CHAPTER III

RESULTS

3.1 Saponification of latex

3.1.1 Optimum conditions for saponificaiton of latex

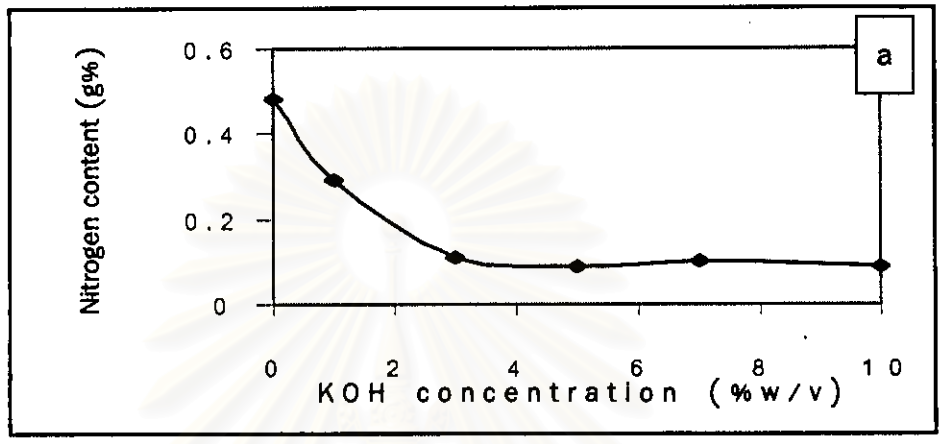
Fresh field latex in this research is stabilized with 0.2% ammonia at the rubber plantation after tapping, 0.15 p.h.r of hydroxylamine hydrochloride, 0.05 p.h.r of sodium metabisulfite and 0.1%v/v of Wing stay-L were added as viscosity stabilize, color control and anti-oxidation respectively. Initial latex in the range of 32-35 %DRC. DRC were adjusted to 30% DRC by adding water. Each saponification was performed in 100 ml of latex at 30%DRC by adding various concentrations of KOH from 0 to 10 % w/v. Control latex was 30% DRC latex containing 5%v/v isopropanol. The saponification was carried out at 70 °C, 3 hours and the efficiency of saponification in protein removal was monitored by measuring % nitrogen content of solid rubber after saponified latex was diluted 1 fold with water and coagulated by formic acid, washed and dried at 70 °C. The minimal percentage of total nitrogen about 0.1 g% was obtained when 5% w/v or more of KOH was used. (Figure 3.1a)

In the following experiment 5% w/v of KOH was therefore used, and the amount of isopropanol was varied from 0-5 %v/v. The solid rubber obtained indicated that isopropanol has no effect on removal of total nitrogen (Figure 3.1 b). Therefore in the next experiments isopropanol was not used. By varying temperature from 40 °C to 80 °C the proper temperature was confirmed at70 °C (Figure 3.1c). In the next experiments the temperature of saponification at 70 °C was used, Figure 3.1d shows when time was varied from 0-5 hours, the minimal percentage of total nitrogen about 0.11 g% was obtained with minimal saponification time of 3 hours (Figure 3.1 d).

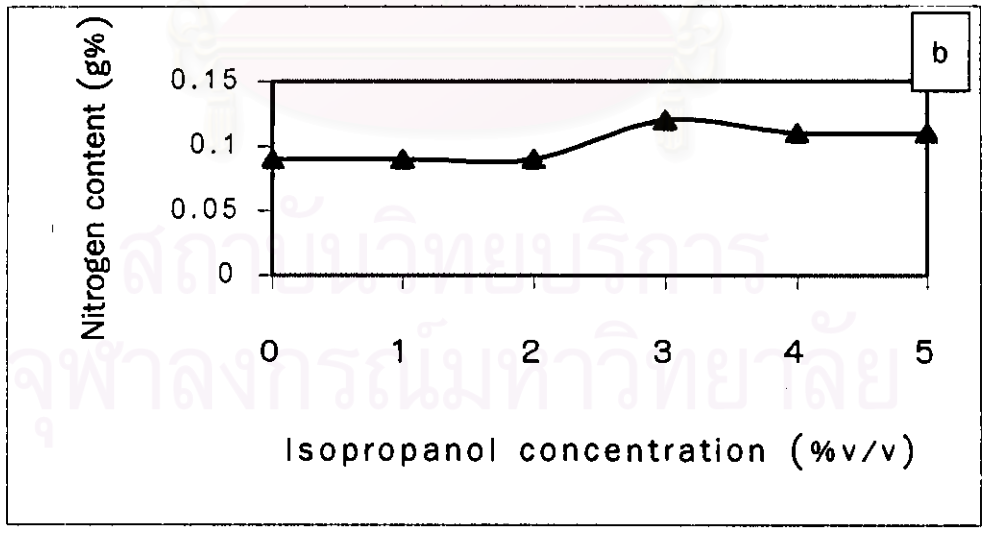
After saponification, latex proteins have been hydrolyzed into small oligopeptides, and soluble amino acids, which are readily soluble in water and easily removed from rubber particles, by washing. Therefore the dilution volume with water was studied for optimal process. Table 3.1 shows that the dilution ratio of latex: water in the range of 1:2 and 1:3 are necessary to obtain the minimum % N of the raw rubber.

Figure 3.1 Optimum conditions for latex saponification

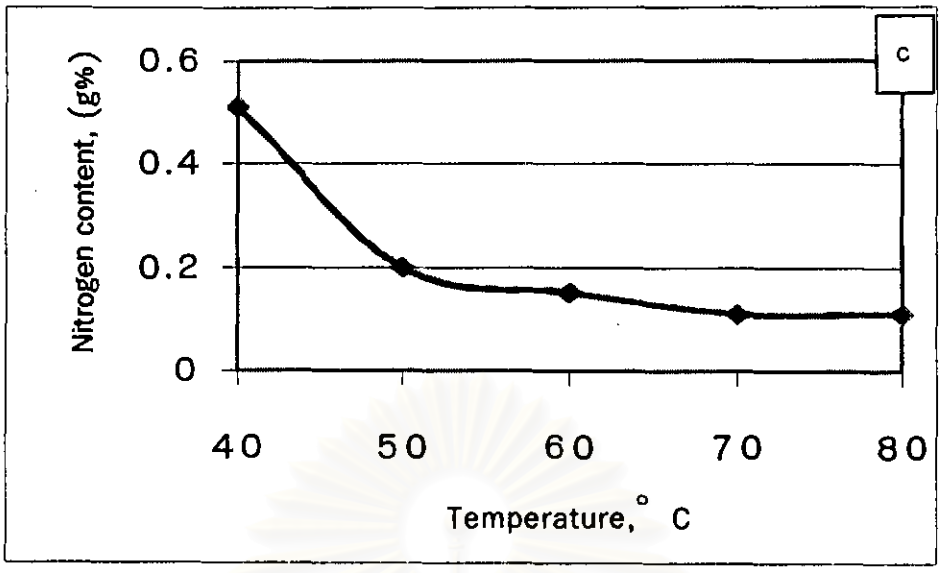
The 30% DRC latex was prepared from fresh field latex as described in 2.3.2 and then saponified at various conditions. The saponified latex was diluted 1 fold with water and coagulated with 2% formic acid. Total nitrogen of dry rubber was determined as described in 2.9.4.



a) The effect of KOH concentration on latex saponification
Treatment of latex at various KOH concentrations was performed at fixed isopropanol concentration at 5%v/v, 3 hours at 70 °C.

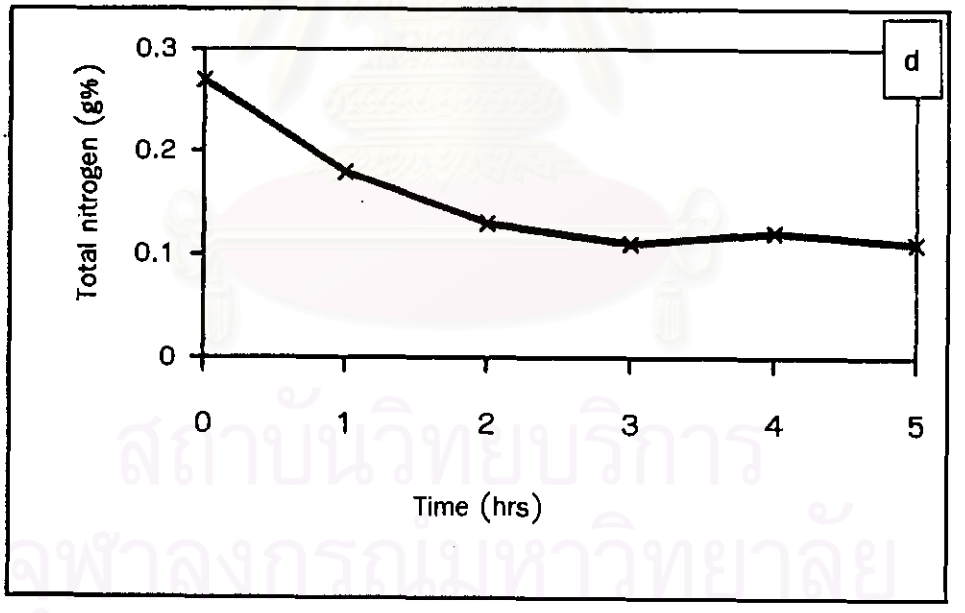


b) The effect of isopropanol concentration on latex saponification
The latex was saponifed with 5% w/v of KOH concentration at 70 °C for 3 hours at various concentrations of isopropanol.



c) The effect of temperature on latex saponification

The latex was saponified in 5%w/v of KOH at different temperature for 3 hrs.



d) The effect of time on latex saponification

The latex was saponified in 5%w/v of KOH, 70 °C at various times Intervals.

Table 3.1 Effect of dilution on latex saponification

After saponification reaction with 5%w/v KOH concentration at 70 °C for 3 hours, the resulting latex was diluted with various amounts of water and coagulated with 2% formic acid. Total nitrogen content of coagulum was determined.

Experiment	Dilution ratio (latex : water)	% Total nitrogen		% Yield
		Control	SAP-L	
1	1:0	0.68	0.21	93
	1:1.5	0.58	0.15	90
	1:2	0.57	0.09	90
	1:3	0.56	0.06	93
	1:6	0.49	0.09	89
2	1:0	0.65	0.20	93
	1:1.5	0.56	0.16	90
	1:2	0.51	0.08	90
	1:3	0.53	0.07	93
	1:6	0.47	0.09	90

* SAP-L: saponified latex

The optimal conditions for saponification of 30 % DRC fresh field latex can be summarized as follows: latex with the volume reaction of 100 ml was saponified with 5% w/v of KOH and incubated for 3 hours at 70 °C. After that, latex was diluted to 10-15 % DRC with water, and coagulated with 2% formic acid. The coagulum was then milled into crepe and washed with water and dried at 60 °C. The yield of saponified rubber starting from 100 ml of 30% DRC was more than 85 % with the nitrogen content less than 0.15 g%. However where the batch size was increased to 15 L under these optimal conditions the yield of SAP-L was 52 % and the quality of saponified rubber obtained was not as good as observed in from small batch. (Table 3.2)

3.1.2 Quality of saponified latex

The properties of raw rubber material indicate that the quality of solid rubber in term of percent non-rubber impurities (total nitrogen content, dirt, ash, volatile matter and color index) and the processibility of rubber (P_o, PRI and Mooney viscosity).

Table 3.2 Raw rubber properties of saponified latex (SAP-L) *

Specification of STR5L	Control	SAP-L	±% Difference from control
Nitrogen content (0.60g% max)	0.75	0.58	-23
Ash (0.40% max)	0.24	6.48	+2,600
Dirt (0.04% max)	0.003	0.009	+200
Volatile matter (0.80% max)	0.15	0.59	+293
Initial plasticity (P_o, 35 min)	31	63	+103
Plasticity Retention Index (PRI, 60 min)	27	26	-
Color Index (6, max)	5	6	+20
Mooney viscosity -	56.9	80	+41
% Yield	82	52	-36

SAP-L: saponifed latex

* The saponified latex was carried out at the batch size of 15 L.

