CHAPTER 4
RESULTS AND DISCUSSION

4.1 Preliminary study

4.1.1 FTIR spectroscopy

FITR spectra of CS and N-PhCS prepared under 1:1 mole ratio of CS:PA at different temperatures and stirring times are presented in Figure 16. The spectrum of CS showed characteristic peaks at 1650 and 1600 cm^{-1} assigned to the C=O stretching (amide I) and the bending of amine ($-NH_2$) functional group, respectively (Figure 16a). The absorption band at 1155 cm^{-1} was an asymmetric stretching of the C-O-C bridge. The peaks at 1081 and 1033 cm^{-1} assigned to the skeletal vibration of C-O stretching [14]. In all the spectra of N-PhCS (Figure 16 b-e), the stretching vibration of –OH overlapped with -NH at 3500-3400 cm^{-1} became narrower and shifted to lower wavenumber, indicating the substitution at amino groups of CS [25]. The new peak at 1647 cm^{-1} assigned to the carbonyl stretching (amide I) of N-phthaloyl amide was also observed, while the peak of ($-NH_2$) bending at 1600 cm^{-1} disappeared. The strong peaks at 1560 cm^{-1} and 1383 cm^{-1} regions attributed to an asymmetric and a symmetric carboxylate anion stretching ($-COO^-$), respectively, indicated that N-PhCS was chitosan-N-phthalamidate sodium (sodium 2-(chitosan-N-carbonyl) benzoate) (Figure 17a). The spectra of N-PhCS prepared under higher temperature (40°C) for 4 and 24 h (Figure 16 d-e), showed the characteristic peaks at 1712 cm^{-1} and 1772 cm^{-1} regions attributed to carbonyl group of phthalimido moieties due to cyclization (Figure 17b) [85] while it was not observed in those prepared at 25°C for 4 and 24 h (Figure 16 b-c). In addition, there was no effect of the stirring time on the FTIR spectra of N-PhCS.

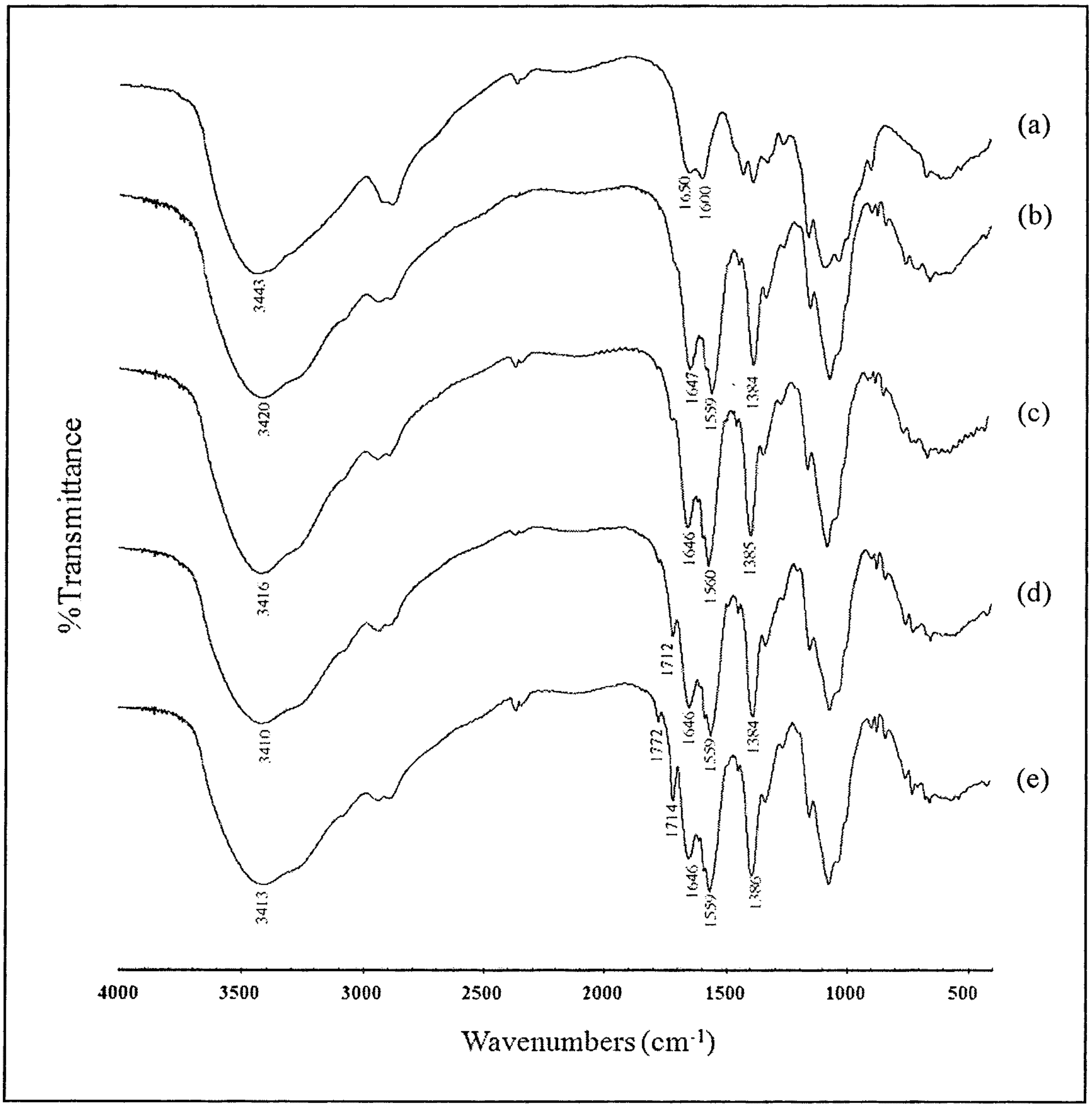


Figure 16 FTIR spectra of N-PhCS prepared under different temperatures and stirring times: (a) CS, N-PhCS at 25°C for (b) 4 h and (c) 24 h, at 40°C for (d) 4 h and (e) 24 h.

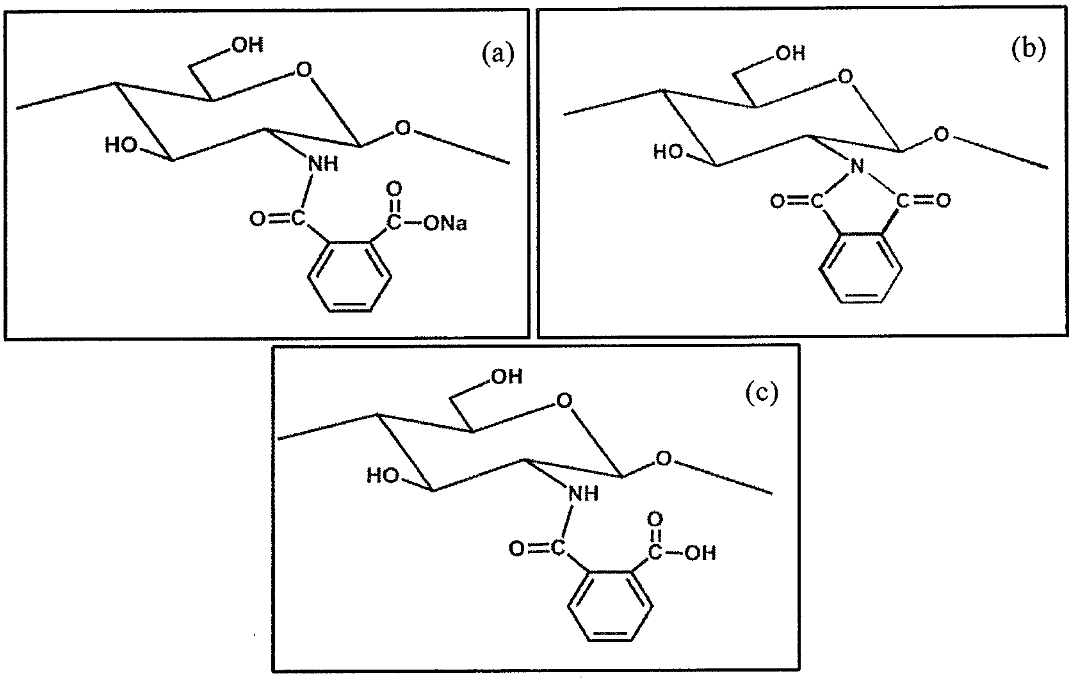


Figure 17 Proposed molecular structure of (a) chitosan-N-phthalamidate sodium, (b) N-phthalimido-chitosan and (c) chitosan-N-phthalamidic acid.

4.1.2 Solubility study

The %transmittance of all N-PhCS in pH 1-10 media are shown in Figure 18. At pH 1, the swelling of N-PhCS due to the protonation of unsubstituted amino group was observed and the %transmittance was around 20%. At pH 2-6, N-PhCS was precipitated according to the decrease of free amino groups. At pH above 7, the solubility of N-PhCS was increased as the % transmittance increased due to salt formation at carboxyl groups of N-PhCS as described in section 4.1.1. The % transmittance of N-PhCS prepared at 25°C was around 90%. At 40°C for 24 h, the solubility of N-PhCS was decreased due to the cyclization of phthalimido moieties and the %transmittance was less than 20%. Therefore, we chose the suitable condition to prepare N-PhCS at 25°C under 4 h stirring for further experiment. In addition, it was also found that the characteristic of N-PhCS depended on the amount of NaOH added in the neutralization pH (step 3 of preparation process) and the effect of pH condition was also studied.

