

**FRACTIONATION OF *HEVEA BRASILIENSIS* LATEX BY  
CENTRIFUGATION:  
(i) A COMPREHENSIVE DESCRIPTION OF THE BIOCHEMICAL  
COMPOSITION OF THE 4 CENTRIFUGATION FRACTIONS**

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**Abstract**

*Biochemical components naturally occurring in Hevea latex are known to impact both superior and inferior properties of natural rubber (NR) but the mechanisms are still not well elucidated up to now. Thus, we established a systematic study to identify and locate the main biochemical components of latex that might drive NR quality inconsistency and dynamic structuration. Fresh latex from RRIM600 and PB235 clones were fractionated into 4 fractions, including cream, skim, C-serum and lutoids, through successive centrifugation steps performed at various accelerations (g). The fractions were further analyzed to provide a comprehensive description of the biochemical composition in comparison with original whole latex. Comparison on a dry weight basis showed that skim was twice more concentrated in lipids and proteins than cream. For non-rubber fractions, lutoid was the richest in lipids, protein and minerals (mainly K and Mg). Similarly, serum also contained high protein and mineral content but no lipids. Nevertheless, when considering the mass balance of each fraction in order to identify the main location of each analyte, the obtained picture was different. Qualitatively, it was noticed that the profiles of biochemical component of fractions were not uniform and dissimilar to that of original latex. This information is essential to further study the quantitative impact of each component on NR structure and properties.*

*Keywords : Biochemical components; Fractionation; Hevea latex; Non-isoprene component*

**INTRODUCTION**

Natural Rubber (NR), a biopolymer produced from the latex of *Hevea brasiliensis*, exhibits very specific properties (low heat build-up, crystallization under strain, etc.) never mimicked by synthetic rubbers. However, NR presents important drawbacks: i) the rather non consistency or variability of its properties, ii) a structuration dynamics or “storage hardening” that is still not fully understood. From this context, an international project supported by French National Research Agency (ANR) called “RUBBex” was launched in 2014. The RUBBex project aims to study and identify the main biochemical components and the mechanisms involved in the structuration of NR in order to optimize the performances of raw NR. The main goal of the project is to generate new knowledge that will allow targeting new treatments before processing for a better control of the variability of NR properties and of the NR structuration dynamics with time. To achieve this goal, a multidisciplinary approach to study this material from fresh rubber particles (organization,

composition) to the raw NR (composition, structures, properties) was established in four scientific work packages.

The present work is dedicated to the identification and location of the main biochemical components of fresh latex so called non-isoprene compounds. These non-isoprene compounds accounts for around 10% of the dry matter of *Hevea* latex or about 5% of the raw dry NR derived from latex. They comprise proteins, carbohydrates, lipids, and inorganic constituents and represent the main composition difference between NR and its synthetic counterparts. The nature and quantity of these non-isoprene compounds can vary greatly depending on the ages, the clones, the season and the environmental conditions [1-5]. Numerous works have been carried out to analyze these compounds for their crucial role in latex biosynthesis as well as latex and NR properties [6-8]. In this work, we provided a comprehensive study of protein, lipid and mineral compounds including quantity and location (fraction) in whole latex which was further compared to the properties of rubber samples made from studied latex [9].

Methodologies for latex sampling, latex fractionation and extraction methods adapted to different properties of fractions and biochemical compounds were developed. The latex was harvested from certified trees of two *Hevea* clones, RRIM600 and PB235, in Chanthaburi province, Thailand. Samples were collected in high (October 2016) and low (January 2017) latex productivity periods. Tree tapping was performed early morning and latex was collected and stored on ice to maintain its native state before centrifugation upon arrival in the laboratory (Bangkok). Different centrifugation parameters were tested (acceleration and time) to provide an efficient method to isolate the four native fractions (cream, skim, serum and lutoids) of fresh latex. The successful separation of rubber fractions (cream and skim) was validated by the distinct particle size distributions measured by dynamic light scattering (DLS) (data not shown). Lutoids are known to be highly sensitive to pH change, osmotic shock, or too high-speed centrifugation. Therefore, their integrity was maintained with a mannitol solution whose concentration was adjusted according to the osmotic pressure of whole latex. After latex fractionation, only proteins were directly extracted from fresh fractions, while other biochemical compounds were extracted from solid samples. The fractions were therefore preserved using freeze drying method. Each compound from the four fractions was analyzed for their biochemical composition in comparison with its original latex. The quantitative and qualitative description of biochemical component, including proteins, lipids and minerals was provided for the four fractions. This information is essential for the subsequent work carried out with the samples fabricated from different combinations of fractions from the same latex origin [9]. Results of both studies will help to answer the possible quantitative effect of each compound on NR structure and properties.

