

Some roles of methanol-soluble fraction of rubber from *Manihot glaziovii*. Part 1. Sitosterol and fatty acids

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Summary

Manihot glaziovii rubber was dissolved in purified chloroform and coagulated with methanol. An oily like material (ACM) remaining from the coagulation process was isolated and submitted to column chromatography over silica gel yielding several fractions. The components of the major fraction, denoted collectively as F-GH, were isolated and characterised by FTIR, ^1H and ^{13}C NMR spectroscopies and GC/MS as sitosterol, stigmasterol and a mixture of palmitic and oleic acid. The effect of ACM itself and the components of F-GH on the thermal oxidation of rubber film at 140 °C was verified by the use of FTIR. The induction time and the rate constant were determined.

Introduction

It has been generally recognised that crude natural rubber (NR) is more resistant toward oxidation than the purified rubber. Arnaud and De Monte (1) observed that the induction time for oxidation of crude *Hevea* NR at 150 °C was about 3 times greater than that of purified NR. The presence of natural antioxidant was also recently associated with the ageing properties of so-called "virgin mature-tree" NR compared with regularly tapped mature-tree NR (2).

Natural antioxidants were isolated from *Hevea brasiliensis* NR at the beginning of the century (3) and have been the subject of a few studies. Free amino acids have been isolated from the crude latex of NR, but were not studied for their stabilising activity (4). Phospholipids, amino acids, phenols, tocotrienols and betaine are also identified as natural antioxidants in *Hevea* NR (5). More recently, free tocotrienols were shown to protect NR against thermal oxidative degradation, but proteins and amino acids did not confer any significant protection (6).

Manihot glaziovii (*Euphorbiaceae*), popularly known in Brazil as "maniçoba", is a plant that produces latex. This rubber latex is constituted mainly of *cis*-1,4-polyisoprene units, with \bar{M}_v lying between 1.0×10^6 and 1.5×10^6 g/mol (7). In this work, the methanol-soluble constituents of *Manihot* NR were isolated and characterised by FTIR, ^1H and ^{13}C NMR spectroscopy and GC/MS. The antioxidant activity of these individual constituents on NR thermal oxidation were studied and compared with synthetic substances.

Experimental

Materials

Experiments were carried out on fresh natural rubber latex, spontaneously coagulated, which were collected in June 1998 from *Manihot glaziovii* trees inhabiting the district of Pacatuba, State of Ceará, northeastern Brazil.

Obtention of the antioxidant crude mixture from natural rubber

53.3 g of the rubber was purified following the method described previously (1). The re-coagulation was carried out with methanol, the remaining solution filtered and the solvent evaporated under vacuum. The mother-liquor was concentrated in a rotary evaporator, at a reduced pressure at 52 °C, yielding an oily like extract, denominated antioxidant crude material (ACM), which represented 9.6 wt% of crude rubber.

Separation and identification of F-GH fraction

ACM (4.960 g) was submitted to column chromatography (2.3 cm diameter) over Merck silica gel 60 yielding several fractions of different polarities. Based on the results from thin layer chromatography (TLC) using Merck silica gel 60 F₂₅₄ pre-coated plates, similar fractions from elution by hexane-CHCl₃ (10:90), EtOAc-CHCl₃ (30:70) and EtOAc-CHCl₃ (50:50) were pooled (2.236 g), and re-chromatographed on a silica gel column. Elution with hexane-CHCl₃ (40:30) (fractions 1-59) led to eight samples: A (20 mg); B (59 mg); C (59 mg); D (36 mg); E (174 mg); F (50 mg); G (526 mg) and H (608 mg). Fractions G and H which were of same polarity were pooled, designated F-GH (1.134 g; 22.8 wt% in relation to ACM) and selected for the investigation described herein.