Table 3.2 shows that when the batch size was increased from 100 ml to 15 L. the % N of saponified latex (SAP-L) is not significantly decreased from control non saponified rubber. The overall quality of SAP-L meets with the STR specifications except ash content and PRI. The results indicated that saponification of latex under these conditions drastically destroy natural rubber anti-oxidant.

High ash content indicated that potassium may be trapped in sapoinified rubber. Furthermore, the low PRI indicated that SAP-L rubber has lower resistance to aging.

Regarding to the yield of saponified rubber, when the batch size was increased from 100 ml to 15 L under these optimal conditions the yield of SAP-L was only 52 % where the yield of 100 ml batch was more than 85 %. These results suggested condition that about half of rubber will become waste together with the alkali solution, and will be discarded as wasted water. To minimize waste, saponfication was therefore attempted with crumb rubber.

3.2 Saponification of crumb rubber

Crumb rubber in this research is prepared from ammoniated latex and skim latex according to 2.3.3. Saponification reaction for ammoniated latex crumb (AL-crumb) and skim crumb (SK-crumb) were carried out with 30 g of crumb rubber in alkali solution volume of 100 ml with 5% KOH, 0.15 p.h.r of hydroxylamine hydrochloride, 0.05 p.h.r of sodium metabisulfite and 0.1%v/v of Wing stay-L. The reaction was carried out at 70 °C for 3 hours, then the resulted rubber was washed and dried at 60 °C.

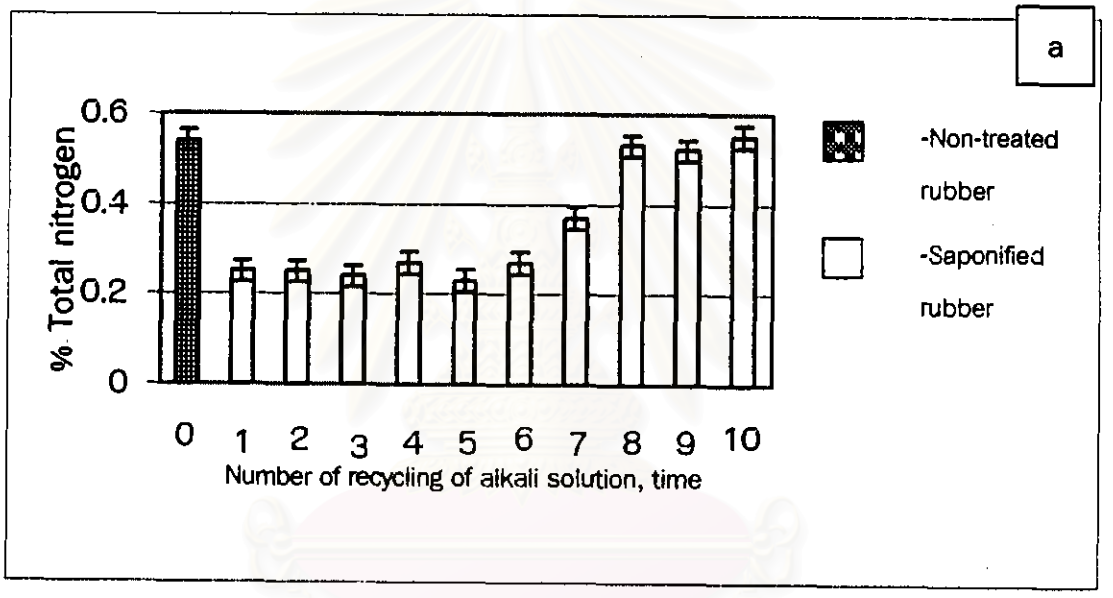
3.2.1 Efficiency of alkali solution in crumb rubber saponification

To minimize the loss of yield, ammoniated latex and skim latex were previously coagulated with 2% formic acid and sulfuric acid respectively. Coagulum was washed out, cut into small pieces before saponification. Furthermore to minimize wastewater, the alkali solution after the first saponification of crumb rubber was reused with the second lot of crumb rubber. The reuses of alkali solution are determined for the efficiency change during each cycle of utilization by monitoring % N in saponified rubber. Figure 3.2a shows consecutive saponfication of ammoniated crumb rubber. The total nitrogen of the saponified ammoniated crumb rubber (SAP-AL) can be reduced about half until the seventh times of recycling of alkali solution.

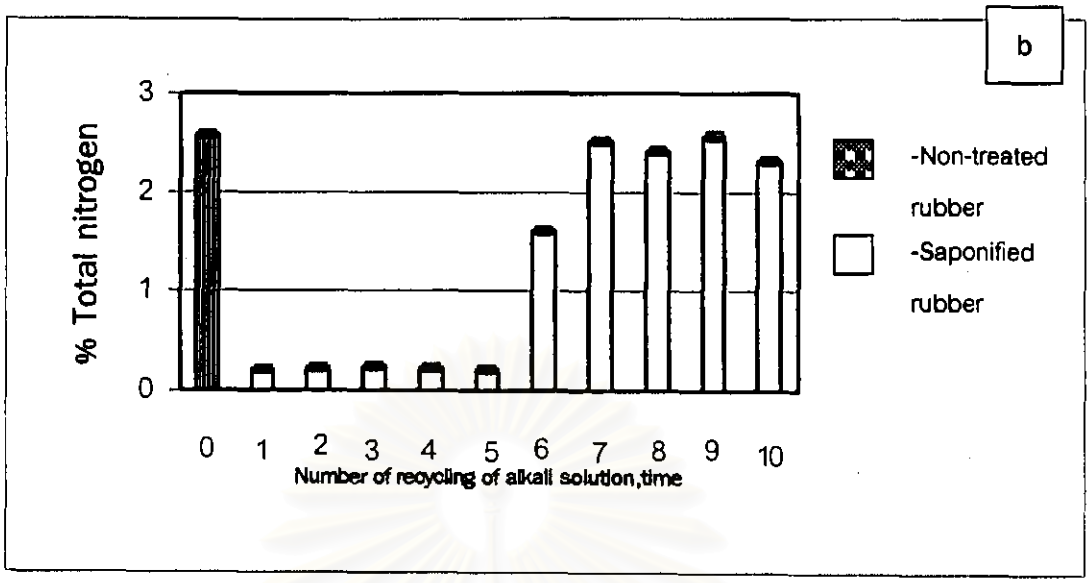
When skim crumb rubber was used, the efficiency of alkali solution recycling was five times. (Figure 3.2b)

Figure 3.2 Efficiency of alkali solution for saponification of solid crumb

Solid crumb rubbers were prepared from ammoniated latex and skim latex respectively and 30 g of crumb rubber was saponified in 100 ml of 5% w/v of KOH concentration, at 70 °C for 3 hours. For the next cycle new lot of ammoniated crumb rubber, another 30 g was incubated in the same alkali solution. The saponified solid crumb was then washed with water. Total nitrogen content of saponified solid rubber was determined.



a) Reutilization alkali solution for consecutive saponification of ammoniated crumb rubber



b) Reutilization of alkali solution for consecutive saponification of skim crumb rubber

The efficiency of alkali solution can be concluded that: ammoniated crumb rubber can be reused the alkali solution 7 times and the number of recycling alkali solution in saponification of skim crumb is 5 times.

3.2.2 Quality of saponified crumb rubber

The saponification of crumb rubber was carried out at the batch size of 1.5 kg instead of 30 g to obtain enough specimen for physical tests. Repeated use of alkali solution were five times for crumb rubber prepared from ammoniated latex. Table 3.3 shows that saponified ammoniated crumb (SAP-AL) have lower total nitrogen content in the range of 0.24-0.36 g%. These means about half of nitrogen was removed. Under ammoniated crumb rubber saponification reaction the ash content still increased but better than that of latex saponification. However there is no significant difference in other contaminants such as: dirt, volatile matter and P_o and Mooney viscosity at 95 % confidence. The aging property, PRI of SAP-AL is significant lower than its control but these values are not much lower than STR5L specification, which means that SAP-AL can replace STR5L in some process.

Table 3.3 Raw rubber properties of saponified rubber, produced from ammoniated crumb rubber

Specifications of STR5L	Control (n =4)	SAP-AL (n=10)	± % Difference from control
Nitrogen content (0.60g% max)	0.49±0.01 [a]	0.29±0.04 [b]	-41
Ash (0.40% max)	0.14±0.01 [b]	0.85±0.11 [a]	+507
Dirt (0.04% max)	0.009±0.001	0.011±0.001	+22
Volatile matter (0.80% max)	0.76±0.01	1.33±0.74	+75
Initial plasticity (P_0 ,35 min)	20±1.63	28±1.71	+40
Plasticity Retention Index (PRI, 60 min)	99±0.58 [a]	64±20.7 [b]	-
Color Index (6 max)	5±0.57	5±1.05	0
Mooney viscosity -	40±1.63	55±4.93	+38

SAP-AL: Saponified ammoniated crumb rubber

Significant difference of physical properties between control and saponified crumb rubber are marked by different letter (a, b) analyzed by t-test at 95 % confidence.

Saponified rubber was produced from skim solid crumb by repeated uses of alkali solution five times and the batch size was increased skim crumb from 30 g to 1.5 kg. Table 3.4 summarizes the raw rubber properties of saponified skim crumb rubber (SAP-SK) in comparison with control untreated raw rubber of identical clone. After saponification, the nitrogen content of SAP-SK was significantly reduced to 0.12 g% when started from 1.85 g%. These results indicated that about 93% of nitrogen was removed. There is no significant difference in other impurities such as dirt and volatile matter after saponification, and these values are not much lower than the acceptable limit of STR-5L specification. The processibility of rubber such as P_0 is not much lower than STR 5L specification. Although ash content is increased but these values are lower than latex and ammoniated crumb saponification. The Moooney viscosity of saponified rubber is always higher than its control. This result suggested that cross-linking

should be increased during acid coagulation and persisted after saponification. The color index of SAP-SK is significantly lower than the control (Figure 3.4).

Table 3.4 Raw rubber properties of saponified rubber, produced from skim crumb rubber

Specifications of STR5L	Control (n = 4)	SAP-SK (n = 10)	± % Difference from control
Nitrogen content (0.60 g% max)	1.85±0.01 [b]	0.12±0.04 [a]	-94
Ash (0.40% max)	0.29±0.02 [b]	0.65±0.11 [a]	+124
Dirt (0.04% max)	0.014±0.001	0.018±0.017	+28
Volatile matter (0.08% max)	0.63±0.01	0.96±0.57	+52
Initial plasticity (P_o,35 min)	18±3.42 [b]	29.8±9.07 [a]	+65
Plasticity Retention Index (PRI , 60 min)	80±1.91	76±20.6	-
Color Index (6, max)	9±0.5 [a]	6±0 [b]	-33
Mooney viscosity -	33±3.4 [b]	55.7±17.8 [a]	+69

SAP-SK: Saponified skim crumb rubber

Significant difference of physical properties between control and saponified crumb rubber are marked by different letter (a, b) analyzed by t-test at 95 % confidence.

