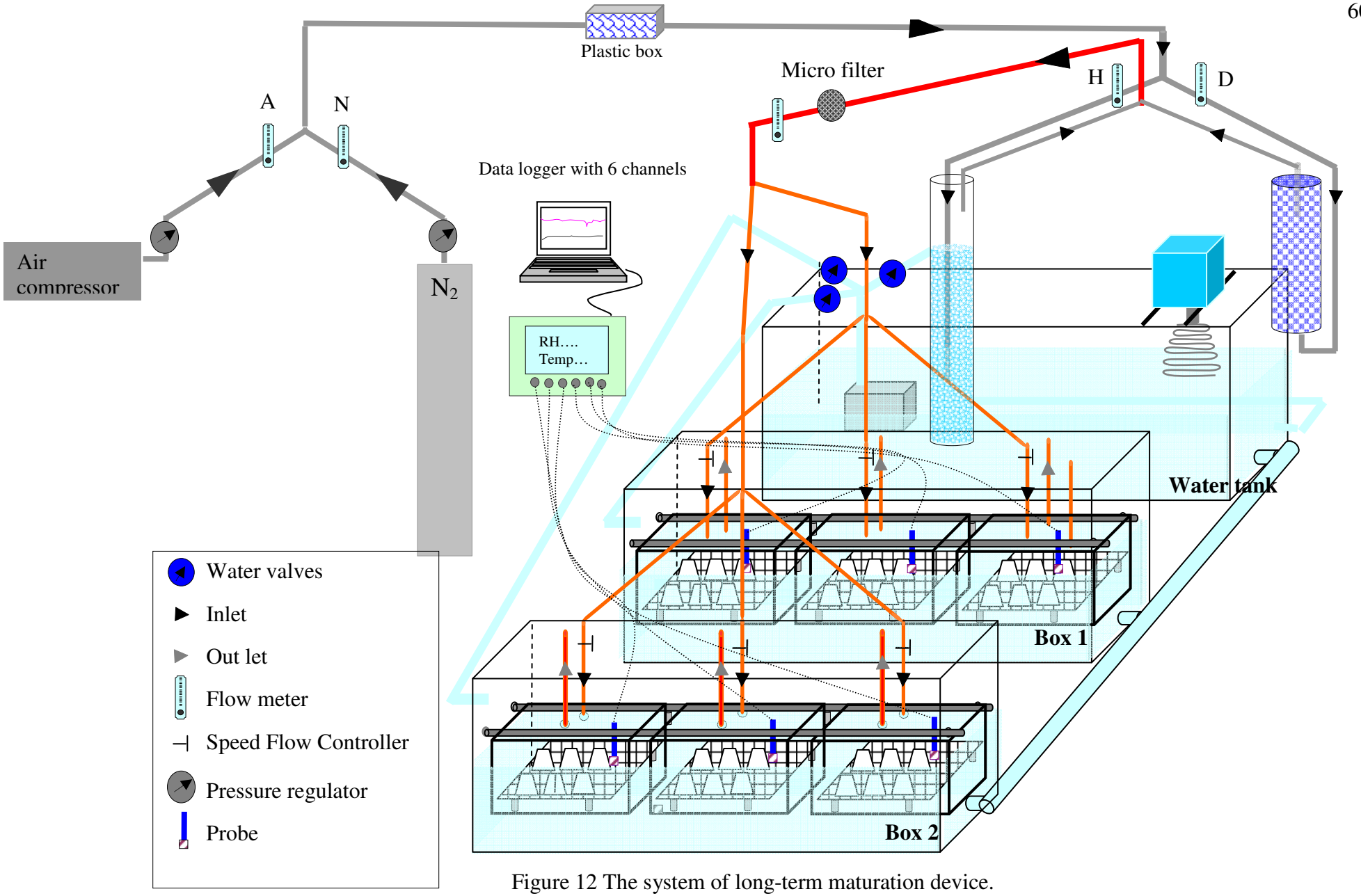


Figure 12 The system of long-term maturation device.

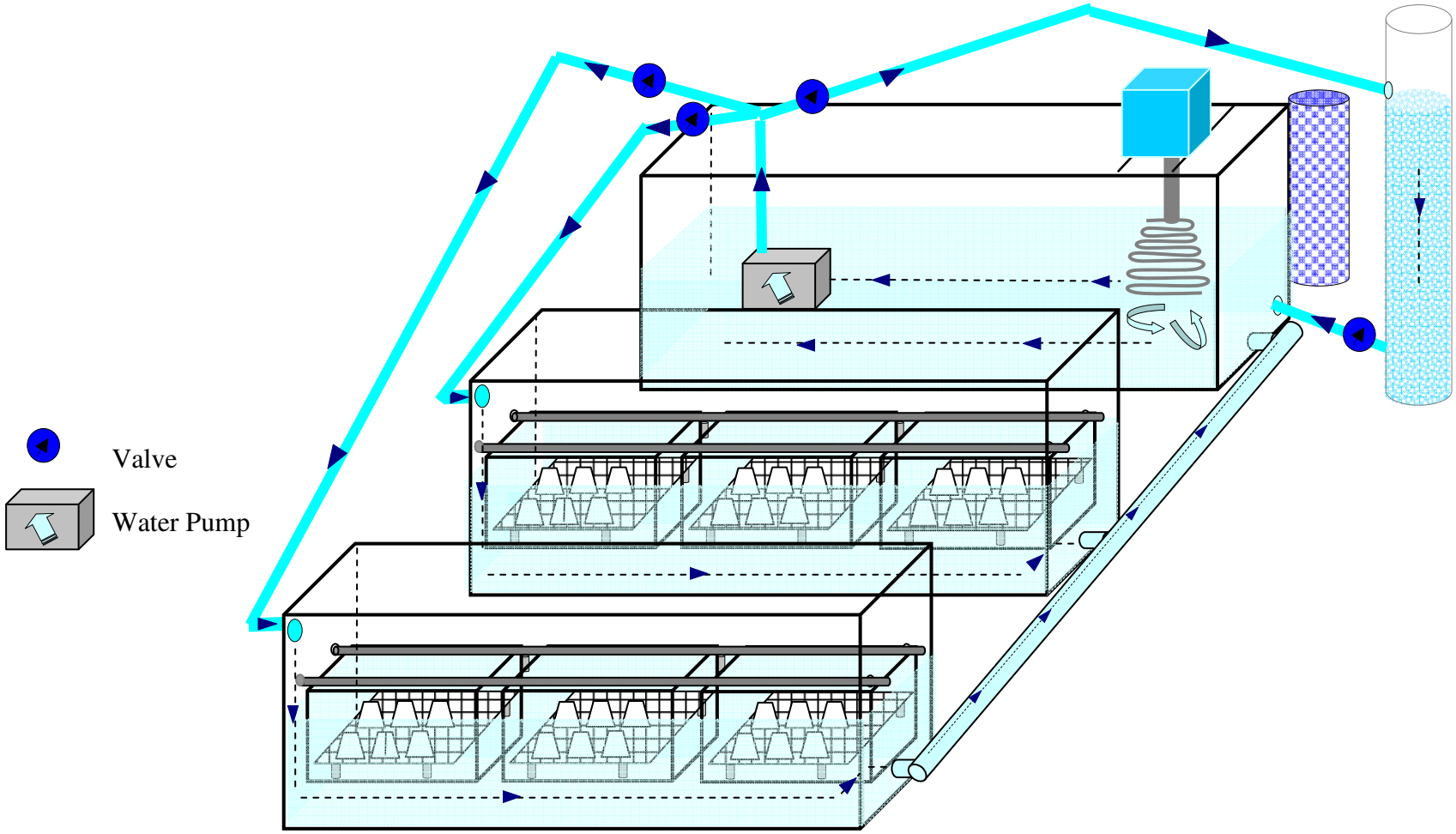


Figure 13 Water circulation system (-►-) of the long-term maturation device.

- **Relative humidity:**

The gas flow with known oxygen content was split into two ways by a "Y" type one-touch connector. One way was directed to a gas humidifying column while the other way was directed to a gas drying column. Each way was equipped with a flow meter ("H" and "D", respectively, see Figures 12, 14). The gas drying column (Figure 15, (B)) consisted of a polycarbonate tube (thickness 2mm, external diameter 10 cm, height 50 cm) equipped with PVC screw cap (diameter 5.5 cm) at the top, and fully filled with silica gel beads (1.75 kg). The air humidifying column (Figure 15, (A)) consisted of a polycarbonate tube (thickness 2mm, external diameter 7.5 cm, height 100 cm) equipped with PVC screw cap (diameter 5.0 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 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. Both "dry" tube and "humid" tube were joined together to obtain gas with a control relative humidity and oxygen content. The balance between "dry" flow and "humid" flow was controlled by the flow meters "H" and "D".

- **Gas flow distribution :**

The gas with a control relative humidity and oxygen content was sterilized by passing through a filter (0.3 microns pore size) and distributed to the maturation units by a system of "Y" connectors. Inlet flow of each maturation unit was adjusted by a speed flow controller.

- **A Posteriori Control :**

The inlet flow, the balance between Nitrogen/Air and the balance between Humid/Dry gases, was adjusted to meet the targeted conditions in maturation units. Gas relative humidity in each maturation unit was measured by a probe connected to a data logger system that linked to a computer. Gas oxygen content was measured with a dedicated probe (Conox 3, VWR GmbH, Weilheim, Germany) connected to a multimeter (Multi 350 model, VWR GmbH, Weilheim, Germany). This "A posteriori" control was especially necessary to buffer the change of material humidity during maturation.

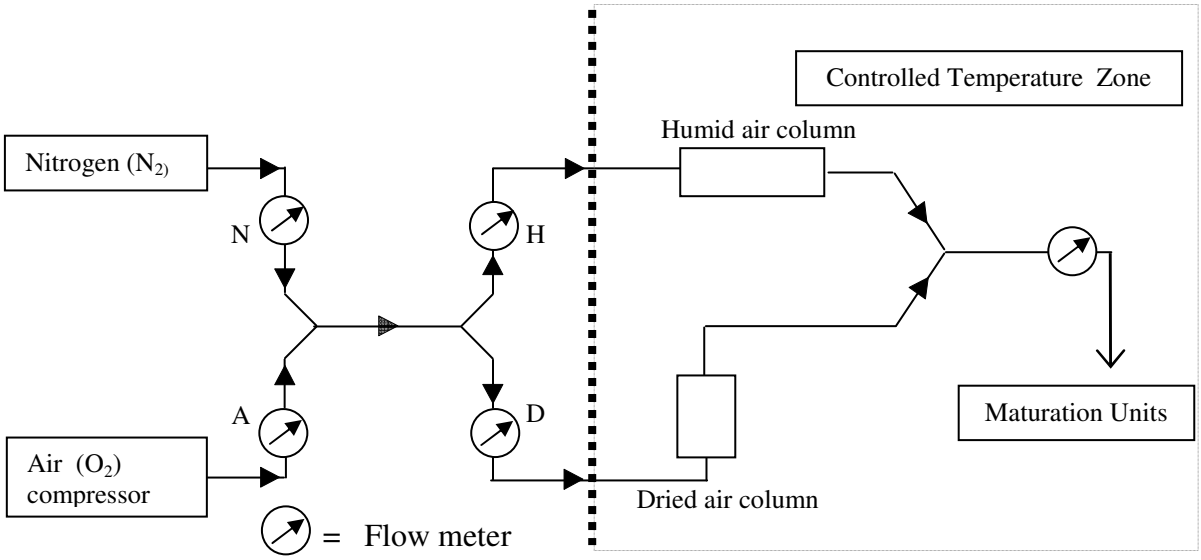


Figure 14 Simplified diagram of the gas flow control system.

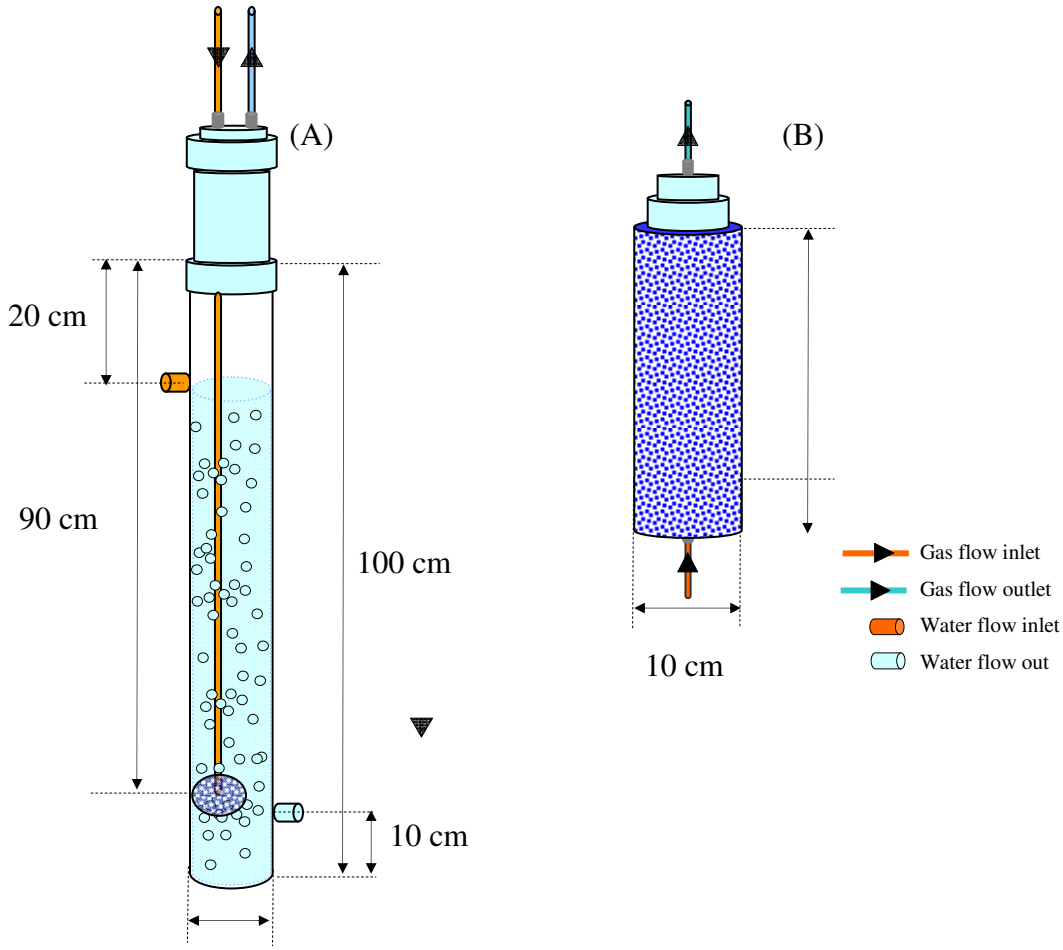


Figure 15 Air humidifying column: (A) and air drying column: (B).

3.2.2 Transferring of the mini-cup coagula in a long-term maturation device

The mini-cup coagula (with formic acid) were kept in hermetically closed plastic containers used for coagulation and placed in 40°C incubator for overnight in order to let the serum drain from the mini-cup coagula. This preliminary step was necessary in order to be able to control further gas relative humidity in maturation units. Afterward, the mini-cup coagula were transferred from the plastic containers to the maturation units. Flame was used to keep the transfer area sterile.

3.3 Maturation conditions and sampling

The following maturation conditions, described in Table 7 were tested during this study:

Table 7 Maturation conditions.

Device	Gas Temperature	Gas Relative Humidity	Composition of gas	Maximum duration	Day of sampling
Short-term	40 +/- 1°C	Saturated	not controlled	6 days	0-1-2-3-4-5-6
Long-term	40 +/- 1°C	90%+/- 10%	Air	45 days	0-3-5-15-30-45
Long-term	40 +/- 1°C	90% +/- 10%	99% Nitrogen	45 days	0-3-5-15-30-45

In order to evaluate the effect of maturation time on the properties in short-term maturation experiments, 3 mini-cup coagula from each closed plastic container were randomly sampled after 0, 1, 2, 3, 4, 5 and 6 days. The sampling days of maturation unit for long term maturation were 0, 3, 5, 15, 30 and 45 days. All sampling were performed in presence of a flame to prevent external contamination. All the treatments and conditions of maturation are described in Figure 16.

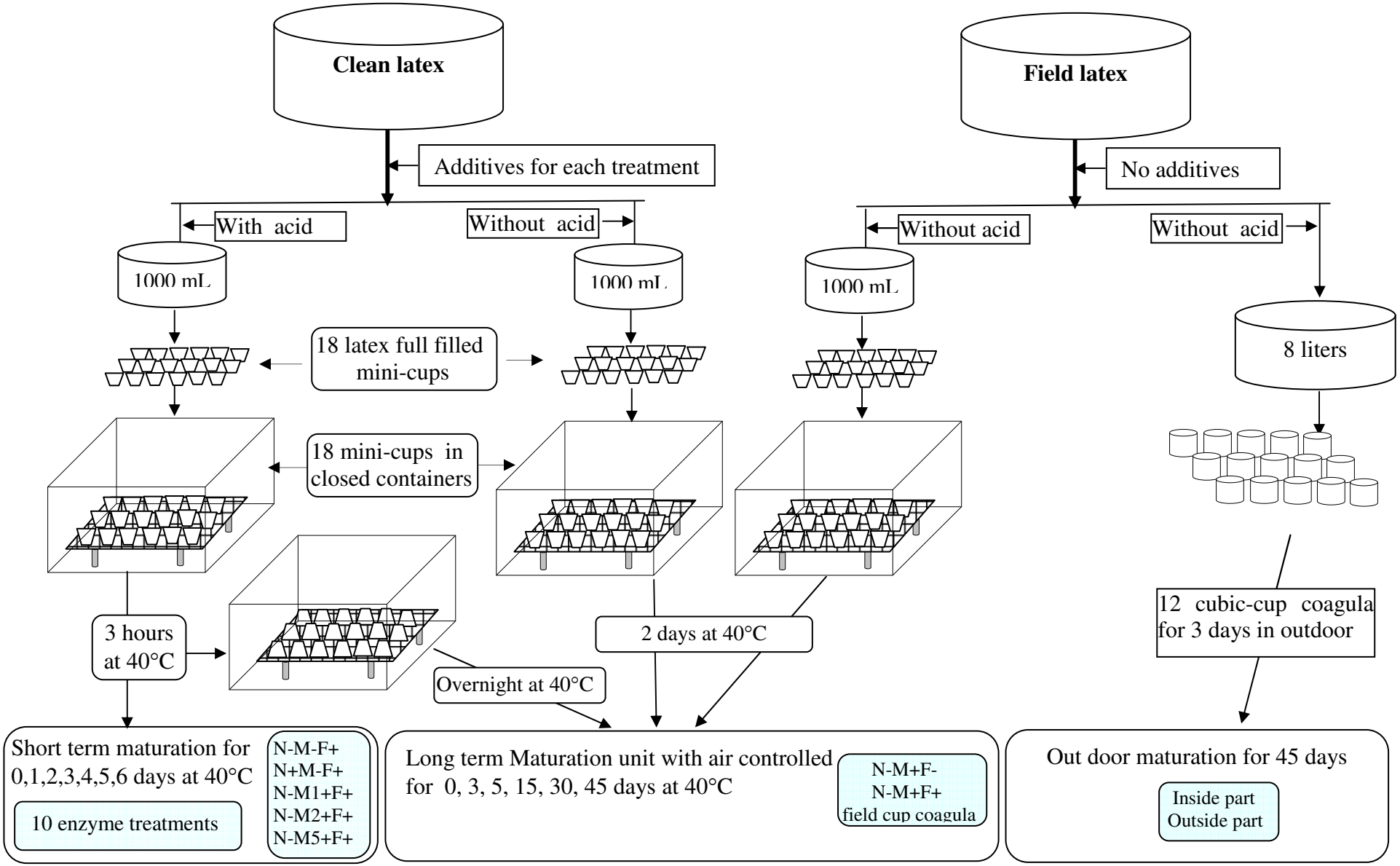


Figure 16 Flow chart of the samples treatments and conditions of maturation

4. Mini processing in the laboratory

To get a dry rubber sample from the maturated mini-cup coagula, and considering the number of sample and the small size of them, a specific laboratory process was developed in order to obtain rubber with similar technological properties as the STR processed industrially. The development steps of this process are detailed in Chapter 2.

This laboratory process is based on a procedure with a specific mini-creper. This equipment has the following specifications : roll diameter 10 cm; roll length 30cm, diamond type groove (space between grooves 6 mm, groove depth 1 mm, groove width 2mm), gap between rolls: 0.04 mm, speed of front roll: 190 rpm/min, friction ratio: 1.3:1 (front:rear), Engine power: 2.2 kW. The creping procedure consisted in 15 double passes and one single pass under water shower placed over the nip of rolls (width 30 cm, flow 6.2 L/min).

The obtained crepes were dried in a hot air oven (UE700, Memmert GmbH & Co. KG., Germany) at 125°C for 2 hours without renewal air.

5. Determinations of cup coagula properties

5.1 Determination of total solid content (TSC) of industrial cup coagula

For industrial cup coagula, three pieces of approximately 1g were cut from one cup coagula and weighted precisely (m_0). Each piece was dried in an oven at 100°C for 3 hours. After cooling down in a desiccator, the dried piece without its whiteness was weighed (m_1).

The TSC of industrial cup coagula was calculated as follows:

$$\text{Total solids content (TSC, \%)} = \frac{m_1 \times 100}{m_0}$$

m_0 = the mass in grams of the test portion before drying

m_1 = the mass in grams of the dried material

5.2 Dry rubber content (DRC) of mini-cup coagula

For mini-cup coagula, the fresh mini-cup coagula were weighed (m_0) before creping and drying (125°C, 2 hours). After cooling down in a desiccator, the dried piece was weighed (m_1).

The DRC of mini-cup coagula calculated as follows:

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

m_0 = the mass in grams of the test portion before creping and drying

m_1 = the mass in grams of the dried material

In both case, three replications were done.

5.3 Determination of pH of cup coagula

Inner pH of fresh and maturated industrial cup coagula or mini-cup coagula was measured with a penetrating probe (Sentix SP S7, VWR, Weilheim, Germany) connected to a Multi 350 data logger (VWR GmbH, Weilheim, Germany). The cup coagula were cut into two parts. For each part of industrial cup coagula, replications of the measurement were performed on five different locations while two repetitions were done for the mini-cup coagula by inserting the probe at the center of each part of the mini-cup coagula.

6. Dry rubber property determinations

6.1 Determination of Initial Plasticity (P_0) , Plasticity Retention Index (PRI)

The P_0 is a standard measurement for raw natural rubber. Minimum level of P_0 is required by manufacturers or international standards ($P_0 > 30$). P_0 value is in relation with the structure of the natural rubber and also energy required during compounding operations; it can provide an idea of the hot-flow behaviour of raw rubber. This measurement requests specific equipment: Wallace Rapid Plastimeter.

The PRI is a measure of the susceptibility of raw natural rubber to thermal oxidative breakdown. The test involves a measurement of the Wallace plasticity before (P_0) and after heating (P_{30}) in an air Wallace oven at 140° C for 30 min. A high value of PRI denotes high resistance to thermal oxidative breakdown.

A dry rubber sample was homogenized following SMR bulletin 7 (1992) part. B.2, standard. Initial plasticity (P_0) and Plasticity retention index (PRI) were determined according SMR bulletin 7 (1992) part. B.8, standard and Accelerated storage hardening test (ASHT) was determined according to SMR bulletin 7 (1992) part. C.1 standard.

6.1.1 Preparation of test pellets

A piece of homogenous rubber was blended twice through the rolls of a cool roller mill with adjusted nip in order to obtain a 1.6-1.8 mm final sheet thickness. The sheet was immediately doubled and the two halves were pressed lightly together by hand. Six test pellets, approximately 3 mm thick and 13 mm in diameter were punched out from the doubled sheet with a Wallace Punch. The test pellets were divided into two sets of three; one set from each was for plasticity determination before ageing and the other set after oven ageing (Figure 17)

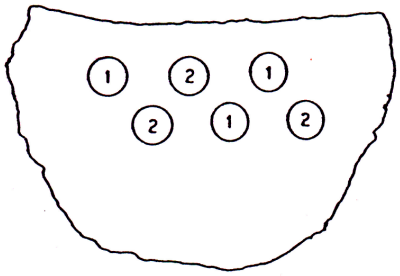


Figure 17 Double rubber sheets with punched test pieces.

6.1.2 Determination of P_0

The test pellet sandwiched between two pieces of cigarette paper, was compressed between the two parallel platens of the Wallace Plastimeter (Wallace Rapid Plastimeter MK V P14, HW, Wallace & Co. Ltd., UK) to a fixed thickness of 1.00 ± 0.01 mm and held for 15 ± 0.2 seconds to reach approximate thermal equilibrium with the platen temperature (100°C ± 1°C). It was subjected to a constant compressive force of 100 ± 1 N for 15 seconds at 100°C ± 1°C. The median value of the thickness of three pellets at the end of this 15 second period was taken as the measure of Initial Plasticity.

6.1.3 Determination of PRI

A set of three test pieces was heated in an air Wallace oven at 140 ± 0.2 °C for 30 ± 0.25 min. After cooling the aged samples at room temperature, the plasticity of the aged pellets was taken according the method used for unaged samples.

The median values of the three unaged and three aged test pieces were used to calculate PRI as follows:

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

6.2 Determination of Mesostructure and Gel content of rubber

6.2.1 Determination of weight average molar mass ($\overline{M_w}$), number average molar mass ($\overline{M_n}$) by size exclusion chromatography coupled with a multi-angle light scattering detector (SEC-MALS)

A 25 ± 2 mg of homogeneous rubber sample was dissolved in 40 mL of tetrahydrofuran (THF) stabilized with 3,5-di-tert-butyl-4-hydroxytoluene (BHT) for 2 weeks at 30° C. The rubber solutions were stored for the first 7 days without stirring and gently stirred for 1 hour daily on the next 7 days. The solution was filtered (disposable filter, Acrodisc 1 µm, glass fiber, Pall) and analysed by a size-exclusion chromatography system consisting of an online degasser (EliteTM, Alltech), a Waters 515 pump, a refractive index detector (Waters 2410) and a multi-angle laser light scattering detector (Dawn DSP, Wyatt technology Corp.). The columns were three inline PLGEL (Polymer Laboratory) mixed beds (20 µm, 7.8 mm ID x 30 cm) with a guard column. These columns were maintained at 45°C. Mobile phase was THF stabilized with BHT at a flow rate of 0.65 mL. min^{-1}; the injected volume was 0.15 mL.

Number-average molar mass ($\overline{M_n}$) and weight-average molar mass ($\overline{M_w}$) were calculated by using ASTRA software (Wyatt technologies Corp.). Fourteen angles, from angle 3 (32°) to angle 16 (134°), were used for extrapolation using Zimm method. The differential refractive index increment (dn/dc) value used was 0.130 $mL.g^{-1}$, as determined by Kim *et al*., (2009).

For a given sample injected into the SEC-MALS, the refractive index increment of solvent and sample solution was measured by a differential refractive index detector (DRI). It represented the incremental refractive index change (dn) of the solution for an incremental change of the concentration of the sample (dc). ATSRA software performed the calculation of the mass of NR (m_1) injected after filtration by integrating the whole NR peak on the chromatogram. Thus, as the exact

concentration of the solution (≈0.625 mg.mL^{-1}) and the injected volume (0.15 mL) were known before and after filtration, the exact initial mass of sample before filtration, in 0.15 mL, was known (m_0). Though, the fraction eliminated by filtration, i.e. the percentage of gel, could be calculated as follows:

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

m_0 = mass of sample in 0.15 mL before filtration

m_1 = mass of injected sample (calculated from SEC-MALS)

7. Characterization of industrial natural rubber cup coagula maturation

Before handling experiment at laboratory scale, a preliminary study was performed to assess the industrial conditions of cup coagula maturation. This preliminary study work focused on the characterization of different conditions of maturation prevailing in cup coagula storage piles of the industrial site as well as the consequences on rubber properties and structures. Physico-chemical conditions [air temperature, air relative humidity (RH) and oxygen content into the pile], rubber properties [total solid content (TSC), pH, initial plasticity (P_0) and plasticity retention index (PRI)] and rubber structure (average molar mass and gel content) were studied at different depths from the top of the cup coagula pile.

The study was carried out in Von-Bundit Co. Ltd. factory (Khun Taley branch, Surat Thani province, Thailand) during February and March 2008. Cup coagula were received on 12/02/2008 from different suppliers and stored as a pile of roughly 3-meter height and 20-meter wide. The pile was stored outdoor and was exposed to rain and sunlight for a total of 24 days of maturation before being transferred to the processing chain.

7.1 Measurement of physico-chemical conditions in cup coagula pile

Upon their delivery to the factory, cup coagula were mixed using a bulldozer in order to homogenize the material within the pile. Two drilled stainless steel tubes (length 3 m, external diameter 5.1 cm, thickness 2 mm, 5 mm-diameter holes, 3000 holes/m2) were placed vertically inside the pile (Figure 18). Distance between drill tubes was 7 m. The measurement of air temperature and oxygen concentration was performed by inserting in the tubes a dedicated probe (Conox 3, VWR GmbH, Weilheim, Germany) connected to a multimeter (Multi 350 model, VWR GmbH, Weilheim, Germany). Calibration of the oxygen probe was realized using mixtures of air (containing 20.95% O2) and nitrogen (99.8%). Plugs (46 mm diameter) were placed 10 cm below and above the probe in order to isolate the measuring zone within the drilled tubes while recording. For relative humidity, measurements were performed with a Digicon (Japan) HT-765-232 humidity/temperature meter. Probes were properly calibrated following supplier's instructions. The measurements were performed at 5 levels from the top: 0, 50, 100, 150, and 200 cm depth every 6 days for 24 days. Each day of measurement, the operation was repeated at 8 AM, 12 AM, and 4 PM.

7.2 Sampling procedure

Sampling procedure is described in Figure 19. On the delivery day, 3 samples of 10 kg of fresh cup coagula were collected in the vicinity of each of the 2 stainless steel tubes as representative samples (total 60 kg) of the fresh cup coagula. These cup coagula were named "fresh cup coagula" and served as a reference to monitor the influence of the storage. They were processed the day of reception and the technological properties were determined in the industrial laboratory. The structure of the samples were determined in the CIRAD laboratory in Montpellier after 2 months ("characterization of raw rubber"). After 24 days of maturation, 10 kg samples of maturated cup coagula were collected around each stainless steel tube on the surface of the pile (directly exposed to sunlight) and at the 4 depths mentioned above (5 baskets of 10 kg per stainless steel tube) as shown in Figure 19.

The samples were processed following factory usual procedures. The 10 kg samples were creped in a Lihoe crepper (Lihoe Co. Ltd, Selangor, Malaysia, gap between nip rolls: 5 mm, friction ratio: 1:1.56, roll length: 71.1 cm, Roll diameter: 38.1 cm, Engine power: 44.7 kW). Cup coagula were creped by 3 single passes followed by 18 double passes. The obtained crepes were dried in an industrial crumb rubber dryer (Golsta, Melaka, Malaysia; 3.5t/h) at 129 °C for 3 hours.

The properties of cup coagula were analyzed [total solid content (TSC), pH]. The dry rubber samples were characterized for their properties: initial plasticity (P_0) and plasticity retention index (PRI)] and structure (average molar mass and gel content) following methods described previously.

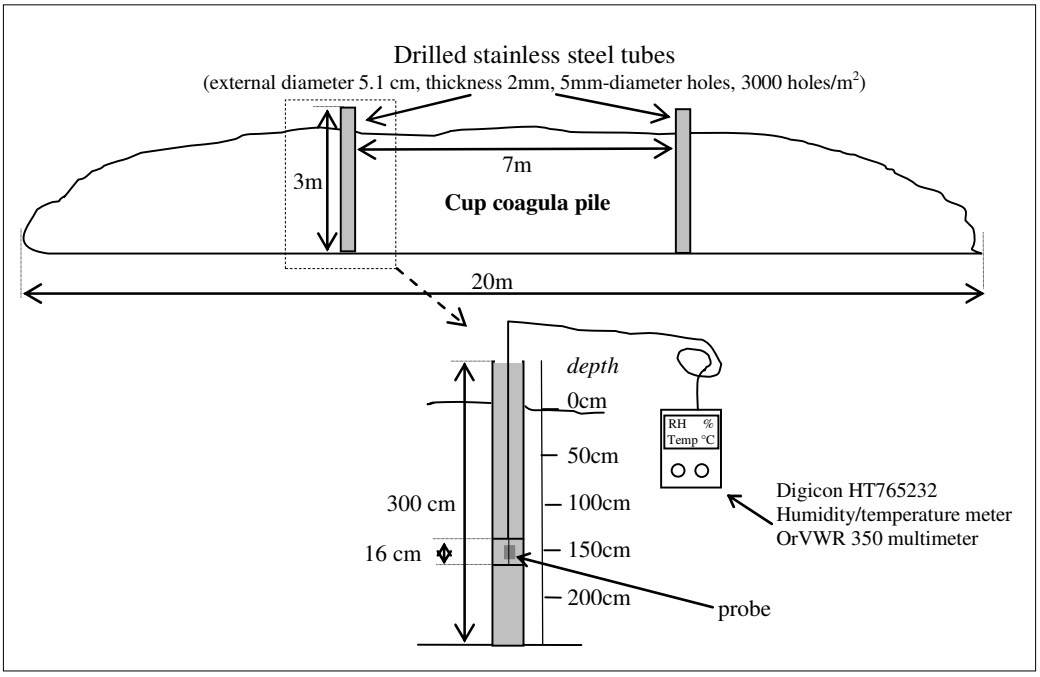


Figure 18 Cup coagula maturation pile equipped with two drilled stainless steel tubes in order to measure humidity, temperature and oxygen content of the air at different depths.

8. Test of homogeneity of rubber properties within a single coagulum.

Cup coagula samples in this experiment were prepared by naturally normal latex coagulation. About 8 L of Fresh Natural Rubber Latex (FNRL) was collected from labelled 32 rubber tress (Materials paragraph 1) and further mixed together.

About 1.5 L of fresh latex was added with formic solution until pH reach to 5.2. A part of this mixture of latex was poured into 3 cubic boxes (8 x 8 x 8 cm^3). After 3 h of coagulation, the cubic coagula were removed and analyzed.

About 6 L was poured into 12 cubic boxes (8 x 8 x 8 cm^3). After 3 days of natural coagulation in the cubic boxes, coagula were removed from the boxes and put on the shelves under shade.

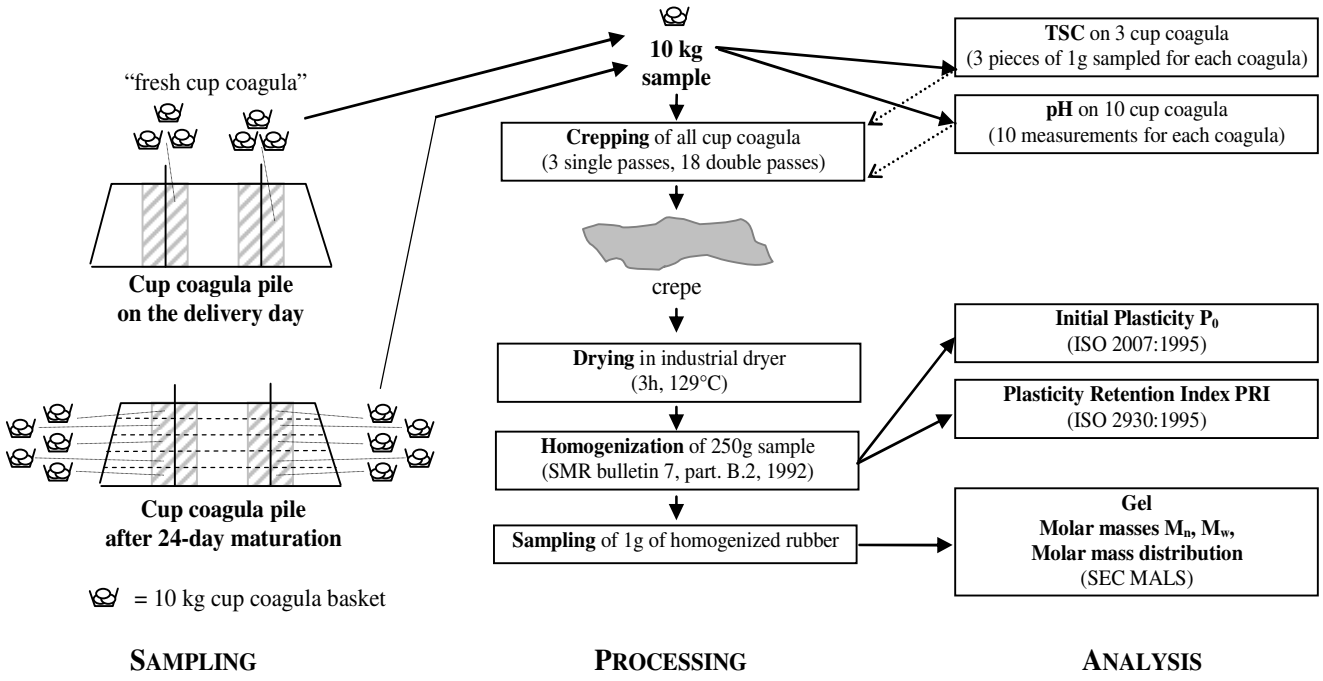


Figure 19 Industrial cup coagula sampling, processing and an analysis of "fresh" and "maturated" cup coagula.

8.1 Inside and Outside parts of a cubic coagula samples preparation

Concerning the cubic coagula after three days of natural coagulation, three of them was analyzed after 4 different maturation times (4, 15, 30, 45 days).Obtained cubic coagula were cut 1 cm from the outer surface on each side leading to "inside" coagula part (6 x 6 x 6 cm^3) and 6 external parts (called "outside part") as shown in Figure 20.

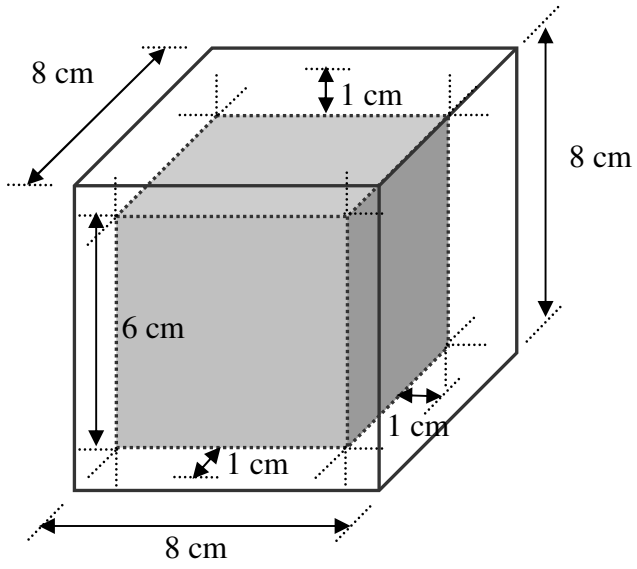


Figure 20 Schematic diagram of inside and outside cubic coagula parts.

8.2 Measurement of dry rubber properties of inside and outside cubic coagula

8.2.1 TSC of inside and outside part of cubic coagula

The TSC of the outside and inside parts were determined following the condition in paragraph 5.1. Approximately 1 g of six small pieces of outside parts and the inside part were cut and dried in an oven at 100°C for 3 hours.

8.2.2 pH of inside and outside part

pH of outside and inside parts were measured by inserting probe at the each piece of cup coagula, repetitions of measurement pH were done in three locations.

8.2.3 P_0 and PRI of inside and outside part.

The inside and outside parts of cubic coagula were processed following the mini-cup coagula processing (see paragraph 4). The technological properties of the obtained rubber were determined (see paragraph 6.)

9. Microbiological techniques

9.1 Preparation of inoculum and concentrated inoculum

About 250 mL latex serum containing microorganisms was sampled from 32 cups of latex coagulation 3 days after tapping by squeezing each cup coagula. The obtained serum containing microorganism cells suspended in liquid phase was filtered through a metallic sieve (1mm pore size). The microorganisms cells were centrifuged and washed as follows: 200 mL of serum was centrifuged at 10,000 g for 15 min (18 tubes x 10 mL). For each tube, the supernatant was removed and the cell pellets was resuspended in 10 mL of sterile 0.9% NaCl (w/v). This operation (centrifugation and resuspension) was repeated once. The inoculum had therefore the same microorganism concentration as the initial concentration presenting in the collected serum (around 10^9 CFU/ml). When "concentrated inoculum 2.5X" was requested the last resuspension was done with 4 mL of 0.9% NaCl (w/v) instead of 10 mL.

9.2 Selective media for identification and counting of microorganisms

The microbial population of inoculum was characterized using selective agar media with appropriate growth temperature. Appropriate 10X dilutions were performed before spreading on agar plate. The followings categories were numbered: Gram-positive, Gram-negative, lactic acid bacteria, total aerobic and total anaerobic, and yeasts. The selective media are indicated in Table 8.

Concerning the prepared latex, only the measurement of total aerobic micro-organisms was performed using as a sample the prepared latex before formic acid addition.

Table 8 Selective media and culture condition.

Group of micro-organisms	Specific media	Culture conditions	Quantity (g) to be dissolved in 1L
Total aerobic	PCA (Difco, USA)	35-37°C for 24-48 h	23.5
Total anaerobic	PCA (Difco, USA)	35-37°C for 24-48 h in anaerobic jar (HP11A, OXIOD)	23.5
Gram positive bacteria	MSA (Difco, USA)	35-37°C for 24-48 h	111.1
Gram negative bacteria	Mac Conkey agar (Merck, Germany)	35-37°C for 24-48 h	50
Lactic acid bacteria	MRS (Difco, USA)	35-37°C for 24-48 h	55
Yeasts and Moulds	MEA (Difco, USA)	25°C for 72 h	33.6

In this study, microorganism content was controlled in different liquid media: in inoculum, in inoculated latex and in serum from cup coagula.

9.2.1 Preparation of media agar

Agar plates were prepared by suspending a quantity of media powder in 1 L of distilled water (in Table 8). This mixture was stirred thoroughly by a magnetic stir-bar and was heated until the powder completely dissolved. The media were autoclaved at 121°C for 15 min. After cooling down to a temperature of 45-50° C, it was slowly poured into sterile Petri dishes. These agar plates were kept in the fridge for further identification and counting microorganisms.

