Denaturation and Viscosity of Whey Proteins Solutions as Affected by Frozen Storage

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Abstract: Concentrated solutions of whey proteins (WPC) were prepared from sweet whey by ultrafiltration technique, and stored at – 18°C up to three months. Denaturation degree and viscosity of WPC solutions were assessed. Denaturation degree of whey protein solutions increased significantly (P<0.05) as affected by duration of frozen storage and protein content. The highest degree of denaturation was found at pH 5.0 and 7.0 after one month of storage. Denaturation percentages of heated and thawed WPC solutions increased significantly (P<0.05) as function of storage, protein content and pH. The flow properties of unheated WPC solutions exhibited a time-independent non-Newtonian behaviour as shear-thickening (dilatants) properties with an increase in the apparent viscosity with increasing the shear rate. Heated thawed WPC solutions behaved as thixotropic fluids with a decrease in the apparent viscosity with increasing shear rate. Apparent viscosities of unheated and heated WPC solutions greatly affected by frozen storage, protein content and pH. [Journal of American Science. 2010;6(12):49-62]. (ISSN: 1545-1003).

Key words: WPC, Frozen storage, Denaturation, Viscosity

1. Introduction

Whey proteins are used as functional ingredients in many food products not only for their nutritional properties, but also for their functional and technological properties. The functional properties of whey proteins may be refered to as: (a) hydration properties that have an important effect on wettability, swelling, adhesion, dispersibility, solubility, viscosity, water absorption and water holding; (b) interfacial properties including emulsification and foaming characteristics; (c) aggregation and gelation properties which are related to protein–protein interactions (Kresic, et al. 2006). These functionalities can be affected by either heat treatment (Mulvihill & Donovan, 1987) or pressure treatment (Patel, et al. 2005).

Preservation of the valuable cheese whey proteins in the form of a whey protein concentrate (WPC) powder has been increasing in recent years. Studies on the functional properties of dried WPC (Harper, 1984; Mangino, 1992; de Wit, 1989; Morr & Foegeding, 1990) indicate variability depending upon the source of whey, extent of protein denaturation during processing, presence of non-protein components and failure to standardize pre-treatment and processing conditions for manufacture.

Storage at freezing temperature is a well established technique for long-term preservation of many foods and other commodities. Surprisingly, very little attention has been paid to the possibility of using frozen storage for long preservation of protein solutions. Freezing of foods may result in undesirable changes, including textural damage, protein denaturation, destruction of cellular membranes mainly due to the freeze-concentration phenomenon. Bhargava & Jelen (1995) investigated the effects of freezing on viscosity of concentrated WPC solutions. They found that a small but statistically highly significant difference in viscosity, showing an effect of the slow freezing rate.

Denaturation of WPI results from a complex mechanism dominated by the denaturation of β-lactoglobulin which has been explained by Simmons *et al.*, (2007) and Schokker *et al.* (2000), by a two-step process. The first step is endothermic; which consists of protein unfolding and changes in the equilibrium between protein dimers and native and non-native monomers, associated with reversible or irreversible intramolecular rearrangements (e.g. disruption of hydrogen bonds). The second step corresponds to aggregation, resulting mainly from an intermolecular –SH to S–S exchange and, to a lesser extent, from non-covalent interactions. Aggregation starts with the formation of non-native dimers and oligomers which rapidly grow as a function of chemical environment and temperature, mainly by incorporation of monomers and smaller aggregates (Le Bon et al., 1999).

The apparent viscosity of WPC determines the potential application of these ingredients with excellent technological functionality and high nutritional value in liquid food preparation as textural ingredients (Patocka et al., 2006).

The main objective of the present work was therefore to investigate the influence of frozen storage followed by heat treatment on the WPC denaturation degree and viscosity of WPC solutions.

2. Materials and Methods:

Materials

Sweet whey from Mozzarella cheese manufacture (pH 5.9 – 6.0) was obtained from Arab Dairy products Co. Kaha, Kalubia. Residual fat and curd were removed from the whey by a cream separator. Clarified whey was directly concentrated by ultrafiltration using a 50,000 molecular weight cut off zirconium oxid membrane installed in a Carbosep pilot plant (modules S 151 UF system, Nova-Sep France). Ultrafiltration was carried out in a batch mode at 45-50°C, inlet and outlet pressure of 5.5 and 3.5 bar, respectively. Ultrafiltration was continued to a concentration factor 20. The retentate (whey protein concentrates) was diluted with an equal volume of water and diafiltered three times to remove most of lactose and minerals from whey retentate.

The WPC solutions were packaged in polyethylene sacs and stored frozen at −18°C. A sample sac was removed from freezer after 1, 2, and 3 months of storage and thawed in a refrigerator at 4°C for analysis.

After thawing WPC was diluted with distilled water to obtain three WPC solutions which containing of different protein ratios. These solutions were adjusted to pH 3.0, 5.0, 7.0 and 8.6 using 1N HCl or NaOH, heated to 80°C for 10 min, and cooled rapidly. Undenatured whey proteins and viscosity for WPC preparing solutions were determined before and after heating.

Methods:

1. **Chemical analysis:**

Whey protein concentrate solutions were analysed for total solids by dry oven at 105°C for 6 hrs as described in AOAC (1990). Fat by Gerber bytrometer and protein nitrogen fractions by micro Kjeldahl method and pH according to Ling (1963). Lactose content by the phenol-sulphuric method of Barrnet and Tawab (1957).

2. **Determination of denaturation of WPC**

The degree of denaturation of WPC was determined according to Andersen *et al.* (1983). Aliquot of the WPC solution was adjusted to pH 4.6 using 1N acetic acid and 1N sodium acetate solutions respectively. The precipitate was removed by filtration and the total nitrogen was determined in the filtrate. Trichloroacetic acid (TCA) solution was added to the supernatant to give 12% TCA in the mixture. The precipitate was removed by filtration, and NPN was determined in the filtrate. The percentage of undenatured whey proteins (UDW%) was calculated as:

$$\%\ UDW = \frac{\text{Percentage of 4.6 soluble N} - \text{NPN}}{\text{Total WPC N before heat treatment} - \text{NPN}} \times 100$$

3. Viscosity of WPC

The viscosity of WPC was measured according to Farrag *et al.* (2006). The apparent viscosity of the thawed and heated WPC solutions were measured using a Bohlin coaxial cylinder viscometer (Bohlin Instrument Inc., Sweden) attached to a work station loaded with software V88 viscometery programme. The system C30 was filled with the WPC solution at the measurement temperature of 20°C. The viscosity was carried out in the up mode at shear rate ranging from 37 to 910 1/s.

The heated WPC solutions samples at 80°C for 10 min were kept at refrigerator at 5-6°C for 24 hrs to examine renaturation of whey proteins again.

Statistical analysis

The data were analysed according to Statistical Analysis System (SAS, 1998). Duncan multiple range test was carried out for separation among means. All experiments were replicated 3 times.

