IMPACT OF STORAGE TIME OF AMMONIA-STABILIZED LATEX ON BIOCHEMICAL AND PHYSICOCHEMICAL INDICATORS OF *HEVEA BRASILIENSIS* LATEX

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Abstract

As ammonia addition is the most widespread latex stabilization method, we investigated the impact of ammonia-stabilization on biochemical and physicochemical indicators of latex in liquid and dry state (thin film) at different storage times (0 to 41 days). For liquid state, measured indicators included surface tension, pH, dry rubber content, total solid content, lipid and protein contents. In addition, semi-quantitative data on lipids and proteins were obtained by TLC and SDS-PAGE, respectively. For latex at dry state, thin films were investigated by AFM to unravel their morphology as well as their elastic and adhesive properties. Analyses were completed by infrared spectroscopy and wettability measurements on non-washed and washed latex films to investigate the impact of film washing as well. The ammonia-stabilization method was shown to quickly and significantly impact the lipid and protein contents resulting in drastic changes regarding the morphology and properties of films.

Keywords: ammonia-stabilization method; Hevea brasiliensis latex; latex biochemical indicators; latex thin film properties; stabilized-latex storage time.

INTRODUCTION

Naturally occurring non-isoprene molecules (*i.e.* lipids, proteins, carbohydrates and minerals, [1]) present in *H. brasiliensis* latex (13% w/w dry latex) and natural rubber (NR) (6% w/w dry NR) are more and more suspected to be involved in the final NR properties [2-5]. A better control of non-isoprenes at latex stage would certainly help to regulate their influence in NR structure and thus properties. However, this task is challenging due to the rapid (few hours) and irreversible coagulation of field latex after tapping.

Several means exist to preserve latex at liquid state including ammonia, sodium sulphite, formalin and tetra methyl thiuram disulphide (TMTD) or zinc oxide (ZnO) [6]. Ammonia has been used in the rubber industry for decades as a stabilizer, regardless of its odor and toxicity. Other products are being developed in industrial laboratories for environmental reasons [7], but their prices remain prohibitive compared to ammonia and their effectiveness remains lower. Ammonia is usually used as the main preservative in both field and concentrated latex. Concentrated latex can be categorized into three types based on ammonia content in latex. Low-ammonia (LA) latex contains ammonia not more than 0.29% (by weight), medium-ammonia (MA) is added with ammonia between 0.30% and 0.59% (by weight), and high-ammonia latex (HA) contains at least 0.60% ammonia (by weight) calculated with respect to the latex [8].

As the most widespread preservation method, latex stabilization by ammonia has been subjected to numerous studies. In 1941, Kemp *et al.* demonstrated that contents of soluble and gel fractions obtained after solubilization of latex films were affected by ammonia treatment, thus resulting in different physical properties, as judged by x-ray and viscosity technics [9]. Latex stabilization by ammonia was later shown to promote the hydrolysis of proteins and lipids. The hydrolysis reactions result in polypeptides, phosphate anions and

long anionic acyl chains (fatty acids). Free fatty acids adsorbed on the surface of rubber particles contribute to the formation of negative charges around them leading to an increase of latex colloidal stability [10,11]. Ho *et al.* deeply investigated the surface of rubber particles in high-ammoniated latex and concluded that the carboxyl groups of surface-adsorbed fatty acids mainly contribute to the increased colloidal stability of latex in comparison to negative charges from adsorbed proteins [12-14]. Proteins in ammoniated latex were essentially studied for their allergenic properties [15-18] as concentrated latex (HA-latex with ammonia content ≥ 0.6%) is used for manufacturing various medical products (gloves, etc.).

The storage time of ammonia-stabilized latex was previously shown to affect both latex composition [11,16] and physical properties [19]. In this study, we investigated the impact of storage time of ammonia-stabilized latex (0.6% NH_3 by weight) on both biochemical and physicochemical indicators in liquid (latex) and dry state (film). For liquid latex, measured indicators included surface tension, pH, DRC, TSC, lipid and protein contents. In addition, semi-quantitative data on lipids and proteins were obtained by thin layer chromatography and SDS-PAGE electrophoresis, respectively. For latex at dry state (latex film), the morphology, surface modulus and surface adhesion of latex films were characterized by atomic force microscopy (AFM) whereas infrared spectroscopy (IR) brought details on composition. The effect of water washing of latex films was also studied. Indeed, the washing process remains a crucial step in the rubber industry and it is thus required to better understand the chemical or biochemical species that might be impacted during this step.

MATERIAL AND METHODS

1. Field latex collection

Hevea brasiliensis rubber trees of certified RRIM600 clone planted in 2002 and taped for the first time in 2009 were selected from a plantation belonging to Visahakit Thai Rubber Co., Ltd., Chanthaburi, Thailand. The tapping system was S/2, 2d/3 since year of opening. Crushed ice was placed in the collection cups of trees which were tapped early morning (5:00 AM) on May 12th 2017. The 10 first drops of latex were discarded and late was then collected in a clean plastic cup placed on ice to keep latex cold during the collection time (1 to 2 hours). When the target volume of latex was reached, latex was filtered through a stainless steel sieve (2 mm pore size) and kept on ice until arrival in laboratory (~3 hours). Presented results are the mean of two independent repetitions made from 2 lots of latex originating from two distinct groups of 13 trees in the same field.

2. Field latex stabilization by ammonia

Fresh latex was stabilized with ammonia solution at a final concentration of 0.6% NH_3 w/v. The concentration of ammonia solution used for stabilization was checked by titration just before stabilizing latex.

