CHAPTER III

RESULTS AND DISCUSSION

This chapter is divided into four sections. The first section is about the synthesis of *N*-alkyl chitosan. Then the alkylated chitosan was methylated by reaction with methyl iodide, which is reported in the second section. The relation between reaction condition and degree of quaternization is discussed. In the third section, solubility values of the quaternized derivatives are determined. In the last section, study for fruit coating application was carried out. Banana was chosen in this study due to its fast change of color.

3.1 Synthesis of *N*-alkyl chitosan

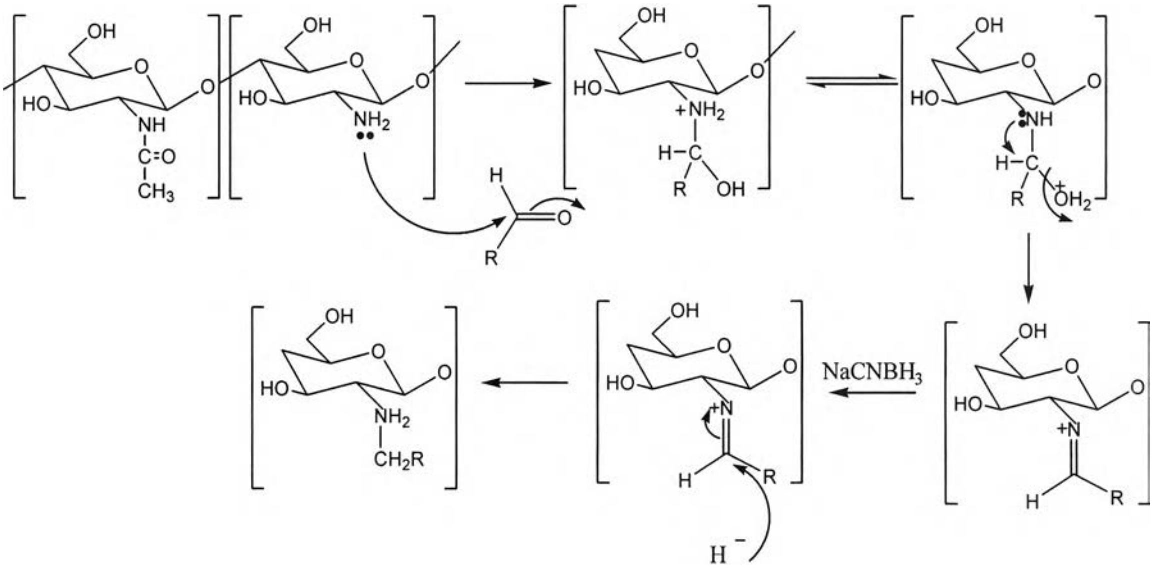
The *N*-alkyl chitosan derivatives were synthesized by the reaction between the amino group of chitosan and two aldehydes; butyraldehyde and benzaldehyde. Since the aldehyde is not soluble in aqueous acetic acid, ethanol was added to help for improving the homogeneity of the reaction mixture. Results are shown in Table 3.1. It should be noted here that product yields were based on the assumption that one amino group reacted with only one aldehyde molecule.

Alkylation at the amino group proceeds as shown in Scheme 3.1. The amino behaves as a nucleophile attacking the carbonyl group of the aldehyde. After loss of water molecules, sodium cyanoborohydride ($NaCNBH_3$) is used to reduce the imine. This reducing agent is more reactive and selective than other reducing agents such as sodium borohydride ($NaBH_4$). When $NaBH_4$ was added into 1% acetic acid (pH 3), it decomposed and bubble formed immediately. Although borohydride works well at a higher pH (~ 6), it is not suitable to do this for the chitosan solution, since chitosan can precipitate at and above this pH. Moreover, there is also a report saying that when using

$NaCNBH_3$, reduction of aldehyde or ketone by $NaCNBH_3$ is negligible in this pH range [1].

Table 3.1 Physical appearance and yield of *N*-alkyl chitosan, prepared by reductive alkylation of chitosan

Aldehyde	Mole proportions NH_2:Aldehyde	Physical Appearance	%Yield
Butyraldehyde	1:0.5	Light yellow powder	72
	1:1	Light yellow powder	79
	1:2	Light yellow powder	97
Benzaldehyde	1:0.5	White powder	62
	1:1	White powder	64
	1:2	White powder	80



