Evaluation of suitability of urea as a preservative in low ammonia centrifuged natural rubber latex

Kalani D. Edirisinghe[1], D.G. Edirisinghe[2], M.K. Mahanama[2], L. Karunanayake[1] and S.D.M. Chinthaka[1]

[1]Department of Chemistry, University of Sri Jayewardenepura, Nugegoda, Sri Lanka.
[2]Rubber Research Institute of Sri Lanka, Telawala Road, Ratmalana, Sri Lanka.

Abstract

Low ammonia centrifuged natural rubber latex is generally preserved with the secondary preservative system, tetramethyl thiuram disulphide (TMTD) and zinc oxide (ZnO). This system is known to be carcinogenic due to the generation of nitrosamines. Hence the aim of this study was to evaluate the suitability of urea as an alternative for the TMTD/ZnO preservative system. Field latex was treated with the TMTD/ZnO system (Control) and varying quantities of urea to produce centrifuged latex. Latex was stored for 0, 15, 45 and 60 days and characterized. Physico-mechanical properties and resistance to thermal degradation were evaluated using cast latex films. The standard test for total extractable proteins, GC/MS analysis for nitrosamines and the standard microbiological examination were conducted. For urea treated latex, most of the characteristics were in accordance with the ISO specifications. Physical properties of the cast films produced with this latex were in accordance with the requirement for cast films. Aging studies of these films indicated higher resistance to thermal degradation than the Control. Also, a marked reduction in the total extractable protein content was observed in comparison to the Control. Microbiological examination further implies the suitability of urea as a preservative agent. In the GC/MS analysis, peaks accountable for N-Nitrosodibutylamine were identified only in the spectrum of the film produced with TMTD/ZnO system, but not in those produced with urea. Results in overall showed that urea, which does not indicate generation of nitrosamines could be an alternative for the TMTD/ZnO preservative system. The 0.021% urea treated latex sample and its cast film showed the best properties.

Introduction

Trees of the species *Hevea brasiliensis* produce the natural latex that consists of cis-1,4 polyisoprene, which is ultimately used in the production of a wide range of rubber articles.[1] Within few hours of collection, fresh natural rubber (NR) latex tends to coagulate spontaneously as a result of destabilization by microbial infection.[1] Hence NR latex should be preserved with long term or short term preservatives depending on the situation. Short term preservatives, also known as anticoagulants, such as ammonia and sodium sulphite are used to prevent coagulation until it reaches the factory.[2] Ammonia itself improves preservation.[3] Ammonia is a bactericide[3,4] and it enhances the colloidal stability of latex by generating the anionic form of the acidic groups at the surface of the rubber particles ensuring repulsion between the colloidal rubber particles.[4] Further, ammonia neutralizes some trace metal ions such as calcium and magnesium, preventing calcium and magnesium salt formation.[4]

High concentration of ammonia can cause irritating vapors, health hazards and also some damages to plant and equipment by means of corrosion.[5,4] Therefore, a reduced amount of ammonia in combination with a bactericide where the concentration of ammonia is reduced to about 0.2% (w/w) on total latex and the amount of bactericide ranging from 0.005 - 0.1% (w/w) on total latex[4] is known as the low ammonia (LA) preservation system. Formaldehyde, alkyl dithiocarbamates, zinc oxide, tetra-alkyl thiuram sulphides are some of the typical bactericides.[4]

