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RasouliI, M, Abbasi, S, Azarkia, F et al. (1 more author) (2020) On the heat stability of whey protein: Effect of Sodium hexametaphosphate. International Journal of Dairy Technology, 73 (1). pp. 46-56. ISSN 1364-727X

https://doi.org/10.1111/1471-0307.12626

On the heat stability of whey protein: Effect of sodium hexametaphosphate

MEHRDAD RASOULI[1], SOLEIMAN ABBASI[1*], FATEMEH AZARIKIA[2], RAMMILE ETTELAIE[3]

[1] Food Colloids and Rheology Lab., Department of Food Science and Technology, Faculty of Agriculture, Tarbiat Modares University, P. O. Box 14115-336, Tehran–Iran

[2] Department of Food Technology, College of Aburaihan, University of Tehran, Tehran–Iran

[3] Food Colloids Group, School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK

*To whom correspondence should be addressed:

sabbasifood@modares.ac.ir

Short Running Title:

Whey protein heat stability development

ABSTRACT

Despite a growing demand for whey protein-based drinks, their instability and lack of solubility during thermal processing is a major challenge for food product formulators. In the present study, effects of different hydrocolloids and sodium hexametaphosphate (SHMP) on the heat stability, rheological properties, microstructure and sensory characteristics of whey protein concentrate (WPC) dispersions were evaluated. The results indicated that at pH 4, xanthan, k-carrageenan, low methoxyl pectin (LMP) and guar stabilized WPC dispersion without heat treatment, all maintained stability during pasteurization but not sterilization. For pH 7, for the same set of hydrocolloids, a similar trend was also observed, albeit at different concentrations. However, by adding optimum ratios of SHMP protein denaturation was retarded, particularly in the case of LMP and i-carrageenan. The highest and lowest apparent viscosities were exhibited by samples containing 0.01% and 0.15% w/w SHMP, respectively. This study highlights the potential capability of SHMP in prevention of protein denaturation. An exact plausible stability mechanism still needs further more detailed investigation.

Keywords: Whey protein, Protein–hydrocolloid interaction, Thermal processing, Stabilization.

INTRODUCTION

Whey proteins, comprising appoxomately 20% of bovine milk protein, are not only important because of their bioactive nature and nutritional value but also for their emulsifying, foaming, gelation and water-binding properties. Differences betweeen functional properties of whey protein products (commercial whey powder, whey protein isolates (WPI) and concentrates (WPC)) have recently been reported by Ghanimahe (2017). Generally speaking, a food with certain added desirable properties, which also includes health-promotion or illness prevention alongside its nutritional value, is called a 'functional food'. According to this definition, whey proteins arising as by-products of cheese-making process can be categorized as nutraceutical and functional milk-based ingredients (Coutinho et al. 2019, Silveira et al. 2019). Todays, there is a general trend for consumption of health-promoting foods arising from an enhanced awarness and knowledge of consumers regarding food products they eat (Amaral et al. 2018; Guimaraes et al. 2018). In other words, whey proteins can be utilized as a substitute for milk in production of beverages in which fruit pulps, chocolate and stabilizers can be added to enhance flavor and overall acceptability of the whey protein based-dairy beverages (Della Lucia et al. 2015; Guimaraes et al. 2018; Cappato et al. 2018; Amaral et al. 2018; Souza et al. 2019; Silveira et al. 2019). There is also a report promoting the production of whey protein-based prebiotic beverage (Guimaraes et al. 2018). Furthermore, whey proteins can be utilized for preparation of fermented whey beverage as a source of protein, B_2 and B_{12} vitamins and several important minerals like Ca and Mg (Souza et al. 2019). Moreover, in the presence of caseins, β-lactoglobulin component of whey proteins can link to κ-casein during heat treatment. This has recently been used to produce an appropriate carrier for hydrophobic nutraceuticals (Moeller et al. 2018).

Like most globular proteins, hydrophobic, covalent and hydrogen bonds play important role in maintaniace of internal structure of whey proteins. However, these proteins are prone to denaturation and thus subsequent aggregation upon heating, arising from conformational changes due to alterations in the degree of exposed hydrophobic regions, as well as modification of protein charge (Mulcahy et al. 2017). It is stated that heat treatment at 70–75 °C leads to formation of small and reversible bonds between protein particles via weak van der Waals interactions while higher temperatures (80–90 °C) strengthen the covalent disulfide bonds. This thermosensitivity against heat processing affects physical stability of WPI-contaning systems (Joyce et al. 2018). Therefore, applicability of WPI in the products needing thermal processing remains as a serious challenge.

Recently, some reports indicated that the prevention of unpleasent effects of heating on whey proteins' bioactivity and functionality is possible using new processing techniques such as cold plasma (Silveira et al. 2019; Coutinho et al. 2019), high-intensity ultrasound (Guimaraes et al. 2018), ohmic heating (Cappato et al. 2018) or the supercritical carbon dioxide technology (Amaral et al. 2018).

Despite the existance of various studies , there is still a lack of sufficient information about finding a way of retarding heat-induced denaturation and aggregation. Nonetheless, there are some reports claiming different methods for stabilization of whey proteins. Amongst these are the application of heat stable WPC, use of hydrocolloids, enzymes, salts and adjustment of effective factors (e.g., pH, ionic strength, protein concentration) but to name a few (Qi et al. 2017; Wagoner and Foegeding 2017; Azarikia and Abbasi 2016; Ryan and Foegeding 2015). Despite the effectiveness of hydrocolloids, the strength of electrostatic interaction between protein and hydrocolloid usually weakens during heat treatment. This results from protein denaturation which eventually can lead to phase separation of these two biopolymers (Jones and McClements 2011). Therefore, it seems that by adding chelating agents (e.g. sodium hexametaphosphate) one may be able to tackle this issue and therefore also the heat induced instability.