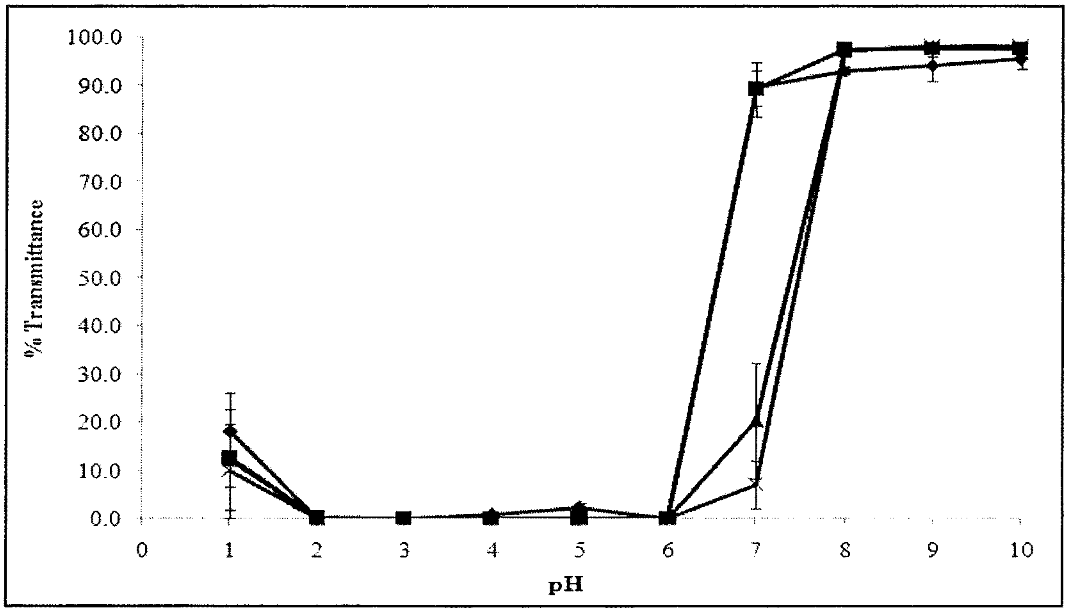


Figure 18 The %transmittance of N-PhCS prepared at different temperatures and stirring times in pH 1-10 media: N-PhCS at 25°C for (♦) 4 h and (■) 24 h, at (▲) 40°C for 4 h and (x) 24 h.

4.2 Characterization of N-PhCS prepared under various conditions

4.2.1 FTIR spectroscopy

The FITR spectra of N-PhCS prepared under various conditions by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 are presented in Figures 19 and 20, respectively. All the spectra of N-PhCS-20 demonstrated the characteristic peaks at 1647, 1560 and 1385 cm^{-1} region assigned to the phthalate moieties of chitosan-N-phthalamidate sodium. At pH 4, the peak at 1716 cm^{-1} attributed to carbonyl stretching of carboxylic functional group [86] indicating the structure of chitosan-N-phthalamidic acid (Figure 17c) was observed only in the spectra of N-PhCS at 1:3 and 1:5 of CS:PA (Figure 19 b-c). It was indicated that at higher mole ratio of CS:PA, the substitution with phthaloyl group was increased resulting in less salt formation of chitosan-N-phthalamidic acid at the same pH of the neutralization step. At pH 6 under 1:5 mole ratio of CS:PA, the intense and narrow band at 3615 cm^{-1} assigned to the stretching vibrations of O–H bonds which might be inferred to the excess NaOH residues (Figure 19i). In addition, the spectra of N-PhCS-200 showed the results similar to those of N-PhCS-20.

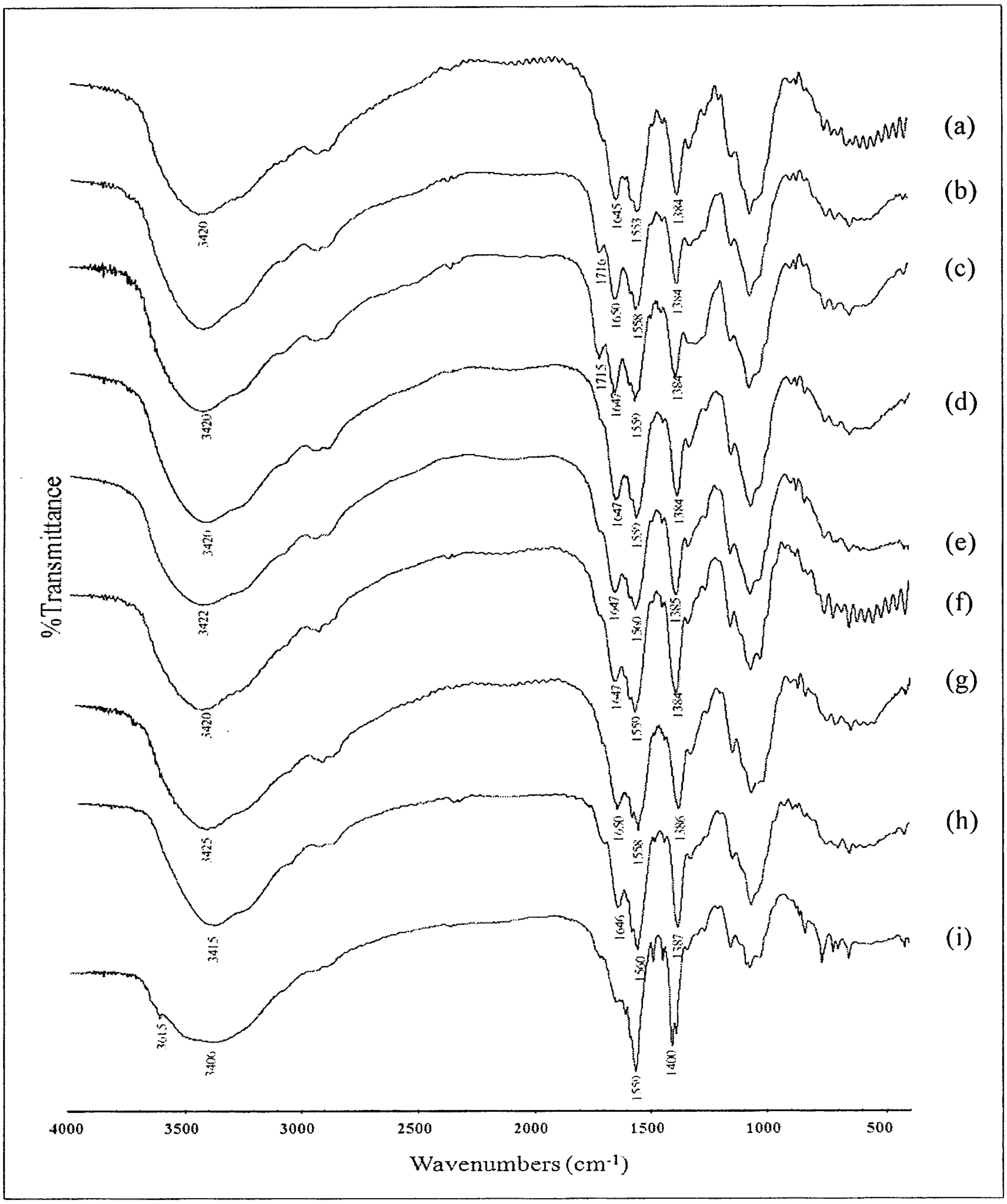


Figure 19 FTIR spectra of N-PhCS-20 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: at pH 4 under CS:PA; (a) 1:1, (b) 1:3 and (c) 1:5; at pH 5 under CS:PA; (d) 1:1, (e) 1:3 and (f) 1:5; at pH 6 under CS:PA; (g) 1:1, (h) 1:3 and (i) 1:5.

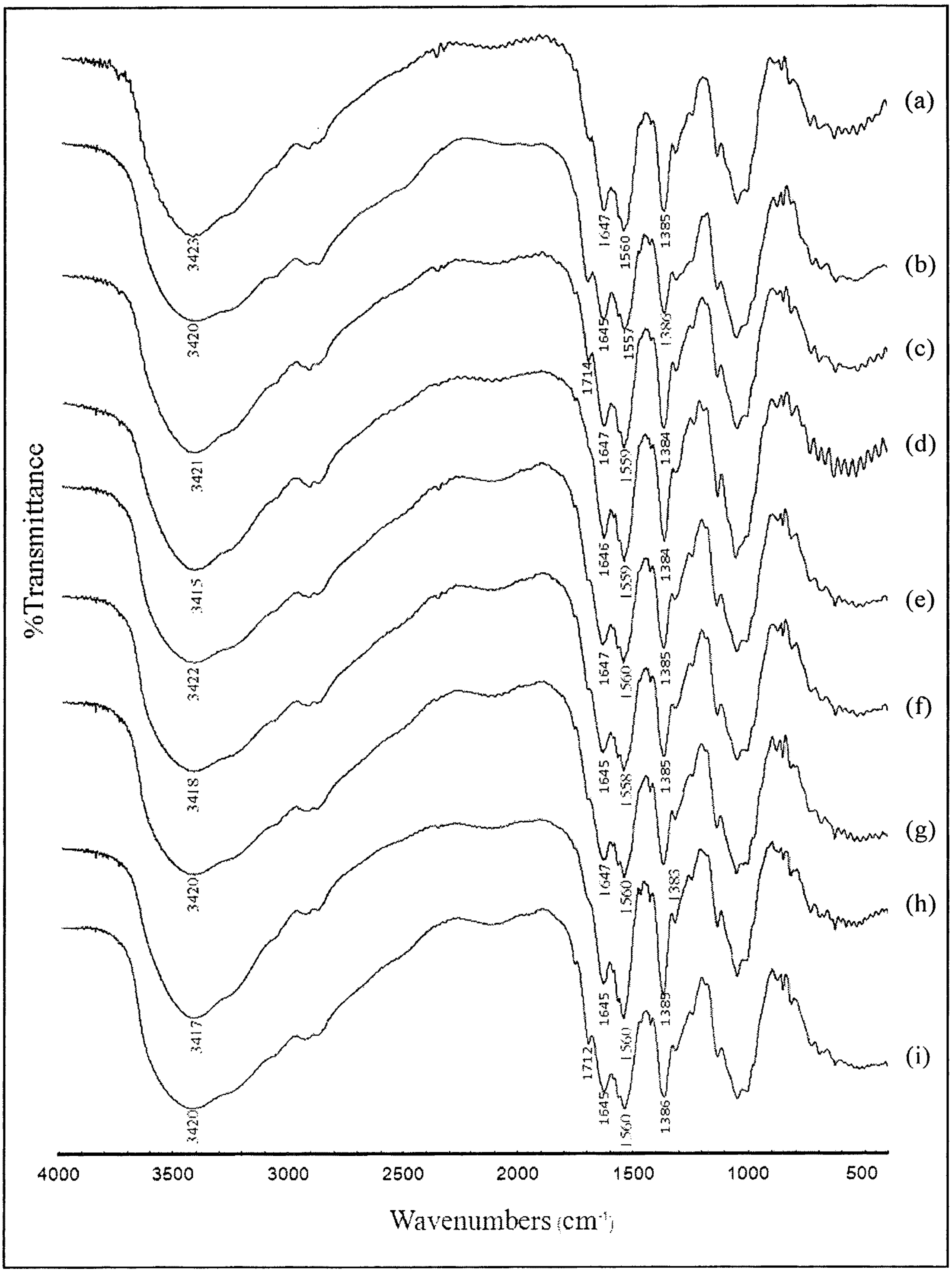


Figure 20 FTIR spectra of N-PhCS-200 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: at pH 4 under CS:PA; (a) 1:1, (b) 1:3 and (c) 1:5; at pH 5 under CS:PA; (d) 1:1, (e) 1:3 and (f) 1:5; at pH 6 under CS:PA; (g) 1:1, (h) 1:3 and (i) 1:5.