3. Results and Discussion

The chemical composition of whey and WPC solutions are shown in table (1). WPC solutions contained total solids (TS) of 9.50, 4.75 and 3.20% for WPC 1, WPC 2 and WPC 3 respectively. Its contained total proteins (TP) of 5.09, 2.5 and 1.7% in the same order. Although the WPC solutions had been subjected to diafiltration, the solutions still contained residual lactose and fat.

Table 1: Chemical composition of whey protein concentrates (WPC) solutions of different protein content.

Concentration Test	Whey	WPC 1	WPC 2	WPC 3
TS %	7.25	9.50	4.75	3.20
TN %	0.220	0.798	0.385	0.259
TP %	1.40	5.09	2.50	1.70
Fat %	0.1	1.8	0.9	0.6
Lactose %	5.5	2.40	1.23	0.83
pH	5.99	5.85	5.75	5.75

Whey protein denaturation as affected by frozen storage

Degree of denaturation of the whey protein concentrates solutions as affected by freezing storage are given in Table 2.

After one month of frozen storage no visible changes was observed in the WPC solutions at all pHs on freezing and thawing. The percentage of denaturation increased significantly ($p<0.05$) compared with fresh or zero time samples. The highest degree of denaturation was found at pH 5.0 and 7.0 being 48.71 and 48.94% compared with that found at pH 3.0 and 8.6 namely 45.10 and 46.46% at WPC solution containing 5.09% protein. The percent of denaturation decreased with decrease in the protein concentration. It was decreased from 45.10 for WPC1 to 41.94% for WPC3 at pH 3.0 and from 48.71 for WPC1 to 39.96 % for WPC3 at pH 5.0. The same trend was found at different pHs. These results were agreement with that reported by Farrag *et al.* 1997.

After two and three months of frozen storage, thawed WPC exhibited protein destabilization with flocculated protein aggregates. At pH 5.0 solutions, a uniform small protein aggregates could be seen in the WPC solution. These observations agreement with that finding by (Bhargava & Jelen, 1995). They found that the protein aggregates seen after thawing appeared to consist of larger clumps along with small floating fragments, indicating possible minor effects of the freezing storage at pH 5.0.

After two months of frozen storage the denaturation degree of WPC solutions increased significantly (p<0.05) compared with fresh or one month samples. After three months freezing storage the highest denaturation was found at pH 8.6 of 56.12, 52.16 and 51.61% for WPC1, WPC2 and WPC3 respectively. Previous studies reported good protein stability in WPC solution stored below -20°C (Antifantakis *et al.*, 1980; Bastian, 1994, Young, 1985). Koschak *et al.* (1981) reported that frozen bovine milk and milk concentrates stored at -20°C or lower remain stable for long periods of time, but stability decreases greatly as the temperature is raised above -20°C.

Heat denaturation of thawed WPC solutions:

Table 3 shows the denaturation percentages of heated WPC solutions as function of freezing storage, protein content and pH. At pH 3 heating denaturation degree increased significantly (P<0.05) from 71.43% at zero time to 85.19% after 3 months of freezing storage of WPC 1 solution. At pH5.0 degree of denaturation increased significantly (P<0.05) of 70.00, 74.44, 82.50 and 85.37% after zero time, 1 month, 2 months and 3 months for WPC2 solution respectively. At pH 7.0 the same trend were found. On the other hand at pH 8.6 no significant differences were found in the denaturation percent of WPCs solutions (different protein content) after 3 months of freezing storage. Higher pH values caused formation of soluble whey proteins aggregates. Vasbinder and de Kruif (2003). Heat treatment at higher pH caused a clear formation of whey protein aggregates, indicating a pH dependent aggregation mechanism. Anema and Klostermeyer (1997) demonstrated using ultracentrifugation that at higher pH more whey proteins remained soluble than at lower pH.

Table (2): Effect of frozen storage on the denaturation % of WPC solutions at different pHs.

	A	B	C
(a) pH 3.0			
Fresh	30.85^{f}	29.17^{f}	28.12^{f}
1 month	45.10^{d}	40.44^{e}	41.94^{e}
2 months	48.91^{bc}	47.81^{bcd}	46.63^{cd}
3 months	52.75^{a}	51.09^{ba}	50.00^{ba}
(b) pH 5.0			
Fresh	30.00^{g}	26.53^{h}	27.29^{h}
1 month	48.71^{cd}	38.30^{f}	39.96^{f}
2 months	52.81^{b}	46.82^{de}	44.79^{e}
3 months	55.68^{a}	50.02^{c}	48.24^{cd}
(c) pH 7.0			
Fresh	26.45^{f}	25.51^{f}	25.81^{f}
1 month	48.94^{b}	39.57^{d}	35.74^{e}
2 months	53.93^{a}	44.68^{c}	39.32^{d}
3 months	55.32^{a}	47.83^{bc}	44.83^{c}
(d) pH 8.6			
Fresh	27.47^{f}	29.65^{f}	30.30^{f}
1 month	46.46^{cd}	41.67^{e}	43.75^{de}
2 months	50.47^{b}	50.00^{b}	48.39^{bc}
3 months	56.12^{a}	52.16^{b}	51.61^{b}

A: WPC 1 Containing 9.50% TS
B: WPC 2 Containing 4.75% TS
C: WPC 3 Containing 3.20% TS

Means with different superscript in the same row are significant a, b, c, and d (P<0.05); Means with different superscript in the same column are significant e, f, g and h (P<0.05).

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Table (3) : Denaturation % of heated thawed WPCs solutions at 80°C/10 min as affected by frozen storage and pHs.

	A	B	C
(a) pH 3.0			
Fresh	71.43^{e}	74.27^{cd}	70.00^{e}
1 month	74.44^{cd}	72.50^{de}	75.84^{c}
2 months	79.07^{b}	81.08^{b}	79.29^{b}
3 months	85.19^{a}	84.20^{a}	85.17^{a}
(b) pH 5.0			
	A	B	C

Fresh	73.09^{fg}	70.00^{h}	71.19^{gh}
1 month	76.78^{de}	74.44^{ef}	77.78^{d}
2 months	79.31^{cd}	82.50^{ab}	80.75^{bc}
3 months	83.33^{ab}	85.37^{a}	84.62^{a}
(c) pH 7.0			
	A	B	C
Fresh	73.61^{d}	71.41^{e}	70.37^{e}
1 month	75.29^{c}	73.67^{d}	73.05^{d}
2 months	80.69^{b}	80.55^{b}	79.98^{b}
3 months	84.71^{a}	83.77^{a}	83.98^{a}
(d) pH 8.6			
	A	B	C
Fresh	72.73^{cd}	70.57^{d}	73.33^{cd}
1 month	75.19^{c}	73.01^{cd}	79.29^{b}
2 months	79.78^{b}	79.99^{b}	82.10^{b}
3 months	85.71^{a}	85.76^{a}	85.71^{a}

A: WPC 1 Containing 9.50% TS
B: WPC 2 Containing 4.75% TS
C: WPC 3 Containing 3.20% TS

By varying the pH prior to heat treatment the mechanism of denaturation is influenced. The degree of denaturation is rather constant (Law & Leaver, 2000), but heat treatment at higher pH results in the formation of more whey protein aggregates while heat treatment at lower pH results in more association of the whey proteins with the casein micelle (Anema & Klostermeyer, 1997; Corredig & Dalgleish, 1996).