Sodium hexametaphosphate (SHMP) is a white, tasteless, water soluble but organic solvent insoluble phosphate salt $(NaPO_3)_6$. It has widespread applications in many industries including food manufacturing. It is considered as GRAS (generally recommended as safe) and is used in foodstuffs for quality improvement, pH adjustment, color preservation, moisture retention and ion chelation (Lanigan 2001). Mizuno and Lucey (2005; 2007) have also examined the influence of different phosphates, including SHMP, on gelation of the milk protein concentrate via cross-linking of the added salts with calcium. Rulliere and co-workers (2012) stated that heating at 120^{o}C for 10 min could decrease the emulsifying and chelating properties of Graham's salt and commercial polyphosphate (long chain phosphates) because of hydrolytic degradation into orthophosphate and trimetaphosphate (short-chain phosphates). Phosphate salt was also used to improve water dispersibility of waxy maize starch. McCarthy and co-workers (2017) used sodium phosphate and SHMP as calcium chelating agents to enhance dissolution and functionality of the milk protein concentrate which reduced the viscosity of SHMP containing dispersions. SHMP has also been used for complex coacervation formation of gelatin (Wang et al. 2014), cheese production (Shirashoji et al. 2010) and to improve

physico-chemical properties of pasteurized soymilk (Pathomrungsiyounggul et al. 2007; Pathomrungsiyounggul et al. 2010).

To the best of our knowledge, the SHMP effect on whey proteins, hydrocolloids, protein–hydrocolloid interactions, as well as thermal stability of whey proteins has never been reported with the exception of a patent (Connoly 1995) in which SHMP was utilized as a thermal improver agent. In the current study, the effect of different adsorbing (i.e., with the capability of adsorbing on protein surface via electrostatic interaction), as well as non-adsorbing hydrocolloids on the stabilization of WPC dispersion both before and after heat treatment (pasteurization and sterilization) was investigated. This was done at various pH values (4 and 7). Following this, the efficacy of SHMP added at different ratios on improving the thermal stability of protein–hydrocolloid systems, their microstructures, zeta potentials, rheological and sensory properties were evaluated.

MATERIALS AND METHODS

Materials

Instant whey protein concentrate (81.6 %w/w protein in dry matter) was obtained from Sachsenmilch Leppersdorf GmbH (Wachau, Germany). High and low methoxyl pectin (HMP and LMP) were provided from CP Kelco Co. (Lille Skensved, Denmark). Xanthan, guar and locust bean gums, as well as sodium hexametaphosphate were purchased from Sigma-Aldrich Chemicals Co. (Roseville, Canada). Both i- and k-carrageenans were supplied by Fluka Chemie GmbH (Buchs, Switzerland). Phosphoric acid, citric acid, NaOH and sodium azide were also provided by Merck Chemicals Co. (Darmstadt, Germany).

Persian gum lumps (PG, Amygdalus scoparia Spach) and gum tragacanth ribbons (GT, Astragalus compactus) were purchased from a local herbal store (Tehran, Iran). They were then grinded, sieved (# -60) and stored in sealed containers as powder. Sugar and cacao powder were purchased from a local supermarket (Tehran, Iran). Aloe vera flavor powder was purchased from flavor and fragrance company (Givaudan, Brazil).

Preparation of protein dispersion

In order to prepare protein dispersion, WPC powder (4, 6 or 8% w/w) was gradually added to distilled water, followed by mixing for 1 h (at 400 rpm) using a magnetic stirrer. To assure

complete hydration, dispersions were maintained at 4ºC overnight. To prevent microbial growth sodium azide (0.04% w/w) was added to the solution (Abbasi and Dickinson 2004). Moreover, the mixture of citric and phosphoric acids (0.5 M) and NaOH (0.1 M) were utilized for pH adjustment (4.0 and 7.0).

Preparation of protein-based beverage with and without hydrocolloid

A WPC-based beverage, used here as a model system, was prepared at pHs 4.0 and 7.0 using WPC (4 % w/w), sugar (10 % w/w) and cacao powder (1 % w/w). The level of the protein (4 % w/w) was then chosen according to the preliminary experiments indicating that protein solubility was a function of concentration: the higher the concentration, the more the precipitation is seen. Since the physical instability and sedimentation of WPC and cacao particles were observed in the prepared beverages, in the next step the effect of different hydrocolloids (LMP, xanthan, k-carrageenan, i-carrageenan and guar) at various concentrations (0.15–0.8 % w/w) were examined to find the appropriate concentration for each hydrocolloid capable of stabilizing the model WPC-based beverage.

During the preliminary tests, it was noticed that the order of addition of ingredients (hydrocolloid, protein, sugar, cacao powder and pH adjustment) at pH 4 could somewhat affect the stability of the dispersion. Therefore, the method of preparation for each hydrocolloid was evaluated and the most appropriate order for obtaining the best stability was chosen (data are not shown). Conversely, primary tests indicated that the order of addition of ingredients did not affect the stability at pH 7. Hence, for preparation of protein-based beverages at this pH, an identical order was followed for all hydrocolloids. With regards to the order of addition of the ingredients and the acidification process, it is worth mentioning that acidifying the protein (pH=4) before addition of hydrocolloids led to formation of protein aggregates, with the added negatively charged hydrocolloids then attached to the surface of these previously aggregated particles. On the other hand, when protein–hydrocolloid mixture was gradually acidified, the electrostatic interaction between WPC and the hydrocolloids retarded protein denaturation at pH 4. However, at pH 7 which is far from isoelectric point of WPC, protein chains have enough negative charge and electrostatic repulsion to hinder their aggregation. Therefore, they are not prone to aggregation and the order of addition and acidification does not impact the physical stability of this colloidal system, at pH=7 (Azarikia et al. 2017).

Addition of sodium hexametaphosphate

To evaluate the order of addition and concentration of SHMP on heat stability of WPC beverage (protein 4%, sugar 10%, cacao powder 1% and the required amount of each hydrocolloid for stabilization), a stock solution of SHMP (5% w/v) was prepared. This was then added at different ratios (from 0.001 to 1% w/w) to WPC dispersions, which had already been stabilized by various hydrocolloids. Finally, the dispersions were mixed (400 rpm for 30 min) prior to any thermal treatment.