F-GH was washed with hexane, yielding a white solid, F-GH_{solid} (230 mg), not soluble in hexane. Methanol addition to the hexane solution precipitated a waxy solid, F-GH_{wax} (250 mg). Solvent evaporation of the supernatant solution yielded a yellowish oil, denominated F-GH_{oil} (292 mg). An analysis of F-GH and all fractions was performed by ¹H and ¹³C NMR spectroscopies on deuterated chloroform solution with tetramethylsilane (TMS) as an internal reference using a Bruker DPX 300 spectrometer operating at 300 MHz (¹H) and 75.5 MHz (¹³C). Methylation of the fatty acids mixture from F-GH_{oil} was performed through reflux with BF₃/MeOH followed by the addition of a saturated solution of NaCl and several washings with hexane. The hexane fraction was washed with distilled H₂O, treated with anhydrous Na₂SO₄ and concentrated under reduced pressure. The methyl ester mixture was characterised by GC/MS on a Hewlett-Packard, HP 5971 and 5890A, II instrument.

Film preparation for oxidation tests

NR films of thickness from 40 to 45 µm on a PTFE base were used for spectroscopy measurements of absorbance changes during thermal oxidation. The samples were prepared by casting and successive solvent evaporation from 1 wt % purified polymer solution in purified CHCl₃ in the absence and in the presence of 0.1 wt% (in relation to NR) of F-GH fractions. The ACM, F-GH_{solid}, F-GH_{wax} and F-GH_{oil} were added to NR and the solution stirred for 24 h before film preparation. The rubber film was analysed by IR spectroscopic measurement of the absorbance corresponding to the C-H stretching vibration in CH₂ and CH₃ groups (1447 cm⁻¹) (8) in order to determine and control its thickness. The films were stored in the absence of light at low temperature (-10° C) before thermal oxidation.

Kinetic study

Thermal oxidation of NR without any antioxidant and in the presence of F-GH constituents at 140 °C in air was studied by FTIR using a Shimadzu model 8300 spectrometer. Experiments were also made in the presence of 0.1 w% of synthetic β -sitosterol and a mixture of palmitic/oleic acid (2:1 wt/wt). The reaction was followed by the individual bands corresponding to the O-H stretching vibration of hydroperoxides (3435 cm^{-1}), to the C=O stretching vibration of aldehydes and ketones (1720 cm^{-1}), and to the bending mode of C=C in the *cis* isoprene group (835 cm^{-1}). The 1447 cm^{-1} band was used as an internal standard for absorbance ratio calculations (9).

Results and Discussion

Spectral characterisation

^{13}C and ^1H spectra of F-GH (Figures 1a and 2a respectively) are compatible with a mixture of sterols and triglycerides. The ^1H NMR spectrum of F-GH (Figure 2b) showed a characteristic profile of the common phytosterols through the carbinolic hydrogen at 3.51 ppm and the vinyl hydrogen absorptions at 5.10 and 5.35 ppm (for stigmasterol and sitosterol, respectively). This was confirmed by ^{13}C NMR analysis through the signals at 140.8 and 121.8 ppm (for sitosterol) plus 138.4 and 129.3 ppm (for stigmasterol) as well as for the carbinol carbon at 71.8 ppm (Figure 1b). As expected the ^1H and ^{13}C NMR spectra of F-GH_{oil} (Figures 2c and 1c respectively) showed a characteristic profile of unsaturated triglycerides with small amounts of sterols.

Analysis of methylated of the fatty acids mixture from F-GH_{oil} by GC/MS indicated that this fraction is constituted predominantly of palmitic acid (23%) and oleic acid (46%). The other minor constituents were not identified.