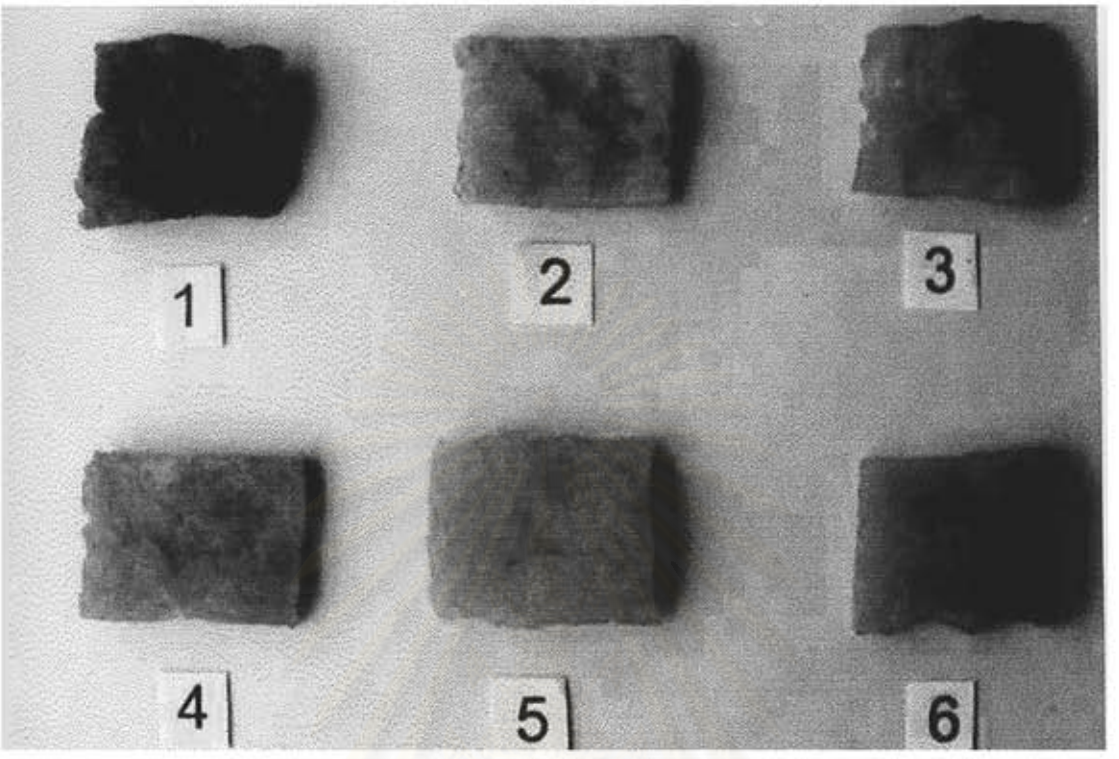


Figure 3.3 Color comparison among non-saponified ammoniated rubber and SAP-AL

1 : Control; non-saponified rubber

2-6 :1st - 5th lot of SAP-AL

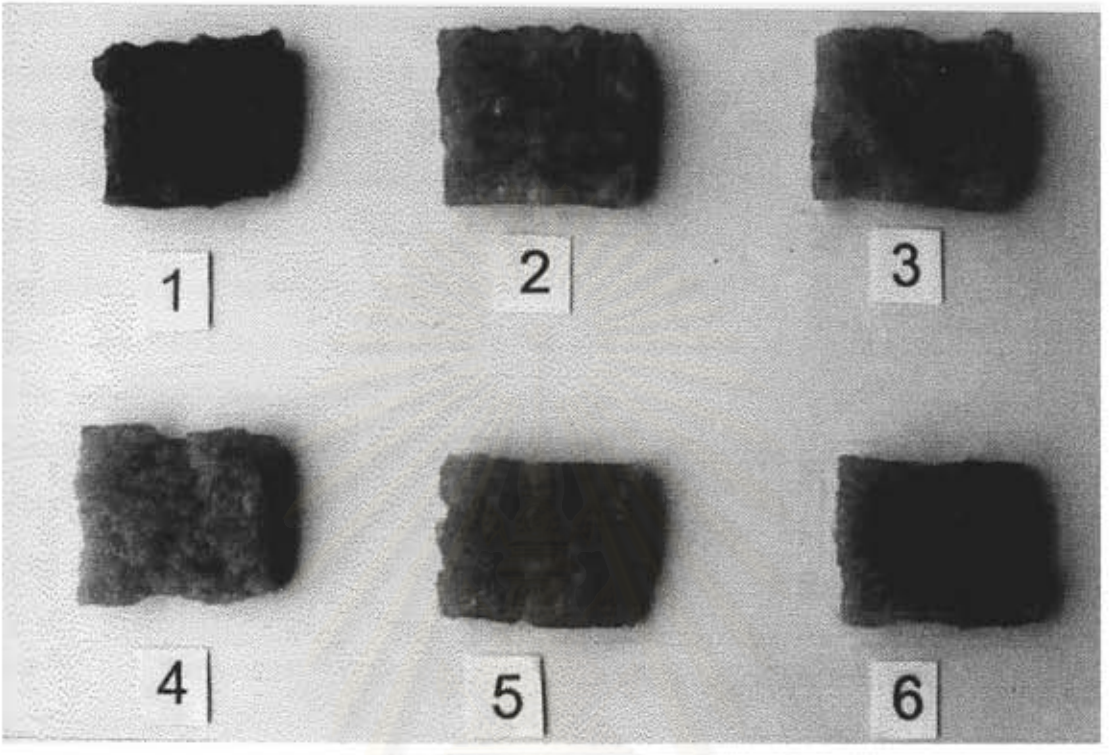


Figure 3.4 Color comparison among non-saponified skim rubber and SAP-SK

1 : Control: non-saponfied rubber

2-6: 1^{st} - 5^{th} of recycling alkali solution rubber

In conclusion, as judged by both retained total nitrogen content, color index and other impurities, the obtained SAP-SK from treatment of skim crumb rubber by saponification give lower non-rubber impurities than treatment of ammoniated crumb rubber especially in the nitrogen content and color. Although this treatment resulted in increase of ash content but they give better ash content than latex saponfication. To determine the effect of saponification on ammoniated and skim crumb rubber, molecular characteristic, gel content and vulcanized properties have been studied.

3.3 Molecular characteristics of rubber by GPC

The number and weight average molecular weights ($\overline{Mn}$, $\overline{Mw}$) of rubber samples from untreated and saponified rubber was determined by GPC using polystyrene standard samples. Table 3.5 shows the $\overline{Mn}$ and $\overline{Mw}$ of NR and SAP-NR before and after saponification, the $\overline{Mn}$ and $\overline{Mw}$ of ammoniated crumb (SAP-AL) are slightly decreased when compared with its control while the $\overline{Mw}$ and $\overline{Mn}$ of skim crumb rubber (SAP-SK) is significantly decreased. The decreasing of these value indicate that skim crumb saponfication affect $\overline{Mn}$ and $\overline{Mw}$. Polydispersity of SAP-AL decreased to a very narrow range where SAP-SK show higher $\overline{Mw}/\overline{Mn}$, the results suggested that proteins involved in cross-linking within the rubber particles are removed. In case of SAP-SK, breakage is evident by drastic reduction of both $\overline{Mw}$ and $\overline{Mn}$.

Table 3.5 The weight average and number average of rubber before and after saponification

Sample	$\overline{Mw}$ x10^{-6}	$\overline{Mn}$ x10^{-5}	$\overline{Mw}/\overline{Mn}$
C-AL	2.47	4.93	5.01
SAP-AL1	1.49	4.66	3.19
SAP-AL2	1.56	4.33	3.59
SAP-Al3	1.35	3.74	3.61
SAP-AL4	1.83	5.26	3.47
SAP-Al5	1.49	3.97	3.77
C-SK	2.97	9.81	3.03
SAP-SK1	0.13	0.23	5.72
SAP-SK2	0.11	0.24	4.58
SAP-SK3	0.10	0.17	5.96
SAP-SK4	0.13	0.21	6.08
SAP-SK5	0.19	0.20	9.58

C-AL and C-SK: Control rubber from ammoniated crumb and skim crumb respectively, SAP-AL and SAP-SK: Saponified rubber from ammoniated crumb and skim crumb respectively, Number 1-5: time of recycling alkali solution

3.4 Gel content and acetone soluble content of NR and SAP-NR

The gel phase is well known as cross-linking of rubber and non-rubber in vivo or during coagulation. The gel content in the rubber prepared from ammoniated crumb rubber was reduced from 5% to 4% after saponification, whereas the saponified rubber prepared from skim crumb (SAP-SK) show a drastic decreased the gel content from 32% to 3-4%. These results mean that about 90 % of gel were removed. These indicated that the cross-links of SAP-SK were destroyed by saponification.

Extraction of solid rubber with acetone removes free fatty acids and their esters. Table 3.6 showed that all saponified ammoniated crumb rubber (SAP-AL) and saponified skim crumb rubber (SAP-SK) show decrease in acetone soluble content about 7% and 40 % respectively. The decreasing of acetone soluble content in both saponified rubbers can imply that: 1) protein and fatty acyl ester in ammoniated crumb rubber are well protected and not destroyed by saponification. 2) protein (90%) and lipid (40%) are involved in gel formation of skim crumb, which can be removed by saponificaiton. 3) this gel structure may protect rubber molecule from oxidation and breakage, without this structure the rubber molecules degrade.

Table 3.6 Gel content and acetone soluble content of rubber before and after saponification

Sample	Gel content, %	Acetone soluble content, %
C-Al	5.70	2.26
SAP-AL1	4.10	2.10
SAP-AL2	4.33	1.71
SAP-AL3	4.13	1.82
SAP-AL4	4.15	2.01
SAP-AL5	5.57	1.64
C-SK	32.2	4.60
SAP-SK1	2.95	2.73
SAP-SK2	3.27	2.47
SAP-SK3	4.97	2.51
SAP-SK4	3.67	2.09
SAP-SK5	3.82	1.69

C-AL and C-SK: control rubber from ammoniated crumb and skim crumb respectively, SAP-AL and SAP-SK: Saponified rubber from ammoniated crumb and skim crumb respectively, Number 1-5: time of recycling alkali solution

3.5 Structural characteristics of NR by FT-IR

The presence of polypeptides and fatty acid can be detected by FT-IR Spectroscopy which can characterize bands of inner N-H of peptide linkage stretching at 3280 cm^{-1}, -(NH) C=O at 1628 cm^{-1}, N-H bending at 1540 cm^{-1} and -(OR)C=O at 1738 cm^{-1}.

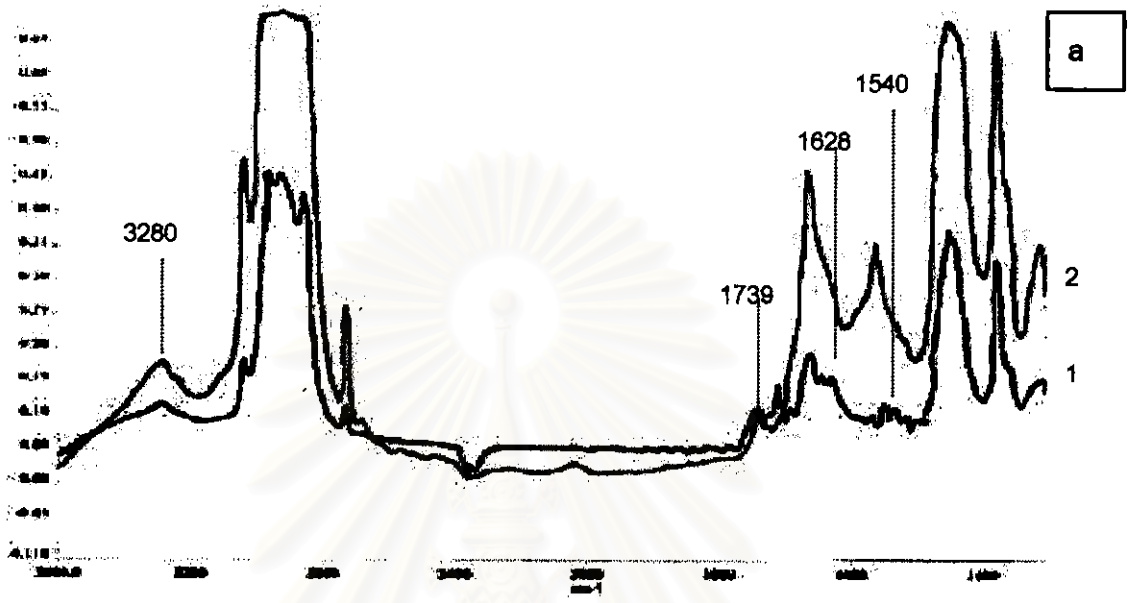
Figure 3.5 a shows the FT-IR spectra of control and saponified ammoniated crumb with the characteristic bands inner N-H units of the peptide linkage of frequency at 3280 cm^{-1} of SAP-AL is persisted after saponfication under these conditions and the band at 1628 which belongs to -(NH) C=O disappeared. These results suggest that ammoniated crumb rubber saponification hydrolyze amide linkage of some proteins, but still consist of polypeptide because the inner N-H units of the peptide linkage is persisted. Furthermore, SAP-AL showed a clear infrared band at 1738 cm^{-1}which is a characteristic of ester groups in fatty acid esters.

