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Effect of freezing on the viscoelastic behaviour of whey protein concentrate suspensions

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ABSTRACT

The effect of freezing on viscoelastic behaviour of whey protein concentrate (WPC) suspensions was studied. Suspensions with total protein content of 5% and 9% w/v were prepared from a commercial WPC (unheated suspensions). A group of unheated suspensions was treated at two temperatures (72.5 and 77.5 °C) during selected times to obtain 60% of soluble protein aggregates (heat-treated suspensions). Unheated suspensions and heat-treated suspensions were frozen at −25 °C (frozen unheated and frozen heat-treated suspensions). Frequency sweeps (0.01–10 Hz) were performed in the region of linear viscoelasticity at 10, 20, 30, 40, and 50 °C. Mechanical spectra of all studied suspensions at 20 °C were similar to viscoelastic fluids and complex viscosity increased with the frequency (ω). Elastic (G') and viscous (G'') moduli were modelled using power law equations ($G' = a\omega^x$, $G'' = b\omega^y$), using fitted parameters a, x, b, and y for statistical analysis. Exponent y was the most influenced by freezing, indicating the existence of a higher degree of arrangement in frozen unheated suspensions and a lower degree of arrangement in frozen heat-treated suspensions. Only characteristic relaxation times (inverse of the crossover frequency) of suspensions with 5% w/v of total protein content were significantly influenced by freezing. Time–temperature superposition was satisfactory applied in unheated whey protein concentrate suspensions only in the range of high temperatures (30–50 °C). However, this principle failed over the complete temperature range in most of the frozen suspensions. It is possible that freezing produced an increase in the susceptibility to morphological changes with temperature during the rheological measurements.

1. Introduction

Whey proteins are used as ingredients in food industry and they are worldwide commercialized as isolates (WPI) and concentrates (WPC). Whey protein concentrates contain between 20% and 89% of protein, being WPC with 35% of protein content the most common product (Tunick, 2008). Whey proteins have functional properties like solubility, emulsification, gelation, and foaming capacity (Ennis & Mulvihill, 2000; de Wit, Hontelez-Backx, & Adamse, 1988). In addition, whey proteins have the advantages of high nutritive value and are generally recognized as safe status (Bryant & McClements, 1998).

Sometimes, heat treatments are used to improve whey protein functionality. When whey protein suspensions are heated over the denaturation temperature, the unfolding of the tertiary structure of the protein molecule (especially α-lactalbumin and β-lactoglobulin) is produced. This phenomenon allows both the exhibition of hydrophobic sites and the sulfhydryl/disulfide interchange reactions that induce the formation of aggregates (Anema, 2009). During heat treatment at high protein concentrations (over 10% or 12%), depending on pH, ionic composition, and temperature, a gel may be formed (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999). Nevertheless, at low protein concentration aggregates can remain in solution.

Heat-treated whey protein suspensions containing soluble protein aggregates can be used as ingredient in food processing due to their specific functional properties. Vardhanabhuti and Foegeding (1999) found that suspensions containing soluble whey proteins aggregates, obtained by heating whey protein isolate suspensions at 80 °C, exhibited high viscosity and had flow behaviours similar to those of hydrocolloids. Also, heat-treated whey protein suspensions can form gels at low temperatures by

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addition of NaCl or $CaCl_2$ (Bryant & McClements, 2000) and by pH lowering (Abd El-Salam & El-Etriby, 1996).

Rheological properties are useful in process engineering calculations, equipment design and optimization. In addition, they can be used for product quality control and sensory assessment of foods (Rao, 1999). Viscoelastic behaviour of whey protein suspensions have been investigated by several research groups (Ikeda & Nishinari, 2001a; Ikeda, Nishinari, & Foegeding, 2001; Lizarraga, De Piante Vicin, Gonzalez, Rubiolo, & Santiago, 2006). Also, the viscoelastic behaviour of heat-treated whey protein concentrate suspensions containing soluble protein aggregates was analysed (Meza, Verdini, & Rubiolo, 2009).

Williams, Landel, and Ferry (1955) developed the time–temperature superposition principle (WLF principle) that equates the effect of time and temperature on rheological properties. This technique, which is sometimes applicable to synthetic polymers and biological materials, has not been widely applied to foods (Steffe, 1996). Isothermal linear viscoelastic data, obtained from frequency sweeps at several different temperatures, are shifted along the frequency axis and overlaid to obtain a single master curve at the reference temperature chosen conveniently (Ferry, 1980). Time–temperature superposition principle was used to investigate the viscoelastic behaviour of bovine serum albumin suspensions (Ikeda & Nishinari, 2000), but it has not been applied in whey protein concentrate suspensions.

Freezing is widely used for food preservation. However, the formation and growth of ice crystals can produce modifications in physicochemical properties of foods like pH, ionic strength, and solute concentration. Freeze-concentration in the unfrozen fraction of the aqueous phase produces changes in the product, including textural modifications, protein denaturation, and cell membrane destruction (Walstra, 2003). The possibility of using freezing to preserve concentrated whey protein suspensions and the effect of freezing on some of their functional properties was investigated by Bhargava and Jelen (1995). However, very little attention has been paid into the use of freezing as a method to preserve heat-treated whey protein suspensions and only few studies about freeze-texturisation of heat-treated whey protein suspensions were found in literature (Lawrence, Consolacion, & Jelen, 1986). Whey protein suspensions can be used as ingredients in foods that can be frozen, like desserts and cheeses. For this reason, it is interesting to study the effects of freezing on the rheological properties of whey protein suspensions.

In this study, the effect of freezing on the viscoelastic behaviour of unheated and heat-treated whey protein suspensions prepared from a commercial WPC was analysed performing frequency sweeps in the region of linear viscoelasticity.

2. Materials and methods

2.1. Whey protein concentrate

Commercial whey protein concentrate (WPC) obtained from sweet cheese whey provided by a local industry was used (Milkaut S.A., Frank, Santa Fe, Argentina).

2.2. Physicochemical analysis of the WPC

The initial composition of the WPC was determined in laboratory with standardized techniques. The total protein content was considered from total nitrogen content determined by the Kjeldahl method, using a Büchi 430 automatic digestor (Büchi, Flawil, Switzerland), a Büchi 322 distillation unit, and a Mettler DL40RC automatic titrator (Mettler Instrumente AG, Greifensee, Switzerland). Ash content was determined after an overnight incineration in a muffle furnace at 540 °C. Moisture content was measured with a CEM AVC 80 microwave (CEM, Mattheus, NC). Fat content was determined using the Standard International Dairy Federation method (IDF, 1969). Lactose content was defined as the difference between the mass of the sample and the amount of protein, ash, moisture and fat. All compositional analyses were determined in triplicate. The initial composition of the WPC was: lactose 48.8%, protein 38.3%, ash 7.5%, moisture 3.2%, and fat 2.2%.

