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Original article

Effect of pH-shift Treatment and Ultrasonication on the Physical Stability and Properties of Hemp Seed Milk

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Abstract

Hemp seed milk is a growing beverage with excellent nutritional content and minimal allergenicity, which offers a tasty substitute for other plant-based milk types. During this research, we investigated the individual and combined impact of pH shift and ultrasound (US) on the stability characteristics of hemp seed milk. The effect of pH shift and US were investigated on the physico-chemical properties of hemp, milk, sedimentation index, rheological properties, color, oBrix, physical stability, titratable acidity, and emulsion stability index (ESI) measurements. According to the obtained data, applying individual US techniques showed the best results, with the highest stability characteristics and better rheological properties, the highest L* (lightness) and oBrix values, and the lowest titratable acidity values. Interestingly, the individual application of the pH-shift technique showed the lowest physical stability results. In comparison, pH shift treatment combined with the US demonstrated moderate stability. Thus, the pH shift and the US are remarkable non-thermal processing methods for producing stable hempseed milk.

Keywords: Hemp Seed Milk, Ph-Shift, Ultrasonication, Viscosity, Rheological Properties.

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INTRODUCTION

The acknowledged nutritional benefits and minimal allergenic potential of hemp seeds have contributed to a surge in the consumption of hemp products in recent years. Hemp seed market size was assessed at $5.1 billion in 2022 and is expected to reach $11.7 billion by 2032, rising at a CAGR of 8.9% between 2023 and 2032, according to a recent research by Allied Market Research titled, "Hemp Seed Market" (Hemp Seed Market).

Since both hemp and marijuana are produced from the same plant, useful or industrial hemp is referred to by the Latin term *Cannabis Sativa*. The primary psychoactive ingredient, delta-9 tetrahydrocannabinol (THC), is present in approximately 0.3% to 1.5% of industrial hemp, but it is present in 5% to 10% or more of marijuana (Besir, A. et al., 2022). About 25% of hemp seeds are protein, and 30% are oil. The oil has a high concentration of polyunsaturated fatty acids (PUFAs), particularly linoleic (-6) and -linolenic (-3) acids (Wang et al., 2018). To provide nutritional advantages, hemp seed milk is made from hemp seeds. Most of the original nutrients remain in hemp seed milk, making it a highly nutritious beverage. Hemp seed milk is regarded as a pleasant substitute for dairy, soy, and nut milk, lactose-free, and low in allergens (Besir, A. et al., 2022).

Hemp seed milk is an oil-in-water (O/W) emulsion system that is unstable and tends to flocculate like other milk substitutes created from plant seeds, decreasing quality and shortening shelf life (Wang et al., 2018). Plant-based milk is a colloidal system that contains large-sized dispersed particles such as fat globules, solid raw material particles, proteins, and starch granules. Because of solid particle sedimentation, it is challenging to produce a stable product that can be kept in stock for an extended period. Therefore, many methods have been applied to increase hemp seed milk's stability, like emulsifiers or stabilizers. However, this method is not recommended due to economic causes and health issues. For instance, several research have indicated that long-term intake of artificial emulsifiers may result in chronic inflammatory diseases linked to obesity and metabolic syndrome. As a result, there is an increasing need for affordable alternative technologies and food items devoid of additives (Cani and Everard, 2015). Such techniques as enzymatic hydrolysis (Yin, S. et al., 2008) and acylation (Yin, S. et al., 2009) have been applied. Wang, Q. et al. (2018), in their published research, studied the application of pH-shift and high-pressure homogenization processes to produce additive-free hemp seed milk with a focus on its physical and oxidative stability (Wang et al., 2018). In recent years, high-pressure homogenization (HPH) has also been developed to assist in creating stable O/W food emulsions. Oil is mechanically divided into tiny droplets by HPH, increasing the overall surface area, uniformizing the size distribution, and enhancing stability.

However, a molten globular or fibrous conformation is produced when a protein solution is brought to a high alkaline or acidic pH and maintained briefly to promote structural unfolding, followed by a brief incubation at the neutral pH to allow partial refolding. It has been demonstrated that this procedure is known as pH shift. The resulting structure exhibits molten globule characteristics, which significantly improves proteins' solubility and emulsifying and film-forming capabilities (Jiang et al., 2018). Hence, pH shift has been effectively used to treat soy and pea proteins to increase their emulsifying capabilities (Wang, Q. et al., 2018).