## **MATERIAL AND METHODS**

### **1. Latex collection**

Clonal certified *Hevea brasiliensis* trees of RRIM600 and PB235 clones from a plantation of Visahakit Thai Rubber Co., Ltd., Chanthaburi, Thailand were selected. The trees were tapped in early morning (5 AM). One hour later, latex that dropped in a clean plastic cup placed on ice (4°C) was collected and stored in ice until further analysis in the laboratory (Bangkok). The samples were collected in October 2016 and January 2017.

### **2. Latex centrifugation and mass balance measurement**

The four native latex fractions (cream, skim, serum and lutoid) were separated via a 4-steps centrifugation method. Fresh latex was centrifuged at 16,000 g for 45 min at 4 °C (centrifugation C1). The top phase (cream fraction) was collected with a spatula while the middle liquid phase (skim suspended in serum) was isolated with a syringe. The serum/skim phase was then subjected to a second centrifugation at 45,000 g for 45 min at 4 °C (centrifugation C2) to separate the skim (top phase) from the serum (bottom phase). The bottom phase obtained after C1 (lutoids) was washed with a mannitol solution by a

third centrifugation at 16,000 g for 30 min at 4 °C (centrifugation C3). The integrity of luteoids was maintained with a suitable concentration of mannitol buffer adjusted according to the osmotic pressure of whole latex measured with an osmometer (Vapro model 5520, Wescor, USA). To get a purer serum fraction for further biochemical analysis, it was centrifuged at 57,000 g for 45 min at 4 °C (centrifugation C4). The mass balance of fractions was obtained by gravimetric method and expressed versus fresh weight of latex.

### **3. Freeze dried sample preparation**

All fractions were diluted to around 20% total solid content with distilled water except serum and stored at -20 °C overnight. The precooled fractions were subjected to freeze drying for 48 hours. The obtained freeze dried fractions were used for total nitrogen, total lipids and mineral analyses.

### **4. Protein analysis**

#### *4.1 Extraction of proteins*

Proteins were extracted from fresh fractions (except serum which was loaded directly into SDS-PAGE well) directly by mixing fresh fractions with extraction buffer (100 mM Tris, 100 mM EDTA, 10% glycerol, 2% Triton X-100, 20 mM DTT and 2 mM PMSF, pH 8.0) at a ratio of 1:2 (w/w) and the mix was agitated on rotating machine at 4°C for 40 min. The solution was then centrifuged at 20000 g for 30 min at 4 °C. The rubber phase was moved aside with a spatula to easily collect an intermediate phase containing proteins. The solution was centrifuged twice at 35000 g for 30 min at 4 °C to remove remaining rubber particles. The obtained protein extracts were stored at -20 °C for further analysis.

#### *4.2 Separation of proteins by 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

The extracted proteins from each fraction were separated through one dimension (*i.e.* molecular weight) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [10] and the gel was analyzed by CLIQS image analysis software (<http://totallab.com/cliqs/>).

#### *4.3 Total nitrogen content determination*

Freeze dried fractions were analyzed by Kjeldahl method to determine their nitrogen content. Known weights of sample were put together with catalyst tablets (Catalyst 1000, Thompson and Capper Ltd.) into digestion tubes and 15 ml of sulfuric acid (H<sub>2</sub>SO<sub>4</sub>, concentration 98%) were added. The digestion was operated at 200°C for 30 minutes and continued at 400°C for another 30 minutes. After digestion, samples were distilled in a Kjeldahl equipment (VAP 30, Germany). A 25 ml of 4% boric acid (added with bromocresol green and methyl red as color indicators) was used to entrap ammonium gas. Then, distilled solutions were titrated with 0.01N HCl solution until end point (pink color, pH 4.6) to determine the nitrogen content (% w/w dry sample).