2.3. Whey protein concentrate suspensions

2.3.1. Unheated suspensions

Whey protein concentrate suspensions at natural pH were prepared to reach 5% and 9% w/v of total protein content. An appropriate amount of WPC was weighed and dissolved in distilled water with vigorous agitation. All suspensions were prepared in triplicate and stored at 5 °C overnight (unheated suspensions). The denatured protein (DP) content of unheated suspensions was 52.8 ± 1.5% and 47.0 ± 1.1% for suspensions with 5% and 9% w/v of total protein content, respectively. The codes of samples are shown in Table 1.

2.3.2. Heat-treated suspensions

Glass tubes (160 by 16 mm) containing 10 mL of unheated suspensions were heat-treated at 72.5 and 77.5 °C for different times to produce 60% of soluble protein aggregates expressed as percentage of DP content, using the previously published procedure (Meza et al., 2009). According to this technique, the protein that remains soluble at pH 4.6 (approximately 40%) is the protein that underwent neither denaturation nor aggregation (Li-Chan, 1983; Verheul, Roefs, & de Kruif, 1998; de Wit, 1990).

All suspensions were prepared in triplicate and stored at 5 °C overnight (heat-treated suspensions). Codes of samples are shown in Table 1.

2.4. Freezing

A group of both unheated and heat-treated suspensions was frozen in the freezer (−25 °C) for 24 h and subsequently thawed at 20 °C. These samples were called frozen unheated suspensions and frozen heat-treated suspensions, respectively. The DP content of frozen unheated suspensions was 52.0 ± 0.6% and 46.4 ± 1.1% for suspensions with 5% and 9% w/v of total protein content, respectively. All suspensions were prepared and stored at each study condition in triplicate. Codes of samples are shown in Table 1.

Table 1
Codes of samples of whey protein concentrate suspensions.

	Unfrozen samples	Frozen samples	Total protein content (% w/v)	Heat-treatment conditions (time and temperature)
Unheated suspensions	5-unheated	5-unheated-F	5	–
	9-unheated	9-unheated-F	9	–
Heat-treated suspensions	5-72.5	5-72.5-F	5	72.5 °C – 15 min
	9-72.5	9-72.5-F	9	72.5 °C – 40 min
	5-77.5	5-77.5-F	5	77.5 °C – 6 min
	9-77.5	9-77.5-F	9	77.5 °C – 12 min

2.5. Rheological analysis

2.5.1. Frequency sweeps

Frequency sweeps were performed in the range of 0.01–10 Hz at 10, 20, 30, 40, and 50 °C using a stress controlled rheometer RheoStress 80 (Haake Inc. Instruments, Karlsruhe, Germany) with a cone and plate geometry test fixture. Diameter and angle of the cone were 60 mm and 0.04 rad, respectively. A thin film of silicone oil was applied to the exposed sample edges to prevent water vaporization during measurements.

The region of linear viscoelasticity was determined prior to each frequency sweep performing stress sweeps in the range of 1.8–9 Pa at 10 Hz to verify the linear relationship between stress and strain. Elastic modulus (G'), viscous modulus (G''), complex viscosity ($|\eta^*|$), and phase angle (δ) were determined at an average stress amplitude of 2 Pa. One frequency sweep was performed for each suspension at each temperature (10, 20, 30, 40, and 50 °C) using an aliquot of the corresponding suspension.

2.5.2. Modelling of the mechanical spectra

Frequency (ω) dependence of G' and G'' at 20 °C was modelled with power law equations:

$$G' = a\omega^x \tag{1}$$

$$G'' = b\omega^y \tag{2}$$

Parameters a, b, x, and y were used for statistical analysis.

2.5.3. Determination of the characteristic relaxation times

Using data modelled with the power law equations (Eqs. (1) and (2)) the crossover frequency, where $G' = G''$, was determined. The crossover between G' and G'' is a useful criterion for comparing products or treatments (Steffe, 1996).

Characteristic relaxation times (τ_c) were calculated as the inverse of crossover frequencies (Kulmyrzaev & McClements, 2000; Wientjes, Duits, Jongschaap, & Mellema, 2000). Values of τ_c were used for statistical analysis.

2.5.4. Time–temperature superposition

Time–temperature superposition overlaps oscillatory isothermal frequency data of viscoelastic variables into a single master curve using a horizontal shift factor a_T. The superposition of G'' data at five temperatures (10, 20, 30, 40, and 50 °C) for each suspension was obtained by calculating:

$$G''_p = G''b_T = G''\left(\frac{T_0\rho_0}{T\rho}\right) \tag{3}$$

where G''_p is the viscous modulus corrected by the vertical shift factor b_T, G'' is the viscous modulus and ρ is the density of the material at the absolute temperature T. Also, ρ_0 is the density of the same material at the absolute reference temperature T_0 that was chosen conveniently (40 °C). The thermal density ratio (ρ_0/ρ) was considered negligible.

The shift factor a_T relates the ratio of viscoelastic variables (for example G' or G'') at a given temperature (T) to the same variable at an arbitrarily selected reference temperature (T_0) (Ferry, 1980). Taking into account the Eq. (3), horizontal shift factor a_T can be expressed as the following expression:

$$a_T \frac{G''_p|_T}{G''_p|_{T_0}} = \frac{G''}{G''_0}\left(\frac{T_0}{T}\right) \tag{4}$$

where G'' is the viscous modulus at the absolute temperature T and G_0'' is the viscous modulus at the absolute reference temperature T_0.

Plotting G''_p versus ω at different temperatures in logarithmical scale, the shift factor a_T corresponds to the horizontal distance between G''_p at temperature T to G''_p at temperature T_0. In this way, a_T can be expressed as:

$$a_T = \frac{\omega|_{T_0}}{\omega|_T} \tag{5}$$

where $\omega|_T$ is the frequency that corresponds to G''_p at temperature T and $\omega|_{T_0}$ is the frequency corresponding to G''_p at temperature T_0.

Values of shift factor a_T determined from Eq. (4) were different depending on frequency ($a_{T(1)}$, $a_{T(2),}\ldots$, $a_{T(n)}$). Therefore, plots of G''_p versus ωa_T were constructed using different values of a_T. The shift factor which superimposes the majority of the G''_p points in the range of studied frequencies was chosen using a numerical shift procedure proposed by Rosati, Van Loon, and Navard (2000). According to this procedure, the shift factor a_T corresponding to the lower value of the sum of the distances between two points was selected to superimpose values of G''_p.