9.2.2 Serial dilution, spreading and cultivation

One mL of liquid sample was transferred to the 9 mL of 0.9% NaCl in a test tube (dilution labelled 10^{-1}). Serial dilutions of the first tube were performed in order to obtain the following dilutions: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. Two aliquots

of 0.1 mL of each serial dilution sample was spreaded onto 2 agar plates respectively. These spread plates were incubated at the temperature indicated in Table 8.

9.2.3 Counting microorganisms

All colonies grown on agar plates containing 30-300 colonies were counted. It corresponded to two dilutions. Colony forming unit (CFU) per milliliter of initial sample were calculated by multiplying the average number of colonies per plate by 10 and by the dilution used.

9.3 Isolation, identification and characterization of bacteria from clean latex and inoculum

9.3.1 Isolation

Viable bacteria in clean latex and inoculum samples were isolated by the plate count agar method with PCA (Difco). For each sample, different colonies were picked randomly from PCA plates and streaked to purify on PCA agar. These plates were incubated for 24 h at 37°C. Each isolated colony was transferred to PCA slant, which were incubated 24h at 37°C before storage at 4°C for further study.

9.3.2 Identification

The isolated strains from PCA slants were identified according to their morphological and biochemical characteristics. The Gram test was performed at PSU Hat Yai, Faculty of Science while the biochemical identification was subcontracted to PSU Hat Yai, Faculty of Medicine.

9.3.2.1 Gram stain technique

A small amount of each isolated colony on PCA slant was fixed on a microscope slide. The slide was covered with crystal violet stain (Merck). After one minute the slide was rinsed with distilled water and drained off. Slide was flooded with iodine solution (0.1% w/v, Merck) for one minute. The iodine acts as a mordant

(fixer) and form a complex with the crystal violet, fixing it into the cell. The slide was rinsed briefly with distilled water and the 95% alcohol decolorizing solution was applied. As the decolorized Gram-negative cells need to be stained in order to be visible, the final step consisted of application of the safranin counter stain (Fluka) for 30 seconds to one minute. The slide was rinsed briefly with distilled water and let dry. The Gram strain was checked with Bright field microscope (Olympus, model CX31) at 100X oil objective, Gram positive cells appearing violet while Gram negative showed a red color.

9.3.2.2 Identification tests

Isolated strains from microorganisms from inoculum and clean latex were sent for identification to PSU Hadyai, Faculty of Medicine, Department of Phathology and Microbiological laboratory.

The following methods were used to identify the strains:

- Test of specific enzymatic activity: Catalase, urease and oxidase
- Test of specific substrate utilization: Triple sugar Iron (TSI), Citrate
- Test of specific metabolite production: Hydrogen sulfide (H_2S), Indole
- Motility test

9.3.3 Screening of hydrolase-producing microorganisms isolated from inoculum and clean latex

Microorganisms, isolated from clean latex and inoculum, were tested for their ability to produce of lipase, protease and phospholipase by a spot method on specific media which contains visible amounts of enzyme substrates. The presence of the enzyme is detected by the appearance of a clear zone around the growing colonies.

9.3.3.1 Specific media preparation

Tributyrine and egg yolk were used as substrates for lipase and phospholipase. The corresponding media were prepared as follows. A weight of 12 g of PCA powder was suspended in 500 mL of distilled water and the mixture was boiled until it

became a clear solution which was further autoclaved. Tributyrin was also autoclaved while egg yolk was sterilely pipeted from a fresh egg. After cooling down of the PCA media, 5 mL of sterile substrates was added to PCA sterile solution bottle to reach a 1% v/w concentration. After gentle shaking, the media were poured into Petri dishes.

(Ref…) Skim milk was used as specific substrate for proteases. Twelve grams of PCA powder and 5g of skim milk powder were suspended in 500 mL of distilled water. This mixture was stirred thoroughly by a magnetic stir-bar and heated until the powder completely dissolved. This medium was autoclaved at 121°C for 15 min. After cooling down at 45-50° C, it was slowly poured into sterile Petri dishes.

9.3.3.2 Culture on specific media

Pure strain of each isolated colony from PCA slant was spotted on specific media (tributyrin agar, skim milk agar and egg yolk agar) and incubated at 37°C either with or without oxygen (in this case, plates were wrapped with Para film) for 4 days in an incubator. The possible clear zone surrounding colony was observed in each specific agar plate and was recorded everyday.

10. Enzyme assay method

10.1 Assay method for lipase activity

Substrate emulsion was prepared by mixing 50 mL of olive oil with 50 mL of 10% Gum Arabic whereas 0.2 % of benzoic acid and 0.2 % of sodium azide were added to this mixture and emulsion was made by agitator.

For testing lipase activity, 5 mL of substrate emulsion was pipetted into 20 mL tube along with 2 mL of phosphate buffer pH 6.5. The tube was shaked and transferred to a water bath regulated at 30°C for a few minutes before addition of 200 µl of enzyme solution. After 20 min, the reaction was stopped by adding 4 mL of stop solution (0.09% w/v of thymolphthalein in a mixture of ethanol/acetone, 1:1) into the reaction tube. This mixture was transferred into 25 mL Erlenmeyer flask and titrated

with 50 mM sodium hydroxide until a light blue color appeared. One unit of enzyme activity was defined as the amount of enzyme for which olive oil was hydrolyzed to 1 μmol of free fatty acid under the control condition.

10.2 Assay method for protease activity

Proteases activity was assayed using the Folin-Ciocalteu method following to the protocol described by Sigma. The reaction mixture contained 2.5 mL of 0.6% (w/v) casein substrate solution and 0.5 mL of protease enzyme solution. First, 2.5 mL of 0.6% casein were pipetted into 25 mL Erlenmeyer flask, then, the solution was equilibrated for a few minutes at 37 °C in water bath. After that 0.5 mL of protease enzyme solution was added and mixed by swirling and incubated at 37 °C for exactly 10 min. A stop solution consisting of 2.5 mL of 110 mM Trichloroacetic acid reagent was added, and finally, mixed by swirling and incubated at 37 °C for about 30 min.

The solution was filtered through 0.45 mm filter and the filtrate was used in color development. The color development was processed as follows: first, 1 mL of test filtrate was added with 2.5 mL of 500 mM sodium carbonate and 0.5 mL of Folin-Ciocalteu's reagent. Next, the mixture was performed by swirling and was incubated at 37 °C for 30 min. The solution was filtered through a 0.45 mm filter immediately prior to reading. Finally, the absorbance at 660 was measured with a spectrophotometer (Double Beam UV-Vis spectrophotometer 2203, Systronics (India) Ltd., India). As a blank test, 110 mM Trichloroacetic acid was added to the reaction mixture before the addition of enzyme solution. The reaction mixture was treated in the same way as above until the absorbance measurement.

11. Amino acid and Free fatty acid determination

11.1 Amino acid contents determination

50 μL of matured cup coagulum serum (0, 1, 2, 3, 4, 5 and 6 days of maturations) samples were added with deionized water 950 μL of deionized water,

2.5 mL of 500 mM sodium carbonate, 0.5 mL of Folin & Ciocalteu's Phenol Reagent. The mixture was homogeneized by swirling then incubated at 30 °C for 30 min. The samples were cleared by filtration through a 0.45 mm filter immediately prior to reading. Amino acid content was measured with a spectrophotometer at 660 nm, and content of amino acid was calculated by using tyrosine as a standard.

11.2 Free fatty acid content determination

Free fatty acids in dried rubber samples were quantified by an adaptation of the method described by Van Autryve *et al.,* (1991) which was used for determining lipase activity in lipase treated cup coagula samples. The principle of this method is based on the specific complexation of free fatty acid with rhodamine 6G. The absorbance of the complex was measured a wavelength of 513 nm. Lipid extract from lipase treated cup coagula was diluted to 60 $\mu g.mL^{-1}$ with n-hexane. Under a fume hood, 0.5 mL of Rhodamine 6G solutions in toluene was added to 3.5 mL of diluted sample in a test tube. Before absorbance measurement at 513 nm using a Hitachi model U-2001 spectrophotometer (Tokyo, Japan), the solution was left for exactly 5 min under a fume hood (25 $\pm$ 2^{o}C) for color stabilization. Free fatty acid concentration was calculated from the response factor obtained from the standard curve.

CHAPTER 1

Characterization of natural rubber cup coagula maturation conditions in industrial plant and consequences on dry rubber properties

Introduction

Before handling any laboratory experiment involving cup coagula maturation, it was necessary to assess precisely the conditions of maturation of cup coagula in an industrial plant. Therefore, this chapter will be dedicated to the assessment of natural rubber cup coagula maturation conditions in factory practice. The consequences on dry rubber properties will also be investigated.

In industrial practice, cup coagula are stored for a variable period of time before processing, ranging from 2 to 4 weeks. This storage time, called "industrial maturation period", follows a variable period comprising natural latex coagulation and initial maturation in the smallholdings or in the middle-men storage areas. The industrial maturation step is sometimes requested in order to improve the consistency and value of specified indicators of rubber quality such as Wallace initial plasticity (P_0) and plasticity retention index (PRI).

The procedure used was that described in the materials and methods section paragraph 7. This experiment was performed as a preliminary study before conducting experiments in laboratory controlled conditions. This work focused on the characterization of the conditions of maturation prevailing in cup coagula storage piles on an industrial site and their consequences on rubber properties and structure. Physico-chemical conditions [temperature, relative humidity (RH) and oxygen content] and rubber properties [total solid content (TSC), pH, initial plasticity (P_0) and plasticity retention index (PRI)] were studied at different depths from the top of cup coagula pile. In order to reach a satisfactory way of data collection (described in the materials and methods section), an optimization of the material used was necessary.

This step is described in the following section 2 of this chapter. Section 3 presents the results concerning the conditions (temperature, oxygen content and relative humidity of the air) while section 4 assesses the consequences of the condition differences to the properties of the dry rubber processed from the cup coagula.

1. Development and optimization of measurement tools

Measuring temperature, humidity and oxygen content in an industrial maturation pile of cup coagula may appear simple provided that appropriate probes are connected to a data logger. However many practical constraints have to be solved before being able to collect the data in a proper manner. First of all, the organization and the coordination of the work: the installation of the drilled tube inside the pile had to be well coordinated with the plant staffs who manipulate tons of rubber with big caterpillar trucks. The second challenge was to find an appropriate resistant material. Indeed, the data collection does not only depend on the procedures of measurement but also on the qualities of materials used.

In order to access the air deeply inside the pile, it was decided to place two vertical drilled tubes located as mentioned in Figure 22 at the time of pile building by the caterpillar trucks. The drilled tube would be later used to insert measuring probe at the desired depth.

The cup coagula in the factory were collected from several sources from smallholders. The delivery was made by different size vehicles, from the small pick-up of smallholders to the bigger trucks of middle-men. They were mixed and piled by factory caterpillar trucks in outdoor storage areas. The size of pile was about 20 m length by 7 m wide with an average height of 3 to 4 m. One pile contained around 100 tons of cup coagula.

1.1 Optimization of drilled tube and supporting blocks

First trial was performed with 2 PVC tubes (height 400 cm, diameter 5.0 cm and thickness 13.5 mm) which were drilled regularly with holes of 5 mm diameter and a density of 3000 holes/m^2.

In addition, cement supporting blocks (base = 50 cm x 50 cm, height 50 cm, upper part = 30 cm x 30 cm) were built in order to support PVC tube [Figure 21 (A)]. The cement blocks were positioned in the appropriate locations (Figure 18 in Materials and Methods section) which were around 7 m from the two ends of the pile. The PVC tubes were inserted in the central hole of the two cement blocks. Then the caterpillar truck delivered cup coagula around the PVC drilled tubes until the height of 3 m was reached.

This system was found to be not satisfactory because of a lack of mechanical resistance of the material used. Indeed, during the loading of the cup coagula, the caterpillar excavator could touch the tube leading to a deformation of its initial shape and a possible bending. Another mechanical constraint is exerted by the weight of the cup coagula. During the maturation process, water is expelled leading to a downward movement of the cup coagula. This movement applies a vertical force to the PVC tube, leading to further deformation of the tube especially if the latter has been inclined during the loading process. This problem affected the measurement of the maturation conditions inside cup coagula pile since the probe could not be inserted properly to the desired depth. Furthermore as the tube was not vertical anymore, assessment of the depth of measurement became delicate.

Therefore, in a second trial it was decided to strengthen the mechanical resistance of the system by using a more resistant drilled tube, and a stronger support. Stainless steel tube (3 m high, 5.1 cm external diameter, 2 mm thickness) was chosen for its strength and resistance to oxidation which may prevail in humid and hot conditions. Drilling was operated to obtain the same density of holes (3000 holes/m^2) as that used with PVC tubes. A special drill bit was used due to the hardness of the

inner surface. Each hole had to be trimmed manually to remove remaining steel left inside the tube after drilling which could have prevented the proper movement of the measuring cell containing the probe.

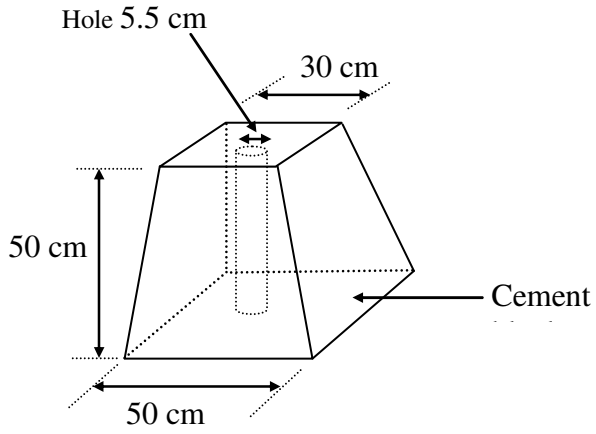
The support was also redesigned using welded metallic materials. It consisted of a larger base (80 cm x 80 cm metallic tray, thickness 2 mm) assembled with a metallic tube (length 100 cm, internal diameter 5.85 cm, thickness 2 mm) joined to the base by 4 metallic rectangular supports (5 cm x 100 cm x 2 mm) as shown in [Figure 21 (B)].

1.2 Measuring cell

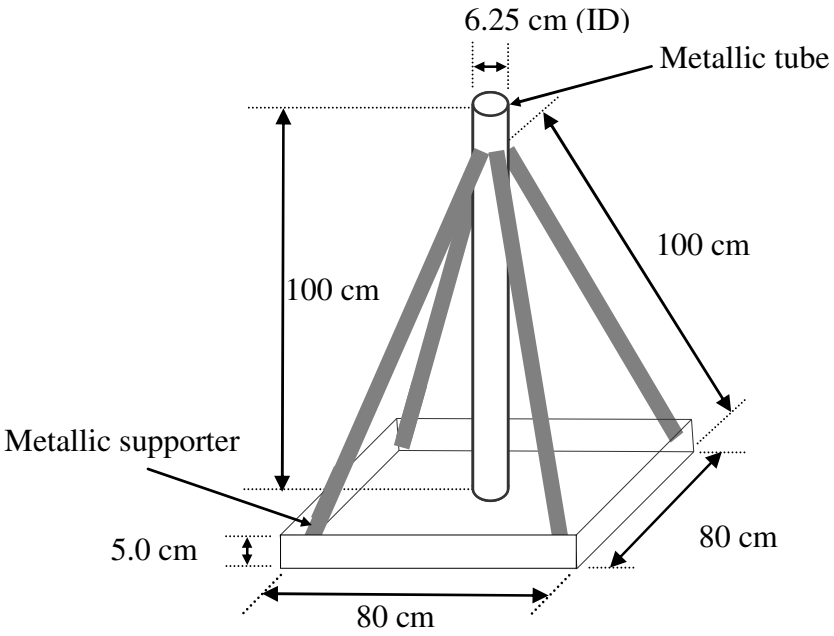
The three investigated parameters of maturation conditions were temperature, relative humidity and oxygen content of the air within the pile. All these parameters were measured with probes connected to the data logger with a connecting cable that was longer than 3 meters. A special cylindrical device called the "measuring cell" was designed as shown in Figure 22. The purpose of this device was to:

- Maintain the probe at the center of the stainless tube and prevent any direct contact with the tube inner surface.
- Isolate the measuring zone within the drilled tubes while recording.

The measuring cell was made from a 3.5 cm external diameter and 24 cm long PVC tube. Two facing windows of 2 cm width and 16 cm height were cut in order to let in the air from the surrounding reach the measurement device. The measuring cell was closed at the bottom by an adapted PVC plug (3.7cm external diameter) and at the top by a rubber plug inserted in the PVC tube. This allowed the isolation of a 16 cm zone of air sampling and the centering of the probe inside the PVC tube in order to maintain it in the right central position. The measuring cell was moved inside the stainless steel tube with the assembly of 1 m PVC tubes with adapted junction PVC tube as shown in Figure 22.



(A) Cement supporting block



(B) Welded metallic supporting block

Figure 21 Optimization of the supporting blocks.

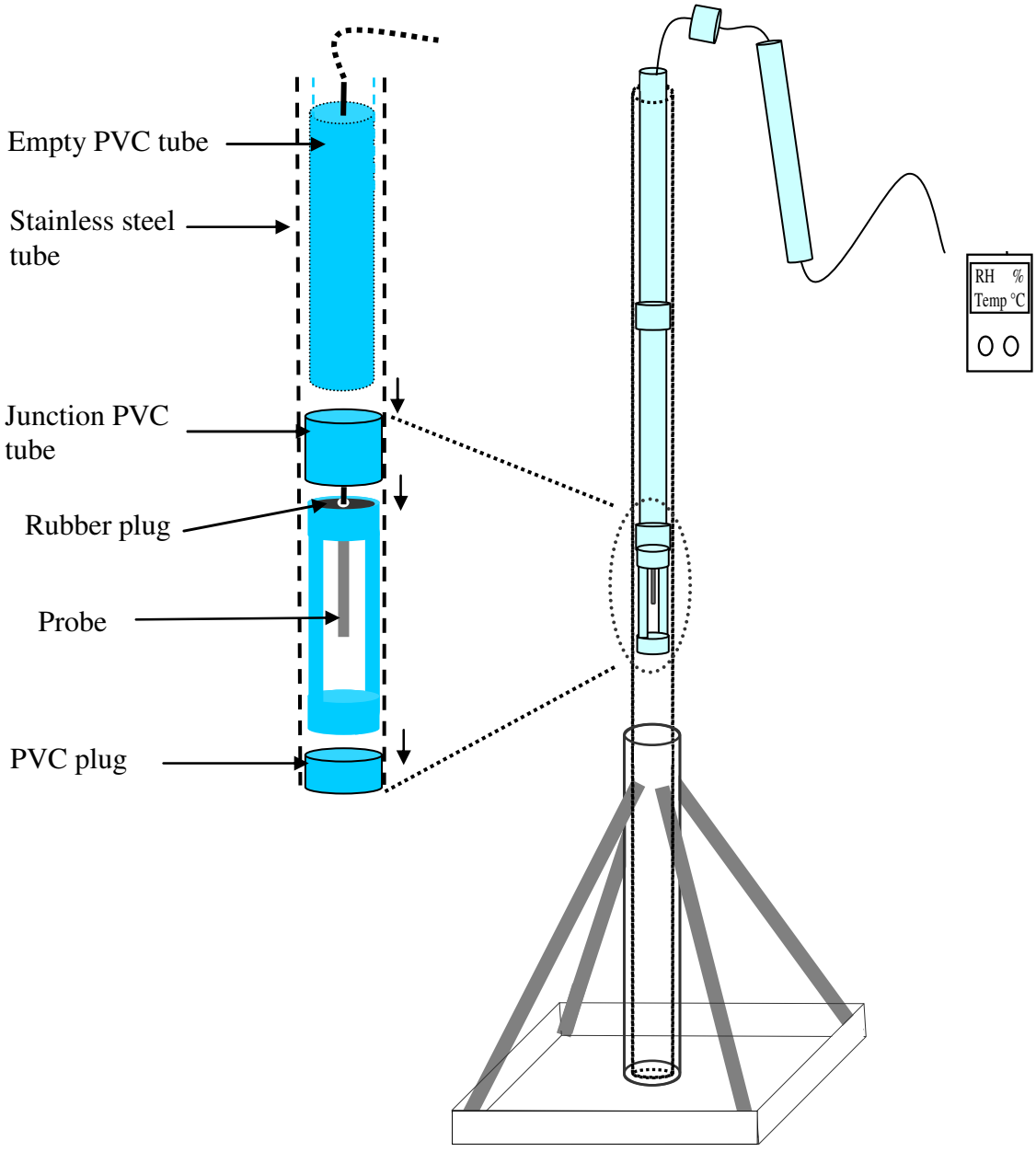


Figure 22 Detail of the measuring cell.

1.3 Processing of the collected sample

The creping and drying procedures used for this experiment are identical to the process used by the factory for industrial cup coagula.

2. Physico-chemical conditions in cup coagula pile

Oxygen content, temperature and relative humidity of air were measured at different depths within cup coagula pile after 1, 7, 13 and 19 days of maturation. The results are shown in Figures 23 to 25. For each maturation time, values are the mean of 2 independent measurements (one in each drilled tube).

The oxygen content of the air contained in cup coagula pile decreased as depth increased (Figure 23). At the top of the pile, oxygen content was 16-18% and decreased to less than 5% below 100 - 150 cm. There were 2 zones of maturation conditions inside industrial cup coagula pile (see in Figure 5): aerobic condition (0-50 cm) and anaerobic condition (deeper than 100 cm). This gradient, most probably driven by oxygen consumption during microbiological oxidation reactions and by the lack of oxygen supply by air renewal, did not change significantly during the studied period (1 to 19 days).

Air temperature in cup coagula pile increased with the depth (Figure 24) according to a gradient with no overall significant change during maturation but with daily variations. Everywhere in the pile, temperature in the morning was 10°C lower than at noon and afternoon; it might be due to heat conduction by the stainless tube, or to air flow inside the tube. At the top of the pile, average temperature was 27 °C at 8 am in the morning and 37 °C at noon and afternoon. This value depended mainly on weather conditions. Temperatures at 50-100 cm and 150-200 cm below the top were respectively 36°C and 39°C on average. This temperature gradient may be caused by a combination of the exothermic metabolic activity of microorganisms inside the pile, solar radiation at the top of the pile and thermal exchanges within the pile by

convection (through stainless steel tube and serum movements) or perhaps by conduction (through the cup coagula).

Relative humidity (RH) in the cup coagula pile also increased with depth, especially at mid-day and in the afternoon with values ranging from 45-55% on the top of the pile to 85-90% at 200 cm below the surface (Figure 25). In the morning, RH was in a closer range (75-95%) in all the locations within the pile. Temperature, humidity and oxygen content are key factors controlling the development and activities of microorganisms. The vertical gradient of values observed for all of these parameters in the pile suggests that the microbial population could differ in terms of species and activities according to the location of the cup coagula within the pile.

From this result, the temperature and humidity prevailing at the center of the pile will be used for maturation of coagula at laboratory scale.

3. Properties of industrial cup coagula

TSC of fresh cup coagula was 73 % in average on the delivery day in the industrial plant (Figure 26). After the maturation period (24 days), TSC increased to around 80-85 %, with slight differences depending on the depth of sampling. Higher TSC measured at the bottom of the pile may be due to compression by upper cup coagula weight, while the high value at the surface most probably resulted from evaporation of water caused by solar radiation and wind and from drainage of serum.

The average pH of the delivered fresh cup coagula was 5.7. After 24 days of maturation, the pH of the cup coagula at the top of the pile rose to 7.4 as shown on Figure 27. This result is in agreement with the observations by Soewarti and Moh (1975), who found that the pH of cup coagula increased from 6.25 to 6.85 after 35 days of maturation. The increase of pH might be due to the release of ammonia during the degradation of latex proteins. Strong “ammonia” odor over the pile backed this hypothesis. Ammonia is indeed one of the co-products of organic matter hydrolysis and fermentation (Mackie *et al.*, 1998). Moreover, a clear vertical pH gradient was

observed in the maturated cup coagula pile, with pH values decreasing gradually from the top to the 200 cm depth (pH 7.5 to pH 6.5). As suggested by Taysum (1961), acid production by anaerobe microorganisms such as *Clostridium* sp., *Lactobacillus* sp., and *Streptrococcus* sp. may explain lower pH in locations with low oxygen content, i.e. in the deeper layers of the pile (Figure 23).

Wallace initial plasticity (P_0) of rubber from cup coagula collected on delivery day was 25 in average (Figure 28). After 24 days of maturation, it was found that P_0 did not increase at the top of the pile but increased with the depth. At the top of pile, P_0 value was 26 and reached a maximum value of 42 at a depth of 200 cm. Higher P_0 deeper in the pile may be related to a lower degradation of polyisoprene chains and/or higher cross-linking between rubber chains. These phenomena may have occurred during maturation and/or drying. Scission and cross-linking are indeed two antagonistic phenomena that play an important role in the modification of the structure of rubber during maturation and drying (Ehabe *et al.*, 2002). It is interesting to note that higher P_0 are measured in layers characterized by a higher temperature and lower pH and oxygen content in the air.

As shown on Figure 29, the mean PRI value of rubber from cup coagula was 25, which is significantly below the TSR20 (Technically Specified Rubber grade 20) standard that requires a minimum value of 40. After 24 days of maturation in the pile,

PRI increased significantly with the depth in the pile, reaching values above 41 when cup coagula were collected deeper than 150 cm under the top (17 units increase).

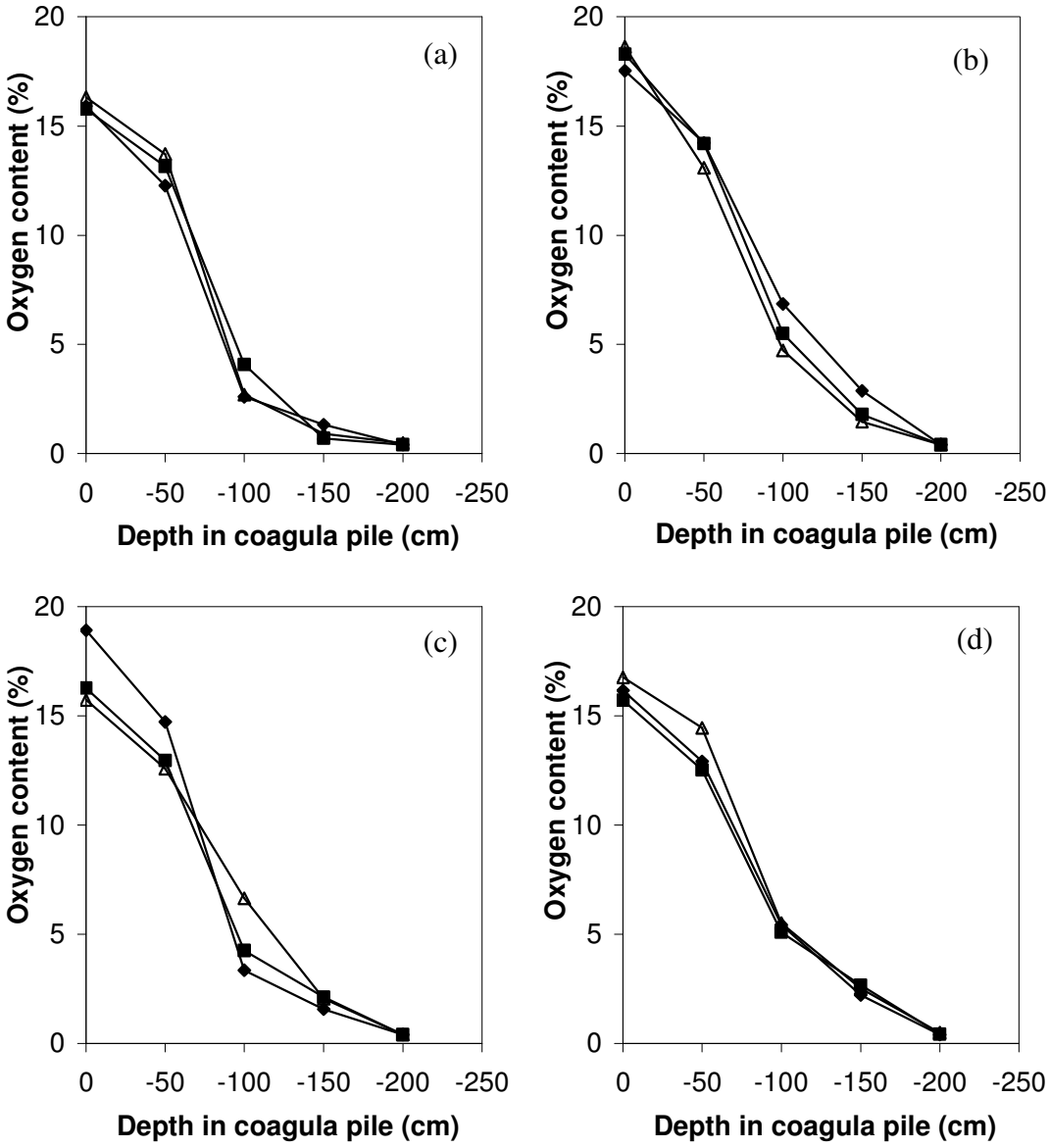


Figure 23 Oxygen content of the air in cup coagula pile at different depths from the top for each maturation duration: (a) 1 day, (b) 7 days, (c) 13 days and (d) 19 days. Measurements were performed at 8 AM (—◆—), 12 AM (—■—) and 4 PM (—△—).

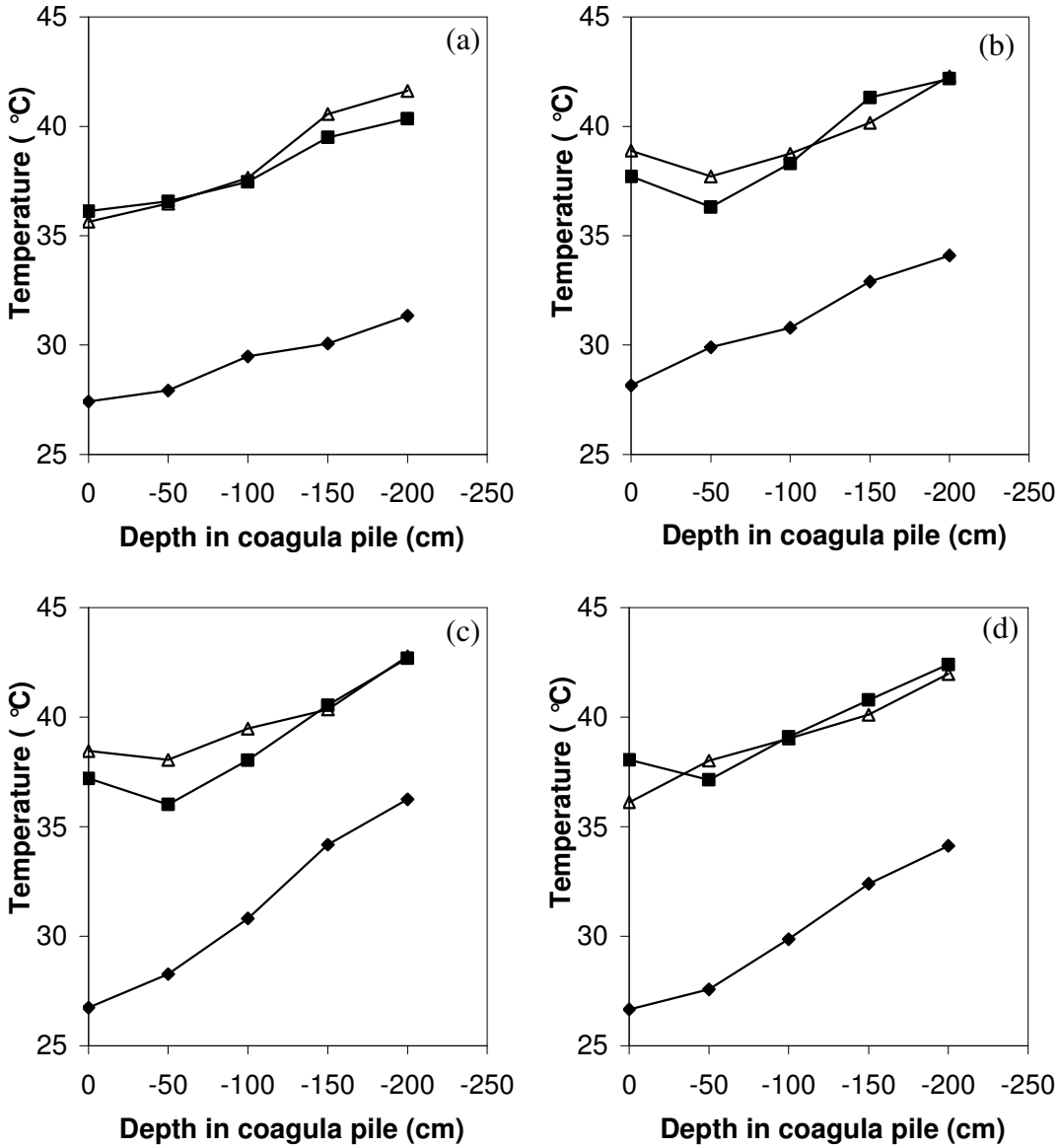


Figure 24 Temperature of the air in cup coagula pile at different depths from the top for each maturation duration: (a) 1 day, (b) 7 days, (c) 13 days and (d) 19 days. Measurements were performed at 8 AM (—◆—), 12 AM (—■—) and 4 PM (—△—).

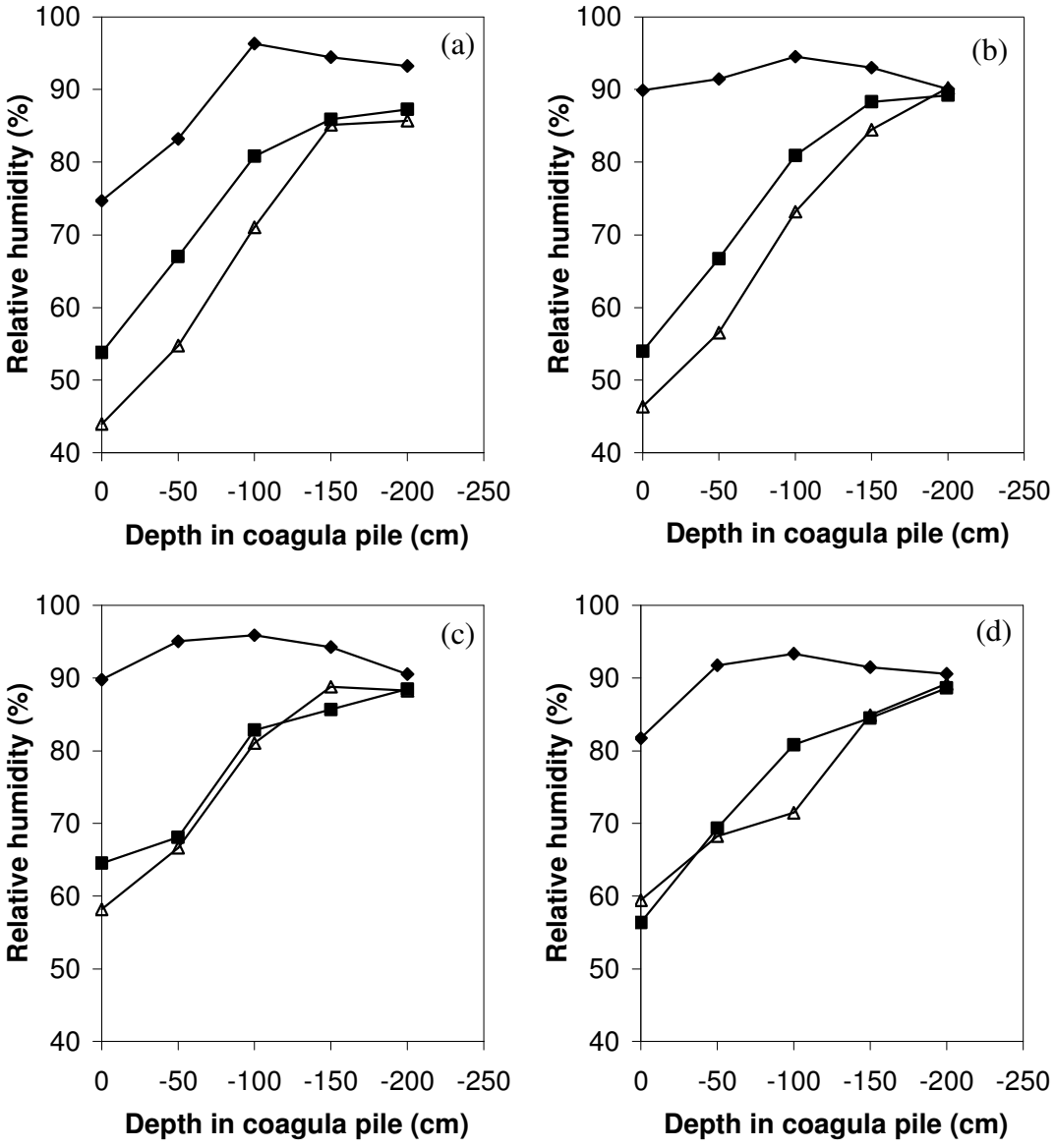


Figure 25 Air relative humidity in cup coagula pile at different depths from the top for each maturation duration: (a) 1 day, (b) 7 days, (c) 13 days and (d) 19 days. Measurements were performed at 8 AM (—◆—), 12 AM (—■—) and 4 PM (—△—).

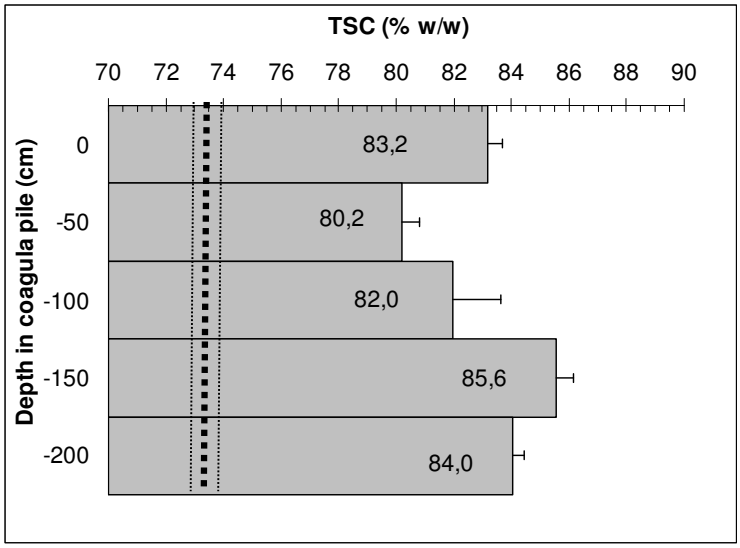


Figure 26 TSC of cup coagula in maturation pile at different depths from the top. Initial TSC on delivery: bold dotted line (▪▪▪▪); TSC after 24 days of maturation: gray histograms. Standard errors are indicated respectively by thin doted line (·····) or error bars.

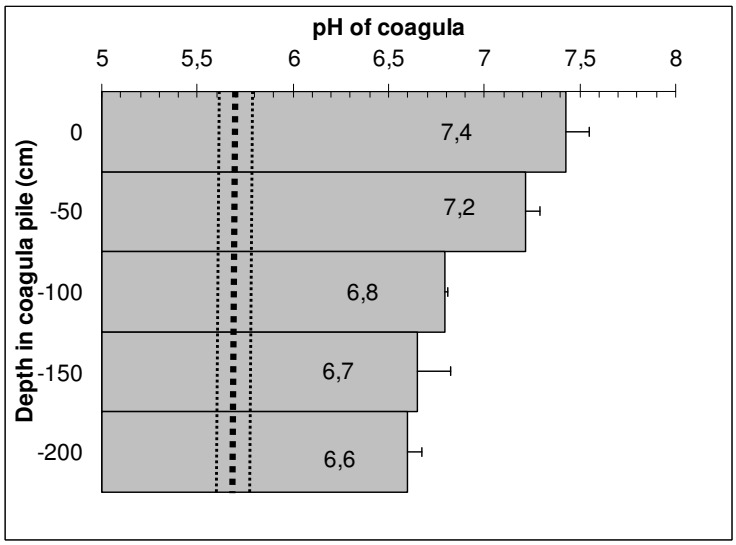


Figure 27 pH of cup coagula in maturation pile at different depths from the top. Initial pH on delivery: bold dotted line (▪▪▪▪); pH after 24 days of maturation: gray histograms. Standard errors are indicated respectively by thin doted line (·····) or error bars.

The environmental conditions at the bottom of the pile (high temperature, low pH and oxygen content in the air) encouraged microbiological/biochemical mechanisms that reduce sensibility of NR to thermo-oxidation. PRI values followed the same trend as those of P_0 within the pile. This similarity indicates that the observed gradient of P_0 can be explained mainly by the effect of depth on the resistance of rubber to thermal treatment during drying.

Mesostructure (average molar masses and gel content) of rubber from fresh and matured cup coagula from different vertical locations in the pile were analyzed by SEC-MALS. As number-average molar mass ($\overline{M_n}$) and weight-average molar mass ($\overline{M_w}$) showed the same trend, only $\overline{M_w}$ is presented (Figure 30). $\overline{M_w}$ values, as the P_0 ones, increased with the depth within the pile. It has to be remained that molar masses measured by SEC-MALS are related to the soluble part of rubber only. Therefore, higher $\overline{M_w}$ deeper in the pile indicates that the soluble poly-isoprene chains maturated at 200 cm depth underwent less scission during the drying process (3 hours – 129°C) than their counterparts from the top of the pile. Initial value of $\overline{M_w}$ of rubber from cup coagula was in the same range as in the layer located 50 cm below the top after maturation. The maturated top layer cup coagula displayed a lower value of $\overline{M_w}$ most probably due to scissions caused by direct sunlight exposure. Concerning gel (Figure 31), and contrary to other parameters, the initial level (47 %, cup coagula) was in the same range as that of the layer located 200 cm below the top. Gel content decreased slightly at the top of the pile (depths 0 and 50 cm), which displayed lower P_0. These results of structural study, as well as the similar trends of P_0 and PRI (increasing values with depth), support the assumption that the P_0 gradient observed in the maturated pile is more due to a higher resistance to scission during the drying process (thermo-oxidation) than to a higher crosslinking.