However, heating does have a definite effect on the whey protein fractions, which consists mainly of β-lactoglobulin (β-lg) and α-lactalbumin (α-la). Upon heating, a reactive thiol group is exposed in β-lg due to conformational changes of the molecule. This reactive thiol group can form disulfide links with other proteins having a reactive thiol group or through thiol group-disulfide bridge exchange reactions. The reaction makes the denaturation process irreversible, in contrast to the reversible denaturation of porcine β -lg which lacks free thiol groups (Burova, et al., 2002; Ugolini *et al.,* 2001).

Renaturation

Table 4 showed that the ability of denatured heated whey proteins to relation its nature after keeping in refrigeration temperature for 24 hrs. The results showed that slight decrease in the ratio of denatured proteins.

The process of denaturation and subsequent aggregation of bovine β -lg resembles a polymerisation process, in which the unfolding represents the initiation step (de Kruif et al., 1995; Roefs & de Kruif, 1994). α-La cannot initiate the polymerization process due to the absence of a free thiol group; however as it has four disulfide bridges it is irreversibly denatured in the presence of β-lg due to thiol group-disulfide bridge exchange reactions (Mulvihill & Donovan, 1987).

According to de Wit (1989), the solubility of whey protein is impaired by heating above 70°C when the pH is 4.0-6.5 due to irreversible unfolding, while at pH 6.8 or 7.0, the increase in aggregation and visible turbidity does not result in the formation of sedimentable particles (Britten *et al.,* 1993). For pH 5.0 samples, the precipitate was in the form of loose aggregates of denatured protein particles; indicating the lack of any major impairment of the structural network formed after heating by the preceding freezing step.

Table (4): Renaturation of Denatured heated WPCs solutions at 80°C/10 min after 24 hrs keeping in refrigerator 4-6°C.

(a) pH 3.0			
	A	B	C
Fresh	69.45^{de}	68.61^{e}	67.83^{e}
1 month	73.04^{c}	70.25^{de}	72.00^{cd}
2 months	78.31^{b}	79.40^{b}	76.90^{b}
3 months	83.12^{a}	82.84^{a}	82.59^{a}
(b) pH 5.0			
	A	B	C
Fresh	71.62^{de}	67.57^{ef}	66.67^{f}
1 month	75.28^{cd}	71.82^{de}	73.91^{cd}
2 months	77.65^{bc}	78.42^{bc}	78.26^{bc}
3 months	81.48^{ab}	83.78^{a}	81.79^{ab}
(b) pH 7.0			
	A	B	C
Fresh	71.83^{ef}	67.63^{g}	67.97^{g}
1 month	73.52^{de}	71.41^{ef}	69.53^{fg}
2 months	78.16^{bc}	76.47^{bcd}	76.00^{cd}
3 months	81.71^{a}	79.46^{ab}	79.17^{abc}
(c) pH 8.6			
	A	B	C
Fresh	70.31^{e}	69.70^{e}	70.37^{e}
1 month	72.22^{de}	70.59^{e}	74.97^{cd}
2 months	78.42^{b}	78.18^{b}	77.78^{bc}
3 months	82.93^{a}	83.86^{a}	81.46^{a}

A: Containing 9.50% TS
B: Containing 4.75% TS
C: Containing 3.20% TS

Means with different superscript in the same row are significant a, b, c and d ($P<0.05$); Means with different superscript in the same column are significant e, f, g and h ($P<0.05$).

Viscosity

Apparent viscosity of WPC samples at different pHs showed different patterns. For all the fresh WPC solutions at different pHs, the apparent viscosities were increased with increasing shear rate.

Also, the apparent viscosities of WPC solutions were increased with increase in the whey protein content. As shown in Fig. 1, at pH values above 5.0 all apparent viscosities were low with little variation, but below this pH at pH 3.0 values increases in apparent viscosity occurred, accompanied by the development of 'greasy' textures (Rattray & Jelen 1995). At shear rate of 185 1/s the apparent viscosities of WPC 1 sample of 4.8, 2.9, 3.6 and 3.2 mPas at pH 3.0, 5.0, 7.0 and 8.6 respectively (Fig 1). On the other hand at decreasing total solids content as sample WPC 3 no differences were records at different pHs uses. At pH 5.0 and 7.0 the highest apparent viscosity was recorded and its increased to 56 and 34.4 mPas for heated WPC 1at shear rate of 185 1/s (Fig. 2). However, at pH 5.0 all WPC solutions were found to be thixotropic fluids as there was a decrease in the apparent viscosity with increasing shear rate. These results were agreement with finding of Howard, (1991).

After one month of frozen storage the apparent viscosity of WPC solution increased at all pHs compared to fresh WPC solutions (Fig. 3). For WPC1, at pH 5.0 the viscosity of 13.3 mPas decreased to 11, 8.4 and 6.6 mPas at pH 3.0, 7.0 and 8.6 respectively at shear rate of 37 1/s. Also apparent viscosities were decreased with decrease of protein content of WPC solutions. Viscosities of heated thawed WPC solutions were illustrated in Fig. 4. The results showed significant differences in the viscosities of different pHs heated WPC solutions stored frozen as compared to the unfrozen control samples. Apparent viscosities were increased significantly of 308, 264 and 269 mPas for WPC 1 at pH 5.0, 7.0 and 8.6 respectively at shear rate of 37 1/s. On the contrary, at pH 3.0 the WPC solutions were observed significantly decreased in apparent viscosity as compared to other pHs. The increase in the viscosity of WPC coincide with the degree of denaturation, Kresic *et al.* (2008) and Meza, *et al.,* (2009) which was confirmed in the present study. The impact of protein denaturation on the development of high apparent viscosities is noticeable. Conceivably, heat treatments of WPC solutions led to some protein denaturation, rendering the proteins more pronounced to pH. (Rattray & Jelen 1995). Fig. 5 and 7 showed the apparent viscosity of WPC solutions after two and three months of frozen storage. Apparent viscosity was increased with increase shear rate. No significant differences in viscosity values were recorded between WPC solutions after two or three months from freezing storage.

Apparent viscosities of heated WPC solutions after two and three months of freezing storage were illustrated in Fig. 6 and 8.

After two months of freezing storage significantly differences were obsereved in apparent viscosity of heated WPC solutions as affected by protein content and pHs (Fig. 6). Viscosities values of WPC1 (highest protein content) record of 8.2, 39.6, 62.4 and 107 mPas at pHs of 3.0, 5.0, 7.0 and 8.6 at shear rate of 185 1/s. These values decreased to 6.5, 8.0, 9.3 and 6.5 for WPC3 sample (lowest protein content) in the same order. The presence of large number of high molecular weight aggregates increase the resistance to flow which, in turn, increases the apparent viscosity (Rattray and Jelen, 1995).