In order for the WLF principle to be valid, the same shift factor a_T must superpose successfully all viscoelastic moduli. For this reason, the selected shift factors used to superimpose the values of G''_p were used to overlap the values of G'_p.

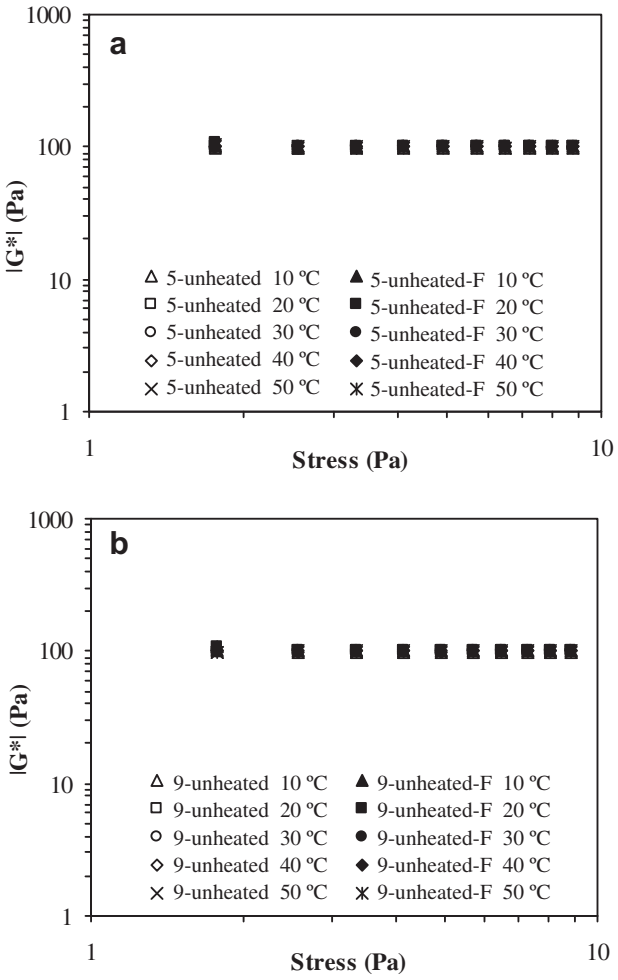

Fig. 1. Complex modulus ($|G^*|$) as function of stress. (a) Unheated suspensions and frozen unheated suspensions with 5% w/v of total protein content. (b) Unheated suspensions and frozen unheated suspensions with 9% w/v of total protein content. Points represent individual values of one of the three replications. See codes of samples in Table 1.

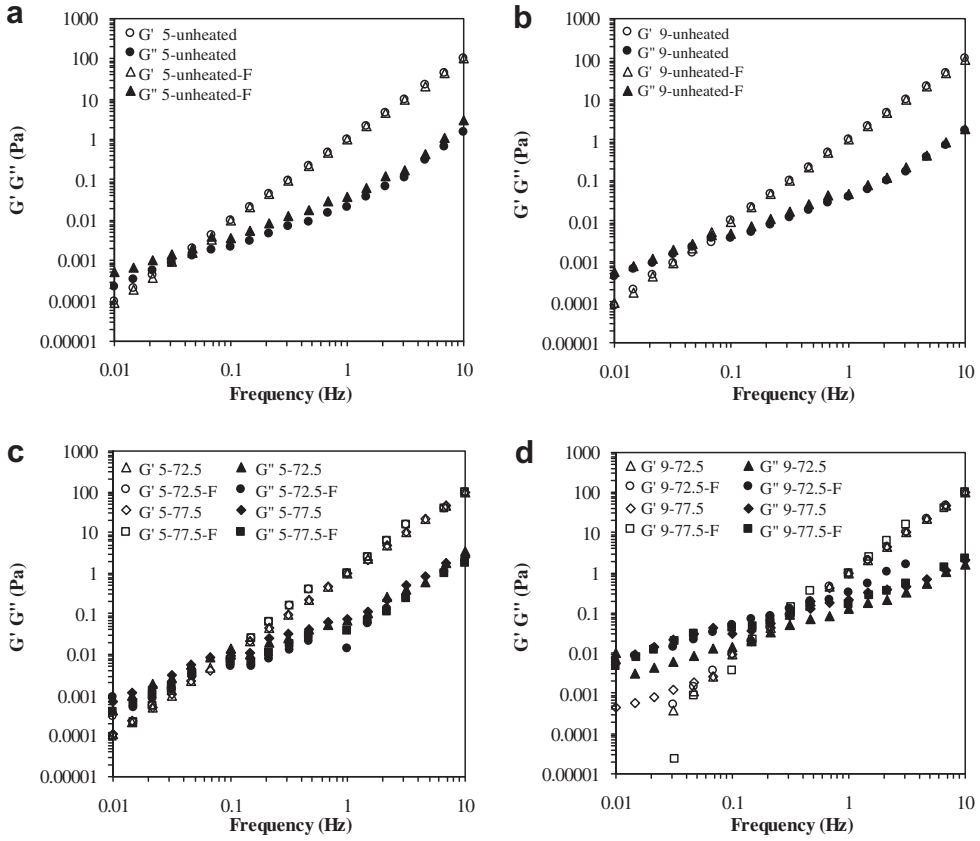

Fig. 2. Elastic (G') and viscous (G'') modulus at 20 °C. (a) Unheated and frozen unheated suspensions with 5% w/v of total protein content. (b) Unheated and frozen unheated suspensions with 9% w/v of total protein content. (c) Heat-treated suspensions and frozen heat-treated suspensions with 5% w/v of total protein content. (d) Heat-treated suspensions and frozen heat-treated suspensions with 9% w/v of total protein content. Points represent individual values of one of the three replications. See codes of samples in Table 1.

Satisfactory application of time–temperature superposition suggests that the material is thermorheologically simple, where relaxation times for all mechanisms change identically with temperature (Ferry, 1980).

2.6. Statistical analysis

Analysis of variance was used ($p < 0.05$) and when the effect of the factors was significant, the test of multiple ranks honestly significant difference (HSD) of Tukey was applied (95% of confidence level). The statistical analysis was performed using Minitab 13.20 (Minitab Inc., State College, PA).