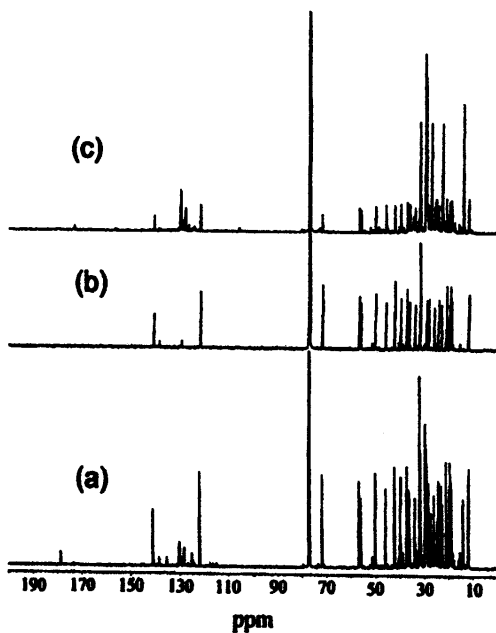


Figure 1. ^{13}C NMR (BB) spectra in CDCl_3
(a). F-GH; (b) F-GH_{solid}; (c) F-GH_{oil}.

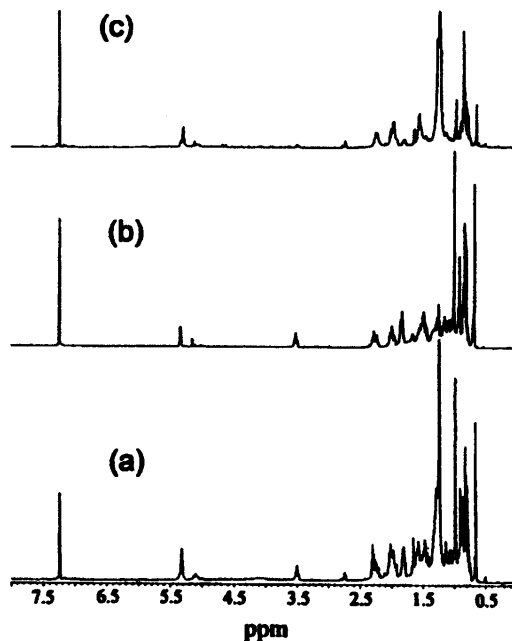


Figure 2. ^1H NMR spectra in CDCl_3
(a). F-GH; (b) F-GH_{solid}; (c) F-GH_{oil}.

Kinetic study

Figure 3a shows the relative absorbance evolution of hydroperoxides ($\nu_{\text{O-H}}$, 3435 cm^{-1}), carbonyl groups ($\nu_{\text{C=O}}$, 1720 cm^{-1}) and isoprene double bond ($\delta_{\text{C=C}}$, 835 cm^{-1}) characteristic bands for purified *Manihot glaziovii* NR. An initial region can be seen where changes in the absorbance do not occur. The subsequent region shows the formation of hydroperoxides and carbonyl groups and a concomitant consumption of the double bond. All the curves have an excellent sigmoidal fit. The formation of C=O and OOH derived from the isoprene chain ceased when all C=C had been consumed.

The formation of hydroperoxides and the consumption of double bonds during early steps of thermal oxidation of polyisoprene rubber obey a first order equation (10). In the case of products formation the most appropriate equation is that which takes into consideration the maximum absorbance achieved at the end of the reaction (A_{∞}) (11). The curve for carbonyl group accumulation shows a self accelerated feature and the formation of these products takes place practically in accordance with the parabolic law ($[\text{C=O}] = kt^2$) (10). Following the adopted method for the determination of the induction time for thermal oxidation (τ_i) and the rate constants by a viscometric method (12), the following equations were obtained:

$$\text{Hydroperoxide formation} \quad \ln(A_{3435(\infty)} - A_{3435(t)}) = B + k_{\text{OOH}}(t - \tau_i) \quad (1)$$

$$\text{Double bond consumption} \quad \ln A_{835(t)} = B' - k_{\text{C=C}}(t - \tau_i) \quad (2)$$

$$\text{Carbonyl formation} \quad A_{1720(t)} = B'' + k_{\text{C=O}}(t - \tau_i)^2 \quad (3)$$

where A_0 is the absorbance at the frequency ν relative to the absorbance at 1447 cm^{-1} , A_t is the absorbance at time of heating t , τ_i is the induction time, the parameters k_{OOH} , $k_{\text{C=C}}$ and $k_{\text{C=O}}$ are the effective rate constants for hydroperoxide formation, double bond consumption and carbonyl formation respectively, and the B, B' and B'' parameters are constants.