On the other hand, further freezing storage to three months led to increase in the viscosity values of 13.8, 18.7 and 18.5 mPas for heated WPC2 and of 7.0, 10.1 and 8.3 mPas for heated WPC3 at pHs of 5.0, 7.0 and 8.6 respectively (Fig. 8). On contrary, apparent viscosity of heated WPC1 was decreased to 49.0, 64.0 and 56.0 mPas compared to that finding in two months of freezing storage.

From the summarized over all viscosities results showed that the apparent viscosities of unheated WPC solutions exhibited a time-independent non-Newtonian character what should be considered as shear-thickening (dilatants) properties. In this type the increase in shear rate results in an increase in apparent viscosity (Kresic et aL. 2008). On the other hand, heated thawed WPC solutions were found to be thixotropic fluids as there was a decrease in the apparent viscosity with increasing shear rate. Generally viscosities results showed that the apparent viscosities of unheated and heated WPC solutions greatly affected by protein content and pH degree. The investigation carried out on the WPC of different protein concentration (Lelas and Herceg, 2002), revealed that the intensity of mentioned increase of water binding properties is proportional to protein concentration.

4. Conclusion:

From the obtained results it can be concluded that frozen storage increased the denaturation degree and viscosity of WPC. However, the protein content and pH play a role in the observed changes in the denaturation and viscosity of stored WPC.

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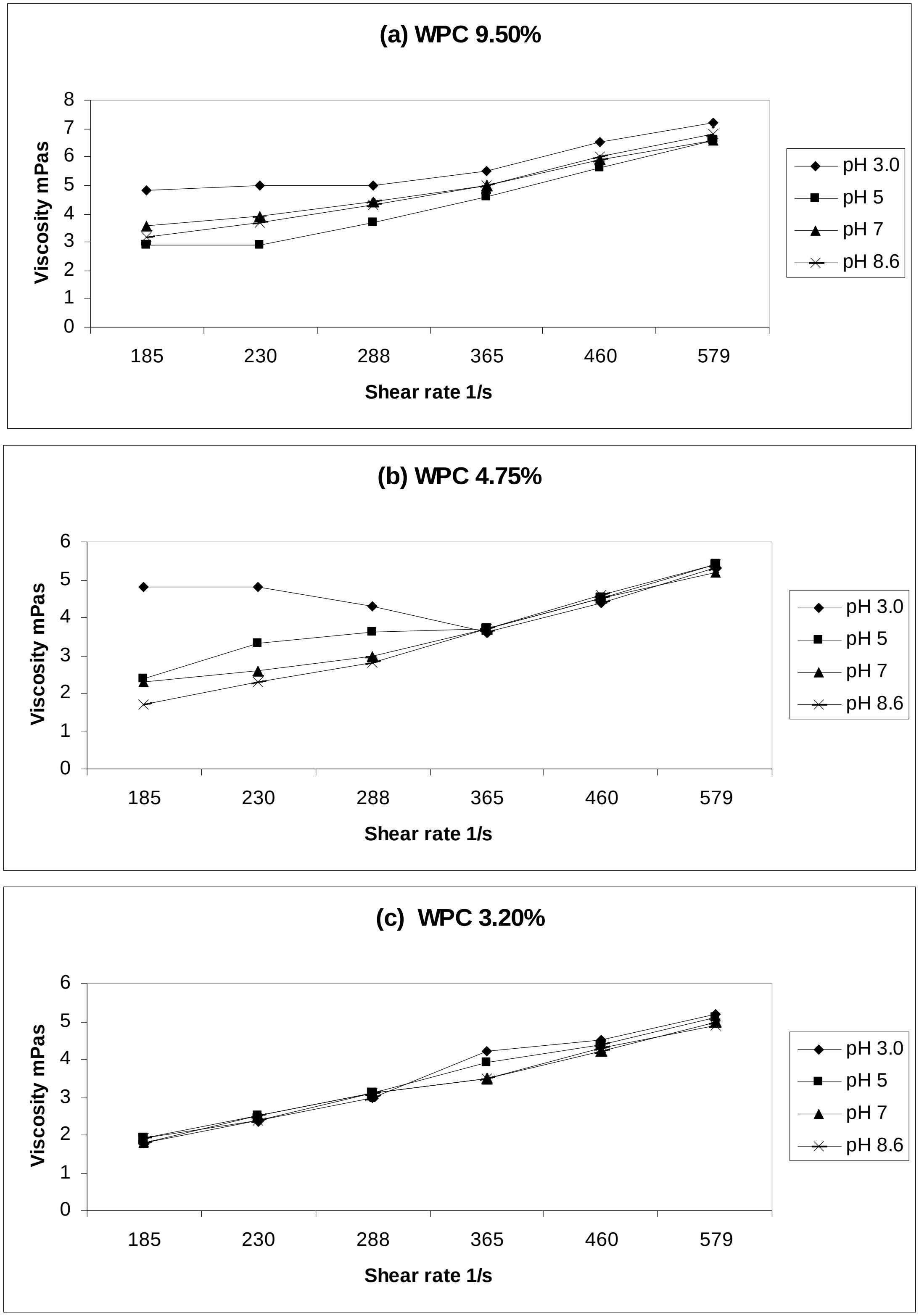


Fig. (1): Viscosity of fresh WPC solutions at different concentration before freezing storage at different pHs