Scheme 3.1 *N*-alkylation mechanism of chitosan via Schiff's base intermediate

3.1.1 Structure characterization

N-butyl chitosan

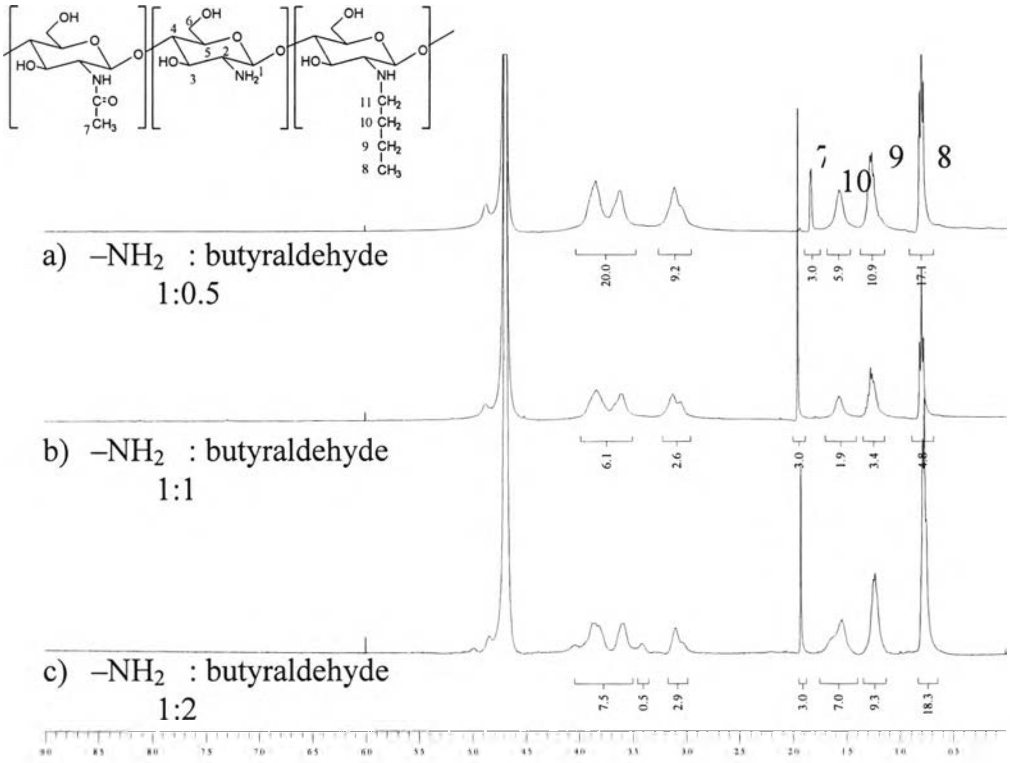


Figure 3.1 ^{1}H NMR spectra of *N*-butyl chitosan

Table 3.2 Signal assignment for *N*-butyl chitosan

Functional group	δ (ppm)
NH-CH$_2$-CH$_2$-CH$_2$–$\underline{CH_3}$	0.7
NH-CH$_2$-CH$_2$-$\underline{CH_2}$–CH$_3$	1.2
NH-CH$_2$-$\underline{CH_2}$-CH$_2$–CH$_3$	1.6
NH-$\underline{CH_2}$-CH$_2$-CH$_2$–CH$_3$	3.6-4.0
H-2 of chitosan	3.1

From the spectra performed on the substituted chitosan (Fig. 3.1, Table 3.2), the area integration of each peak can indicate the degree of substitution. Nevertheless, ratio of peak area no. 8, 9, and 10 does not fit with the theoretical value of 2:2:3. It seems that the areas of the signals for C-3 (CH_2) and C-4 (CH_3) are more intense than they should be. This indicates one possibility that there is butyraldehyde or its reducing form left with the product. (*Note*- A signal for proton at C-1 of butyl alcohol, a reducing form of butyraldehyde can be observed in spectrum 3.1c at 3.4 ppm.) Therefore the proton signal at C-2 is used to calculate degree of butylation. The degree of alkyl substitution determined from NMR analysis is shown in Table 3.3.

Table 3.3 Degree of substitution (DS) of butyl group on chitosan

Equivalent of butyraldehyde	Integration value		%DS
	H-2 of chitosan	$\underline{CH_2}$-CH_2-CH_3 protons of butyl group.	
0.5	9.2	5.9	32
1	2.6	1.9	37
2	2.9	7.0	121

The degree of substitution of chitosan/butyraldehyde ratio at 1:0.5 and 1:1 are 32 and 37% respectively. But when the butyraldehyde equivalent was increased to 2, %DS was over 100%. This suggest that disubstitution at the amino group took place.

N-benzyl chitosan

From the spectra obtained from *N*-benzyl chitosan (Fig. 3.2, Table 3.4), the degree of benzylation can be determined by comparing proton signal for CH_2 of the benzyl group with the proton signal at C-2 of chitosan. Results are shown in Table 3.5.

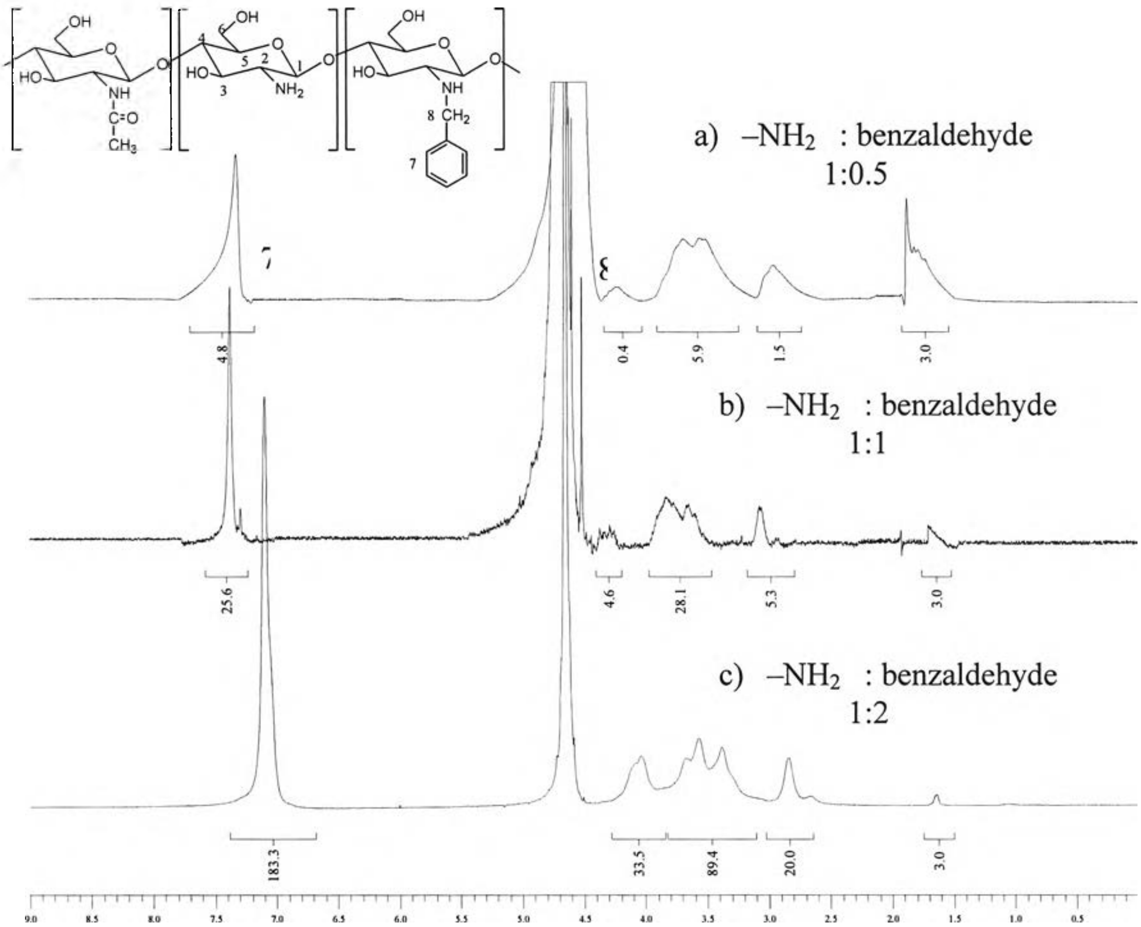


Figure 3.2 ^{1}H NMR spectra of *N*-benzyl chitosan

Table 3.4 Signal assignment for *N*-benzyl chitosan

Functional group	δ (ppm)
H2-chitosan	2.8-2.9
NH-$\underline{CH_2}$-Ar	4.1-4.3
NH-CH_2-$\underline{Ar}$	7.0-7.3