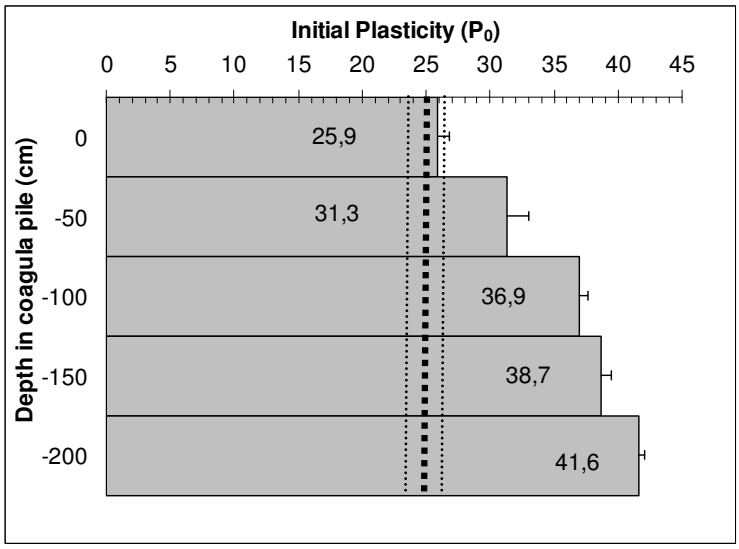


Figure 28 Initial Plasticity (P_0) of cup coagula in maturation pile at different depths from the top. Initial P_0 on delivery: bold dotted line (▪▪▪▪); P_0 after 24 days of maturation: gray histograms. Standard errors are indicated respectively by thin doted line (·····) or error bars.

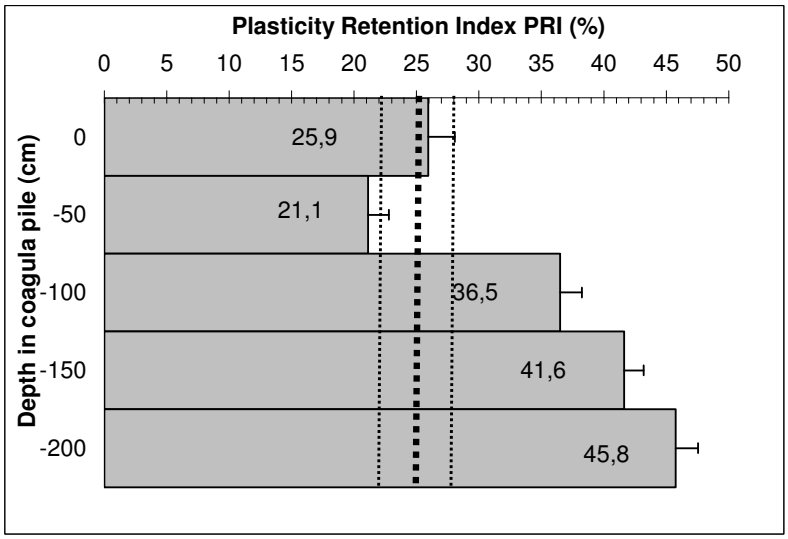


Figure 29 PRI of cup coagula in maturation pile at different depths from the top. Initial PRI on delivery: bold dotted line (▪▪▪▪); PRI after 24 days of maturation: gray histograms. Standard errors are indicated respectively by thin doted line (·····) or error bars.

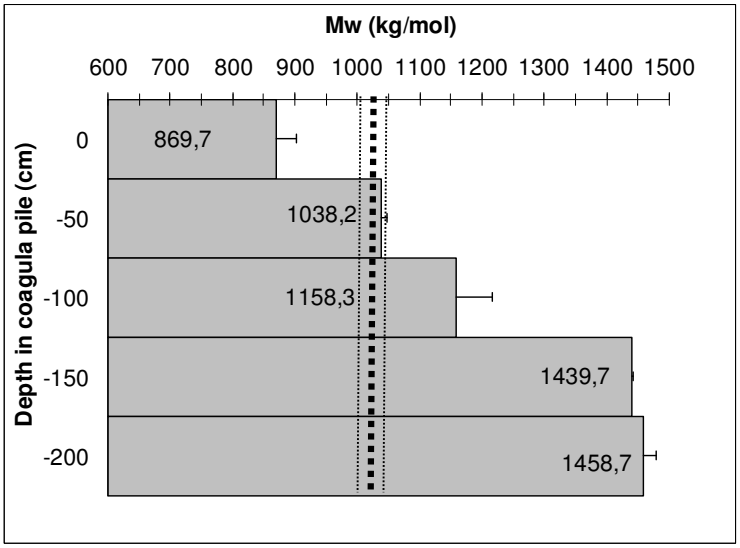


Figure 30 $\overline{M_w}$ of cup coagula in maturation pile at different depths from the top. Initial $\overline{M_w}$ on delivery: bold dotted line (▪▪▪▪); $\overline{M_w}$ after 24 days of maturation: gray histograms. Standard errors are indicated respectively by thin doted line (·····) or error bars.

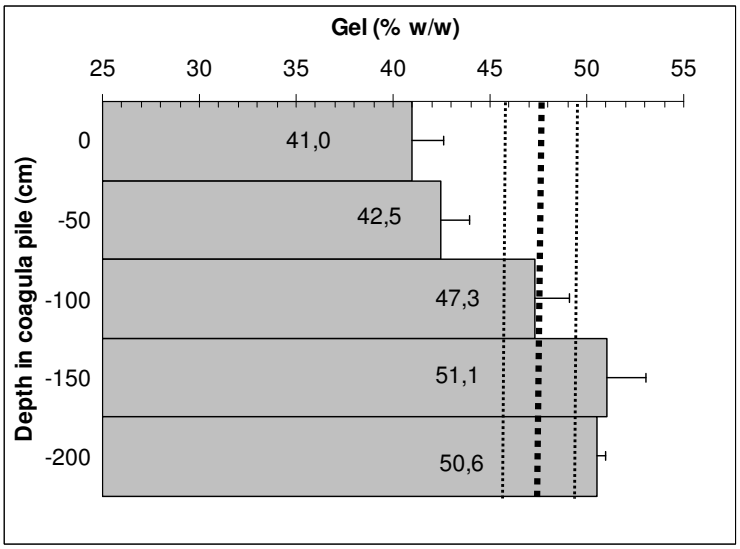


Figure 31 Gel of cup coagula in maturation pile at different depths from the top. Initial gel on delivery: bold dotted line (▪▪▪▪); Gel after 24 days of maturation: gray histograms. Standard errors are indicated respectively by thin doted line (·····) or error bars.

4. Conclusion

This preliminary study was undertaken in order to characterize the maturation conditions of cup coagula pile in an industrial plant and to monitor the influence of maturation on some technological properties of processed cup coagula at different depths from the top of a pile.

Methodological development will be necessary concerning the measurement tools in order to obtain repeatable and consistent data. Using the optimized measurement system, it was found that temperature, relative humidity of the air content increased with depth: from the top to 2 m below the top, temperature changed from 34°C to 42°C while relative humidity increased from 50 % to 90%. In contrast, oxygen content was found to decrease as the depth increased (20 % at top and zero at a depth of 200 cm).

Effects of 24-day maturation on rubber technological properties were found to depend on the position of cup coagula within the pile. The pH of maturated cup coagula decreased significantly with the depth within the pile (7.4 at top to 6.6 at 200 cm deep). An inverse pattern was observed for initial plasticity (P_0), plasticity retention index (PRI), gel content and $\overline{M_w}$. At 200 cm below the top, maturation period (24 days) showed a positive effect on cup coagula properties with increases of P_0 (26 to 42), PRI (26 to 46), gel content (41 to 51), and $\overline{M_w}$ (870 to 1460). At this depth, rubber was more resistant to thermo-oxidation undergone during drying. This phenomenon was probably linked to the environmental conditions at the bottom of the pile which affected the microbiological/biochemical mechanisms involved during the maturation process. Figure 12 summarizes all the measured gradient related either to the physico-chemical characterization of the air in the pile or the properties of the rubber obtained from the maturated cup coagula.

Cup coagula are stored in different locations and conditions from the day of coagulation to the day of processing. Indeed, cup coagula are stored in the farm, then

possibly in the middle-men premises and finally are delivered to the processing factory where they undergo “industrial maturation” in storage pile. The results presented in this chapter showed that the structure and properties of rubber changed during this “industrial maturation” and that these changes depended on the condition of storage.

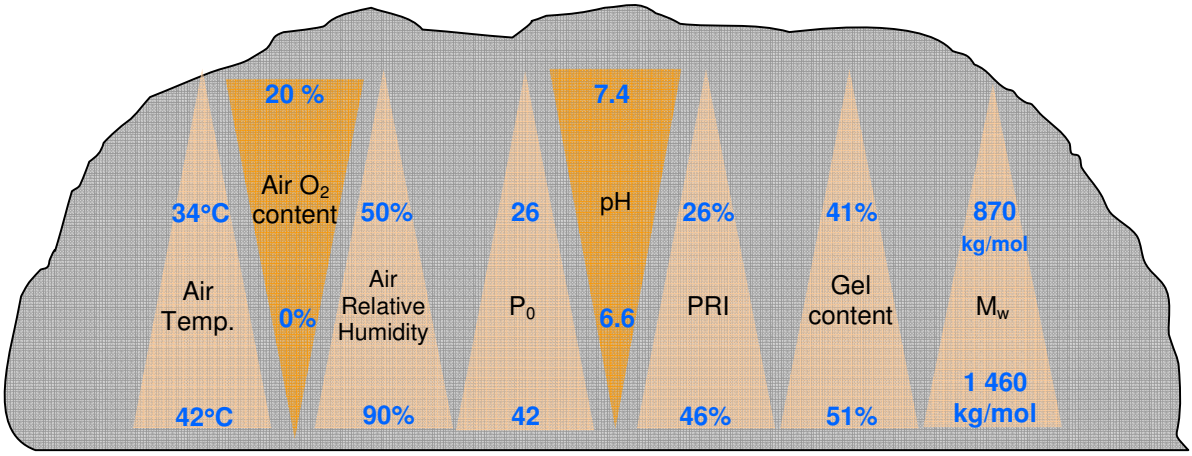


Figure 32 Evolution of cup coagula properties in maturation pile after 24 days of maturation at the top and the bottom.

These results underlined the crucial role that maturation plays on the quality on rubber. Nevertheless, conducting experimental work at factory scale has major drawbacks such as a lack of control of the quality of the cup coagula delivered at the factory, unknown and mixed clones, unknown history (tapping date, exploitation system used by the smallholders, condition of coagulation, duration and condition of initial storages). Other constraints included:

- Experimental installation disturbed the normal operation of the factory and cannot therefore be repeated too often.
- Study dealt only with the maturation which occurred in factory and did not take into account the initial period of maturation that occurs just after coagulation.

It was therefore necessary to envisage a study at the laboratory scale where the three main parameters - temperature, humidity and oxygen content of the air - may be

controlled and monitored. A scaling down of the processing - from the cup coagula to the dry rubber – had also to be developed. Clones, duration of maturation and level of microbial contamination are other parameters to be controlled. These requested methodological developments are the main purposes of the following Chapter 2.

From results obtained in the observed maturation pile, it was decided that the following reference temperature and relative humidity which would be used for further laboratory scale maturation experiments would be 40°C and 80% respectively.

CHAPTER 2

Methodological development of a maturation device and a dry rubber process at laboratory scale

Introduction

The results presented in the previous chapter showed that maturation period affected the structure and the properties of the obtained dry rubber. In order to understand the involved mechanisms, it was necessary to perform experiment on a large and repeated number of samples at laboratory scale under controlled conditions. Testing different conditions, different maturation times and working with a minimum number of three repetitions for each combination supposed indeed to be able to handle few tens of samples for each experimental trial. In these conditions, using normal size mini-cup coagula (around 13 cm maximum diameters) was not possible for practical reasons related either to the laboratory device maximum size or to the availability of latex. It was therefore envisaged to work with small mini-cup coagula called "mini-cup coagula".

In this context, several constraints have been taken into account to handle this scaling down process and would be described in details in this chapter. The addressed methodological questions can be listed as follows:

1. Is there an effect of the reduction of the sample size? : When reducing the size of the cup coagula, the proportion of rubber which is in contact with the air is much more important. A set of experiments was performed to check whether there is a significant difference in terms of properties between the rubber issued from the external part of the coagula, in contact with air and, the rubber issued from the central part of the coagula. It was indeed necessary to check that the reduction of sample size would not be an artifact which would have biased the results.

2. How to concomitantly regulate the temperature and relative humidity of different maturation units atmosphere? The developed device would have indeed to allow operations where temperature and relative humidity are controlled over a long period lasting up to 45 days. Preliminary tests were organized to put to test the loaded or not maturation devices. An additional preliminary test was also set to verify whether the water loss kinetic of the mini-cup coagula was comparable to that occurring outdoor.
3. How to optimize a laboratory process in order to obtain dry rubber with comparable quality as the one made from industrial process? Once maturated in controlled condition the reduced size cup coagula had to be mechanically sheared and dried with laboratory dedicated equipments. It was important to adjust the parameters of these latter operations in order to obtain dry rubber with similar properties as it would have been processed in the factory. Different parallel experiments have been therefore performed concomitantly in laboratory and in factory.
4. How to assess the effect of antimicrobial agent on properties of rubber and especially distinguish between its antimicrobial activity and others? Using antimicrobial chemical agent was chosen to prevent the development of microorganisms. It was necessary to check that the action of such chemical do not interfere with the properties of the obtained rubber by other means that its action on microbial population.

1. Impact of the reduction of sample size : assessment of the homogeneity of properties within a cup coagula

The volume of latex collected in a fresh cup coagulum in real field practice can reach 300 to 500 mL. As mentioned before, considering the minimum number of sample requested and for a practical point of view it was necessary to reduce the volume of each cup coagula. The envisaged volume of mini-coagula was 45 mL. In order to validate that this mini-cup coagula would be a good representative of the normal cup coagula, it was decided to assess the homogeneity of properties of rubber

issued from surrounding part of coagula (in contact with air) and from the inside of coagula. If the properties of rubber issued from these two parts do not show significant difference, the reduction of size would be validated. For this purpose the experimental protocol described in the section 8 of Materials and Methods was performed.

1.1 pH of coagula

From the average pH of the fresh latex (day 0) which was 6.8 ± 0.2 (Figure 33), pH of inside and outside part of the coagula decreased to 5.3 and 5.6 respectively after 4 days of maturation as shown on Figure 33. As suggested by Taysum (1969) acid production by anaerobic microorganisms such as *Clostridium* sp., *Lactobacillus* sp. and *Streptrococcus* sp. may explain this higher decrease of pH inside cup coagula with low oxygen content inside the coagula. At 45 days of maturation, pH of inside and outside part of the coagula increased to 7.8 and 8.1 respectively. The increase of pH might be due to the release of ammonia related to the degradation of latex nitrogenous compound such as proteins. Ammonia is indeed one of the co-products of organic matter hydrolysis and fermentation (Mackie *et al.*, 1998). Even if the inside pH tended to be lower than the one from outside part, those differences were not found to be statistically significant.

1.2 Total solid content (TSC)

TSC of fresh latex was about 40% (Figure 34). After latex coagulated, TSC of inside and outside part of cup coagula reached 75% and 72% respectively after 4 days of maturation and continuously increased until 81% and 91 % respectively at 45 days of maturation. Logically TSC of the outside part of the coagula which is in contact with air was found significantly higher that the one from inside part from 15 to 45 days of maturation. It is known that the remaining water can not migrate easily from the inside towards the outside because of a dry layer of rubber which surrounded the coagula.

1.3 Wallace rapid plasticity (P_0) and Plasticity retention index (PRI)

The evolutions of P_0 and PRI during maturation time are presented in Figures 35 and 36 respectively. For the rubber which was processed on the day of coagulation (Day 0), the average of P_0 and PRI were 34.2 and 97.7. No difference was observed between the rubbers originating from outside part and inside part of the coagula at this stage. After 4 days of maturation, P_0 remained high while PRI decreased by around 70 points. Over maturation time P_0 decreased until 25 while PRI remained at a low level. Except for 15 days of maturation, where the "inside" values of Po and PRI were lower than the "outside" one, no significant difference was observed for those two properties between the rubber originating from the inside part of the coagula and that originating from the outside one.

1.4 Conclusions

This preliminary experiment was intended to assess whether the evolution of the properties of the rubber originating from the peripheral part of the cup coagula was the same as the one that originating from the central part of the cup coagula. Concerning pH of the coagula before processing and P_0, PRI of rubber obtained after processing, no significant difference was observed between this two locations. Concerning TSC, the peripheral part was found significantly dryer than the central one. However, this difference seemed not to have any effect on the properties of rubber.

Consequently, the reduction of the size of sample, from a volume of latex of approximately 400 mL per cup coagula to 45 mL per mini-cup coagula, which would increase the relative importance of "outside" part in the coagula, should not affect the properties of the obtained rubber.

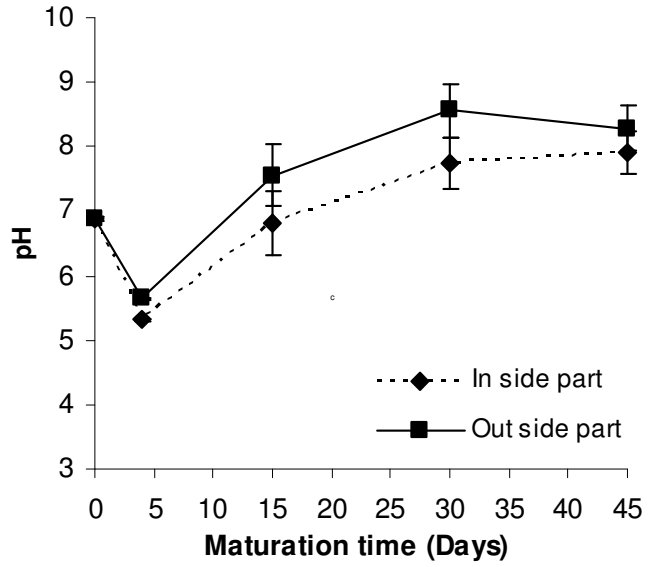


Figure 33 pH of inside and outside parts of cubic coagula.

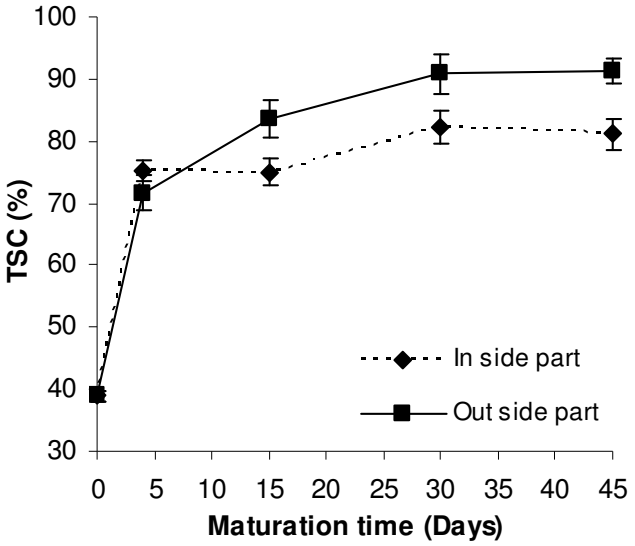


Figure 34 TSC of inside and outside parts of cubic coagula.

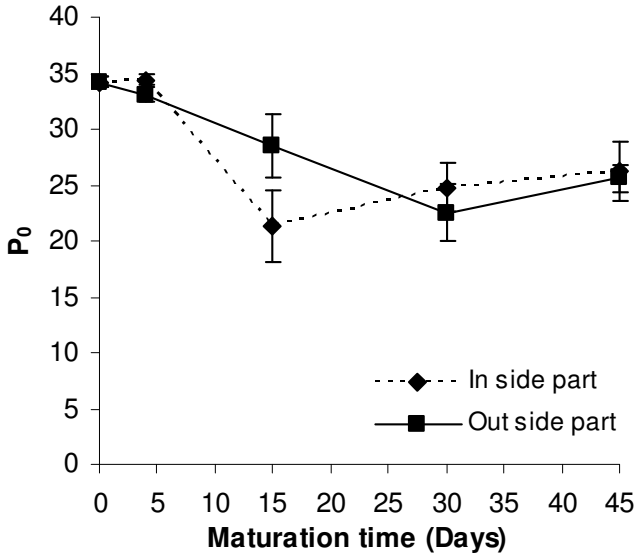


Figure 35 P_0 of inside and outside parts of cubic coagula.

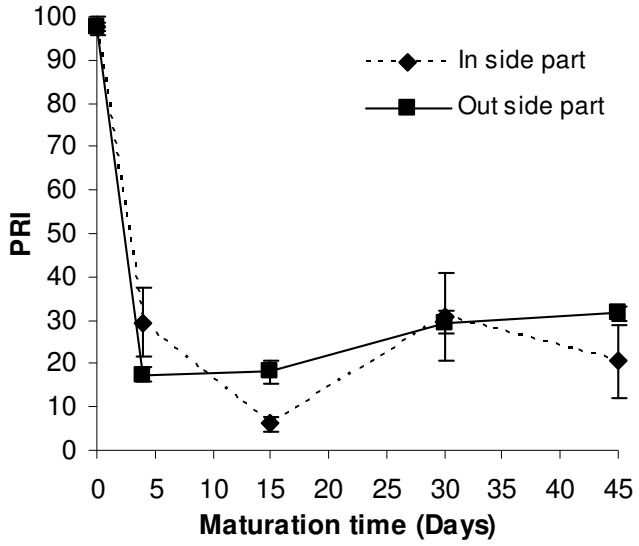


Figure 36 PRI of inside and outside parts of cubic coagula.

2. Methodological development of maturation device at laboratory scale

The maturation device which has been developed is presented in details in the Materials and Methods section (cf. paragraph 3.2). It consisted in several independent maturation units. Temperature was controlled by immersion of the units in a temperature controlled bath. Humidity of the air was controlled by the mix of dry air

(D flow) and humid air (H flow). When experiment without oxygen was performed, air flow from air compressor was replaced by Nitrogen flow from a 9 m^3 tank.

Before using the device to study the maturation of mini-cup coagula, few experiments have been performed to assess the performance of this device. The first task was to determine the appropriate flow rate balances between humid and dry channels in order to regulate in a proper manner the relative humidity inside the maturation units. Flow rates were tested on empty maturation units first (9.1 L internal air volume). Maturation units were then loaded with mini-cup coagula to test the regulation performance over a longer period (45 days).

Eventually, once the flow rates were optimized, it was necessary to check whether those latter would not create an artificial increase of the drying rate of the mini-cup coagula.

2.1 Optimization of the gas flow rate for relative humidity control and assessment of temperature regulation on empty maturation unit.

It was decided from the data obtained from industrial conditions that maturation experiment at laboratory scale should be conducted at a relative humidity of 90±10% and a temperature of 40±1°C. Before focusing on those specific values, it was decided to assess the working maximum range of relative humidity of the developed maturation device. First assessment was performed on empty maturation devices.

Different combinations of humid air flow rate (H) and dry air flow rate (D) have been tested in the range from 0 to 3 L air/min (0 to 0.33 liter air per liter free internal volume of box per min, *i.e.* 0-0.33 L/L/min). Three temperatures have been used during this test: 45°C, 40°C and 35°C successively. Figure 37 presents the obtained results which are summarized in Table 9 where equilibration times are indicated in bracket. The test began at room temperature (32°C) with a RH of air of 80%, then humid air was circulated inside the maturation unit at a flow rate of 1.5

L/min (0.16 L/L/min) while the temperature of the water bath was set at 45°C (action 1, Figure 37).

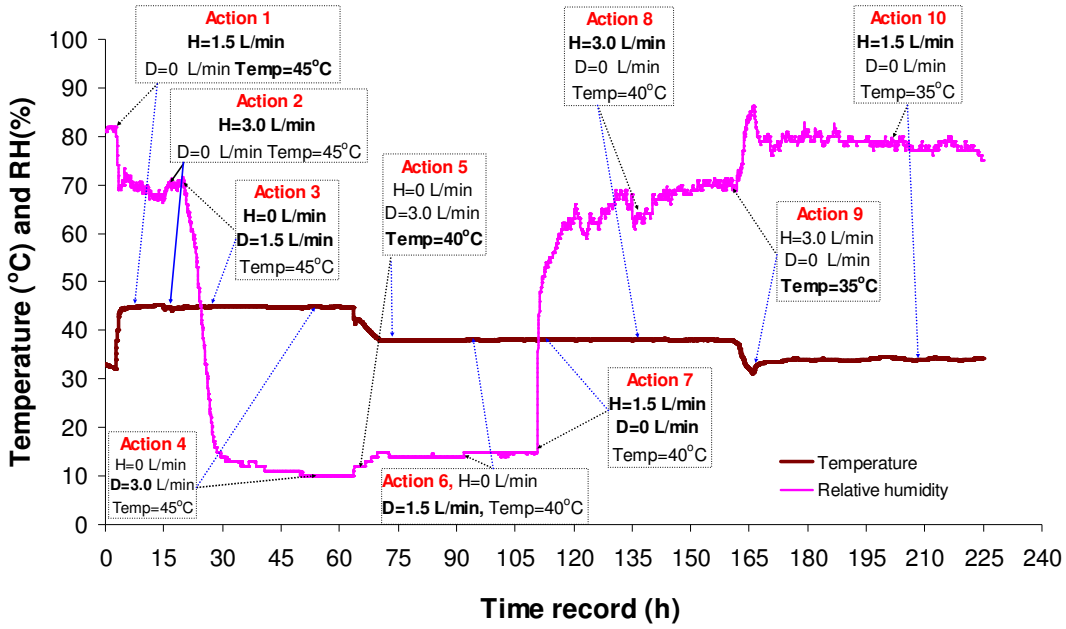


Figure 37 Example of a temperature and relative humidity regulation sequence in an empty maturation unit (H: humid air, D: dry air), box free internal volume was 9.1 L.

Table 9 Example of a temperature and relative humidity regulation sequence in an empty maturation unit (9.1 L internal air volume).

Action number	Targeted Temperature (°C)	Humid air (H) flow rate (L/min)	Dry air (D) flow rate (L/min)	Temperature obtained in maturation unit (equilibration time in bracket) (°C)	Relative humidity obtained in maturation unit (equilibration time in bracket) (%)
1	45	1.5	0	45±1 (2 h)	70±3 (3.0h)
2	45	3.0	0	45±1	70±3 (3.5h)
3	45	0	1.5	45±1	12±3(21.5h)
4	45	0	3.0	45±1	10±3 (30 min)
5	40	0	3.0	40± (8 h)	14±1(10 h)
6	40	0	1.5	40±	15±1(15 min)
7	40	1.5	0	40±1	65±5 (21 h)
8	40	3.0	0	40±1	70±3 (5 h)
9	35	0	3.0	35±1(5 h)	80±3(13 h)
10	35	0	1.5	35±1	80+5(4.5h)

The temperature reached the targeted one after 2 hours, and the measured RH was around 70%. Increasing the humid air flow rate to 3 L/min (action 2, Figure 37) did not increase much the RH in maturation unit. The humid air inlet was shut down while dry air inlet was set to 1.5 L/min (action 3, Figure 37). The RH decreased until around 12%, and this new equilibrium was reached after 21.5 hours period. Increasing the dry air flow rate to 3 L/min (action 4, Figure 37) decreased slightly the RH of air in maturation unit down to 10% which was the minimum RH value reached during the present test. At this point, the targeted temperature, which was well regulated at 45°C so far, was set to 40°C (action 5, Figure 37). The equilibration time was 8 hours, which corresponded to the time necessary for the water to be cooled down by thermal exchanges with the room temperature. This was accompanied by an increase of RH, even if the inlet of dry air was kept at 3 L/min. It is to be remained that water saturated hot air contains higher amount of water per volume of air than colder air. At a constant quantity of water in the air, RH is logically negatively correlated with temperature. This was clearly observed at each change of temperature (action 1, 5 and 9, Figure 37). A decrease of dry air flow rate to 1.5 L/min (action 6) did not impact on RH of the air in maturation unit. To test humid condition at 40°C, dry air inlet was closed and humid air inlet was set to 1.5 L/min (action 7). This led to a pseudo equilibrium of RH at around 65% while 70% could be later reached when adjusting the humid air inlet flow rate to 3 L/min (action 8). Change of temperature down to 35°C (action 9) was performed this time with a partial renewal of the circulating water with tap water (at a temperature of around 25°C). It decreased the equilibration time to 13 hours after which the RH of the air stabilized at around 80%. The last action (action 10) confirmed that a flow rate of 1.5 L/min (0.16 L/L/min) was sufficient to maintain this equilibrium.

This example of regulation sequence showed that the developed device can offer quite a good regulation capacity for working in the range from RH 10% (at 45°C) to RH 80% (at 35°C). Lower than 1.5L/min flow rate were tested, but were found to generate very high equilibrium times which could prevent an accurate and efficient regulation capacity. However, this test was performed with empty maturation

units. Next step of the methodological validation was to check the efficiency of the regulation on maturation units loaded with humid mini-cup coagula.

2.2 Control of relative humidity and temperature on loaded maturation units on a long-term run

Three maturation units, each containing 18 mini-cup coagula were equipped with RH and temperature probes connected to a computerized data-logger in order to follow the evolution of those two parameters during a long run maturation experiment (43 days). The purpose of this trial run was to try to assess the manual regulation of the relative humidity and temperature in maturation units, loaded with mini-cup coagula, with the following respective targeted values : 90±10% and 40°C±1.

Temperature of maturation was controlled through the circulation of automatically thermostated water coming from a water bath (Figure 13 in the Materials and Methods section). Relative humidity inside the maturation units was controlled by balancing manually the flow rates of humid and dry air inlets in the range of 0 to 0.22 L/L/min (0 to 2 L/min with an internal gas volume of 8.4 L per box). The volume of internal gas inside the box varied with the number of mini-cups, but this was not taken into account in the flow control because the variation was at maximum 7.4% between full and empty box. The data logger recorded both parameters inside each of the three boxes every 30 min for 43 days.

The parameters recorded concomitantly on the three channels from three maturation units are shown in Figure 38. While the regulation of temperature was performed automatically by the water bath regulator, the regulation process of humidity was more complex and performed manually. This manual regulation was necessary to compensate the evaporation of water from the samples which interacted obviously with the relative humidity of the atmosphere in the maturation unit.

The regulation of temperature and especially relative humidity is a delicate process which can be described in five main different phases (Figure 38):

Phase 1: 0-5 days

Fresh mini-cup coagula obtained after acid coagulation in mini-cup contained exuding serum. It resulted in the presence of serum puddle at the bottom of maturation unit in the first days of maturation which increased drastically the RH. In order to compensate, dry air was introduced with a high flow rate without any humid air flow (D=2.0 L/min, H=0 L/min). In the three maturation units, RH exceeds 90% during this first period.

Phase 2: 6-13 days

After 6 days, the serum puddle was evaporated. The dry air flow was reduced to 1.5 L/min while humid air flow was started up to 0.5 L/min when necessary in order to maintain RH at an approximate level of 90%. The regulation was more delicate for the maturation units 2 and 3 where the mini-cup coagula were contaminated with microorganisms contrary to maturation unit 1. The daily manual adjustment allowed anyway to stay in the initially targeted range of RH (90 ± 10%).

Phase 3: 14-23 days

In the period from 14 to 22 days of maturation, humid air was adjusted in the range 1.0 to 1.5 L/min and dry air flow in the range from 0.5 to 1.0 L/min in order to stay in the requested RH range.

Phase 4: 23 to 37 days

At this stage the inlet flows consisted mainly on humid air (1.5-2 L/min) as the mini-cup coagula were drier and their own water content did not influence much anymore the water content of the air flowing through the maturation unit.

Phase 5: 38 to 43 days

During this period, in order to maintain a RH at 90%±10 it was necessary to flow exclusively humid air through the maturation units. The significant drop of humidity during after 36 days in the third maturation unit (channel 3, Figure 38) was due to an abnormal stop of inlet air flow which lasted for 4hours. When the abnormal

RH was discovered, the flow was started again with only humid air at 2 L/min. Almost 2 days were necessary to recover the initial RH of approximately 90%.

These examples of regulation sequence over a long period showed clearly that:

1. The regulation of temperature and humidity is possible though more delicate when mini-cup coagula are introduced in maturation unit
2. The regulation consisted mainly of a progressive change of the humidity of the air inlet (from 100% dry air to 100% humid air) in order to compensate the change of water content of the sample which interact less and less over the time with the humidity of the air contained in the maturation unit.
3. The regulation has to be adapted to the nature of the sample contained in the maturation unit which may change the kinetic of water release.
4. The regulation operation by flow adjustment need to be performed on a very regular basis, as the equilibrium time requested after abnormal event may be long.

In conclusion, this new set of tests showed that the system can be regulated in the targeted range of humidity and temperature. The release of water by the sample during maturation appeared as an important parameter, and the purpose of the last experiment would be to check whether that this release is not artificially accelerated by the relative importance of the air flow inside the maturation unit (2 L/min).

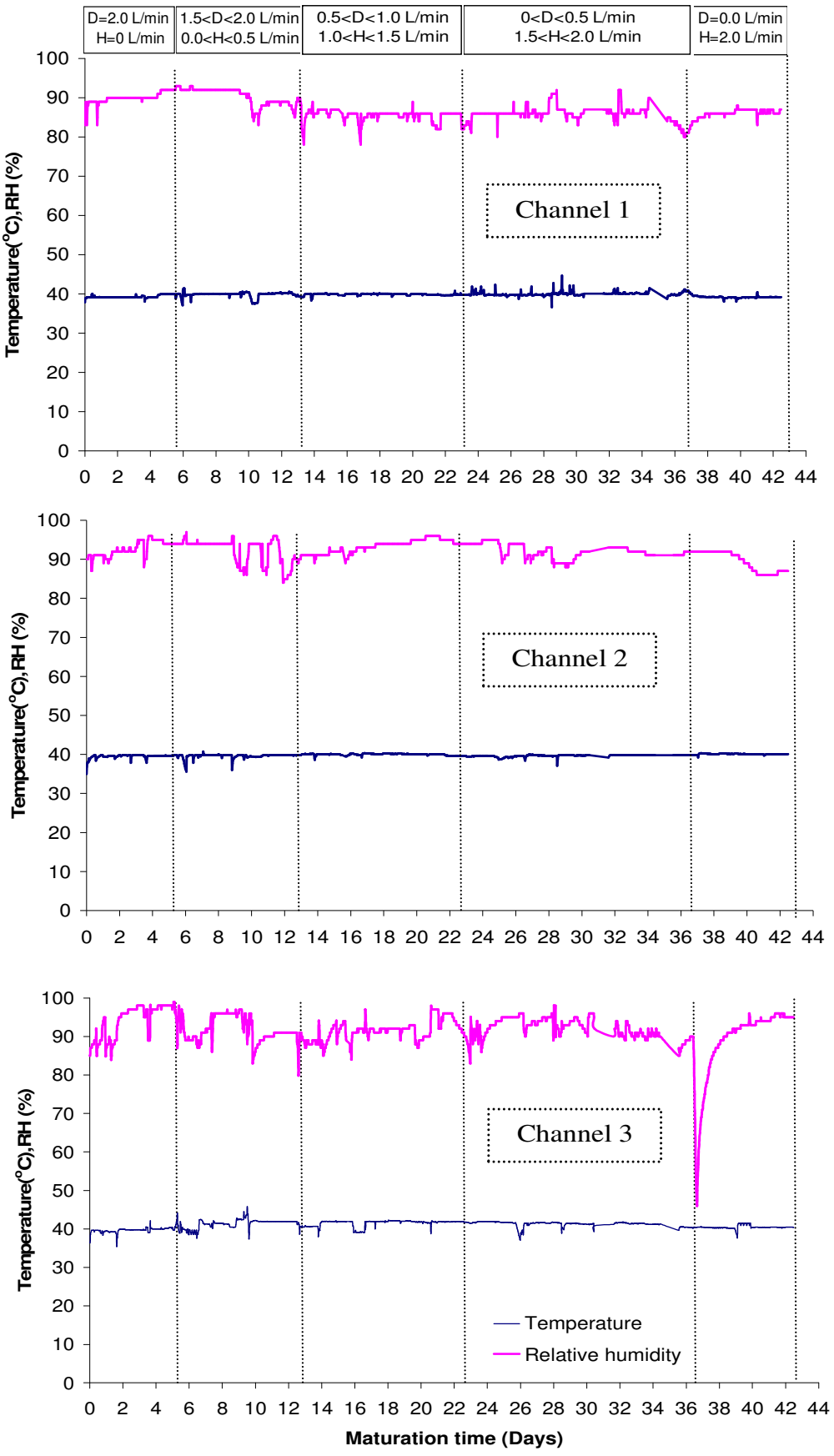

D=2.0 L/min
H=0 L/min
1.5<D<2.0 L/min
0.0<H<0.5 L/min
0.5<D<1.0 L/min
1.0<H<1.5 L/min
0<D<0.5 L/min
1.5<H<2.0 L/min
D=0.0 L/min
H=2.0 L/min
Channel 1
Channel 2
Channel 3
Temperature(°C),RH (%)
Temperature
Relative humidity
Maturation time (Days)

Figure 38 Maturation conditions of three maturation units. The free air volume inside each unit was 9.1 L; total air flow was 0.22 L/L/min.

2.3 Comparative study of the water release during maturation of mini-cup coagula

It was necessary to check whether the high flow rate used (2.3 to 3.0 L/min; 0.25 to 0.33 L/L/min) could not artificially increase the drying of mini-cup coagula during maturation. The increase of total solid content (DRC) of mini-cup coagula was followed over a 7 days period and compared with that from coagula let dried outdoor.

For that purpose, twenty eight mini-cup coagula were prepared with what we called "clean latex" coagulated with formic acid. Clean latex was obtained with a special care to collect latex with a minimum of microorganisms (cf. the Materials and Methods section, *paragraph 1.2*) and no sodium azide was added in the latex. The set of mini-cup coagula was divided into two lots of 14. The first set was matured in maturation unit under controlled condition (total of air flow mixed between humid air and dry air was 2.3 – 3.0 L/min, *i.e.* 0.25 to 0.33 L/L/min; RH and temperature were controlled at 80-90% and 40°C respectively). The second set of mini-cup coagula was placed on an outdoor plastic grid under shade for 7 days (outdoor temperature range was from 26 to 32°C while relative humidity range was from 70 to 90%). For each condition (maturation unit and outdoor), two mini-cup coagula were collected concomitantly every 12 hours. The DRC of the 4 collected mini-cup coagula was measured as described in Materials and Methods (*5.2 – Determination of Dry rubber content of mini-cup coagula*)

2.3.1 Follow up of the conditions prevailing in maturation unit

Figure 39 shows the evolution of temperature and relative humidity in maturation unit during the test over a period of 7 days, as well as the detailed adjustment of flow rate. Small daily drops of temperature correspond to the opening

of the maturation unit for sampling purpose. After about 84 hours of maturation, the increase of humid air flow rate was not fast enough which leaded to a period of 10 hours where relative humidity decreased under 80%. A second phase of RH inferior to 80% appeared between 110 and 132 hours. It appears that after 84 hours the relative humidity was very unstable and rather often under 80%. This was probably due to the decrease of the number of mini-cup coagula.

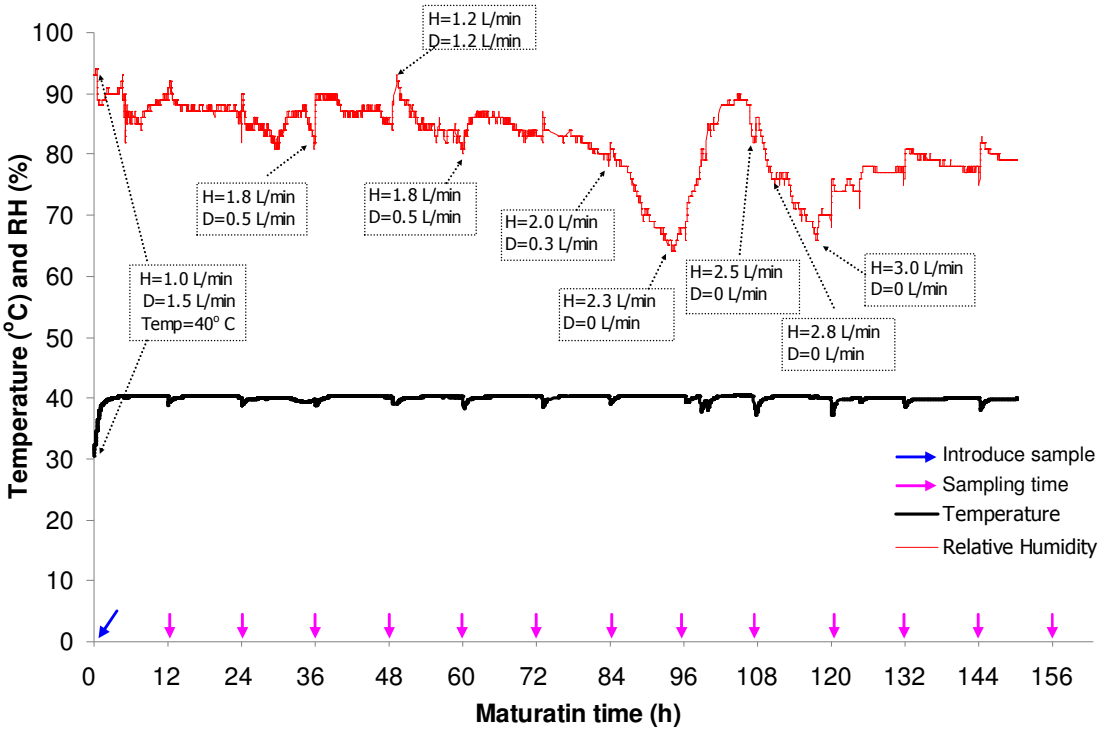


Figure 39 Maturation conditions of mini-cup coagula inside maturation unit.