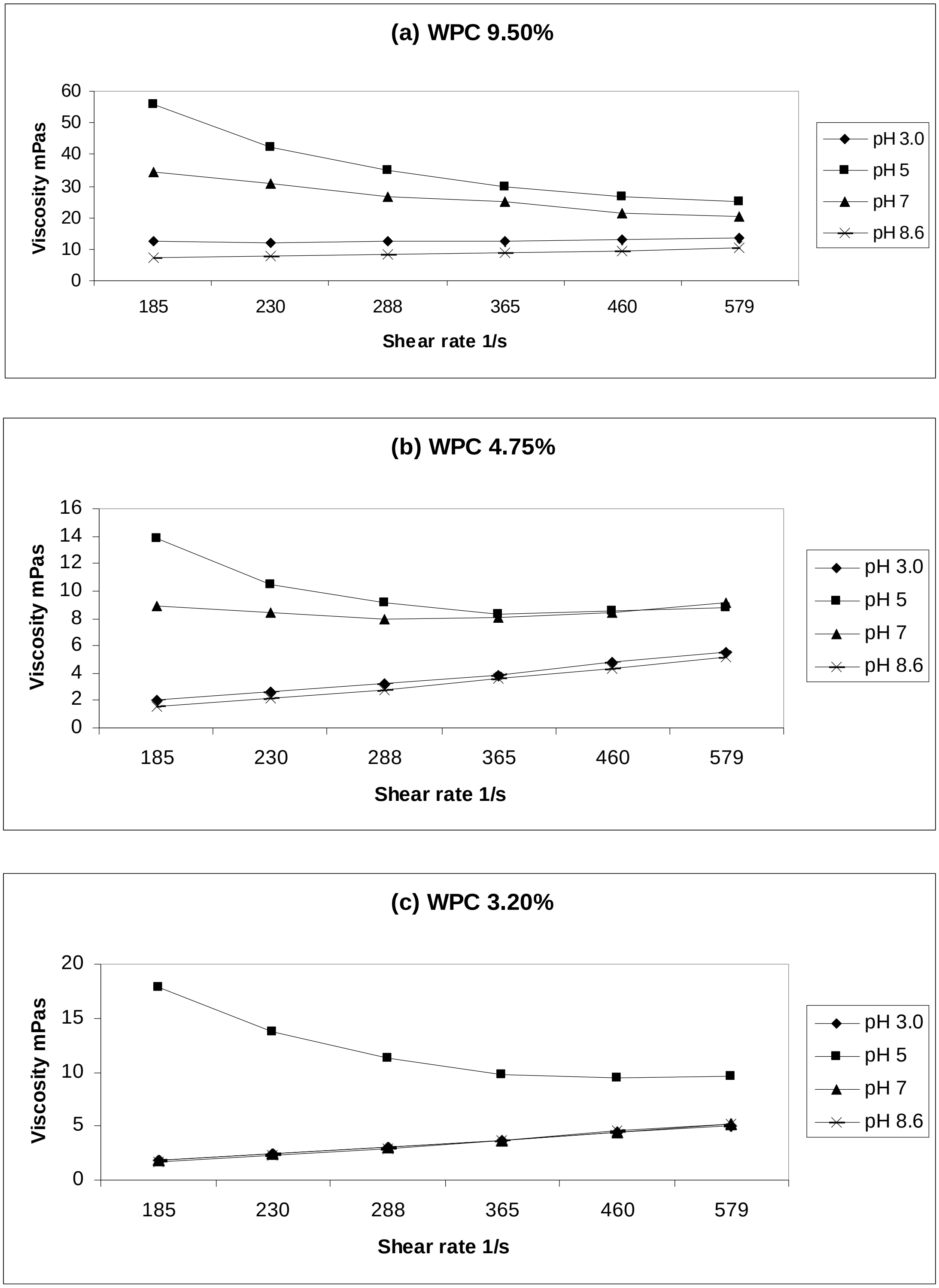


Fig. (2): Viscosity of heated fresh WPC solutions at different concentration before freezing storage at different pHs

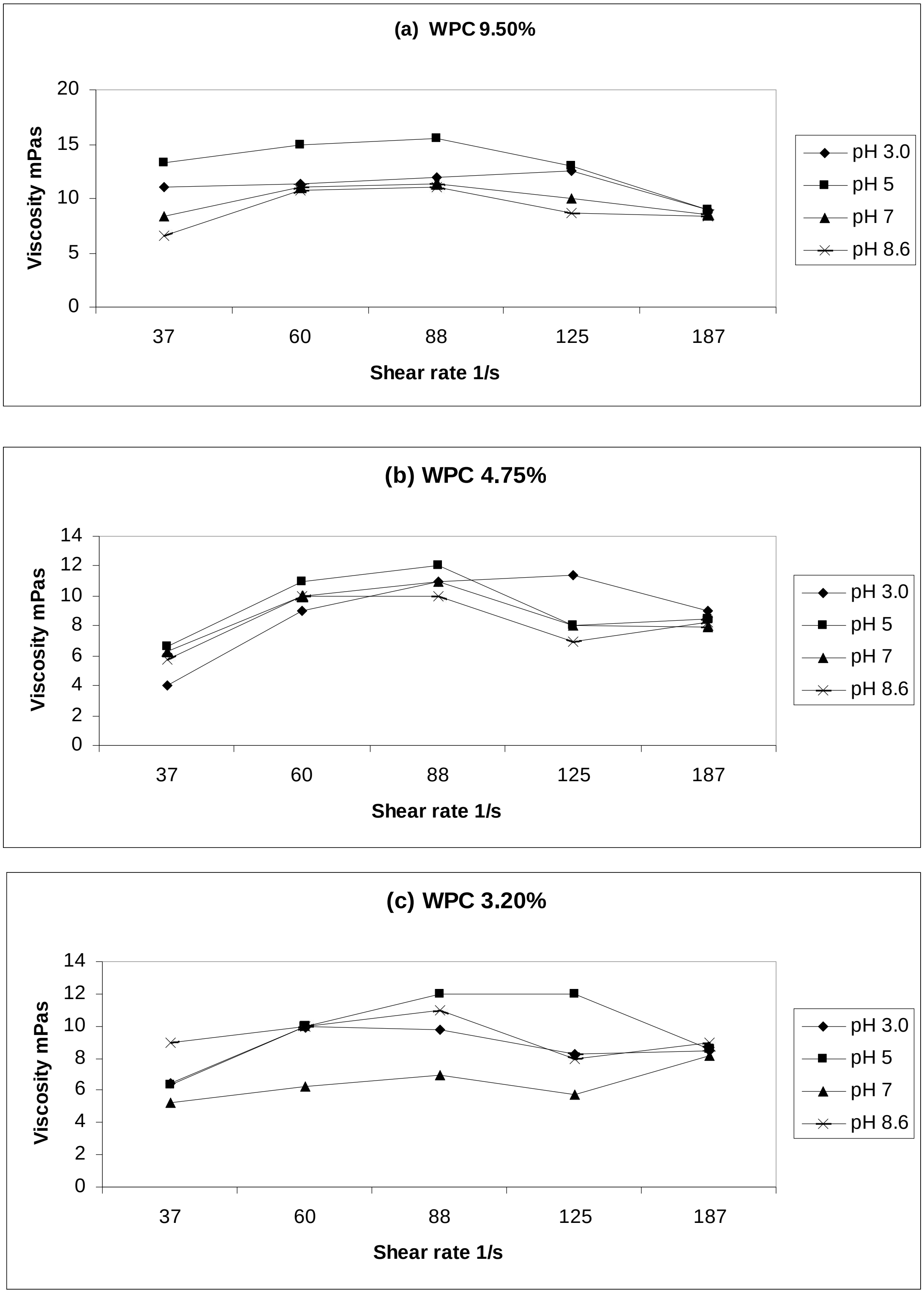


Fig. (3): Viscosity of WPC solutions at different concentration after one month freezing storage