### **5. Lipid analysis**

#### *5.1 Extraction of lipids*

Lipids of freeze dried samples were extracted with the method previously developed for dry rubber [11]. The pieces of 2.5 g of freeze dried fractions were ground under liquid nitrogen and transferred into 50 ml of chloroform: methanol (2:1; v/v). After 6 hours of agitation at 200 rpm, rubber was removed by filtration and total lipids was concentrated using rotary evaporator. Total extract was redissolved with 4 mL of the same solvent and water soluble components were removed with 1 mL of 0.9% NaCl solution. The lipid containing bottom layer was taken and evaporated to obtain the lipid extract.

## 5.2 Lipid class separation by solid phase extraction (SPE)

Neutral lipids (NL), glycolipids (GL) and phospholipid classes were separated. A Si SPE cartridge (900 mg, 45-150  $\mu\text{m}$ , 1 cm diameter x 2.7 cm length, VertiPak, Thailand) inserted in a 12-port vacuum manifold was activated by rinsing successively with 2.7 mL of methanol and 2.7 mL of chloroform. Lipid extract (0.5 mL of 60 mg.mL<sup>-1</sup> solution in chloroform) was loaded into the activated Si SPE cartridge. NL, GL, and PL were eluted with 8.1 mL of chloroform, 8.1 mL of a mixture of acetone/methanol (9:1; v/v) and 8.1 mL of methanol, respectively. The flow rate was controlled approximately at 3 mL.min<sup>-1</sup> using a vacuum pump. Lipid class was verified by thin layer chromatography (TLC) as described by Liengprayoon [12].

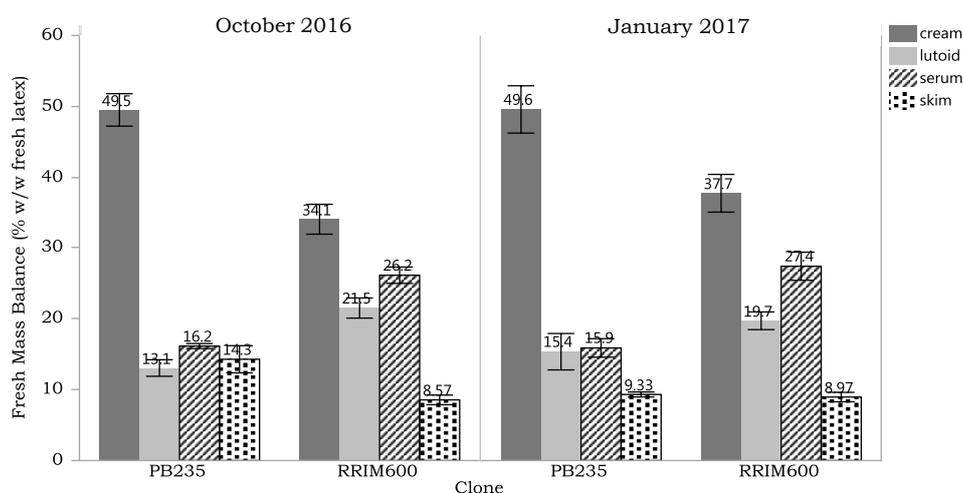
## 6. Mineral analysis

Minimum 50 mg of fractions in 10 ml of 50% nitric were mineralized using microwave reaction systems with 3 successive cycles: 1) 30 min at 140 °C; 2) 30 min at 170 °C and 3) 30 min at 190 °C. After mineralization, samples were diluted to reach 10% nitric acid concentration. Sulfur (S), phosphorus (P), potassium (K), magnesium (Mg) and calcium (Ca) in the samples were analyzed using inductively coupled plasma atomic emission spectroscopy (ICP-AES).

## RESULTS AND DISCUSSION

### Mass balance of the 4 centrifugation fractions of *Hevea* latex

The freshly tapped latex from two *Hevea* clones, RRIM600 and PB235 were separated into four fractions through successive centrifugations. The mass balance of each fraction (cream, serum, lutoids and skim) against fresh latex weight from two clones and two sampling periods is shown in Figure 1. Clonal differences were noticed with higher rubber content for PB235 (average of cream 49.5% and skim 11.8%) than for RRIM600 clone (average of cream 35.9% and skim 8.8%). In addition, no obvious seasonal effect was observed.