2.3.2 DRC evolution of mini-cup coagula

Figure 40 shows the evolution of the DRC according to each storage conditions (outdoor and maturation unit). DRC of fresh mini-cup coagula was 52.2 % in average after coagulation. When the mini-cup coagula were placed outdoor under shade (Temperature 26-32°C and RH 70-90%) the DRC increased gradually: after 48 hours DRC reached 80% and then slightly increased to 90% after 156 hours of maturation outdoor. Concerning the mini-cup coagula which were maturated in

maturation unit and submitted to air flow ranging from 2.3 to 3L/min, the DRC evolution was similar. The water loss kinetic was slightly slower for the initial period from 0 to 96hours (4days). Afterwards the DRC of mini-cup coagula were identical wherever they were maturated (outdoor or maturation unit) probably because of lower RH conditions in the maturation unit after 84 hours (Figure 40).

This experiment confirmed that the airflow used in the maturation units in order to regulate the RH would not lead to an increase of the water loss kinetic compared to that of mini-cup coagula maturated outdoor.

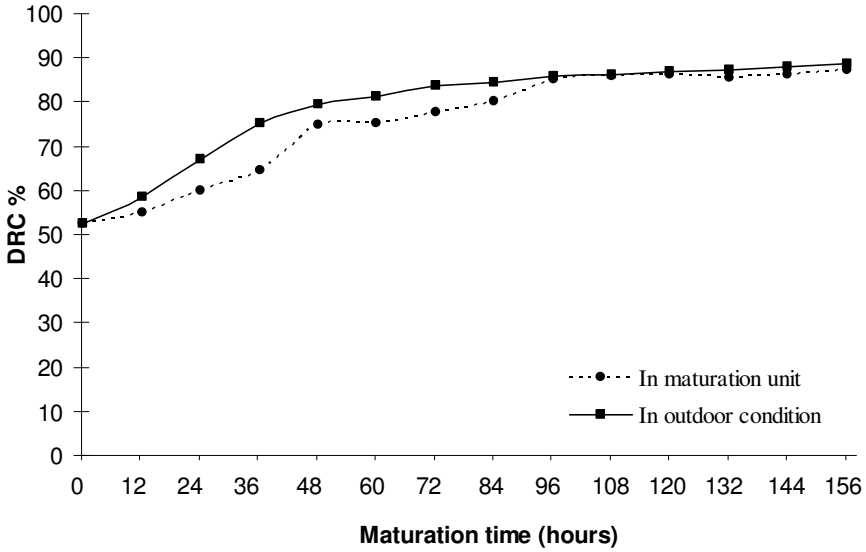


Figure 40 DRC evolution of mini-cup coagula in maturation unit and outdoor condition.

3. Optimization of the mini-cup coagula processing conditions in laboratory

After the optimization of the controlled maturation device, it was necessary to develop, at laboratory scale, a process to obtain from the mini-cup coagula a dry raw natural rubber with properties similar to that of the rubber processed in the TSR20 factory. It was indeed crucial no to introduce any artifact by using a laboratory scale process (creping and drying of the mini-cup coagula) that could influence the properties of the obtained rubber by over-creping or over-drying. The reference industrial process was that used by Von Bundit Co. Ltd., Surat Thani factory to assess

the quality of cup coagula in storage pile (TSR20 rubber). This reference process consisted in the creping of 10 kg of cup coagula (16 passes) followed by industrial drying (129°C, 3 hours). It was the one previously described in Chapter 1 and Materials and Methods section *7.2-sampling procedure*.

The envisaged strategy to scale down this process at laboratory scale was the use of a mini-creper followed by a drying in a ventilated laboratory oven (see Materials and Methods section *4-Mini processing in the laboratory*). The aim of this preliminary experiment was to determine the correct creping and drying parameters in order to obtain rubber with similar properties (P_0 and PRI) as that creped and dried in the factory. Variable creping parameter consisted in number of passes while drying parameters were temperature and duration.

The choice of the tested temperature was done following preliminary tests which showed that 130°C led to highly thermooxidized rubber while 115°C led to rubber containing white spots (not dried zones inside rubber). The two tested temperature were therefore 120°C and 125°C. The preliminary tests gave also indications to choose three drying duration to be tested: 2 hours, 2.5 hours, and 3 hours.

The other parameter was the number of passes in the mini-creper. Before using the mini-creper, a pre-creping was compulsory using the industrial creper in order to form a pre-crepe from the 10 kg cup coagula sample. Preliminary test allowed choosing two numbers of passes in the mini-creper: 12 and 16 passes.

As main objective to set up a laboratory process scale which will give the same quality NR samples as the reference industrial process, two tests were performed. The first test objective was to set up the drying conditions in the laboratory oven. This first experiment compared drying conditions between industrial dryer (129°C, 3h) and hot air oven in laboratory (120°C and 125°C for 2 hours, 2.5 hours or 3 hours) and was operated on a crepe obtained with industrial creper (Figure 41). The main objective of the second test was to set up the mini-creping

conditions, keeping the same drying condition used in the first test. For this second test cup coagula were pre-creped in industrial creper to form a pre-crepe. The pre-crepe was cut in two, on half being process industrially (industrial creper + industrial drying (129°C, 3 hours)) while the other half was creped by 12 or 16 passes in a laboratory mini-creper and dried in laboratory oven. All combination of temperature and duration used in test 1 were tested, (Figure 42).

For this experiment, the cup coagula originated from the industrial coagula pile described in Chapter 1 and were sampled in a ready to be processed lot.

As indicated in Figures 41 and 42, control samples for test 1 and 2 consisted in an industrial crepe dried in industrial dryer (129°C, 3 hours). Three replicates were performed for each treatment and for control samples.

TEST 1: Effect of drying conditions on the properties of an industrial rubber crepe

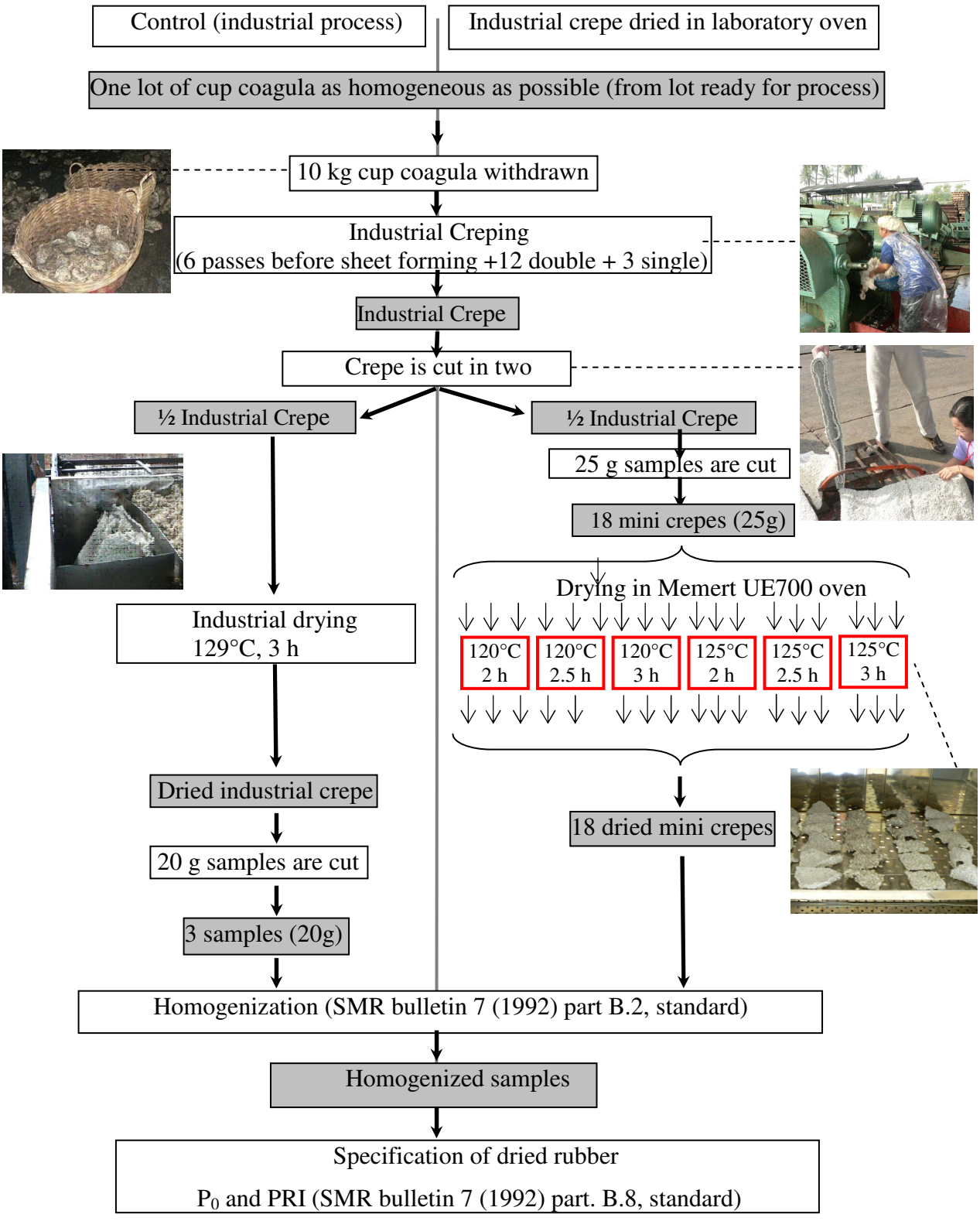


Figure 41 Cup coagula sampling and processing for studying drying conditions.

TEST 2: Effect of mini-creping and drying conditions on the properties of rubber crepe

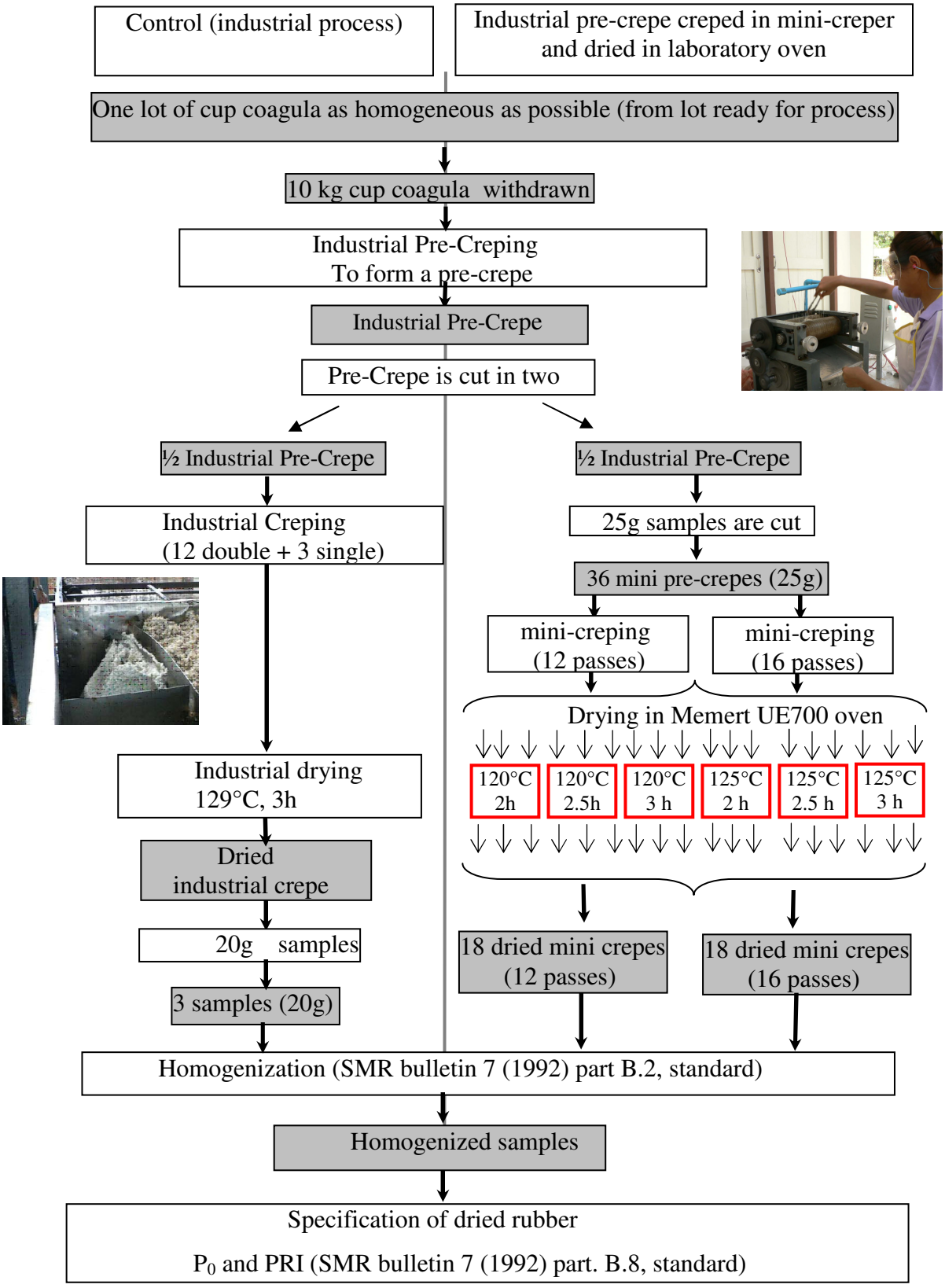


Figure 42 Procedure of cup coagula creping and drying.

3.1 Optimization of drying temperature between hot air oven and industrial dryer

Figure 43 and 44 show the P_0 and PRI of the samples obtained with the test 1. Control sample, obtained by industrial process, had an average P_0 of 39 and a PRI of 25. When submitted to a drying in the laboratory oven for 2 to 3 hours at 120°C or 125°C, P_0 values tended to decrease with temperature and drying duration. The maximum P_0 (39) were obtained at 120°C, for 2 or 2.5 hours. Drying at 120°C for 3 hours or 125°C for 2 or 2.5 hours led to a P_0 of around 35 while rubber dried for 3 hours at 125°C had a P_0 less than 25.

PRI of the control was about 25. Drying at 120°C for 2 or 2.5 hours produced a rubber with higher PRI than control (around 29) while drying 3 hours at that temperature led to PRI value below 20. At 125°C, drying for 2.5 hours gave the highest PRI (around 30) compared to that obtained after 2 hours (approx 24) and 3 hours (approx. 14).

From this first test, it can be concluded that 3 hours drying seemed too long as both PRI and P_0 were significantly lower than those of the control. If the comparison is based on P_0 only, drying at 120°C for 2 or 2.5 hours are the two combinations leading to a similar than control rubber. Based on PRI, 125°C for 2 hours would be the optimal combination.

At this stage it was decided to perform the same drying test on rubber processed with mini-creper.

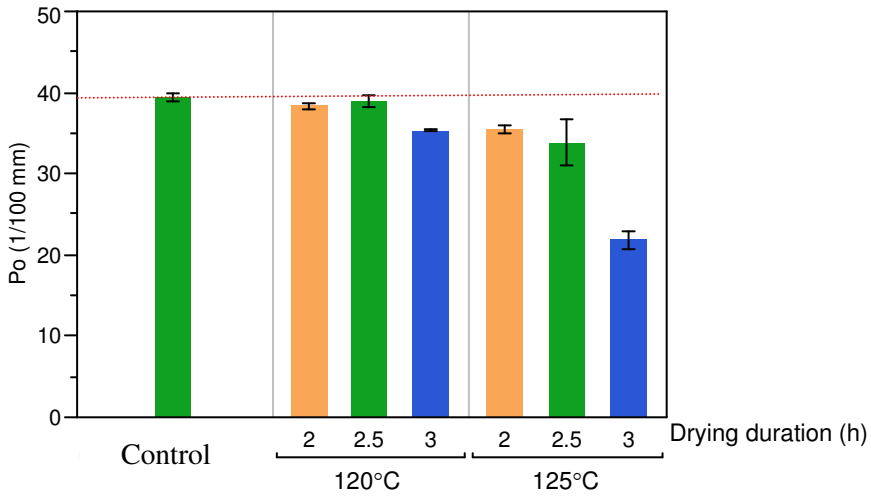


Figure 43 P_0 of cup coagula with various drying conditions.

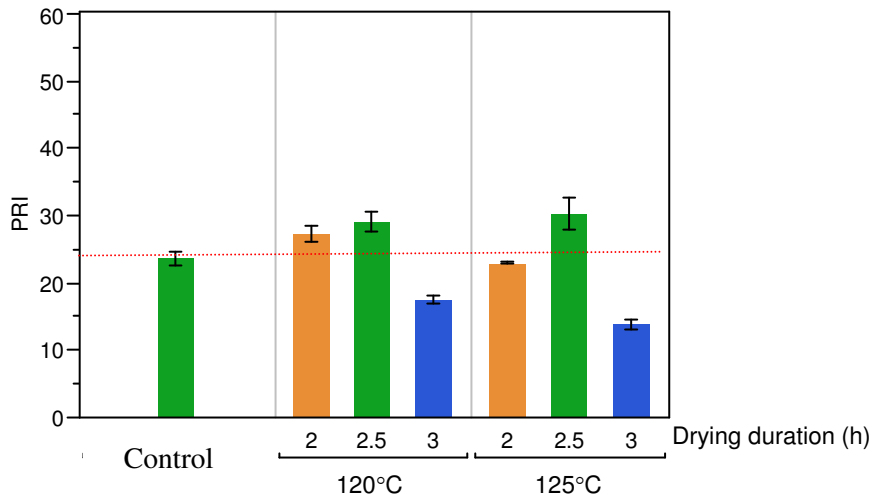


Figure 44 PRI of cup coagula with various drying conditions.

3.2 Optimization of mini processing (drying temperature and number of passes in mini-creper) in laboratory scale

This part was performed in order to optimize both drying temperature and number of mini-creper passes in laboratory scale. Figures 45 and 46 presented P_0 and PRI respectively for all the combinations of number of passes (12 and 16), drying duration (2 hours, 2.5 hours and 3 hours) and drying temperature (120°C and 125°C).

The rubber obtained after 12 or 16 passes in the mini-creper and dried in laboratory oven showed a range of P_0 from 20 to 42 and a range of PRI from 14 to 48. Those values are in agreement with the value obtained in test 1 where the rubber was creped with industrial creper only. The mini-creper seemed therefore to provide same kind of mechanical treatment than that provided industrially.

The effect of number of passes was not significant on P_0 while PRI of rubber creped 16 passes in mini-creper were significantly lower than those creped by 12 passes for most of temperature/duration combinations.

P_0 and PRI values of control sample were 41 and 30 respectively. Only two combinations of temperature, duration, and number of passes showed a PRI similar to that of the control one: 120°C / 3 hours /12 passes and 125°C / 2 hours / 16 passes (indicated with arrows on Figure 46). When observing P_0 value for the two latter combinations it was found that the drying for three hours at 120°C led to P_0 value lower than control which is in agreement with the observations made during test 1. Drying at 125°C for 2 hours with 16 passes produced rubber with P_0 similar to the control. The latter combination was therefore considered as the best compromise.

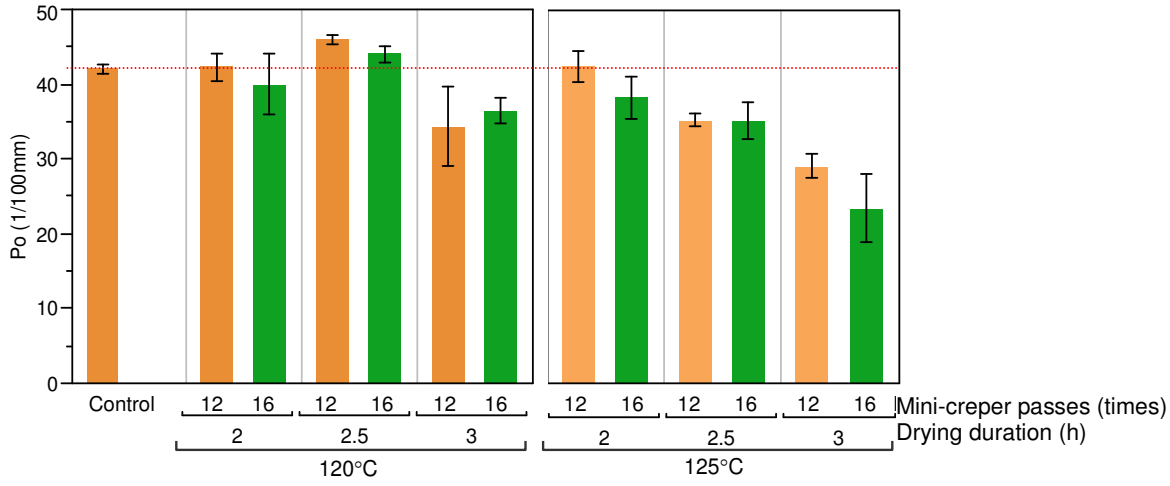


Figure 45 P_0 of cup coagula with various creping and drying conditions.

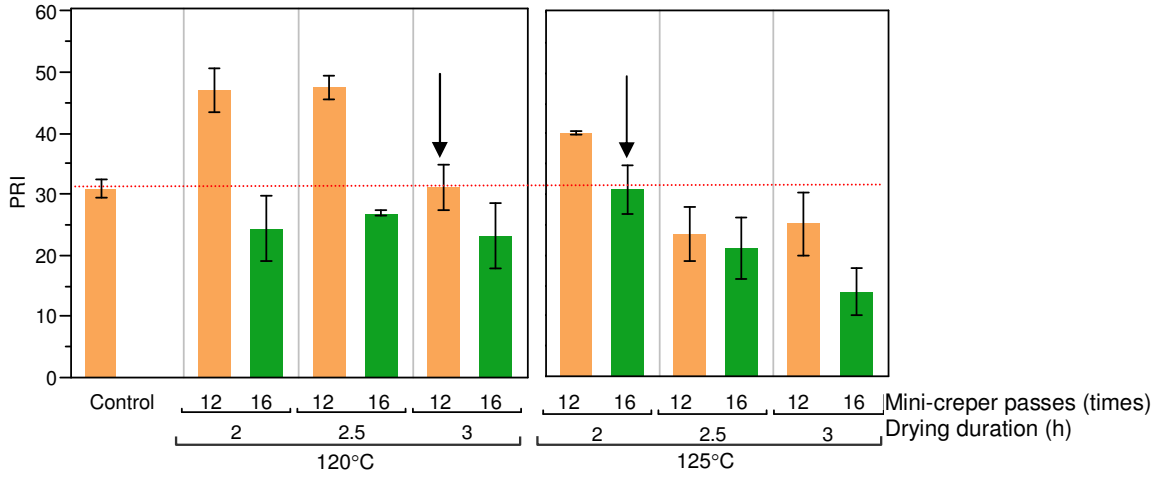


Figure 46 PRI of cup coagula with various creping and drying conditions.

In conclusion of these optimization experiments, it was decided that the mini-process consisting in a mini-creper and a ventilated laboratory oven will be used with the following parameters for all further laboratory processing of mini-cup coagula:

- Mini-creping: 16 passes
- Drying: 125°C, 2 hours

4. Control of the microbial population by adding an antimicrobial agent

Apart from physico-chemical parameters constituted by temperature, relative humidity, and oxygen concentration of the air, a crucial biological parameter was controlled during laboratory scale maturation experiments: microorganism population. It was proposed to control this population by working with either low micro-organisms field latex, added by antimicrobial agent or on the contrary, known quantity of microorganisms (inoculum) as described in Methods section *2.2.1- Mini-cup coagula with controlled microbial population.*

Preliminary tests showed that sodium azide was a good candidate to act as a broad spectra antimicrobial agent. It was shown that adding sodium azide prevented

from the drop of PRI observed in the initial stage of maturation of field coagulum. The purpose of this preliminary experiment was to prove that the action of such chemical do not interfere with the properties of the obtained rubber by other means that its action on microbial population. For that reason three experiments were performed:

1. Verify the antimicrobial activity of sodium azide by observing the relation between added antimicrobial agent concentration and living microorganism in treated latex.
2. Verify that the same effect on rubber properties (inhibition of PRI drop) are obtained with another well known antimicrobial agent such as formaldehyde
3. Verify that those antimicrobial agents do not display antioxidant properties: for that purpose, low PRI creped were soaked in antimicrobial agent solutions to observe an eventual increase of PRI.

4.1 Study of the effect of added sodium azide concentration on microorganisms content in treated latex

The aim of this study was to assess the quantitative effect of sodium azide addition on microorganism growth in latex. For that, five lots of fresh clean latex, around 100 mL, (obtained as described in Materials section *1.2-Clean natural latex*) were added with various volumes of a 10% aqueous solution of sodium azide, leading to final concentrations in latex of 0.05, 0.1, 0.2, 0.3 and 0.4% w/v latex (Table 10). After 1 hour of gentle agitation latex containing sodium azide was sampled and counted for living microorganisms by PCA method (Materials and Methods, paragraph 9.2). The results are shown in Table 11 and Figure 47

Table 10 Preparation of clean latex containing different sodium azide concentrations

Treatments	A	B	C	D	E	Control
Clean Latex (mL)	99.5	99	98	97	96	100
Antibiotic solution 10% (w/v) (mL)	0.5	1	2	3	4	0
Final concentration of sodium azide (% w/v latex)	0.05	0.1	0.2	0.3	0.4	0

Table 11 Total count of living microorganisms (CFU/mL) in latex containing different concentrations of sodium azide.

Label	$[NaN_3]$	TOTAL COUNT (CFU/mL)		
	(%w/v)	Average	SD	CV (%)
Control	0	1.18×10^7	5.52×10^5	4.7
A	0.05	5.56×10^4	4.78×10^3	8.6
B	0.1	5.37×10^4	4.11×10^3	7.7
C	0.2	2.62×10^4	1.17×10^3	4.5
D	0.3	2.71×10^4	1.82×10^3	6.7
E	0.4	2.62×10^4	3.53×10^3	13.5

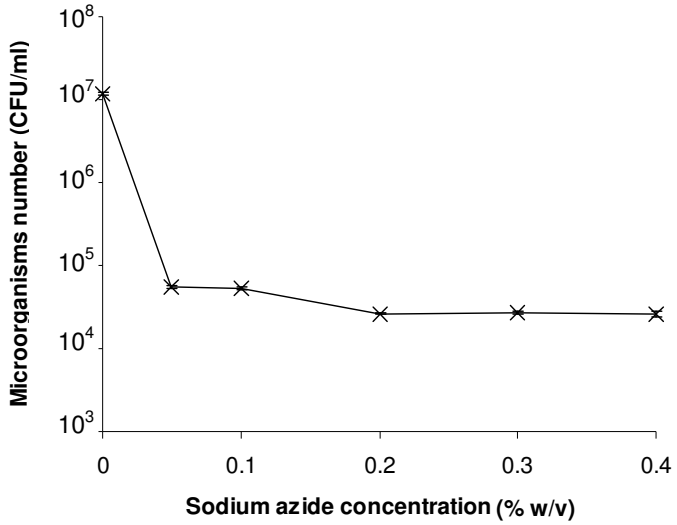


Figure 47 The effect of sodium azide concentration on microorganisms content in latex (Standard error mean of each point is very low).

The presence of the lowest concentration of sodium azide (0.05%) resulted in a reduction of microorganism amount of about 200 times when compared with fresh clean latex. The microorganism amount reached its minimum with a concentration of 0.2% (2.62 x 10^4 CFU/mL) and higher concentration did not reduce more the number of living microorganisms. The concentration of 0.2% seemed therefore to be optimal.

This preliminary test confirmed the antimicrobial activity of sodium azide in clean latex. Furthermore, the concentration of 0.2% w/v was selected for any further experiment with antimicrobial agent treated treatments.

4.2 Effect of formaldehyde on PRI of mini-cup coagula

Preliminary tests showed that addition of sodium azide in latex prevented the initial drop of PRI of the rubber obtained from from latex. Formaldehyde is another antimicrobial agent, with different mechanism than sodium azide. If this chemical has the same effect as azide (inhibiting the drop of PRI) that would reinforce the idea that micro-organisms are involved in the drop of PRI.

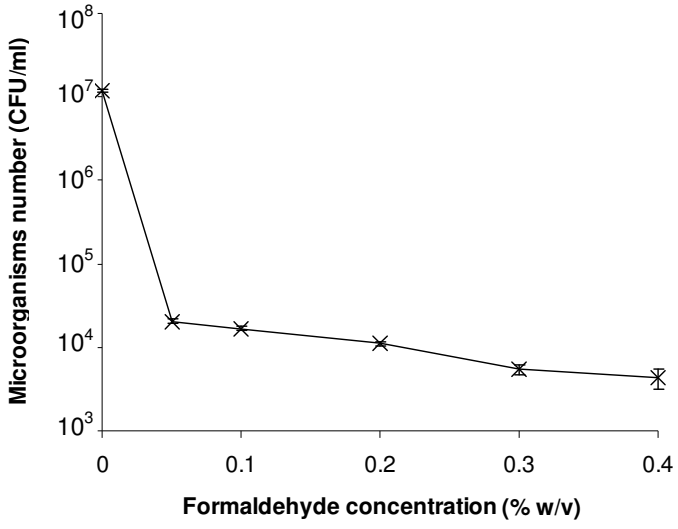


Figure 48 The effect of formaldehyde concentration on microorganism content in latex.

In order to choose the appropriate concentration of formaldehyde to be used for that comparative test, same experiment as that described in the previous section 5.1 was conducted. Figure 48 shows that formaldehyde effect had similar effect on the reduction of microorganism population and that both antimicrobial agents can be used at the same concentration in latex, i.e. 0.2%.

Mini-cup coagula were prepared from clean fresh latex added with 0.2% antibiotic solution (formaldehyde or sodium azide) following N+M1+F+ treatment described in Materials and Methods, paragraph 2.2.1. An inoculated treatment was used as control and was prepared following N-M1+F+ treatment. Mini-cup coagula obtained from these three treatments were matured in maturation units for a short-term maturation duration of 5 days (Materials and Methods, Paragraph 3.1). At 0 and 5 days of maturation, three replicated mini-cup coagula from each treatment were withdrawn and processed following mini-processing procedure (Materials and Methods, section 4). The obtained dry rubber was submitted to P_0 and PRI analyses and the results are shown in Figure 49 and 50 respectively.

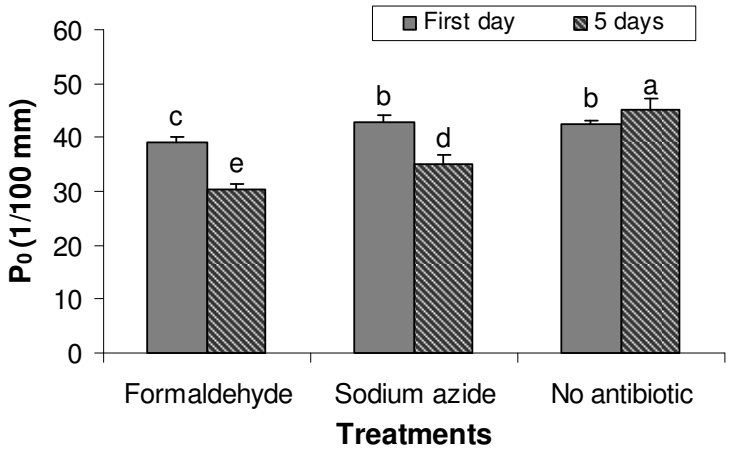


Figure 49 Effect of antibiotic addition and maturation time on P_0 of mini-cup coagula.

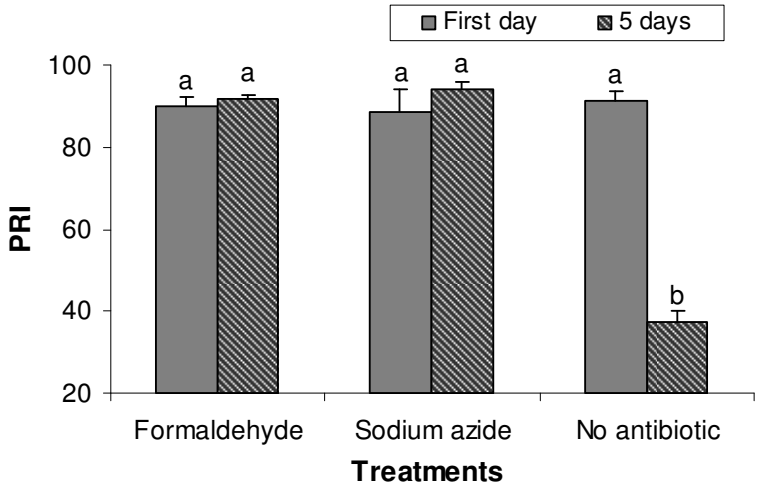


Figure 50 Effect of antibiotic addition and maturation time on PRI of mini-cup coagula

The values of PRI were not significantly different between treatments for the rubber processed just after coagulation (day 0). After 5 days of maturation, the PRI of rubber without antimicrobial agent dropped drastically (from PRI 90 at day 0 to PRI lower than 40 at day 5). This phenomenon will be studied in details in Chapter 3. The presence of formaldehyde resulted in the same effect as the presence of sodium azide: the PRI was maintained at high level after 5 days of maturation.

Concerning P_0 before maturation (day 0) the value was the same for sodium azide and control rubber (around 40) while the rubber treated with formaldehyde showed a slightly lower P_0 (39). After 5 days maturation, P_0 decreased for the rubber treated with antimicrobial agent.

In conclusion, the effect on PRI was the same for both sodium azide and formaldehyde which may indicate that they acted indirectly on this parameter by reducing the microorganism population thanks to their antimicrobial activity. To confirm further that assumption it was to be checked that they both do not have any antioxidant activity. This is the purpose of next section.

4.3 To control the antioxidant activity of sodium azide and formaldehyde

The aim of this experiment was to test an eventual antioxidant activity of sodium azide and formaldehyde. Based on bibliographic references (Tuampoemsap *et al.*, 2007 and Varghese *et al.*, 2005) assumption was made that, if they have such antioxidant activity, soaking in their solution potential low PRI crepes obtained with maturated mini-cup coagula would increase PRI value.

For that purpose, 15 mini-cup coagula were prepared clean latex inoculated with microorganisms (N-M1+F+, see Materials and methods section, part 2.2). They were maturated for 10 days and were creped with mini-creper (Materials and Methods, section 4). Referring to bibliography, mini-crepes were soaked in 1% antimicrobial agent solution (either sodium azide or formaldehyde) in order to test the antioxidant activity of antimicrobial agent solution. Tested durations of soaking were 0 (no soaking, control) and 8 hours. The 4 treatments involving soaking as well as the control treatment without soaking were repeated 3 times. After soaking, mini-crepes were drained off and dried in hot air oven at 125°C for 2 hours. P_0, PRI of rubber from dried crepes were determined and are presented in Figures 51 and 52 respectively.

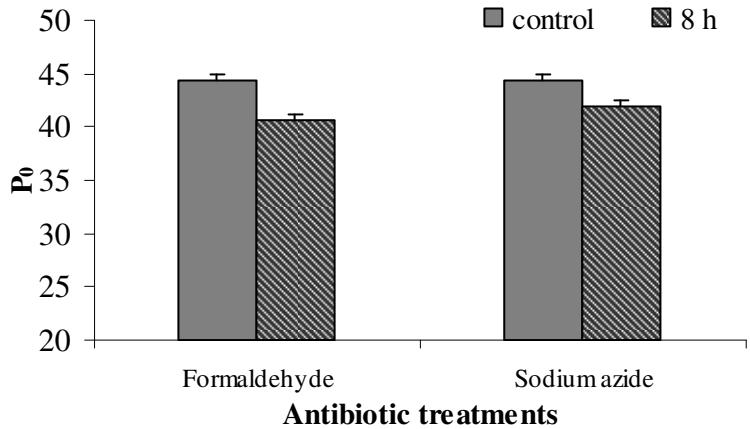


Figure 51 Effect of antibiotic solution soaking and soaking time on P_0 of mini-cup coagula maturated 10 days.

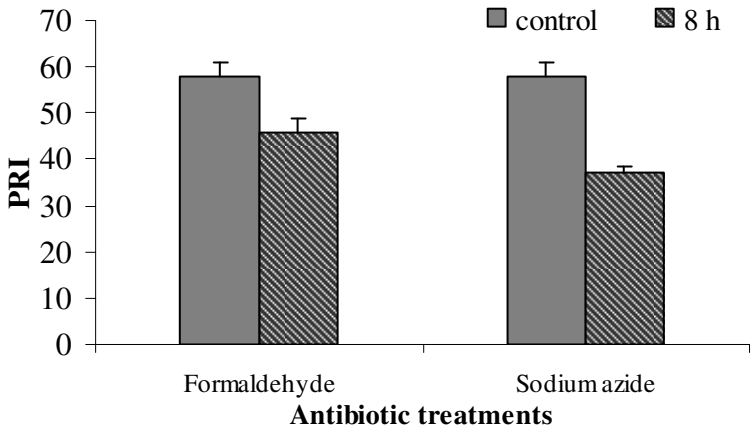


Figure 52 Effect of antibiotic solution soaking and soaking time on PRI of mini-cup coagula maturated 10 days.

The value of P_0 and PRI of rubber from non-soaked treatment (control) were 44 and 58. With soaking in 1% formaldehyde solution for 8 h, P_0 values slightly decreased down to 41 while PRI values were 46. With 8h soaking in sodium azide solution, P_0 and PRI decreased down to 42 and 37, respectively. It was found that soaking in both tested antimicrobial agents did not improve PRI. In addition, the soaking tended to decrease both P_0 and PRI. Considering that soaking in antioxidant of coagulated non dried rubber has been proven previously to improve PRI, it can be concluded that sodium azide which will be used in our further experiment to control microorganisms population, has no antioxidant activity and, therefore, that the high PRI observed on maturated sample treated with that chemical is only due to a reduction of microorganism activity.

5. Conclusion

The purpose of the work reported in this chapter was to set up the necessary experimental devices to be able to control in a proper manner the maturation of mini-cup coagula at laboratory scale as well as the process of transformation of the maturated mini-cup coagula to dry rubber.

Is there an effect of the reduction of the sample size? It was observed that there was no significant difference of P_0 and PRI when sampling rubber at the surface or inside a maturated cup coagula. These results validated the possibility of reducing the size of coagula (from normal cup coagula used in the rubber field to mini-cup coagula (45 mL)). The mini-cup coagula can be considered as a good representative of the rubber field coagula.

How to concomitantly regulate the temperature and relative humidity of different maturation units atmosphere? The maturation device containing several maturation units was tested. It was shown that the regulation possibilities offered by the system were satisfactory with empty or loaded units. The water release from fresh mini-cup coagula followed a decreasing kinetic and had to be compensated by an increasing humid air share in the air inlet. It was also shown that this water release, illustrated by an increase of the dry rubber content (DRC) of the mini-cup coagula, was similar between the outdoor and the laboratory conditions.

How to optimize a laboratory process in order to obtain dry rubber with comparable quality as the one made from industrial process? Two main sets of comparative experiments allowed to optimize parameters of the laboratory process of transformation of the maturated mini-cup coagula to dry rubber. The mini-cup coagula would be creped 16 passes in the mini-creper and dried for 2 hours at 125°C in a ventilated laboratory oven.

How to assess the effect of antimicrobial agent on properties of rubber and especially distinguish between its antimicrobial activity and others? Sodium azide antimicrobial activity was proven in latex. The result of the quantitative effect of this chemical agent on the amount of microorganisms amount in latex allowed to select the default concentration of 0.2% w/v for the antimicrobial agent treatment of mini-cup coagula. Comparison with another well known antimicrobial agent such as formaldehyde and soaking test confirmed that sodium azide activity do not interfere with the properties of the obtained rubber - especially resistance to thermo oxidation illustrated by PRI - by other means that its action on microbial population

CHAPTER 3

Study of coagula maturation in controlled conditions

Introduction

The duration of cup coagula storage in industrial conditions and the physicochemical conditions occurring during this storage period have been identified as key parameters influencing the quality of the produced dry rubber (Chapter 1). To assess more precisely the phenomena occurring during this phase, a laboratory scale maturation device as well as a scaled down process to obtain dry rubber have been set up and validated by comparison with an industrial process (Chapter 2). The purpose of the study described in the present chapter was to use the developed device to study more precisely the involvement of the microorganisms during the maturation of cup coagula.

The depressive effect of microorganisms presence on PRI was previously observed at the early stage of maturation (Figure 36 and 50, Chapter 2). It was therefore decided to study in details the quantitative effect of the presence of microorganisms on the properties and structure of natural rubber issued from mini-cup coagula maturated during a short period (Materials and Methods, Section 3.1 Short-term maturation in closed plastic containers). After this characterization, the influences of the mode of coagulation (formic acid/ natural coagulation) and of the presence of oxygen (aerobic or anaerobic conditions) were checked over a long period of maturation in maturation units. Microbiological and enzymatic characterizations of the inoculum were performed and some of the identified enzymatic activities were tested in a preliminary study by adding pure enzyme in latex instead of inoculum.

Therefore this chapter is organized in four main parts: i- Quantitative effect of the presence of microorganisms on the structure and properties of natural rubber, ii- Study of the influences of the mode of coagulation (formic acid/ natural coagulation)

and of the presence of oxygen (aerobic or anaerobic conditions) ; iii- Characterization of the microorganisms present in the inoculum including an assessment of main extracellular enzymes produced by those strains; and finally iv- Study of the effect of pure enzyme addition to the latex on the properties of rubber issued from maturated mini-cup coagula.

1. The effect of living microorganisms quantity on structure and properties of rubber over a short-term maturation period (6 days)

It was observed in the experiments described in the previous chapter that the presence of microorganisms drastically affects PRI during the early stage of maturation. To understand further this phenomenon, it was decided to study whether this early drop of PRI would be proportional to the initial quantity of microorganisms inoculated in clean latex. In order to identify the mechanisms involved in the drop of PRI, a structural analysis of the obtained rubber was performed by the assessment of the evolution of the mesostructure (soluble isoprene molar masses and gel quantity).

Clean latex was treated with different inoculum quantities before acid coagulation (See Table 5, Materials and Methods section). The maturation of mini-cup coagula was studied from 0 to 6 days after inoculation with different quantities of microorganisms (see Materials and Methods section 3.1 Short-term maturation in closed containers). Considering the internal volume of the containers, the frequency of samplings (full renewal of air inside the container) and the quantity of microorganism in presence, it can be considered that even this experiment was performed under uncontrolled oxygen content condition that there was no deficit of oxygen.