4.2.2 Powder X-ray diffraction characterization

Powder X-ray diffraction (PXRD) patterns of N-PhCS prepared under various conditions by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 are presented in Figures 21 and 22, respectively. The PXRD patterns of CS-20, CS-200, PA and physical mixtures of CS-20:PA, CS-200:PA are also demonstrated. The crystalline peaks at around 11° and 20° (2θ) were observed in the PXRD pattern of CS while physical mixtures expressed the sharp peaks of PA at 13°, 17°, 20°, 22°, 23°, 27° and 28° (2θ) and the broad peaks related to the peaks of CS. A lot of strong intermolecular and intramolecular hydrogen bonds (H-bonds) make CS form crystalline regions and results in being insoluble in water [25]. After modification, halo diffraction patterns all of N-PhCS were observed. It was suggested that its ability of forming hydrogen bond might be decreased after the amino groups of CS were substituted with phthaloyl groups, resulting in the formation of amorphous solids of N-PhCS.

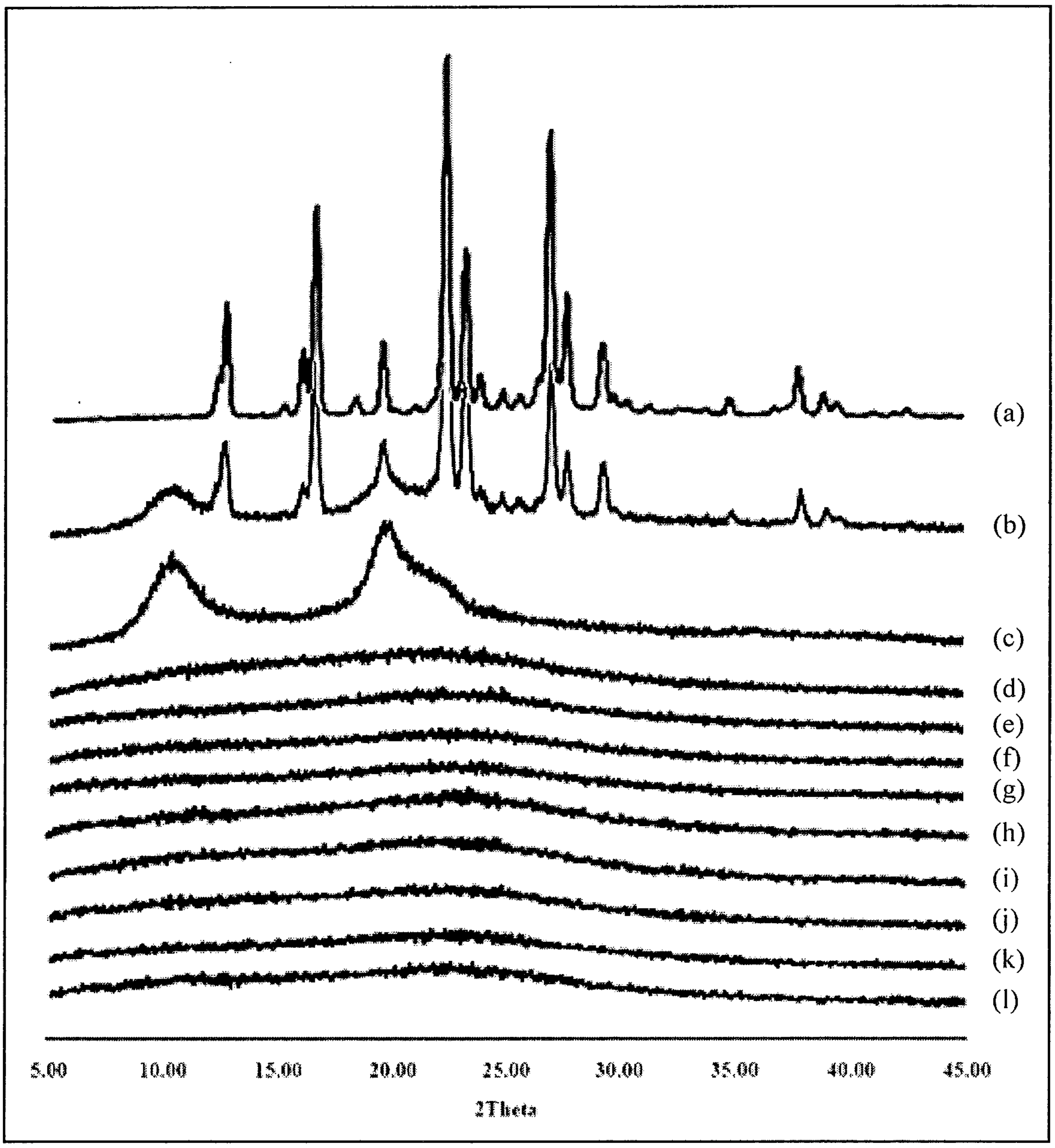


Figure 21 PXRD patterns of N-PhCS-20 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) PA, (b) physical mixture of CS:PA, 1:1, (c) CS-20, at pH 4 under CS:PA; (d) 1:1, (e) 1:3 and (f) 1:5; at pH 5 under CS:PA; (g) 1:1, (h) 1:3 and (i) 1:5; at pH 6 under CS:PA; (j) 1:1, (k) 1:3 and (l) 1:5.

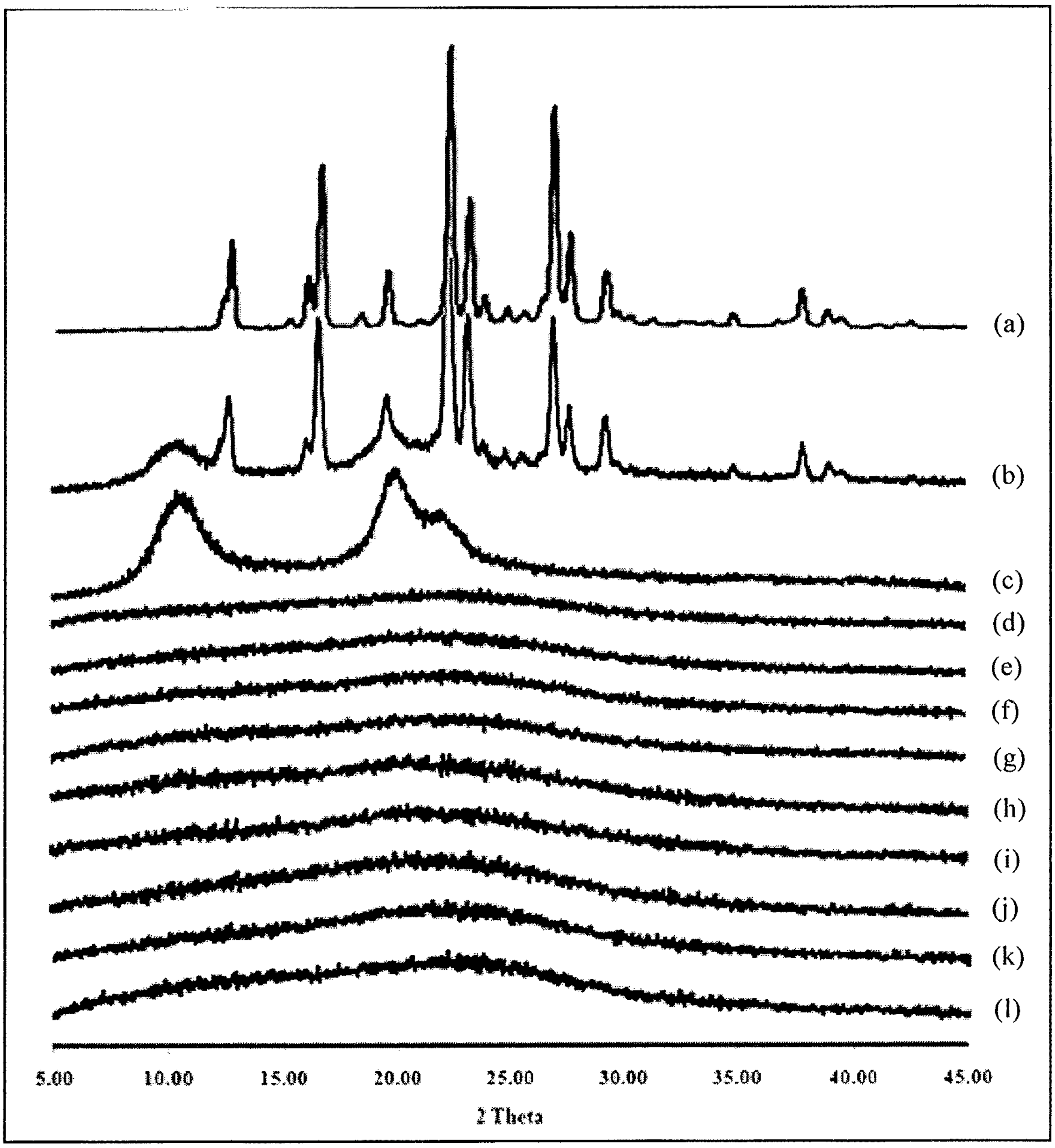


Figure 22 PXRD patterns of N-PhCS-200 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) PA, (b) physical mixture of CS:PA, 1:1, (c) CS-200, at pH 4 under CS:PA; (d) 1:1, (e) 1:3 and (f) 1:5; at pH 5 under CS:PA; (g) 1:1, (h) 1:3 and (i) 1:5; at pH 6 under CS:PA; (j) 1:1, (k) 1:3 and (l) 1:5.