Tetramethylthiuram disulphide (TMTD) with zinc oxide (ZnO) in 0.025% on latex[2] is the most common secondary preservative system for NR latex. TMTD, during vulcanization process can produce reactive intermediate structures that contain secondary amines.[6] When the intermediates react with nitrosating agents, nitrosamines are formed.[6] Nitrosamines are identified as potential carcinogens for animals including humans.[7,8] These substances have been demonstrated as carcinogenic for several species of animals and therefore

identified as related to human cancers.[8] More than 90% of the identified 300 nitrosamines are classified as carcinogens or mutagens, and the target organs of nitrosamine attack are liver and lungs.[9] Moreover, most nitrosamines are volatile at low temperatures and the leaching of nitrosamines in production areas and warehouses can affect the planet's air quality.[9] Therefore, the world today is more and more concerned about nitrosamines and ASTM standard F 1313-90 limits the inclusion of N-nitrosamines to 10 µg/kg or less per chemical compound.[8] Considering the rapidly developing standards for rubber products all over the world, the purpose of this study was to evaluate the suitability of urea as a replacement for TMTD which would eliminate the generation of nitrosamines.

Urea has bacteriostatic properties.[10,11] The two scientists Symmers and Kirk have recommended urea as an antiseptic in 1915[10], and it is widely used as a disinfectant. Specially, urea is known for its ability for the deproteinization of natural rubber latex.[12] Urea can chemically denature proteins by changing the conformation of proteins through interactions.[13,14] The first step in unfolding proteins is the expansion of the hydrophobic core and then the core is solvated by water and later by urea.[13] Urea has the ability to interact directly with polar residues and the peptide backbone, and thereby stabilizes non-native conformations, thus urea can denature proteins by both direct and indirect mechanisms.[13,14] Exposure to proteins derived from NR latex can cause allergies.[15] Hence, the use of urea as a preservative is expected to be beneficial in eliminating nitrosamine generation as well as in deproteinization of NR latex. In aqueous solution, urea gradually degrades into ammonia and carbon dioxide in one of the three possible non-enzymatic hydrolysis pathways, acid catalyzed, base catalyzed or neutral.[16] Thus urea can improve preservation. Moreover, urea is known to be a cheap, readily available and non-toxic chemical and essentially no danger to the environment, animals, plant life and human beings.[17] Urea dissolves in water completely making it easier to incorporate with NR latex.

Materials and methodology

Field latex was obtained from the Dartonfield Estate of Rubber Research Institute of Sri Lanka, Agalawatta from selected healthy rubber trees of the same clone. All the other chemicals and compounding ingredients were purchased from a local supplier. A 15% solution of diammonium hydrogen phosphate (DAHP) was added to field latex and kept overnight for magnesium sedimentation in order to reduce the magnesium content to 80-100 ppm level[2]. A quantity of three litres of field latex was used for each system where TMTD/ZnO was added as the Control and varying quantities of urea from 0.016% w/w to 0.036% w/w of latex were added for the samples 1 to 5. The preservative systems used are given in Table 1. The samples were centrifuged at a speed of 8000 rpm using a centrifuge. Characteristics of the six centrifuged latex samples were evaluated as per the relevant ISO/EU standards. Thus, pH was determined according to ISO 976:2013 (E)[18]. Total solids content (TSC), dry rubber content (DRC), volatile fatty acid (VFA) number and mechanical stability time (MST) were determined according to ISO 124:2014 (E)[19], ISO 126: 2005[20], ISO 506:1992(E)[21] and ISO 35:2004 (E)[22], respectively. Characterization was repeated at 15, 30, 45- and 60-days intervals.

Table 1: Chemical components of preservative systems

System	Sample	TMTD (%)	Urea (%)	ZnO (%)	NH_3(%)	Amm-onium laurate (%)
LA-TMTD/ZnO	Control	0.013	-	0.013	0.3	0.05
Urea	1	-	0.016	-	0.3	0.05
Urea	2	-	0.021	-	0.3	0.05
Urea	3	-	0.026	-	0.3	0.05
Urea	4	-	0.031	-	0.3	0.05
Urea	5	-	0.036	-	0.3	0.05

Latex compounding was carried out for each system to produce cast films. Compounding was followed by stirring the latex samples at 300 rpm for 30 minutes using a mechanical stirrer. The formulation used in compounding is given in Table 2. Compounded latex was poured into glass moulds with 3 mm depth and kept undisturbed for 3 days to form dried films, before vulcanizing in the oven at 100 °C for 30 minutes.