Heat treatment

In order to investigate the effects of heat treatment on the stability of stabilized-WPC-based model beverage, the samples were transferred to sealed tubes, heated in water-bath at 85^{o}C for 2 min while being stirred. The samples were then cooled down to 5^{o}C using a water–ice bath (Abbasi and Mohammadi 2013; Behbahani and Abbasi 2017). For sterilization purpose, the stabilized dispersions were autoclaved using a static rotor (121.5^{o}C for 15 min), then inverted several times and were immediately cooled down using a water–ice bath (Azarikia and Abbasi 2016; Azarikia et al. 2017).

Viscosity measurement

To evaluate the effect of different hydrocolloids and SHMP on the apparent viscosity of WPC dispersions, measurements were conducted at 10^{o}C using a Brookfield DV III ULTRA, LV viscometer (Brookfield Engineering Laboratories, Stoughton, USA), equipped with concentric cylinder geometries: SC4-34, SC4-31 and SC4-18 (Abbasi and Mohammadi 2013; Teimouri et al. 2017; Mohammadi et al. 2017). To determine the flow behavior, the shear stress and viscosity were measured as a function of shear rate, increasing from 25 to 85 s^{-1} during the tests.

Zeta potential and particle size measurement

The heat treatment impact on the particle size distribution and zeta potential of stabilized samples were investigated using a dynamic light scattering (Nano-ZS90, Malvern Instruments, Worcestershire, UK) technique. To get reliable results and avoid multiple scattering effects, the samples were diluted (1:30) using double distilled water at the same pH prior to measurements (Azarikia et al. 2017).

Sensory evaluation

Five formulations containing different hydrocolloids (xanthan, guar, LMP, i-carrageenan and k-carrageenan) were prepared. Then, aloe vera powder (0.02% w/w) and SHMP (at optimum concentrations) were added and formulations sterilized (121.5^{o}C, 15 min). The sensory attributes including color, odor, taste, consistency and mouth feeling were evaluated by 15 semi-trained panelists (with ages ranging from 25 to 50 years old, including both men and women) using five-level hedonic test (Azarikia and Abbasi 2010; Behbahani and Abbasi 2017).

Statistical analysis

All of the formulation experiments and measurements were repeated at least three times. For determining the significant differences between mean values, ANOVA and Duncan's multiple range tests were applied using SPSS software (Version 14.0, SPSS Inc.). EXCEL and MATLAB soft wares were used for reproducing the charts and for examining the fitting of the experimental viscosity data to rheological models.

RESULTS AND DISCUSSION

Effect of pH, protein content and heating on solubility and stability of WPC

As a first step, the solubility of WPC at different concentrations (4, 6 and 8% w/w) and pHs (4 and 7) was investigated. Shortly after preparation, phase separation occurred regardless of concentration and pH. As expected, a high protein content (8% w/w) led to a larger amount of precipitate (Fig. 1). There was two choices for comparing the turbidity of colloidal systems at pHs 4 and 7, either after being vigorously mixed or without mixing. Then, the visual turbidity of supernatant was reported as an obvious evidence that the aggregation at pH=4 (near to pI of

proteins) caused phase separation. In addition, at pH 7 the turbidity of supernatant was seen to be higher than pH 4. Since pH 4 is adjacent to the isoelectric point (pI) of WPC, electrostatic repulsion between protein molecules is rather weak and therefore their precipitation is expected. WPC has a higher negative charge at pH 7 in comparison to pH 4 (Azarikia and Abbasi 2016). Hence, it is expected that this leads to a higher electrostatic repulsion between proteins. It seems that turbid appearance of supernatant phase at pH 7 (Fig. 1a) was the macroscopic evidence of higher electrostatic repulsion forces between protein particles. In contrast, weak repulsion forces at pH 4 caused the supernatant phase to look more transparent, with a much larger degree of sedimentation than their counterparts at pH 7 (Fig. 1a & b). This implied that most of the protein was no longer present in the supernatant at pH=4, giving rise to a clearer appearance.

Not surprisingly, as has already been well established in the literature (Azarikia et al. 2017; Flett and Correding 2009), the results also confirmed the detrimental impact of heat treatment (pasteurization and sterilization) on denaturation and desolubilization of WPC upon heating. In other words, the precipitation significantly increased at all concentrations for both studied pH values (7 and 4). The heat instability (> 65 ^{o}C) of whey proteins is attributed to the exposure of their buried hydrophobic and sulfhydryl groups, formation of inter-molecular S–S bonding and hydrophobic interactions which itself results from partial or complete denaturation and lack of functionality (Azarikia et al. 2017; Wijayanti et al. 2014).

In agreement with the previous reports (Baier and McClements 2001; Cornacchia et al. 2014), the sediment part turned to a gel at 8% (w/w) of protein, at both pHs. It is stated that if prior to heat treatment (> 60^{o}C) the pH is adjusted to values close to pI, aggregation and eventually gelation will take place (Azarikia et al. 2017).

Effect of hydrocolloids and heating

Due to the phase separation of WPC dispersions (prior and post heating), utilization of stabilizers in the formulation was seen as inevitable. Hence, the influence of different adsorbing and non-adsorbing hydrocolloids on the stabilization of individual proteins or small aggregated particles at pH 7 and 4 was perused. According to the observations (Table 1), the system was stabilized by different concentrations (0.15–0.80% w/w) of the examined hydrocolloids without being heated. It seems that the charge density of hydrocolloids is a crucial factor for

their suitability as an appropriate hydrocolloid in the current application. For instance, at pH 7, 0.35, 0.3 and 0.15% w/w of guar (non-ionic), xanthan (moderate negative charge), and carrageenan (high negative charge) required to provide stability, respectively. Relation between the charge density of hydrocolloids and the requested amount to induce sufficient stability has been reported previously (Zhang et al. 2014). Table 1 also demonstrates that the required amount of charged hydrocolloid was noticeably higher at pH 4 (< pI) than the corresponding value at pH 7. The reason is attributed to positive charge on protein molecules at pH values below pI. The amount of negatively charged hydrocolloids not only has to be enough to neutralize the positive charge of the proteins, but it also needs to be large enough to provide charge reversal and a sufficient dominant electrostatic repulsion between particles via negatively charged groups.