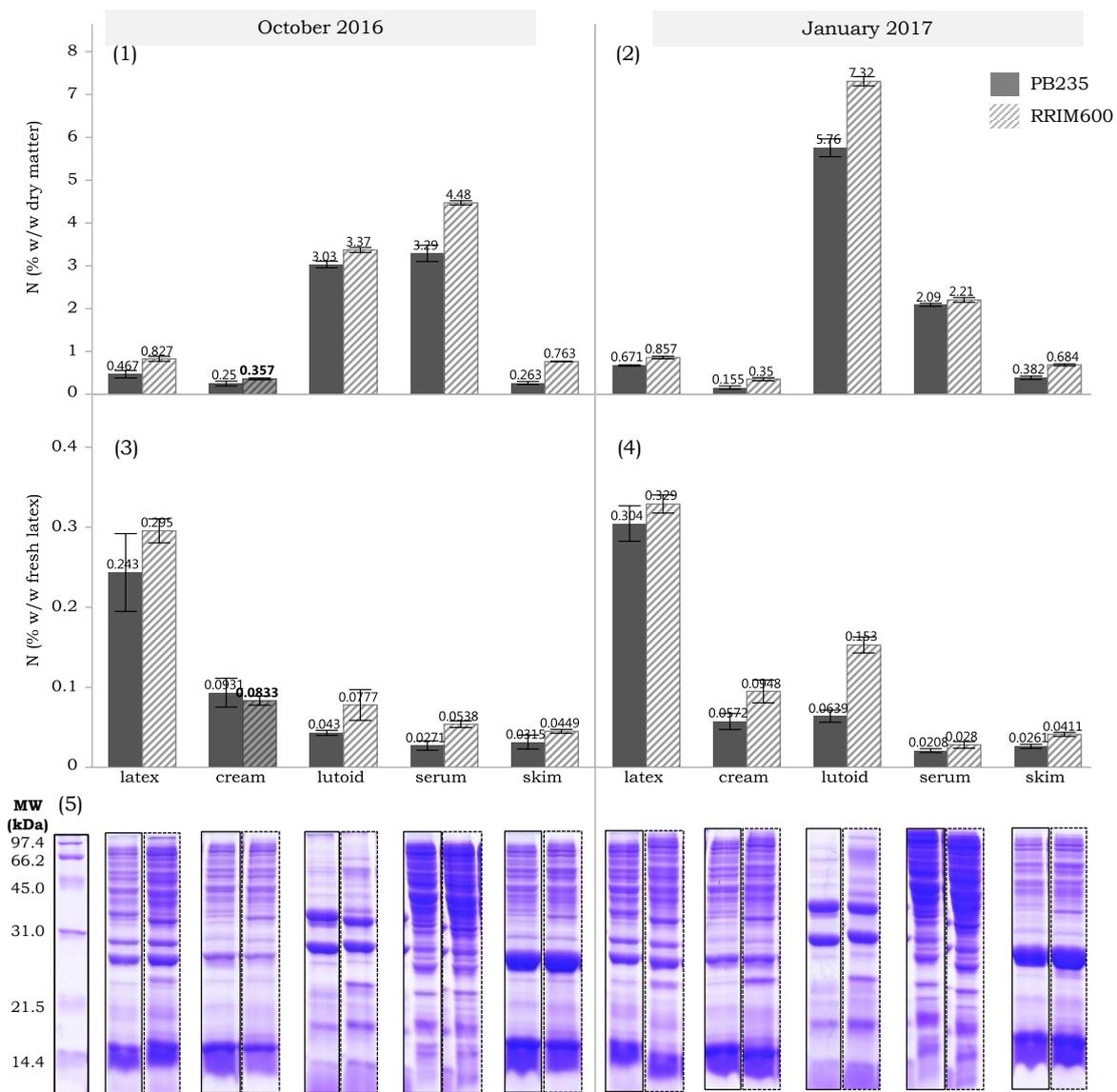
**Figure 1.** Mass balance of each fractions (%w/w/ fresh latex) from high (October 2016) and low (January 2017) latex productivity periods.