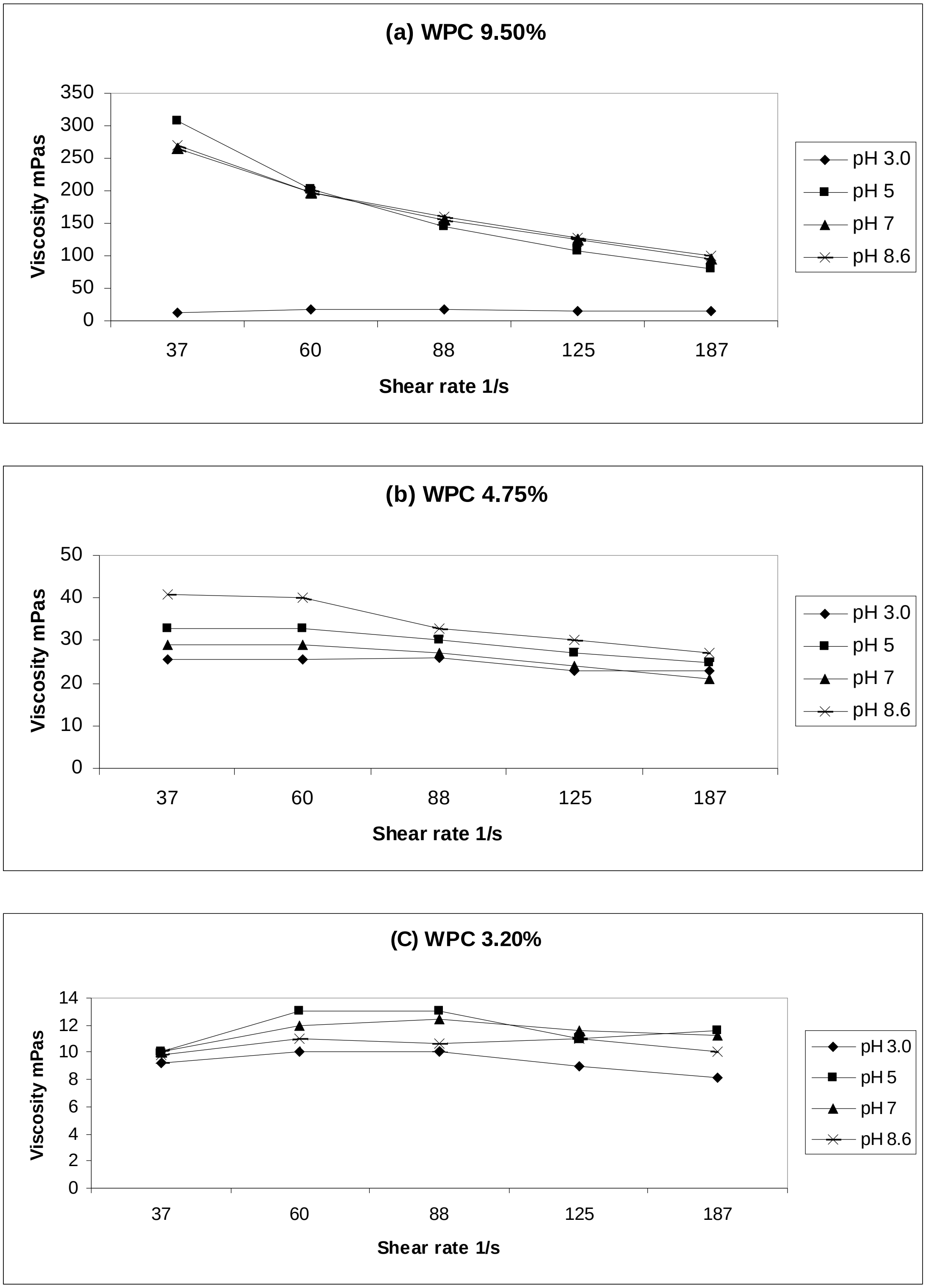


Fig. (4): Viscosity of heated WPC solutions at different concentration after one months freezing storage

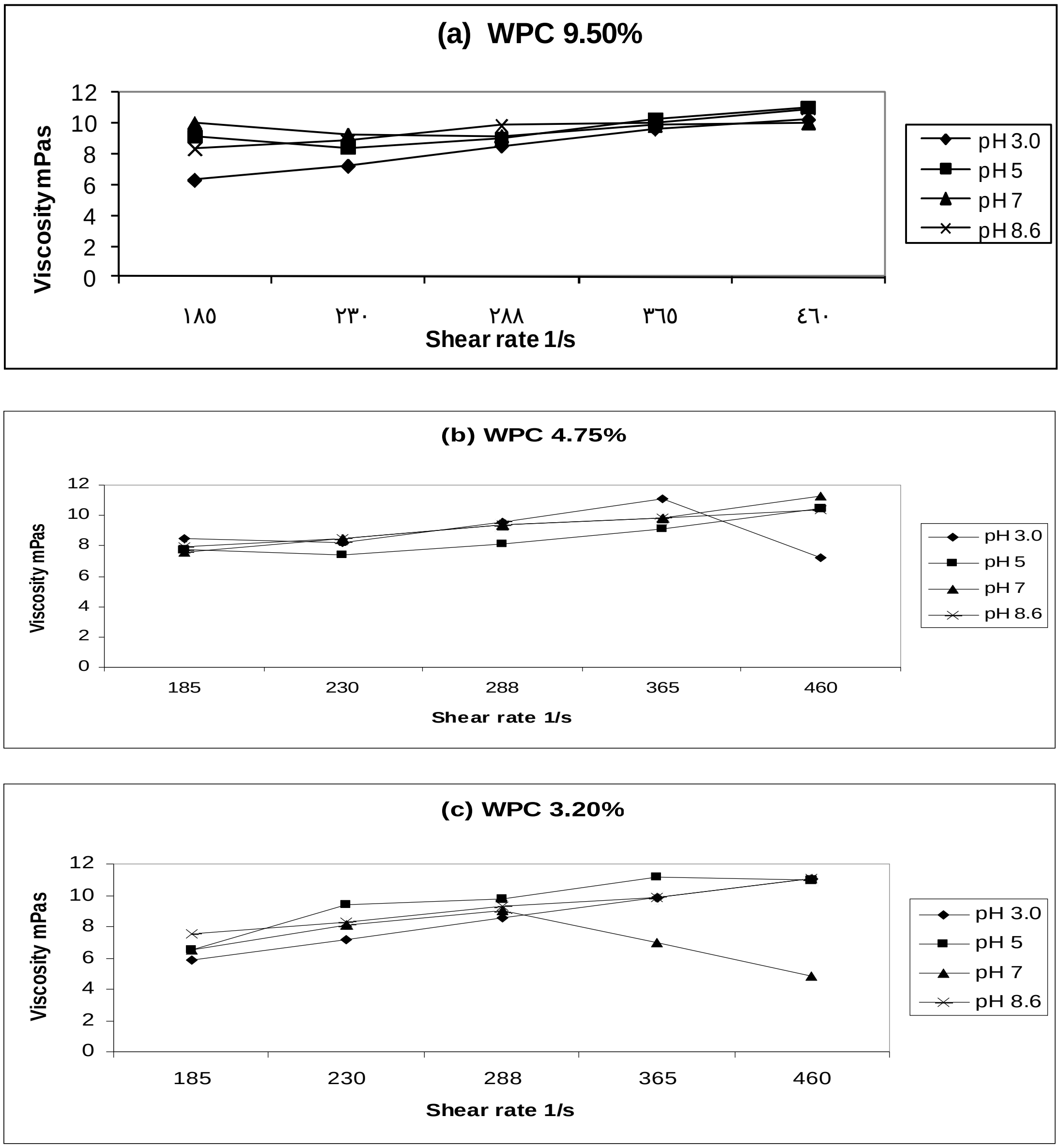


Fig. (5): Viscosity of WPC solutions at different concentration after two months freezing at different pHs