Table 3.5 Degree of substitution (DS) of benzyl group on chitosan

Equivalent of benzaldehyde	Integration		%DS.
	H-2 of chitosan	Chitosan-NH-$\underline{CH_2}$-Ar	
0.5	1.5	0.4	13
1	4.6	5.3	57
2	20	33.5	56

The degree of substitution of chitosan/benzaldehyde ratio at 1:0.5, 1:1 and 1:2 are 13, 57 and 56% respectively. The %DS is not high for the system of *N*-benzylation, possibly due to the electronic effect and the bulky of phenyl group that hinders the approaching of $-NH_2$ from chitosan chain toward the neighboring carbonyl.

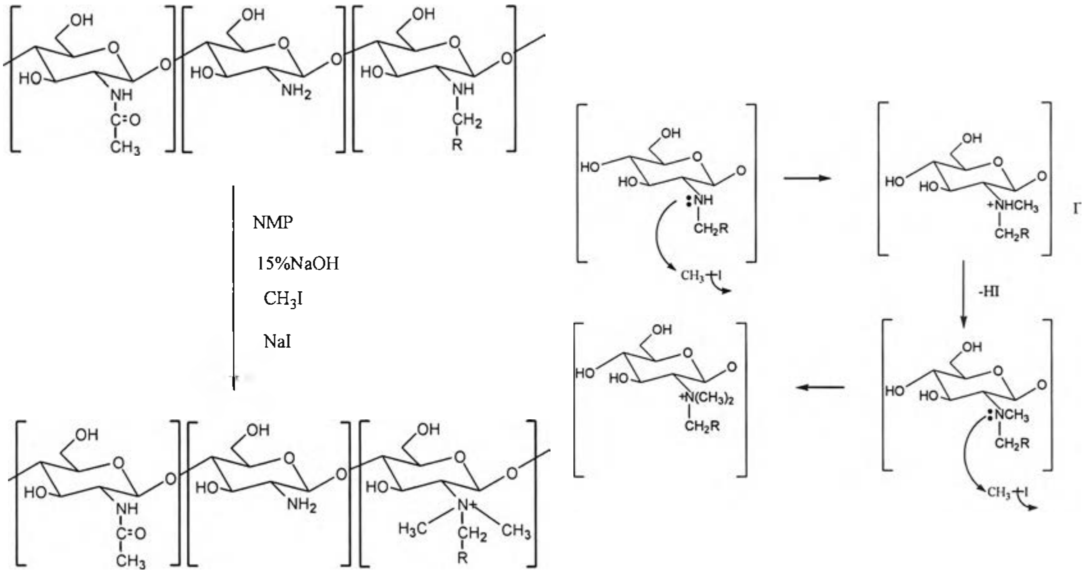
From these results, it is shown that the degree of alkylation at chitosan depends largely on the amount of aldehyde used in the reaction.

3.2 Synthesis of quaternary ammonium salt of *N*-alkyl chitosan

Quaternary ammonium salt of *N*-alkyl chitosan was synthesized by methylation of *N*-alkyl chitosan based on the method previously described [8] with some modifications (Scheme 3.2). A mixture of H_2O/NMP is an appropriate solvent for the quaternization of chitosan. The amount of H_2O during the reaction should be equal to 10% (v/v) for a homogeneous reaction since methyl iodide is insoluble in H_2O [1]. Thus the concentration of water in this reaction is important. It must be as low as possible, to avoid difficulties in recovering the quaternized chitosan.

As the reaction was carried out the charge density in reaction increased and the rate of quaternization was affected by electrostatic hindrance of the molecule. To reduce this charge density, sodium iodide (NaI) was added in the reaction. Sodium hydroxide was added to avoid protonation of the amino group and fix the iodohydric acid liberated.

The addition of a key methylating agent CH3I was varied from 1 to 3 adding steps, with a fixed amount of CH3I at 12 equivalents of all amino groups in chitosan chain.



Scheme 3.2 Reaction scheme and mechanism Methylation of *N*-alkyl chitosan

For *N*-butyl chitosan, the products yields are about 30-40%. No significant difference of product yield was observed when changing the adding step from 1 to 3 steps. Methylation at *N*-benzyl chitosan that was obtained from the reaction of chitosan with 1 and 2 equivalent of benzaldehyde was, however, not successful. The reason could be the bulky phenyl group that transforms chitosan amino group to be a bulky nucleophile. This bulky group thus cannot attacks methyl iodide efficiently.

Table 3.6 Appearance and yield of quaternized *N*-alkyl chitosan

Sample no.	Product code	Alkylation step		Methylation step			Physical appearance	%Yield
		Mole equivalent of		Mole equivalent of CH_3I				
		Butyl	Benzyl	1 step	2 steps	3 steps		
1	Q1 *N*-but (0.5)	0.5	-	12	-	-	White solid	40
2	Q2 *N*-but (0.5)	0.5	-	8	4	-	White solid	37
3	Q3 *N*-but (0.5)	0.5	-	8	2	2	White solid	36
4	Q1 *N*-but (1.0)	1	-	12	-	-	White solid	34
5	Q2 *N*-but (1.0)	1	-	8	4	-	White solid	32
6	Q3 *N*-but (1.0)	1	-	8	2	2	White solid	32
7	Q1 *N*-but (2.0)	2	-	12			White solid	30
8	Q2 *N*-but (2.0)	2	-	8	4	-	White solid	28
9	Q3 *N*-but (2.0)	2	-	8	2	2	White solid	28
10	Q1 *N*-ben (0.5)	-	0.5	12			White solid	20
11	Q2 *N*-ben (0.5)	-	0.5	8	4	-	White solid	20
12	Q3 *N*-ben (0.5)	-	0.5	8	2	2	N/R	N/R
13	Q1 *N*-ben (1.0)	-	1	12	-	-	N/R	N/R
14	Q2 *N*-ben (1.0)	-	1	8	4	-	N/R	N/R
15	Q3 *N*-ben (1.0)	-	1	8	2	2	N/R	N/R
16	Q2 *N*-ben (2.0)	-	2	12	-	-	N/R	N/R
17	Q3 *N*-ben (2.0)	-	2	8	4	-	N/R	N/R
18	Q1 *N*-ben (2.0)	-	2.	8	2	2	N/R	N/R

3.2.1 Structure Characterization

From the spectra performed on quaternized *N*-butyl an *N*-benzyl chitosan (Fig. 3.3 and 3.4; Table 3.7 and 3.8), the integration of each peak allow to calculate of degree of quaternization.