3. Storage conditions of stabilized-latex

Each lot of stabilized latex was separated into two samples of equal volumes to be stored in Thailand (LBTNR, Bangkok) and France (IS2M, Mulhouse). Both samples were stored in similar conditions: samples were protected from light and kept in a water bath at 35°C. Experiments were simultaneously carried out in Thailand and France at different storage times (0 to 41 days). Film characterizing was performed on the same day as film preparation to prevent the film from ageing. This protocol allowed considering only the effect of the storage time of ammonia-stabilized latex and getting rid of the effect of film ageing which might induce morphological and/or property modifications.

4. Thin stabilized-latex film preparation

Non-washed latex films were prepared by spreading latex on glass supports (1 cm^2, 2.5 µL/support) and by letting the films dry at room temperature until complete drying, *i.e.* when films became transparent. To prepare washed films, latex films were washed for 30 minutes in a water bath at 50°C and dried under cold air until complete drying.

5. Biochemical indicators of latex

5.1 Dry Rubber Content (DRC) and Total Solid Content (TSC)

Dry rubber content (DRC) and total solid content (TSC) of latex was determined according to ISO 126:1995 [20] and ISO 124:1992 [21] international standards, respectively.

5.2 Nitrogen content

Dry latex thin films 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_2SO_4, 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).

5.3 Characterization of extractible proteins by Bradford method and SDS-PAGE electrophoresis

Proteins were extracted from field and ammonia-stabilized latex directly by mixing latex 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 [22]. The obtained protein extracts were kept at -20°C until they were used for further analysis.

Protein concentration in extracts was determined according to Bradford's procedure by using bovine serum albumin (BSA, Sigma) as reference protein [23]. Briefly, the calibration curve was obtained by measuring with a spectrophotometer (Shimadzu UV-1800) the absorbance at 595 nm of BSA solutions with amounts varying from 0 to 10 µg. This calibration curve was later used to determine the protein concentration of extracts and results were expressed in µg equivalent BSA.

Proteins were separated by 1D SDS-PAGE electrophoresis, performed at 13% acrylamide resolving gel on a Thermo Fisher apparatus regulated at 15°C. Volumes of 7.5 µL of protein extracts were loaded per well. Running was first conducted at 15 mA per gel all along the stacking gel and 20 mA per gel until the run ends. Gels were then stained with colloidal CBB R-250 (Biorad). A low molecular weight calibration kit (Biorad), ranging from 14.4 to 97.0 kDa, was included in each electrophoretic run. Image elaboration and analysis were carried out with Cliqs 1D software (http://totallab.com/cliqs/). Image analysis was used to assign molecular weight to the staining bands and to calculate its "volume". This volume (product of the band area by the intensity of each pixel within this area) is proportional to the protein amount in each band provided saturation of the coloration has not been reached.

From the SDS-PAGE gel of field latex (control), two groups of bands (N°1 and N°4) and four bands (N°2, N°3, N°5, N°6) were selected and their volume variations (%) over storage time were normalized with regard to the control.

5.4 Lipid content

Lipids were extracted from fresh latex as previously described [24]. Some modifications were made for ammonia-stabilized latex. A volume of 10 mL of stabilized latex was diluted with distilled water at the ratio of 1:1. The diluted latex was mixed with 100 mL of chloroform: methanol (2:1, v/v) by adding dropwise in to continuously stirred solvent for 4 min. After that, pH of the mixture was adjusted to 3 with 2N HCl. The bottom chloroform phase containing lipid was taken using micropipette and the coagulum was rinsed 3 times with 5 ml of the same solvent. In a separating funnel, the lipid containing phase was washed with 1/5 of its volume of 0.9% NaCl solution. After decantation, the organic phase was concentrated by evaporation. Lipid extracts were weighed and dissolved in chloroform for further analysis.

5.5 Characterization of extractible lipids by Thin Layer Chromatography (TLC)

Neutral lipid profiles of extractable lipids from fresh and stabilized latex were compared. A volume of 5 uL of solution of lipids, at a concentration between 1 and 10 $mg.mL^{-1}$ that is proportional to lipid extract, was deposited on silica gel 60G TLC plate and placed in TLC chamber. Mobile phase for neutral lipid separation was composed of n-hexane/ diethyl ether/ acetic acid (80:20:1; v/v/v). The developed TLC plate was sprayed with a mixture of 40% orthophosphoric acid and saturated copper acetate aqueous solution (1:1; v/v), then heated at 180 °C for 10 min in an oven. The lipid spots appear brownish on a white background.

5.6 Quantification of free fatty acid with Rhodamine-6-G

Free fatty acids in lipid samples were quantified by the adaptation of the method described by Van Autryve *et al.* [25]. The quantification was based on the specific complexation of free fatty acid with rhodamine 6G which was measured at a wavelength of 513 nm. Lipid extracts was diluted to 20 $\mu g.mL^{-1}$ with n-hexane. Under a fume hood, 0.5 mL of Rhodamine 6G (0.025% w/v solution in toluene) was added to 3.5 mL of diluted sample in a test tube. Before absorbance measurement using a spectrophotometer (Model UV-VIS 1800, Shimadzu Tokyo, Japan), the solution was left for 5 minutes for color stabilization. Linoleic acid was used as a calibration standard. Results were presented as the fatty acid content variations (%) over storage time normalized with regard to the control (fresh latex).

6. Physical properties of liquid latex and thin latex films

6.1 Pendant drop and sessile drop methods applied on latex and thin latex films

Wettability measurements were performed on a Drop Shape Analysis (DSA 100) from Krüss®. Pendant drop method was used to determine latex surface tension. Sessile drop method was applied to find out components (polar and dispersive parts) of latex and thin latex films.