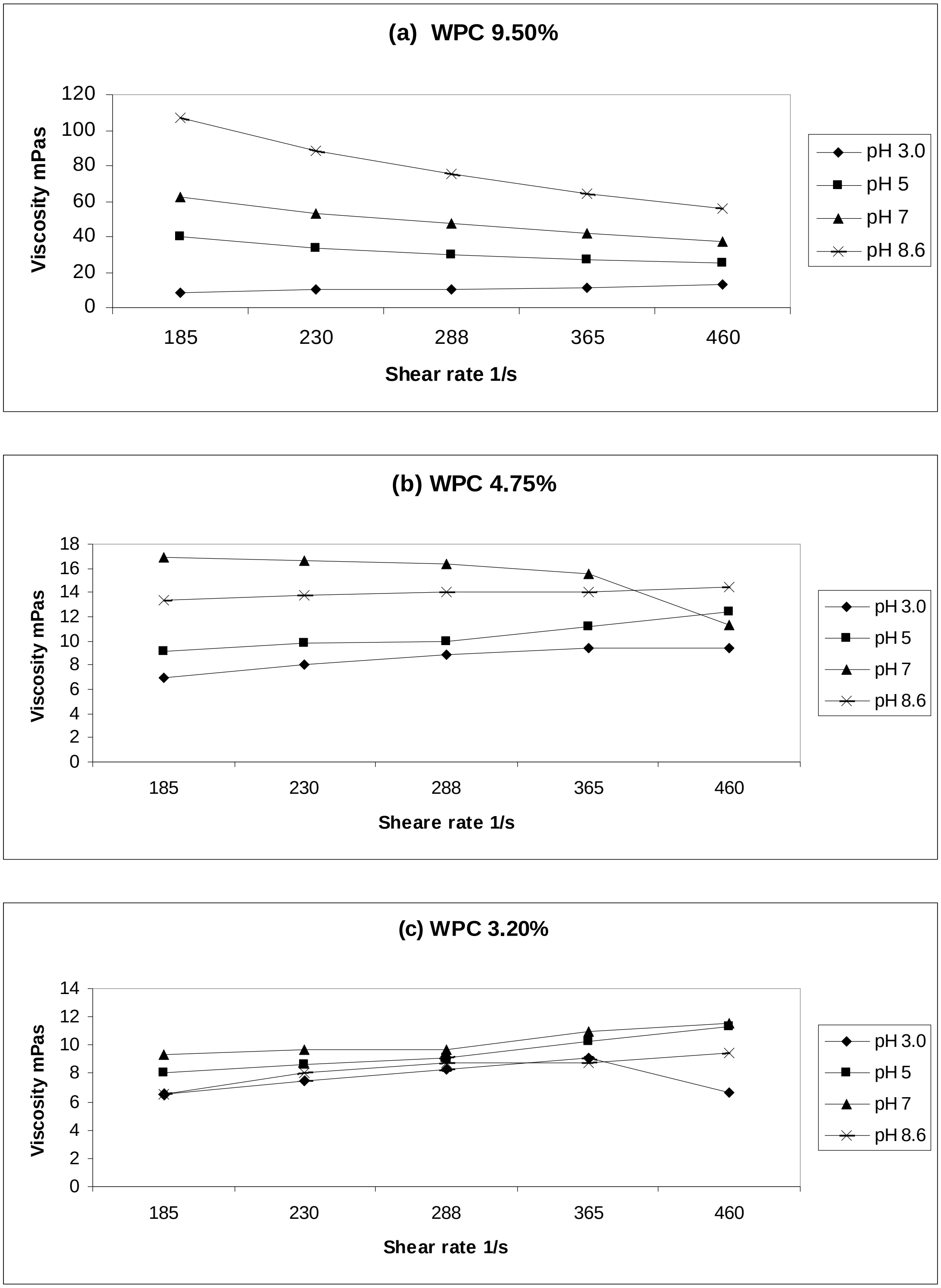


Fig. (6): Viscosity of heated WPC solutions at different concentration after two months freezing at different pHs