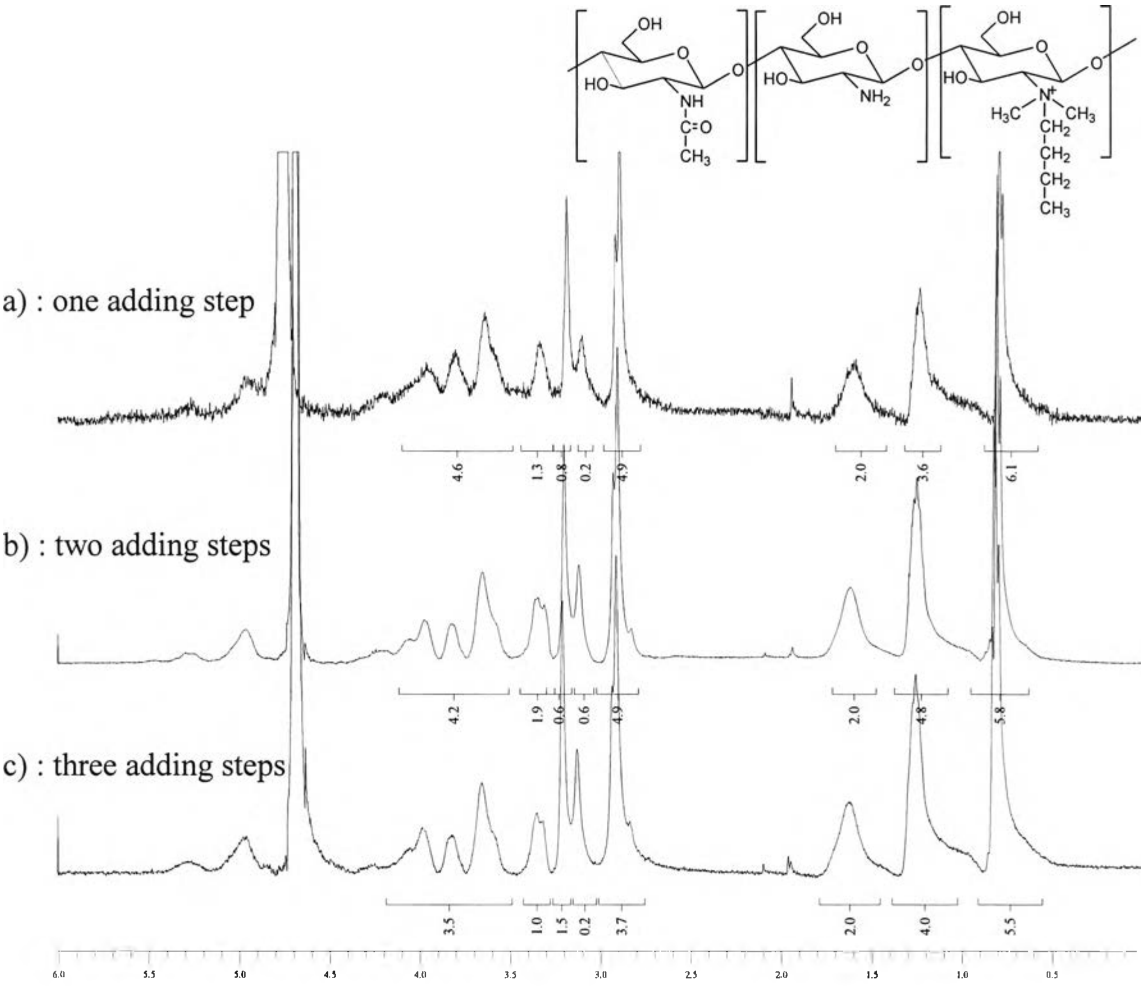


Figure 3.3 ^{1}H NMR spectra of quaternized *N*-butyl chitosan

Table 3.7 Signal assignment for quaternized *N*-butyl chitosan

Functional group	δ (ppm)
$-NH-C\underline{H}_3$	2.9
$-NR(C\underline{H}_3)$	3.1
$-N^+R(C\underline{H}_3)_2$	3.2
$-O-C\underline{H}_3$	3.4

Note: R = butyl ($-CH_2CH_2CH_2CH_3$)