The main technological properties assessed during maturation were PRI and P_0 which were measured, as usual, within 24 hours after obtaining dried rubber. Furthermore, analyses of the mesostructure (macromolecular structure and gel content) of raw rubber were performed to understand the evolution of properties during maturation (see Materials and Methods, section 6.2 Determination of

mesostructure). The latter analyses were performed in France 80 days after drying. To evaluate any property change during that delay, P_0 and PRI were measured again at the time of mesostructural analysis.

1.1 Initial microorganism amount in inoculated latex

Clean latex (N-M-) contained $4.8x10^5$ CFU/mL of total aerobic microorganisms while the addition of sodium azide (N+M-) reduced this amount to $3.41x10^4$ CFU/mL. Addition of inocula (N-M1, N-M2 and N-M5) raised this amount to $2.16x10^6$, $9.52x10^6$ and $2.36x10^7$ CFU/mL respectively (Figure 53). This fact confirmed that the quantity of counted aerobic microorganisms in inoculated latex was proportional to the quantity of added inoculum.

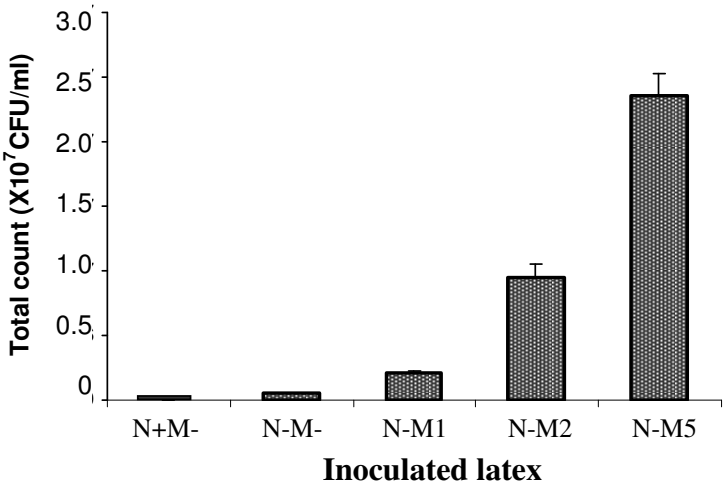


Figure 53 Initial microorganism amount in inoculated lattices for each treatment.

1.2 Evolution of dry rubber content of mini-cup coagula during maturation

At the first day, the average dry rubber content of all mini-cup coagula inoculated was 40.5%. After 6 days of maturation, dry rubber content of mini-cup coagula increased up to a range of 62% to 72%, depending of the initial number of microorganisms (Figure 54). Indeed, increasing the quantity of microorganisms decreased proportionally the rate of serum release during maturation as well as the

level of the obtained plateau. The physical aspects of mini-cup coagula after six days of maturation were very different (Figure 55). The presence of growing microorganisms was clearly illustrated by the generation of bubbles inside the coagula. On a qualitative point of view, size and number of bubbles seemed to increase with the initial quantity of microorganisms. The differences between calculated DRC values relied mainly on a difference of fresh weight, the dry weight being roughly the same for all treatment at a given maturation time. Observed DRC differences could therefore be explained by a higher serum retention within the mini cup coagula where microorganisms has been inoculated.

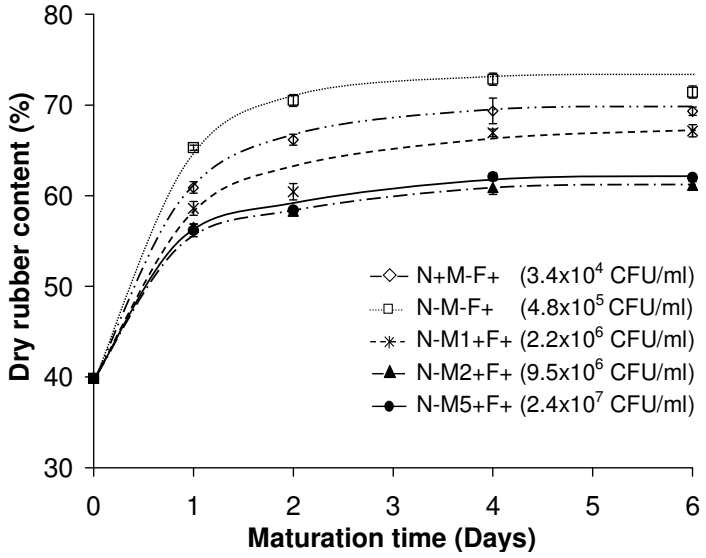


Figure 54 Dry rubber content of treated mini-cup coagula evolution with maturation time. Initial microorganism counts are indicated in brackets. *The solid lines are a guide for eyes.*

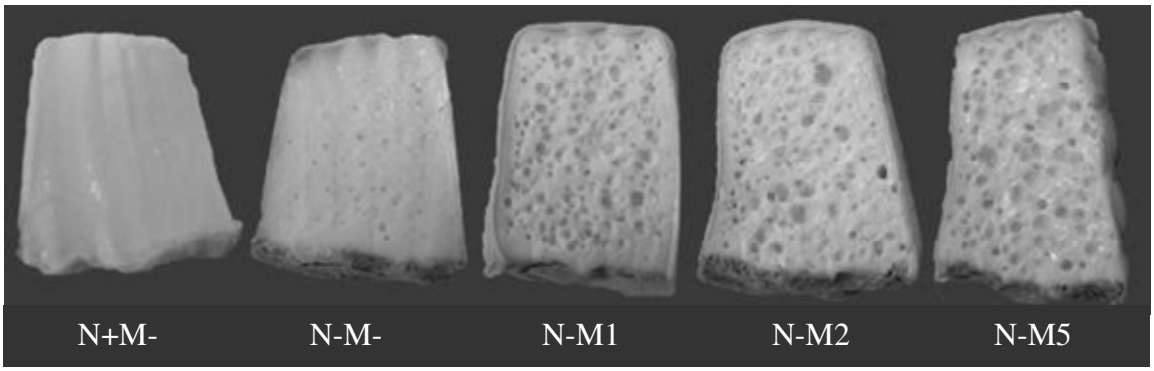


Figure 55 Central sections of mini-cup coagula after 6 days of maturation, with different initial level of microorganisms inoculation.

Superior water retention in inoculated coagula can be explained by two reasons: (i) gas produced by the microbiological metabolism formed bubbles inside the mini-cup coagula (Figure 55). The cavities formed by the bubbles, bigger and more numerous with the quantity of microorganisms, could act as a reservoir and trap water; (ii) microorganism activity may have degraded non isoprene compounds, especially proteins, which are known to be a vector of water in the hydrophobic rubber medium (Sainte-Beuve *et al*., 2000). Water may thus have been retained inside the mini-cup coagula because of a lack of carrier.

1.3 Wallace plasticity (P_0) and Plasticity retention index (PRI) measured within 24 hours after drying

Figure 56 presents the evolution of Wallace plasticity during maturation time (0 to 6 days) for all treatments described previously, the main difference between treatments being the initial quantity of microorganisms as described previously. The control sample for all treatments (0 day maturation time) displayed a Wallace plasticity (P_0) value about 34.1 (standard error of the mean or SEM=0.35), whatever the microorganisms quantity (Figure 56). When observed over maturation time, the evolution of P_0 was clearly depending on the quantity of microorganisms. Indeed, when microbial activity was prevented (treatment N+M-F+), P_0 did not change significantly while for the non inoculated treatment (N-M-F+) P_0 increased up to 45 after 6 days of maturation. With inoculated ones (N-M1+F+, N-M2+F+ and

N-M5+F+), an increase of P_0 was observed for the first 2 days of maturation and was followed by a drop down to 30.5, 27.5 and 27.0 respectively after 6 days of maturation.

Initial PRI of all mini-cup coagula processed 3h after coagulation was similar with an average value of 97.6 (SEM=1.4) (Figure 57). The evolution of PRI over maturation time was found to be clearly dependent of the initial quantity of microorganisms in latex. Indeed, when microbial growth was prevented, no change of PRI was observed. For the three inoculated treatments (N-M1+F+, N-M2+F+ and N+M5+F+) the drop rate of PRI was proportional to the initial microorganism concentration, even though the PRI reached after 6 days was found similarly very low (<10). Concerning the non-inoculated treatment (N-M-F+), which contained a low initial number of microorganism, a less important drop was observed over maturation time, PRI value reached after 6 days being 70.

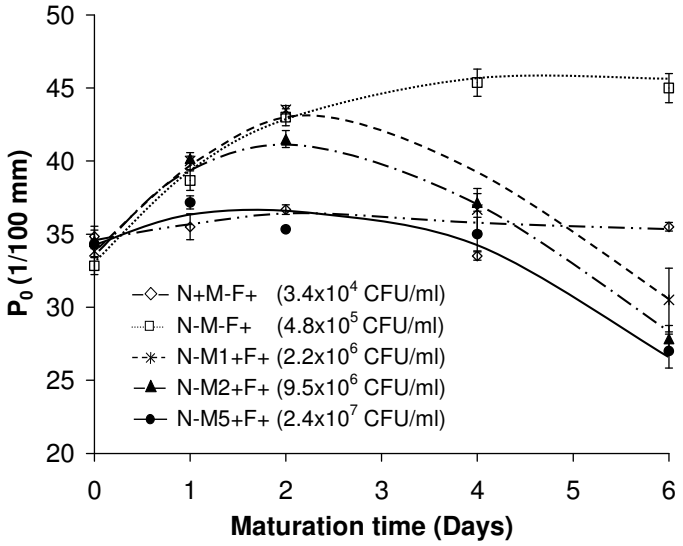


Figure 56 Wallace plasticity evolution with maturation time for each treatment. Initial microorganism counts are indicated in brackets. *The lines are a guide for eyes.*

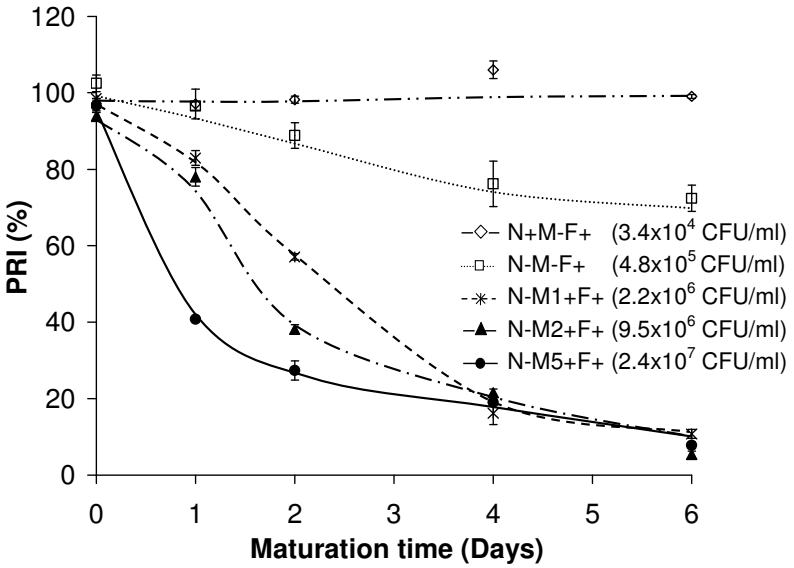


Figure 57 Plasticity retention index evolution with maturation time for each treatment. Initial microorganism counts are indicated in brackets. *The lines are a guide for eyes.*

The presence of microorganisms in latex before coagulation clearly led to a proportional decrease of PRI. The more inoculated the latex; the more sensitive to thermo-oxidation the rubber. This may be explained by two phenomena: (i) microorganisms activity released pro-oxidant molecules such as free metallic ions (Hasma and Othman, 1990) or fatty acids (Tuampoemsab and Sakdapipanich, 2007); (ii) this activity degraded or inactivated some of the latex native anti-oxidants such as tocotrienols (Hasma and Othman, 1990). On-going studies of this anti-oxidant are in progress but it has already been observed on these samples that free tocotrienol disappearance is associated with the decrease of PRI (data not shown).

Concerning the P_0 values measured just after drying (Figure 55) the observed variations could be due to a balance between hardening of rubber by crosslinking of polyisoprene chains and softening by oxidative scission of rubber chain during drying. In inoculated samples (N-M1+F+, N-M2+F+ and N-M5+F+), despite a decrease of PRI from the first day, crosslinking seemed to counteract and even exceed scissions during the 2 first days, leading to an increase of P_0. Later on, after the 4^{th} day, scission

became prominent, leading to a decrease of P_0. For non-inoculated samples (N-M-F+) the crosslinking phenomenon was higher than scission during the 6 days of maturation. Rubber added with antimicrobial agent (N+M-F+) showed a constant P_0, which illustrates equilibrium (or non-existence) of those two phenomena. The involvement of microorganisms in the crosslinking phenomenon is difficult to assess. It can be supposed that microbial activity interacts with abnormal groups as well as with non-isoprene constituents, which leads to new interactions between rubber chains. Microorganisms interestingly seemed to promote both phenomena – crosslinking and scission – which have contrary effect on P_0. Mesostructural information (gel and molecular masses), which will be discussed below are needed to complete those assumptions.

1.4 Wallace plasticity (P_0) and Plasticity retention index (PRI) measured 80 days after drying

Measurements of P_0 and PRI were repeated after storing the samples 80 days at room temperature for all treatments but the treatment (N-M2+F+). Considering the low variance among repetitions for P_0 and PRI measured just after drying, and the fact that three repetitions of the SEC analysis are realized, only one repetition of each treatment and each maturation time was taken for the re-measurement of P_0 and PRI and for the assessment of polyisoprene molar masses and gel. Figure 58 and 59 presents respectively the re-measured P_0 and PRI (plain line) in comparison with the initial measure just after drying (dotted line). The results obtained showed the same global trends as the ones obtained without storage, but P_0 was clearly increased because of storage hardening. This hardening, assessed by the P_0 measured 80 days after drying minus by that measured within 24 hours after drying, was in the range of 10 to 15 P_0 units for non-inoculated samples (N-M-F+ and N+M-F+). With the inoculated samples (N-M1+F+ and N-M5+F+), the shift was more important (between 15 and 22) and increased with maturation time. This is shown on Figure 60 which presents the evolution of this increase of P_0 with maturation time for treatments N+M-F+, N-M1+F+ and N-M5+F+. A good linear correlation between maturation time and increase of P_0 was observed for inoculated samples.

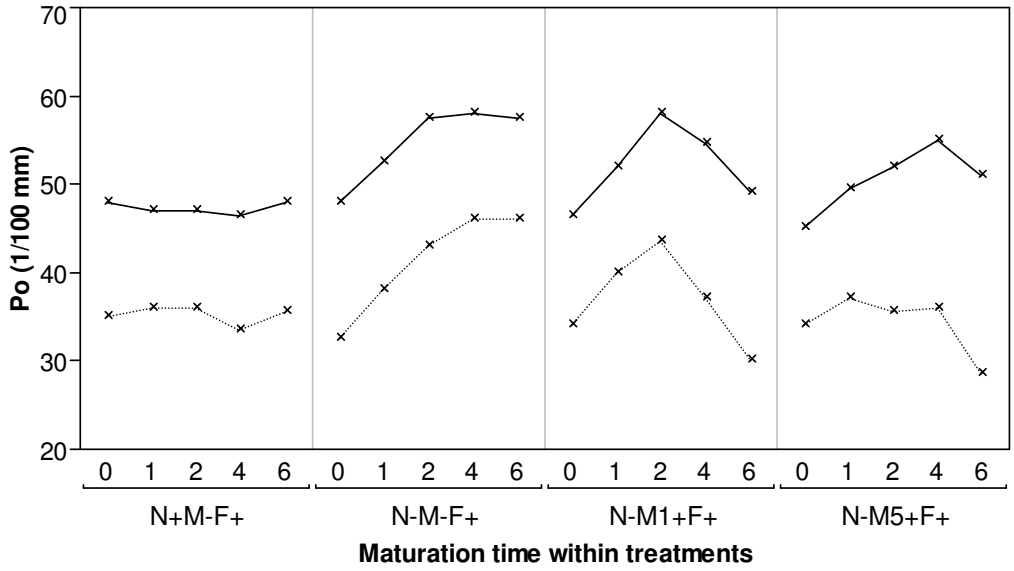


Figure 58 Wallace plasticity (P_0) evolution for different maturation times for each treatment. Measurements performed after 80 days of storage (solid lines) compared with initial ones.

The samples used for mesostructural measurement underwent obviously storage hardening. However, the trends discussed previously on P_0 and PRI are roughly conserved with the harden rubber. It was nevertheless observed that the presence of microorganisms in latex seemed to promote storage hardening, and that this increase of P_0 was more pronounced for inoculated samples maturated for 4 or 6 days. This is again an illustration of the impact of microbial activity on the crosslinking of rubber chains even after drying, i.e. during storage time.

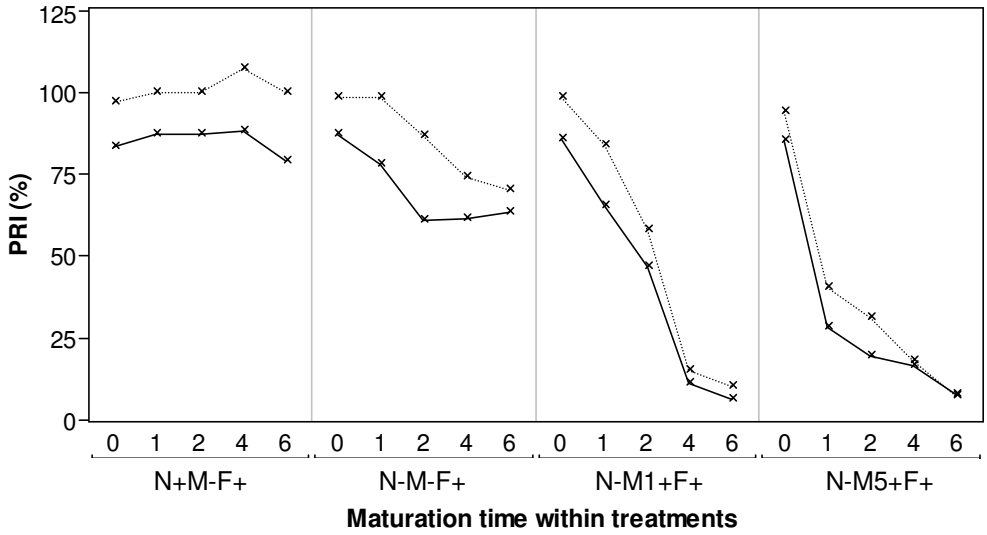


Figure 59 Plasticity retention index (PRI) evolution for different maturation times for each treatment. Measurements performed after 80 days of storage (solid lines) compared with initial ones (dashed lines).

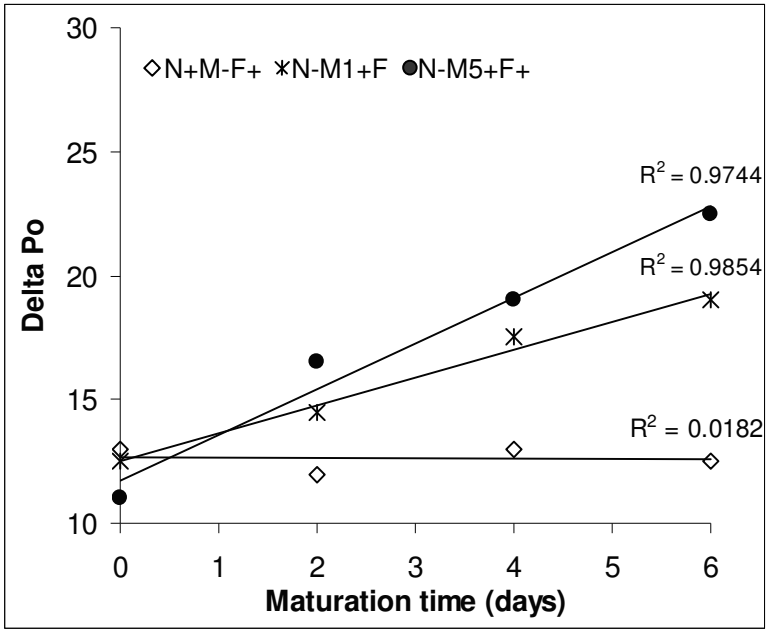


Figure 60 Effect of the maturation time on the increasing of P_0 during 80 days storage for each treatment. The solid lines are linear regressions, Coefficient of determinationR^2 are indicated nearby the corresponding straight lines.

1.5 Mesostructure of rubber

1.5.1 Gel content

Figure 61 presents the evolution of gel content during maturation time (0 to 6 days) in latex inoculated with different initial quantities of microorganisms. Measures were realized after 80 days of storage for all natural rubber samples. The control sample for all treatments (0 day maturation time, (Figure 56)) displayed a very similar initial gel quantity with an average value of 30% (SD=1.3%). For all treatments, gel quantity increased with maturation time and reached a plateau after 2 days of maturation. The value associated with this plateau was proportional to the initial quantity of microorganisms, from 45% for antibiotic treated rubber (N+M-F+) to 55% for the treatment with the highest initial microorganism content (N-M5+F+).

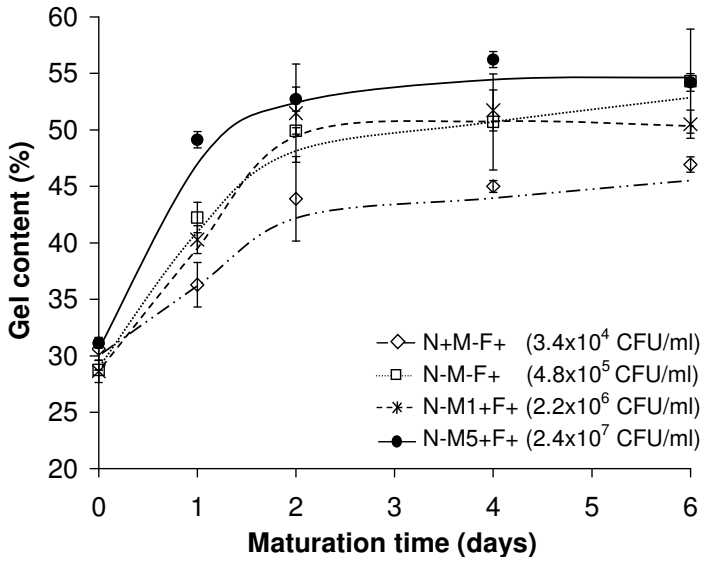


Figure 61 Gel content evolution with maturation time for each treatment. Measurements were performed after 80 days of storage. *Initial microorganism counts are indicated in brackets. The lines are a guide for eyes.*

1.5.2 Molar masses

It is worth noting that, contrarily to the other measured parameters, the initial value of molar masses from samples processed 3 hours after coagulation were slightly

different between treatments, inoculated rubber showing slightly lower values of $\overline{M_w}$ and $\overline{M_n}$ (Figure 10 and 11 respectively).

A slight decrease in weight averaged molar mass ($\overline{M_w}$, Figure 62) was observed with maturation time for the antibiotic treated rubber (N+M-F+). $\overline{M_w}$ values dropped from 1500 kg/mol down to 1400 kg/mol after 6 days of maturation. The $\overline{M_w}$ from N-M-F+ treatment (no inoculum, no antibiotic) showed a similar trend with a faster drop. For inoculated treatments (N-M1+F+ and N-M5+F+), this drop was more important, $\overline{M_w}$ reaching a minimum value of around 1150 kg/mol. Interestingly, the drop rate was proportional to the quantity of microorganisms. The minimum value was reached after 1 day for N-M5+F+ and after 4 days for N-M1+F+.

Number-average molar mass $\overline{M_n}$ (Figure 63) followed a different trend than $\overline{M_w}$ over maturation time. Indeed, inoculated rubber showed a stable value of $\overline{M_n}$ staying around 700 kg/mol while non inoculated samples (M0+N, M0) showed an increase of $\overline{M_n}$ over the maturation time, up to 900 kg/mol.

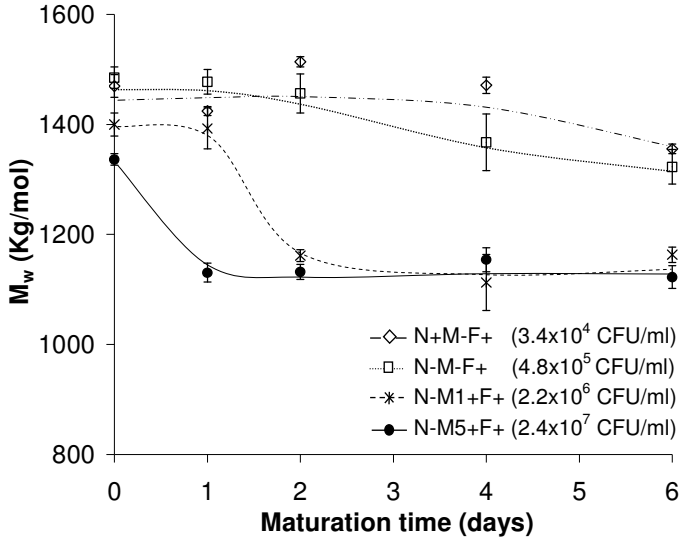


Figure 62 Weight average molar mass ($\overline{M_w}$) evolution with maturation time for each treatment. Measurements were performed after 80 days storage. *Initial microorganism counts are indicated in brackets. The lines are a guide for eyes.*

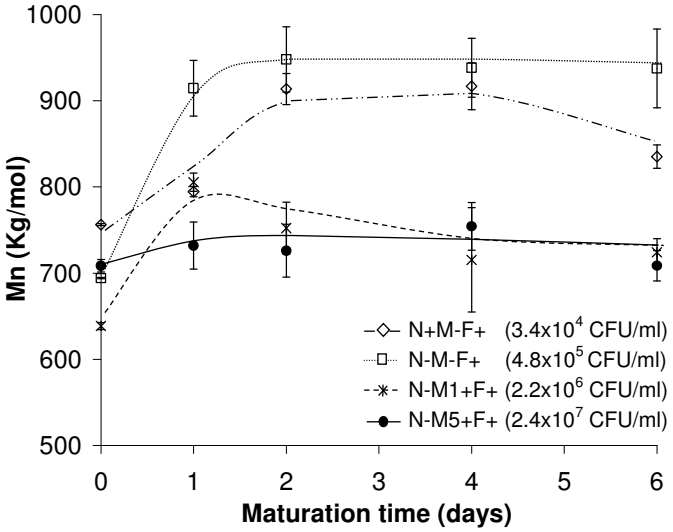


Figure 63 Number average molar mass ($\overline{M_n}$) evolution with maturation time for each treatment. Measurements were performed after 80 days storage. *Initial microorganism counts are indicated in brackets. The lines are a guide for eyes.*

The weight-average molar mass ($\overline{M_w}$), which preferentially illustrates the high molecular mass-isoprene population, confirmed that the presence of microorganisms in latex before coagulation promoted scissions. Considering the similarity of $\overline{M_w}$ and PRI evolution, those scissions are most probably due to a thermo-oxidative phenomenon that may have occurred during drying.

Concomitantly, it is obvious that microorganisms are also involved into crosslinking interactions between isoprene chains during and/or after maturation (drying and/or storage) because this phenomenon increases with maturation time and initial quantity of microorganisms, as illustrated in the gel evolution (Figure 9). Nevertheless, for samples containing an antimicrobial agent (N+M-F+), an increase of gel content was also observed at a lesser extent, which means that gel formation may occur without microbial activity. This gel may be of a different nature as it did not impact on the P_0 of N+M-F+ samples, which remained stable over maturation time. This could be a physical gel, or thermo-reversible gel, and be destroyed at 100°C, the temperature used for P_0 measurement. Indeed, Voznyakovskii et al. (1996) studied gel

in NR by dynamic light scattering and showed that increasing temperature of the solutions from 20°C to 70°C led to a decrease of the mean size of the aggregates.

Gel formation during storage hardening of natural rubber has been studied in many works concerning natural rubber. Sekhar (1962) attributed gel formation to interactions between aldehyde groups located on the polyisoprene and bifunctional amine compounds. Mineral components such as divalent metallic ions have been also suspected to cause ionic bounds between rubber chains under low humidity, leading to storage hardening (Gan, 1996). More recently, Yunyongwattanakorn *et al*., (2003) concluded that proteins were not involved in gel formation under low humidity conditions, citing fatty ester groups from the phospholipid located at alpha terminal end of polyisoprene chain as a causal agent. Molar mass distribution of the polymer is also an important factor short chains being apparently more involved in the crosslinking process (Ngolemasango *et al*., 2003). However, most of these authors do evaluate gel quantity only with the non-soluble part separated by centrifugation what it is usually called “macrogel”. In our study, the “microgel”, the gel part which is not separated by centrifugation but removed by filtration previously to SEC analysis, has also been included. In fact, most of previous studies took only macrogel into account as gel content while in our study we took into account the total gel content (macrogel + microgel). Therefore, comparisons have to be done with caution. Microorganisms enzymatic activity may catalyze reactions involved in gel formation such as the oxidation of polyunsaturated fatty acid leading to formation of aldehyde groups and the release of amino acids or metallic ions. In order to better understand the mode of action of microorganisms, the study of in situ enzymatic activity is necessary.

$\overline{M_n}$ data are more delicate to interpret. Indeed, on one hand, scissions would logically produce more short chains and decrease $\overline{M_n}$ even if the produced short chains may be involved into gel and therefore not counted in the $\overline{M_n}$ assessment. The latter would therefore be overestimated (Ngolemasango *et al*., 2003). On the other hand, crosslinkings during or after maturation could increase $\overline{M_n}$. However, there was no significant branching difference observed in the conformation plots obtained by SEC-MALS (data not shown). In our case it seems that the high gel containing rubber

samples, which are the inoculated ones, keep a stable $\overline{M_n}$ over maturation. This could be due to a balance between scissions and the crosslinkings illustrated by gel content. Concerning the antibiotic treated rubber which seemed protected from scission (high PRI and slight decrease of $\overline{M_w}$ over maturation time), the increase of $\overline{M_n}$ may be due to the formation of gel not countered by polyisoprene short chain release through oxidative reaction.

1.6 Conclusion

This experiment dealt with the initial stages of the maturation of natural rubber mini-cup coagula (6 first days). It has been shown that phenomena occurring during the first stage of maturation of rubber are complex and clearly depend on the presence and the amount of microorganisms in the initial latex. At the initial stage of maturation, microorganisms clearly enhance the sensitivity of rubber to thermal oxidation while concomitantly seeming to promote crosslinking between rubber chains. However, the exact mode of action of these micro-organisms is not understood, hence the need to focus on the role of enzymes they secrete.

For that purpose, microbiological and enzymatic characterization of the microorganisms present in the inoculum, will be described in part 3 of the present chapter, while part 4 will present the study of the effect of pure enzyme addition in initial latex on rubber properties issued from a short-term maturation.

Before dealing with microbiological studies, it was decided to check over a long maturation (45 days) using maturation device the effect of the mode of coagulation (natural or formic acid) and the presence of oxygen (anaerobic or anaerobic conditions).

2. Effect of the mode of coagulation and the oxygen presence on a long run maturation (45 days) in maturation units

In our experimental procedure it has been decided to coagulate the latex with addition of formic acid until pH 5.2. This was done for two main reasons:

1. When comparing different treatments (quantity of inoculum, addition of Sodium azide and addition of enzyme) it appeared important to synchronize the coagulation to make the comparison reliable.
2. No previous coagulation would have implied the handling of liquid samples in maturation units waiting for natural coagulation to occur which would have been practically not realistic for routine procedure.

The present experiment was set up to assess the eventual effect of the mode of coagulation together with the influence of oxygen presence. Indeed, the study of the industrial maturation pile showed a significantly decreasing the gradient of oxygen concentration in the air while going deeper into the pile. Two conditions were therefore tested during this experiment: aerobic (under air flow) or anaerobic (under nitrogen flow).

For that double purpose, the inoculated and formic acid coagulated mini-cup coagula (N-M+F+) were compared with inoculated cup coagula not added with formic acid (N-M+F-) as well as with a control mini-cup coagula obtained with normal field latex called “field coagula”. For the latter treatment, “clean latex” procedure was not followed and the tapper was asked to follow his routine way of harvesting. The formulation of each samples are shown in table 3 in Materials and Methods.

The experiment was performed over a 45 days maturation period and was repeated twice: once under aerobic condition and under anaerobic condition (under pure nitrogen flow). During maturation, sampling of three replicates was performed after 0, 3, 5, 15, 30 and 45 days of maturation. Withdrawn mini-cup coagula were characterized by pH and DRC measurements and the P_0 and PRI of the obtained rubber could be determined providing that the coagulum was formed which allowed further process (i.e. creping and drying). For natural coagulation, this occurred 3 days after tapping which explain the absence of “0 days” data for DRC, P_0 and PRI.

2.1.1 pH evolution of mini-cup coagula

As shown in red color in Figure 64, the formic acid coagulated mini-cup coagula pH (either with or without oxygen) followed a slightly increasing trend (increase from 5.4 to 6.5) over 45 days of maturation. Under anaerobic condition, and after an initial drop from pH 6.7 (fresh latex) to 5.1 (3 days after tapping), that trend was followed as well by the naturally coagulated mini cup coagula (either issued from inoculated clean latex or from field latex). Similar slight increases of pH were observed in another long-term maturation experiment with or without oxygen where Sodium azide was added to formic acid coagulated (data not shown).

However, under aerobic condition the evolution of pH of naturally coagulated latex was obviously different: after an initial decrease from pH 6.7 (pH of fresh latex) to 5.2 (pH of the naturally coagulated mini cup coagula three days after tapping), the pH value increased up to 9 after 15 days of maturation and stay at this high level until 45 days of maturation. The presence of oxygen seems therefore to be a factor of influence of the pH evolution of naturally coagulated rubber. Indeed when oxygen is present and the coagulation is natural, and only in this case, the pH increased drastically after 5 days of maturation. This must be linked with the aerobic degradation of proteins leading to ammonia production. This phenomenon was also observed in-situ by placing a pH probe connected to a data logger inside a field cup coagula (data not shown).

In addition it is interesting to be noted that the inoculated clean lattices followed the same trends as the corresponding “normal” field lattices, which meant that this artificially inoculated latex could be considered as a representative of field latex.

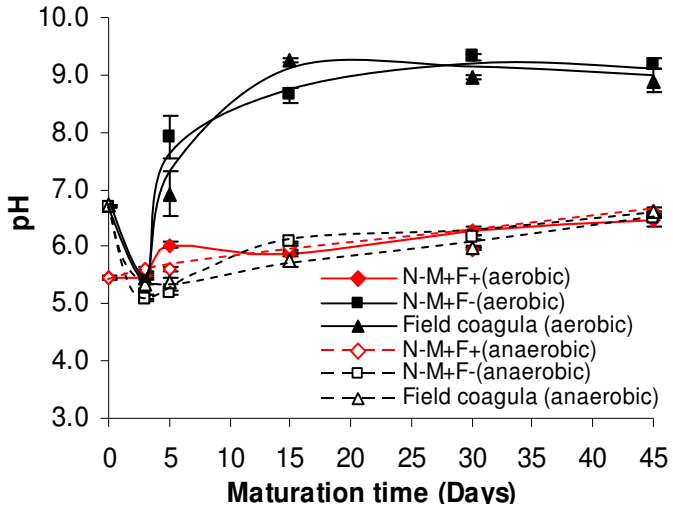


Figure 64 pH evolution with maturation time for each treatment (N-M-F-: fresh latex; N-M+F-: clean latex added with microorganisms and without formic acid; N-M+F+: clean latex added with microorganisms and formic acid), *the lines are a guide for eyes.*

2.1.2 DRC evolution of mini-cup coagula

DRC evolutions of mini-cup coagula are shown in Figure 65. DRC of mini-cup coagula increased with maturation time, the TSC value of latex being 38 % in average and reaching a plateau at DRC 80-90% after 30 days of maturation. For the first 5 days of maturation, DRCs of formic acid coagulated mini cup coagula (N-M+F+ with or without oxygen, represented with red color in figure 65) were higher than those of naturally coagulated samples. Nevertheless this difference disappeared after 15 days of maturation. Moreover, the DRCs of mini-cup coagula maturated under aerobic conditions tended to be higher those of the corresponding coagula maturated under anaerobic condition.

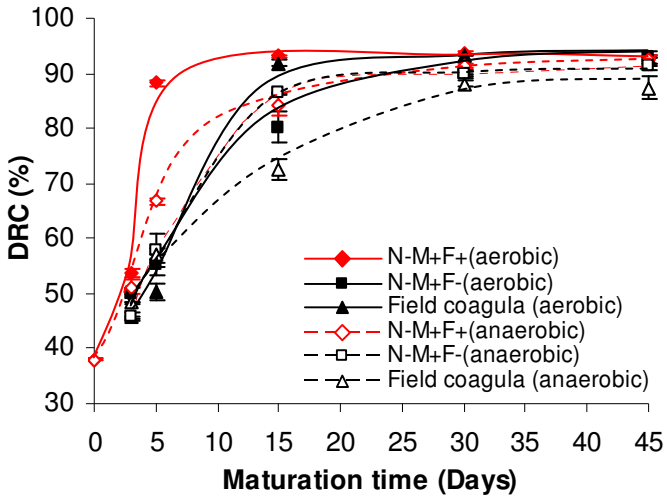


Figure 65 DRC evolution with maturation time for each treatment (N-M-F-: fresh latex; N-M+F-: clean latex added with microorganisms and without formic acid; N-M+F+: clean latex added with microorganisms and formic acid), *the lines are a guide for eyes.*

When compared with the results reported in previous section, the difference of the plateau value (plateau values ranging between 55 and 70%) can be explained by the facts that i-the maturation was performed under air or nitrogen flow (contrarily to closed containers) and ii- the maturation lasted 45 days (contrarily to 6 days). Moreover, referring to that previous result the lower DRC observed for naturally coagulated sample could be explained by a higher growth of microbial population and larger bubble formation (caused by a slower coagulation) which allowed a higher serum retention within the mini-cup coagula (see figure 55).

2.1.3 P_0 evolution of mini-cup coagula

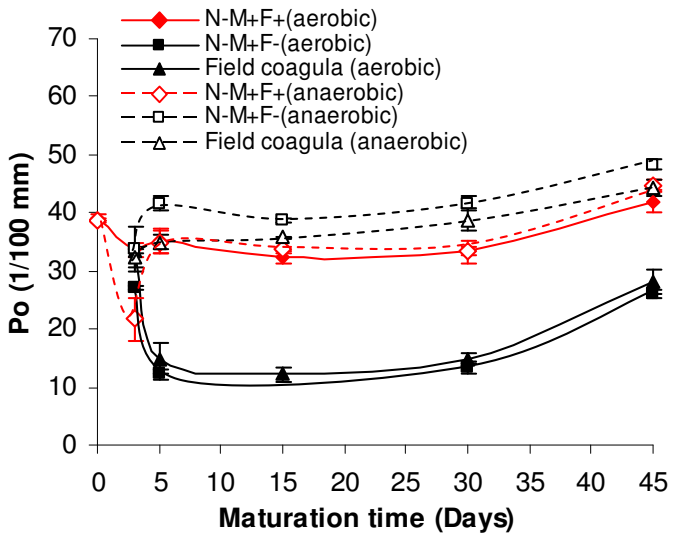


Figure 66 P_0 evolution with maturation time for each treatment (N-M-F-: fresh latex; N-M+F-: clean latex added with microorganisms and without formic acid; N-M+F+: clean latex added with microorganisms and formic acid), *the lines are a guide for eyes.*

As shown in figure 66, P_0 of the formic acid coagulated (N-M+F+) mini-cup coagula with or without oxygen did not change much over maturation conditions as observed in the previous section. The values stayed in the range of 32 to 42. Under anaerobic conditions, the observed P_0 of naturally coagulated followed also a steady evolution during maturation time. The values were slightly higher than the formic coagulated one. But in presence of oxygen, the P_0 of naturally coagulated samples did show a singular trend. Indeed, a drastic drop of that indicator was observed from 0 to 5 days of maturation reaching values close to 12 which generally illustrates high scission of the polymer chains. This low level stayed steady until 30 days of maturation and a final increase to P_0 28 was observed after 45 days of maturation.

As observed in the study of maturation pile (chapter 1) it is interesting to note that the evolution of the P_0 is inversely correlated to that of the pH. When the coagula were naturally coagulated, the presence of oxygen seemed to favor a huge increase of pH accompanied by a drastic drop of P_0. Further studies of the aerobic metabolism of

microorganisms in cup coagula, and especially the aerobic protein catabolism should be performed to understand what could be the source of this crucial difference. As the presence of oxygen in the atmosphere surrounding the maturating coagula seems to be of importance, oxygen concentration gradient inside the coagula should also be studied further.

Two non exclusive assumptions can be made to explain the fact that when formic acid is added in presence of oxygen, these important changes of pH and P_0 are not observed:

1. ***Selective initial pH***: the initial pH (5.2 when formic acid is added and 6.8 when it is not) could be a factor of selection of the aerobes which will grow inside the cup. The "pH 6.8" aerobes could be able through their metabolism to provoke the observed changes while the "pH 5.2" aerobes could not.

2. ***Physical oxygen barrier***: apparently the oxygen presence seems to be compulsory (no changes observed under anaerobic conditions). An early coagulation due to formic acid addition could prevent physically the oxygen to enter the coagulum, while in the case of natural coagulation; the latex keeps its liquid state for the first days which allows an easier diffusion of oxygen inside the coagulating latex.

2.1.4 PRI evolution of mini-cup coagula properties

For formic acid coagulated rubber, PRI of mini-cup coagula were 95 in average at the first day of maturation (Figure 67) and dropped to 15 in average after 5 days of maturation. Concerning naturally coagulated rubber, the first measurement of PRI which occurred 3 days after tapping showed that PRI is already low (<30).

After 5 days of maturation and for mini-cup coagula maturated under anaerobic conditions, the PRI value increased progressively to reach an average value

of 40 after 45 days of coagulation. Concerning mini-cup coagula maturated under aerobic condition, the PRI from formic acid coagulated sample followed the same kind of evolution (progressive increase up to PRI 32) while the PRI from naturally coagulated sample were lower and stayed below 20. For those 2 latter samples it is to be reminded that their P_0 were very low which makes the interpretation of PRI delicate.