Saponified skim crumb (SAP-SK) shifted vibrational band corresponding to inner units of peptide linkage from 3280 cm^{-1} to 3417 cm^{-1} (Figure 3.5 b). The N-H at 3417 cm^{-1} group is a terminal amino acid of oligopeptide , these can imply that skim crumb saponfication hydrolyze peptide linkage as protease do in the rubber latex. The characteristic bands of the amide and amine bonding at 1628 $cm^{-1}$and 1540 cm^{-1} also disappeared in SAP-SK. Furthermore, the band at 1738 cm^{-1}which is a characteristic of ester groups in fatty acid esters diminished after saponification of skim crumb rubber.

In conclusion, saponified crumb rubber prepared from skim crumb (SAP-SK) better than saponified ammoniated crumb (SAP-AL) due to reducing number of peptide linkage of rubber, that resulted in oligopeptide. The ester group in fatty acid ester diminishes after saponification under these conditions.

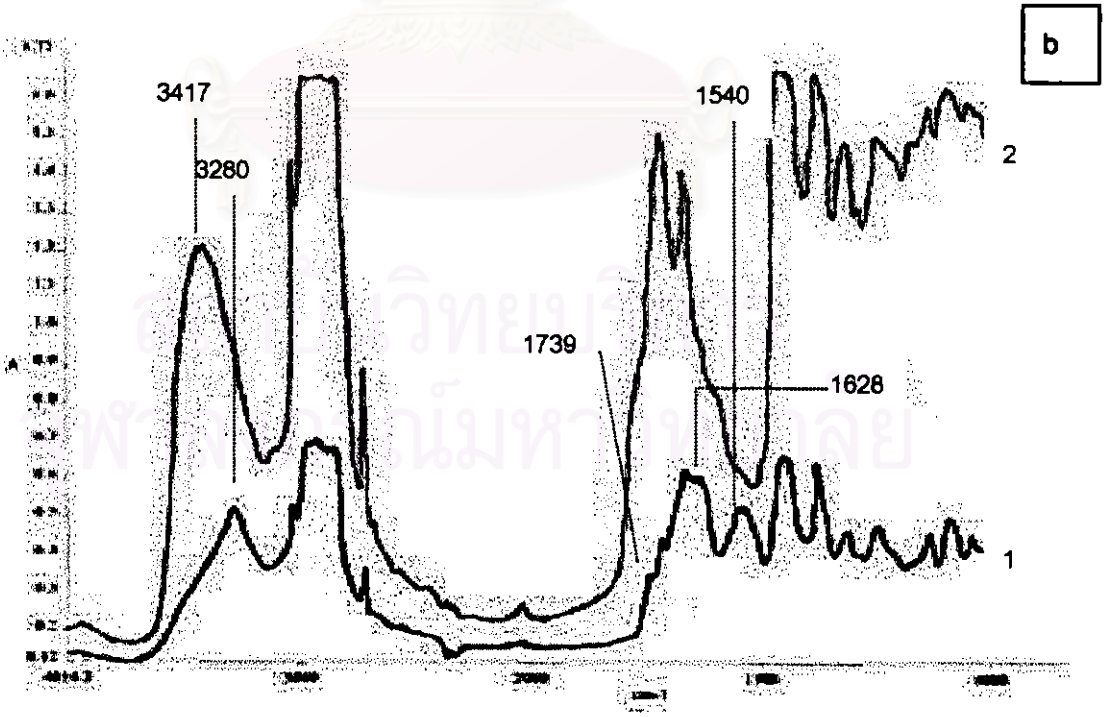
A typical FT-IR spectrum of NR obtained after saponification is shown in Figure 3.5.

Figure 3.5 FTIR spectra of the rubber from control and saponified rubber



—— 1: Control ammoniated crumb rubber

—— 2: Saponified ammoniated crumb rubber



___ 1; Control skim crumb rubber

___ 2: Saponified skim crumb rubber

3.6 Processibility of saponified rubber

Processibility of the rubber in the factory is usually performed by mixing the rubber with chemical ingredients to obtain the compound rubber and followed by curing or vulcanization. The compounding formula used in this research is normal formulation for making the outsole of sport shoe, where the high modulus, high resilience, high durability rubber and lighter color are required.

Since in this process, the compound rubber should flow throughout a complicate mold during the short precise time duration and this process, the appearance physical properties of vulcanizate should be uniform.

The existence of high nitrogen in NR has been believed to affect the cure behavior of rubber compound. Theses proteins can interfere with the homogenization of vulcanizing ingredients resulting in reduction of cure rate.

Deproteinization by saponification of ammoniated crumb rubber has retarded the rate of cure by extending the scorch times and a slight decrease in cure time which normally are in range of 3.20-3.59 min and 3.56-4.32 min respectively (Figure3.6 a, Figure 3.6 b). These suggest that ammoniated crumb saponification is not enough to remove non-rubber especially proteins so that the rubber not enough homogenized rubber. Figure 3.7 shows cure characteristic of control and saponified skim crumb (SAP-AK), the cure rate of SAP-SK shows a trend to increase by extending in cure time and slightly decreasing in scorch time. These can imply that skim crumb saponification resulting in improve homogeneous vulcanization.

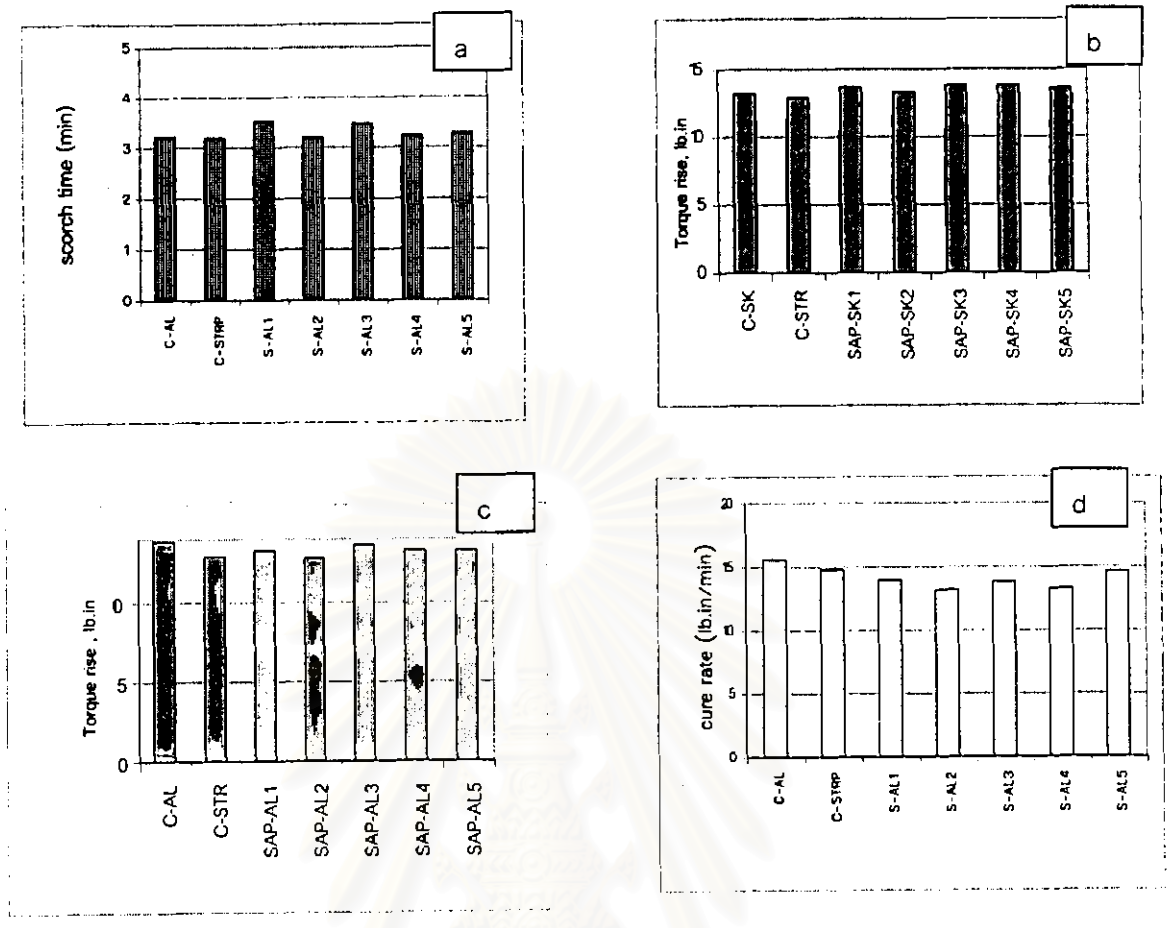


Figure 3.6 Rheological properties of saponifed ammoniated crumb rubber (SAP-AL)

C-P: Control rubber from production

C-AL: Control non-saponified rubber from ammoniated crumb

SAP-AL: Saponified rubber from ammoniated crumb rubber

Numbers 1-5: lot of SAP-AL

a) Scorch time b) Cure time

c) Torque rise (MH-ML) d) Cure rate

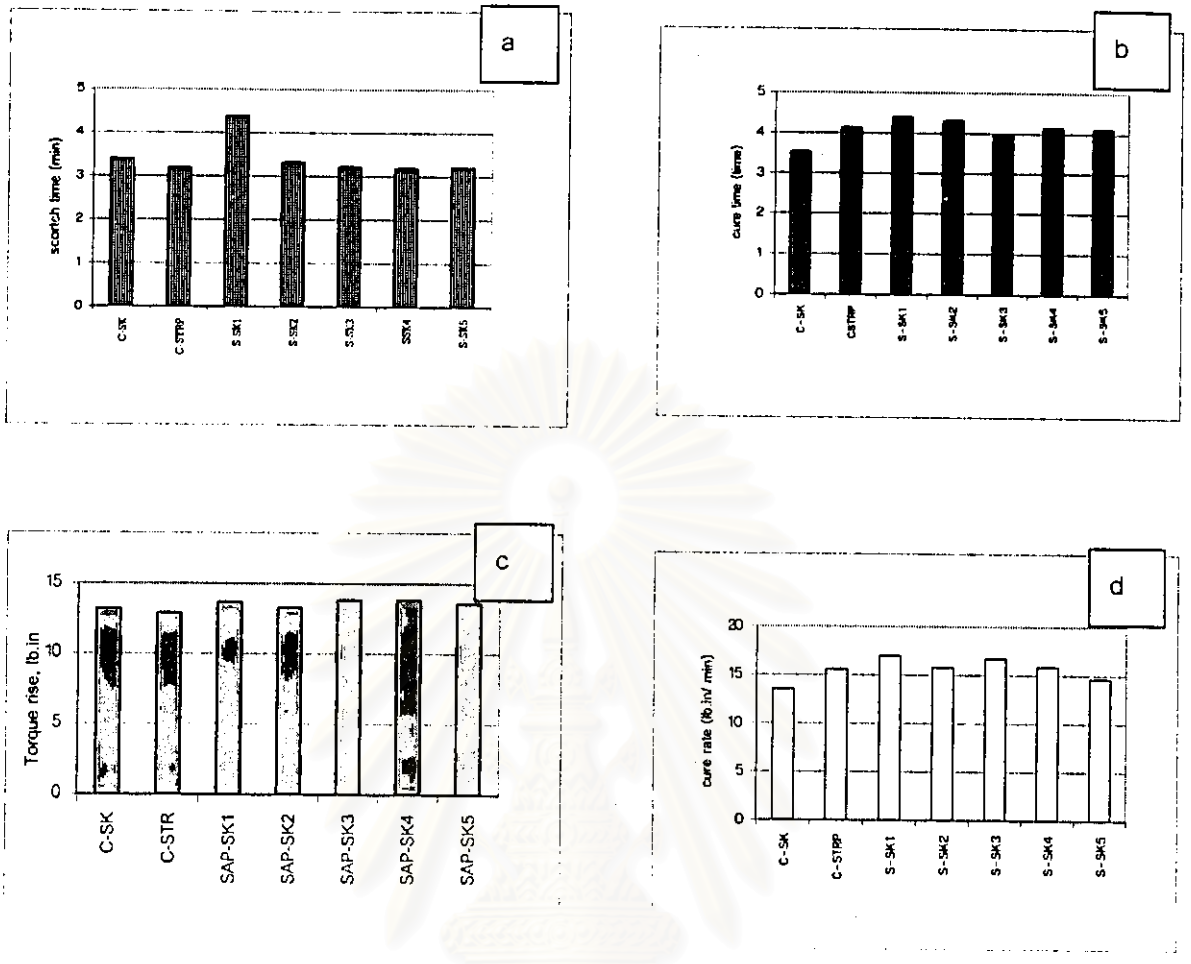


Figure 3.7 Rheological properties of saponifed skim crumb rubber (SAP-SK)

C-P: Control rubber from production

C-SK: Control non-saponified rubber from skim crumb

SAP-SK: Saponified rubber from skim crumb rubber

Numbers 1-5: lot of SAP-SK

a) Scorch time b) Cure time

c) Torque rise (MH-ML) d) Cure rate

Figure 3.8 shows the scattered plot between the total nitrogen content and its cure rate, the cure rate increases when the reduction of the total nitrogen contents are lower than 0.2 g% for this compound formulation.