There is no doubt that protein denaturation could easily happen upon heating, even when this was not macroscopically detectable (Fig. 2). However, macroscopic instability (formation of aggregates which are observable with the naked eye) was observed in the samples containing k-carrageenan and LMP. Regarding the former, it could be speculated that the heating not only denatured the protein but also weakened or destroyed any weak interactions as there might be between protein and sulfate groups of carrageenan. It has been pointed out that protein: carrageenan ratio and the heating are extremely important parameters in determining the effective stability. The protein denaturation, separation of protein from hydrocolloid and also formation of hydrophobic interactions between proteins take place at temperatures higher than 80^{o}C (Zhang et al. 2017). Furthermore, it is reported that a higher temperature (approximately 90^{o}C) could lead to thermodynamic instability of protein–k-carrageenan system as a consequence of bridging aggregation of the formed intermediate whey protein aggregates in the presence of negatively charged hydrocolloids (Flett and Correding, 2009).

With regards to LMP, Qi and co-workers (2017) reported that by heating soluble complexes of β-lactoglobulin–LMP mixture (3:1), at 80^{o}C for 10 min, denaturation and aggregation of protein was observed. In contrast, in a different study the presence of LMP (0.3% w/w) was found to improve the heat stability of WPI (3% w/w). In this latter study, the researchers also stated that the destruction of alpha structure of protein could be prevented by LMP (Zhang et al. 2012). These contradictory behaviors could be partially attributed to different protein:LMP ratios used in these two reports. Nonetheless, the exact mechanism for such behavior is still largely unclear. The protein:LMP ratio used in the present study was very similar to that used in the study of Zhang and co-workers (2012). However, the structural and compositional

differences of WPC and WPI, pH and the ionic strength are other important parameters which could equally affect the overall observed behavior.

Upon sterilization, some degree of visible protein aggregation at both pH values (4.0 and 7.0) were observed (Fig. 2). However, the aggregation rate was not the same for different hydrocolloids. For instance, in the presence of a non-adsorbing hydrocolloid (e.g. guar), the aggregation was found to be particularly slower than other hydrocolloids. In addition, it was found that pasteurized samples (Fig 2a, b) kept their physically uniform state in each phase and remained liquid, while aggregated proteins (Fig. 2c, d) formed a semi-solid state with a non-uniform morphology. Hidden hydrophobic and sulfhydryl groups of whey proteins were exposed due to heating which consequently caused unfolded proteins to form these particulate aggregates (Azarikia and Abbasi, 2017).

Concerning the influence of pH (Fig 2), as alluded, the lower stability at pH 4 seems to be related to lower charge of proteins which could not cause adequate electrostatic repulsion to prevent protein molecules from approaching each other and subsequently aggregating.

Effect of sodium hexametaphosphate

As discussed above, the formulated WPC dispersions (containing protein, cacao powder, sugar and hydrocolloid) did not tolerate thermal treatments (Fig. 2b, d), particularly the sterilization process where clear phase separation occurred. For this reason, the potential capability of SHMP in enhancing the thermal stabilization of model formulation was examined. An inverse relation between the concentration of SHMP and dispersion viscosity was observed over the studied range (Section 3.4).

It was also found that SHMP was not effective in preventing heat instability at pH 4. For instance, in the case of dispersions which were stabilized with k-carrageenan or LMP (pH 4), the addition of SHMP ($\leq$ 0.5% w/w) initially caused a reduction in viscosity (before heat treatment). However, after pasteurization, the phase separation was perceived. On the contrary, a group of researchers have reported that conjugation of WPI with maltodextrin at 60^{o}C for 24 h enhanced the protein solubility at pH 3.5–4 (Mulcahy et al. 2017). Interestingly, the system did stabilize when SHMP concentration was increased to 1% w/w. This behavior may be attributed to **a)** the SHMP's hygroscopicity which enables it to easily entrap water molecules, **b)** the high content of salt (1 %w/w), because high positive charges (comparing to acidic pH)

of protein at pH near pI requires more negatively charged ions (phosphate groups of salt), and **c)** degradation of SHMP since it is already reported that, under acidic conditions in particular, SHMP could easily hydrolyze to sodium trimetaphosphate and sodium orthophosphate. This hydrolytic degradation of phosphates decreases its functional properties (Rulliere et al. 2012). Moreover, at high temperatures (≥120^{o}C), the majority of SHMP could be degraded (50% orthophosphate and 25% 3-metaphosphate) so that its chelating properties can significantly alter. Polyphosphates are more efficient than orthophosphates in this respect (Rulliere et al. 2012). A direct relation between water availability and protein denaturation (Zayas 1997) can be another reason for the above mentioned observations. In addition, electrostatic interaction of positively charged residues of protein with negative sites (phosphate ions) of SHMP can also be a further factor. On the contrary, SHMP was unable to stabilize other formulations. For instance, samples with xanthan and guar, even at the presence of very high SHMP concentrations exhibited formation of large aggregates. Regarding the effect of SHMP on viscosity reduction, one may speculate that the electrostatic interaction between negatively charged (phosphate) residues of SHMP and positively charged patches of biopolymers resulted in the same level of charge neutralization and therefore low repulsion and consequently low viscosity. Similarly, it is reported that there is a relation between the charge density of colloidal particles and apparent viscosity of the system. Indeed, the higher electrostatic repulsion between already stabilized particles could cause a higher viscosity by increasing the resistance against flow (i.e., magnifying the effective volume fraction of particles). Such phenomenon has been extensively investigated in relation to polysaccharides and proteins individually and in a rather systematic manner.