4.2.3 Differential scanning calorimetry (DSC)

DSC thermograms of N-PhCS prepared under various conditions by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 are illustrated in Figures 23 and 24, respectively. The thermograms of CS 20 and 200 kDa showed the broad endothermic peak around 90°C due to the water vapor that the CS contains and the exothermic decomposition peaks at onset at 290-320°C [87]. The DSC thermograms all of N-PhCS expressed endothermic dehydration peaks around 60-80°C and the decomposing peak at onset around 200-250°C were observed, correspond to its thermal decomposition. The results indicated that the decomposition temperature of N-PhCS was lower than CS base due to the structure of CS chains has been changed after the introduction of phthaloyl groups and the reduced ability of crystallization was in agreement with PXRD results.

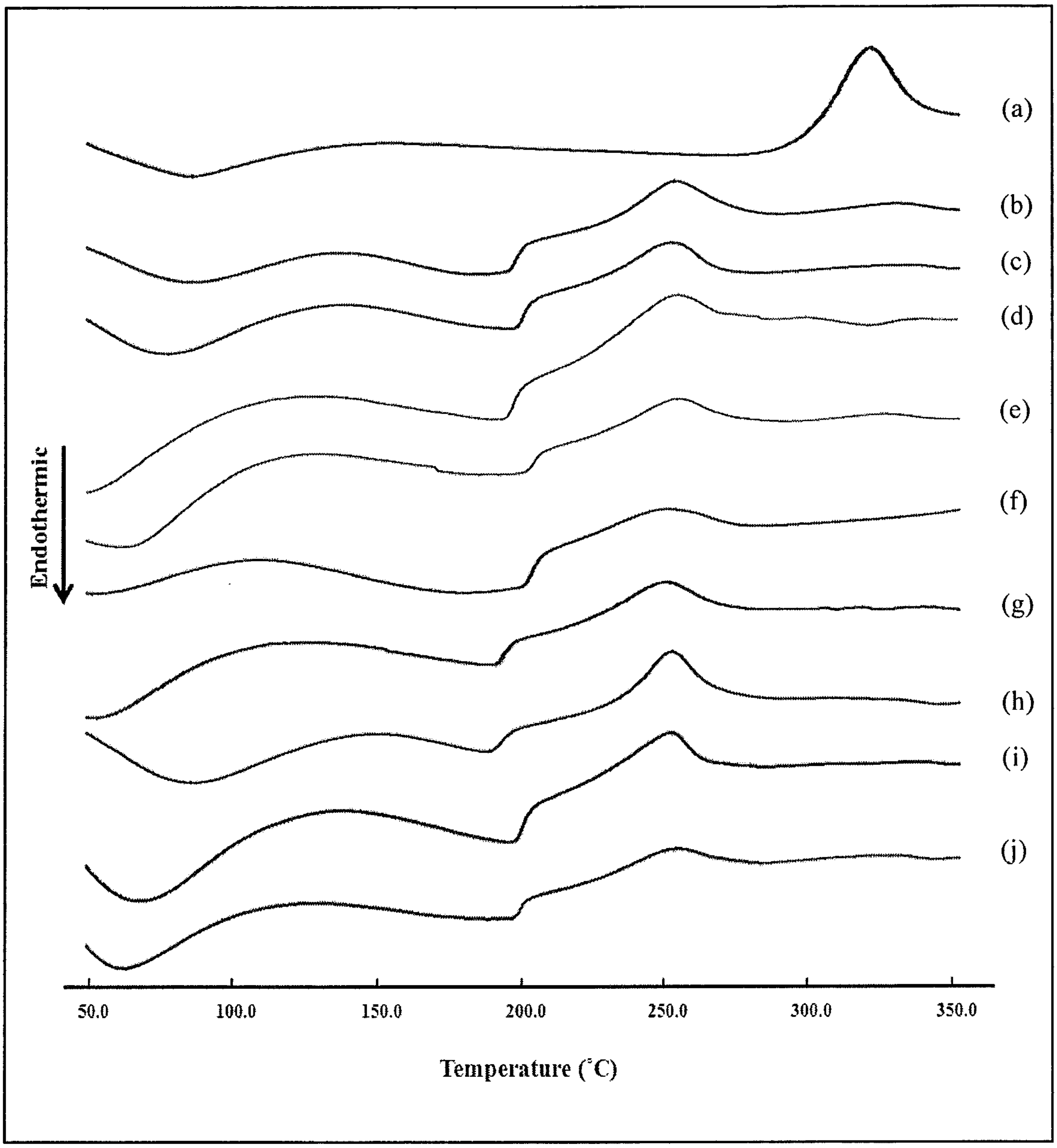


Figure 23 DSC thermograms of N-PhCS-20 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) CS-20, at pH 4 under CS:PA; (b) 1:1, (c) 1:3 and (d) 1:5; at pH 5 under CS:PA; (e) 1:1, (f) 1:3 and (g) 1:5; at pH 6 under CS:PA; (h) 1:1; (i) 1:3 and (j) 1:5.

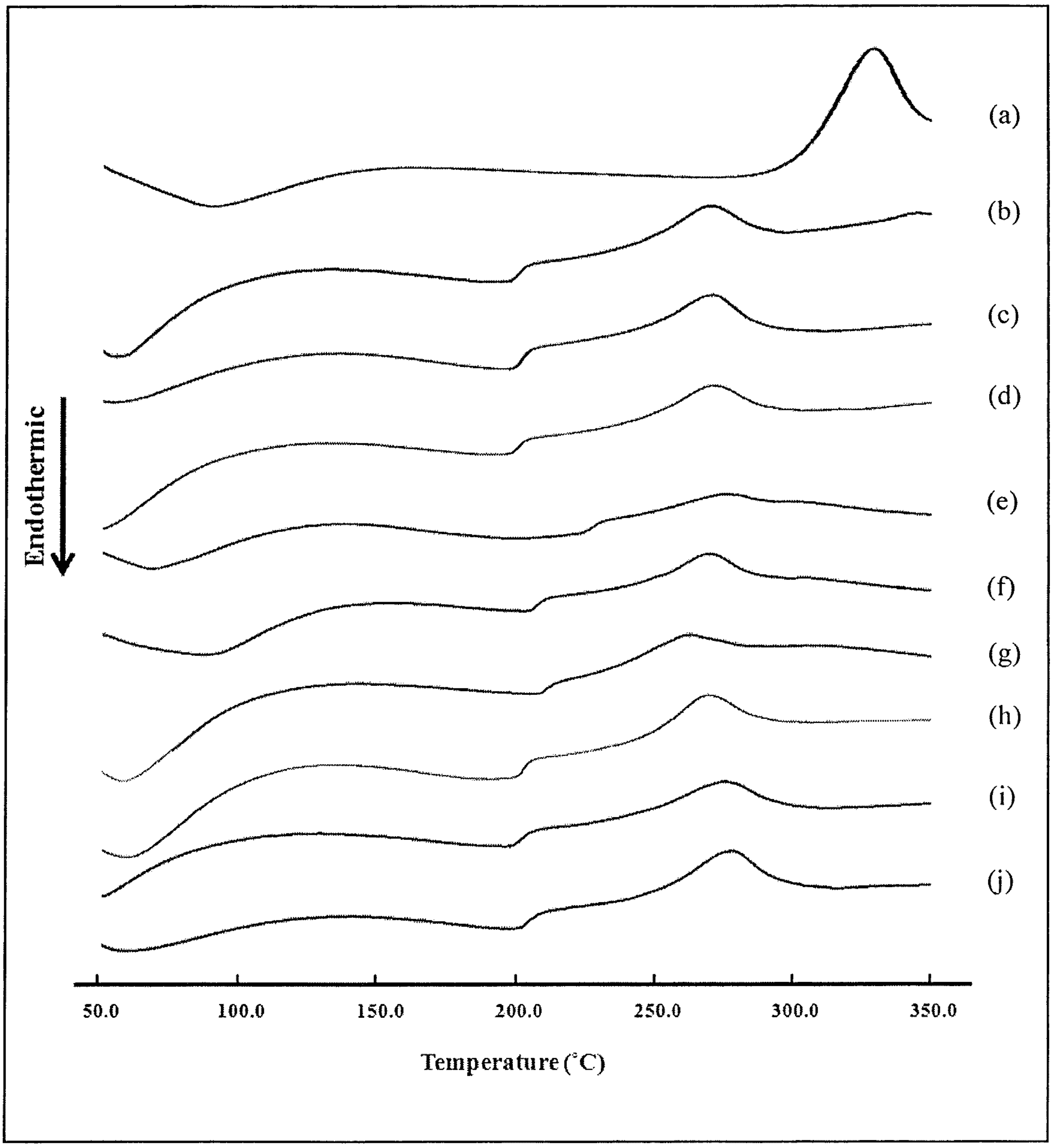


Figure 24 DSC thermograms of N-PhCS-200 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) CS-200, at pH 4 under CS:PA; (b) 1:1, (c) 1:3 and (d) 1:5; at pH 5 under CS:PA; (e) 1:1, (f) 1:3 and (g) 1:5; at pH 6 under CS:PA; (h) 1:1, (i) 1:3 and (j) 1:5.