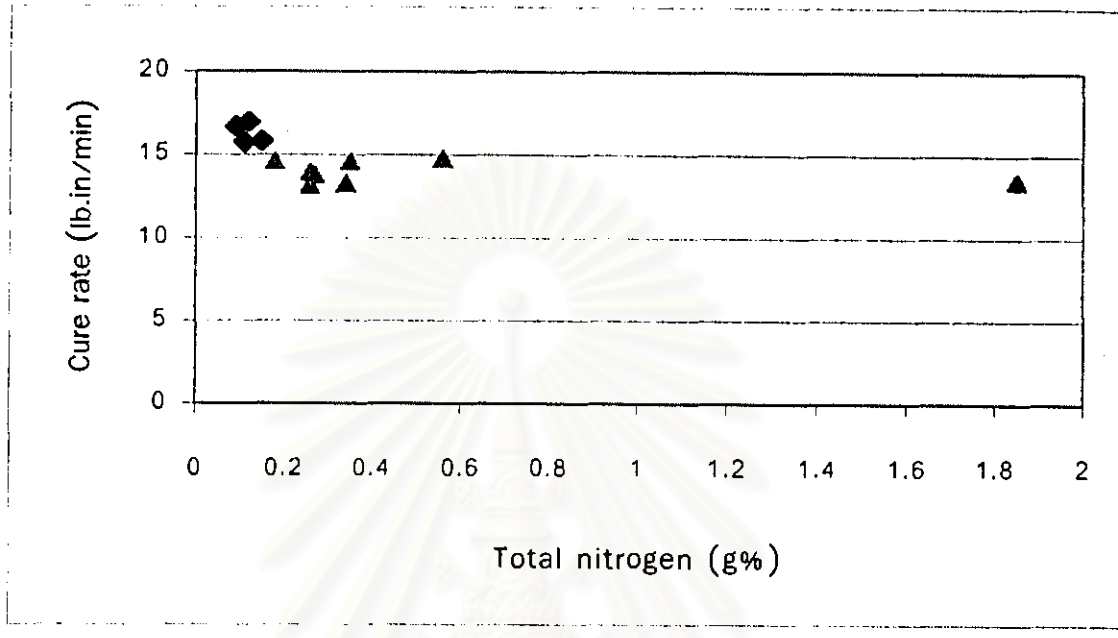


Figure 3.8 Relationship between the total nitrogen content and the cure rate

Technological properties of saponified NR vulcanizate have been compared with its control and commercial TTR-5 (production) as shown in Figure 3.9, the properties of SAP-NR prepared from ammoniated crumb rubber such as hardness, 300% modulus, tensile strength, tear strength, and specific gravity were not evidently changed after treatment while elongation at break decreased.

The properties of saponified NR vulcanizate prepared from skim crumb are summarizes in Figure 3.10. After treatment, like SAP-AL that all of the properties were not significantly changed except decreasing in elongation at brake.

The elongation at brake values reflects the cross-linking density of the rubber. The decrease in these value of SAP-AL and SAP-SK indicates that the cross-linking density of the SAP-AL and SAP-SK rubber decrease.

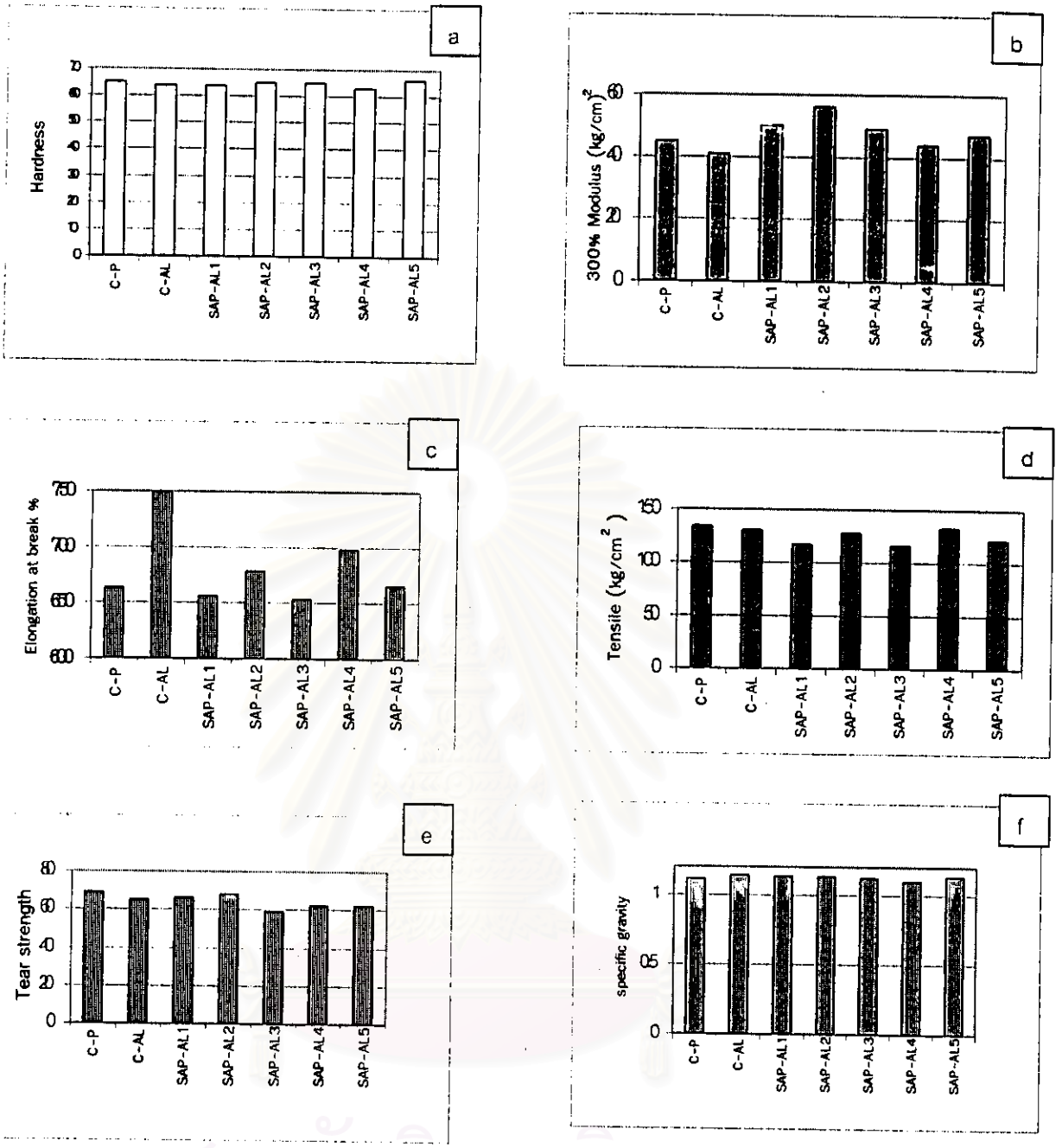


Figure 3.9: valcanizate properties of saponified produced from ammoniated crumb

C-P: Control untreated rubber of production, C-AL: Control untreated rubber of experiment, SAP-AL: saponified rubber from ammoniated crumb, Number 1-5: time of recycling alkali solution

a) Hardness (shore A) b) 300% Modulus c) Ultimate elongation

d) Tensile strength e) Tear strength f) Specific gravity

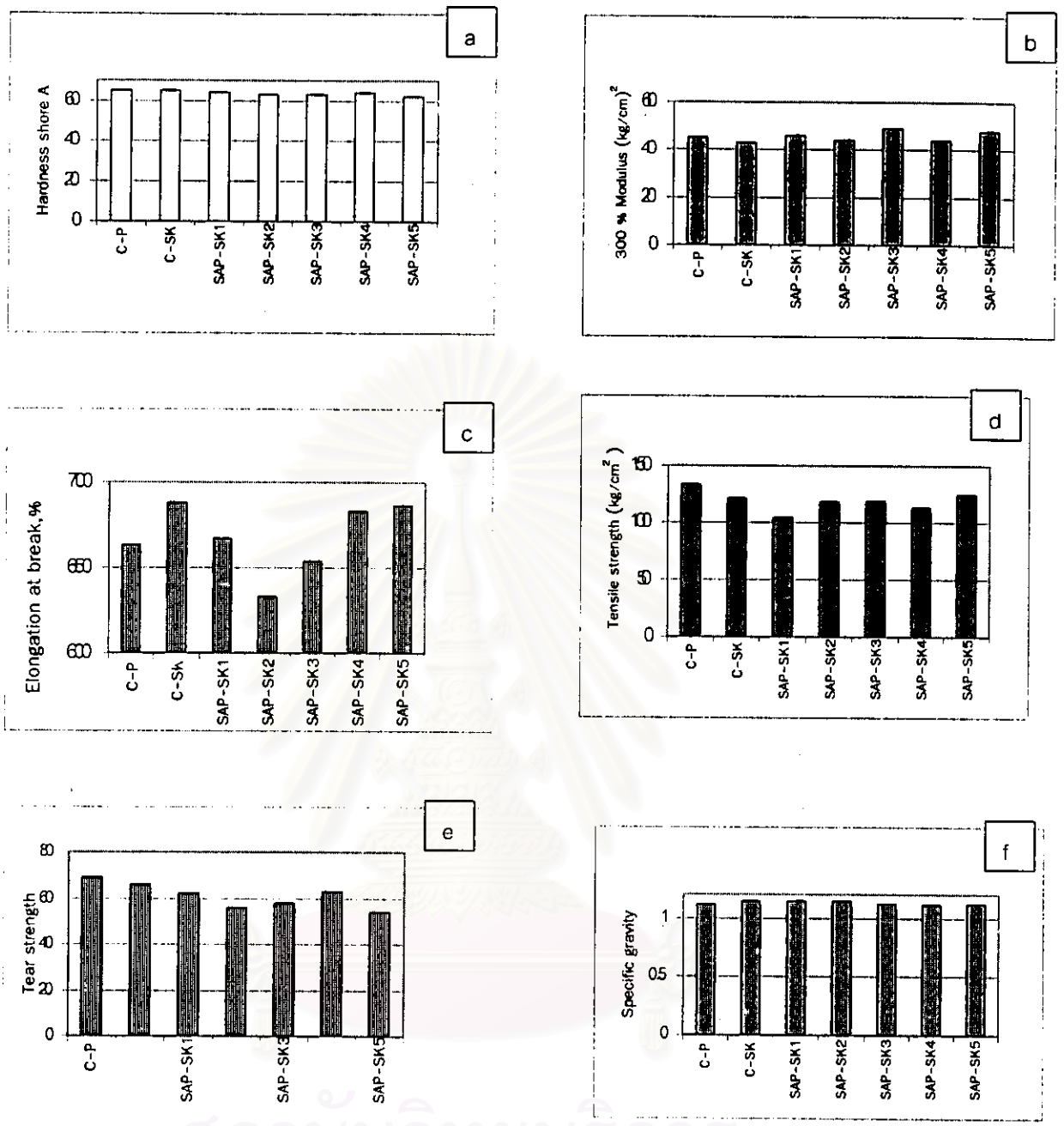


Figure 3.10: valcanizate properties of saponified produced from skim crumb

C-P: Control untreated rubber of production, C-SK Control untreated rubber of skim crumb, SAP-SK: saponified skim crumb, Number 1-5: time of recycling alkali solution

a) Hardness (shore A) b) 300% Modulus c) Ultimate elongation

d) Tensile strength e) Tear strength f) Specific gravity

The color of vulcanized from ammoniated crumb rubber and skim crumb rubber are shown in Figure 3.11(a-b), it sit noted that the color all of SAP-NR from ammoniated crumb rubber, skim crumb rubber and non-treated rubber shown light color as well as the commercially used TTR-5L.