At pH 7, the heat stability was improved effectively by the presence of a comparably lower concentration of SHMP. In other words, no phase separation was observed neither after heating (pasteurization and sterilization) nor during any subsequent cold storage. More interestingly, the viscosity of studied samples was less than their counterparts not containing SHMP (data are not shown). It is worth noting that the optimum SHMP required strongly depends on the structural features of protein, hydrocolloid, their actual contents, pH, and the ionic strength. That is why various quantities (0.04, 0.15, 0.03, 0.01 and 0.03% w/w) of SHMP were added to stabilize systems which contained either xanthan (0.2%), LMP (0.5%), i-carrageenan (0.15%), k-carrageenan (0.15%) or guar (0.35%). Also it needs to be emphasized that despite the constant protein content (4%w/w), the ash contribution of various hydrocolloids with different concentrations was not the same. This could be another possible reason why the amount of

SHMP required for optimum stabilization were unequal. These findings imply that ionic strength and pH play important role on the functioning of SHMP, since upon heating under acidic pH, the functionality of SHMP diminishes, most likely due to structural degradation as was alluded earlier. It has also been reported that the most desirable pH for extraction of whey, flaxseed and cottonseed proteins is around 7.0, whereas for complex coacervation of gelatin with SHMP it is 4.7. Similarly, the optimum protein:SHMP ratio is reported to be anywhere in the range 3:1 to 30:1 depending on the pH, the ionic strength and the type of protein involved (Wang et al. 2014). Phosphates can also bind to polysaccharides via electrostatic interactions. The higher the charge density of interacting species, the stronger is the binding. This is why pH should be considered as the most influential factor (Pascual and Sher 2013). Similarly, the higher concentrations of background electrolyte can reduce the electrostatic interactions through screening.

Cross-linking of casein molecules via polyphosphate ions is reported as a probable mechanism of heat stability enhancement. Based on such mechanism, polyphosphates are being used in formulation of processed and cream cheese to create smooth texture and an even casein distribution. It is speculated that phosphates, by interrupting the exposure of hydrophobic parts during thermal unfolding, could postpone aggregation of denatured proteins (Shirashoji et al. 2010). Additionally, high temperatures (such as those encountered in sterilization process) could cause discernible change in functioning of SHMP, probably due to degradation and dissociation of the latter (Rulliere et al. 2012).

Rheological properties of stabilized dispersions

The flow behavior of thermally treated and stable dispersions (pH 7) were also evaluated at 10^{o}C. Fig. 3 illustrates apparent viscosity of dispersions as a function of shear rate in the presence of different hydrocolloids and specified amount of SHMP. It is seen that the lowest and the highest apparent viscosities belong to dispersions which were stabilized with LMP and guar gums, respectively. The viscosity of the latter, as a non-adsorbing hydrocolloid, was significantly higher than the former one, which is an adsorbing (on protein) hydrocolloid. In addition, by increasing shear rate the viscosity is reduced, indicating a slight shear thinning behavior. It has already been reported that by adding a certain concentration of SHMP to soymilk the viscosity can be reduced. However, any further increment beyond this specific concentration caused no significant further changes to the kinematic viscosity

(Pathomrungsiyounggul et al. 2007). The significant effect of protein concentration on the viscosity of acidified milk-based beverage, due to dry material content increment, has already been confirmed (Azarikia and Abbasi 2010).

Generally, a relation between apparent viscosity of dispersions and the concentration of SHMP was observed in the current work. The sample with 0.5 %w/w LMP and 0.15% w/w of SHMP had the lowest apparent viscosity, whereas the apparent viscosity of the formulation with 0.15%w/w k-carrageenan and 0.01% w/w SHMP was higher than those of others containing 0.03 and 0.04% w/w SHMP. To some extent, this can be attributed to the fact that a higher SHMP concentration leads to more ion chelating, lower electrostatic repulsion and a lower viscosity. However, at this stage this is just a supposition which needs to be investigated more thoroughly in the future. It is worth noting that a viscosity increase upon inclusion of hydrocolloids, added for stabilization purposes, can usually cause undesirable changes such as viscosity rise. In this respect, SHMP seems to play a very important role in viscosity reduction in addition to improving the thermal stability of the formulation. Joyce et al. (2018) used different temperatures and Ca ion to modify the viscosity of WPI-containing system. According to their results, heat induced protein denaturation and the ability of Ca ion in aiding the aggregation of protein into WPI–Ca–WPI complexes, were effective in enhancing the apparent viscosity.

Particle size and zeta potential

At the absence of SHMP and without any heat treatment, it can be seen (Table 2) that zeta potential of all samples were in the range -21 to -36 mV just about sufficient to provide the required physical stability. Moreover, the highest zeta potential (-35 and -36 mV) belonged to the samples containing carrageenan, despite its lower concentration in comparison with other hydrocolloids. As stated before (section 3.2), carrageenan molecules have the highest negative charge amongst the studied hydrocolloids. As expected, the smallest level of electrical charge belonged to guar, a non-adsorbing hydrocolloid (-21 mV). With regard to LMP, being an adsorbing hydrocolloid, the zeta potential was smaller than carrageenan, even though it was used at higher concentrations in comparison with carrageenan. This is attributed to the lower charge density of LMP. These findings confirm that the optimum concentrations of various hydrocolloids for stabilization purposes depends on their charge density, with the higher charge density requiring a lower concentration.

By adding the required amounts of SHMP, the zeta potential increased (around -1 to -6 mV) depending on the type of hydrocolloid and SHMP concentration used. It has already been demonstrated that a SHMP solution (0.08%w/w) has a zeta potential of around -12 mV, almost independent of pH (Wang et al. 2014). Hence, once a certain concentration of SHMP was added to the dispersions, the alteration of zeta potential could be expected due to the contribution of SHMP. Again, these samples were quite stable despite the reduction in their viscosity. This finding indicates the lesser role played by the viscosity, as opposed to the electrostatic repulsion, in providing the required stability for the dispersion, with one exception to this trend possibly being guar (Azarikia and Abbasi 2010; Abbasi and Mohammadi 2013; Teimouri et al. 2017).