4.2.4 Thermogravimetric analysis (TGA)

TGA thermograms of N-PhCS prepared under various conditions by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 are illustrated in Figures 25 and 26, respectively. The thermogram of CS showed two steps of weight loss in which, first step at about 90°C corresponded to the dehydration of water. The second step at around 280-320°C, was attributed to the decomposition of the polymer [85]. The thermograms of all of N-PhCS showed three steps of weight loss related to the dehydration of bound water around 60-100°C, the melting behavior of impurities such as, sodium phthathalate, phthalic acid sodium acetate and acetic acid around 100-200°C and the decomposition of N-PhCS at around 200-250°C (as shown in Figures 36-53 of Appendix). It was indicated that N-PhCS was less stable than CS, which was due to the weakening of the hydrogen bonding as a result of introduction of substitution groups [25]. These behaviors were in good agreement with the DSC results.

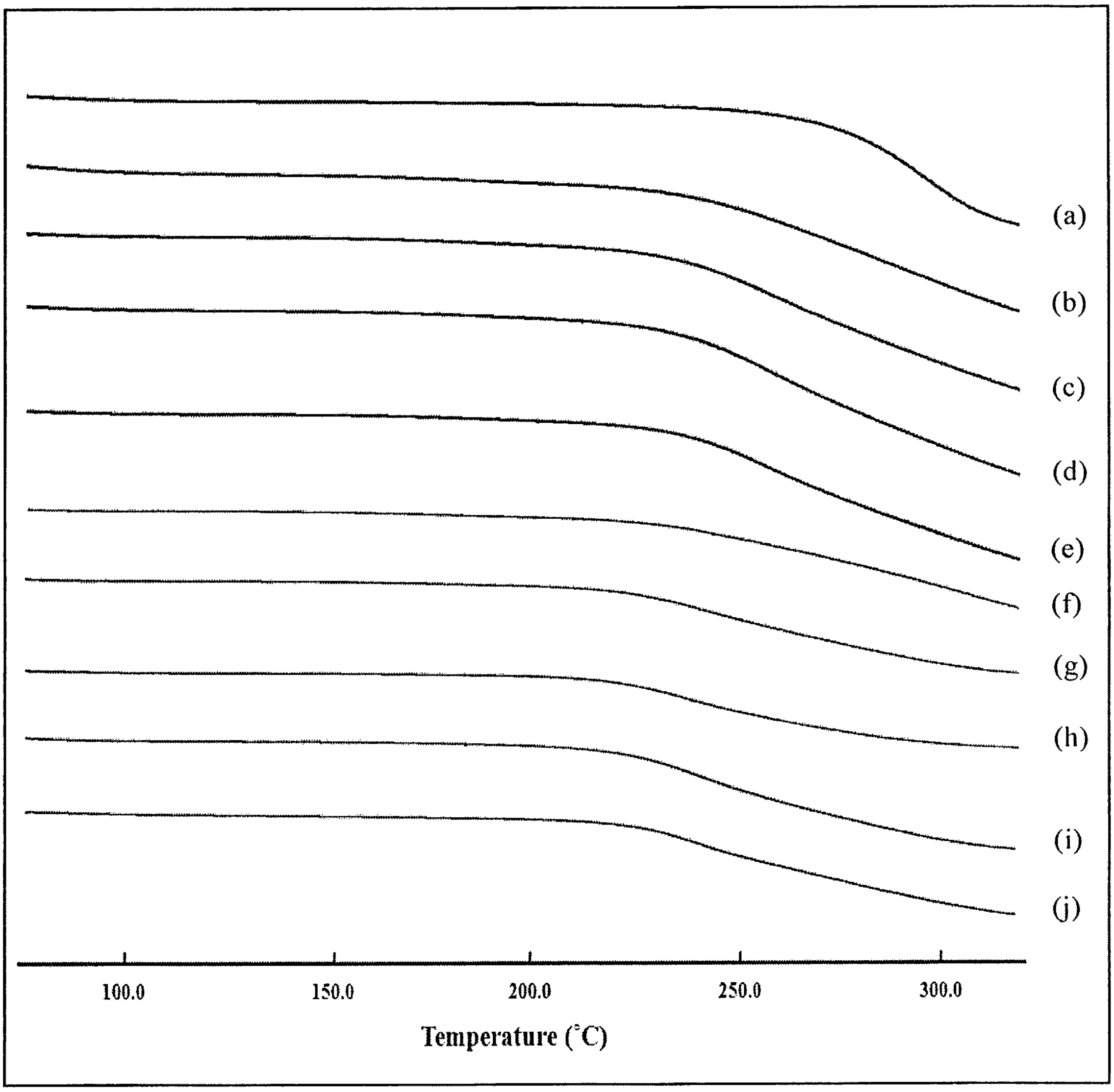


Figure 25 TGA thermograms of N-PhCS-20 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) CS-20, at pH 4 under CS:PA; (b) 1:1, (c) 1:3 and (d) 1:5; at pH 5 under CS:PA; (e) 1:1; (f) 1:3 and (g) 1:5; at pH 6 under CS:PA; (h) 1:1, (i) 1:3 and (j) 1:5.

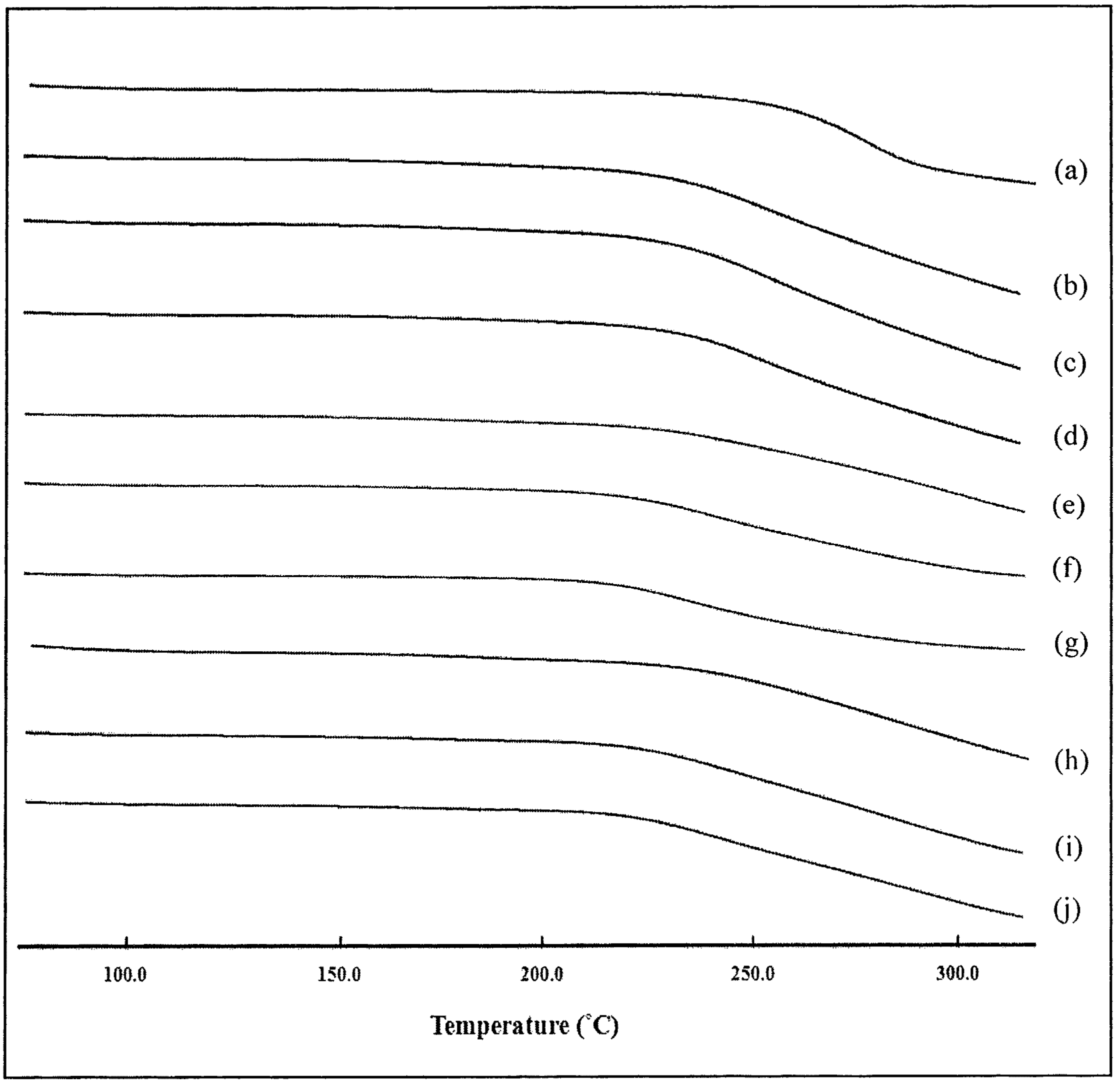


Figure 26 TGA thermograms of N-PhCS-200 prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) CS-200, at pH 4 under CS:PA; (b) 1:1, (c) 1:3 and (d) 1:5; at pH 5 under CS:PA; (e) 1:1, (f) 1:3 and (g) 1:5; at pH 6 under CS:PA; (h) 1:1,CS:PA; (i) 1:3 and (j) 1:5.

4.2.5 Solubility study

The %transmittance of N-PhCS prepared by varying mole ratio of CS:PA at 1:1, 1:3 and 1:5mole ratio, neutralization pH at pH 4, 5 and 6 and different molecular weights of CS-20 and CS-200 in pH 1-10 media are presented in Figures 27 and 28, respectively. As the mole ratio of CS:PA increased, the precipitation of N-PhCS was observed at pH range 1-5 with zero %transmittances. N-PhCS gradually swelled at pH 5-6 and the % transmittance was around 10-30%. The solubility of N-PhCS was increased at pH above 7 with nearly 100% transmittances. At 1:1 mole ratio, the partial swelling of N-PhCS at pH 1 was observed, indicating the protonation of unsubstituted amino group as already described in section 4.1.2. At pH 5-6, the partial swelling was not observed which might be due to the less substitution of N-phthaloyl groups of N-PhCS [29]. In case of pH 4 in neutralization pH, the %transmittance of N-PhCS at pH range 6-7 was lower than those of pH 5 and 6 which might be due to the less salt formation of chitosan-N-phthalamidic acid as already described in section 4.2.1. The % transmittance of N-PhCS prepared from both different molecular weights demonstrated the similar results but the N-PhCS with high molecular weight exhibited the higher viscosity [88].