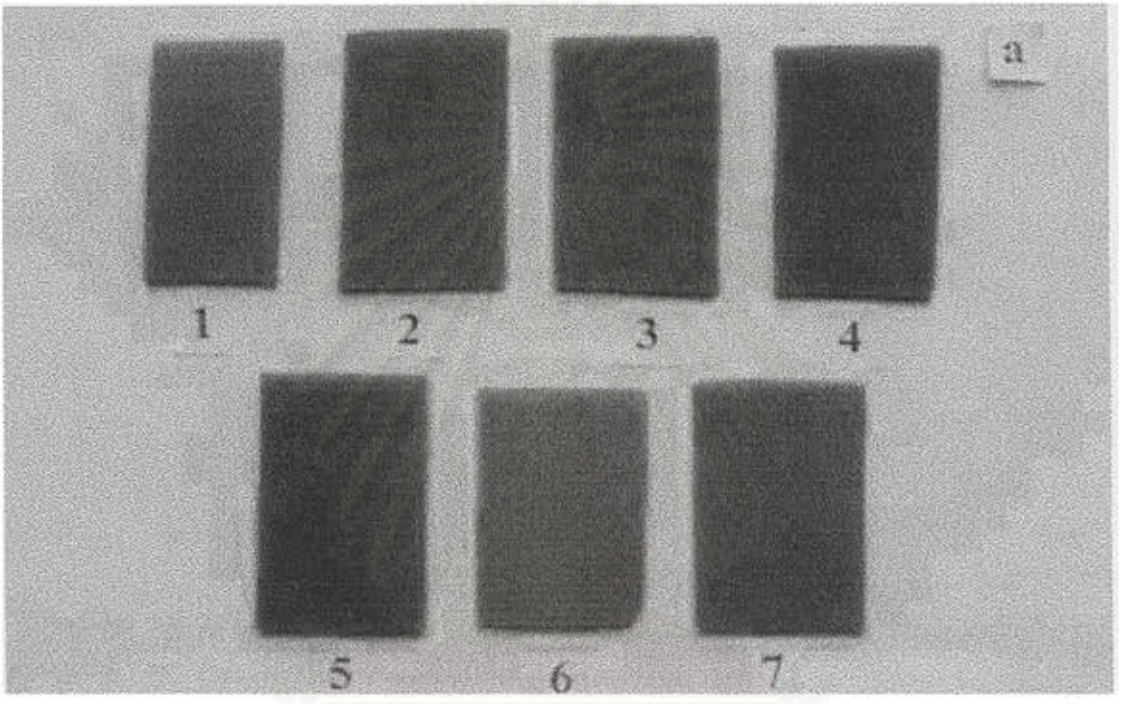


Figure 3.11 Color of ammoniated crumb rubber vulcanize

Compound rubber from high ammoniated solid crumb either saponified or control, were cure in similar compression mold at 155 °C for optimal cure time and compared for color.

1:CP; Untreated rubber from production (STR-5L),

2:C-AL; untreated rubber,

3-7: SAP-AL1-SAP-AL5; saponified rubber from the1th of recycling alkali solution -saponified rubber from the 5th of recycling alkali solution

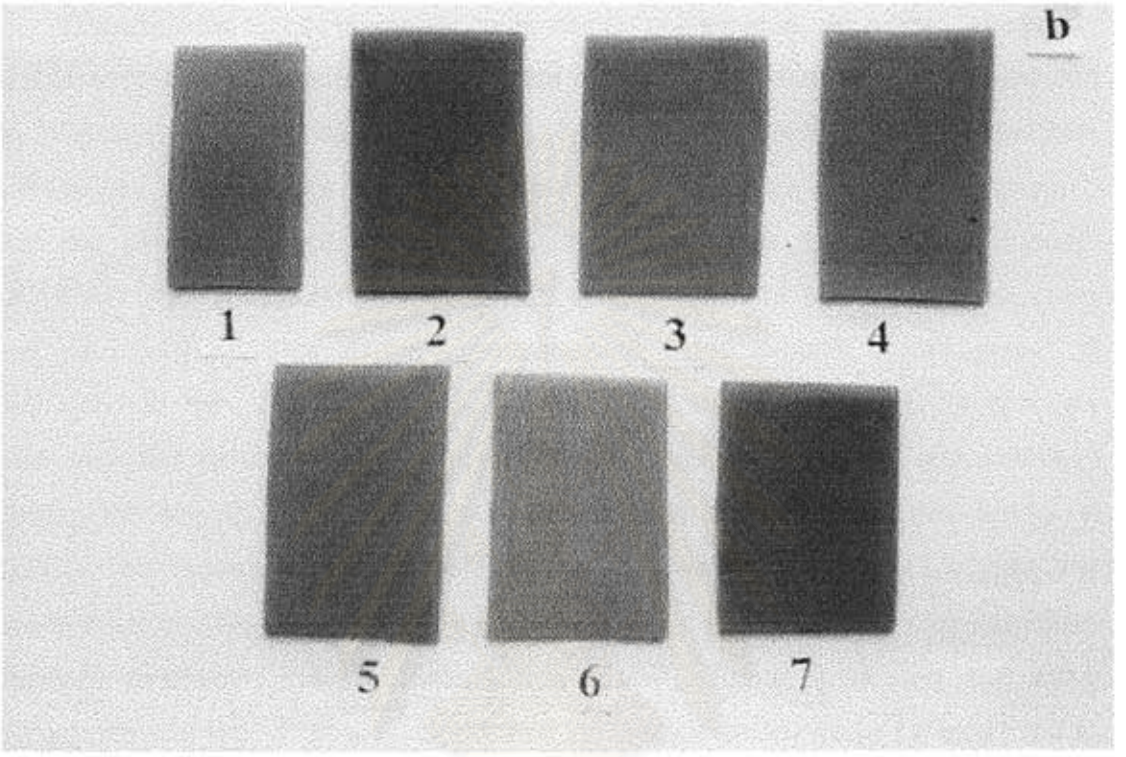


Figure 3.12 Color of skim crumb rubber vulcanize

Compound rubber from skim crumb saponified or control, were cure in similar compression mold at 155 °C for optimal cure time and compared for color.

1:C-P; untreated rubber from production (STR-5L),

2:C-SK: Untreated rubber,

3-7: SAP-SK1 -SAP-SK5; saponified rubber from the1th of recycling alkali solution - saponified rubber from the 5th of recycling alkali solution

In conclusion, the over all properties obtained SAP-AL and SAP-SK, was not significantly changed after saponification. In the next step, the water extractable protein, which has been believed protein allergen, will be observed.

3.7 Comparison of water extractable protein of non-saponified rubber and saponified rubber

Since solid rubber is usually compounded and molded into various shapes of rubber product and there is no standard method for extractable protein prepared as in gloves and other dipping products. The method to extract water extractable protein was done by raw material specimens were cut into small pieces about 5 mm and about 10 g extracted with 10 volume of water at 37 °C for 2 hours (2.12.1). The water extractable protein can be determined by modified Lowry method (2.12.3). This method involved determined protein in the preset of $CuSO_4$and in the absent of $CuSO_4$. Figure 3.13 shows $O.D_{750}$ of the standard ovalbumin that determined by Modified Lowry method in the present and absent of $CuSO_4$, that the $O.D_{750}$ in the present of $CuSO_4$ is higher than in the absent of $CuSO_4$. The results indicated that the higher value is due to $CuSO_4$ so the absorbance at 750 nm is high. Thus water extractable protein was evaluated from standard ovalbumin that subtracting from $CuSO_4$. These suggested that water extractable protein, which was determined by Lowry method, might be interfered by divalent cation contaminants, resulting in the high absorbance without protein per se. Therefore to obtain the correct result, cation should be removed by dialyzing the sample or precipitation protein before determination by Lowry' s method.

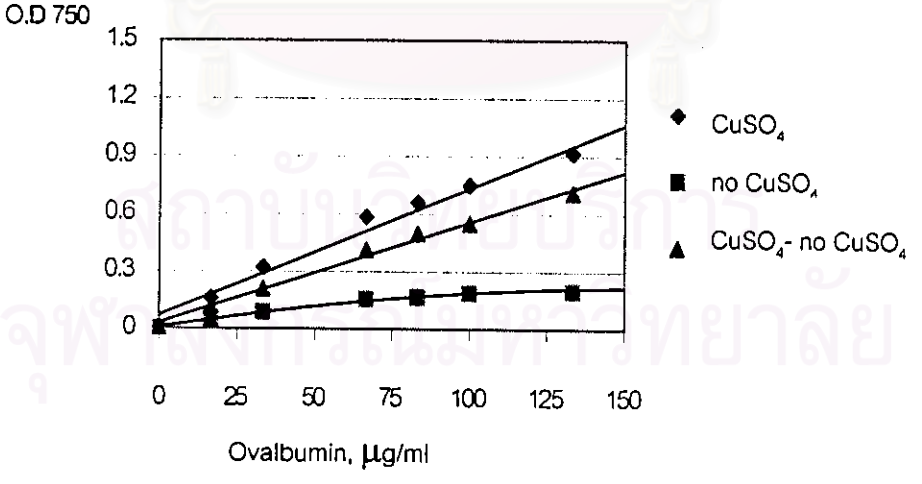


Figure 3.13 Effect of $CuSO_4$ on standard ovalbumin protein determination by Lowry method.

Table 3.7 shows that the nitrogen content of rubber decreased substantially after saponification and extraction with 10-volume of water. The water extractable protein from C-AL and SAP-AL are about the same, but the water extractable protein of SAP-SK is significantly lower after saponification.

Table 3.7 The nitrogen content and water extractable protein prepared from saponified and non-saponified rubber

Sample	% Nitrogen content		The water extractable protein, μg/g
	Before extract	After extract	
C-AL	0.49	0.44	32.5
SAP-AL	0.29	0.21	47.2
C-SK	1.85	1.53	131.2[a]
SAP-SK	0.13	0.11	< 10[b]

C-AL and C-SK: are control rubbers from ammoniated crumb and skim crumb; SAP-AL and SAP-SK: are saponified ammoniated crumb and skim crumbs respectively.

Significant difference of water extractable protein between control and saponified crumb rubber are marked by different letter (a, b) analyzed by t-test at 95 % confidence.

Since the nitrogen content before and after saponification of both rubber were not significant change, and the amount of water extractable protein of both rubber seem to be low especially in SAP-SK, these may be the surface area of the rubber may be difference. Therefore the surface characteristics of these rubber was studied.