A drop of polar (water) and non-polar (diiodomethane) liquids were deposited on thin latex films (washed and non-washed). A latex drop was deposited on the Polytetrafluoroethylene (purely non-polar) from Goodfellow®. Then, contact angle was measured on different surfaces. To calculate polar and dispersive components of latex solution from dry latex films, we used a previously described method [26-29]. This method is standard for calculating the surface free energy of a solid from the contact angle with several liquids.

To calculate Polarity percentage of latex thin film we used equation 1 below, where γ_L is the surface tension and γ_L^P the polar component of the latex film:

$$\text{Equation 1: } \frac{\gamma_L^P}{\gamma_L} X100$$

6.2 Atomic Force Microscopy (AFM) applied on thin latex films

The latex solution was deposited onto a glass slide by drop-casting. Microscale adhesion was measured by a Bruker Multimode 8 AFM with the Peakforce Quantitative Nanomechanics (PeakForce QNM) mode. PeakForce QNM mode is a recent advancement in AFM method providing quantitative nanomechanical mapping mode with the simultaneous measurement of the sample's adhesion between tip and sample surface, Young's modulus (DMT model), deformation and energy dissipation along with the surface topography.

QNM etched silicon probes were used with a nominal spring constant k ~ 6 N/m. All used tips were calibrated according to Bruker's recommendations. The results were analyzed by Gwyddion software. Each sample have been scanned over a 5x5 μm^2 at least in 3 different areas. Adhesion and Young Modulus were investigated as mechanical properties.

6.3 Infrared spectroscopy applied on thin latex films

Latex films were analyzed by transmission on a IFS66S from Bruker®. Germanium crystal was used as a reference and subtracted to the same crystal with latex film. A previously described method was used for data processing [30].

RESULTS AND DISCUSSION

Protein and lipid contents of ammonia-stabilized latex evolve dramatically during storage

Indicators including pH, DRC (dry rubber content) and TSC (total solid content) were regularly recorded over storage time of ammonia-stabilized latex and are gathered in **Table 1**. While fresh latex exhibited a pH of 6.6, alkalinity of stabilized latex was strongly increased at day 0 after addition of ammonia (0.6% by mass) reaching a value of 10.5. Then pH values remained constant over the storage period with a pH of 10.1 measured after 41 days of storage. At day 0, DRC and TSC values measured on fresh and ammonia-stabilized latex indicated that ammonia-preservation method did not affect the dry rubber nor total solid contents which remained constant until 41 days of storage.

Table 1. pH, DRC and TSC values of fresh latex measured on the day of collection (0 day storage) and evolution of pH, DRC and TSC values of NH_3-stabilized latex over storage time. SD: standard deviation.

Type	Storage Time (days)	pH (± SD)	DRC (%) (± SD)	TSC (%) (± SD)
Fresh latex	0	6.6 ± 0.1	34 ± 3	38 ± 3
0.6% NH3-stabilized latex	0	10.5 ± 0.1	33 ± 3	37 ± 3
0.6% NH3-stabilized latex	4	10.2 ± 0.1	34 ± 2	38 ± 3
0.6% NH3-stabilized latex	6	10.2 ± 0.1	33 ± 2	37 ± 2
0.6% NH3-stabilized latex	10	9.8 ± 0.1	33 ± 3	37 ± 3
0.6% NH3-stabilized latex	12	9.8 ± 0.1	33 ± 3	37 ± 3
0.6% NH3-stabilized latex	17	10.1 ± 0.1	33 ± 3	37 ± 4
0.6% NH3-stabilized latex	19	10.3 ± 0.1	33 ± 2	37 ± 2
0.6% NH3-stabilized latex	25	10.1 ± 0.1	33 ± 3	37 ± 3
0.6% NH3-stabilized latex	41	10.1 ± 0.1	32 ± 2	36 ± 3

Ammonia-stabilization method is known to impact the latex composition mainly by hydrolysis reactions of proteins and phospholipids/glycolipids [11,16]. In this work, both quantitative and qualitative analysis were carried out to understand whether specific molecular species were affected by the ammonia-preservation treatment. Lipids and proteins were regularly extracted over the storage period of latex and extracts were analyzed by TLC and SDS-PAGE methods, respectively.

The total lipid content of fresh latex at day 0 was 3.24% ± 0.32% (w/w dry rubber), in agreement with data previously reported for RRIM600 clone, *i.e.* 3.36% [30]. The total lipid content of ammonia-stabilized latex was shown to decrease over the storage period to reach a value of 2.22% ± 0.49% (w/w dry rubber) after 41 days of storage. Lipid extracts were deposited on TLC plates and the lipid profiles of both independent repetitions are presented in **Figure 1**. Note that a contamination by poly-isoprene was clearly detected through the dark smear on top of TLC plates. This spot was almost absent from fresh latex profile. The obtained result of lipid extracts (data not shown) were therefore not interpretable as part of the "lipid" weight is made of contaminating poly isoprene.

Several spots were detected on TLC plates corresponding to sterol ester, triglycerides, α- and γ-tocotrienol, free fatty acids and free sterols. Two main effects were visually identified: an increase of free fatty acid content attributed to hydrolysis reactions of fatty acid-containing lipids and a decrease of α- and γ-tocotrienol over storage time, as previously observed [31]. Those effects appear very quickly, as soon as 4 days of storage. Tocotrienols were reported to act as antioxidants in NR [32] and to increase the heat resistance of raw and vulcanized NR [33]. The decrease of tocotrienol over storage time of ammonia-stabilized latex indicates a decrease of the pool of native antioxidants contained in latex.