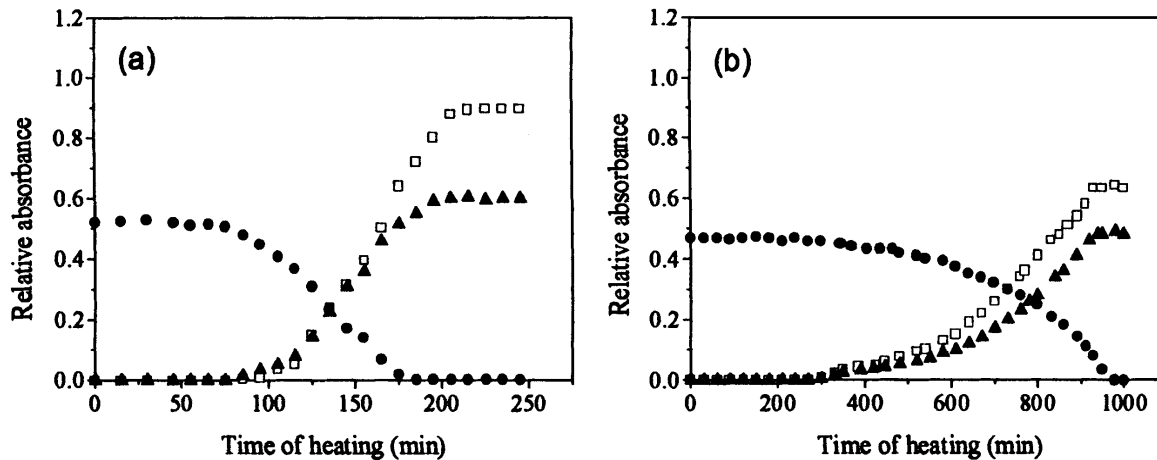


Figure 3. Evolution of double bond, hydroperoxides and carbonyl groups characteristic bands during thermal oxidation of purified *Manihot* NR at 140 °C. (a) with no antioxidant (b) in the presence of 0.1 wt% antioxidant crude material (ACM). (□) OOH; (●) C=C; (▲) C=O.

The data for the rate constant and induction time for the purified NR, obtained from two experiments (in order to check its reproducibility), are presented in Table I. It can be seen that the rate of consumption of the double bond is greater (in this case approximately double) than the rate of hydroperoxides formation. The rate for carbonyl products formation cannot be compared with the others, due to their different kinetic. The differences between the induction times are negligible and lie within the experimental error. The data indicate that it can be considered just an average value of τ_i associated with thermal oxidation as a whole. For the purified NR, this value is 113 ± 8 min. This induction time is comparable to the value determined for *Hevea* NR film (130 min) at 130 °C, thickness 23-25 μm , based on the absorbance of the carbonyl group (8).

The presence of ACM 0.1 wt% displaces all three curves (Figure 3b) for longer times of reaction indicating a delay on the thermal oxidation. It can be seen that, a region exists (approximately between 300 and 500 min) in which the formation of both hydroperoxides and carbonyl groups is very slow. From this point, the process is fast and resembles the one observed for purified NR. Similar behaviour was observed previously (1, 13) and the authors assumed that the products formed in this region, were due to the consumption or transformation of residues from antioxidants, not from NR oxidation. The rate constants for NR with ACM 0.1 wt% are respectively $2.1 \times 10^{-3} \text{ min}^{-1}$, 2.3×10^{-3} and $7.5 \times 10^{-7} \text{ min}^{-2}$ for k_{OOH} , $k_{\text{C=C}}$ and $k_{\text{C=O}}$. It can be verified that the addition of ACM reduced all the rate constants and provoked a delay of the beginning of the reaction (increase in τ_i for 519 min). The formation of hydroperoxide groups, the consumption of double bond and the formation of carbonyl group were reduced by 4, 16 and 32 times, respectively. The induction time increased by 5 times approximately.