Figure 3.14 show the surface characteristics of C-AL (a, b) which are rough and show several pores (size $\geq$ 100 μm). Saponified AL (c, d) have smooth surface and smaller pore size comparing to control non-saponified rubber. After water extraction both C-AL and SAP-AL (b, d) show particulate matter (10-20μm) migrated to the surface from inside, most likely from the pores. The surface characteristics of control skim (c) show many big pores and very rough comparing to saponified skim (g). After water extraction C-SK (f) with 1.85% nitrogen content show significant swelling but still contain big pores, while SAP-SK with 0.11% nitrogen content shows smooth surface (g), and less particle migration on the surface after water extraction (h).

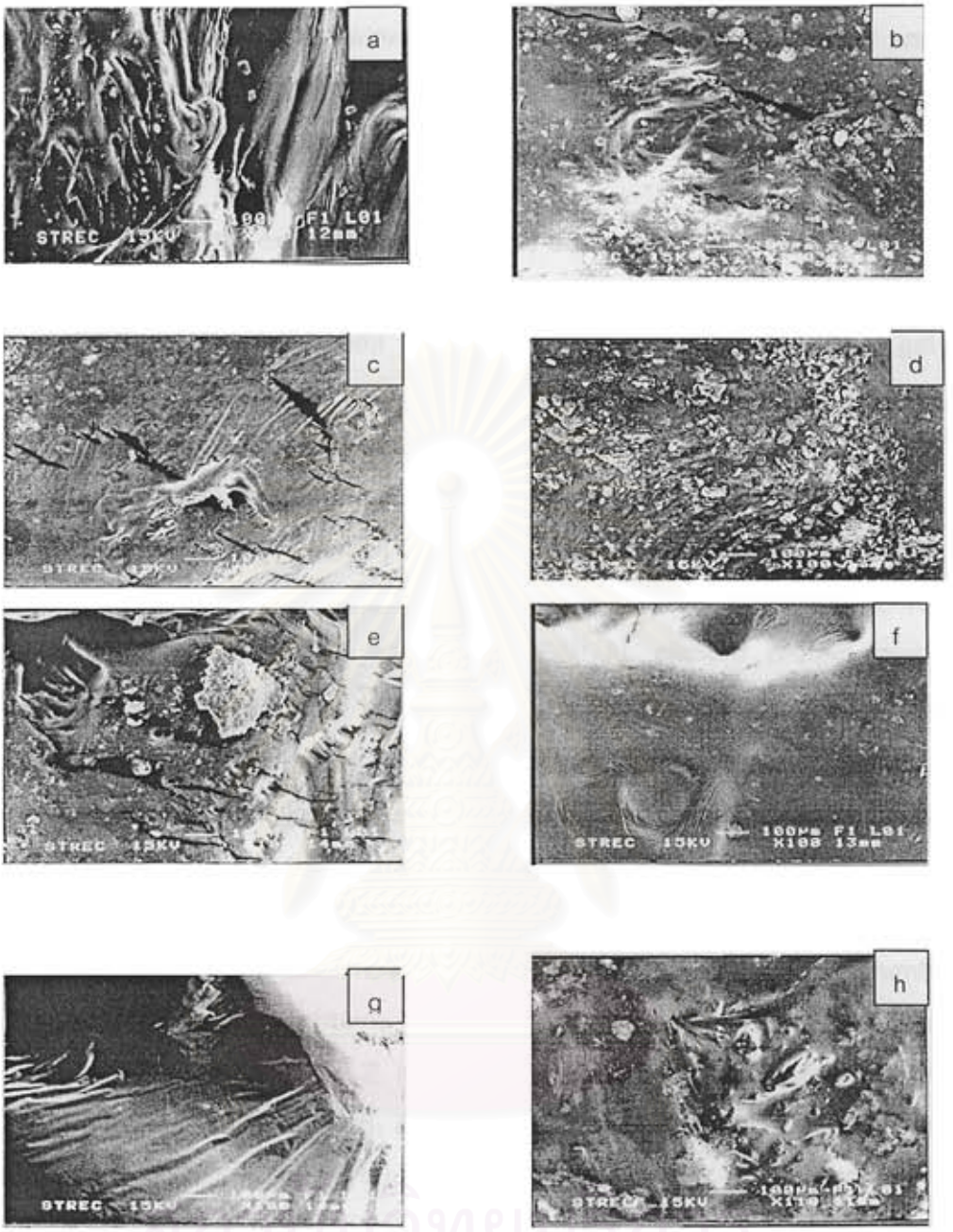


Figure 3.14 Scanning electron micrographs (SEM) showing the surface of control and saponified rubber specimens before and after extraction.

—— : 100μm

a) C-AL : control ammonia rubber before extraction

b) C-AL : control ammonia rubber after extraction

c) SAP-AL : saponified ammoniated before extraction

d) SAP-AL : saponified ammoniated after extraction

e) C-SK: control skim rubber before extraction

f) C-SK: control skim rubber after extraction

g) SAP-SK: saponified skim rubber before extraction

h) SAP-SK: saponified skim rubber after extraction

Since there is difference of water extractable protein between control and saponified crumb rubber, the further the pattern protein distribution of water extractable protein will be identified.

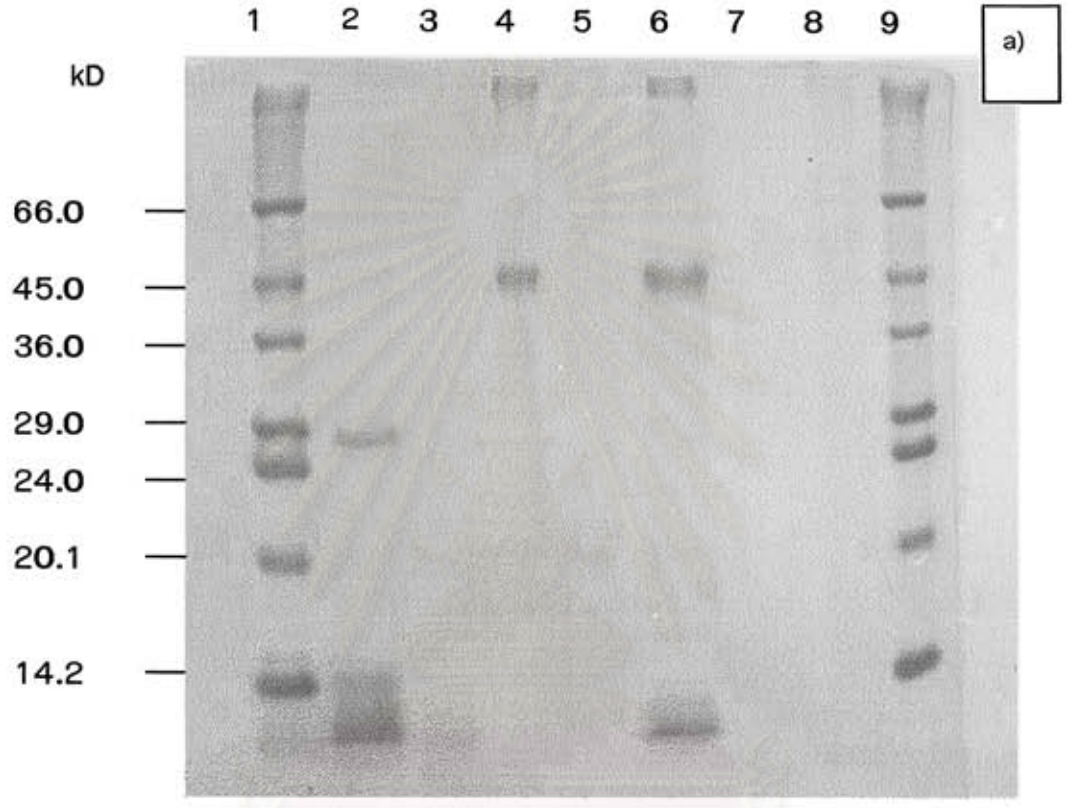
3.7 Identification of water extractable proteins by sodium dodecel sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Figure 3.15a shows separation of water extractable proteins by SDS gel electrophoresis before and after saponification of ammoniated crumb rubber. Control ammoniated rubber (C-AL) displays several protein bands of a slightly lower molecular weight (MW) than the 14.2 kD MW marker, which could be region. Four clear bands at 14.2, 20, 29 and 45 kD were observed (Figure 3.15a lane 2). After saponfication two of six samples of SAP-AL showed a clear band at 45 kD (Figure 3.15a lane 4 and 6) and two of SAP-AL showed protein lower molecular weight than 14.2 kD. This indicated that although each lane was loaded with equal amount of water extractable protein (50 μg by modified Lowry method), the $O.D_{750}$ may come from small peptides or due to disadvantage of modified Lowry method that may give false positive results.

Figure 3.15b demonstrated distribution patterns of water extractable proteins from non-saponified skim crumb (C-SK) and saponified skim crumb (SAP-SK). There are several proteins band of C-SK with the major bands are slightly higher mobility than the 14.2 kD protein marker, 14.2 kD and 45 kD (Figure 13.5b lane 2). While SAP-SK, protein bands observed from 3 samples of SAP-SK (Figure 3.15b lane3, 4 and 6) display a persist band at 45 kD and three sample of SAP-SK showed protein lower molecular than 14.2 kD. These protein bands no correlation with equal amount of protein determination by modified Lowry method.

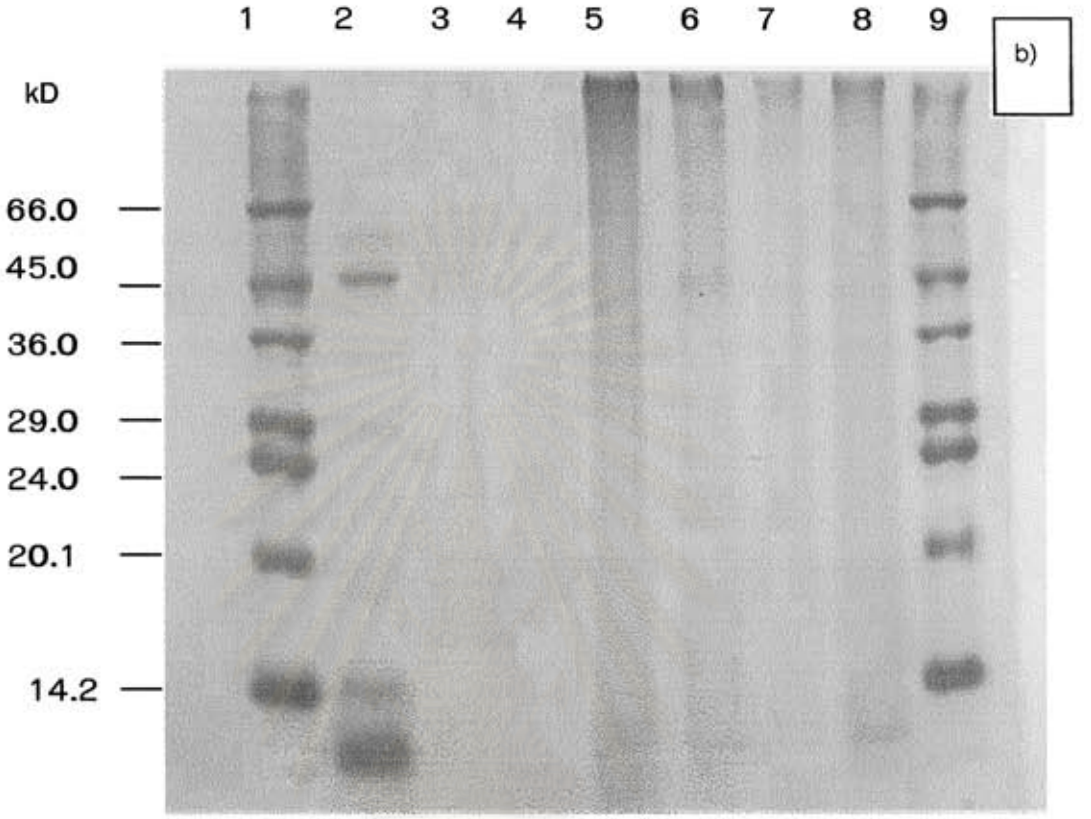
It is concluded that 45 kD and protein lower molecular weight than 14.2 kD are the major water extractable protein in SAP-AL and SAP-SK. The band also exits in control rubber. The presence of protein bands do not correlate with equal amount loaded.