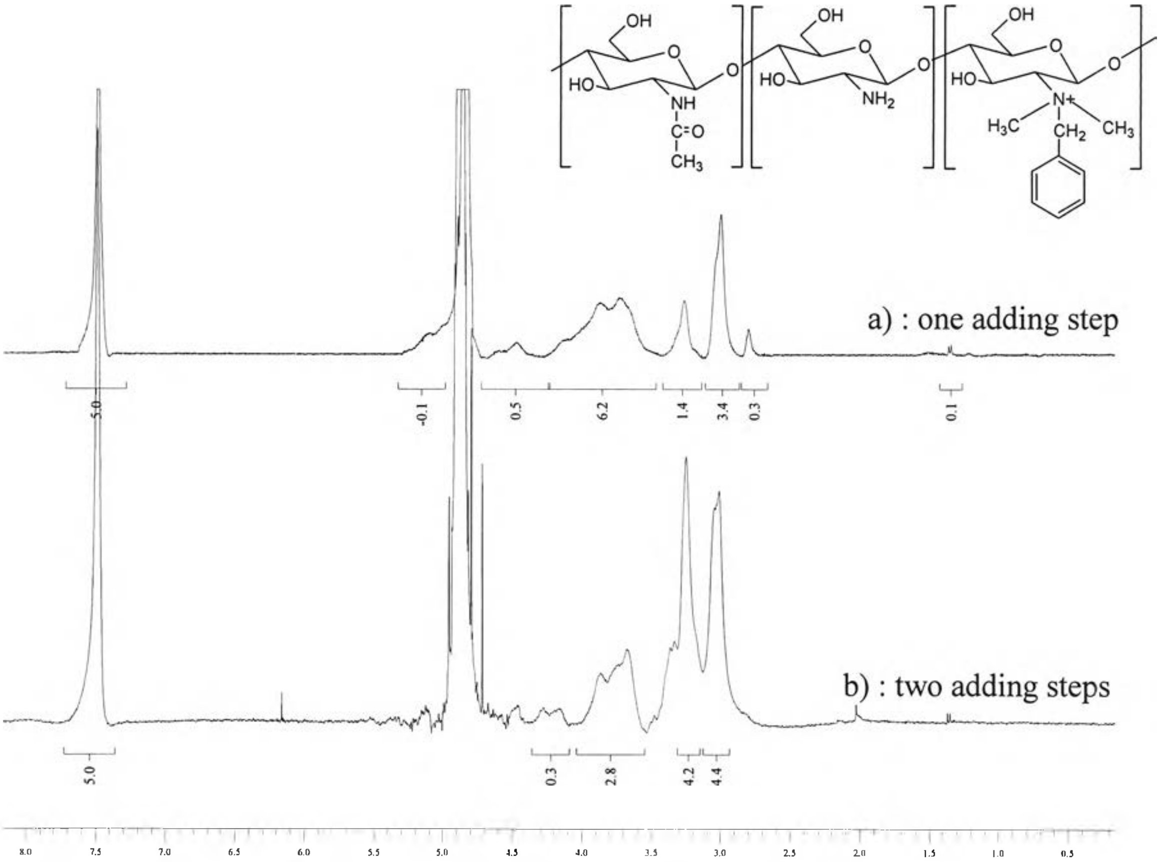


Figure 3.4 ^{1}H NMR spectra of quaternized *N*-benzyl chitosan

Table 3.8 Signal assignment for quaternized *N*-benzyl chitosan

Functional group	δ (ppm)
-NH–C$\underline{H}_3$	2.7-2.8
-NR-(C$\underline{H}_3$)	3.0-3.1
-N$^+$R(C$\underline{H}_3$)$_2$	3.2

Note: R = benzyl (-CH$_2$Ph)

The degree of quaternization determined form NMR analysis is shown in Table 3.9

Table 3.9 Degree of methylation of quaternized *N*-butyl and *N*-benzyl chitosan

Sample	Quaternized *N*-alkyl chitosan	%$NHCH_3$	%$NR(CH_3)$	%$N^+R(CH_3)_2$	%OCH_3
1	Q1 *N*-but (0.5)	65	3	13	13
2	Q2 *N*-but (0.5)	58	6	10	25
3	Q3 *N*-but (0.5)	48	10	6	17
4	Q1 *N*-but (1.0)	55	5	5	13
5	Q2 *N*-but (1.0)	30	10	3	8
6	Q3 *N*-but (1.0)	15	13	5	15
7	Q1 *N*-but (2.0)	10	6	1	5
8	Q2 *N*-but (2.0)	18	6	3	2
9	Q3 *N*-but (2.0)	18	12	3	3
10	Q1 *N*-ben (0.5)	7	35	10	-
11	Q2 *N*-ben (0.5)	-	57	32	40

From Table 3.9, % quaternization depends on the type of *N*-alkyl group. Methylation took place more at the butyl chitosan than the benzyl derivative. It is also found that adding step does not affect the degree of quaternization. Two factors that influence this reaction are steric and electronic effect. The steric effect seems to be a major contribution that lowers the degree of methylation as shown by sample no. 5 to 9.

The quaternization step is difficult for the *N*-benzyl chitosan possibly due to the steric hindrance of the benzyl group in the chitosan chain. The methylation of *N*-benzyl chitosan (0.5) is higher than *N*-butyl chitosan because of the electronic effect of benzene ring.

In all cases, *O*-methylation is present. These results in fact agree well with other reports that methylation at the hydroxyl group of chitosan is unavoidable. Especially in basic condition, the nucleophilicity of oxygen is enhanced. Oxygen thus competes with nitrogen of the amino group to react with CH_3I.

3.3 Solubility of quaternized *N*-alkyl chitosan

The solubility values of the quaternized chitosan derivatives in DI water are shown in Table 3.10.

Table 3.10 The solubility of quaternized *N*-alkyl chitosan

Sample	Quaternized *N*-alkyl chitosan	Solubility (mg/ml)
Chitosan	QAC	10
1	Q1 *N*-but (0.5)	2
2	Q2 *N*-but (0.5)	1
3	Q3 *N*-but (0.5)	1
4	Q1 *N*-but (1.0)	-
5	Q2 *N*-but (1.0)	-
6	Q3 *N*-but (1.0)	-
7	Q1 *N*-but (2.0)	-
8	Q2 *N*-but (2.0)	-
9	Q3 *N*-but (2.0)	-
10	Q1 *N*-ben (0.5)	-
11	Q2 *N*-ben (0.5)	-

The solubility of QAC synthesized by one adding step is 10 mg/ml, which is higher than all *N*-alkylated QAC. The solubility also depends on %quaternization and %*N*-alkylation. Only the quaternized derivatives obtained from 0.5 mole ratio of butyraldehyde can be soluble in DI water. It is possibly caused by the presence of hydrocarbon group (see Table 3.3). For the quaternized *N*-benzyl chitosan, it is insoluble since very low degree of methylation was obtained.

3.4 Fruit coating study for quaternary ammonium chitosan derivatives

Chitosan seems to be a good choice for use as a preservative coating material for fruits. It is safe and shows antifungal activity against several fungi. Moreover, the antifungal activity was found to be enhanced by the cationic characteristic of quaternary ammonium chitosan. This thesis thus focuses on studying the use of quaternized derivatives of chitosan for fruit coating. These quaternary ammonium derivatives are also water soluble. Therefore, no acidic solution is needed to prepare the polymer solution.