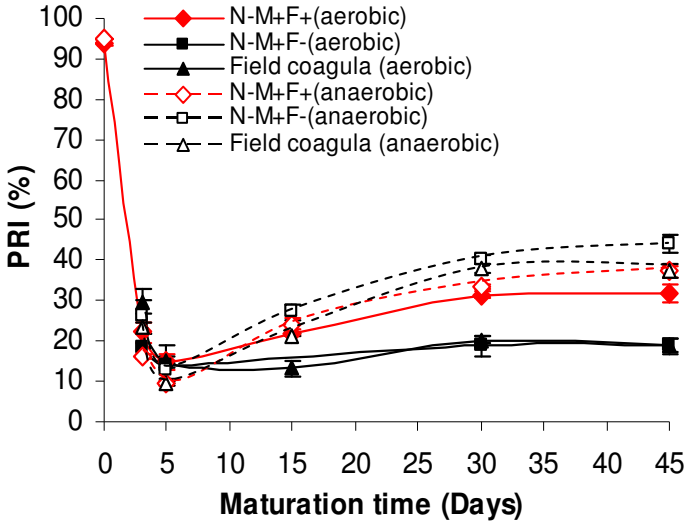


Figure 67 PRI evolution with maturation time for each treatment (N-M-F-: fresh latex; N-M+F-: clean latex added with microorganisms and without formic acid; N-M+F+: clean latex added with microorganisms and formic acid), *the lines are a guide for eyes.*

Moreover, it is interesting to be noted that the PRI values were ranked in the same order as the P_0 ones: *Anaerobic conditions* [naturally coagulated (N-M+F-) > field coagula > formic acid coagulated (N-M+F+)]; *Aerobic conditions* [formic acid coagulated (N-M+F+) > naturally coagulated (N-M+F-) = field coagula].

For 30 and 45 days of maturation, the PRI of anaerobic maturated mini cup coagula (whatever the mode of coagulation) was higher than that of coagula which were maturated in presence of oxygen: it is in agreement with what was observed in maturation pile where PRI of coagula maturated at a depth of 2 meters (where oxygen content was less than 5%) were significantly higher than that of coagula located at the upper layer of the pile (Chapter 1).

2.1.5 Conclusion

Under anaerobic condition the properties of the rubber issued from mini-cup coagula either naturally coagulated or with formic acid showed similar values. However, in the presence of oxygen, significant differences were observed between the two modes of coagulation: indeed, naturally coagulated mini-cup coagula showed a higher pH (>9 vs 6 for acid coagulated), and a lower P_0 (<20 vs 35 for acid coagulated sample). Coagulating the inoculated latex with formic acid and maturating in presence of oxygen seems therefore to generate a rubber different from that issued from natural coagulation. However that concern is limited only to initial plasticity, the initial drop of PRI being present for all treatments with or without oxygen.

It is also interesting to note that the treatment consisting of a natural coagulation of "clean latex" inoculated with microorganisms (N-M+F-) gives a rubber with similar properties as that obtained from raw field latex. This inoculated latex could therefore be a good representative of the field normal latex with the additional advantage that its microbial population is quantitatively and qualitatively known.

3. Characterization of microorganisms in inoculum

In order to control the initial microorganism concentration in latex, clean latex was inoculated with known amounts of microorganisms. This allowed studying the effect of inoculum concentration on mini-cup coagula properties. The results described in paragraph 2.3 showed that increasing microorganism concentration in latex before coagulation resulted in a decreased resistance of rubber to thermo-oxidation. This effect was then further investigated by isolation, counting and identification of the main groups of microorganisms present in an inoculum sample collected from a pool of coagulated latex from 32 trees under plantation (uncontrolled) conditions (see Materials and Methods, paragraph 9). The secretion of lipolytic and proteolytic activities by the isolated strains was also studied.

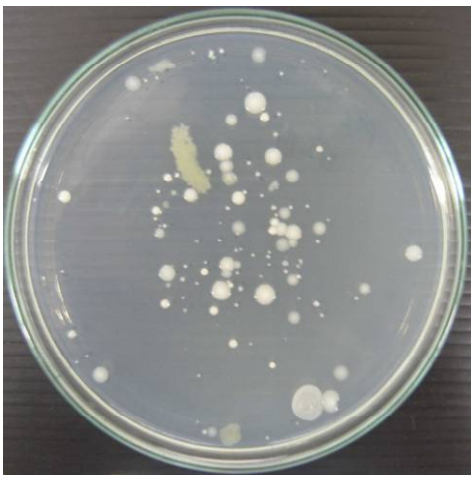
3.1 Main groups of microorganisms in inoculum

The microbial population in the inoculum was characterized using selective agar media. Appropriate dilutions were performed before spreading on agar plates. The following categories were numbered: total aerobic and total anaerobic bacteria, yeasts, Gram-positive, Gram-negative, and lactic acid bacteria. The number and aspect of colonies of each group of microorganisms are given in Figure 68. The number of microorganisms of inoculum incubated under anaerobiosis was $1.07x10^9 \pm 0.47x10^9$ CFU/mL, while under aerobic condition were $8.65x10^8 \pm 1.8\ x10^8$ CFU/mL. Amounts of yeasts, Gram-positive bacteria, Gram-negative bacteria and lactic acid bacteria were $4.23x10^8 \pm 0.02\ x10^8$ CFU/mL, $3.22x10^8 \pm 0.07\ x10^8$ CFU/mL, $4.1x10^7 \pm 0.47\ x10^7$ CFU/mL and $8.5x10^7 \pm 0.28\ x10^7$ CFU/mL, respectively.

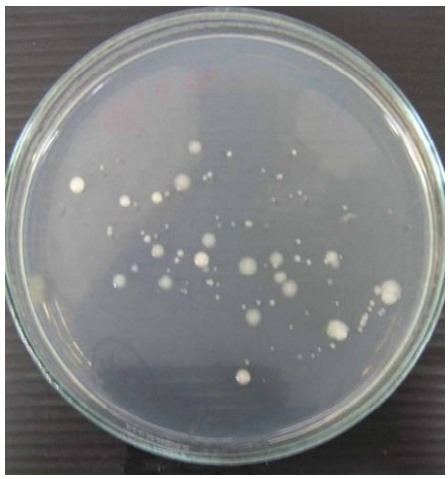
3.2 Identification of bacterial strains

Individual colonies of differing aspects grown on PCA agar media for 24 h at 37°C in aerobiosis were picked and subcloned by restreaking on new PCA agar plates. The subcloned colonies were then picked, streaked on PCA slant tube and given a unique code (No. 1, 2, 3,…) before further use.

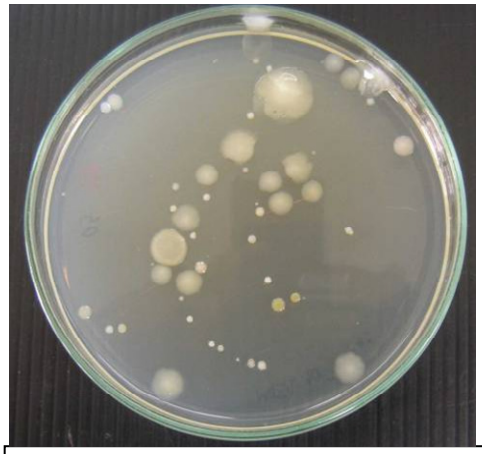
Each strain was analyzed at the Microbiology Laboratory Unit of Faculty of medicine (Prince of Songkla University, Thailand) as described in Materials and Methods. The lists of isolated strains are shown in Table10.



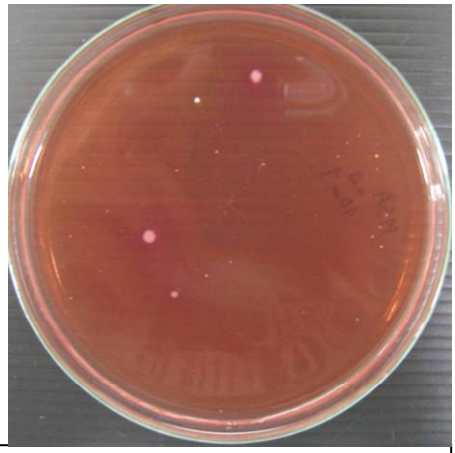
Total anaerobe on PCA agar



Total aerobe on PCA agar

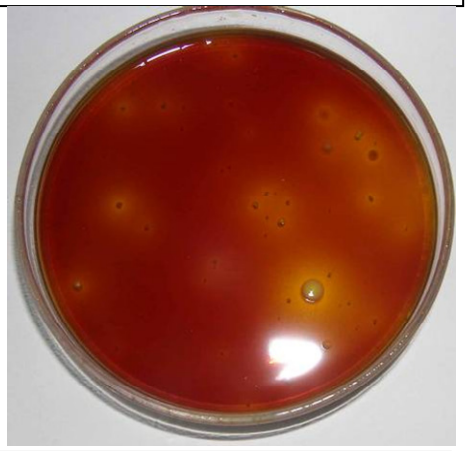


Yeasts and Moulds on MEA agar



Gram positive bacteria on MSA

Gram negative bacteria on MAC



Lactic acid bacteria on MRS

Figure 68 Colonies appearance of microorganisms in inoculum on selective media.

Table 10 Genera and species of bacteria isolated from inoculum.

No.	Gram type	Cell shape	Genus, species	General characters
1	+	Rod	*Bacillus* sp.	Lactose fermented, Produces acid and gas
2	-	Rod	*Klebsiella pneumoniae*	Lactose fermented, Produces acid and gas
3	-	Rod	*Acinetobacter* sp.	Stricly aerobic, non-fermentating
4	-	Rod	*Enterobacter cloacae*	Lactose fermented, Produces acid and gas
5	-	Rod	*Klebsiella oxytoca*	Lactose fermented, Produces acid and gas
6	-	Rod	*Citrobacter freundii*	Lactose fermented, Produces acid and H_2S
7	+	Coccoid	*Υ-Streptococcus* sp.	Production of lactic acid
8	-	Rod	*Pseudomonas alcaligenes*	Non-fermentative, Citrate positive, Produces ammonia
9	-	Rod	*Escherichia coli*	Lactose fermented, Produces acid and gas
10	+	Cocci	*Staphylococcus*	Glucose fermented, produces acid
11	+	Rod	Enterococci	Production of lactic acid
12	+	Rod	*Sphingobacterium* spp.	

Twelve different species were isolated from this inoculum. The most abundant microorganisms isolated were Enterobacteriaceae (rod type and Gram-negative). There were 5 strains of Gram-positive bacteria species with three rod shaped, one coccus shaped and one coccoid shaped. From these results, 4 families were found that included Lactobacteriaceae, Enterobacteriaceae, Pseudomonadaceae, and Bacillaceae. This is to be compared with the results of Taysum (1957) who identified a wide variety of bacteria in field latex, belonging to the genera *Staphylococcus, Streptococcus, Propionibacterium, Microbacterium, Bacillus, Micrococcus, Corynebacterium* and *Flavobacter*. Boonsatit *et al.* (2007) concluded that the type of

bacteria found in fresh natural latex after 5 hours of storage may cause the putrefaction of fresh natural rubber (FNR) latex. The bacterial species found were *B. coagulans, Burkholderia cepacia, B. licheniformis*, *B. cereus, B. subtilis, Serratia ficaria, B. amyloliquefaciens*, *Brevibacillus brevis* and *Enterobacter aerogenes*. The presence of these microorganisms in latex was influence the evolution of mini-cup coagula properties during maturation.

3.3 Hydrolase-production by isolated microorganisms

The strains were tested for hydrolase production by spotting cells onto the surface of specific media agar. The details of testing are described in Materials and Methods in paragraph 10. The appearance of a clear zone around each colony was investigated. Testing hydrolase production by the 16 isolated strains showed that 15 strains could produce phospholipase, 8 strains produced protease and 6 isolates produced lipase (Table 11). A strain of *Enterobacter cloacae* seemed to produce none of the hydrolase activities tested (protease, lipase and phospholipase) during the 96 h of incubation in the conditions tested. It is worth noting that *K. oxytoca* and *P. alcaligenes* could produce hydrolase only under anaerobic conditions, while *Bacillus* sp., *K. pneumoniae* and some strains of *E. cloacae* could produce phospholipase only under aerobiosis.

4. The effect of hydrolytic enzymes on mini-cup coagula properties

This preliminary study focused on protease and lipase activities, for which enzymes with broad substrate specificity are available and a potential effect has been observed in non-controlled maturation conditions. Indeed, correlations between lipid composition and hydrolytic degradation and rubber properties (P_0, PRI) have been recently shown (Liengprayoon, 2008; Rodphukdeekul *et al*., 2008). Moreover, an effect of proteases on rubber properties has been described in literatures (Yunyongwattanakorn *et al.*, 2003; Tuampoemsab and Sakdapipanich, 2007), with for example a positive effect of a papain treatment of skim latex on P_0 and PRI (Georges *et al*., 2009). In the present work, the enzymes (papain from *C. papaya* and lipase

from *C. parapsilosis*) were chosen for their broad substrate specificity and their high activity in the pH range observed during maturation. Mini-cup coagula were prepared by adding various concentrations of the hydrolases (Table 11) before coagulation with formic acid solution (see Materials and Methods paragraph 10).

Table 11 Target enzyme activity added in latex.

	Target enzyme activity in latex (U/L latex)			
Enzyme concentration*	1X	5X	20X	100X
Lipase	50	250	1000	5000
Protease (papain)	11	55	220	1100

* The amount of enzyme for concentration 1X was determined on the basis of lipid and protein content of fresh latex before treatment, so that the added enzyme could potentially hydrolyse 100% of these substrates in 3 hours.

4.1 Determination of the activity of enzymes in cup coagula

The effect of added enzyme activity in cup coagula during maturation was estimated by the determination of free amino acid and free fatty acid (FFA) concentrations as described in Materials and Methods (paragraph 11). The addition of protease in cup coagula resulted in up to a 50% increase of free amino acids in 4 hours compared to the control without added enzyme (Figure 69). A general decrease in amino acid content was observed during maturation, probably due to assimilation by the microbial flora.

Table 12 Hydrolase-production test of each isolated bacterial strain cultivated in aerobic or anaerobic conditions.

Incubation time	24 h.						48 h.						72 h.						96 h.					
	Lipase		Protease		Phospho-lipase		Lipase		Protease		Phospho-lipase		Lipase		Protease		Phospho-lipase		Lipase		Protease		Phospho-lipase	
Aerobiosis(■) Anaerobiosis (□)	■	□	■	□	■	□	■	□	■	□	■	□	■	□	■	□	■	□	■	□	■	□	■	□
Acinetobacter sp.	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+
Bacillus spp.	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-
Citrobacter freundii	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-
Enterobacter cloacae	+	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+
Enterobacter cloacae	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-		-	+	-
Enterobacter cloacae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterococci sp.	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+
Escherichia coli	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	+
Klebsiella pneumoniae	-	+	-	-	+	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	-	+	+
Klebsiella oxytoca	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+
Klebsiella oxytoca	-	-	-	+	-	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+
Pseudomonas alcaligenes	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	+
Staphylococcus	-	+	-	-	+	+	-	+		-	+	+	+	+	-	-	+	+	+	+	-	-	+	+
Sphingobacterium sp.	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
γ-Streptococcus sp.	+	-	+	+	-	-	+	-	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-

The symbols - and + represent negative and positive hydrolase production respectively.

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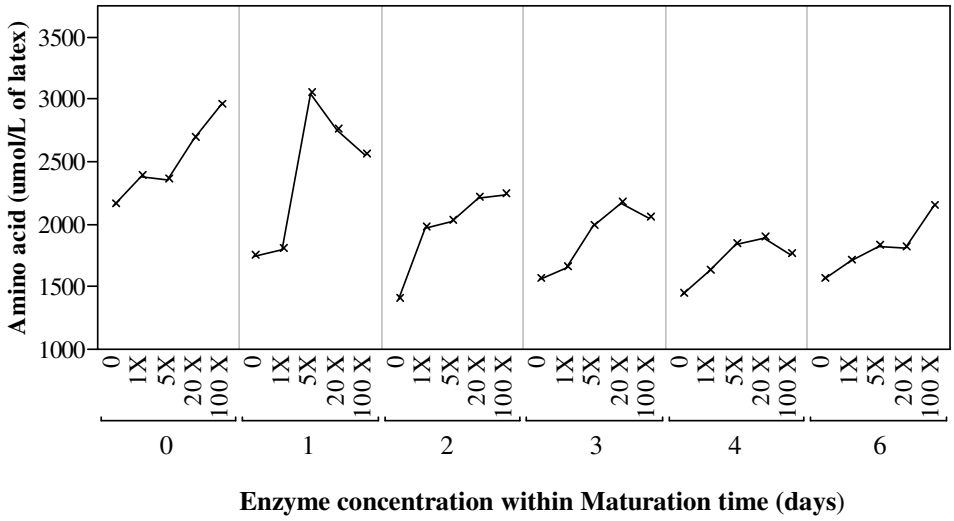

Figure 69 Amino acid content in cup coagula for each treatment.

The amount of FFA increased after treatment with 5X and 100X lipase concentration and maturation time, as shown in Figure 70. A significant endogeneous lipase activity was also observed, leading to FFA release even without lipase addition.

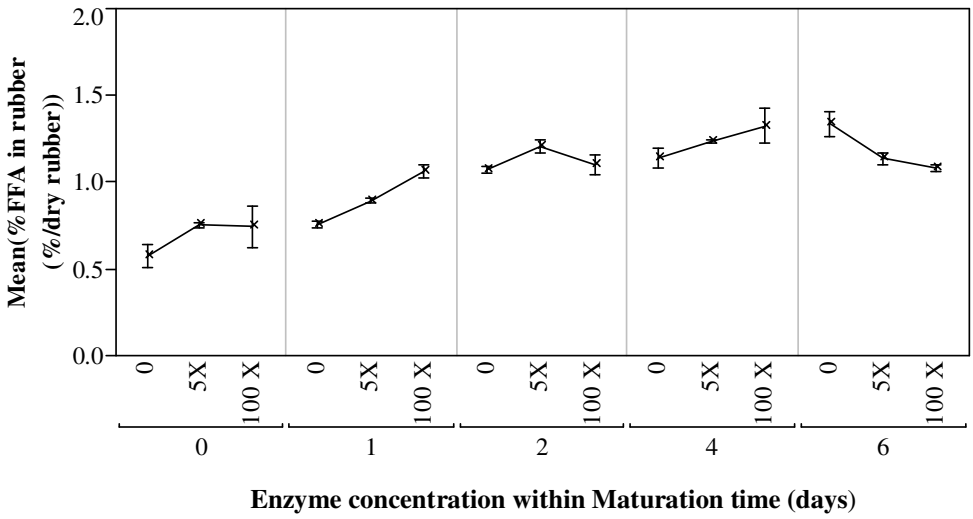

Figure 70 Fatty acid content of dried cup coagula for each treatment.

4.2 Properties of rubber from enzyme-treated mini-cup coagula

Two rubber properties, P_0 and PRI, were measured on the different samples during maturation in the presence of added enzymes.

4.2.1 Effect of protease

P_0 of mini-cup coagula treated with protease increased with enzyme concentration (Figure 71). This effect seemed to be very fast as no significant change was observed after the first day of maturation for each enzyme concentration. Statistic analyses showed a significant dose-effect of enzyme addition on P_0 for protease concentrations higher than 1X. The mean P_0 increase in rubber from mini-cup coagula treated with protease was of about 20%, from P_0=34 to P_0=41. The dose-effect on PRI was less direct except for the first hours (day 0) where PRI reached 110 for the two highest enzyme amounts. On average, papain addition resulted in a 10% increase of PRI from 91 to 97-102 with 1X-20X protease addition (Figure 72). Such a positive effect has been attributed in literature to the extraction of copper ions from rubber allowed by the digestion of proteins (Hasma and Othman, 1990; Georges *et al*., 2009). This effect may be more specifically due to the antioxidant property of short peptides, especially containing histine, which are able to act through both the chelation of metals such as copper, cobalt, nickel and zinc ions, and by free radical scavenging including singlet oxygen (Shahidi, 1997). It is possible that a very high protease activity, such as in the 100x samples, would result in a too complete degradation of protein into free amino acids at the expense of antioxidant peptides. The effect of protease may thus be to release water soluble peptides complexed to metals, which would be eliminated during washing in raw rubber processing, and also to provide rubber with radical scavenging and metal chelating peptides that could, if remaining in raw rubber, provide protection against polyisoprene and lipid oxidation in rubber. Through this mechanism, protease secretion by microorganisms during maturation could thus contribute positively to the improvement of rubber quality.

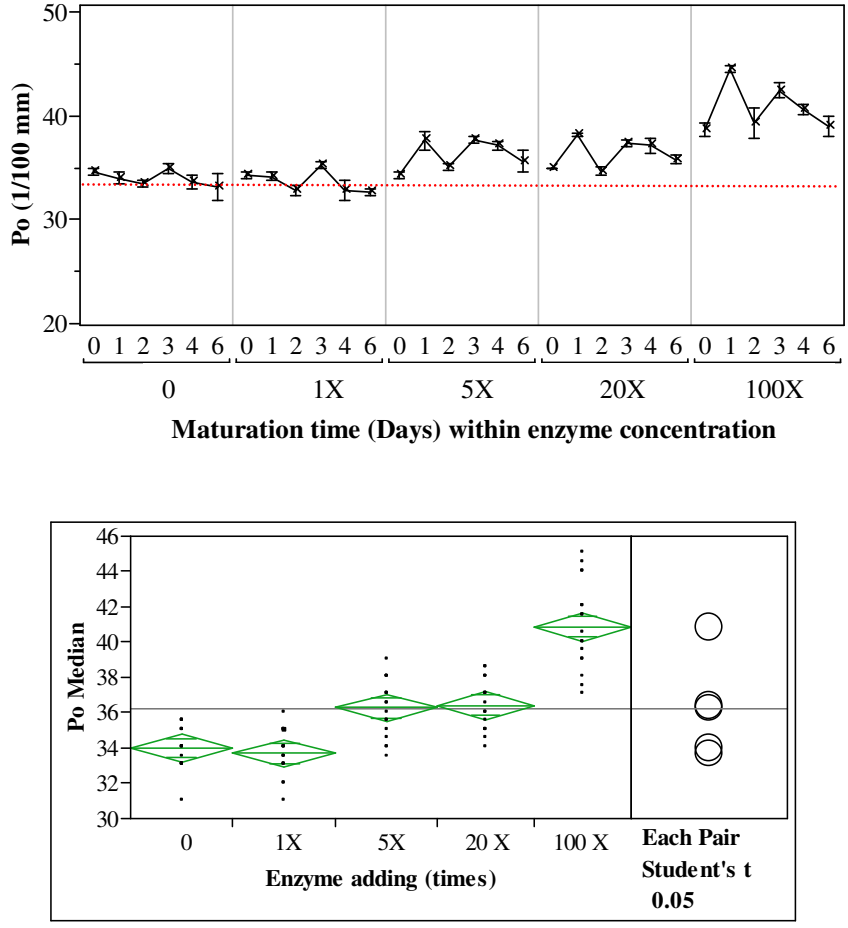

Figure 71 The effect of protease concentration and maturation time on P_0 evolution of mini-cup coagula.

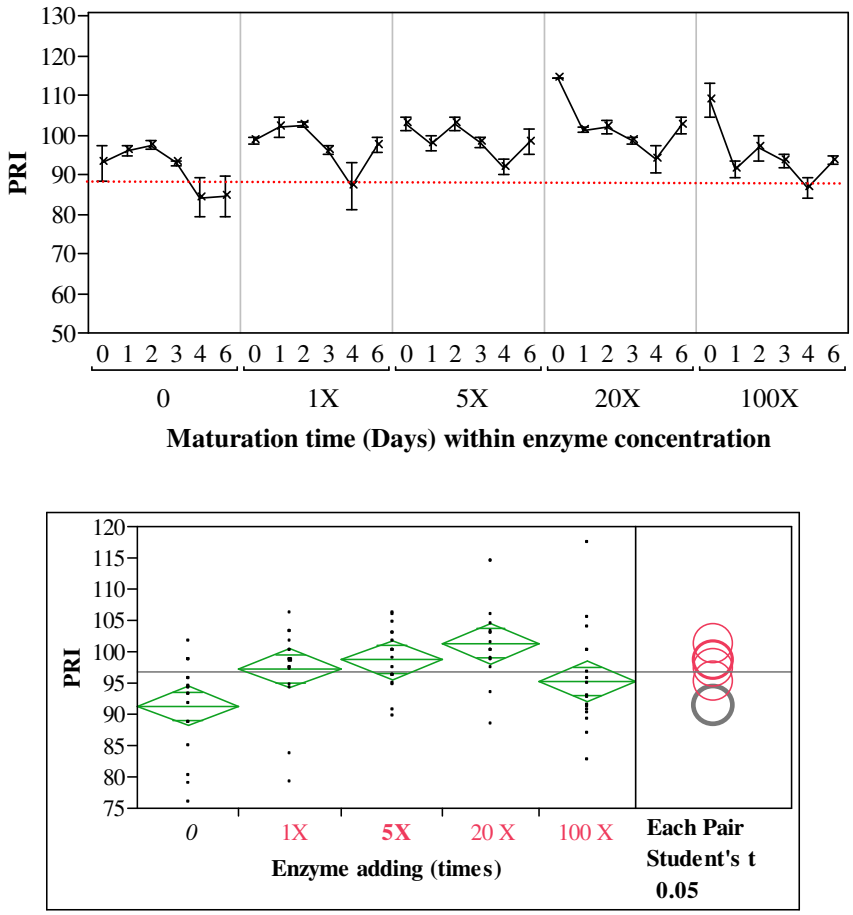

Figure 72 The effect of protease concentration and maturation time on PRI evolution of mini-cup coagula.

4.2.2 Lipase

No significant effect of enzyme addition was observed on the P_0 of rubber from mini-cup coagula treated with lipase although significant amounts of free fatty acids were released during maturation. As observed earlier, an important endogenous lipase activity was present in latex, which may have masked the effect of enzyme addition. P_0 increased with maturation time during the 4 first days from 34 to 38 (Figure 73), while at the same time a lowering of PRI was observed in all samples (Figure 74). This might be related to the pro-oxidant effect of the released free fatty acids, which could activate rubber degradation under PRI test (Tuampoemsab, *et al*, 2007).

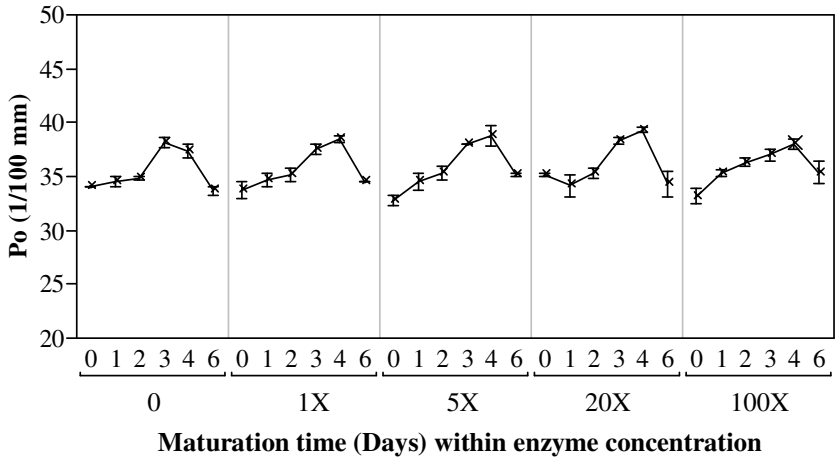

Figure 73 The effect of lipase concentration and maturation time on P_0 evolution of mini-cup coagula.

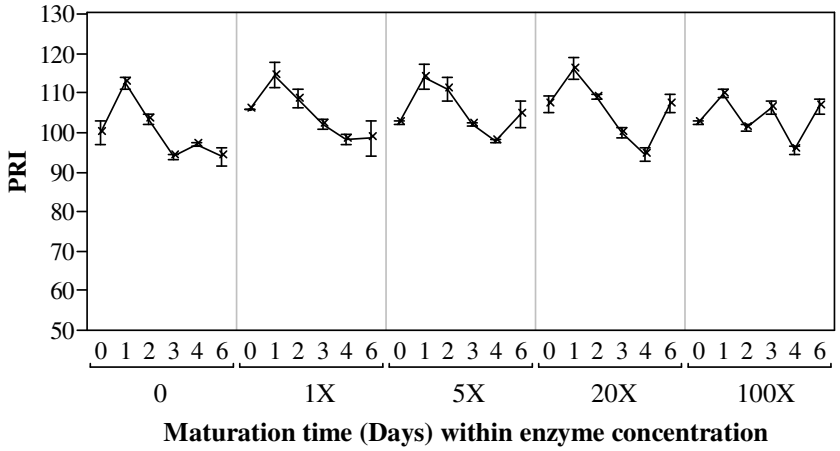

Figure 74 The effect of lipase concentration and maturation time on PRI evolution of mini-cup coagula.

5. Conclusion

The total number of microorganism in the inoculum was about $1x10^9$ CFU/mL, among which yeasts, gram positive, gram negative and lactic acid bacteria represented $4.23x10^8$ CFU/mL, $3.22x10^8$ CFU/mL, $4.1x10^7$ CFU/mL and $8.5x10^7$ CFU/mL respectively. Fifteen of the 16 bacterial strains isolated from the inoculum used in latex treatments produced hydrolytic enzymes such as lipase, phospholipase

and protease when inoculated in specific media, thus potentially participating to lipid and protein degradation in latex and coagula.

The addition of lipase in mini-cup coagula resulted in an increased release of free fatty acids but had no significant effect on PRI evolution during the first 6 days of maturation. The main effect was observed in the presence of the protease papain with, a clear positive dose-effect on enzyme concentration on P_0. This enzyme also had an improving effect on PRI.

The addition of the two enzymes thus did no result in the strong negative effect of microbial activity observed during maturation, whereas both enzymes proved active in latex as evidenced by a dose-effect of enzyme concentration on the release of amino acids and fatty acids. Further studies will be required in order to understand the effect of microorganisms and enzymes during maturation. For example, phospholipase activity, that was secreted by most of the bacterial strains tested, could have an impact on PRI by destructing of phospholipids, that are considered as metal-scavenging antioxidants (Shahidi, 1997). The effect of microorganisms appears to be complex and a more comprehensive identification and following of the microbial flora during maturation will be necessary to provide useful information in view of the understanding and the control of the effect of free enzymes and microbial metabolism on rubber properties.

GENERAL CONCLUSION

The purpose of this research was to investigate the effects of physico-chemical parameters and the role of microorganisms on the evolution during the maturation period of key properties (P_0, PRI, molar mass and gel content) of processed rubber.

The first part of our experimental work consisted in the study of the industrial coagula maturation conditions and in the determination of correlations between physico-chemical conditions of industrial coagula storage and rubber properties. In order to be able to separately study the effect of the different parameters, to control their value and to be able to perform repeatable experiments, procedures and devices for the study of maturation at laboratory scale under controlled conditions were developed and optimized.

Physico-chemical conditions of storage of latex cup coagula [temperature, relative humidity (RH) and oxygen content] and their relations with the properties of processed raw rubber obtained from these coagula [total solid content (TSC), pH, initial plasticity (P_0), plasticity retention index (PRI), gel content and weight average molar mass ($\overline{M_w}$)] were studied at different positions within cup coagula storage piles on an industrial site. It was found that temperature and relative humidity of the air within the pile increased with depth: from the top to 2 m below the top, temperature changed from 34°C to 42°C while relative humidity increased from 50 % to 90 %. In contrast, oxygen content was found to decrease as depth increased (20 % at top and zero at a depth of 200 cm). After 24 days of storage of cup coagula on the industrial site, rubber properties were found to vary with the position of coagula within the pile, in correlation with the values of physico-chemical parameters. The pH of maturated cup coagula decreased significantly with depth in the pile, while P_0, PRI, gel content and $\overline{M_w}$ of processed raw rubber increased with depth. The properties of rubber obtained from cup coagula located at the middle of the storage pile were significantly modified by the maturation period, with an increase of P_0 (26 to 42), PRI (26 to 46), gel content (41 to 51), and $\overline{M_w}$ (870 to 1460). The resulting rubber was more resistant

to thermo-oxidation undergone during drying. Overall, maturation of latex coagula in the industrial storage conditions was found to have a positive effect on the standardized properties of rubber such as P_0 and PRI.

Scientific study of the relationships between physico-chemical conditions prevailing during maturation, microbial activities and rubber properties required to control independently the various parameters and to be able to reproduce experiments, which was not possible on the industrial site and with cup coagula of unknown origin. Methodological and technical developments of a controlled latex collection procedure, of a controlled maturation device and of a dry rubber process at laboratory scale were thus performed. Reducing the size of industrial cup coagula (~500g) to mini-cup coagula (45g) was validated after a study of the homogeneity of properties of rubber issued from internal and peripheral parts of industrial and laboratory-made coagula, that showed no significant difference in pH before processing nor in P_0 and PRI of rubber after processing, although the peripheral part was significantly dryer than the internal one.

Maturation device in laboratory was set up with 6 parallel maturation units. Relative humidity (10-90%), oxygen amount (0-21%) and air flow (0-0.3 L/L/min) can be controlled independently for each unit. Temperature regulation is common to all units.

After the optimization of the controlled maturation device, a laboratory-scale process was developed to obtain from the mini-cup coagula a dry raw natural rubber with properties similar to that of the rubber processed in the TSR20 factory, used as reference.

As it is not possible in practice to obtain sterile latex by tapping trees, a procedure for controlling microbial population in the collected latex without side effects on rubber quality was developed. First, "clean latex" was obtained by tapping the trees in a sterile environment and collecting latex in ice-cooled sterile bags, yielding a product with a low microbial population (~5.10^5 CFU/mL). For maturation

experiments without active microorganisms, 0.2% (w/v) sodium azide was used as an antimicrobial agent in latex. We showed that azide had no specific effect on P_0 and PRI by itself, and that the differences observed in these rubber properties with or without azide were due to microbial activity only with a direct dose-effect of microbial inoculum on the evolution of P_0 and PRI.

The presence of active microorganisms was shown to have a negative impact on the drying rate of coagula, with dry rubber content of coagula increasing from 40% to 72% after 6 days of maturation without microbial development and to 62% in the presence of the highest concentration of microbial inoculum. The evolution of P_0 was also clearly dependent on the quantity of microorganisms, with a constant value in the absence of microorganisms and a significant variation (up to 38% increase and down to 20% decrease) during time and according to inoculum concentration. The same way, the evolution of PRI over maturation time was found to be clearly dependent on the initial quantity of microorganisms in latex. Indeed, the drop rate of PRI seemed to be proportional to the initial microorganism concentration. Only a slight decrease in weight averaged molar mass of rubber was observed with maturation time for the antibiotic treated rubber, while $\overline{M_w}$ values dropped from 1500 kg/mol down to 1150 kg/mol after 6 days of maturation in the presence of active microorganisms. For all treatments, gel quantity increased with maturation time and reached a plateau after 2 days of maturation but the value associated with this plateau increased with the initial quantity of microorganisms, from 45% for antibiotic treated rubber to 55% for the treatment with the highest initial microorganism content.

The effect of oxygen concentration in the atmosphere of the maturation units on mini-cup coagula properties was also studied. Under anaerobic conditions the properties of the rubber issued from mini-cup coagula, either naturally coagulated or with formic acid, showed similar values. However, in the presence of oxygen significant differences were observed between the two modes of coagulation, with a higher pH value in naturally coagulated coagula (>9 vs 6 for acid coagulated), and a lower P_0 (<20 vs 35 for acid coagulated sample). Coagulation of the inoculated latex

with formic acid and maturation in the presence of oxygen thus seems to generate a rubber different from that issued from natural coagulation. However that concern is limited only to initial plasticity, the initial drop of PRI being present for all treatments with or without oxygen.

The total number of microorganism in serum from maturating industrial coagula was 10^9 CFU/mL, among which yeasts, gram positive, gram negative and lactic acid bacteria represented $4.23x10^8$, $3.22x10^8$, $4.1x10^7$ and $8.5x10^7$ CFU/mL, respectively. Fifteen of the 16 bacterial strains isolated from the inoculum used for latex treatments produced hydrolytic enzyme activities such as lipase, phospholipase and protease when inoculated in specific media, thus potentially participating to lipid and protein degradation in latex and coagula.

The addition of lipase in mini-cup coagula resulted in an increased release of free fatty acids but had no significant effect on PRI evolution during the first 6 days of maturation. The main effect was observed in the presence of papain, with a clear positive dose-effect of the concentration of this protease on P_0. The activity of papain also had an improving effect on PRI. It is to be noted that the evolution of the properties of rubber from coagula treated with pure enzymes showed not similarity with that observed during maturation with microorganisms, which confirms that the phenomena involved are complex.

PERSPECTIVES

Further studies will be required in order to understand the mechanisms of actions of microorganisms and enzymes during maturation. The present work has provided bases for such studies through an analysis of the conditions of maturation in an industrial environment, and through the conception and development of protocols and tools for scientific laboratory-scale studies of the evolution of rubber properties during maturation in a controlled physico-chemical and biological environment. Our preliminary study of the complex microbial flora present in maturating cup coagula should be followed up by a more comprehensive approach in terms of characterization of the microbial ecosystem in cup coagula, its evolution during maturation. The use of molecular biology tools such as 16S RNA analysis and metagenomics approaches would be peculiarly pertinent for such studies. An analysis of the kinetics of evolution of key biochemicals such as peptides, lipids, antioxidants and pro-oxidants would also be required for the comprehension of the molecular mechanisms involved in the modifications of rubber properties. Our observations of a positive effect of papain treatment on rubber properties suggest for example that a special attention should be given to the evolution of the peptide composition of coagula during maturation, and to microorganisms secreting proteases of various substrate specificities.

Future developments of this work should aim, besides the scientific understanding of the interactions between polyisoprene polymer and non-polyisoprene components, at defining optimal handling and storage conditions for latex coagula in industrial conditions. The practical possibilities of control of maturation in the industry could concern aeration of the coagula piles, exposure to sun and rain or the inoculation of latex or fresh coagula with selected microbial strains.

More generally, we hope that the controlled mini maturation and processing device, setup in our laboratory in Thailand in a close vicinity to *Hevea* trees, will serve as a basis for further works on biological maturation of rubber products, for example for the study of the effects of maturation on the properties of stabilized latex or dry rubber, or for studying the release of volatile malodorous compounds that largely impair the environment of rubber industries.

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APPENDICES

VITAE

Name Miss Jutharat Intapun

Student ID 4910230003

Educational Attainment

Degree	Name of Institution	Year of Graduation
B.Sc (Chemistry Science)	Thaksin University	1998
M.Sc (Polymer Technology)	Prince of Songkla University	2002

Scholarship Awards during Enrolment

1. Commission of Higher Education, Ministry of Education, Thailand and the French Embassy in Bangkok.
2. The Graduate school scholarship, Prince of Songkla University.

Current Position

Lecturer at Faculty of Technology and Management, Prince of Songkla University

List of Publications and Communications

Scientific papers:

Intapun, J., Sainte-Beuve, J., Bonfils, F., Tanrattanakul, V., Dubreucq, E. and Vaysse, L. 2009. Characterization of natural rubber cup coagula maturation conditions and consequences on dry rubber properties. Journal of Rubber Research. 12 (4), 171-184.

Intapun, J., Sainte-Beuve, J., Bonfils, F., Tanrattanakul, V., Dubreucq, E. and Vaysse, L. 2009. Effect of micro-organisms during initial coagula maturation of *Hevea* natural rubber. Journal of Applied Polymer Science. (Accepted on 23 February, 2010)

Conference proceeding:

Intapun J, Tanrattanakul V., Dubreucq E, Bonfils F., Sainte-Beuve J, Vaysse L. (2007). Maturation of natural rubber cup coagula: Characterization of industrial conditions. IRRDB Symposium proceedings. 12-14 Nov 07, Siem Reap, Cambodia. pp. 529-537. (Oral presentation)**.**

Intapun J, Dubreucq E, Bonfils F., Sainte-Beuve J, Vaysse L. and Tanrattanakul V. (2008). Characterization of industrial natural rubber coagula maturation and dry rubber properties. Mae Fah Luang Symposium on the Occasion of the 10th Anniversary of Mae Fah Luang University, 26-28 November, Chiangrai Thailand. pp. 185-195. (Oral presentation).

Intapun, J., Vaysse, L., Bonfils, F., Tanrattanakul, V. and Dubreucq, E. 2008. Study of the role of micro-organisms on the properties of natural rubber from coagula. Journee de l'ecole doctorale "Science des Procedes-Science des Aliments" Montpellier SupAgro, June, 2008, Montpellier, France. (Poster presentation)

APPENDIX A

Scientific paper

Journal of Rubber Research. 12(4), 171-182: 2009

J. Rubb. Res., **12(4)**, 171–184

Characterisation of Natural Rubber Cup Coagula Maturation Conditions and Consequences on Dry Rubber Properties

J. INTAPUN[*,**], J. SAINTE-BEUVE[***], F. BONFILS[****], V. TANRATTANAKUL[*], E. DUBREUCQ[**], L. VAYSSE[****,#]

The maturation conditions of cup rubber coagula stacked in the form of a stocking pile in an industrial plant have been characterised. It was found that temperature and relative humidity of the air increased with depth within the pile. In contrast, oxygen content of the air within the pile decreased as depth increased. The effects of these parameters on the properties of processed cup coagula maturation were studied. Effects of 24-day maturation on rubber properties were found to vary with the position of cup coagula within the pile. The pH of maturated cup coagula decreased significantly with depth in the pile. An inverse pattern was observed for Wallace initial plasticity (P_0), Plasticity retention index (PRI), gel content and weight average molar mass (M_w). Moreover, the maturation period, especially at the deepest layers, was found to have a positive effect on the standardised properties of rubber such as P_0 and PRI.

Keywords: *Hevea brasiliensis*; natural rubber; cup coagula; maturation; plasticity retention index; initial plasticity

Thailand is currently the biggest producer and exporter of natural rubber in the world. The most exported type is Standard Thai Rubber or STR, which is block rubber, the major grade being STR 20. It is mainly used in tyre industries. The raw materials used for STR production are cup coagula and low grade rubber sheets produced from smallholder plantations. Both raw materials are bought by STR factories either directly or through middlemen.