The kinetic curve corresponding to NR in the presence of 0.1 wt% of F-GH_{oil} (Figure 4a) reveals clearly the consumption period of antioxidant (between 250 and 550 min) showed in Figure 3b. It was observed that the curve was displaced for higher times of reaction when compared to the purified NR curve (Figure 3a). The rate constants (except k_{OOH}) and, specially, the induction time indicate the antioxidant action of this component.

The fraction F-GH_{oil} was predominantly composed of: 23 wt% palmitic acid (C16:0) and 46 wt% oleic acid (C18:1) as free fatty acids and/or triglycerides. Fatty acids are already known as pro-oxidants in *Hevea* (14) and *Guayule* rubber (15). The authors proposed that the fatty acids oxidise rapidly to form hydroperoxide and act as an initiator in the rubber degradation. The rate of degradation was found to increase with the number of conjugate double bonds of the fatty acids (14).

Table I. Kinetic parameters for thermal oxidation at 140 °C of purified *Manihot* NR

Reaction	Rate constant *			τ_i (min)		
	Exp 1	Exp 2	Average	Exp 1	Exp 2	Average
OOH formation	1.0×10^{-2}	1.3×10^{-2}	$1.2 \pm 0.1 \times 10^{-2}$	127	115	121 ± 6
C=C consumption	2.1×10^{-2}	2.5×10^{-2}	$2.3 \pm 0.2 \times 10^{-2}$	123	98	111 ± 12
C=O formation	2.1×10^{-5}	2.6×10^{-5}	$2.4 \pm 0.3 \times 10^{-5}$	115	101	108 ± 7

* min^{-1} for OOH formation and C=C consumption; min^{-2} for C=O formation
The curve on Figure 3a represents the experiment 2.

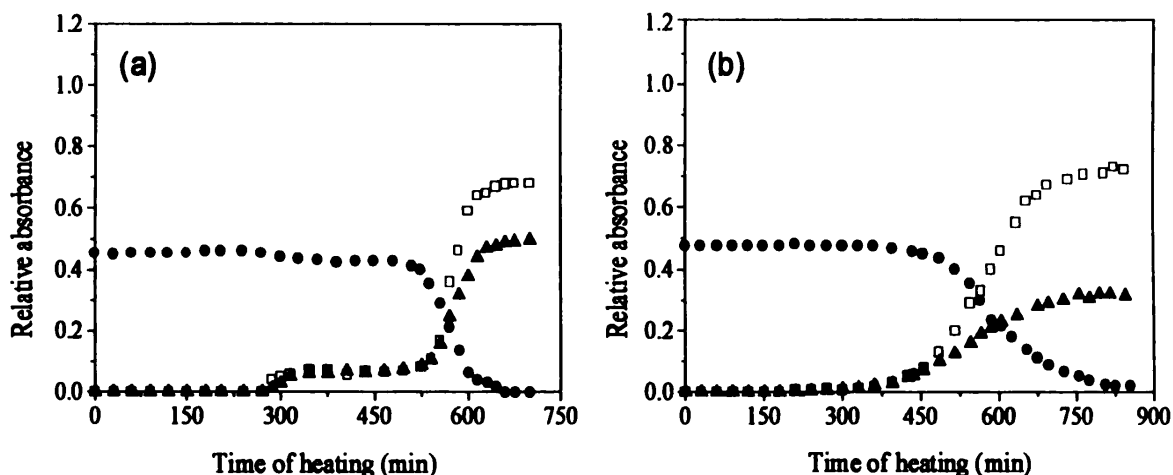
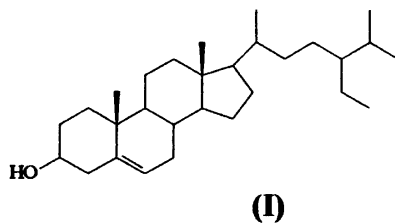