Two quaternary ammonium salts of *N*-butyl and *N*-benzyl chitosan were subjected to test for their usage in fruit coating. Two aspects of investigation were focused; weight loss and appearance change during storage at ambient atmosphere.

3.4.1 Weight loss during storage

Loss of weight was determined by weighing banana samples every 2 days. Results are shown in Table 3.11.

Table 3.11 Averages of % weight loss during storage of banana samples coated with various chemicals (4 repetitions)

Storage days	Blank	0.5% Acetic acid	Chitosan (0.5% acid)	Quaternized *N*-alkyl chitosan		
				Q1 *N*-butyl (0.5) chitosan (0.5% acid)	Q1 *N*-benzyl (0.5) chitosan (0.5% acid)	Q1 *N*-butyl (0.5) chitosan (DI water)
2	2.59	3.19	2.65	2.78	2.89	2.73
4	5.73	6.58	5.70	6.04	5.80	5.93
6	14.77	14.45	11.86	13.96	13.94	13.58
8	24.54	25.02	21.64	22.84	22.85	22.99
10	34.69	35.23	31.66	32.29	32.71	32.51

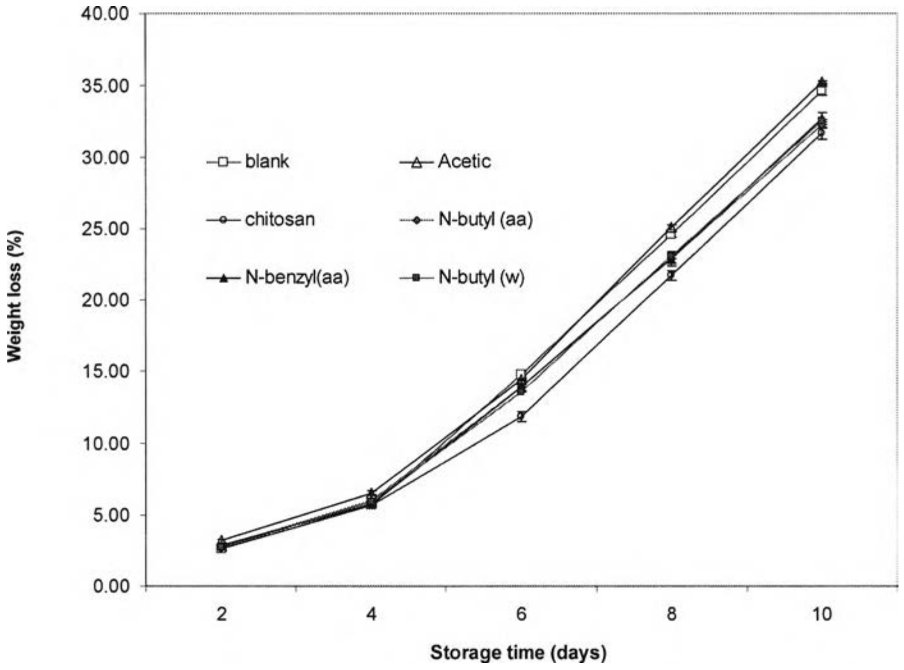


Figure 3.5 Comparison of weight loss of banana samples coated with various chemicals (4 repetitions, error bar is included but too narrow to be seen)

The weight loss of non-treated banana and treated banana with 0.5% acetic acid are not difference. When compared with coated banana, the blank and acid-coated bananas show the highest weight loss after storage for 6 days (Fig. 3.6). Loss of weight seems to slow down from the bananas coated with chitosan and its derivatives. These findings fit well with other studies [15] that chitosan acts as a barrier, delaying the evaporation of moisture and other gases.

3.4.2 Appearance change during storage

Color change

The appearance of banana is judged by visible inspection of color change and peel browning (Table 3.12). After 8 days, the non-coated and acid-coated banana turned brown, and became black all over after 10 days. The bananas coated with chitosan and quaternized derivatives turned their color at a slower rate. (Fig. 3.6 to 3.11).

Table 3.12: Physical appearance of banana samples coated with various chemicals

After storage (days)	Blank	0.5% acetic acid	Chitosan (0.5% acid)	Q1 *N*-butyl (0.5) chitosan (0.5% acid)	Q1 *N*-benzyl (0.5) chitosan (0.5% acid)	Q1 *N*-butyl (0.5) chitosan (DI water)
2	Green	Green	Green	Green	Green	Yellow
4	Green	Yellow	Yellow	Yellow	Yellow	Yellow
6	Yellow & Brown spot	Yellow	Yellow	Yellow	Yellow & Brown spot	Yellow
8	Black	Black	Yellow & Brown spot	Yellow & Brown spot	Yellow & Brown spot	Brown spot to black
10	Black	Black	Black	Brown spot to black	Brown spot to black	Brown spot to black

Texture change

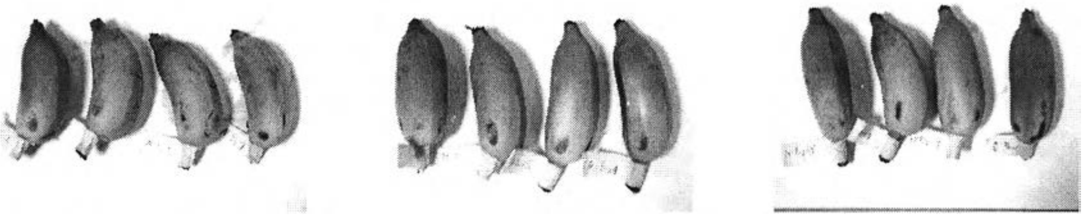
The texture change of banana is judged by visible inspection after 10 days (Fig. 3.12-3.13). Results are shown in Table 3.13.