In industrial practice, cup coagula are stored for a variable period of time before processing, ranging from 2 to 4 weeks. This storage time is referred to as "maturation period". This step is sometimes requested in order to improve the consistency and value of specified indicators of

[*]Polymer Science Program, Faculty of Science, Prince of Songkla University, Hadyai 90112, Thailand
[**]Montpellier SupAgro, UMR IATE, 2 Place Viala, 34060 Montpellier Cedex, France
[***]Cirad, UMR IATE, TA B 62/16 - 73, Rue Jean François Breton - 34398 Montpellier Cedex 5, France
[****]Cirad, UMR IATE, Hevea Research Platform in Partnership, KAPI, Kasetsart University, Bangkok 10900, Thailand
[#] Corresponding author (e-mail: laurent.vaysse@cirad.fr)

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rubber quality such as Wallace initial plasticity (P_0) and plasticity retention index (PRI). PRI reflects the susceptibility of the product to thermo-oxidation, whereas P_0 provides information on the rheological flow behavior of the raw material. The conditions and the phenomena that occur during this maturation period are still not well characterised. Watson[1] noticed that soaking the maturated cup coagula in aqueous solution of oxalic acid or phosphoric acid could increase the PRI. Chin *et al*.[2] stated that the maturation of cup coagula and their storage environment have a marked effect on the viscosity and thermo-oxidative degradation of processed dry rubber. Hasma and Othman[3] attributed drop of PRI to the deterioration of natural antioxidants in the cup coagula during maturation. Le Roux *et al*.[4] found relationships between tree physiological parameters and eco-climatic conditions on technological properties of raw rubber but the maturation step which occurred under different climatic conditions was not suspected as a source of property variability. More recently, Varghese *et al*.[5] showed clearly that the treatment of the cup coagula by a bactericide could retain the PRI at a high level and reduce the storage hardening of the processed rubber.

Understanding these phenomena could allow industries to control and eventually shorten this costly storage period. For this purpose, before conducting experiments in laboratory controlled conditions, the present work focused on the characterisation of the conditions of maturation prevailing in cup coagula storage piles on an industrial site and their consequences on rubber properties and structure. Physico-chemical conditions (temperature, relative humidity (RH) and oxygen content) and rubber properties (total solid content (TSC), pH, initial plasticity (P_0) and plasticity retention index (PRI)) were studied at different depths from the top of the cup coagula pile.

MATERIALS AND METHOD

Cup coagula pile

The study was carried out in the Von-Bundit Co. Ltd. factory (Surat Thani province, Thailand) during February and March 2008. Cup coagula were received on 12/02/2008 from different suppliers and stored as a pile of 3-meter height and 20-meter width (*Figure 1*). The pile was stored outdoor and was exposed to sunlight for a total of 24 days of maturation before being transferred to the processing chain.

Measurement of physico-chemical conditions in cup coagula pile

Upon their delivery to the factory, cup coagula were mixed using a bulldozer in order to homogenise the material within the pile. Two drilled stainless steel tubes (length 3 m, external diameter 5.1 cm, thickness 2 mm, 5 mm-diameter holes, 3000 holes/m^2) were placed vertically inside the pile (*Figure 1*). Distance between drill tubes was 7 m. The measurement of temperature and oxygen concentration was performed by inserting in the tubes a dedicated probe (Conox 3, VWR GmbH, Weilheim, Germany) connected to a multimeter (Multi 350 model, VWR GmbH, Weilheim, Germany). Calibration of the oxygen probe was realized using mixtures of air (containing 20.95% O_2) and nitrogen (99.8%). Plugs (46 mm diameter) were placed 10 cm below and above the probe in order to isolate the measuring zone within the drilled tubes while recording. For relative humidity, measurements were performed with a Digicon (Japan) HT-765-232 humidity/ temperature meter. Probes were properly calibrated following supplier's instructions. The measurements were performed at 5 levels from the top: 0, 50, 100, 150 and 200 cm depth every 6 days for 24 days. On each day

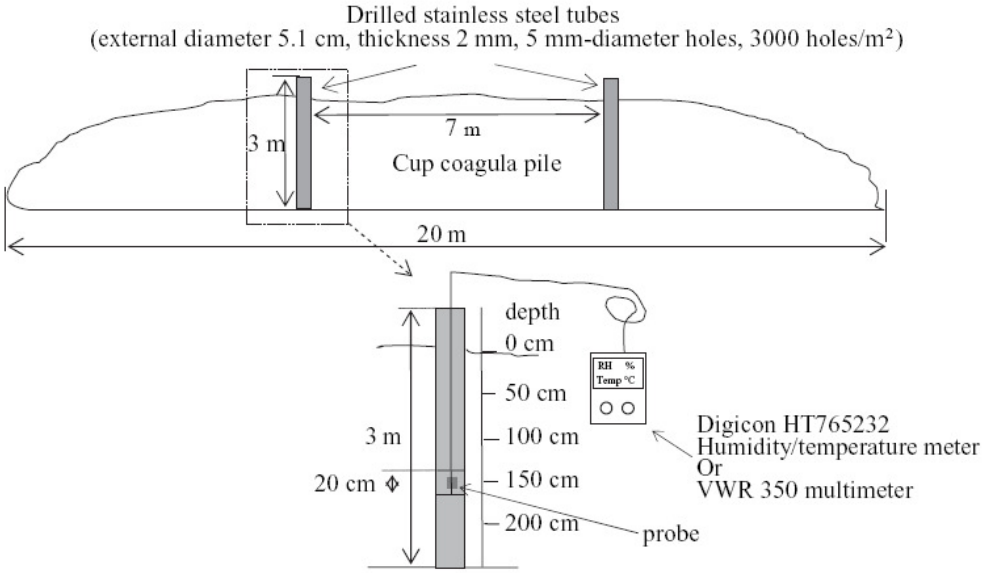

Figure 1. Cup coagula maturation pile equipped with two drilled stainless steel tubes in order to measure humidity, temperature and oxygen content of the air at different depths.

of measurement, the operation was repeated at 8 a.m., 12 a.m. and 4 p.m.

Raw rubber sample preparation

Sampling. Sampling procedure is described in *Figure 2*. On the delivery day, 3 samples of 10 kg of fresh cup coagula were collected in the vicinity of each of the 2 stainless steel tubes as representative samples (total 60 kg) of the fresh cup coagula. These cup coagula were named "fresh cup coagula" even though they have undergone a preliminary storage period before arrival in the factory and served as a reference to monitor the influence of the storage. They were processed the day of reception and the technological properties and structure of obtained rubber were determined (see "characterisation of raw rubber"). After 24 days of maturation, 10 kg samples of maturated cup coagula were collected around each stainless steel tube on the surface of the pile (directly exposed to sunlight) and at the 4 depths mentioned above (5 baskets of 10 kg per stainless steel tube) as shown in *Figure 2*.

Cup coagula creping and drying. The samples were processed following factory usual procedure. The 10 kg samples were creped in a Lihoe crepper (Lihoe Co. Ltd, Selangor, Malaysia, gap between nip rolls: 5 mm, friction ratio: 1:1.56, roll length: 71.1 cm, Roll diameter: 38.1 cm, Engine power: 44.7 kW). Cup coagula were creped by 3 single passes followed by 18 double passes. The obtained crepes were dried in an industrial crumb rubber dryer (Golsta, Melaka, Malaysia; 3.5t/h) at 129ºC for 3 hours.

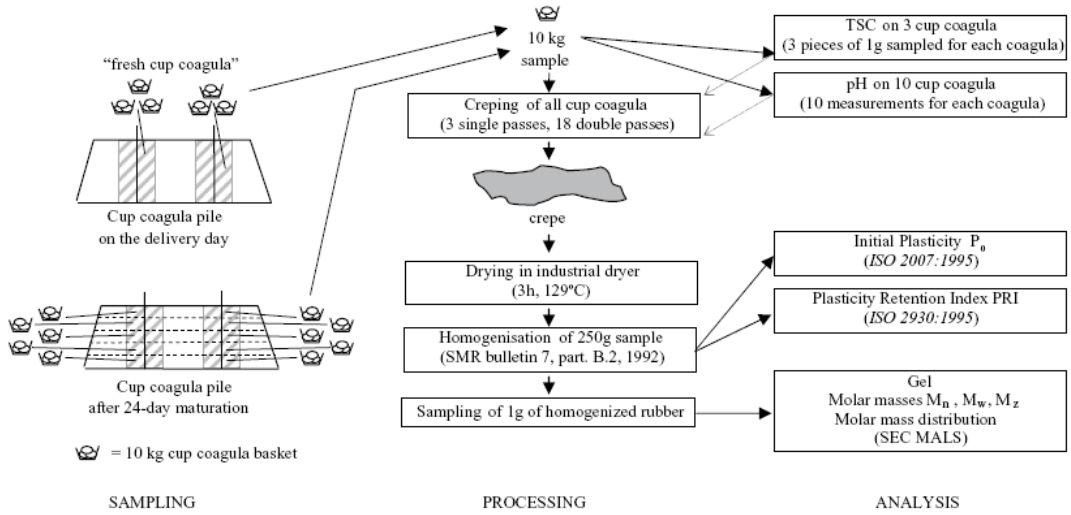

Figure 2. Flow chart of sampling, processing and analysis of "fresh" and "maturated" cup coagula.

Characterisation of raw rubber

pH measurement. Inner pH of fresh and maturated cup coagula was measured with a penetrating probe (Sentix SP S7, VWR, Weilheim, Germany) connected to a Multi 350 data logger (VWR GmbH, Weilheim, Germany). The probe was inserted at the center of 10 cup coagula from each of the 10 kg samples. For each cup coagulum, ten repetitions of the measurement were performed.

Total solid content (TSC) measurement. Three cup coagula were randomly taken from each 10 kg sample. Three pieces of 1 g were cut from each cup coagulum. Each 1 g piece was dried in oven at 100ºC for 3 h. After cooling down in a desiccator, the dried piece was weighed. Additional 30 min drying was performed until constant weight was reached. Total solid content (TSC) was calculated as the dry weight/fresh weight ratio and expressed as a percentage.

Wallace initial plasticity (P_0) and plasticity retention index (PRI) measurement. A 250 g sample was cut from the dry rubber crepe obtained previously. This sample was homogenised following SMR bulletin 7, part. B.2, 1992 standard. Initial Wallace plasticity (P_0) was determined according to *ISO 2007:1995* while plasticity retention index (PRI) was determined according to *ISO 2930:1995*. The PRI standardised method (*ISO 2930*) consists in determining the Wallace plasticity of a disc of natural rubber with standardised dimensions before (P_0) and after aging for 30 min (P_{30}) at 140ºC in an oven with controlled air circulation. The PRI, percentage of Wallace plasticity retained, is then given by *Equation 1*:

$$PRI = \left(\frac{P_{30}}{P_0}\right) \times 100 \qquad \ldots 1$$

Mesostructure and gel content of rubber. A 25 ± 2 mg of homogeneous rubber sample was dissolved in 40 mL of tetrahydrofuran (THF) stabilised with 3,5-di-tert-butyl-4-hydroxytoluene (BHT) for 2 weeks. The rubber solutions were stored at 30°C for 7 days without stirring and gently stirred for 1 hour daily for 7 days after. The solution was filtered (Acrodisc 1 µm, glass fiber, Pall) and injected into a size-exclusion HPLC system consisting of an online degasser (EliteTM, Alltech), a Waters 515 pump, a refractive index detector (Waters 2410) and a multiangle laser light scattering detector (Dawn DSP, Wyatt technology Corp.). The columns were three inline PLGEL (Polymer Laboratory) mixed beds (20 µm, 7.8 mm ID × 30 cm) with a guard column. The columns were thermostated at 45°C. The mobile phase was THF stabilised with BHT at a flow rate of 0.65 mL min^{-1}, the injected volume was 0.15 mL. Number-average molar mass (M_n), weight-average molar mass (M_w) and z-average molar mass (M_z) were calculated using ASTRA software (Wyatt technologies Corp.). Fourteen angles, from angle 3 (32°) to angle 16 (134°), were used for calculation using Zimm method. The differential refractive index increment (dn/dc) value used was 0.130 mL g^{-1}.

For a given sample injected into the SEC-MALLS chain, the refractive index increment of solvent and sample solution was measured by a refractive index detector (DRI). It represents the incremental refractive index change (dn) of the solution for an incremental change of the concentration (dc). ASTRA software performed the calculation of the injected quantity of natural rubber (NR) after filtration by integrating the whole NR peak on the chromatogram. Thus, as the concentration of the solution (0.625 mg mL^{-1}) and the injected volume (0.15 mL) were known before and after filtration, the fraction eliminated by filtration, *i.e.* the percentage of gel, could be calculated as follows (*Equation 2*).

$$\text{Total gel content (\%)} = \left(\frac{m_0 - m_1}{m0}\right) \times 100 \quad \ldots 2$$

m_0 = mass of sample in 0.15 mL before filtration
m_1 = mass of injected sample (calculated from SEC-MALLS)

RESULTS AND DISCUSSION

Physico-chemical conditions in cup coagula pile

Oxygen content, temperature and relative humidity of air were measured at different depths within the cup coagula pile after 1, 7, 13 and 19 days of maturation are shown in *Figures 3* to *5*. For each maturation time, values are the mean of 2 independent measurements (one in each drilled tube).

The oxygen content of the air contained in the cup coagula pile decreased as depth increased (*Figure 3*). At the top of the pile, oxygen content was 16% – 18% and decreased to less than 5% below 100 cm –150 cm. This gradient, most probably driven by oxygen consumption during microbiological oxidation reactions and by the lack of oxygen supply by air renewal, did not change significantly during the period of study.

Air temperature in the cup coagula pile increased with depth (*Figure 4*) according to a gradient with no overall significant change during maturation except for daily variations. Throughout the pile, temperature in the morning was 10°C lower than at noon and afternoon. At the top of the pile, average temperature was 34°C. This value depended mainly on weather conditions and time of measurement. Temperatures at 50 cm – 100 cm and 150 cm – 200 cm below the top were respectively 36°C and 39°C on average. This temperature gradient may be

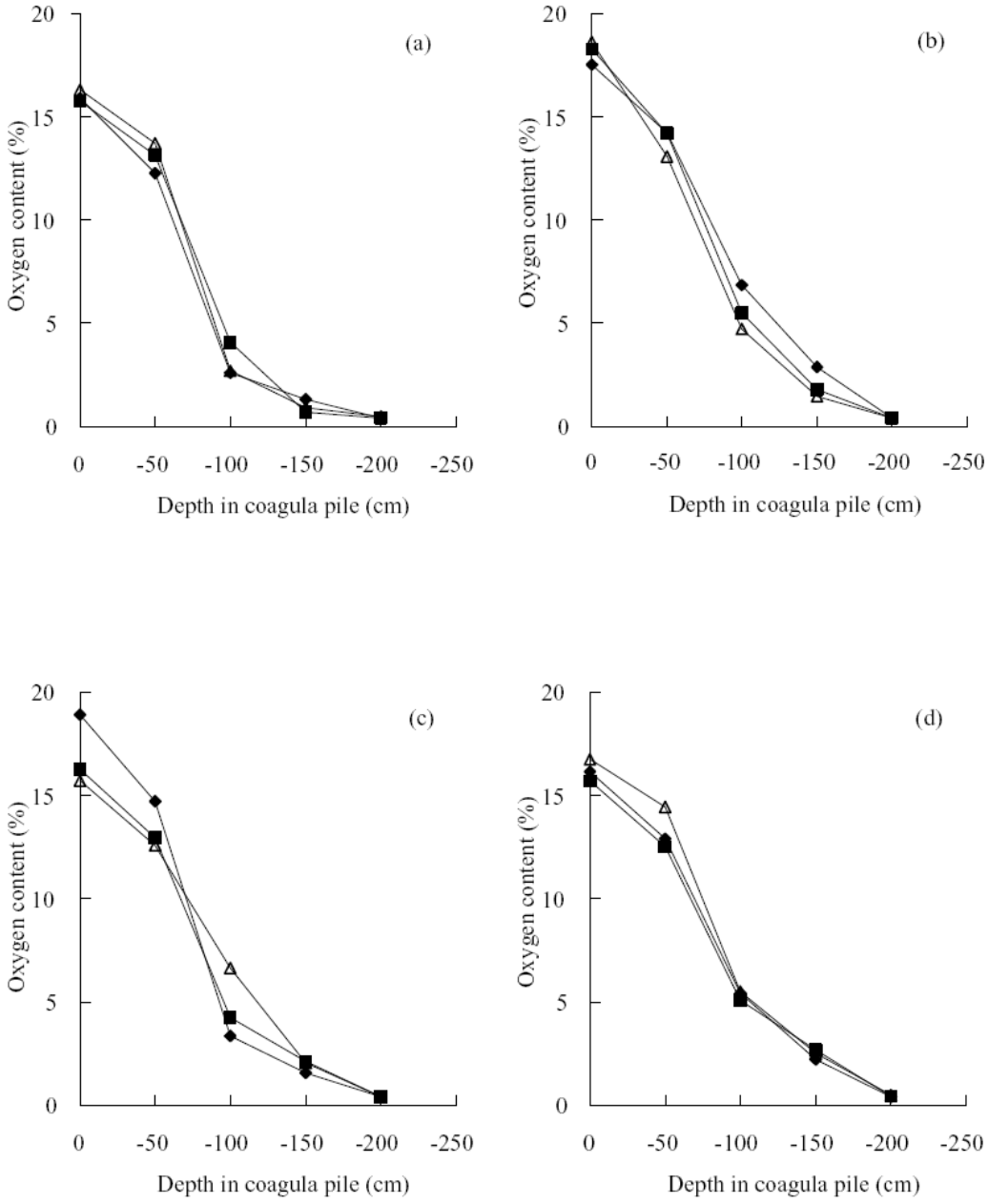

Figure 3. Oxygen content of the air in cup coagula pile at different depths from the top for each maturation duration: (a) 1 day, (b) 7 days, (c) 13 days and (d) 19 days.
Measurements were performed at 8 a.m (—◆—), 12 a.m (—■—) and 4 p.m (—△—)

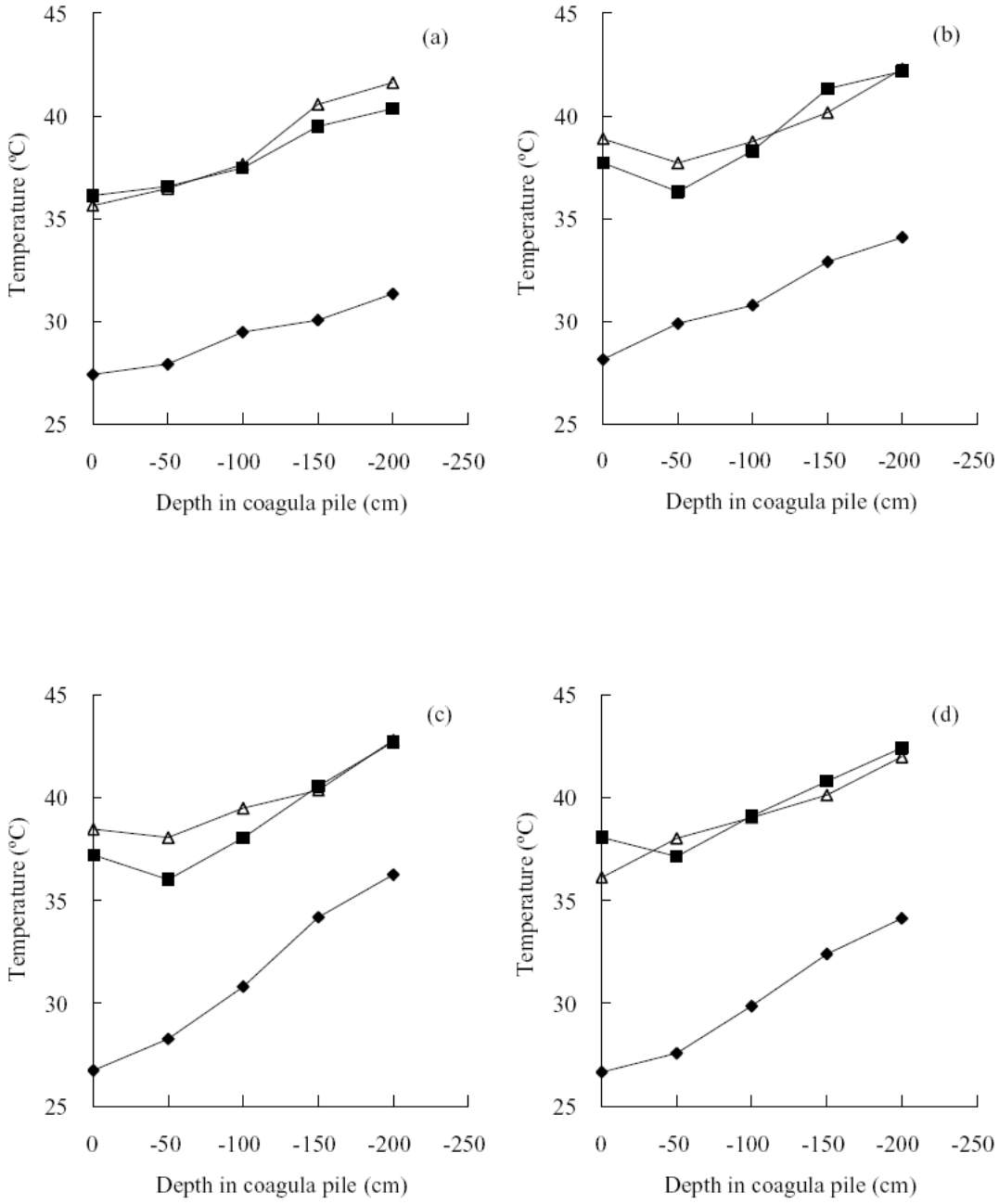

Figure 4. Temperature of the air in cup coagula pile at different depths from the top for each maturation duration: (a) 1 day, (b) 7 days, (c) 13 days and (d) 19 days.
Measurements were performed at 8 a.m (—◆—), 12 a.m (—■—) and 4 p.m (—△—)

caused by a combination of the exothermic metabolic activity of microorganisms inside the pile, solar radiation at the top of the pile and thermal exchanges within the pile by convection (through air and serum movements) or conduction (through cup coagula).

Relative humidity (RH) in cup coagula pile also increased with depth, especially at mid-day and in the afternoon with values ranging from 45%–55% on the top of the pile to 85%–90% at 200 cm below the surface (*Figure 5*). In the morning, RH was in a closer range (75%–95%) in all the locations within the pile, probably due to a lower evaporation rate on pile surface and the lower temperature of the air.

Temperature, humidity and oxygen content are key factors controlling the development and activities of microorganisms. The vertical gradient of values observed for all of these parameters in the pile suggests that the microbial population could differ in terms of species and activities according to the location of cup coagula within the pile.

Properties of cup coagula

Total solid content (TSC) of fresh cup coagula was 73% in average on the delivery day in the industrial plant (*Figure 6*). After the maturation period, TSC increased to around 80% – 85%, with slight differences depending on the depth of sampling. Higher TSC measured at the bottom of the pile may be due to compression by upper cup coagula weight, while the high value at the surface most probably resulted from evaporation of water caused by solar radiation and wind as well as from drainage of serum.

The average pH of the delivered fresh cup coagula was 5.7. After 24 days of maturation, the pH of cup coagula at the top of the pile rose to 7.4 as shown on *Figure 7*. This result is in agreement with the observations by Soewarti and Moh[6], who found that the pH of cup coagula increased from 6.25 to 6.85 after 35 days of maturation. The increase of pH might be due to the release of ammonia during the degradation of latex proteins. Strong "ammonia" odor over the pile backed this hypothesis. Ammonia is indeed one of the co-products of organic matter hydrolysis and fermentation[7]. Moreover, a clear vertical pH gradient was observed in the maturated cup coagula pile, with pH values decreasing gradually from the top to the 200 cm depth (pH 7.5 to pH 6.5). As suggested by Taysum[8], acid production by anaerobe microorganisms such as *Clostridium* sp., *Lactobacillus* sp. and *Streptrococcus* sp. may explain lower pH in locations with low oxygen content, *i.e.* in the deeper layers of the pile (*Figure 3*).

Wallace initial plasticity (P_0) of rubber from fresh cup coagula collected on delivery day was 25 in average (*Figure 8*). After 24 days of maturation in the pile, it was found that P_0 increased with the depth. At the top of the pile, P_0 value was 26 and reached a maximum value of 42 at a depth of 200 cm. Higher P_0 deeper in the pile may be related to a lower degradation of polyisoprene chains and/or higher cross-linking between rubber chains. These phenomena may have occurred during maturation and/or drying. Scission and cross-linking are indeed two antagonistic phenomena that play an important role in the modification of the structure of rubber during maturation and drying[9]. It is interesting to note that higher P_0 are measured in layers characterised by a higher temperature and lower pH and oxygen content in the air.

Plasticity retention index (PRI) is a standardised parameter to determine sensitivity of natural rubber to thermo-oxidation. It has to be mentioned that for natural rubber there is a balance between scission and cross-linking during the thermo-oxidative process. In most

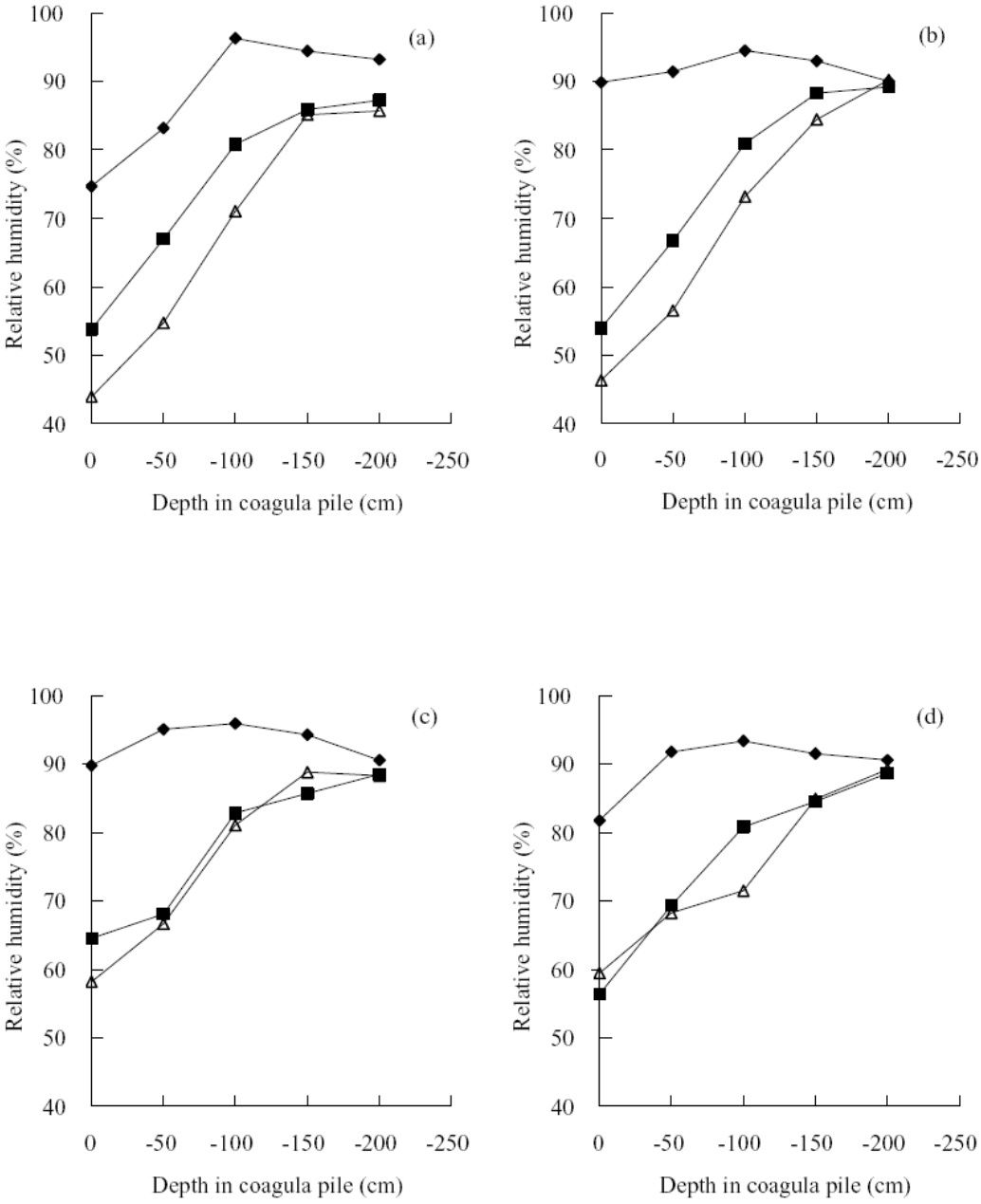

Figure 5. Air relative humidity in cup coagula pile at different depths from the top for each maturation duration: (a) 1 day, (b) 7 days, (c) 13 days and (d) 19 days. Measurements were performed at 8 a.m (—◆—), 12 a.m (—■—) and 4 p.m (—△—)

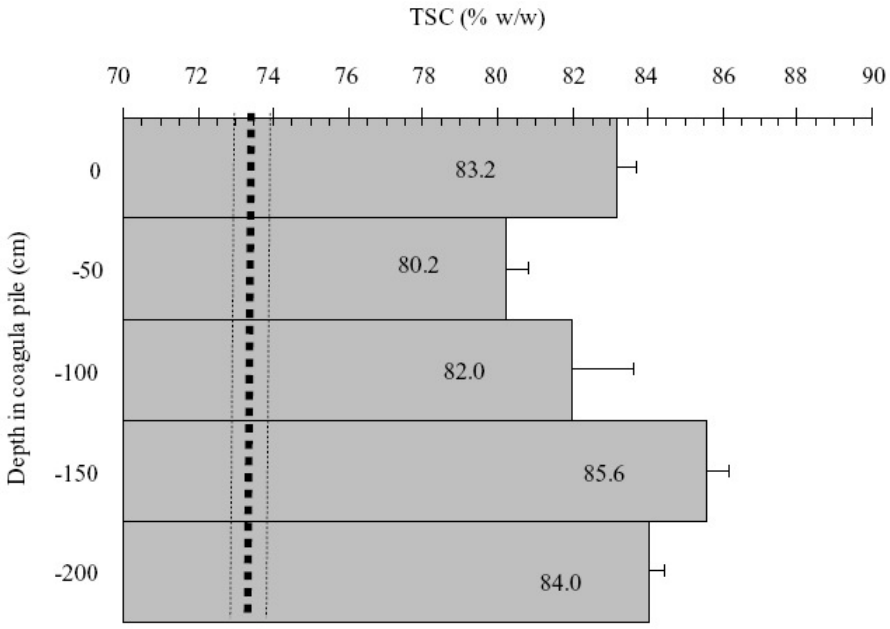

Figure 6. TSC of cup coagula in maturation pile at different depths from the top
Initial TSC on delivery: bold dotted line (▪▪▪); TSC after 24 days of maturation: gray histograms.
Standard errors are inidicated respectively by thin doted line (-----) or error bars.

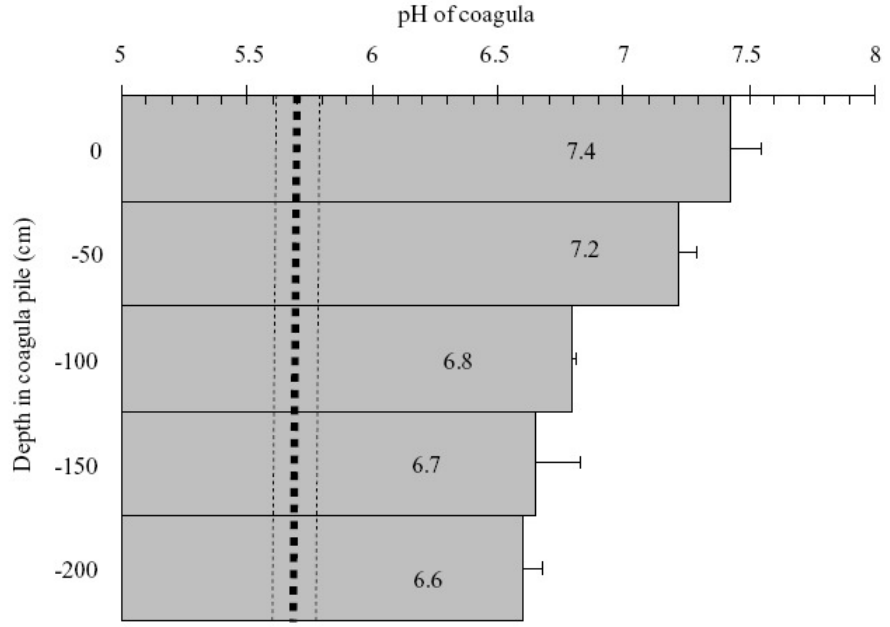

Figure 7. pH of cup coagula in maturation pile at different depths from the top
Initial pH on delivery: bold dotted line (▪▪▪); pH after 24 days of maturation: gray histograms.
Standard errors are inidicated respectively by thin doted line (-----) or error bars.

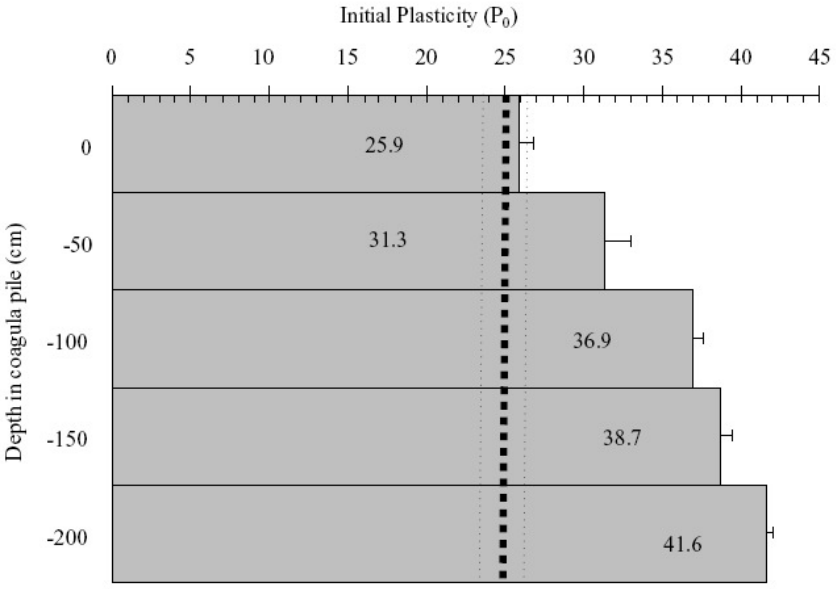

Figure 8. P_0 of cup coagula in maturation pile at different depths from the top
Initial P_0 on delivery: bold dotted line (▪▪▪); P_0 after 24 days of maturation: gray histograms.
Standard errors are inidicated respectively by thin doted line (·····) or error bars.

of the cases, scissions are prominent. As shown in *Figure 9*, the mean PRI value of rubber from fresh cup coagula was 25, which is significantly below TSR 20 standard (Technically Specified Rubber grade 20) that requires a minimum value of 40. After 24 days of maturation in the pile, PRI increased significantly with the depth in the pile, reaching values above 41 when cup coagula were collected deeper than 150 cm under the top (17 units increase). The environmental conditions at the bottom of the pile (high temperature, low pH and oxygen content in the air) encouraged microbiological/ biochemical mechanisms that reduce sensibility of NR to thermo-oxidation. However, these mechanisms remain to be characterised: they may involve a change in the balance between antioxidant molecules (proteins, amino acids, tocotrieniols, *etc*) and pro-oxidant molecules (unsaturated free fatty acids, free metallic ions, *etc*)[3,10]. PRI values followed the same trend as those of P_0 within the pile. This similarity indicates that the observed gradient of P_0 can be explained mainly by the effect of depth on the resistance of rubber to thermal treatment during drying.

Mesostructure (average molar masses and gel content) of rubber from fresh and matured cup coagula from different vertical locations in the pile were analysed by SEC-MALLS. As number-average molar mass (M_n), weight-average molar mass (M_w) and z-average molar mass (M_z) showed the same trend, only M_w is presented (*Figure 10*). M_w values, as the P_0 ones, increased with the depth within the pile. Bearing in mind that molar masses measured by SEC are related to the soluble part of rubber only, therefore, higher M_w deeper in the pile indicates that the soluble polyisoprene

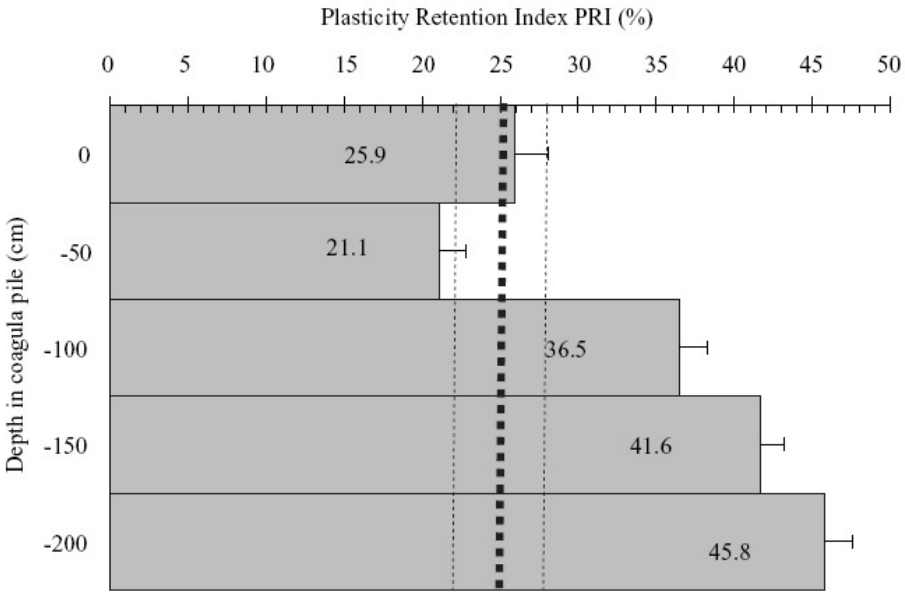

Figure 9. PRI of cup coagula in maturation pile at different depths from the top
Initial PRI on delivery: bold dotted line (▪▪▪); PRI after 24 days of maturation: gray histograms.
Standard errors are inidicated respectively by thin doted line (------) or error bars.

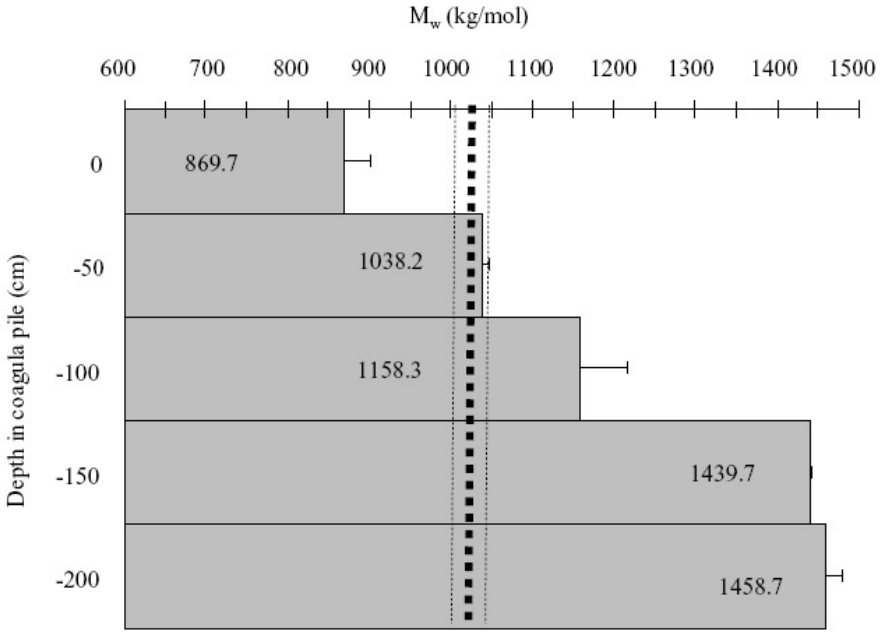

Figure 10. M_w of cup coagula in maturation pile at different depths from the top
Initial M_w on delivery: bold dotted line (▪▪▪); M_w after 24 days of maturation: gray histograms.
Standard errors are inidicated respectively by thin doted line (------) or error bars.

chains maturated at 200 cm depth underwent less scission during the drying process (3h – 129°C) than their counterparts from the top of the pile. Initial value of M_w of rubber from non-maturated fresh cup coagula was in the same range as in the layer located 50 cm below the top after maturation. The maturated top layer cup coagula displayed a lower value of M_w most probably due to scissions caused by direct sunlight exposure. Concerning gel (*Figure 11*), and contrary to other parameters, the initial level (47.7%, fresh cup coagula) was in the same range as that of the layer located 200 cm below the top. Gel content decreased slightly at the top of the pile (depths 0 and 50 cm), which displayed lower P_0. These results of structural study, as well as the similar trends of P_0 and PRI (increasing values with depth), support the assumption that the P_0 gradient observed in the maturated pile is more due to a higher resistance to scission during the drying process (thermo-oxidation) than to a higher cross-linking.