### Analysis of biochemical compounds of centrifuged latex fractions

Total nitrogen content of each fraction expressed versus dry matter and fresh latex weight are presented in Figure 2. The results on dry basis showed that lutoids and serum are the two fractions mostly concentrated in proteins. Both seasonal and clonal effects were

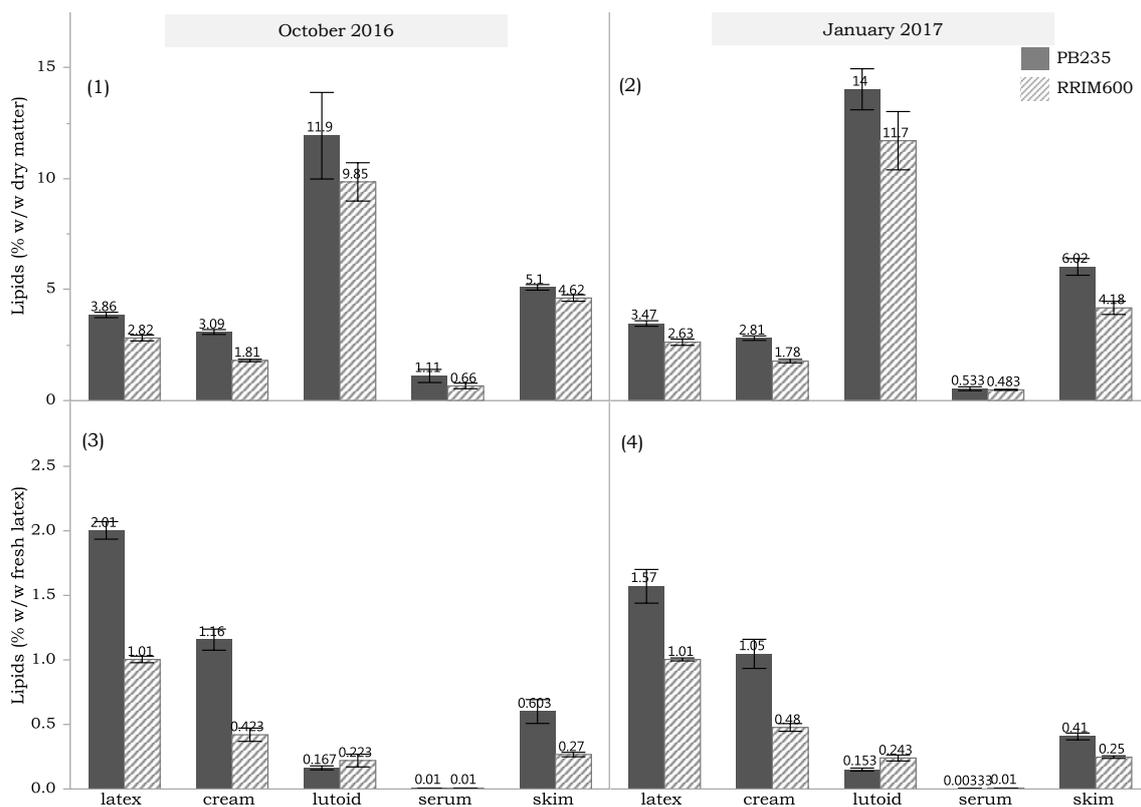
observed. From the period of high (October 2016) to low (January 2017) production of latex, the nitrogen content (% w/w dry matter) in lutoids increased from 3.03% to 5.76% for PB235 clone and from 3.37% to 7.32% for RRIM600 clone. A contrary tendency was observed for serum as its nitrogen content decreased in low latex productivity period (Figure 2 (1) and (2)). This high nitrogen content observed in October 2016 (around 4%) and a lower one in January 2017 (around 2%) are consistent with the work of Archer *et al.* [13] and d'Auzac *et al.* [14], respectively. As compared to other fractions, cream and skim contained much less nitrogen. Nevertheless, taking into account a large proportion of cream in fresh latex (Figure 1), cream and lutoids represent the major location of proteins (see results expressed versus fresh latex, Figure 2, (3) and (4)).

Proteins extracted from fresh fractions were qualitatively characterized using SDS-PAGE electrophoresis and obtained gels are presented in Figure 2 (5). For latex and its four fractions, the distribution of protein bands within gels seemed to be slightly affected by season or clone. However, in some cases, some specific bands were present or absent depending on clone or season. Image and proteomic analysis of SDS-PAGE gels are currently under progress and will later provide additional information to investigate possible differences originating from clone or season.



**Figure 2.** Total nitrogen content of whole latex and its four fractions expressed versus dry matter in October 2016 (1) and January 2017 (2) and expressed versus fresh latex in October 2016 (3) and January 2017 (4); SDS-PAGE protein profile of each latex fraction of PB235 and RRIM600 clones (5).