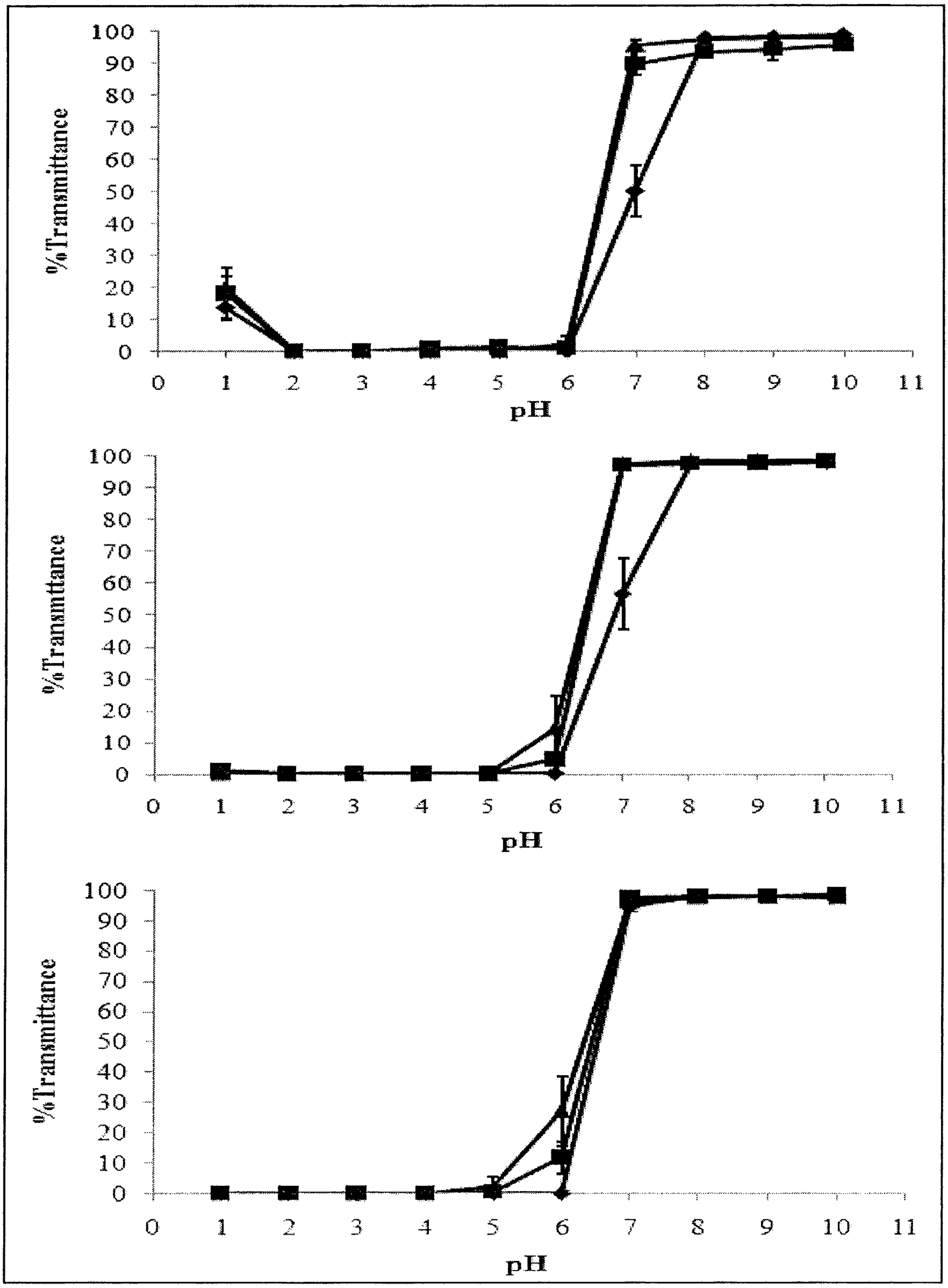


Figure 27 The %transmittance of N-PhCS-20 kDa prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) 1:1, (b) 1:3 and (c) 1:5 mole ratio of CS:PA and (♦) pH 4, (■) pH 5 and (▲) pH 6.

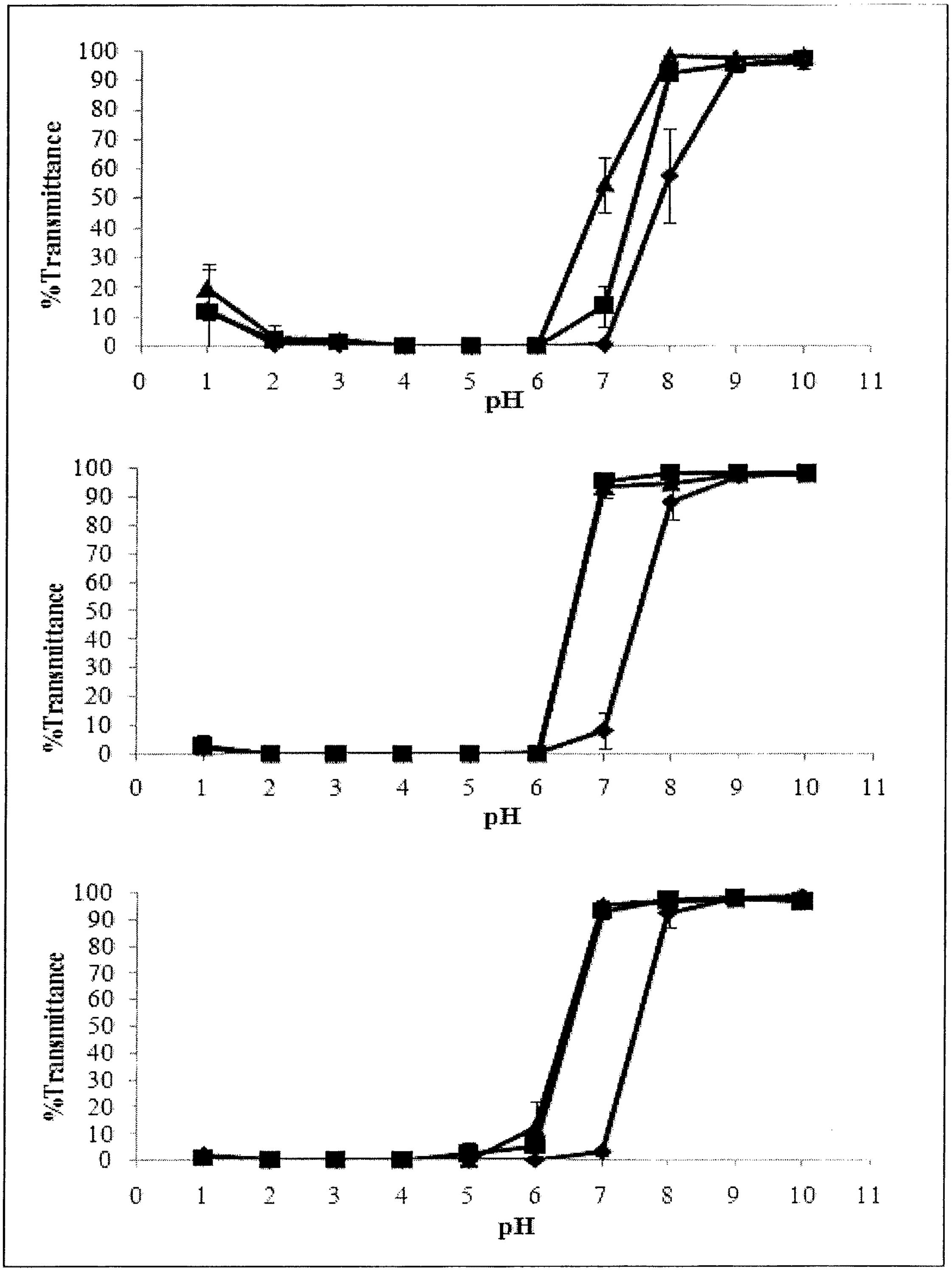


Figure 28 The %transmittance of N-PhCS-200 kDa prepared under various conditions by varying mole ratio of CS:PA and neutralization pH: (a) 1:1, (b) 1:3 and (c) 1:5 mole ratio of CS:PA and (♦) pH 4, (■) pH 5 and (▲) pH 6.