Table 3.13 Appearance of banana flesh after 10 days of storage

Banana	Texture appearance
Non-treated	Brown and soft
Non-treated, 0.5% acetic acid	Dark brown and soft
20% chitosan in 0.5% acetic acid	Light brown and normal
20% Q1 *N*-but (0.5) in 0.5% acetic acid	White and normal
20% Q1 *N*-ben (0.5) in 0.5% acetic acid	Light brown and normal
20% Q1 *N*-but (0.5) in DI water	White and normal

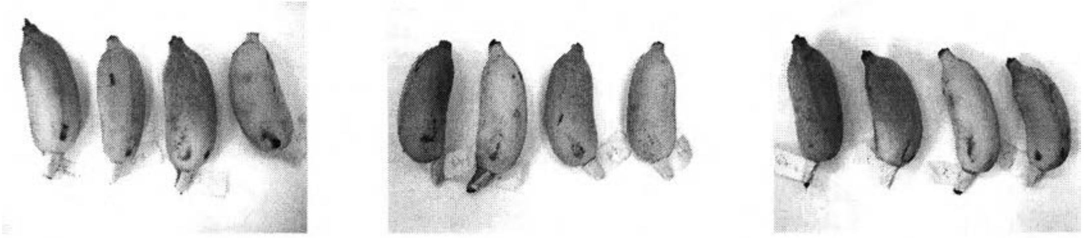
From this study, it is feasible to use chitosan and the quaternized derivatives of chitosan as fruit coating substances for short-term storage (up to 10 days).

a) blank b) 0.5% acetic acid c) chitosan

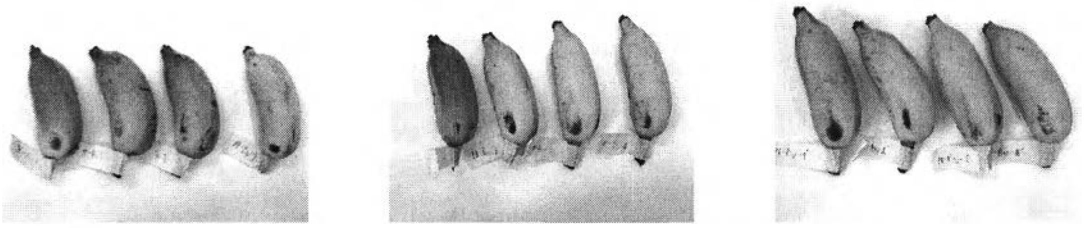


d) *N*-butyl chitosan e) *N*-benzyl chitosan f) *N*-butyl chitosan (water)

Figure 3.6 The appearance of bananas at 0 time point



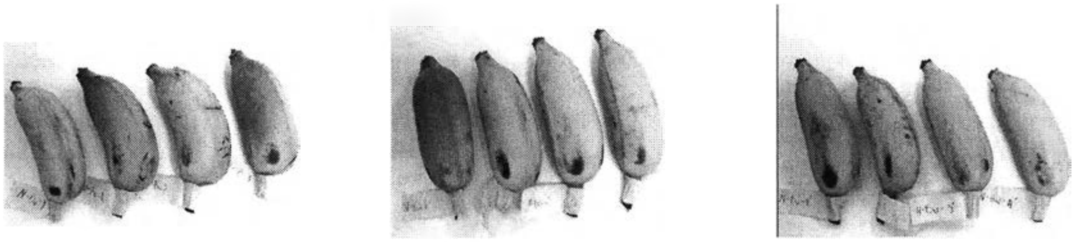
a) blank b) 0.5% acetic acid c) chitosan



d) *N*-butyl chitosan e) *N*-benzyl chitosan f) *N*-butyl chitosan (water)

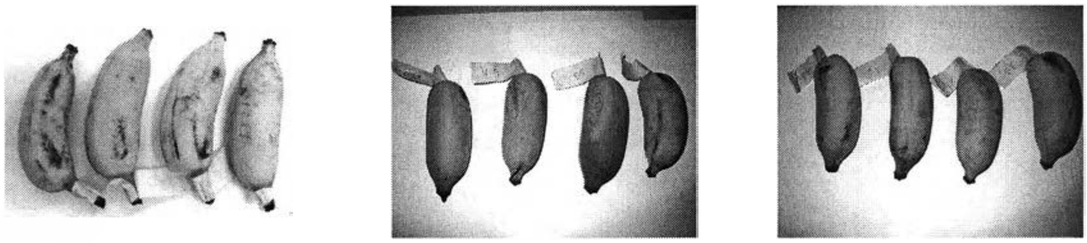
Figure 3.7 The appearance of bananas after 2 days

a) blank b) 0.5% acetic acid c) chitosan

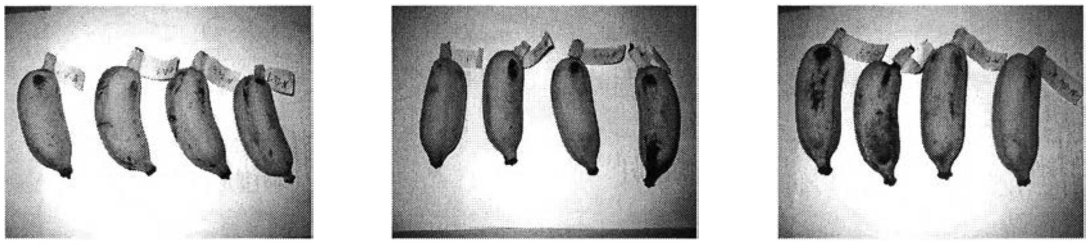


d) *N*-butyl chitosan e) *N*-benzyl chitosan f) *N*-butyl chitosan (water)

Figure 3.8 The appearance of bananas after 4 days

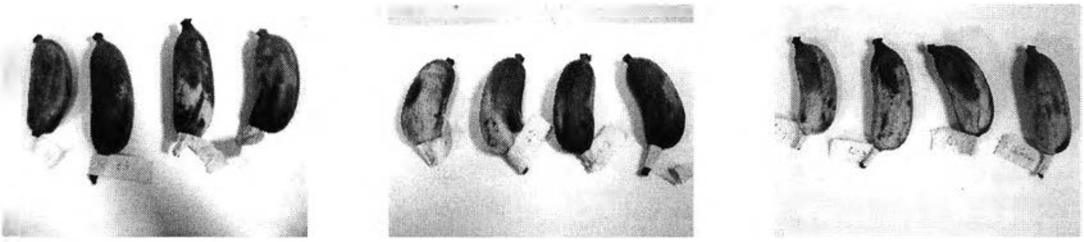


a) blank b) 0.5% acetic acid c) chitosan

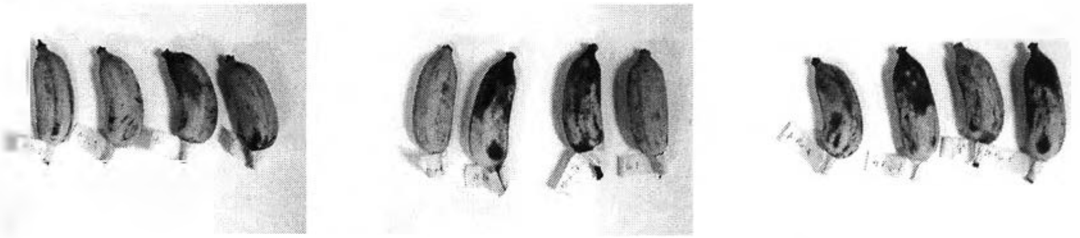


d) *N*-butyl chitosan e) *N*-benzyl chitosan f) *N*-butyl chitosan (water)