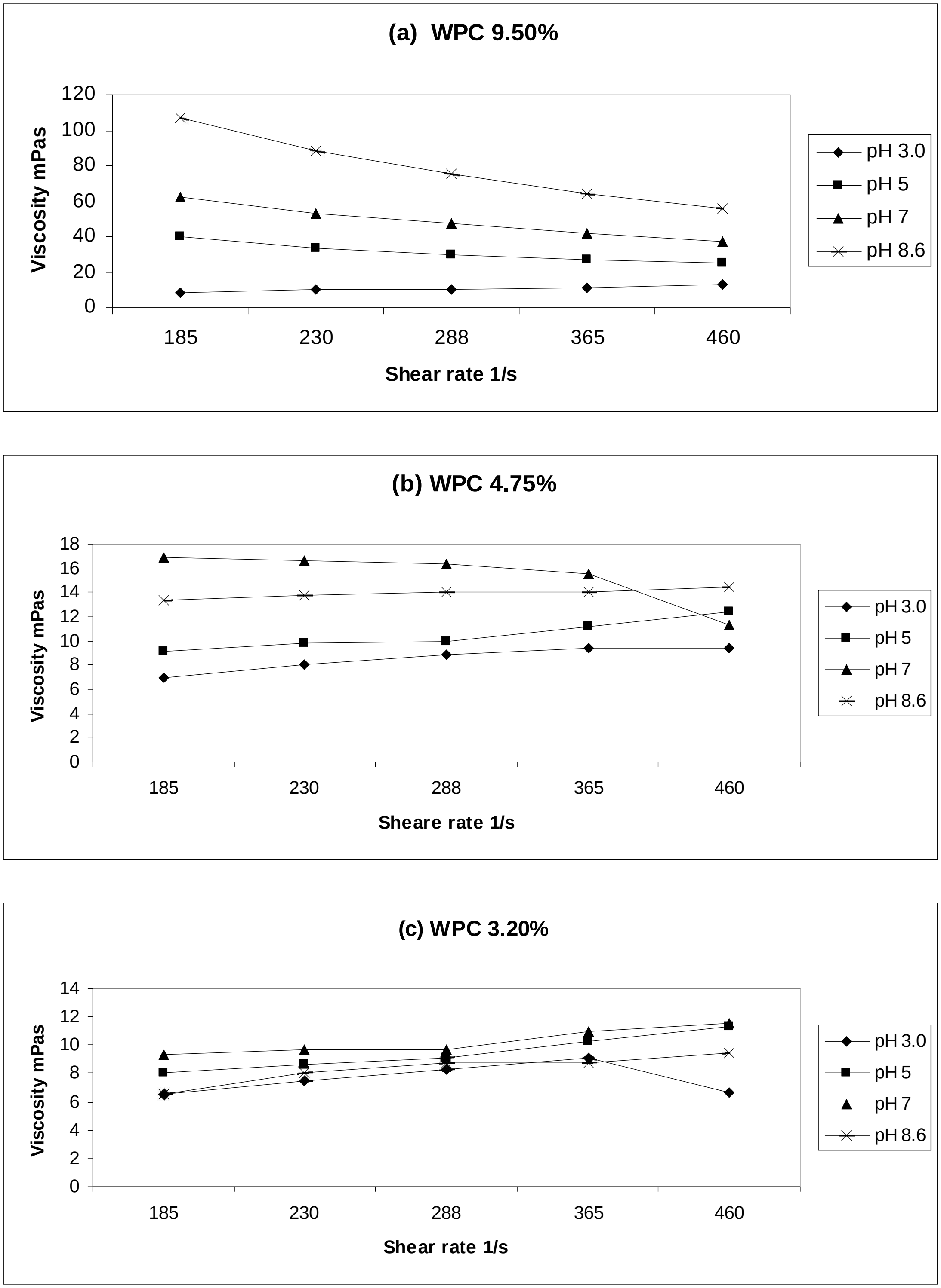
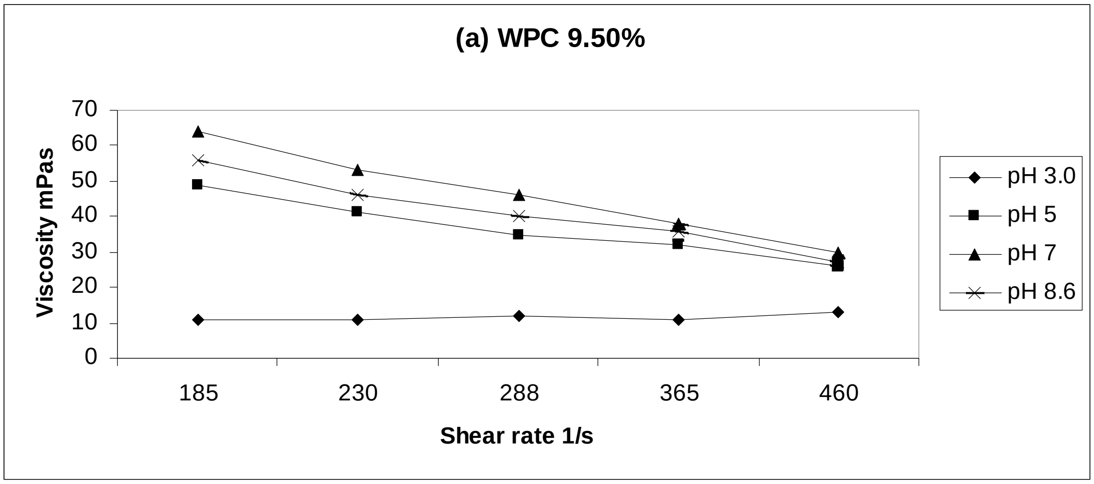
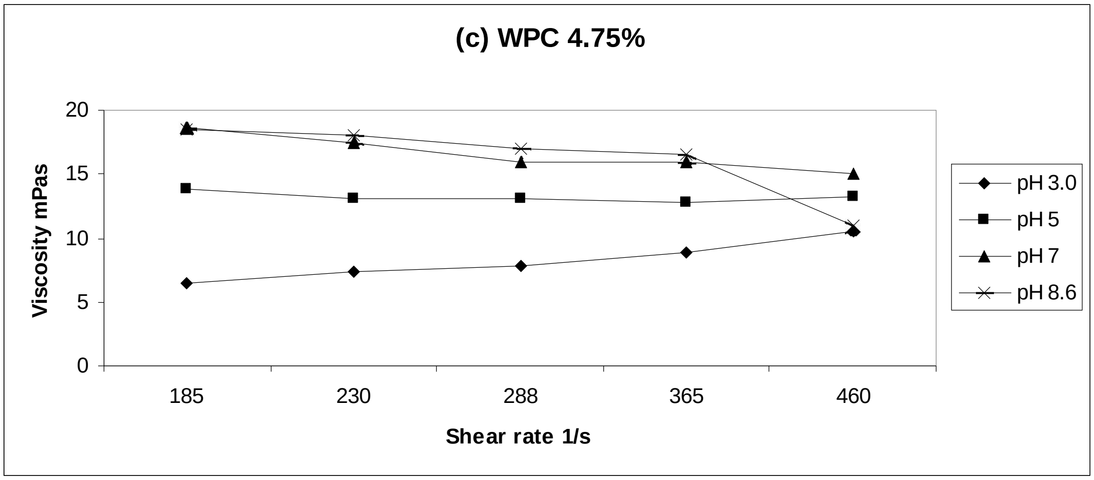


Fig. (7): Viscosity of WPC solutions at different concentration after three months freezing at different pHs





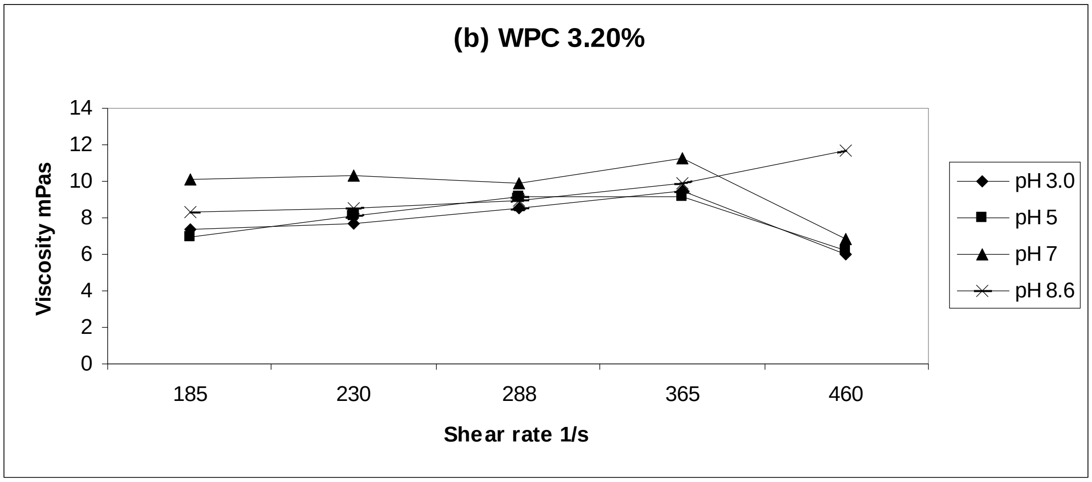


Fig. (8): Viscosity of heated WPC solutions at different concentration after three months freezing at different pHs

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