3. Results and discussion

3.1. Region of linear viscoelasticity

Values of complex modulus ($|G^*|$) of unheated suspensions and frozen unheated suspensions were plotted as function of applied stress at the frequency of 10 Hz (Fig. 1). Complex modulus was not affected by the magnitude of the applied stress and a linear relationship between stress and strain was observed. Heat-treated suspensions and frozen heat-treated suspensions presented similar description as unheated suspensions. These results indicate that both unfrozen and frozen samples behaved as a linear viscoelastic material during dynamic tests at stresses below 9 Pa at 10, 20, 30, 40, and 50 °C.

3.2. Analysis of mechanical spectra

Values of elastic (G') and viscous (G'') modulus as function of frequency at 20 °C are shown in Fig. 2. At low frequencies a fluid-like behaviour was observed ($G'' > G'$), at the middle of the frequency range there was a crossover between G'' and G' curves, and at frequencies larger than the crossover frequency a solid-like behaviour was visualized ($G' > G''$). This description of the mechanical spectra is characteristic of viscoelastic fluids (Steffe, 1996).

A shear-thickening behaviour was observed, where $|\eta^*|$ increases with frequency, in unheated suspensions (Fig. 3) and in heat-treated suspensions. At high frequencies, the time scale of observation can be much higher than the relaxation time scale of the material (high Deborah number). This leads to a higher $|G^*|$ and therefore to a higher $|\eta^*|$. The structure of the whey protein concentrate suspensions may not be able to deform fast enough to follow the applied shearing flow at a higher frequency and behaviours as a fixed or ordered structure (solid-like and high viscosity) because the relaxation times of molecules become relatively longer compared to the observation time scale, which is shorter at a higher frequency.

Changes in the phase angle (δ) at 20 °C are shown in Fig. 4. At very low frequencies, δ was relatively high showing the dominant effect of the viscous component. The phase angle decreased with increasing frequency and reached a very low level at a high frequency, showing a dominant effect of the elastic component.

Unheated suspensions presented lower values of δ than heat-treated suspensions (Fig. 4), indicating that heat treatments

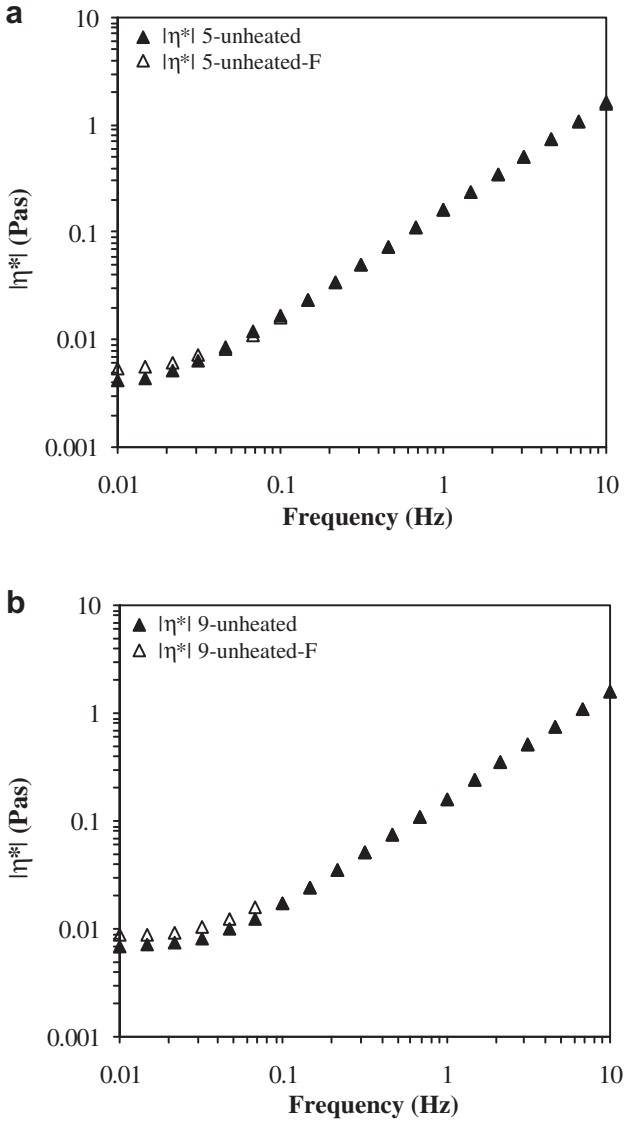

Fig. 3. Complex viscosity ($|\eta^*|$) at 20 °C. (a) Unheated and frozen unheated suspensions with 5% w/v of total protein content. (b) Unheated and frozen unheated suspensions with 9% w/v of total protein content. Points represent individual values of one of the three replications. See codes of samples in Table 1.

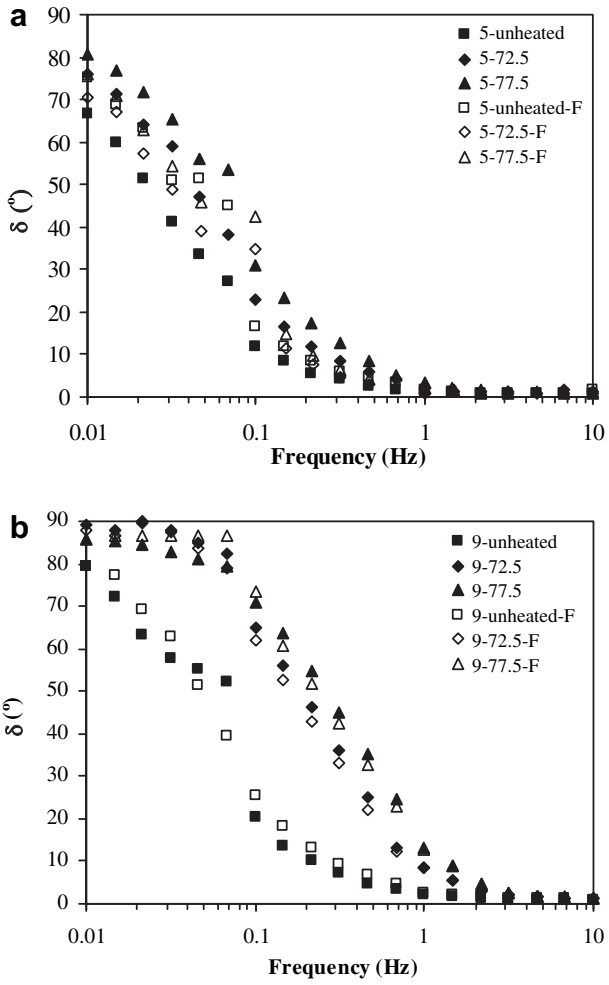

Fig. 4. Dependency of the phase angle (δ) with frequency at 20 °C. (a) Suspensions with 5% w/v of total protein content. (b) Suspensions with 9% w/v of total protein content. Points represent individual values of one of the three replications. See codes of samples in Table 1.

produced an increase in the extent of viscous behaviour. This result was more noticeable in suspensions with 9% w/v of total protein content where an almost purely viscous behaviour at very low frequencies, with δ equal or close to 90°, was observed in both 9-72.5 and 9-77.5 (Fig. 4b).