CONCLUSION

This study was undertaken in order to characterise the maturation conditions of cup coagula pile in an industrial plant and to monitor the influence of maturation on some technological properties of processed cup coagula at different depths from the top of a pile. It was found that temperature, relative humidity of the air and total solid content increased with depth. In contrast, oxygen content of the air within the pile decreased as depth increased. The effects of 24-day maturation on rubber technological properties were found to depend on the position of cup coagula within the pile. The pH of maturated cup coagula decreased significantly with the

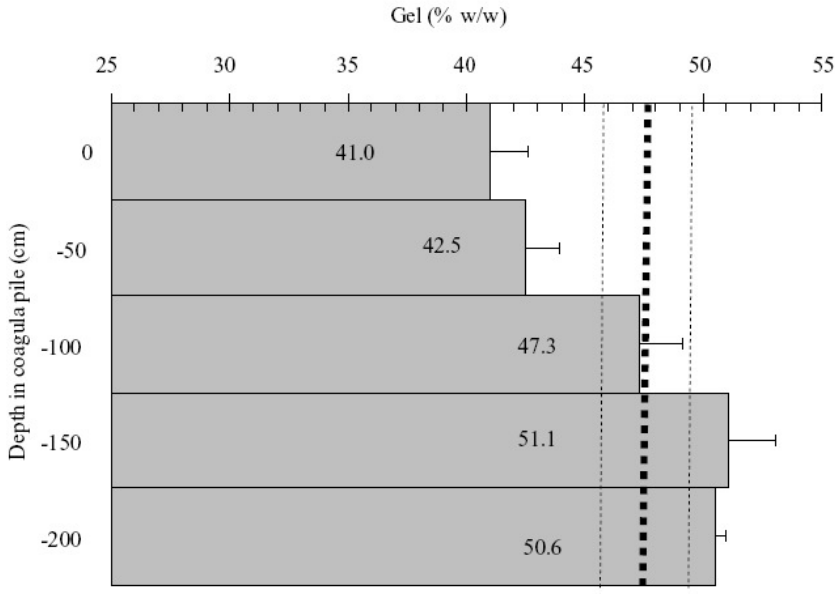

Figure 11. Gel of cup coagula in maturation pile at different depths from the top
Initial gel on delivery: bold dotted line (▪▪▪); Gel after 24 days of maturation: gray histograms.
Standard errors are inidicated respectively by thin doted line (-----) or error bars.

depth within the pile. An inverse pattern was observed for initial plasticity (P_0), plasticity retention index (PRI), gel content and M_w. At 150 cm below the top, maturation period showed a positive effect on cup coagula properties (P_0, PRI, M_w and gel content). At this depth, rubber was more resistant to thermo-oxidation undergone during drying. This phenomenon was probably linked to the environmental conditions at the bottom of the pile which affected the microbiological/ biochemical mechanisms involved during the maturation process.

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APPENDIX B

Scientific paper

Journal of Applied Polymer Science

(Accepted on 23 February, 2010)

Effect of micro-organisms during initial coagula maturation of Hevea natural rubber

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Manuscript ID:	APP-2009-12-3783.R1
Wiley - Manuscript type:	Research Article
Keywords:	structure-property relations, crosslinking, molecular weight distribution / molar, rubber

Effect of micro-organisms during initial coagula maturation of *Hevea* natural rubber

J. Intapun[1,2], J. Sainte-Beuve[3], F. Bonfils[3], V.Tanrattanakul[1], E. Dubreucq[2], L. Vaysse[4]

[1] *Polymer Science Program, Faculty of Science, Prince of Songkla University, Hadyai 90112, Thailand*
[2] *Montpellier SupAgro, UMR IATE, 2 Place Viala, 34060 Montpellier cedex, France*
[3] *Cirad, UMR IATE, TA B 62/16 - 73, Rue Jean François Breton - 34398 Montpellier Cedex 5, France*
[4] *Cirad, UMR IATE, Hevea Research Platform in Partnership, KAPI, Kasetsart University, Bangkok 10900, Thailand*

Corresponding author
Dr Laurent VAYSSE
Rubber Technology Laboratory
Agro-Industry Building 3, 8th floor
Kasetsart University
Chatuchak - Bangkok 10900 - Thaïland
laurent.vaysse@cirad.fr

Keywords

natural rubber; maturation; structure-properties relation; molar masses, crosslinking.

Abstract

The involvement of micro-organisms in the initial stage of maturation of natural rubber coagula was assessed by five latex treatments varying initial quantity of micro-organisms: from latex added with antimicrobial agent (3.4×10^4 CFU/mL) to strongly inoculated latex (2.4×10^7 CFU/mL). After 0 to 6 days of maturation, the obtained rubber was characterized for its physical and structural properties. Wallace plasticity (P_0) and plasticity retention index (PRI) remained constant during maturation for antibiotic added treatment. PRI was found to decrease with maturation time and the rate proportional to the initial micro-organism concentration. P_0 of all inoculated rubber increased for the first 2 days of maturation and decreased after 6 days of maturation. Concerning structural parameters, higher initial micro-organisms content corresponded to higher gel content and lower weight-average molar mass ($\overline{M_w}$) after maturation, drying and storage. Inoculated rubber showed a stable value of number-average molar mass ($\overline{M_n}$) contrary to non inoculated samples where an increase of $\overline{M_n}$ during maturation was observed. The quantity of micro-organisms significantly affected the physical properties and structure of processed dry rubber. The mechanisms occurring at the initial stage of maturation are complex and micro-organisms are not only involved in the increase of sensitivity to thermo-oxidation but also in crosslinking phenomenon between isoprene chains.

1. INTRODUCTION

The TSR 10 and TSR 20 are the most produced commercial grades of raw Technically Specified natural rubber. The main raw materials for producing these two grades are the "coagula" or "cuplumps", obtained by natural (or auto) coagulation of latex in the collection container a few hours after tapping. The time between tapping the trees and processing the coagula in TSR10 or 20 raw natural rubber can reach several weeks: it is called the maturation time. During this period, coagula undergo biochemical intrinsic modifications in which micro-organisms are supposed to play an important role.[1,2] This maturation time is crucial for the processors as it impacts the quality of the processed rubber. Indeed, the quality of natural rubber (NR) obtained from coagula available for the processors from grower sector is inconsistent especially in terms of sensitivity to thermo-oxidation.[3]

Natural rubber latex is the cytoplasm of laticiferous cells located in the bark of *Hevea brasiliensis* tree. Its composition, rich in mineral salts, sugars, amino acids, proteins and lipids, is a highly favourable medium for microbial growth. Taysum[4] identified about 100 species of micro-organisms in *Hevea* latex and in its commercial derivatives. Bacteria in fresh latex may come from several sources (bark and cut of tapped trees, collection cup, etc.). According to Taysum[5], primary infection could reach, according to cases, 10^6 to 10^7 total bacteria per mL and coagulation starts when bacterial population reached 10^9 to 10^{10} per mL. Micro-organisms could account for spoilage of latex and ammoniated latex concentrate. Some like Gordonia sp.[6], Nocardia sp.[7], several bacteria species [8,9] and fungi [10] are known to degrade dry rubber and rubber products.

Few researchers have studied the effect of microorganisms in latex on the properties of raw NR. Soewarti and Moh[11] have shown that coagula obtained after inoculation of skim latex with some micro-organisms lead to rubber of lower quality than when coagulation was provoked by addition of formic acid. Increasing maturation time usually increased sensitivity of NR to thermo-oxidation.[1,2,12,13] Hasma and Othman[14] also attributed the lower resistance to thermo-oxidation of auto-coagulated coagula to the bacterial decomposition of proteins and other non-rubber components by bacteria, leading to the release of strong pro-oxidants such as free copper.

The conditions of storage of coagula between the day of delivery in a factory and the day of processing has been studied recently by Intapun and coworkers.[15] It was observed

that coagula arriving in the factory, after a few weeks of maturation in the farms, were rather sensitive to thermo-oxidation (PRI 22-28). It was demonstrated that the industrial storage conditions have a significant impact on properties of dry rubber. After 24 days of storage in a 3m-high pile, the sensitivity to thermo-oxidation of coagula interestingly decreased as illustrated by an increase of PRI up to 46. Soaking the crumb rubber in phosphoric or oxalic acid solution before drying is another industrial way to increase PRI.[1]

The initial quantity of micro-organisms in latex before coagulation and its effect on the structure and properties of rubber have not been reported. This is the main purpose of the present study which was performed under controlled maturation conditions at laboratory scale. The maturation of coagula was studied from 0 to 6 days after inoculation with different quantities of micro-organisms sampled in the rubber field. The main bulk properties assessed during maturation were PRI and Initial Wallace plasticity (P_0). Furthermore, analyses of the mesostructure (macromolecular structure and gel content) of raw rubber were performed to understand the evolution of properties during maturation.

2. MATERIALS AND METHODS

2.1 Clean latex

Five liters of latex were collected from 32 *Hevea brasiliensis* trees from RRIM600 clone located in the rubber plantation of Prince of Songkla University, Surat Thani, Thailand. A special tapping procedure was developed for the collection of latex samples with an as low as possible level of microbial contamination. The collection material was autoclaved at 121°C for 15 min before use. Sterilized material included a knife, a spout, a plastic tube connected with a collection plastic bag (15 cm x 23 cm), a plastic sheet (60 cm x 70 cm), metallic blade (5 cm x 12 cm) and water (1L). The bark was slightly scrapped with the metallic blade downward from the cut to 3 cm below. The scrapped area was cleaned successively with water, ethanol and water, using commercial sterile cotton. The clean panel was covered with a plastic sheet fixed on the tree by a rubber band, 10 cm up the tapping cut. Previously to tapping a new spout was fixed on the tree. Latex was directed to flow through the spout and the plastic tube into the collection bag. The bag was placed inside an ice-containing cup. When the latex flow stopped (around 3h after tapping), bags containing latex were transferred to a laminar flow cabinet in the nearby laboratory. The latex harvested through this special tapping system is called “clean latex” in this paper.

2.2 Inoculum

Thirty-two trees, identical to those used for latex collection, were tapped following the traditional procedure and the harvested latex allowed to coagulate naturally. Three days after tapping, the 32 coagula were squeezed and approximately 250 mL of serum containing suspended micro-organisms were collected and filtered through a metallic sieve (1 mm pore size). Microbial cells were then centrifuged and washed as follows: 80 mL of serum was centrifuged at 10,000 g for 15 min (8 tubes x 10 mL). For each tube, the supernatant was removed and the cell pellet was resuspended in 10 mL of 0.9% NaCl (w/v). The washing operation was repeated twice. The cell suspensions were then pooled in a capped flask and the obtained inoculum was stored at 4°C until further use. The "2.5X concentrated" inoculum was obtained following the same procedure except that the last resuspension was performed with 4 mL instead of 10 mL of 0.9% NaCl (w/v). The microbial population in the inoculum was estimated as described in section 2.4.

2.3 Latex preparation : 5 treatments

The amount of micro-organisms in latex was controlled either through the volume of inoculum added to latex or, by the addition of sodium azide as an antimicrobial agent. Fresh clean latex was split into 5 different lots of 1 L each corresponding to the five treatments. Antibiotic (16 mL of 10% sodium azide solution) was added to the first lot while the others received different amounts of inoculum as detailed in Table 1. Preliminary tests had shown that sodium azide provided anti-microbial activity without interfering with the rubber's properties.

2.4 Counting of micro-organisms

The microbial population in the inoculum was characterized using selective agar media. Appropriate 10X dilution were performed before spreading on agar plate. The following categories were numbered: gram positive, gram negative, lactic acid bacteria, total aerobic and total anaerobic bacteria, and yeasts. Micro-organisms present in inoculum are indicated in Table 2.

Counting of micro-organisms in latex before coagulation was limited to total aerobic population on PCA media (Table 2).

2.5 Coagula preparation

About 9 ml of 5% formic acid solution were added to each lot of latex to reduce their pH to 5.2. The pH change was monitored by a disinfected pH probe (Sentix SP S7, VWR,

Weilheim, Germany) connected to a multi 350 data logger (VWR GmbH, Weilheim, Germany). Each latex mixture lot corresponding to one treatment was poured into 45 mL sterile glass cups (15 mini cups per treatment). Mini coagula in glass cups were stored in sterile hermetically closed boxes in an incubator (BD Series 53, Binder GmbH, Germany) at 40°C for 3 h. Under a laminar flow hood, coagula were retrieved, placed on sterile stainless steel trays, and kept in sufficiently large hermetically sealed plastic boxes.

2.6 Coagula maturation

Coagula with different microbial populations were matured under saturated humidity in the closed sterile plastic boxes (1 maturation box per treatment) and incubated at 40°C. This temperature was chosen with respect to that prevailing in the coagula maturation piles. To evaluate the effect of maturation time, 3 coagula from each box were randomly sampled and weighed from the start (day 0) to the 6^{th} day after coagulation.

2.7 Creping and drying of coagula

A laboratory-scale creper (mini-creper) was built with the following specifications: gap between nip rolls 0.04 mm, speed of front roll: 190 rpm, friction ratio: 1:1.3, roll length: 20 cm, Roll diameter 10 cm, Engine power: 2.2 kW. Each cup coagulum was creped by 16 double passes in this mini-creper (crepe was folded end to end between each pass) followed by 1 single final pass.

The obtained crepes were dried in a hot air oven (UE700, Memmert GmbH & Co. KG., Germany) at 125°C for 2 h, cooled in a desiccators and weighed. The creping and drying process parameters were chosen after a comparative study with the industrial STR20 process used at the Von Bundit Co. Ltd., Surat Thani factory.

2.8 Bulk characterization of coagula and rubber

Considering that, during creping, only water soluble components were leached, the dry rubber content of coagula (DRC_c) was assessed as the percentage ratio of the weight of the crepe to the fresh weight of the coagula.

About 20 g of crepe were homogenized following the SMR bulletin No. 7 (1992), B2 Standard. Wallace rapid plasticity (P_0) and Plasticity Retention Index (PRI) were measured following SMR bulletin No. 7 (1992), B8 Standard. P_0 and PRI measurements were performed twice: within 24 hours and 80 days after drying.

2.9 Mesostructure and gel content testing

The method described by Kim *et al.*[16] was followed with slight modifications. A 25 ± 2 mg sample of homogeneous rubber was dissolved in 40 mL tetrahydrofuran (THF) stabilized with 3,5-di-tert-butyl-4-hydroxytoluene (BHT) for 2 weeks. The rubber solutions were stored at 30°C for 7 days without stirring then gently stirred for 1 hour daily for 7 other days. The solution was filtered (Acrodisc 1 µm, glass fiber, Pall) and injected into a size-exclusion HPLC system consisting of an online degasser (EliteTM, Alltech), a Waters 515 pump, a refractive index detector (Waters 2410) and a multiangle laser light scattering detector (Dawn DSP, Wyatt technology Corp.). The columns were three inline PLGEL (Polymer Laboratory) mixed beds (20 µm, 7.8 mm ID x 30 cm) with a guard column. The columns were thermostated at 45°C. The mobile phase was THF, stabilized with BHT. Flow rate was 0.65 mL.min^{-1}. The injected volume was 0.15 mL. Number-average molar mass ($\overline{M_n}$), and weight-average molar mass ($\overline{M_w}$) were calculated using the ASTRA software (Wyatt Technologies Corp.). Fourteen angles, from "Angle 3" (32°) to "Angle 16" (134°), were used for calculation using the Zimm method. The differential refractive index increment (dn/dc) value used was 0.130 mL.g^{-1}.

For a given sample injected into the SEC-MALS chain, the refractive index increment of solvent and sample solution was measured by a refractive index detector (DRI). This represents the incremental refractive index change (dn) of the solution for an incremental change of the concentration (dc). ASTRA software was used for the calculation of the injected quantity of natural rubber (NR) after filtration by integrating the whole NR peak on the chromatogram. Thus, as the concentration of the solution (0.625 mg.mL^{-1}) and the injected volume (0.15 mL) were known before and after filtration, the fraction eliminated by filtration, i.e. the percentage of total gel, could be calculated as follows (equation 1) :

$$\text{Total gel content (\%)} = \left(\frac{m_0 - m_1}{m_0} \right) \times 100 \qquad (1)$$

m_0 = mass of sample in 0.15 mL before filtration

m_1 = mass of injected sample (calculated from SEC-MALS)

3. RESULTS

3.1 Initial micro-organism amount in inoculated latex

Clean latex (M0) contained 4.82 x 10^5 CFU/ml of total aerobic micro-organisms while the addition of sodium azide (M0+N) reduced this amount to 3.41 x 10^4 CFU/ml. Addition of inocula (M1, M2 and M5) raised this amount to 2.16 x 10^6, 9.52x10^6 and 2.36x10^7 CFU/ml respectively (Fig. 1). The quantity of counted aerobic micro-organisms in inoculated latex was therefore proportional to the quantity of added inoculum.

3.2 Evolution of dry rubber content of coagula during maturation

At the first day of maturation, the average dry rubber content of all coagula inoculated was 40.5%. After 6 day of maturation, dry rubber content of coagula (DRC_c) increased up to a range of 62% -72% depending on the initial number of micro-organisms (Fig. 2). Indeed, increasing the quantity of micro-organisms decreased proportionally the dry rubber content during maturation as well as the level of the obtained plateau. The physical aspects of coagula after six days of maturation were very different (Fig. 3). The presence of growing micro-organisms was clearly illustrated by the generation of bubbles inside the coagula. On a qualitative point of view, size and number of bubbles seemed to increase with the initial quantity of micro-organisms.

The difference of DRC_c between treatments was mainly explained by a difference of fresh weight before drying (results not shown), which illustrates a higher serum retention from the coagula containing higher amount of micro-organisms. Superior water retention in inoculated coagula can be explained by two reasons: (i) gas produced by the microbiological metabolism formed bubbles inside the coagula (Fig. 3) ; the cavities formed by the bubbles, bigger and more numerous with the quantity of micro-organisms, could act as a reservoir and trap water; (ii) micro-organism activity may have degraded non isoprene compounds, especially proteins, which are known to help water migration in the hydrophobic rubber medium.[17] Water may thus have been retained inside the coagula because of a lack of carrier.

3.3 Evolution of Plasticity retention index (PRI) and Wallace plasticity (P_0) during maturation

Initial PRI of rubber originating from all coagula processed 3h after coagulation were similarly high with an average value of 97.6 (SEM=1.4) (Fig. 4A). The evolution of PRI

during maturation was clearly dependent on the initial quantity of micro-organisms in latex. Indeed, when microbial growth was prevented (M0+N), no change of PRI was observed. For the three inoculated treatments (M1, M2 and M5) the drop rate of PRI was proportional to the initial micro-organism concentration, even though the PRI reached after 6 days was found similarly very low (<10). Concerning the non-inoculated treatment (M0), which contained a low initial number of micro-organism, a less important drop was observed during maturation, PRI value reached after 6 days being 70. The presence of micro-organisms in latex before coagulation clearly led to a proportional increase of sensitivity to thermo-oxidation (decrease of PRI). This may be explained by two phenomena: (i) micro-organisms activity released pro-oxidant molecules such as free metallic ions[14] or fatty acids[18]; (ii) this activity degraded or inactivated some of the latex native anti-oxidants such as tocotrienols.[14] On-going studies of this anti-oxidant are in progress but it has already been observed on these samples that free tocotrienols disappearance is associated with the decrease of PRI (data not shown).

Figure 4B presents the evolution of Wallace plasticity during maturation time (0 to 6 days) for all treatments described previously, the main difference between treatments being the initial quantity of micro-organisms. The control sample for all treatments (0 day maturation time) displayed a Wallace plasticity (P_0) value about 34.1 (standard error of the mean or SEM=0.35), whatever the micro-organisms quantity. When observed during maturation, the evolution of P_0 was clearly dependent on the quantity of micro-organisms. Indeed, when microbial activity was prevented (treatment M0+N), P_0 did not change significantly while for the non inoculated treatment (M0) P_0 increased up to 45 after 6 days of maturation. With inoculated ones (M1, M2 and M5), an increase of P_0 was observed for the first 2 days of maturation and was followed by a drop down to 30.5, 27.5 and 27.0 respectively after 6 days of maturation. The observed variations of P_0 could be due to a balance between hardening of rubber by crosslinking of polyisoprene chains and softening by oxidative scission of polyisoprene chain during drying. In inoculated samples (M1, M2, M5), despite a decrease of PRI from the first day, crosslinking seemed to counteract and even to exceed scissions during the first two days, leading to an increase of P_0. Later on, after the 4th day, scission became prominent, leading to a decrease of P_0. For non-inoculated samples (M0) the crosslinking phenomenon was higher than scission during the 6 days of maturation. Rubber added with antimicrobial agent (M0+N) showed a constant P_0, which illustrates equilibrium (or non-existence) of those two phenomena. The involvement of

micro-organisms in the crosslinking phenomenon is difficult to assess. It can be supposed that microbial activity interacts with polymer chain abnormal groups as well as with non-isoprene constituents, which leads to new interactions between rubber chains. Micro-organisms interestingly seemed to promote both phenomena – crosslinking and scission – which have contrary effect on P_0. Mesostructural information (total gel and molar masses), which will be discussed below, are needed to complete those assumptions.

3.4 Evolution of P_0, PRI and mesostructure of rubber after storage

For all treatments but M2 (considered similar as M1), measurements of P_0 and PRI were repeated before structural analysis by SEC analysis after storing the samples 80 days at room temperature. Considering the low variance among repetitions for P_0 and PRI measured just after drying, and the fact that three repetitions of the SEC analysis are realized, only one repetition of each treatment and each maturation time was taken for the re-measurement of P_0 and PRI and for the assessment of polyisoprene molar masses and total gel.

Figure 5A and 5B presents respectively the re-measured PRI and P_0 (plain line) in comparison with the initial measure just after drying (dotted line). The results obtained showed the same global trends as the ones obtained without storage, but P_0 was clearly increased because of storage hardening. This hardening, assessed by "Delta P_0" (P_0 measured 80 days after drying minus P_0 measured within 24 hours after drying) was in the range of 10 to 15 P_0 units for non-inoculated samples (M0 and M0+N). With the inoculated samples (M1 and M5), the shift was more important (between 15 and 22) and increased with maturation time. This is shown on Figure 6, which presents the evolution of "Delta P_0" with maturation time for treatments M0+N, M1 and M5. A good linear correlation between maturation time and increase of P_0 was observed for inoculated samples (p<0.01). Therefore, the presence of micro-organisms in latex promoted storage hardening, and the increase of P_0 was more marked for inoculated samples matured for 4 or 6 days. This is again an illustration of the impact of microbial activity during maturation on the crosslinking of rubber chains even after drying, i.e. during storage time.

The mesostructure of the rubber after storage was also studied. Concerning the weight-average molar mass ($\overline{M_w}$) and the number-average molar mass ($\overline{M_n}$), it is worth noting that, contrarily to the other measured parameters, the initial value of molar masses from samples processed 3 h after coagulation were slightly different between treatments,

inoculated rubber showing slightly lower values of $\overline{M_n}$ and $\overline{M_w}$ (Fig. 7). $\overline{M_n}$ followed a different trend than that of $\overline{M_w}$ during maturation. Indeed, inoculated rubber showed a stable value of $\overline{M_n}$ staying around 700 kg/mol while non inoculated samples (M0+N, M0) showed an increase of $\overline{M_n}$ over the maturation time, up to 900 kg/mol. $\overline{M_n}$ data are delicate to interpret. Indeed, on one hand, scissions would logically produce more short chains and decrease $\overline{M_n}$ while on the other hand, short chains may be involved into gel and therefore not be counted in the $\overline{M_n}$ assessment leading to its overestimation[19]. A slight decrease of $\overline{M_w}$ was observed with maturation time for the antibiotic treated rubber (M0+N). $\overline{M_w}$ values dropped from 1500kg/mol down to 1400kg/mol after 6 days of maturation. The $\overline{M_w}$ from M0 treatment (no inoculum, no antibiotic) showed a similar trend with a faster drop. For inoculated treatments (M1 and M5), this drop was more important, $\overline{M_w}$ reaching a minimum value of around 1150 kg/mol. Interestingly, the drop rate was proportional to the quantity of micro-organisms, the minimum value being reached after 1 day for M5 and after 4 days for M1. The weight-average molar mass ($\overline{M_w}$), which preferentially illustrates the high molecular mass-isoprene population, confirmed that the presence of micro-organisms in latex before coagulation promoted scissions. Considering the similarity of $\overline{M_w}$ and PRI evolution, those scissions are most probably due to a thermo-oxidative phenomenon which occurred during drying.

Concomitantly, it is obvious that micro-organisms are also involved into crosslinking interactions between isoprene chains during and/or after maturation (drying and/or storage) as illustrated in the total gel evolution (Fig. 8). This total gel included both "microgel and macrogel" as defined by Allan and Bristow[20], contrary to other authors who determined only macrogel by centrifugation when studying the mechanisms of gel formation during storage hardening of natural rubber [21,22,23]. The control sample for all treatments (0 day maturation time) displayed a very similar initial gel content with an average value of 30% (SEM=0.64%). For all treatments, gel quantity increased with maturation time and reached a plateau after 2 days of maturation. The value associated with this plateau was proportional to the initial quantity of micro-organisms, from 45% for antibiotic treated rubber (M0+N) to 55% for the treatment with the highest initial micro-organism content (M5). Nevertheless, for samples containing an antimicrobial agent (M0+N), an increase of gel content was also observed at a lesser extent, which means that gel formation may occur without microbial activity. This gel may be of a different nature as it did not impact on the P_0 of M0+N

samples, which remained stable during maturation. This could be a thermoreversible physical gel which could have appeared during storage and disappeared during P_0 measurement at 100°C. Indeed, Voznyakovskii et al.[24] studied gel in NR by dynamic light scattering and showed that increasing temperature of the solutions from 20°C to 70°C led to a decrease of the mean size of the aggregates. Micro-organisms enzymatic activity may catalyze reactions involved in gel formation such as the oxidation of polyunsaturated fatty acid leading to formation of aldehyde groups and the release of amino acids or metallic ions. In order to better understand the mode of action of micro-organisms, the study of in situ enzymatic activity is necessary.

4. CONCLUSION

The initial stages of the maturation of natural rubber coagula were investigated. The obtained structure and properties of dried rubber were further studied. It has been shown that phenomena occurring during the first stage of maturation of rubber are complex and clearly depend on the presence and the amount of micro-organisms in the initial latex. At the initial stage of maturation, micro-organisms clearly enhance the sensitivity of rubber to thermal oxidation while concomitantly seeming to promote crosslinking between rubber chains. However, the exact mode of action of these micro-organisms is not understood, hence the need to focus on the role of enzymes they secrete.

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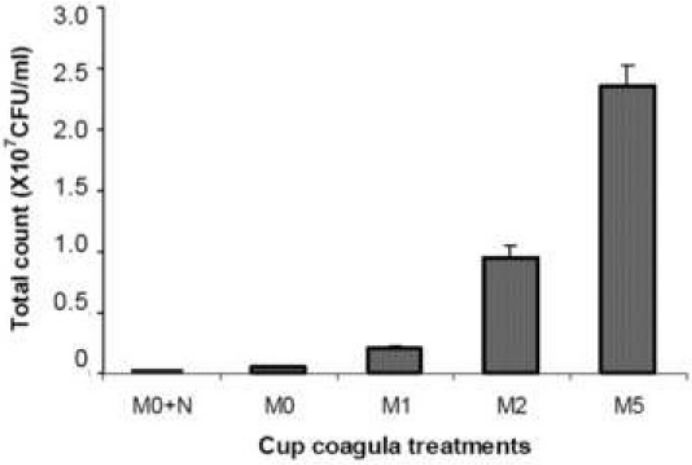

1 Initial microorganism amount in lattices for each treatment. Error bars are SEMs of three replicates.
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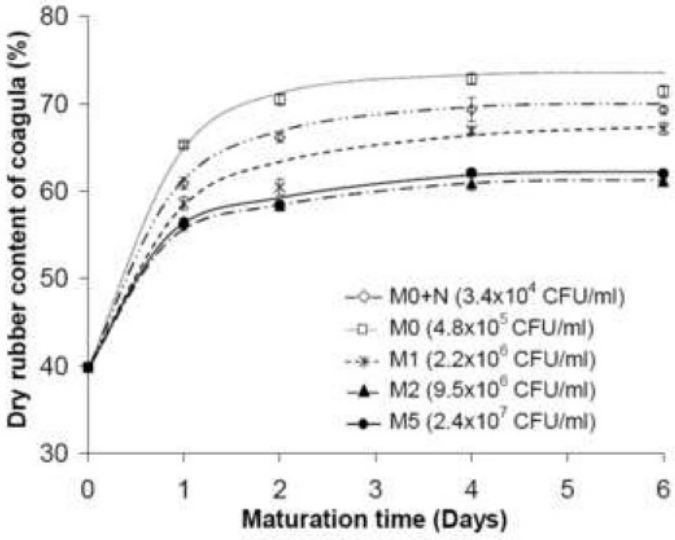

ution of dry rubber content of coagula (DRCc) with maturation time. Error bars are SEMs of three replicates.
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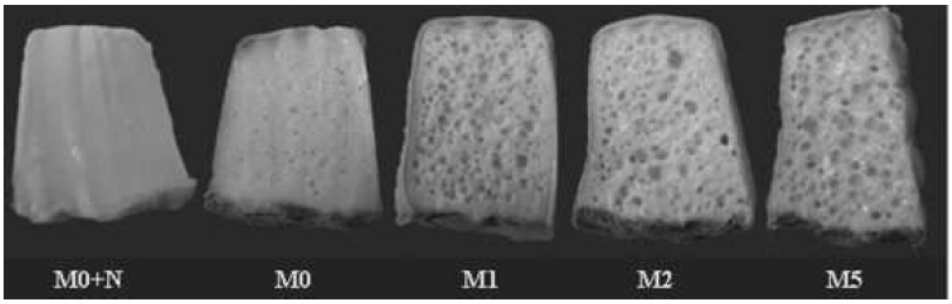
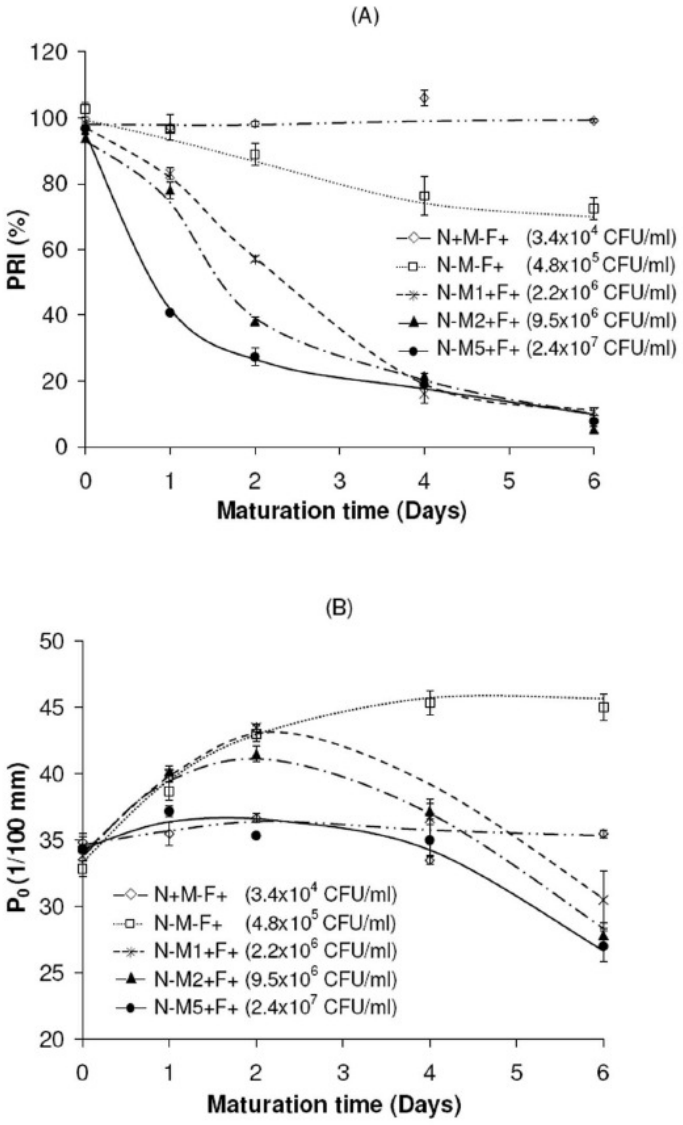
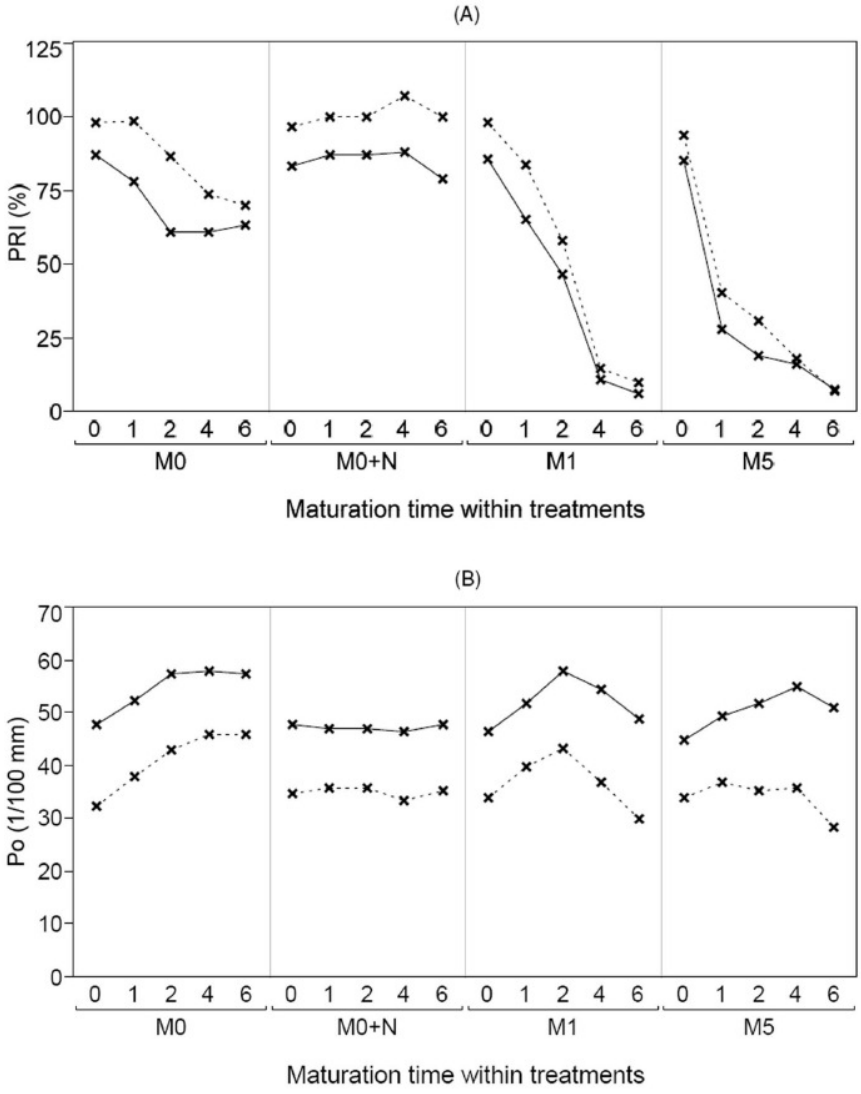


Central sections of cup coagula after 6 days of maturation, with different initial levels of micro-organisms inoculation.
22x7mm (600 x 600 DPI)



Evolution of PRI (A) and P0 (B) with maturation time. Measurement performed within 24h after drying (Error bars are SEMs of three replicates).
104x173mm (600 x 600 DPI)



Evolution of PRI (A) and P0 (B) with maturation and storage time. Measurements performed after 80 days of storage (solid lines) compared with initial ones (dashed lines).
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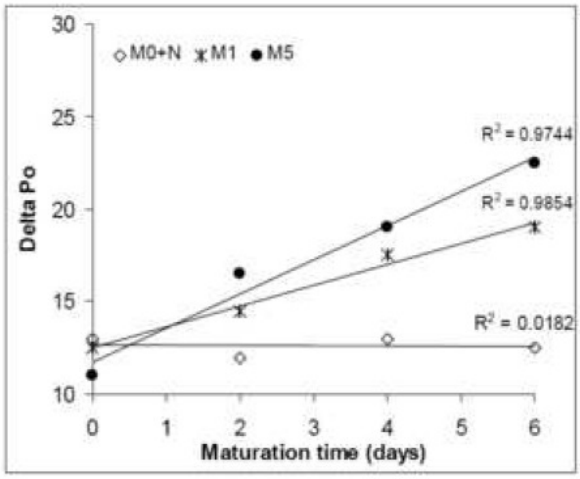

Effect of the maturation time on the increase of P0 during 80 days storage. The solid lines are linear regressions, Coefficient of determination R2 are indicated nearby the corresponding straight lines.
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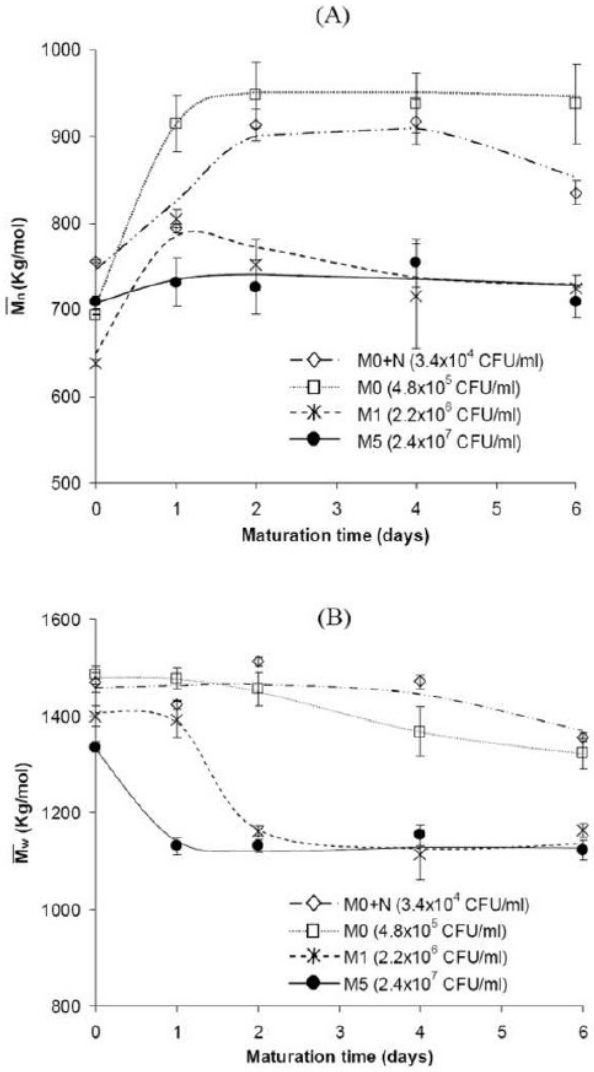
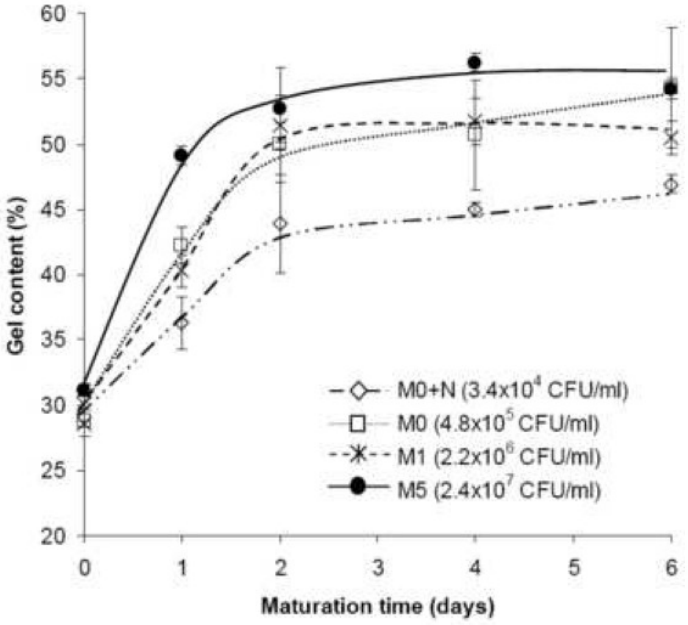


Evolution of Mn (A) and Mw (B) with maturation time after 80 days of storage. Error bars are SEMs of three replicates.
26x45mm (600 x 600 DPI)



Evolution of Total Gel content with maturation time after 80 days of storage. Error bars are SEMs of three replicates.

18x16mm (600 x 600 DPI)

Figure 1 Initial microorganism amount in lattices for each treatment. *Error bars are SEMs of three replicates.*

Figure 2 Evolution of dry rubber content of coagula (DRC_c) with maturation time. *Error bars are SEMs of three replicates.*

Figure 3 Central sections of cup coagula after 6 days of maturation, with different initial levels of micro-organisms inoculation.

Figure 4 Evolution of PRI (A) and P_0 (B) with maturation time. Measurement performed within 24h after drying (*Error bars are SEMs of three replicates*).

Figure 5. Evolution of PRI (A) and P_0 (B) with maturation and storage time. Measurements performed after 80 days of storage (solid lines) compared with initial ones (dashed lines).

Figure 6 Effect of the maturation time on the increase of P_0 during 80 days storage. *The solid lines are linear regressions, Coefficient of determination R^2 are indicated nearby the corresponding straight lines.*

Figure 7 Evolution of $\overline{M_n}$ (A) and $\overline{M_w}$ (B) with maturation time after 80 days of storage. *Error bars are SEMs of three replicates*.

Figure 8 Evolution of Total Gel content with maturation time after 80 days of storage. *Error bars are SEMs of three replicates*.

Table I Preparation of applied treatments

Treatment	M0+N	M0	M1	M2	M5
Clean Latex (mL)	1000	1000	1000	1000	1000
10% Sodium azide solution (mL)	16	-	-	-	-
Inoculum (mL)	-	-	14	28	-
Concentrated inoculum 2.5X (mL)	-	-	-	-	28
Sterile 0.9% NaCl (mL)	12	28	14	-	-
Total volume (mL)	*1028*	*1028*	*1028*	*1028*	*1028*

Table II Microbial population in the inoculum

Micro-organisms	Specific media	Culture conditions	Total count (CFU/ml)
Total aerobic	PCA (Difco, USA)	35-37°C for 24-48 h	8.7×10^8
Total anaerobic	PCA (Difco, USA)	35-37°C for 24-48 h in anaerobic jar	1.1×10^9
Gram + bacteria	MSA (Difco, USA)	35-37°C for 24-48 h	4.2×10^8
Gram – bacteria	Mac Conkey agar (Merck, Germany)	35-37°C for 24-48 h	3.2×10^8
Lactic acid bacteria	MRS (Difco, USA)	35-37°C for 24-48 h	4.1×10^7
Yeasts and Moulds	MEA (Difco, USA)	25°C for 72 h	8.5×10^7