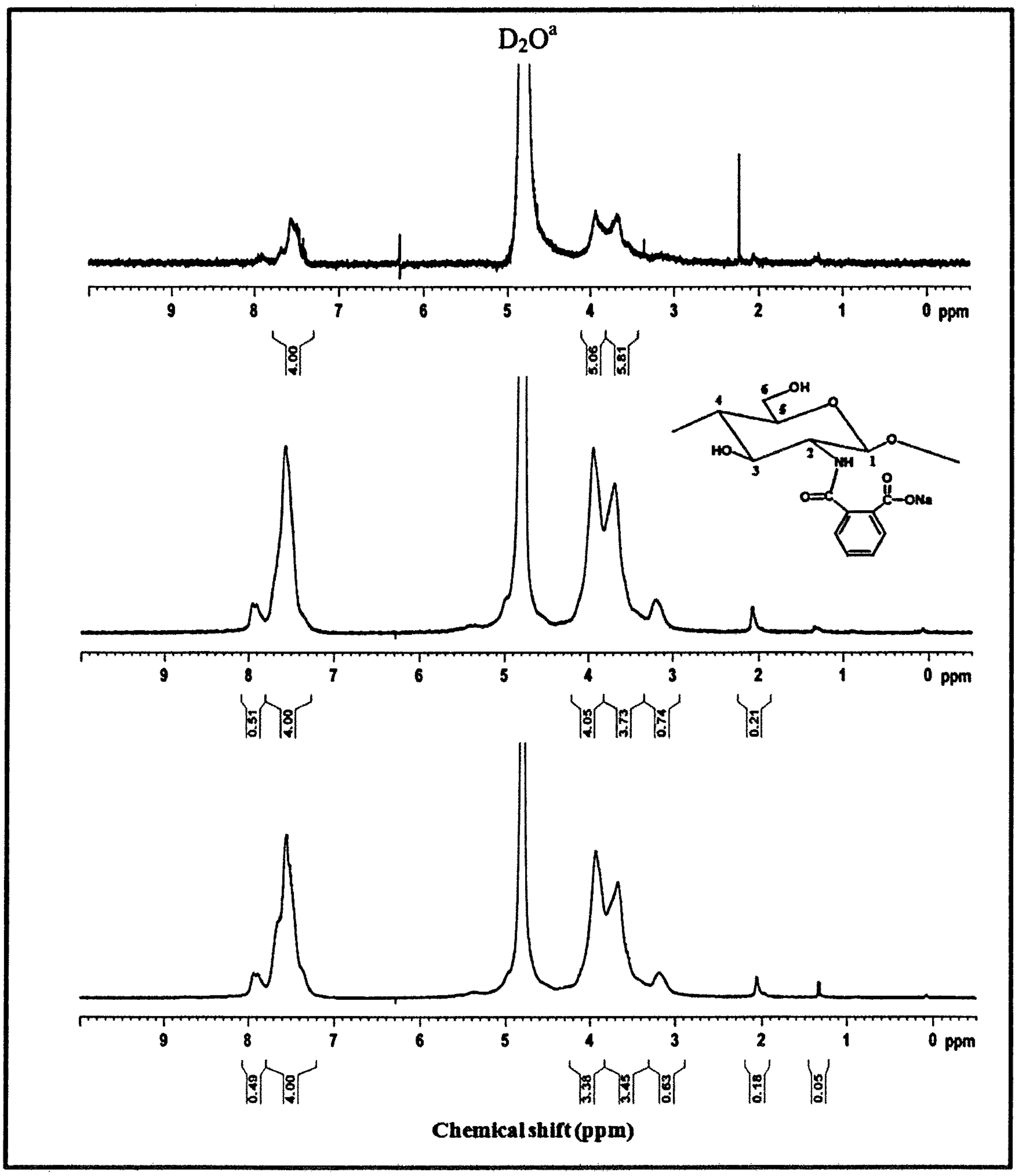
4.2.6 Measurement of degree of substitution (DS)

The DS of all N-PhCS determined by FTIR assay is shown in Table 8. The %DS was around 10-20% and not correlated with mole ratio of CS:PA. It was suggested that no significant difference was noticed between the values obtained by FTIR assay. This was because the %DS of chitosan derivatives was calculated from the ratio of absorbance at 1650 cm^{-1} (described to amide I as probe band) and the hydroxyl band at 3450 cm^{-1} (reference band) [16, 45, 71]. In the spectra of N-PhCS, the hydroxyl band became narrower and shifted to lower wavenumber as described in section 4.1.1. Thus, FTIR assay was not suitable for estimation the DS of N-PhCS.

From the results of FTIR and solubility study, it was suggested that N-PhCS prepared from CS-20 under the condition of pH 5 in neutralization and 1:5 mole ratio of CS:PA gave the solubility best fitted to enteric polymer property. Therefore, the exact %DS of N-PhCS with 1:1, 1:3 and 1:5 mole ratio of CS:PA under pH 5 in neutralization pH was confirmed by ^1H-NMR assay (Figure 29). The assignments of ^1H in the NMR spectra of N-PhCS were as follows, the signals at 3.1 ppm (H_2) and 3.5-3.9 ppm (H_3, H_4, H_5, H_6) were corresponding to the ring methenyl protons of CS [26]. The signals at 7.4-7.9 ppm were assigned to the aromatic proton of phthaloyl group [85]. The %DS of N-PhCS with 1:1, 1:3 and 1:5 mole ratio of CS:PA, calculated from $[(I_{\text{aromatic proton}}/ 4) /(I_{(H3+ H4+ H5+H6)} / 5)]$ x100, were 46.0%, 64.3% and 73.2%, respectively. It was suggested that the substitution of N-phthaloyl group increased, as the mole ratio of CS:PA increased. Therefore, N-PhCS prepared at 1:5 mole ratio of CS:PA was chosen for stability and cytotoxicity as well as film forming properties study.

Table 8 The degree of substitution of N-PhCS prepared under various conditions using FTIR and ^{1}H-NMR methods.

Sample	%DS	
	FTIR assay	^{1}H-NMR assay
N-PhCS-20 1:1;CS:PA, pH4	19.58 ± 0.39	
N-PhCS-20 1:1;CS:PA, pH5	18.70 ± 0.15	46.0
N-PhCS-20 1:1;CS:PA, pH6	13.61 ± 0.57	
N-PhCS-20 1:3;CS:PA, pH4	20.07 ± 0.39	
N-PhCS-20 1:3;CS:PA, pH5	17.98 ± 0.33	64.3
N-PhCS-20 1:3;CS:PA, pH6	14.99 ± 0.39	
N-PhCS-20 1:5;CS:PA, pH4	16.78 ± 0.85	
N-PhCS-20 1:5;CS:PA, pH5	16.94 ± 1.01	73.2
N-PhCS-20 1:5;CS:PA, pH6	-	
N-PhCS-200 1:1;CS:PA, pH4	12.34 ± 0.81	
N-PhCS-200 1:1;CS:PA, pH5	14.53 ± 1.26	
N-PhCS-200 1:1;CS:PA, pH6	12.45 ± 1.07	
N-PhCS-200 1:3;CS:PA, pH4	15.79 ± 0.55	
N-PhCS-200 1:3;CS:PA, pH5	12.04 ± 1.54	
N-PhCS-200 1:3;CS:PA, pH6	13.71 ± 1.86	
N-PhCS-200 1:5;CS:PA, pH4	12.32 ± 1.37	
N-PhCS-200 1:5;CS:PA, pH5	13.95 ± 0.71	
N-PhCS-200 1:5;CS:PA, pH6	8.73 ± 1.22	



[a]The chemical shifts at 4.8 ppm referred to D_2O [89].

Figure 29 ^{1}H NMR spectra of N-PhCS prepared at 25°C for 4 h under various mole ratios: (a)1:1, (b) 1:3 and (c)1:5.

4.3 Stability study

Figure 30 demonstrates the FTIR spectra of N-PhCS stored at 60, 80 and 120 ± 2°C for 6, 12 and 24 h. After storage at 120°C, the peaks at 1712 cm^{-1} and 1772 cm^{-1} attributed to carbonyl stretching of phthalimido moieties were observed and tended to increase when the exposure time increased. This indicated the cyclization of chitosan-N-phthalamidic acid after exposure to high temperature. The results suggested the applications of N-PhCS should be aware of dealing with high temperatures.

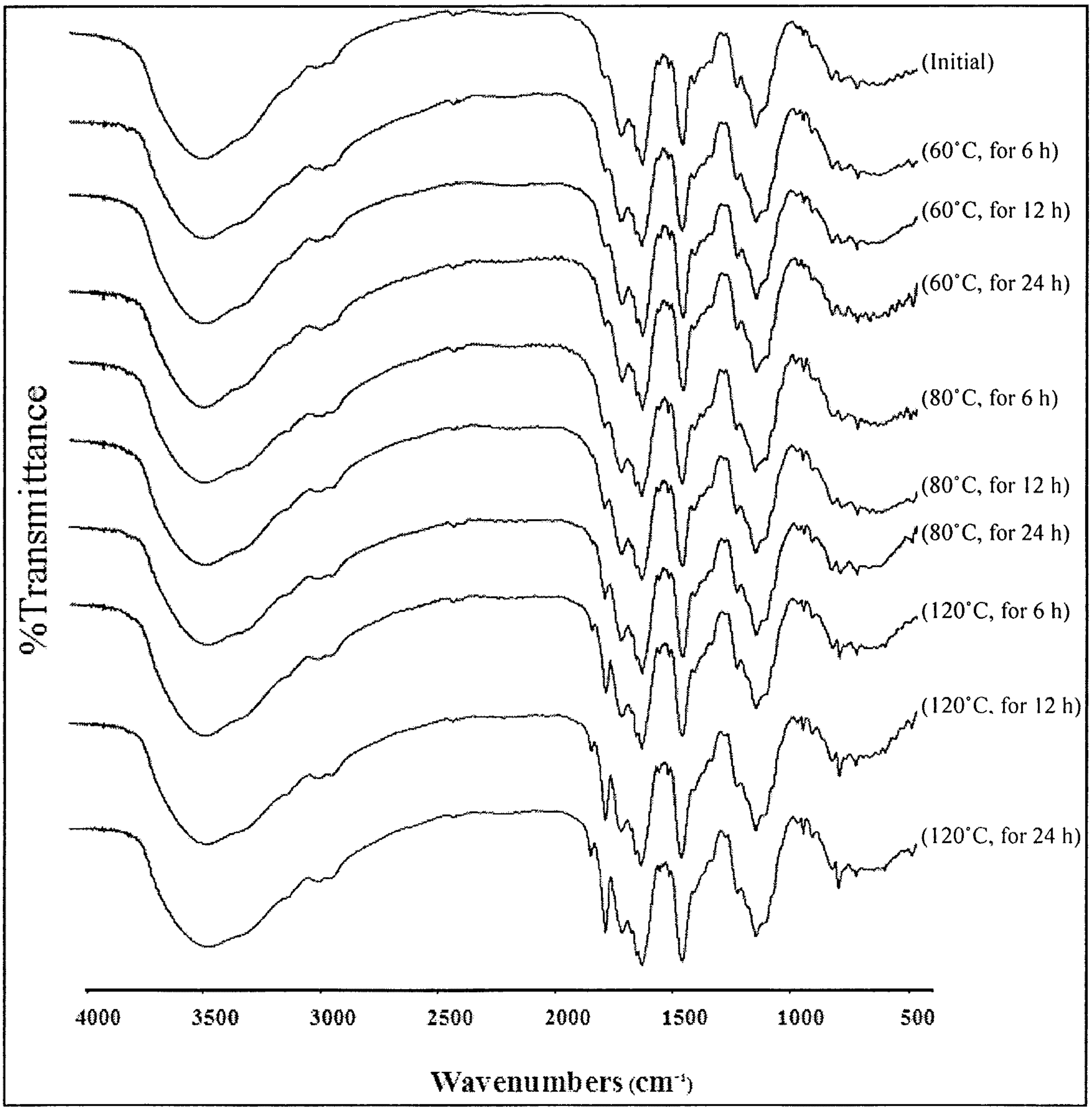
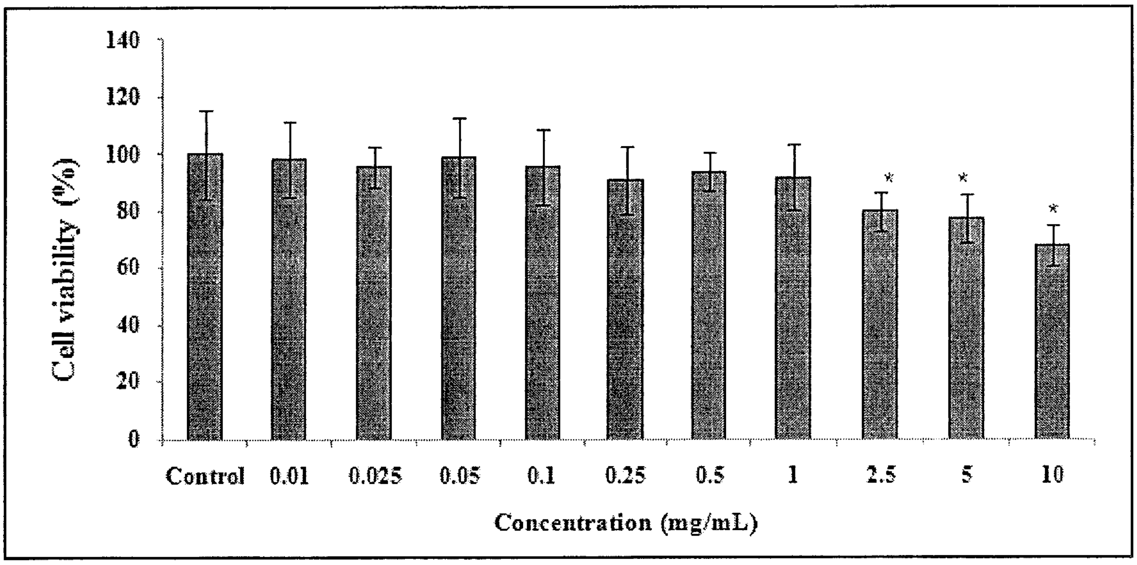