Table 2: Formulation used in the preparation of cast latex films

Ingredient	Wet weight (pbw)[(a)]
60% NR latex	167
20% Solution of KOH (stabilizer)	2
50% Dispersion of sulphur	4
50% Dispersion of ZDC (accelerator)	2
50% Dispersion of ZnO	3
50% Dispersion of WSP (phenolic antioxidant)	2
50% Dispersion of whiting	40

pbw[(a)] = parts by weight

Tensile properties namely, tensile strength, elongation at break and modulus at 100% and 300% elongations were measured according to the standard ASTM D 412[23] and tear strength was measured according to ASTM D 624[24].Hardness of the specimens was measured by a type A durometer as per the standard ASTM D 2240[25]. The dumbbell shaped specimens for determination of tensile properties and angle shaped specimens for determination of tear properties were cut using metal dies. The thickness of the specimens was measured by a Wallace thickness gauge before subjecting to testing using an Instron 5943 standard universal testing machine at a grip separation rate of 500 mm / minute.

Also, the vulcanized latex films were aged for 72 hours at 70°C in an aging oven as per ASTM 3578-91[26]and tensile properties of the aged films were evaluated.

The EU directive standard EN 12868[27], a method for the isolation, identification and determination of N-nitrosamines was carried out for the cast films after aging. The nitrosamines, if present, were extracted to a nitrite containing artificial saliva salt solution and the solution was examined by gas chromatography (GC). The artificial saliva was prepared as described by the EN 12868 method, by dissolving 4.2g sodium hydrogen carbonate, 0.5g of sodium chloride, 0.2g of potassium carbonate and 30mg of sodium nitrite in water and by diluting to 900 ml with distilled water. Thereafter, the pH was adjusted to 9 with 0.1 M NaOH and diluted with distilled water to obtain a total volume of 1000 ml. Latex film samples of 10g were dipped into artificial saliva solution and the nitrosamines were extracted according to the conventional liquid-liquid extraction technique with dichloromethane as described in EN12868. The samples (1.0 µl aliquot) were analyzed by Agilent Technologies 7890A GC system coupled with an Agilent 5975C mass selective detector (Shimadzu GCMS-QP2010). A HP-5MS capillary column of 30 m x 0.25 mm internal diameter x 0.25µm film thickness was used. The flow rate of helium was 1.0 mL/min. The injector port was in split mode with split ratio of 1:1. The injector port temperature was 120 °C and the column temperature was programmed in two steps from 50 °C (held for 2 min.) to 160 °C at a rate of 10 °C/min and to 250 °C at a rate of 10 °C/min. The mass spectra were obtained by electronic impact at 70 eV and detection of compounds was carried out in selective ion monitoring (SIM) mode.

The standard test for proteins ASTM-D 5712-15[28] was carried out to determine the amount of total extractable proteins associated with NR latex films. Cut pieces of each test film was extracted in 0.01M phosphate buffer (pH 7.4) at 25 °C for 2 hours. The extracts were centrifuged at 500 rpm for 25 minutes to sediment particles. Protein precipitation was carried out using trichloroacetic acid (TCA) and phosphotungstic acid (PTA) solution followed by sodium deoxycholate (DOC) solution. The solution was centrifuged at 6000 rpm for 15 minutes and the resulting pellet was re-dissolved in 0.2 M sodium hydroxide, and the concentration of proteins was determined by Lowry micro-assay. Absorbance recorded at 750 nm were calibrated against standard Ovalbumin solution (1mg/ml) and the results were expressed as µg/g of latex film.