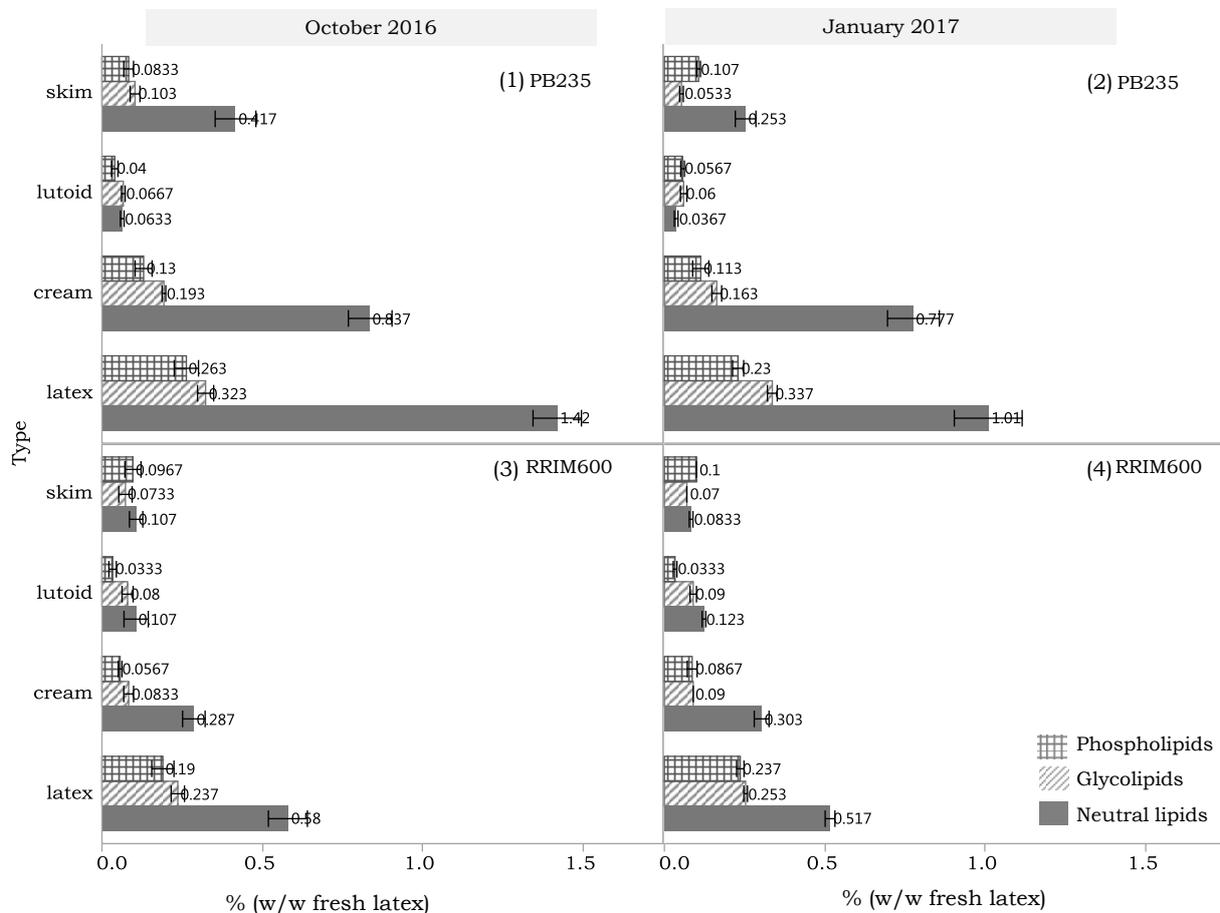
A common feature was noted for both clones and seasons: different protein profiles were clearly highlighted between fractions. Both cream and skim fractions displayed an intense band at 14.7 kDa suggesting the presence of rubber elongation factor (REF, 14.6 kDa) in these bands. For skim, a more intense band was visible around 27.7 kDa suspected to contain small rubber particle protein (SRPP). For cream, this band was much less intense. These observations are in agreement with the presence of REF on mainly large rubber particles (cream) and SRPP only on small rubber particles (skim) [15-18]. In the lutoid fraction, two intense protein bands were highlighted, as observed by other authors [19; 20]. Molecular weights of 28.0 and 32.8 kDa were assigned to these bands suggesting that they respectively contain hevamine (chitinase, 29 kDa) and  $\beta$ -1-3 glucanase (32-35 kDa), two enzymes identified in lutoids [21; 22]. Note that hevein (4.7 kDa), the main protein of lutoids [23] did not appear on gels due to non-adapted migration conditions. For serum, while many protein bands were concentrated in the region of high molecular weights (97-25 kDa), several protein bands appeared below 25 kDa as well. This is in agreement with authors detecting serum protein bands in a large range of molecular weights (12-186 kDa) [24].