Figure 4. Evolution of the double bond, hydroperoxides and carbonyl groups characteristic bands during the thermal oxidation of *Manihot* purified rubber at 140 °C in the presence of 0.1%: (a) F-GH_{oil}; (b) Mixture of palmitic acid and oleic acid. (□) OOH; (●) C=C; (▲) C=O.

Because the F-GH_{oil} may contain free fatty acids that act, in general, as pro-oxidant, the effect of the addition of 0.1 wt% of the commercial available mixture of palmitic acid/oleic acid (1:2 wt/wt) on the thermal oxidative degradation of rubber was tested (Figure 4b). The kinetic parameters obtained ($k_{\text{OOH}} = 4.0 \times 10^{-3} \text{ min}^{-1}$, $k_{\text{C=C}} = 8.9 \times 10^{-3} \text{ min}^{-1}$, $k_{\text{C=O}} = 1.1 \times 10^{-6} \text{ min}^{-2}$ and average $\tau_i = 437 \text{ min}$) were not so close to those obtained for NR in the presence of F-GH_{oil} ($k_{\text{OOH}} = 1.9 \times 10^{-3} \text{ min}^{-1}$, $k_{\text{C=C}} = 1.5 \times 10^{-2} \text{ min}^{-1}$, $k_{\text{C=O}} = 4.1 \times 10^{-6} \text{ min}^{-2}$ and average $\tau_i = 524 \text{ min}$). This indicates that the triglycerides from F-GH_{oil} or the not identified minor constituents (31%) may have a significant effect on NR degradation. The pronounced variation observed in the pattern of carbonyl groups formation may suggest the occurrence of different reaction mechanisms, to be investigated further. In any case, the comparison with the parameters of purified NR indicates that the mixture of fatty acids, in concentration of 0.1 wt%, acts as an antioxidant in *Manihot* rubber, by at least a threefold decrease in rate constants and a fourfold increase in induction time, approximately.

One of the possible explanations for the catalytic activity of free fatty acids is the reaction of pro-oxidant metals such as copper, cobalt, iron and manganese to produce metal carboxylates (14). Differences in the composition of metallic ions present in *Manihot* rubber (16) when compared with *Hevea* rubber may be associated with the behaviour observed. Experiments are being performed to verify this association.

Figure 5a presents the curves for NR in the presence of F-GH_{solid}. The main constituent of this fraction was identified as β -sitosterol (I) and, so, the effect of this synthetic compound was also tested (Figure 5b). β -Sitosterol is one of the components of neutral lipids from fresh *Hevea* latex (17). From these lipids only tocotrienols were identified as natural antioxidants.