The standard test ISO-9252 was carried out for the examination of the rubber latices for presence and approximate concentration of viable aerobic and facultative anaerobic organisms. The test was done in the 60th day of latex storage. Identification of micro-organisms was outside the scope of this study. A nutrient medium was selected on the basis of the pH of the latex. Since pH of latex was above 7, beef extract agar was used. The composition of beef extract agar was 300g of beef extract, 5g of peptone, 5g of NaCl, and 10g of agar in 1 liter of water. Quarter strength (one part of the solution diluted with 3 parts of water) Ringer's solution containing 9 g of NaCl, 0.42g of anhydrous $CaCl_2$ and 0.20g of $NaHCO_3$ was used as the dilution fluid. A test portion of latex was serially diluted, and aliquots of each dilution were spread over the surface of the nutrient medium in a petri dish, followed by incubation at 30 °C for 3 days. The number of colonies of micro-organisms that developed were counted, multiplied by the dilution factor and recorded as colony-forming units per cm^3 of the original sample.

Results and Discussion

1. Characterization of Latex

pH

The graph of pH versus storage period was constructed using the average pH of 3 trials. The pH of the 0.016% urea solution is 7 and overall pH increases upon the concentration of urea samples. With the increase of storage period, there is a decrease in pH in all the samples including the Control. With the release of NH_3 to the outer environment, the basicity of the latex decreases. Possible release of amino acids by hydrolysis of the proteins and hydrolysis of lipids in basic medium to higher fatty acids, the total acid content could go up during storage.[2] Thus, the pH value of latex decreases. Bacterial action on non-rubbers such as proteins and carbohydrates present in latex can result in the formation of volatile fatty acids, which can also lower the pH of the latex.[2,4] All the five samples, which were treated with urea using different concentrations, show a higher pH than the Control (Figure 1). This could be because urea can be enzymatically hydrolyzed to ammonia and carbon dioxide by urease[29], which might be present in latex in minor quantities, in turn may contribute to the increase of pH.

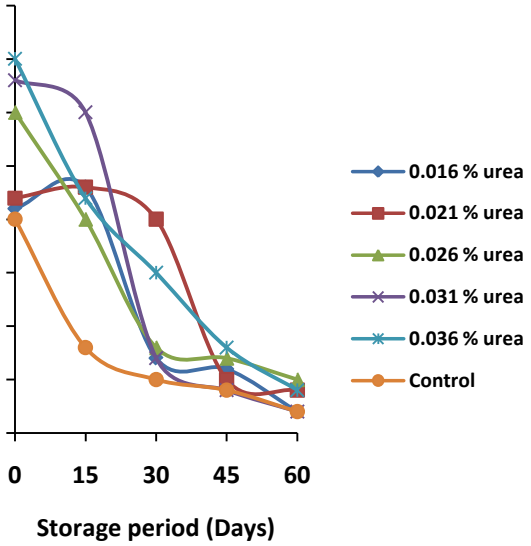

Figure 1. Variation of pH with storage period

Volatile Fatty Acid (VFA) Number

NR latex contains about 4-5% non–rubber constituents consisting mainly of carbohydrates, proteins and lipids.[30] VFA number of NR latex is an indication of bacterial action on carbohydrates.[31] Most predominantly a sugary substance present in latex namely, quebrachitol is consumed by bacteria in the presence of oxygen to form volatile fatty acids such as acetic and formic acids,[2] which increase the VFA number resulting in coagulation. Even though addition of DAHP can remove Mg^{2+} ions as magnesium ammonium phosphate $[Mg(NH_4)PO_4]$, there can be residual ions, which could enhance bacterial growth.

Variation of VFA number with storage period is shown in Figure 2. VFA number does not exceed the ISO 2004:2010(E)[32] specified value of 0.06 for concentrated latex.