Interestingly, sterilization process diminished the zeta potential except those with LMP and i-carrageenan. This reduction was more intense in the case of guar, being a non-adsorbing hydrocolloid. In line with the observed changes in zeta potentials, the particle sizes were also altered. In the case of samples containing LMP and i-carrageenan, the average size slightly decreased, but for xanthan and guar it actually increased by almost 3 to 5 times, whereas the minimum change was observed for the k-carrageenan based system. It is evident that the particle size increment occurred due to the diminishment of electrostatic repulsion between the particles, as well as the denaturation of the proteins. Both of these led to the aggregation of the proteins into larger particles. It has been suggested that due to the polyanionic nature of SHMP, this molecule can be attracted and oriented along the charged sites of other polyelectrolytes, such as proteins or hydrocolloids. As a result, the charge repulsion between molecules (protein–protein, hydrocolloid–hydrocolloid, and protein–hydrocolloid) at pH values above their isoelectric points could increase. Conversely, at pH values < pI, SHMP can induce protein precipitation by cation–anion interactions (Rulliere et al. 2012). There is also a possibility for binding of the negatively charged phosphate ions of SHMP to positively charged residues of proteins or hydrocolloids, as well as carboxyl or phosphoseryl groups via divalent ion bridging (Zittle 1966). The direct binding of SHMP to positively charged residues of casein proteins and the indirect binding via calcium bridges have also been suggested as potential mechanisms for enhancing aggregation (Mizuno and Lucey 2005; 2007; de Kort et al. 2012). These findings reveal that SHMP is not only a chelating agent but also a deflocculation or denaturation prohibitory agent with probable effects on hydrocolloids and their interactions with protein molecules. Further systematic studies are needed to elucidate the possible underlying mechanisms involved in such a modification of the hydrocolloid–protein interactions.

Sensory evaluation

Despite the minor effect of hydrocolloid type on the color and odor of the dispersions (Fig. 4), the differences were not statistically significant ($p>0.05$). Such results were expected since no coloring agent was used and the formulation of all dispersions were similar, with only difference being the type of hydrocolloid used. In contrast, the consistency was significantly different ($p<0.05$) as those with LMP and guar had the lowest and the highest, respectively. Furthermore, the consistency of those formulations using xanthan, i- and k-carrageenans were almost similar to each other. In terms of consistency and taste, the sample stabilized with LMP was the most preferred one due to its similarity to commercially available dairy based drinks. On the other hand, the lowest consistency and taste scores belonged to formulations with guar, because of its high viscosity and exhibiting a specific flavor. The scores for mouth feel followed approximately a similar trend to the consistency results.

CONCLUSIONS

Based on visual, rheological, microstructural and sensory evaluations, it was found that fabrication of mildly heat stable (85^{o}C, 2 min) WPC dispersions (4% w/w) at pH 7.0 is promising if specified amount of xanthan, HMP, guar or locust bean gum are used. However, such a thermal processing, at these lower temperatures, cannot always guarantee the required standard of safety. Therefore, the formulations should be sterilized at 121.5^{o}C for 15 min in order for the food safety obligations to be met. However, this caused the structural and colloidal stability of the product to fail. The findings of the present study demonstrated that using optimum amounts of SHMP alongside specific ratios of either LMP, i-carrageenan, k-carrageenan, xanthan or guar gums led to a satisfactory physical stability of the dispersion, as well as ensuring microbiological safety. However, amongst the various gums, only LMP and i-carrageenan showed the desirable rheological, particle size and sensory properties. The accurate concentration of needed SHMP was a key parameter in reducing the viscosity and prevention of protein denaturation in the presence of various hydrocolloids. The provision of these functions is very dependent on the structural features of proteins and hydrocolloids used, their ratios, intensity of the thermal process, the ionic strength and pH. This is one of the handful reports in this field that provides extensive research to elucidate the plausible

mechanisms of SHMP function on reduction of viscosity and the enhancement of heat stability necessary in formulation of whey protein based beverage. Further work is underway in our food colloids lab to further investigate the potential of SHMP in viscosity decrease and stabilization of whey proteins at fundamental and application levels.

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Table 1. The required concentration of different hydrocolloids for stabilizing WPC dispersion (4% w/w) at different pHs (4.0 and 7.0) with no heat treatment.

Hydrocolloid type	Hydrocolloid	pH	Concentration (% w/w)
Adsorbing	LMP	4.0	0.80
		7.0	0.50
	Xanthan	4.0	0.30
		7.0	0.20
	k-Carrageenan	4.0	0.60
		7.0	0.15
	i-Carrageenan	4.0	NA
		7.0	0.15
Non-adsorbing	Guar	4.0	0.40
		7.0	0.35

NA=Not applicable as sample was gelled.

Table 2: The effect of various hydrocolloids, presence (✓) or absence (x) of sodium hexamethaphosphate (SHMP) and sterilization process on the particle size and zeta potential of WPC-based beverage (protein 4%, sugar 8% and cacao powder 1% w/w) at pH 7.0.

Hydrocolloid type	SHMP (% w/w)	Sterilized (121.5 °C, 15 min)	Volume (%)	Particle Size (nm)	Avg Size (nm)	Zeta potential (mV)
LMP (0.50% w/w)	**0.15**	✓	63	144	217	-33
			37	362		
	0.15	**x**	75	284	234	-33
			25	75		
	0	**x**	-	-	-	-27
i-Carrageenan (0.15% w/w)	**0.03**	✓	97	262	357	-39
			3	2137		
	0.03	**x**	24	1150	440	-39
			76	217		
	0	**x**	-	-	-	-36
k-Carrageenan (0.15% w/w)	**0.01**	✓	80	493	326	-29
			20	85		
	0.01	**x**	99	271	279	-34
			1	5144		
	0	**x**	-	-	-	-35
Xanthan (0.20% w/w)	**0.04**	✓	79	289	900	-26
			21	39		
	0.04	**x**	68	290	247	-33
			32	114		
	0	**x**	-	-	-	-31
Guar (0.50% w/w)	**0.03**	✓	16	1071	1404	-16
			84	60		
	0.03	**x**	53	358	262	-27
			47	96		
	0	**x**	-	-	-	-21