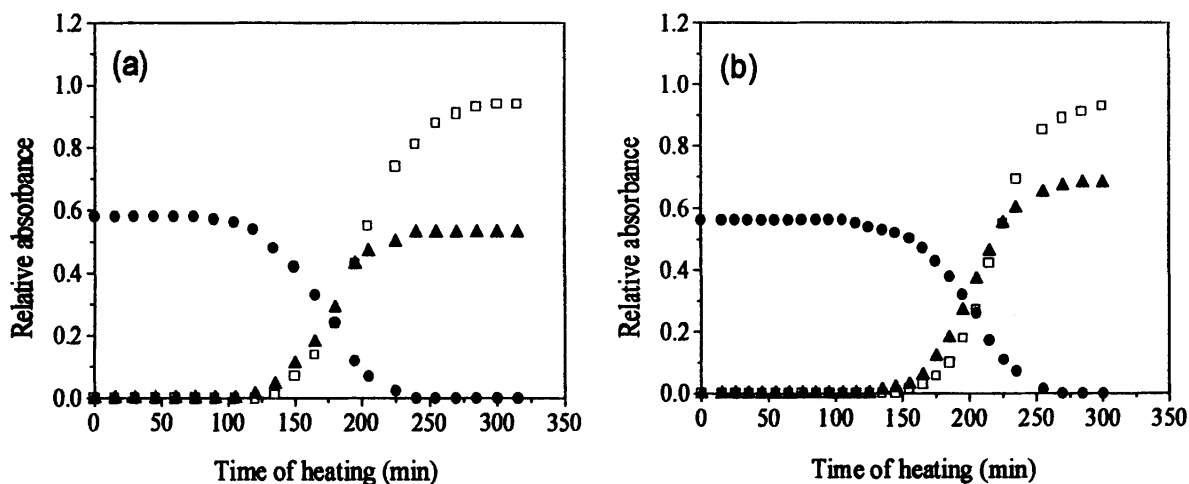


Figure 5. Evolution of the double bonds, hydroperoxides and carbonyl groups characteristics bands during the thermal oxidation of *Manihot* purified rubber at 140 °C in the presence of 0.1 wt%: (a) F-GH_{solid}; (b) synthetic β -sitosterol. (\square) OOH; (\bullet) C=C; (\blacktriangle) C=O.

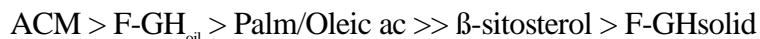
It can be observed that the curves from Figures 5a and 5b are very similar. The variation between each rate constant (Table II) is small and can be considered within the experimental uncertainty. The induction time is however significantly different, indicating that some impurity in the F-GH_{solid} fraction has a lower antioxidant effect than the synthetic β -sitosterol. Comparing the kinetic parameters from NR in the presence and in the absence of synthetic β -sitosterol, the rate constants are on average 1.4 times lesser than for the purified NR, while τ_i is 1.4 times greater. Table II summarises the kinetic parameters for all the studied systems.

Table II. Kinetic parameters for the thermal oxidative degradation of NR from *Manihot glaziovii* at 140 °C without any antioxidant and in the presence of 0.1 wt% of fraction GH constituents and of similar substances

NR	Rate constant			τ_i (min)			
	k_{OOH} (min ⁻¹)	$k_{\text{C=C}}$ (min ⁻¹)	$k_{\text{C=O}}$ (min ⁻²)	OOH	C=C	C=O	Average
without addition	1.2×10^{-2}	2.3×10^{-2}	2.4×10^{-5}	121	111	108	113 \pm 8
ACM	2.1×10^{-3}	2.3×10^{-3}	7.5×10^{-7}	517	534	507	519 \pm 10
F-GH _{oil}	1.9×10^{-2}	1.5×10^{-2}	4.1×10^{-6}	536	522	515	524 \pm 7
Palm/Oleic ac	4.0×10^{-3}	8.9×10^{-3}	1.1×10^{-6}	440	503	368	437 \pm 47
F-GH _{solid}	6.2×10^{-3}	1.6×10^{-2}	1.9×10^{-5}	134	125	129	129 \pm 5
β -sitosterol	7.7×10^{-3}	1.5×10^{-2}	2.0×10^{-5}	164	156	160	160 \pm 4

Conclusion

- 1) The crude antioxidant material, even in concentration 100 times inferior to the one existing in the crude rubber, has shown a significant antioxidant action on the thermal oxidation of *Manihot glaziovii* rubber thin films at 140 °C.
- 2) β -sitosterol, a neutral lipid, has presented little effect as antioxidant.
- 3) A mixture of palmitic acid and oleic acid has acted as a strong antioxidant, with comparable activity to the crude antioxidant material.
- 4) In general, the rate constant for double bond consumption was twice the rate constant for hydroperoxide formation.
- 5) The order of antioxidant activity on the thermal oxidation of *Manihot* NR, in the concentration of 0.1 %, was:



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