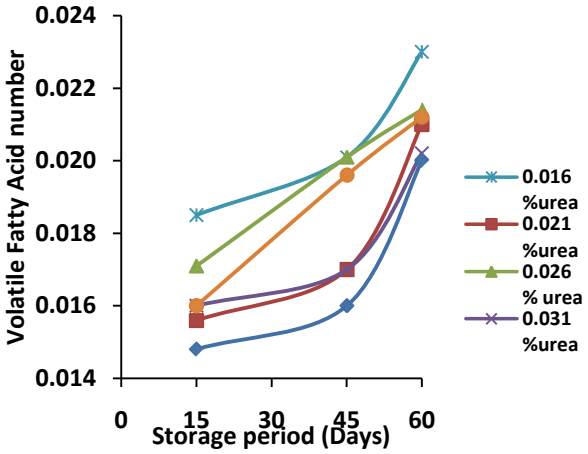

Figure 2. Variation of VFA number with storage period

The 0.016% urea preserved latex shows the highest VFA number (0.0185) at 15 days. This can be because of the comparatively low concentration of the secondary preservative urea, causing a lower bactericidal effect. Also, there is a remarkable decrease of VFA number in the sample prepared with 0.036% urea at all three storage periods and this indicates a better preservation. In the sample which 0.026% urea was added, a VFA number similar to that of the Control can be observed.

Mechanical Stability Time (MST)

The MST values determined were in between 800 to 1150 seconds and were found to be higher than the specified value of 650 seconds. Figure 3 below represents the variation of MST with storage period.

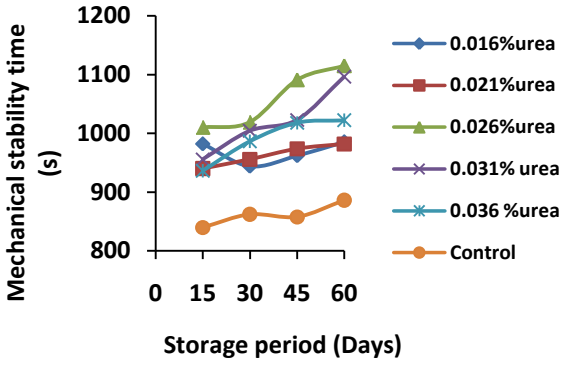

Figure 3. Variation of MST with storage period

An MST value higher than the expected is probably an outcome of higher pH. Samples containing 0.016% and 0.021% of urea, are the samples with low MST. More

the anionic charges around the rubber particles, more the stability of latex towards mechanical agitation. Anions around the rubber particles keep them apart because of repulsive forces between each other. This leads to higher stability towards mechanical agitation. During the storage of latex, naturally occurring lipids present in NR latex undergo hydrolysis in a basic medium to form long chain fatty acids, which in turn enhance the negative charge density around the rubber particles increasing the stability of latex with time.[2]

Total Solids Content (TSC)

The total solids content of all six samples do not vary considerably (Table 3) and this suggests that TSC and in turn viscosity of latex is not affected by urea. Average viscosity of urea preserved samples is 79 cP, while that of the Control is 80 cP. This would then suggest that urea as the secondary preservative does not affect the total solids content or other parameters such as particle size distribution that could affect viscosity.

Table 3. Variation of TSC with storage period

Concentration of urea (%)	Total solids content (%)				
	0 days	15 days	30 days	45 days	60 days
0.016	63.30	63.48	63.45	63.38	63.46
0.021	63.40	63.45	63.34	63.41	63.40
0.026	63.40	63.32	63.36	63.42	63.38
0.031	63.40	63.41	63.21	63.30	63.38
0.036	63.42	63.54	63.32	63.48	63.42
Control	63.46	63.37	63.43	63.52	63.50

Dry Rubber Content (DRC)

Dry rubber content, which is the amount of rubber present in latex, gives a lower amount than TSC as expected. Moreover, the DRC of all samples do not show a significant alteration with storage period (Table 4).