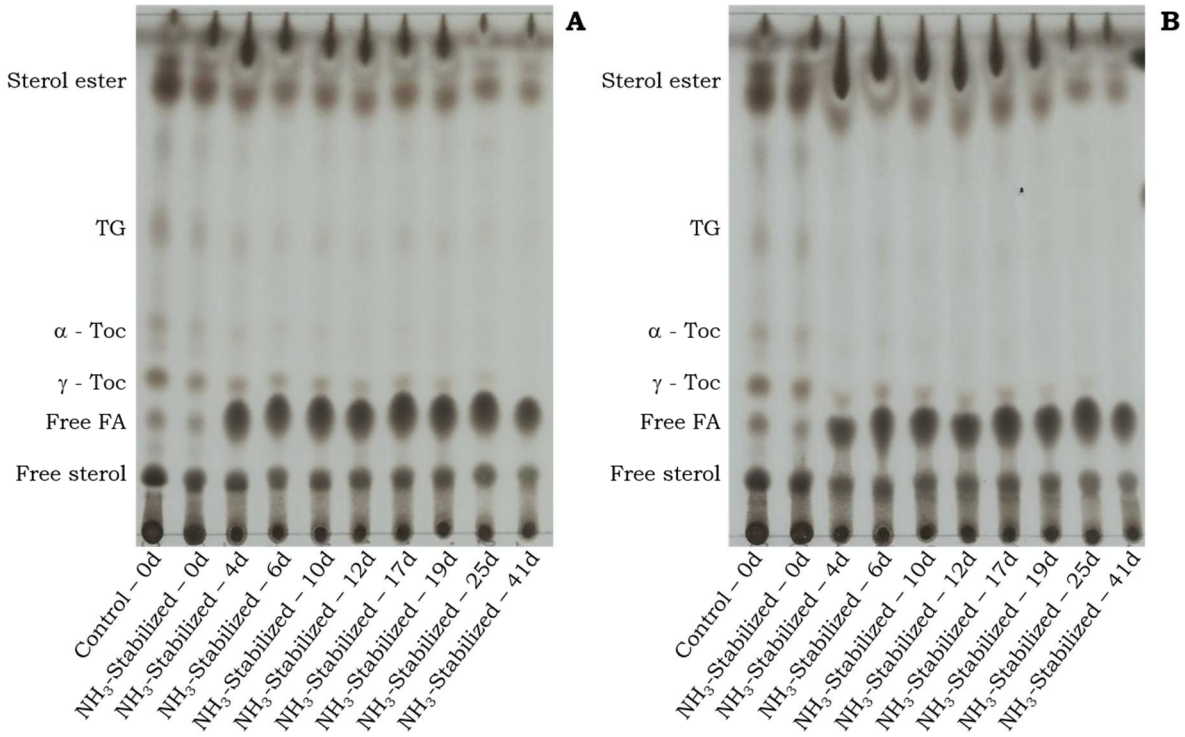

Figure 1. TLC plates of repetition 1 (A) and repetition 2 (B) showing the lipid profiles of field latex (control, 0 day only) and ammonia-stabilized latex from 0 to 41 days of storage. Standard deposit allowed to assign spots to known species: sterol esters, triglycerides (TG), alpha-tocotrienol (α-Toc), gamma-tocotrienol (γ-Toc), free fatty acids (FA) and free sterols.

The variation over storage time of fatty acid content (% w/w total lipids) was quantified in ammonia-stabilized latex and is presented in **Figure 2**. The rather high standard deviations of fatty acid content expressed versus total lipid extract is due to the variable contamination of that extract by polyisoprene. Despite this effect, a clear increase a fatty acid content was observed. The rise in fatty acids tended to 200% after 41 days of storage, *i.e.* the level was tripled as compared to fresh latex. As Chen *et al.*, we observed a rapid increase of fatty acid content within the 10 first days of storage then it seems to tend to a limit [11]. These authors observed for RRIM701 clone a fatty acid content which was doubled in ammonia as compared to fresh latex.

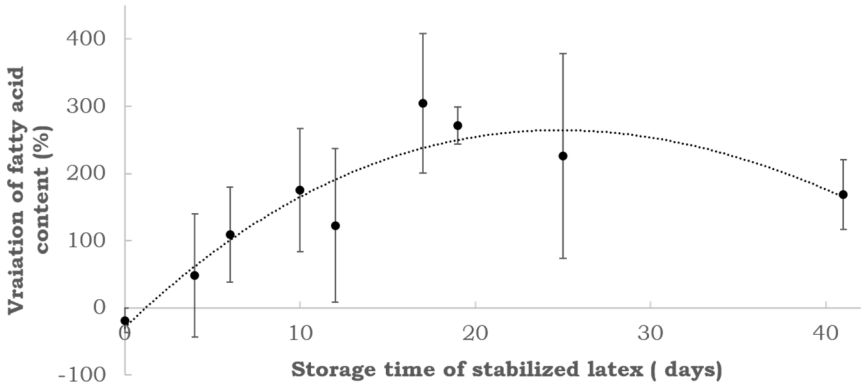

Figure 2. Variation (%) of the fatty acid content over storage time. Error bars correspond to standard deviations.

Proteins were the second biochemical indicator to be analyzed in this work. Nitrogen contents were measured in both non-washed and washed field latex films by Kjeldahl method and were found to be 0.68 ± 0.04 and 0.59 ± 0.04 % w/w dry latex, respectively. The washing of latex films resulted in a nitrogen loss of 13% which is lower than the 34% of nitrogen leached when latex is processed into unsmoked rubber sheet (USS) [34]. This indicates that latex washing with mechanical stress induces a higher leaching of nitrogen.

Kjeldahl method could not be applied to ammonia-stabilized latex as ammonia addition affects their nitrogen content. Thus, extractible protein contents of ammonia-stabilized latex over storage time were monitored in protein extracts by Bradford method and are presented in **Figure 3A**. Field latex displayed a content of 4000 µg protein/g rubber, very close from the one determined by Ratnayake *et al.* [18]. At 0 day, the extractible protein content of ammonia-stabilized latex was slightly impacted (3500 µg protein/g rubber) as compared to field latex while it decreased rapidly within the 12 first days of storage until it reached a plateau at 2500 µg protein/g rubber.