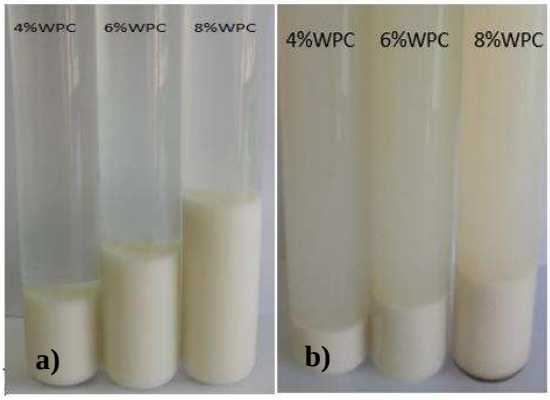

Fig. 1. Effect of pH and WPC concentration (4, 6 and 8% w/w) on the protein solubility at **a**) pH 4 and **b**) pH 7.

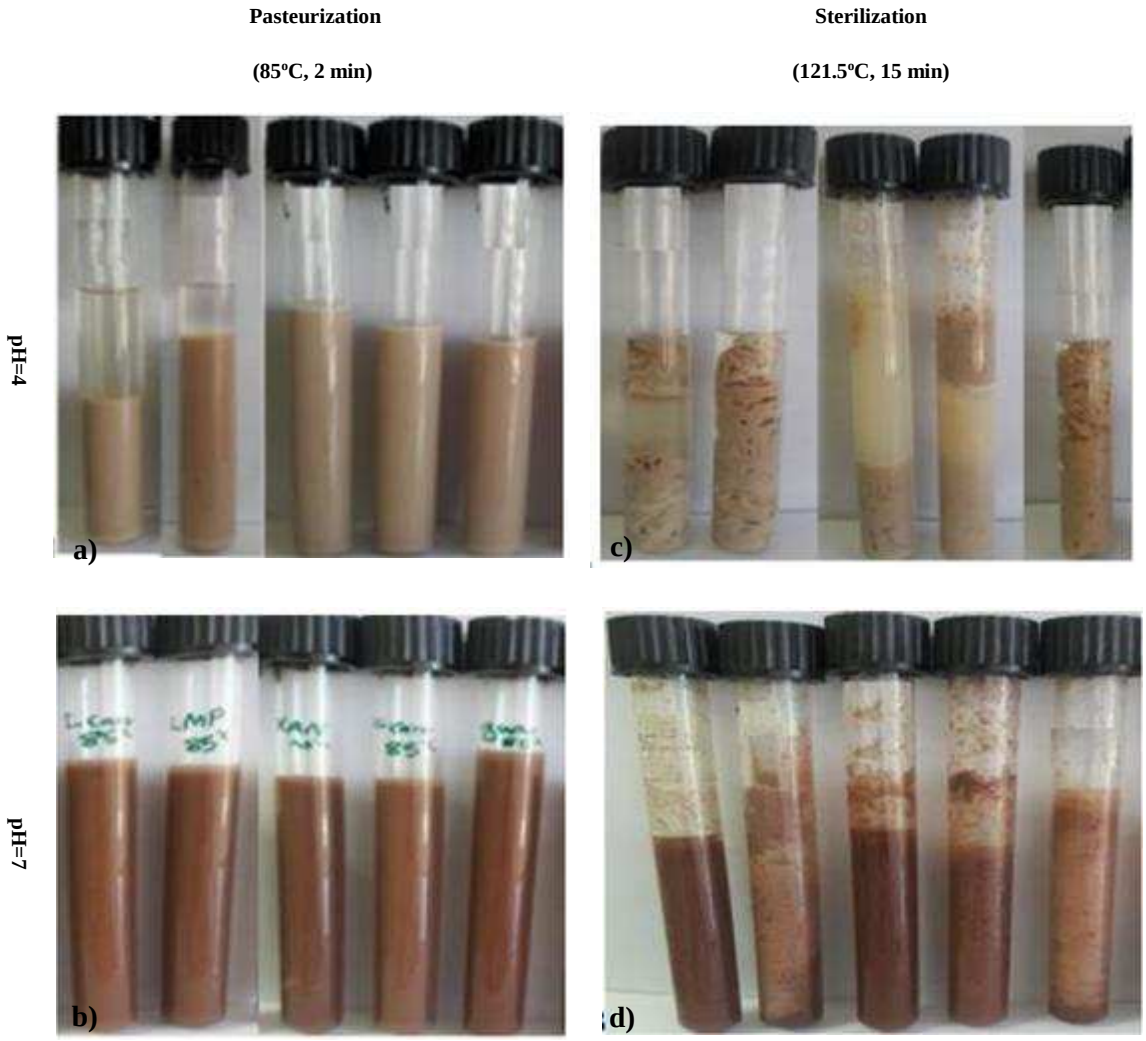

Fig. 2. Effect of pasteurization (a and b) and sterilization (c and d) processes on the stability of WPC-based beverage (protein 4%, sugar 8% and cacao powder 1% w/w) at the presence of various hydrocolloids: i-carrageenan (0.15% w/w), LMP (0.50% w/w), xanthan (0.20% w/w), k-carrageenan (0.15% w/w), and guar (0. 50% w/w) from left to right at pHs 4.0 (a and c) and 7.0 (b and d).