Table 4. Variation of DRC with storage period

Concentration of urea (%)	Dry Rubber Content (%)				
	0 days	15 days	30 days	45 days	60 days
0.016	62.32	62.06	62.21	62.34	62.28
0.021	62.22	62.12	62.41	62.28	62.32
0.026	62.20	62.21	62.40	62.34	62.32
0.031	62.45	62.36	62.40	62.44	62.42
0.036	62.33	62.30	62.25	62.41	62.21
Control	62.12	62.20	62.34	62.26	62.16

2. Vulcanizate Properties of Latex Films

Tensile strength

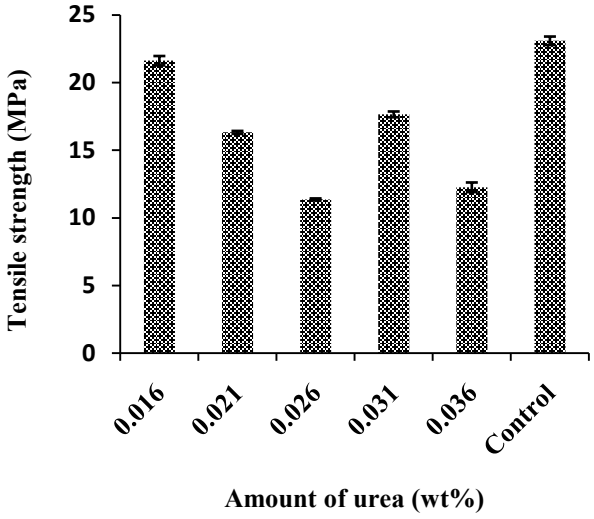

Figure 4. Variation of tensile strength with amount of urea

Ultimate tensile strength is known to be as the maximum stress the test pieces can withstand while being stretched, without fracturing**.** The Control shows a tensile strength of 23.08 MPa, which is higher than that of the urea treated samples (Figure 4). Control containing TMTD and ZnO can react together to form the active sulfurating agent facilitating vulcanization.[33] Thus, there will be more sulphur crosslinks forming in the Control. Failure properties like tensile strength increases upto a maximum value as crosslink density increases and then decreases.[33,34]the fraction p of cross-linked units in the products ranging from 0.10x 10?2 to 8.0 x 10?2. In spite of the small dimensions of the test specimen (cross section 0.10 in. by about 0.005 in. Crosslink density might be affected by addition of urea.

Modulus at 100% elongation

Variation of modulus at 100% elongation with the percentage of urea is presented in Figure 5.

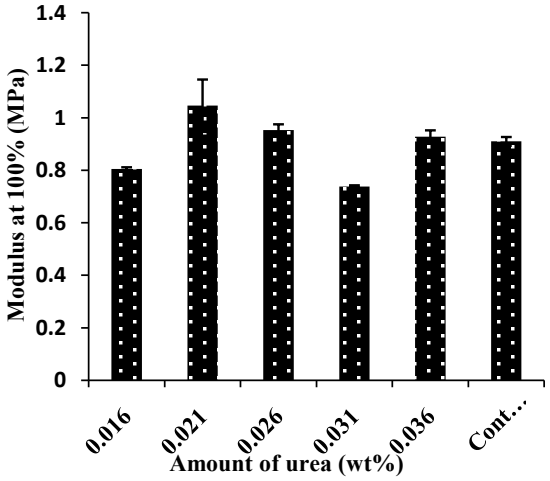

Figure 5. Variation of modulus at 100% elongation with amount of urea

Generally, modulus at 100% elongation is directly proportional to the stiffness or hardness. Higher the hardness, higher the amount of force required to elongate the film. The highest hardness is recorded for the 0.021% urea treated film, which also records the highest modulus at 100% elongation. The average of three experiments has been used in developing the graphs. As expected, modulus at 100% elongation shows a direct correlation with hardness.