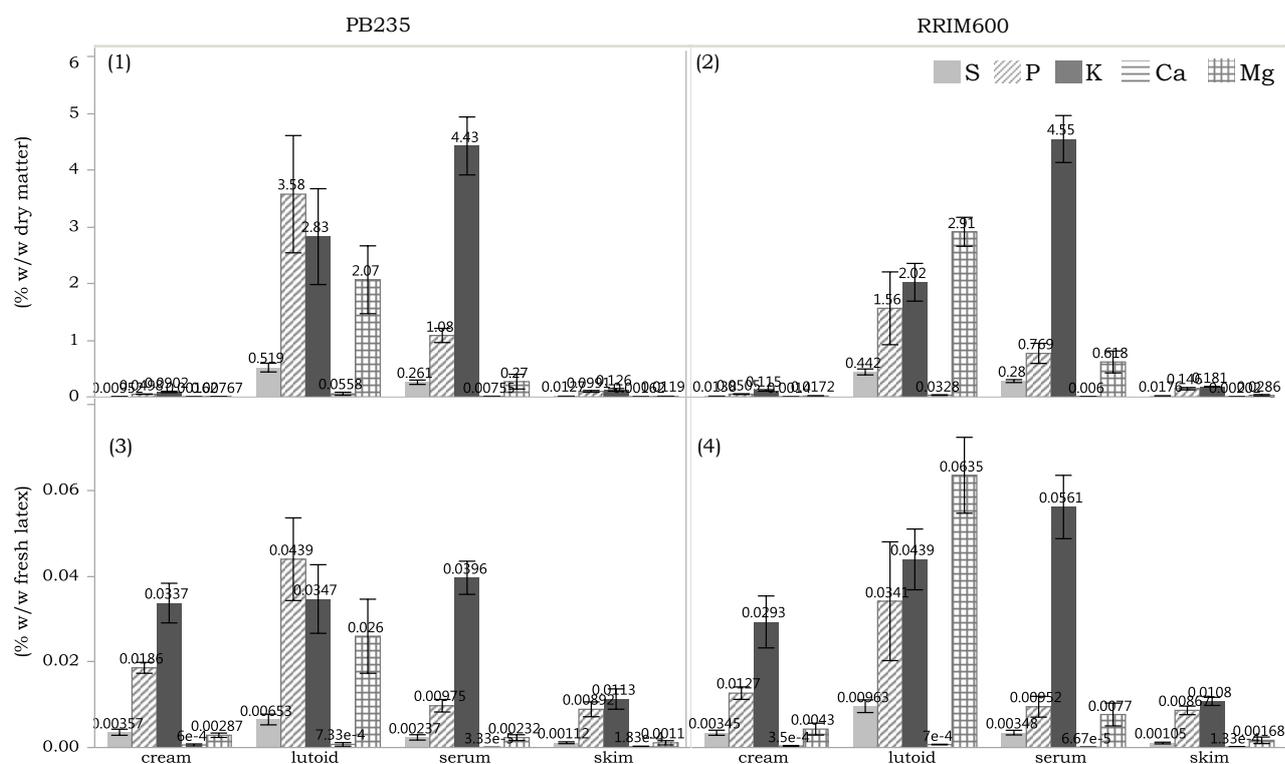
**Figure 3.** Total lipids content (%w/w dry matter) from whole latex and fractions in October 2016 (1) and January 2017 (2) and deduced total lipid content (%w/w fresh latex) in October 2016 (3) and January 2017 (4).

For lipids, the clonal effect was more important than the seasonal one as previously reported (Figure 3) [4]. Every fraction from PB235 latex had higher lipid content than that of RRIM600 clone. Only negligible lipid quantity was detected in serum. Indeed, as a hydrophobic compound, this amount of lipids in serum might be due to contamination from other fractions. In the same manner as proteins, lutoids was the most concentrated in lipids (up to 10% w/w dry matter). It was observed that skim contained two times more lipids than cream for both clones (Figure 3 (1) and (2)). Specific surface of the lipid-containing particle membranes that is higher in small particles than in large ones may explain partly this difference. However, considering its larger proportion in fresh latex, the cream fraction was still the main location of lipids in whole latex.