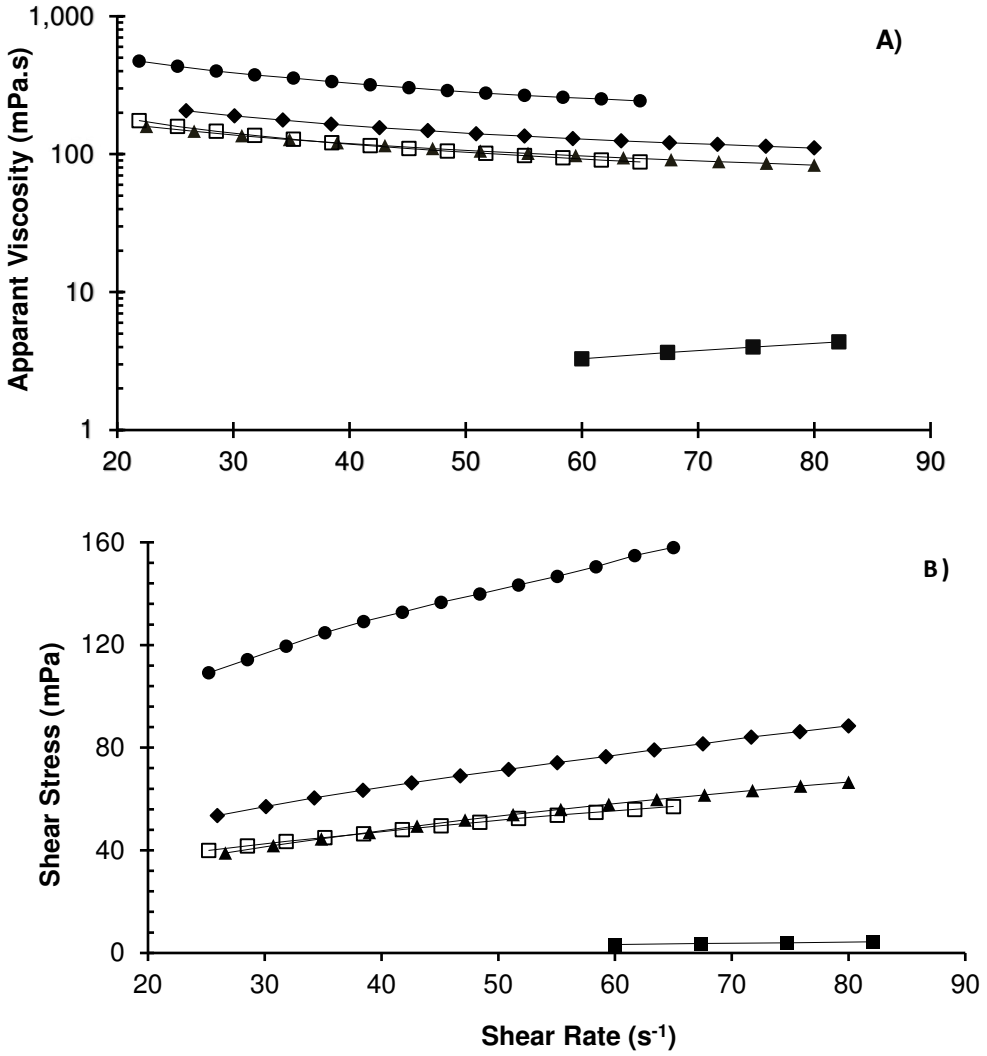

Fig. 3. Comparison of the effect of hydrocolloid type and SHMP concentration (●: guar 0.35 + SHMP 0.03, □: xanthan 0.2 + SHMP 0.04, ▲: i-carrageenan 0.15 + SHMP 0.03, ♦: k-carrageenan 0.15 + SHMP 0.01 and ■: LMP 0.5 + SHMP 0.15) on **A)** apparent viscosity and **B)** flow behavior (shear stress vs shear rate) of the sterilized WPC-based beverage (protein 4%, sugar 8% and cacao powder 1% w/w).

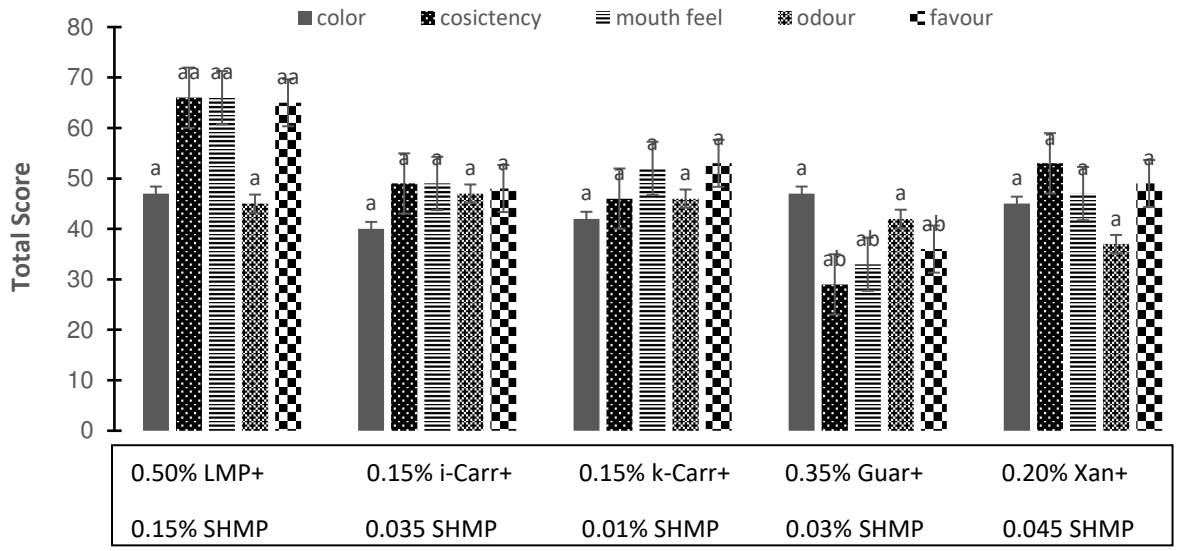

Fig. 4. Comparison of sensory characteristics (from left to right: color, consistency, mouth feel, odor, and taste) of the sterilized (121.5ºC, 15 min) WPC-based drink (protein 4%, sugar 8% and aloe vera powder 0.02% w/w) pH 7.0. Different letters show significant difference ($p < 0.05$).