Figure 30 FTIR spectra of N-PhCS under storage at various conditions (stability study).

4.4 Cytotoxicity test

The MTT assay was performed to assess the cytotoxic activity of the N-PhCS in Caco-2 cell lines used. As presented in Figure 31, N-PhCS in concentrations ranges 0.01-1 mg/mL exhibited cell viability around 90% and it was not significantly different from the control (*p*>0.05). As the concentrations of N-PhCS increased higher than 2.5 mg/mL, the cell viability decreased lower than 80%. The cytotoxicity result agrees with a previous research by Huanbutta [78] that CS in the form of nano/microparticles was non-toxic, and cell-compatible when using in the concentration 0.01-0.1 mg/mL.



*p<0.05 (Turkey)

Figure 31 Cytotoxicity of N-PhCS in Caco-2 cells under the MTT assay.

4.5 Characterization of N-PhCS films

The films prepared from N-PhCS were light yellow clear transparent films with no odor and the thickness was controlled in a range of 110 ± 20 µm (Figure 32).

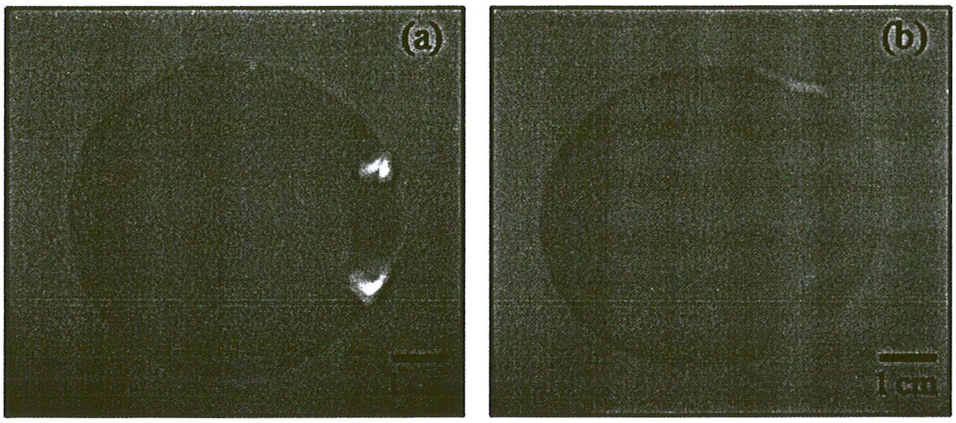


Figure 32 Images of (a) N-PhCS film and (b) CSA film (digital camera).

4.5.1 Mechanical properties

The tensile strength, including the elongation at break and gradient stress-strain of N-PhCS and CSA films, are summarized in Table 9. The tensile strength of N-PhCS films were not significantly different from CSA films ($p>0.05$) whereas percent of elongation at break of N-PhCS was significantly lower than that of CSA films ($p< 0.05$) due to the rigid aromatic ring (phthaloyl moiety) reduced the polymer chain mobility [90]. In addition, gradient stress-strain of N-PhCS was significantly higher than that of CSA films ($p< 0.05$). The results demonstrated that N-PhCS films were more brittle than CSA films, characterized by having small elongation and high values of gradient stress-strain.

Table 9 Mechanical properties of N-PhCS and CSA films.

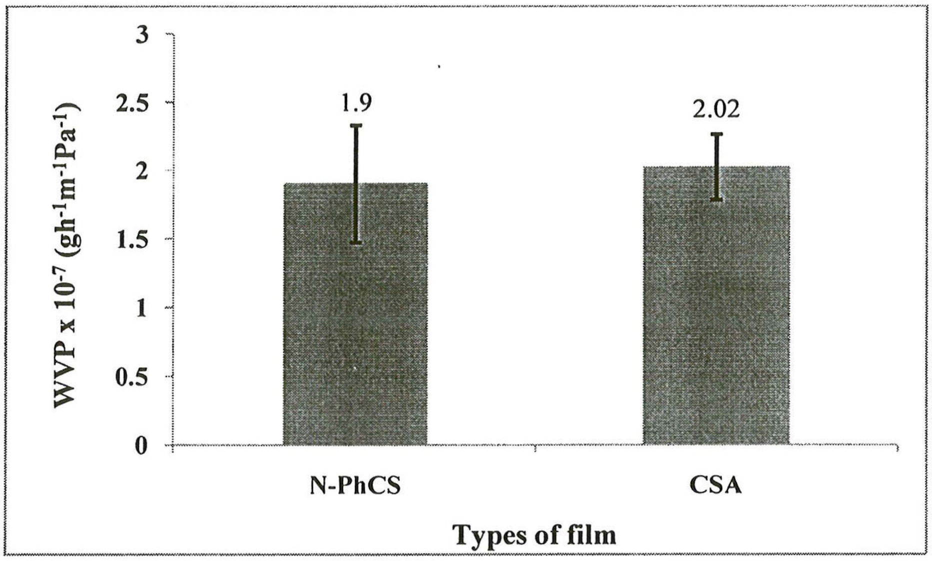
Sample	Thickness[a] (mm)	Tensile strength[a] (MPa)	elongation at break[a] **(%)**	Gradient St-Strain[a] (MPa/%)
N-PhCS	**0.117**±0.008	**49.891**±11.4	**2.881**±0.79*	**19.534**±1.96*
CSA	0.113±0.004	46.672±5.5	8.802±2.45	17.665±1.52

[a] All values were mean ± SD of ten samples.

*$p<0.05$ (t-test)

4.5.2 Water vapor permeability

The water vapor permeability (WVP) of N-PhCS and CSA films are illustrate in Figure 33. N-PhCS films demonstrated not significantly different moisture permeability (1.90 x 10^{-7} g × h^{-1} × m^{-1} × Pa^{-1}) as compared to that of CSA film (2.02 x 10^{-7} g × h^{-1} × m^{-1} × Pa^{-1}) ($p > 0.05$). It was indicated that moisture protection of N-PhCS films was not deteriorated after introducing the phthaloyl group.



^{a}All values were mean ± SD of six samples.

$^{*}p > 0.05$ (t-test)

Figure 33 Water vapor permeability of N-PhCS and CSA films.

4.5.3 Moisture content and pH solubility

The moisture content and percent dissolved of N-PhCS and CSA films in SGF (pH 1.2) and SIF (pH 6.8) are presented in Table 10. The result showed that the moisture content of N-PhCS was significantly higher than that of CSA film ($p < 0.05$). The percent dissolved of N-PhCS films in SGF was 12.43 ± 1.28 while in SIF, the films were completely dissolved. The results were in consistent with the solubility study of N-PhCS powder as described in section 4.2.5. On the other hand, CSA films were completely dissolved in SGF and 23.21 ± 3.11% in SIF. It was suggested that N-PhCS film may be suitable for using as a film-forming polymer for gastro-resistant or enteric coating.

Table 10 Moisture content and percent dissolved of N-PhCS and CSA films in SGF and SIF (pH 6.8).

Films	% Moisture content	Percent dissolved[a]	
		in SGF pH 1.2	in SIF pH 6.8
N-PhCS	11.51 ± 0.93*	12.43 ± 1.28	100
CSA	9.54 ± 0.55	100	23.20 ± 3.11

[a]All values were mean ± SD of six samples.

*p<0.05 (t-test)