Using the DRC of field latex, the protein content in field latex could be expressed versus fresh latex, *i.e.* 0.14 % w/w fresh latex. Similarly, using the DRC value as well as the factor of 6.25 to transform nitrogen content into protein content, the nitrogen content of non-washed field latex films determined by Kjeldahl (N % w/w dry latex) was expressed in protein % w/w fresh latex and was found to be 1.44 %. Thus, it appeared than only a tenth of proteins were extracted from fresh latex. This low extraction yield could be explained by a large part of particle-bound proteins that would be eliminated during protein extraction process, as previously quantified by Ratnayake *et al.* [18]. Moreover, caution has to be taken when comparing results from different analytical methodologies: Bradford calibrated with BSA versus nitrogen determination multiplied by arbitrary factor (6.25).

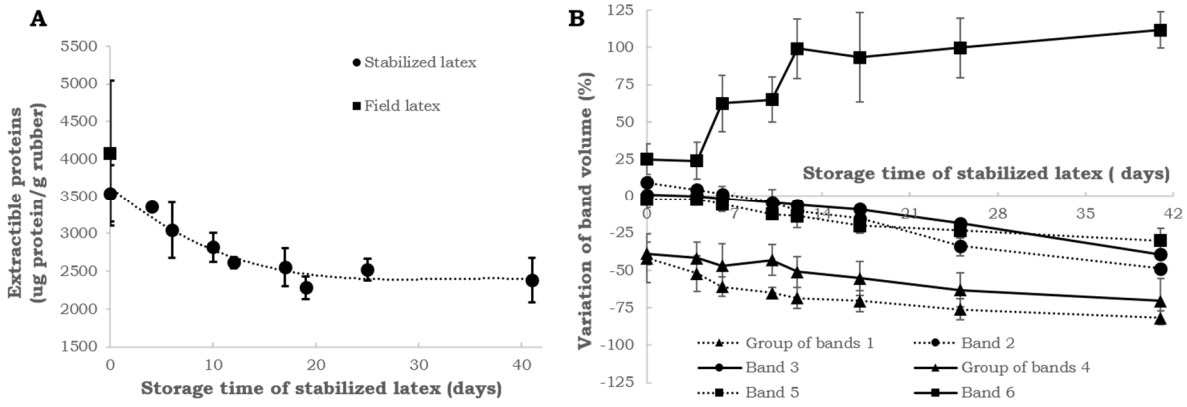

Figure 3. A) Concentration of extractible proteins of field latex (control, 0 day only) and ammonia-stabilized latex from 0 to 41 days. B) Variation (%) of the volumes of the six selected protein bands or group of bands shown in Figure 4B. Error bars correspond to standard deviations.

SDS-PAGE electrophoresis gels of both repetitions are presented in **Figure 4**. Numerous protein bands were observed in fresh latex (control, **Figure 4A**). These bands were categorized as shown by black dotted frames in **Figure 4B**. Many bands of high molecular weights were detected within the 97-31 kDa region (group of bands N°1). Then, two well defined and intense bands were detected at 29.4 and 27.3 kDa (N°2 and N°3, respectively). A strong band (N°5) was observed at 14.7 kDa while a band located at 12.5 kDa (N°6) appeared clearly from 12 days of storage. At 0 day, visual observation of gels indicated that the stabilization of latex by ammonia does not impact the protein composition in agreement with similar extractible protein contents measured on both field and ammonia-stabilized latex (**Figure 3A**). After 4 days of storage, a strong decrease in intensity was observed for groups of bands N°1 and N°4. In contrast, single bands N°2, N°3 and N°5 seemed to resist better to ammonia treatment as they were still clearly visible after 41 days of storage.

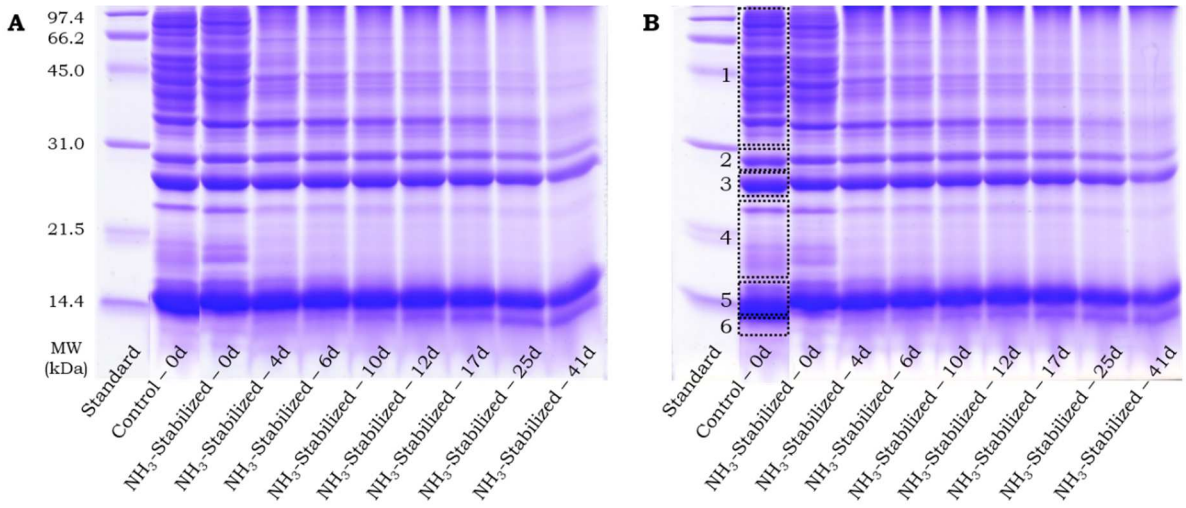

Figure 4. SDS-PAGE gels of repetition 1 (A) and repetition 2 (B) showing the protein profiles of field latex (control, 0 day only) and ammonia-stabilized latex from 0 to 41 days of storage. Molecular weights of used standard are indicated on (A). The six selected groups of bands (N°1: 97-31 kDa and N°4: 26-17 kDa) and bands (N°2: 29.4 kDa, N°3: 27.3 kDa, N°5: 14.7 kDa N°6: 12.5 kDa) are indicated on (B) by black dotted frames with corresponding numbers.