Freezing produced interesting changes in the values of δ, with more noticeable changes in suspensions with 5% w/v of total protein content (Fig. 4a). Frozen unheated suspensions presented higher values of δ in comparison to unheated suspensions, indicating that freezing produced an increase in the extent of viscous behaviour. However, frozen heat-treated suspensions showed lower values of δ than heat-treated suspensions (5-72.5-F < 5-72.5, 5-77.5-F < 5-77.5). These results indicate that freezing produced a decrease in the extent of viscous behaviour in heat-treated suspensions.

3.3. Modelling of the mechanical spectra

Power law parameters used to describe the frequency dependence of G' and G'' of whey protein concentrate suspensions are shown in Table 2. Parameter a was higher than b, indicating that G' at the frequency value of 1 Hz predominates in both unfrozen and frozen samples.

Exponent x obtained from the elastic modulus was always higher than y obtained from the viscous modulus, indicating that G' increases faster than G'' as frequency increased in both unfrozen and frozen samples. The magnitude of x is approximately 2 (Table 2), suggesting that elastic modulus increases proportionally to the square of the frequency. Taking into account that $|\eta^*| = (|G^*|/\omega)$ and $|G^*| = [(G'')^2+(G')^2]^{1/2}$, the shear-thickening behaviour observed in all suspensions may be related to the faster increasing rate of G' with frequency (Rao & Tattiyakul, 1999).

The power law parameters a, x, and b were not significantly influenced by freezing for all suspensions except for 9-77.5. In this case, frozen heat-treated suspensions (9-77.5-F) presented lower values of a, higher values of x, and lower values of b than unfrozen heat-treated suspensions (9-77.5). The decrease of parameters a and b suggest lower values of G' and G'' at 1 Hz and the increase in exponent x indicates a faster increasing rate of G' with frequency after freezing the 9-77.5 suspension.

Exponent y was the most influenced by freezing. Frozen unheated suspensions showed lower values of exponent y than unheated suspensions. This decrease in exponent y indicates that freezing produced a reduction in the increasing rate of G'' with

Table 2
Power law parameters for the elastic ($G' = a\omega^x$) and viscous moduli ($G'' = b\omega^y$) of whey protein concentrate suspensions.

Samples	a (Pa s)	x	R^2	b (Pa s)	y	R^2
5-unheated	0.025 ± 0.000^a	2.011 ± 0.007^b	0.99	0.005 ± 0.000^a	1.177 ± 0.016^g	0.98
9-unheated	0.024 ± 0.000^a	2.019 ± 0.007^b	0.99	0.008 ± 0.000^a	1.119 ± 0.008^f	0.99
5-72.5	0.025 ± 0.001^a	2.005 ± 0.009^b	0.99	0.008 ± 0.002^a	1.106 ± 0.007^f	0.99
9-72.5	0.019 ± 0.000^a	2.114 ± 0.008^b	0.99	0.033 ± 0.002^b	0.872 ± 0.021^c	0.99
5-77.5	0.026 ± 0.000^a	1.987 ± 0.003^b	0.99	0.011 ± 0.000^a	1.054 ± 0.006^e	0.99
9-77.5	0.041 ± 0.009^b	1.786 ± 0.093^a	0.99	0.066 ± 0.009^c	0.728 ± 0.039^a	0.96
5-unheated-F	0.024 ± 0.000^a	2.041 ± 0.007^b	0.99	0.006 ± 0.000^a	1.031 ± 0.005^e	0.99
9-unheated-F	0.025 ± 0.000^a	2.014 ± 0.005^b	0.99	0.009 ± 0.001^a	1.000 ± 0.007^e	0.99
5-72.5-F	0.023 ± 0.000^a	2.033 ± 0.003^b	0.99	0.006 ± 0.000^a	1.193 ± 0.008^g	0.97
9-72.5-F	0.021 ± 0.000^a	2.066 ± 0.021^b	0.99	0.026 ± 0.001^b	0.924 ± 0.003^d	0.99
5-77.5-F	0.023 ± 0.000^a	2.028 ± 0.001^b	0.99	0.008 ± 0.000^a	1.140 ± 0.009^f	0.98
9-77.5-F	0.022 ± 0.001^a	2.045 ± 0.004^c	0.99	0.054 ± 0.002^d	0.803 ± 0.011^b	0.98

Mean values and standard deviations of three samples. Values with different letters in each column are significantly different ($p < 0.05$).

frequency. This phenomenon could be related to the presence of a higher degree of arrangement in frozen unheated suspensions. The freeze-concentration of solutes in the aqueous phase of the food system could have disturbed the stability of proteins (Franks & Hatley, 1991) and the increase in protein concentration during freezing can induce aggregation (Franks & Hatley, 1991; Tolstoguzov & Braudo, 1983). It is interesting to notice that heat treatments produced a similar effect to freezing on exponent y. Heat-treated suspensions showed lower values of exponent y than unheated suspensions (Table 2), showing that heat treatments may produce an improvement on the degree of arrangement of the suspensions, probably due to the formation of protein aggregates. On the other hand, frozen heat-treated suspensions presented higher values of exponent y than unfrozen heat-treated suspensions. The increase in exponent y indicates that freezing produced a rise in the increasing rate of G'' with frequency. This result can be related to a lower degree of arrangement in frozen heat-treated suspensions, where the formation of ice crystals during freezing could lead to the modification or disruption of the structure of soluble protein aggregates.

3.4. Analysis of the characteristic relaxation times

Characteristic relaxation times (τ_c) of whey protein concentrate suspensions are shown in Table 3. The τ_c of 5-unheated suspensions was significantly higher than the τ_c of 9-unheated suspensions, indicating that suspensions with the lower total protein content have a higher range of elastic behaviour in the mechanical spectra.