Table 5. Hardness of cast latex films

Sample	Modulus at 100% elongation (MPa)	Hardness (Shore A)
0.016% urea	0.81	34
0.021% urea	1.05	47
0.026% urea	0.95	30
0.031% urea	0.74	34
0.036% urea	0.93	41
Control	0.91	43

Elongation at break

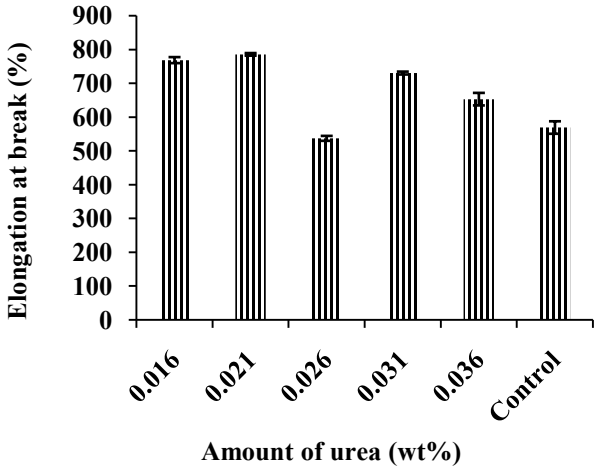

Figure 6. Variation of elongation at break with amount of urea

Urea treated cast latex films except that treated with 0.026% urea, show a higher elongation at break in comparison to the film prepared with TMTD/ZnO preserved latex (Control). As stated earlier, in the Control there could be more crosslinks formed due to the presence of TMTD and ZnO and this in turn would reduce elongation at break.

Tear strength

It is known that hardness increases with increase in crosslink density[33]. Hence out of the urea treated samples, the maximum crosslink density is expected in 0.021% urea treated sample. Figure 7 shows the expected variation in tear strength with the amount of urea. The highest tear strength shown by the Control could be attributed to thiuram accelerated vulcanisation operative in the presence of TMTD/ZnO[33], which enhances crosslink formation.

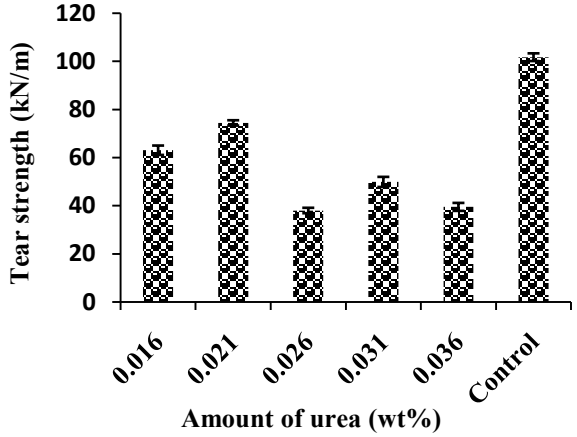

Figure 7. Variation of tear strength with Amount of urea

Tensile properties of cast films after ageing

Percentage retention of modulus, tensile strength and elongation at break after ageing at 70°C for 72 hours are tabulated in Table 6. Figure 7 shows the variation in tear strength of each sample.

Table 6. Percentage retention of tensile properties after aging

Concentration of urea (%)	0.016	0.021	0.026	0.031	0.036	Control
% retention of tensile strength	94	91	113	58	125	53
% retention of modulus at 100% elongation	101	70	123	96	88	137
% retention of modulus at 300% elongation	96	77	109	76	59	157
% retention of elongation at break	99	116	97	104	100	87

Urea treated NR vulcanizates show a higher percentage retention of tensile strength and elongation at break compared to the Control. However, the Control sample shows a much higher retention of moduli compared to urea treated samples, probably due to more crosslink formation during ageing due to thiuram acceleration.