The volumes of the 6 protein bands shown in **Figure 4B** were determined by image analysis and their variation over storage time are plotted in **Figure 3B**. The two groups of protein bands N°1 (dotted line, triangle markers) and N°4 (solid line, triangle markers) were strongly impacted by ammonia. As early as day 0, the volumes of both groups of protein bands of ammonia-stabilized latex were reduced by 40% and decreased until 80% (N°1) and 70% (N°4) after 41 days of storage. Our data are in agreement with the diminution of protein bands in the high molecular weight region observed by other authors [35,36]. Moreover, the SDS-PAGE profile of serum fraction displayed many bands in the high molecular weights region [37] suggesting that the rapid decrease of protein bands in group N°1 was mainly attributed to hydrolysis of hydrophilic serum proteins.

As observed on SDS-PAGE gels, the 3 bands N°2 (dotted line, circle markers), N°3 (solid line, circle markers) and N°5 (dotted line, square markers) were much less affected; after 41 days of storage, their volumes were decreased by 50%, 40% and 30%, respectively (**Figure 3B**). According to their assigned molecular weights, bands N°3 (27.3 kDa) and N°5 (14.7 kDa) were assumed to contain SRPP (Small Rubber Particle Protein) and REF (Rubber Elongation Factor), respectively. These two proteins are known to be located at the surface of rubber particle [38] and their presence in ammonia-stabilized latex after long storage time might impact the properties/quality of manufactured rubber products. Finally, band N°6 (solid line, square markers) was shown to appear from 12 days of storage of ammonia-stabilized latex and was clearly visible after 41 days. The appearance of this band located at 12.5 kDa might be attributed to a degradation of proteins located in the band above (N°5).

Physico-chemical properties of latex

The surface tension (wettability) and the percentage of polarity variation of ammonia-stabilized latex were measured regularly during the 41 days of storage. Regarding the surface tension evolution during storage period (**Figure 5a**), the results clearly showed a drop of the surface tension of latex from 48 (0 day) to 43 $mJ.m^{-1}$ (41 days). This exponential decrease occurred during the first two weeks after latex harvest. Then, a stabilization of the surface tension of the latex solution at around 43 $mJ.m^{-1}$ was observed. This physicochemical parameter is representative of the ageing of the latex colloidal solution, which can be explained by a reorganization of the colloidal particles among the latex solution. A phase separation between the polar (non-rubber) and non-polar (polyisoprene rubber) moieties occurred during the first 15 days after harvest (data not shown). This effect then seemed to be stabilized in the presence of ammonia. Indeed, the presence of ammonia avoids and limits

the coagulation of the latex, leading ultimately to a phase separation between the polar and apolar parts of the solution.

A drop of the percentage polarity of ammonia-stabilized latex was observed in **Figure 5b** where values evolved from 55 % down to 45 % during the first two weeks of storage. Indeed, between the 5th and the 15th day after harvest, wettability measurements displayed a decrease of the polar component and during the same period an increase of the dispersive component (data not shown). Latex was then stabilized after two weeks. Such a decrease in polarity might correspond to a decrease in the amount of proteins or in the concentration of amino acids (polar part) of latex. This phenomenon can be also explained by a possible degradation (breakage of chains) of the molecules and/or a reorganization of these molecules (surfactant effect) in latex during storage. Analysis of the proteins contents as depicted in **Figures 3 and 4** concluded to a strong decrease of hydrophilic serum proteins confirming these hypotheses. The physicochemical modifications of ammonia-stabilized latex during the two weeks following its harvest also induced a strong impact on the chemical and mechanical properties of the corresponding latex thin film, as described below.

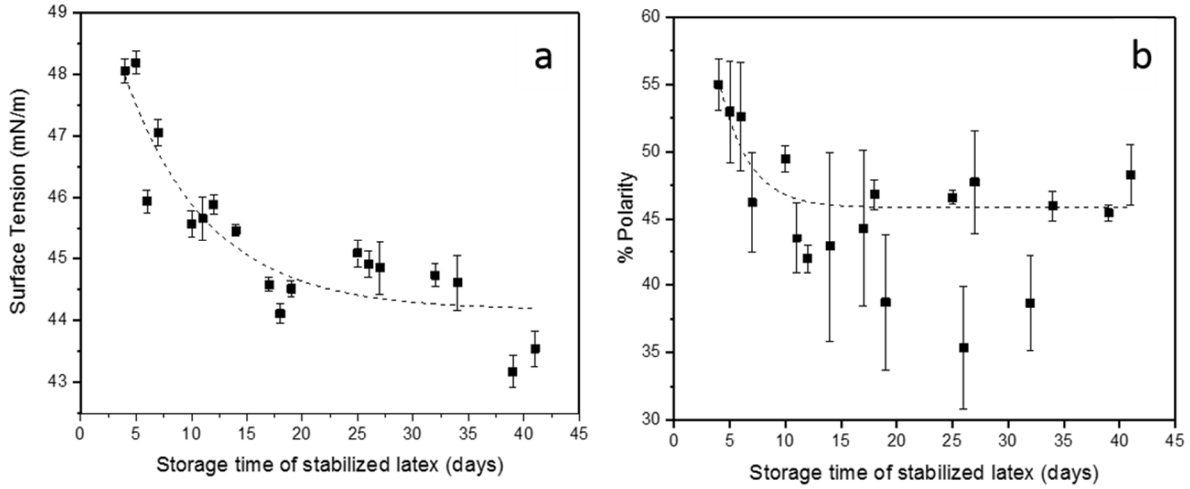

Figure 5. Evolution of the surface tension (a) and percentage of polarity (b) of ammonia-stabilized latex along the 41 days of storage following its harvest. Dashed curves: exponential fitting of the curves.