The rheological behaviour of globular protein suspensions is similar to a colloidal crystal. It has been known for years that charged colloidal particles in an aqueous solution can arrange into a crystalline lattice (Ikeda & Nishinari, 2000; Lindsay & Chaikin, 1982). Such colloidal system, designated as a colloidal crystal, mechanically responds to oscillating small strains as if it was a solid. However, the reason why globular protein suspensions behave as colloidal crystals is not known with certainty. These phenomena could be due to the existence of repulsive forces of hydration, where polar interactions orient water molecules on the surface of the protein and generate repulsions between molecules. These polar forces could be far greater than van der Waals attractive or electrostatic repulsive forces (Ikeda & Nishinari, 2001b). Also, it has been observed that colloidal crystals flow relatively easily at high concentrations, but do not flow easily at low concentrations (Inoue & Matsumoto, 1996). In our study, unheated suspensions have a high content of denatured protein content (>40%) that may be related to the high temperatures used in some steps of the commercial WPC manufacture, like heating and drying (de la Fuente, Singh, & Hemar, 2002; Roufik, Paquin, & Britten, 2005). However, it is possible that globular proteins that remain in native state showed some mechanical characteristics of colloidal crystals like the difficulty to flow at low contents of total protein content, represented by high values of τ_c in unheated suspensions containing 5% w/v of total protein content.

Characteristic relaxation times of heat-treated suspensions were smaller than those of unheated suspensions for the same content of total protein (Table 3), indicating a smaller range of elastic behaviour for heat-treated suspensions. The repulsive forces that are present in globular protein suspensions are dominated by a molecular surface force (such as the hydration force) and may be lost by denaturation due to exposure of the hydrophobic core of the protein (Ikeda & Nishinari, 2000). This phenomenon could explain the diminution of τ_c in the heat-treated suspensions.

Only characteristic relaxation times of suspensions with 5% w/v of total protein content were significantly influenced by freezing. Frozen unheated suspensions showed lower values of τ_c than unheated suspensions (5-unheated-F < 5-unheated). Taking into account that lower values of τ_c are related to important viscous characteristics in the material, this result indicates that the range of viscous behaviour in the mechanical spectrum increased in unheated suspensions after freezing. As previously discussed, the relative increase in protein concentration during freezing may induce the formation of protein aggregates (Franks & Hatley, 1991; Tolstoguzov & Braudo, 1983). This protein aggregates may generate a similar effect on the rheological properties of suspensions as heat denaturation, explaining the differences observed in the mechanical spectra of unheated suspensions after freezing.

Frozen heat-treated suspensions had higher values of τ_c than unfrozen heat-treated suspensions (5-72.5-F > 5-72.5; 5-77.5-F > 5-77.5). In agreement with the results discussed in Section 3.3, these observations indicate that frozen heat-treated suspensions have a lower range of viscous behaviour in the mechanical spectra

Table 3
Characteristic relaxation times (τ_c) of whey protein concentrate suspensions.

Samples	τ_c (s)
5-unheated	7.25 ± 0.73^f
9-unheated	3.51 ± 0.02^{dc}
5-72.5	3.68 ± 0.29^d
9-72.5	0.64 ± 0.04^a
5-77.5	2.43 ± 0.08^b
9-77.5	0.63 ± 0.05^a
5-unheated-F	3.94 ± 0.02^d
9-unheated-F	2.83 ± 0.04^{bc}
5-72.5-F	5.00 ± 0.44^e
9-72.5-F	0.85 ± 0.05^a
5-77.5-F	3.38 ± 0.04^c
9-77.5-F	0.48 ± 0.02^a

Mean values and standard deviations of three samples. Values with different letters are significantly different ($p < 0.05$).

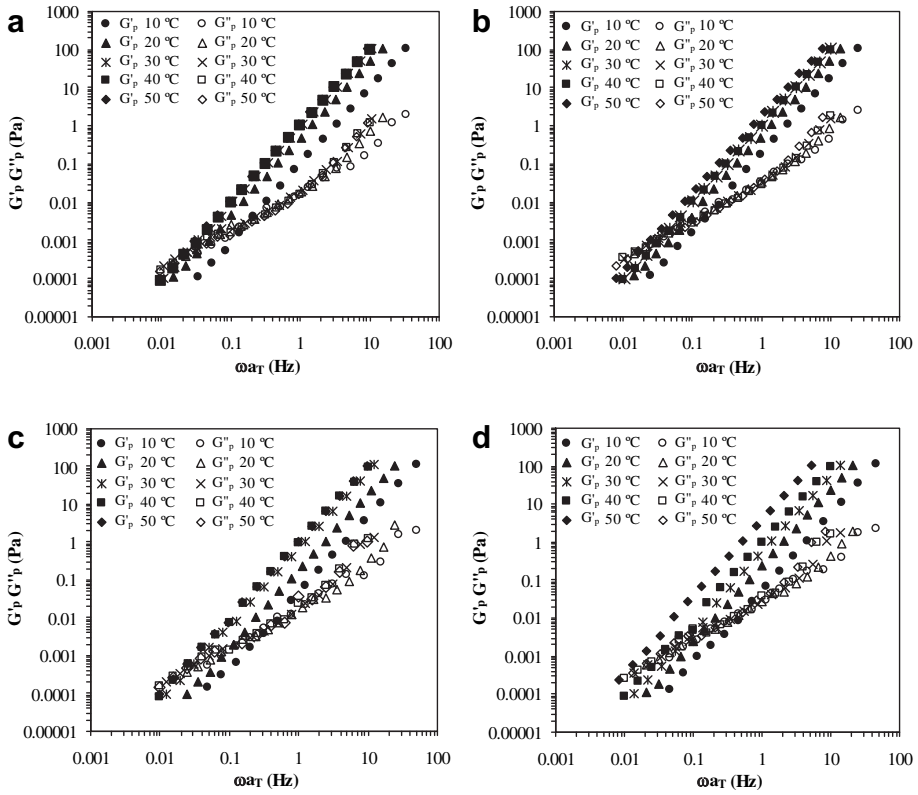

Fig. 5. Application of WLF principle at the reference temperature of 40 °C. (a) Unheated suspensions with 5% w/v of total protein content. (b) Unheated suspensions with 9% w/v of total protein content. (c) Frozen unheated suspensions with 5% w/v of total protein content. (d) Frozen unheated suspensions with 9% w/v of total protein content. Points represent individual values of one of the three replications.