Figure 3.14 Proteins pattern bands of rubber before and after saponification



a) Proteins pattern of ammoniated crumb rubber before and after saponification

Lane 1 and 9 = standard MW markers (14.2-66 KD), lane 2 = C-AL rubber, lane 3-8 = SAP-AL. Each lane was loaded with latex protein 50μg.



b) Proteins pattern of skim crumb rubber before and after saponification

Lane 1 and 9 =standard MW markers (14.2-66 KD), lane 2 =C-SK, lane 3-8 = saponified rubber. Each lane was loaded with latex protein 50μg.

Since protein of 45 kD and protein lower molecular weight than 14.2 kD persisted in both SAP-AL and SAP-SK, the next step the allergenic latex protein of these samples will be observed.

3.10 Prevalence of latex-specific IgE antibodies

Latex-specific IgE antibodies can be detected by Enzyme Allergosorbent Test (EAST). EAST positive results are evident by the yellow-color wells (Figure 3.16).

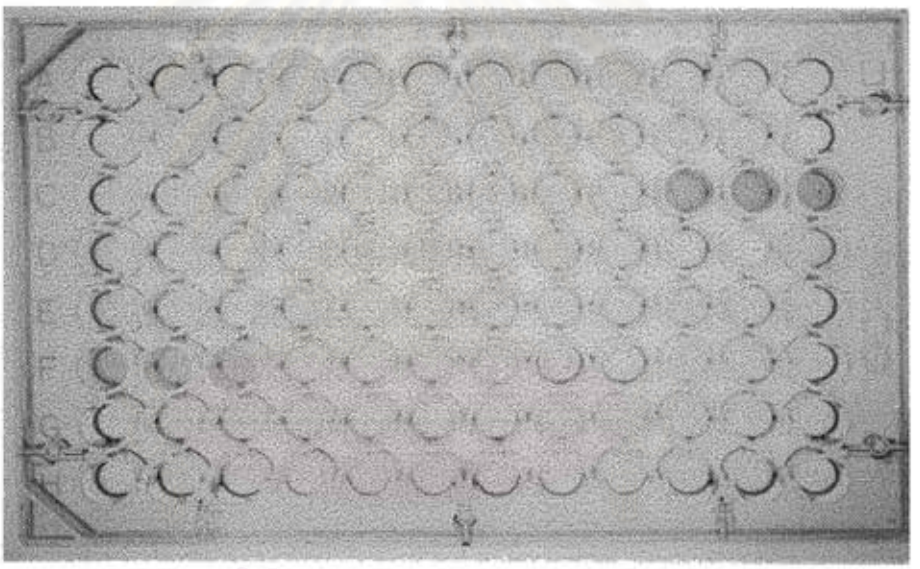


Figure 3.16 Positive EAST of the test sera: the serum that gave positive results were seen as yellow-color wells F1, F2, F3, C10, C11 and C12.

In this study, sample sera are 3 groups: control's sera were collected from Thai Red Cross; sera from patients who came to Chulalongkorn Hospital on general-purpose; and atopic healthcare workers (2.13). Table 3.8 shows EAST positive results 5 % in general blood donors with general-purpose' sera there were

8.3 % EAST positive. There was high prevalence in atopic healthcare workers that showed 66 %EAST positive.

Table 3.8 EAST positive result from the general population with different levels of risk factors.

Populations	Sample size	No. EAST positive (%)
Blood donors	100	5 (5)
General patients	12	1 (8.3)
Atopic healthcare workers	6	4 (66)
Total	118	10 (8.5)

Four sera samples with positive EAST test are healthcare workers in the hospital. They were interviewed to study risk factors and history of atopic symptoms, which are summarized in Table 3.9. Their age ranged from 27 to 39 years old. These atopic cases can be categorized by general allergic symptoms into 3 stages; 1) contact urticaria, 4 cases; 2) asthma, 1 case; and 3) conjuntivitis, 1case. Two of four subjects (AA, BB) are food allergy whereas CC is alcohol allergy. Two subjects (BB, CC) had family history of atopic symptoms. The working duration with latex range from 3-192 month, and average working hours more than 20 hours per week. Three of four subjects had contact with latex for more than 20 hours per week.

Table 3.9 Evidence of risk factors and history atopic patients with latex allergy

Characteristics	Patients			
	AA	BB	CC	DD
Sex Male				
Female	/	/	/	/
Age (year)	33	37	39	27
Prior allergic diseases				
Yes	/	/		
No			/	Unk
Family history of atopy				
Present	/			/
Absent		/	/	
Prior surgery				
Yes			/	
No	/	/		/
Occupation	Cleaner	Cleaner	Health care worker	Civil officer
Working Duration (mo.)	3	36	192	60
Contact with latex gloves				
≥ 20 hour/week	/	/		/
< 20 hour/week			/	
Number gloves/day				
≥ 3 pairs			/	/
< 3 pairs	/	/		
Allergenic symptoms				
-Urticaria	/	/	/	/
-Asthma	/			
-Conjuntivitis				/
-Food allergy	/	/		
-Alcohol allergy				

Unk – unknown

3.11 Allergen detection by Skin Prick test (SPT)

The Human Rights and Ethics Committee of dermal clinic at Srinakharinwirot University, Prasarnmitr campus approved this study. Only one latex allergenic patient, subject DD has volunteered for SPT that was kindly made available by Dr. Suwirakom Ophaswongse. Figure 3.16 shows that a positive SPT was evident as a wheal equal to or greater than that of a positive control. Table 3.10 shows that subject DD gave positive SPT results when using latex proteins prepared from non-saponified crumb rubber (C-AL, C-SK). In contrast both latex proteins prepared from saponified rubber (SAP-AL, SAP-SK) showed negative results. These results indicated that the latex proteins though visible by SDS-PAGE are not allergen and could not response in DD.

Table 3.10 Allergen detection by Skin Prick test

Test solution	Protein, μg/ml	Skin prick testing
Normal saline (negative control)	-	NEG
Histamine phosphate(Positive control)	10^3	POS
Std. Latex protein allergen	10	POS
Powdered glove 1	ND	POS
Powdered glove 2	58	POS
C-AL	3.2	POS
C-SK	1.3	POS
SAP-AL	4.7	NEG
SAP-SK	<1	NEG

C-AL and C-SK : Untreated rubber from ammoniated crumb and skim crumb respectively, SAP-AL and SAP-SK : Saponified rubber from ammoniated crumb and skim crumb respectively. POS: Positive, NEG: Negative, ND: not determined.

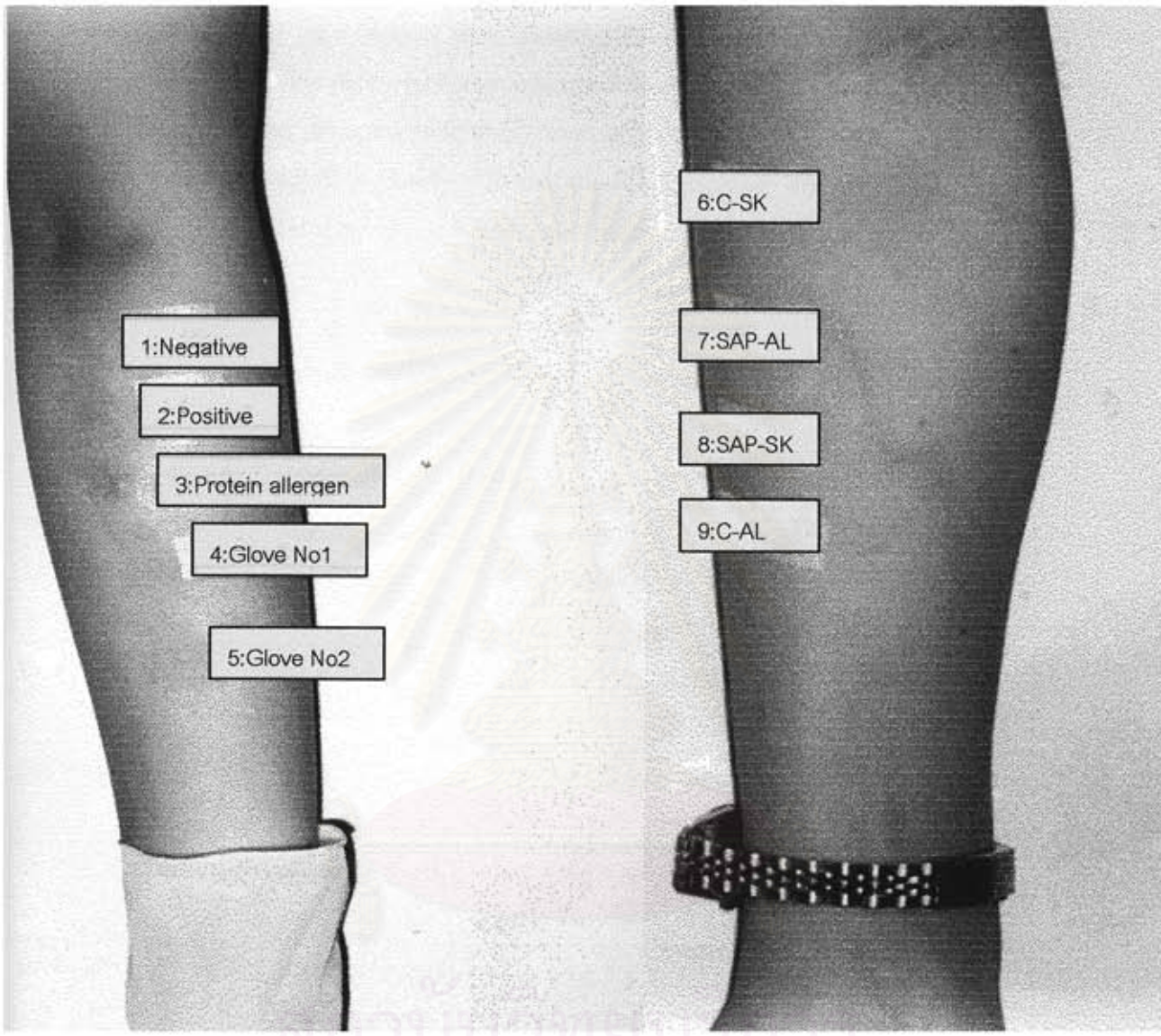


Figure 3.17 **Positive Skin prick test of protein allergen: the samples that gave positive results were seen as a wheal equal to or greater than that of a positive control.**

3.12 Allergen detection by EAST test

Positive EAST sera from 4 atopic healthcare worker and 1 positive EAST from general patients were used for allergen detection of latex proteins that were extracted from control non-saponified rubber (C-AL, C-SK) and saponified rubber (SAP-AL, SAP-SK). Table 3.11 shows that all 5 positive EAST cases gave significant positive EAST results when using latex proteins prepared from non-saponified (C-AL, C-SK). In contrast both latex proteins prepared from saponified rubber (SAP-AL, SAP-SK) showed significant negative results. These results indicated that the latex allergen could not be detected in saponified rubber with respects this subject.

Table 3.11 Allergenic response by EAST test

Source of latex antigen	$O.D_{405}$ (Mean ±S.D)		No. of Positive EAST/total no. of patients
	Control group (n=9)	Patient (n=5)	
C-AL	0.014 ^{b} ± 0.007	0.104 ^{a} ± 0.052	5/14
SAP-AL	0.028 ^{b} ± 0.010	0.043 ^{a} ± 0.011	0/14
C-SK	0.028 ^{b} ± 0.015	0.102 ^{a} ± 0.052	5/14
SAP-SK	0.020 ^{b} ± 0.009	0.028 ^{a} ± 0.014	0/14

C-AL and C-SK: control non-saponfied prepared from ammoniated crumb and skim crumb respectively, SAP-AL and SAP-SK: Saponified rubber prepared from ammoniated crumb and skim crumb respectively.

Significant difference of allergenic response by EAST test between control and saponified crumb rubber are marked by different letter (a, b) analyzed by Wilcoxon Signed-Rank Test at 99 % confidence.