Mechanico-chemical properties of thin latex films

Figure 6 shows AFM images corresponding to the mapping of the adhesion forces between an AFM probe and the surface of ammonia-stabilized latex films which were non-washed (a) and washed (b). The bright areas of the AFM images correspond to the maximum of adhesion between the tip and the zones containing non-rubber components (polar molecules). At the opposite the dark areas correspond to the rubber part.

A homogeneous phase separation was observed on top of the non-washed film (**Figure 6a**) displaying circular zones of hundreds of nanometers gathering non-isoprenes and polar molecules (lipids and proteins). However, the washing process has completely damaged the regular and circular distribution of the non-rubber components into the rubbery matrix. A random morphology of phase separation can be observed on **figure 6b**. In addition, the amount and the intensity of the bright part has also strongly decreased due to the washing process that has removed a large part of the non-isoprenes and decreased the adhesion forces induced by the presence of polar molecules.

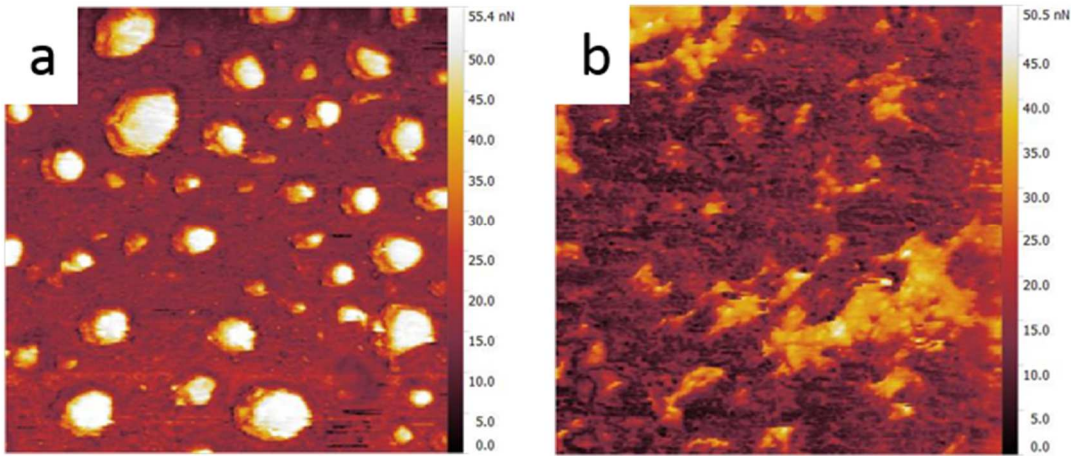

Figure 6. AFM images corresponding to adhesive forces between the latex film and the AFM probe after collection of latex (4th day). A) non-washed latex film, B) washed latex film. Scale frame: 5 µm × 5 µm.

The study of the Young's modulus of films that were either non-washed or washed on zones presenting (or not) rubber compounds emphasized that values of modulus of washed films are of the order of MPa compared to those obtained on non-washed films which were of the order of the GPa (**Figure 7**). The modulus values of the washed films were generally divided by 1000 relative to those of the unwashed films. The washing process thus had a real effect on the rigidity of the film. This can be explained by the elimination of a large part of the non-rubber compounds with small molecules such as lipids and amphiphilic molecules providing a certain rigidity to latex films.

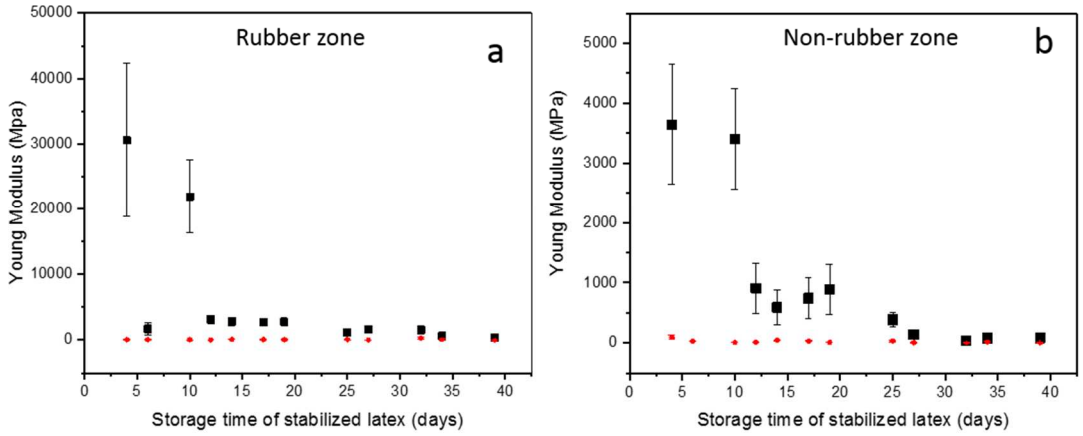

Figure 7. Evolution of the Young's modulus of a latex thin film measured on rubber areas (a) and non-rubber areas (b) during the 41 days following its harvest. Dark filled squares correspond to non-washed film, red filled small circles to washed film.