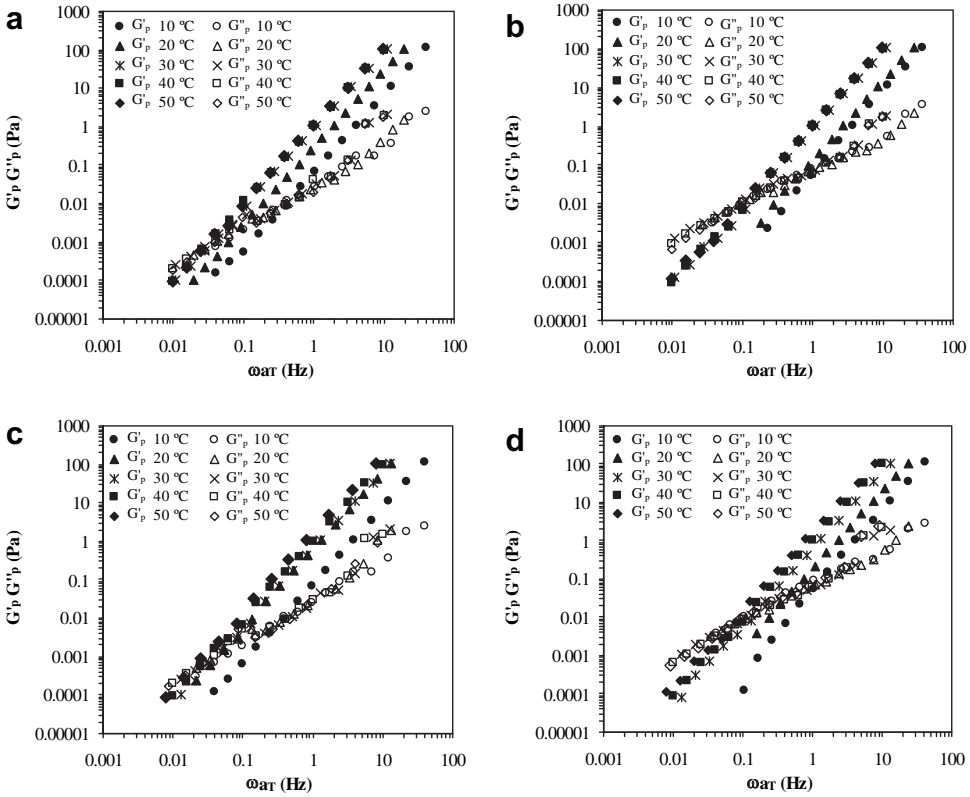

Fig. 6. Application of WLF principle at the reference temperature of 40 °C. (a) Heat-treated suspensions at 72.5 °C with 5% w/v of total protein content. (b) Heat-treated suspensions at 72.5 °C with 9% w/v of total protein content. (c) Frozen heat-treated suspensions at 72.5 °C with 5% w/v of total protein content. (d) Frozen heat-treated suspensions at 72.5 °C with 9% w/v of total protein content. Points represent individual values of one of the three replications.

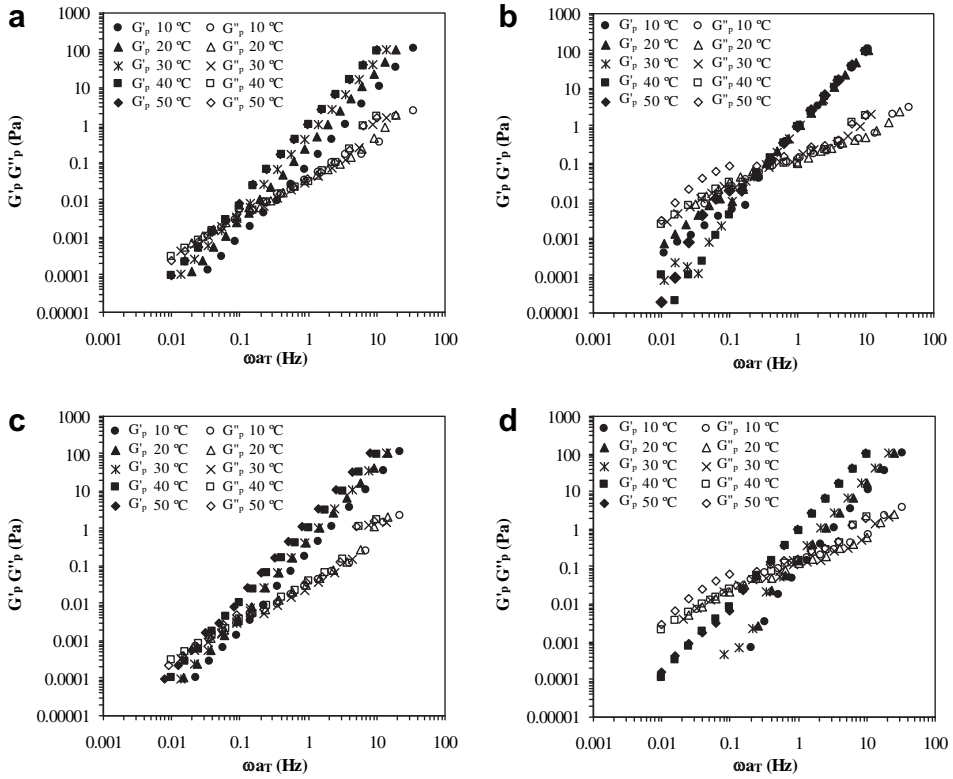

Fig. 7. Application of WLF principle at the reference temperature of 40 °C. (a) Heat-treated suspensions at 77.5 °C with 5% w/v of total protein content. (b) Heat-treated suspensions at 77.5 °C with 9% w/v of total protein content. (c) Frozen heat-treated suspensions at 77.5 °C with 5% w/v of total protein content. (d) Frozen heat-treated suspensions at 77.5 °C with 9% w/v of total protein content. Points represent individual values of one of the three replications.

than unfrozen heat-treated suspensions. Freeze-concentration and ice crystal formation during freezing may produce modification or disruption of the structure of soluble protein aggregates that were produced by heat treatment.

It is interesting to notice that only the characteristic relaxation times of suspensions with low total protein content were influenced by freezing. Whey proteins are able to bind a great number of water molecules mainly though hydrogen bonds. By increasing the concentration of proteins in the solution, the interstitial water becomes less available for freezing. The stability of frozen aqueous food systems, such as whey, depends mainly on the physical state of their constituents and the proportionality between them in the initial solution (Aider, de Halleux, & Akbache, 2007). At the same time, the physical state of proteins depends on the composition, temperature, and water content. The stability of proteins in aqueous solution increases with protein concentration due to self-stabilization effect. This phenomenon has been observed in enzymes and in β-lactoglobulin dispersions (Pikal-Cleland, Rodríguez-Hornedo, Amidon, & Carpenter, 2000; Xiaolin & Pikal, 2005).