Figure 3.9 The appearance of bananas after 6 days

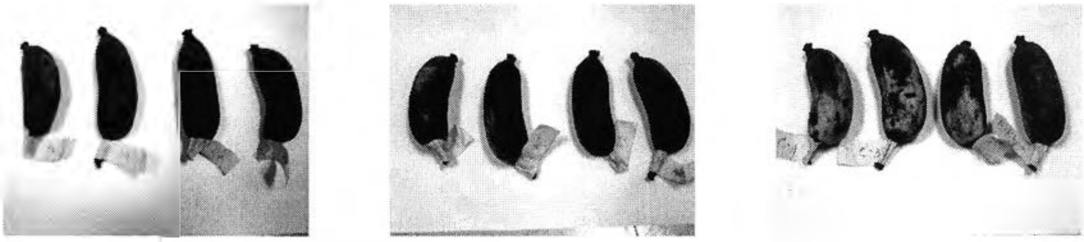


a) blank
b) 0.5% acetic acid
c) chitosan

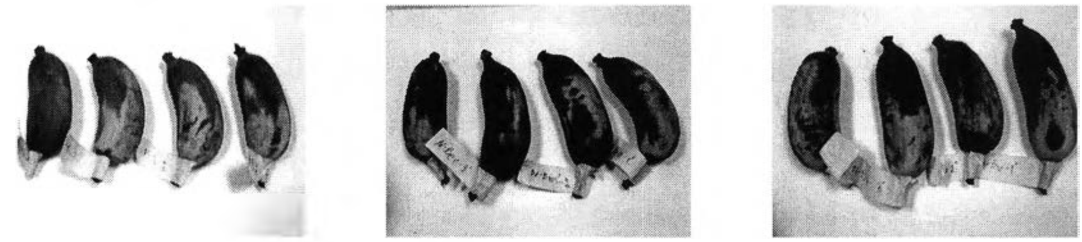


d) *N*-butyl chitosan
e) *N*-benzyl chitosan
f) *N*-butyl chitosan (water)

Figure 3.10 The appearance of bananas after 8 days

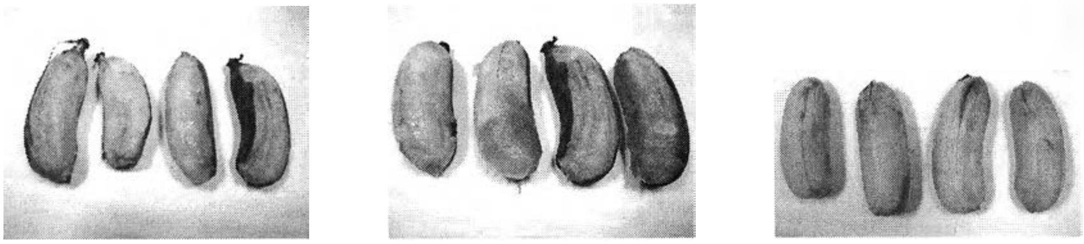


a) blank
b) 0.5% acetic acid
c) chitosan



d) *N*-butyl chitosan
e) *N*-benzyl chitosan
f) *N*-butyl chitosan (water)

Figure 3.11 The appearance of bananas after 10 days



a) blank
b) 0.5% acetic acid
c) chitosan

d) *N*-butyl chitosan
e) *N*-benzyl chitosan
f) *N*-butyl chitosan (water)

Figure 3.12 The appearance of banana texture after 10 days

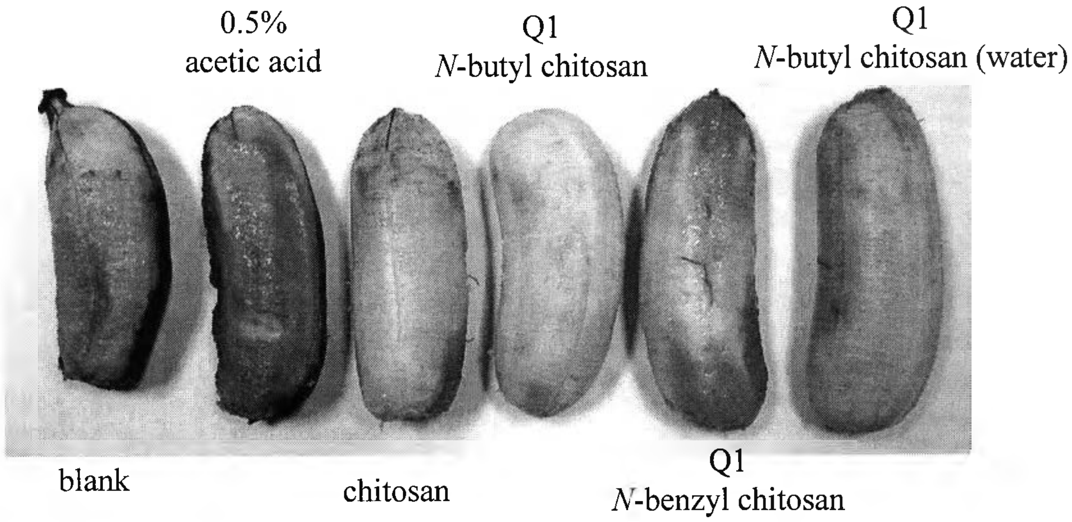


Figure 3.13 The comparison of banana texture after 10 days