Figure 7a depicts the evolution of the DMT Young's modulus of the rubber zones of washed and non-washed latex films observed on AFM images (shown in **Figure 6**). Results showed that Young's modulus of the rubber areas has been greatly reduced due to the elimination of non-rubber molecules after film washing of a large part of the molecules originally constituting these zones. This specific behavior can be explained by the fact that only small molecules of lipids could have been easily removed due to the washing effect. Long proteins with a relatively low modulus (flexibility of the zone) should have remained in the natural rubber thin film. This hypothesis is in good agreement with biochemical analytical measurements described in the 1st part of this paper.

In addition, a drop of the Young's modulus of non-washed film occurred during the first two weeks of storage, as observed by wettability. This severe decrease can be explained by a possible degradation of non-rubber molecules present in the latex along the time. The final plateau value of the young modulus corresponding to poly-isoprene is of the order of 70 MPa, value in agreement with the literature concerning a natural rubber (around 100 MPa) [39].

The DMT module corresponding to the non-rubber area (**Figure 7b**) also decreased in the first two weeks after harvesting and then stabilized at a value of less than 1GPa. The stabilization of the module can be emphasized from the 15th day of analysis. A similar tendency could have also been observed for the adhesion forces (data not shown). This phenomenon might appear to be related to a reorganization or chemical reaction (degradation) within the rubber and non-rubber compounds of the latex film, as discussed previously.

Finally, the results obtained by AFM showed a correlation between the adhesion and the module (data not shown). Indeed, when the adhesion of our samples was high, the module was weak and vice versa. However, although we observed this trend, it is not possible yet to propose a mechanism of ageing explaining this phenomenon.

Infra-Red characterization

Figure 8 presents the infra-red curves corresponding to rubber latex films (washed and non-washed) and analyzed between 1500 cm^{-1} and 1800 cm^{-1}. Analysis of thin films was done after latex collection (4th day), by transmission mode, which implies that the whole film is analyzed and not only its surface. As a consequence, surface changes might have been hidden by the spectroscopic fingerprint of the bulk. In addition, there is no significant variation along the time (data not shown).

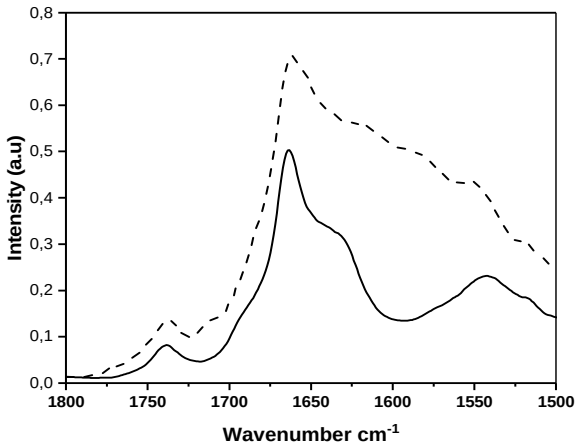

Figure 8. Infra-Red spectra of ammonia-stabilized latex: non-washed film (dashed curve) and washed latex film (solid curve) carried out after collection of latex solution.

Both spectra displayed several peaks which were assigned according to a previous study [40]. A peak at 1740 cm^{-1}, which was assigned to the presence of lipids, a stronger one at 1660 cm^{-1}, corresponding to poly-isoprene and finally a broad one at 1548 cm^{-1} due to the presence of secondary amides (**Table 2**).

Table 2. Assignments of the specific peaks of the latex chemical compounds [38].

Wavenumber (cm^{-1})	Characteristic	Functional group
1740	Esters stretching	Lipid
1710	Carboxyl stretching	Fatty acid
1660	C=C stretching	Cis 1,4 isoprene
1630	Primary amid stretching	Protein
1548	Secondary amid stretching	Protein

The results showed that there was no effect of increased storage time on washed latex films (data not shown). The analysis of IR spectra has been realized before and after washing showing the disappearance of 2 main peaks after washing: one at 1710 cm^{-1} corresponding to the fatty acids and a second at 1590 cm^{-1} corresponding to the amine function (amino acids) (**Table 2**). The washing process has clearly removed the fatty acids (lipids) as well as the amino acids and other molecules of the family of lipids and proteins present in the non-rubber compounds. This result is also in good agreement with the results obtained on the evolution of the Young's modulus of the latex film before and after washing.

CONCLUSION

All measurements indicated a strong impact of ammonia-stabilization of latex on lipid and protein contents which both decreased quickly (4 days) after ammonia-stabilization. A release of free fatty acids was observed whereas specific proteins remained, even after long storage time (> 41 days). A drop in surface tension appeared after short storage time while high-ammoniated latex displayed lower surface tension. In addition, the morphology of stabilized latex films strongly evolved during the first days after preparation, towards a phase separation between poly-isoprene particles and non-rubber components. Young's modulus and adhesion of these films were investigated showing a high increase of their average Young's modulus, compared to a lower effect on average adhesion of the films.

As a conclusion, the ammonia-stabilization method was shown to quickly and significantly impact the lipid and protein contents. When possible, the use of fresh latex should be preferred to ammoniated latex if the objective is to evaluate the impact of non-isoprenes on NR properties. Indeed, though ammonia stabilizes the physical state of latex allowing the latex to remain at a liquid state made of a suspension of rubber particles, it does not stabilize the biochemical composition that is evolving dramatically during storage. The modification of the biochemical composition of stabilized latex over storage time is assumed to be linked to the drastic changes observed in morphology and properties (adhesive, elastic) of thin latex films.

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