Analysing τ_c and power law exponent y, several common changes were observed. While freezing decreased values of y and τ_c of unheated suspensions, it increased values of y and τ_c of heat-treated suspensions. Taking into account this observation, a positive linear correlation between τ_c and y was found (correlation coefficient = 0.84; $p < 0.01$) suggesting that the change of τ_c could be correlated to the change of the slope of the G'' versus frequency (exponent y).

3.5. Time–temperature superposition

Success in the application of time–temperature superposition suggests that no morphological changes occurred in the material, and moduli displayed similar temperature dependence (Ferry, 1980). On the other hand, failure of this principle is an indicator of a relaxation time (or moduli) with nonuniform temperature dependence, such as those observed either in multiphase systems or in systems that undergo physical changes during the rheological measurements (Mavridis & Shroff, 1992; Muliawan & Hatzikiriakos, 2007; Van Gurp & Palmen, 1998).

The application of WLF principle in whey protein concentrate suspensions are shown in Figs. 5–7. Plotted results represent the general trends of all replications. The shift factors which superposed the majority of the G''_p points at each temperature were used to construct a master curve of viscous modulus (Table 4). The same shift factors a_T used to superimposed the values of G''_p were used to overlap the values of G'_p.

Master curves could be obtained only in a range of frequencies depending on the sample (Figs. 5–7). The WLF principle was satisfactory applied in unheated suspensions only in the temperature range from 30 to 50 °C (5-unheated) and 30 to 40 °C (9-unheated) (Fig. 5a and b). These results suggest that unheated suspensions exhibited thermorheologically simple behaviour in the high-temperature region of the mechanical spectrum.

In the case of heat-treated suspensions at 72.5 °C (5-72.5 and 9-72.5), time–temperature superposition was achieved successfully in the temperature range from 30 to 50 °C (Fig. 6a and b). Also, in heat-treated suspensions at 77.5 °C, this principle was valid in the temperature range from 40 to 50 °C only in suspensions with 5% w/v of total protein content (5-77.5) (Fig. 7a). Time–temperature superposition failed over the complete temperature range in heat-treated suspensions at 77.5 °C with 9% w/v of total protein content (9-77.5) due to the linear viscoelastic data could not be overlapped using the same shift factor (Fig. 7b). In this case, it is possible that the heat treatment of suspensions with 9% w/v of total protein content at 77.5 °C could produce aggregates that are susceptible to morphological changes during rheological measurements at

Table 4
Time–temperature shift factors (a_T) of G''_p as function of temperature.

Samples	a_T				
	10 °C	20 °C	30 °C	40 °C	50 °C
5-unheated	3.35	1.51	1.08	1.00	0.97
9-unheated	2.47	1.41	1.02	1.00	0.87
5-72.5	4.10	1.95	1.10	1.00	0.97
9-72.5	3.71	2.72	1.10	1.00	0.98
5-77.5	3.48	1.95	1.39	1.00	0.98
9-77.5	4.45	3.15	1.20	1.00	0.98
5-unheated-F	4.95	2.45	1.25	1.00	0.98
9-unheated-F	4.50	2.11	1.37	1.00	0.85
5-72.5-F	3.91	1.35	1.29	1.00	0.85
5-77.5-F	2.25	1.49	1.10	1.00	0.81
9-72.5-F	4.50	2.36	1.32	1.00	0.91
9-77.5-F	3.30	2.52	2.10	1.00	0.98

Values correspond to one of the three replications.

different temperatures. These phenomena suggest that both high protein content and high heat-treatment temperature can significantly influence the rheological properties of whey protein concentrate suspension.

The rheological behaviour of globular protein suspensions is similar to a colloidal crystal (Section 3.4). Such colloidal system mechanically responds to oscillating small strains as if it was a solid. These phenomena could be due to the existence of repulsive forces of hydration, where polar interactions orient water molecules on the surface of the protein mainly via hydrogen bonds and generate repulsions between molecules. These polar forces could be far greater than van der Waals attractive or electrostatic repulsive forces (Ikeda & Nishinari, 2001). In this work, when rheological measurements were performed at low temperatures, it is possible that hydrogen bonds were strengthened in whey protein concentrate suspensions, explaining the thermorheological behaviour of samples (5-unheated, 9-unheated, 5-72.5, 9-72.5, 5-77.5) at low temperatures.

The WLF principle was satisfactory applied only in frozen unheated suspensions with 5% w/v of total protein content (5-unheated-F) in the temperature range from 40 to 50 °C (Fig. 5c). However, this principle failed over the complete temperature range in frozen unheated suspensions with 9% of total protein content (9-unheated-F) and in all frozen heat-treated suspensions (Figs. 5d, 6c, d, and 7c, d). In this study, it was proposed that freezing may increase protein aggregation in unheated suspensions and modify or disrupt protein aggregates in heat-treated suspensions. Consequently, freezing may change the surface of proteins and therefore influences the repulsive hydration forces among protein molecules. These phenomena could produce an increase in the susceptibility to morphological changes with temperature during the rheological measurements of frozen samples (9-unheated-F, 5-72.5-F, 5-77.5-F, 9-72.5-F, 9-77.5-F) in the complete range of analysed temperatures.

4. Conclusions

In this work, the effect of freezing on the viscoelastic behaviour of whey protein concentrate suspensions was analysed. The effect of freezing on characteristic relaxation times was most important in whey protein suspensions with the low total protein content (5% w/v). Freezing produced an increase in the range of viscous behaviour observed in the mechanical spectrum of unheated suspensions, possibly due to protein aggregation that occurred during freezing. However, freezing produced a decrease in the range of viscous behaviour of heat-treated suspensions. In this case, freeze-concentration and ice crystal formation could have produced the modification or disruption of the structure of soluble protein aggregates.

Power law exponent of viscous modulus (y) was the parameter most influenced by freezing, where all frozen unheated suspensions showed lower values of exponent y than unheated suspensions. This phenomenon could be due to the presence of a higher degree of arrangement in frozen unheated suspensions. On the other hand, frozen heat-treated suspensions presented higher values of exponent y than heat-treated suspensions. These results indicate that a lower degree of arrangement can exist in frozen heat-treated suspensions. Possibly, the formation of ice crystals during freezing can lead to the modification or disruption of the structure of soluble protein aggregates.

In general, WLF principle was satisfactory applied in whey protein suspensions only in a range of temperatures from 30 to 50 °C. However, time–temperature superposition failed over the complete temperature range in most of the frozen suspensions. It is possible that freezing may change the surface of proteins and therefore influences the repulsive hydration forces among protein molecules. This phenomenon could produce an increase in the susceptibility to morphological changes during the rheological measurements at different temperatures of frozen samples.

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