3. Total Extractable Protein Content

Natural rubber products contain two kinds of proteins; proteins that are adsorbed to the rubber particles and serum derived soluble proteins, which contain most of the extractable proteins.[35]"container-title":"Journal of Bioscience and Bioengineering","page":"628-634","volume":"111","issue":"6","abstract":"ABSTRACT\nNon-rubber components present in natural rubber (NR The Lowry method involves an extraction and precipitation procedure followed by an assay of protein content, where a reduction of peptide bonds is identified.[36] The extractable protein content vary from 30 to 700 µg/g in latex based products.[36] Water extractable proteins are responsible for most allergic problems and urea brings an added advantage when extractable proteins are removed.

Figure 8 shows the protein concentration of each film. Urea treated samples show a lower amount of extractable proteins than the Control and can be attributed to protein denaturing ability of urea.

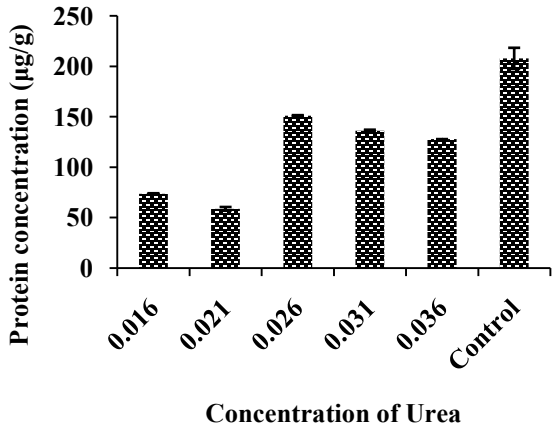

Figure 8. Total extractable protein content of cast films

4. Anti-microbial Activity of Urea

As per the Tables 7 and 8, urea treated latex samples do not show a significant contamination of bacteria.

Table 7. Microbial count

Urea concentration	Dilution	Rough count (visual)	Dilution factor	Count x dilution factor
0.016%	10^{-1}	1	X10	10
0.021%	10^{-1}	1	X10	10
0.026%	10^{-1}	1	X10	10
0.031%	10^{-1}	1	X10	10
0.036%	10^{-1}	0	X10	0
Control	10^{-1}	3	X10	30
Blank	10^{-1}	0	X10	0

Table 8. Interpretation of microbial count

Count	Interpretation
Below 30	No significant contamination
= 10^3	Slight contamination
Above 10^4	Contamination
Above 10^5	Heavy contamination

The antimicrobial effect of urea could be because of its protein denaturing ability, where the portentous membranes of bacteria can be destroyed by urea.

5. GC/MS Analysis for Nitrosamines

As per the standard EN 12868, 11 kinds of N-Nitrosamines that can be identified from the analysis were targeted (Table 9).

Table 9. Nitrosamines targeted from the analysis [27]

N-Nitrosamines	
NDMA	N- Nitrosodimethylamine
NDEA	N-Nitrosodiethylamine
NDiPA	N-Nitrosodiisopropylamine
NDPA	N-Nitrosodipropylamine
NDBA	N-Nitrosodibutylamine
NPIP	N-Nitrosopiperidine
NPYR	N-Nitrosopyrrolidine
NMOR	N-Nirosomorpholine
NEPhA	N-Nitroso N-ethyl N-phenylamine
NMPhA	N-Nitroso N-methyl N-phenylamine
NDBzA	N-Nitrosodibenzylamine
NDiNA	N-Nitrosodiisononylamine

Based on the SIM (Selective Ion monitoring) chromatogram of the standard solution and the test solutions, NDMA (N- Nitrosodimethylamine) was detected only in the Control. The following m/z values[37] were selected for each compound, in which the base peak is in bold. NDMA (42,43,**74**), NDEA (42,44,**102**), NPYR (41,42,**100**), NMOR (56,86,**116**), NPIP (42,55,**114**) and NDiPA (43,70,**130**). No any nitrosamines were found in urea-based films.

Conclusion

Results in overall indicate that urea could be an alternative to the conventional secondary preservative system TMTD/ZnO used in the production of low ammonia centrifuged latex. The 0.021% urea treated latex sample and its cast film showed the best properties.

Acknowledgement

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