Quantitative comparison of lipid classes (neutral lipids, glycolipids and phospholipids) from each fraction and from original latex is shown in Figure 4. For PB235 clone, lipid classes of latex, and rubber particle-containing fractions (cream and skim) were similarly mainly composed of neutral lipids. This clone has been reported to have a high content of triacylglycerols of furan fatty acids (TGF) [4] which belong to the neutral lipid family. TGF might be present in rubber particles or stored in specific lipid droplets (LDs). A LD is described as an organic core comprising neutral lipids (mainly triacylglycerols and sterol esters) that are surrounded by a phospholipid monolayer [25]. In both cases (specific LDs or rubber particles), considering the density of TGF and the size of plant LDs, the location of TGF should be in the upper fractions (cream or skim) which can explain why both fractions are enriched in neutral lipids for PB235 clone. Meanwhile lutoids contained similar quantities of all classes (approximately one third of each) (Figure 4 (1 and 2)). For RRIM600 clone, latex and cream lipid profiles are also similar (mainly neutral lipids) but that of skim contained same amount of neutral lipids and phospholipids. Lutoids contained same amount of neutral lipids and glycolipids (Figure 4 (3 and 4)). Phospholipids have been reported to be a structural lipid of rubber particles and lutoid membrane [26; 27]. In fact, glycolipids and some neutral lipids (such as sterols) are also often reported to take part of membranous structures [28; 29], but they are barely mentioned as component of *Hevea* rubber particle membrane as compared to phospholipids.



**Figure 4.** Lipid classes (%w/w fresh latex) of PB235 fractions and latex from October 2016 (1) and January 2017(2) and that of RRIM600 from October 2016 (3) and January 2017 (4).



**Figure 5.** Averaged mineral content of PB235 (1 and 3) and RRIM600 (2 and 4) fractions from two samplings expressed in dry weight basis (%w/w dry matter) and fresh latex weight basis (%w/w fresh latex)

Mineral content in dry weight basis indicated that B-serum-containing lutoids and serum were the two major sources of minerals for both clones (Figure 5). Lutoids of PB235 clone was rich in phosphorus (P: 3.58% of dry matter), potassium (K: 2.83%) and magnesium (Mg: 2.07%) while potassium was the major mineral in serum (4.43% or 73% of all detected minerals in this fraction). The mineral profile in serum of RRIM600 clone was consistent with the work of d'Auzac et al. [14]. Nevertheless, reverse order of minerals was found in lutoids as compared to PB235. Mg (2.91%) was the main mineral in lutoids of RRIM600 clone followed by K (2.02%) and P (1.56%), respectively.

## CONCLUSION

Fractionating complex whole *Hevea* latex into its four fractions using centrifugation allowed us to locate the main non-isoprene components within the fractions. Seasonal and clonal impacts were also studied. The analyses of biochemical composition of cream, skim, lutoids and serum indicated a heterogeneous distribution and profile of lipids, proteins and mineral. When the analyte concentrations are expressed versus dry matter, lutoids and serum fractions are the richest in all biochemical compounds. When considering the mass balance of each fraction in order to identify the main location of each analyte, the obtained picture was different: lipids are mainly located in rubber-containing fraction (skim and cream), mineral in serum-containing fractions (serum and lutoids) and nitrogen (protein indicator) in lutoid and cream fractions. Further repetitions are on-going to consolidate this first set of data.

This study provides complementary basic knowledge on the complex biochemical composition of natural rubber latex which can feed upstream (biosynthesis) and downstream (e.g. postharvest phenomena) investigations. Moreover, it provides detailed information to the next study, presented as a second part of this publication that consisted in using the very same latex fractions to make ADS rubber samples and assess their structure and physical properties.

## ACKNOWLEDGEMENT

The authors thank ANR agency for financial support of the RUBBex-ANR 14-CE07-0026-02 project. Thailand International Cooperation Agency (TICA) is acknowledged for financial support of the field trips. The authors would like to thank Visahakit Thai Rubber Co., Ltd. for kindly providing samples. Agropolis Fondation is thanked for the support to the creation of LipPolGreen-Asia platform in Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Bangkok. This work was undertaken as part of the Hevea Research Platform in Partnership (HRPP).

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