Moreover, another promising technique for increasing the stability of plant-based milk is ultrasonication. Ultrasonication is applying high-power ultrasound to a specific product; it may cause cavitation. Cavitation is a process wherein the propagation of high-power sound waves causes the emergence of small gas or vapor bubbles that develop inside the sample. Extreme temperatures and pressures are created as a result of this process. These extreme conditions have the potential to deactivate enzymes and change the secondary structure of proteins, altering both their nutritional value and functional capabilities (Vanga et al., 2020). However, ultrasound is divided into two types: low-intensity ultrasound (with a power intensity of less than one wcm-2 and a frequency of five to ten MHz) and high-intensity ultrasound (with a power intensity of ten to one hundred wcm-2 and a frequency of twenty to one hundred kHz), the latter of which is used in food processing technologies (Sarangapany, A. et al., 2022). Hence, the effect of US on plant proteins has been evaluated in many previous studies on soy, pea, black bean, almond, and wheat proteins (Vanga, S. et al., 2020) and peanut (Salve et al., 2019). However, no study has evaluated the effect of ultrasonication on the structural properties, organoleptic, and functional properties of hemp seed milk. The objective of the current study was to make additive-free hemp seed milk using a pH shift and US procedure while examining its physical, organoleptic, and functional characteristics.

MATERIALS and METHODS

Seed material

During this research, defatted hemp seeds have been utilized for milk production. The oil has been previously extracted by cold pressing technique.

Oil extraction by cold pressing

According to the literature, the high lipid content of seeds and nuts may result in undesirable phase separation and decreased product stability; thus these components are eliminated during processing (Tangyu, M. et al., 2019). The current research used cold pressing to extract oil from the hemp seeds. In a technical procedure known as pressing, oil is mainly extracted (drained) from the hemp seeds using mechanical pressure. Cold pressing is performed by directly pressing raw/dried seeds on a continuous screw press at low temperature (Rabrenović et al., 2014).

Hemp Seed Milk Preparation

Each experimental run began with fresh hemp seed milk preparation, according to the method described by Wang, Q. et al. (2018), with slight modifications. Briefly, defatted seeds were ground in a 1:8 w/v ratio of deionized water with an ultra-turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany) at 12000 rpm for 10 min, then filtered using Stainless Steel Sieve, to ensure the consistency of milk and to remove particles.

pH Shifting and Ultrasonication Treatments

The prepared hemp seed milk underwent three treatments: pH shifting alone, ultrasonication alone, and a combination of both. The control sample had not been modified, only stirred. For pH shift, the pH of hemp seed milk samples was adjusted to 12 using 1 M NaOH at room temperature (The pH value of the freshly produced hemp seed milk was 6.95 ± 0.02) and then brought back to pH 7 using 1 M HCl. US treatment was applied using a VibraCell VC750 ultrasonic processor (Sonics & Materials, Inc., Newtown, CT, USA) at 20 kHz and 750 W. Using a 13 mm diameter probe, the sonic energy was transferred into hemp seed milk. An ice bath was used to prevent overheating from ultrasonication, which might result in protein denaturation (Kahraman, O. et al., 2022).

The sonication pulse duty cycle (5 s on, 5 s off) was set to 100% amplitude. The hemp seed milk samples were sonicated for 5 or 10 minutes during the treatment with ultrasonication alone or with pH shift. For the samples of pH shifting and ultrasonication combined treatments, the hemp seed milk samples were exposed to pH shift and then immediately sonicated for 5 or 10 min.

The produced milk was immediately kept at 4°C in airtight containers, and the analysis carried out within 24 hr after milk production.

Physical stability/ Creaming index

Two 20 mL of processed hemp seed milk were immediately transferred to graduated tubes, sealed, and kept in a refrigerator (4 ± 2°C) to calculate the sedimentation index. According to the Indu, C. et al. (2019) approach, measurements were taken every 24 hours until the samples indicated total separation, at which point the separation index (SI) was determined using the equation given below (Indu, C. et al., 2019).

$$\text{Creaming index (\%)} = \frac{\text{Height of the aqueous layer HA}}{\text{Total height of emulsion HE}} \times 100$$

Color

A colorimeter (CR-400, Konica Minolta, Inc., Japan) was used to measure color properties. The study used the CIE-L^* a^* b^* color coordinate system. L^* value is a lightness measuring factor, ranging from blackness (0) to whiteness (100). a^* value varies from greenness (-60) to redness (+60), whereas b^* value goes from blueness (-60) to yellowness (+60) (Zaaboul et al., 2019).

Total soluble solids (Brix)

On a Brix scale of 0-100, the total soluble solids of the samples were calculated using a refractometer at room temperature (Salve, A. et al., 2019).

Solid particle sedimentation (SPS)

Solid particle sedimentation was conducted according to the method described by (Gul et al., 2017). Each sample was centrifuged at 2500 g for 20 min using 10 mL of the solution. The amount of solid deposition at the bottom of the tube was given as a percentage (w/w)(Gul et al., 2017).

Titratable acidity

The samples (10 mL) were mixed with 10 ml of deionized water after that, titrated with 0.1 N sodium hydroxide (with indicator phenolphthalein) in order to assess the effect of treatment on the titratable acidity of hemp seed milk (Salve, A., et al., 2019).

Rheological properties

Rheological characterization of hemp seed milk was carried out by using Haake Mars III rheometer (Thermo Scientific, Germany) with a cone and plate system (35 mm diameter, 0.105 mm gap, 2_ angle). The temperature was maintained constant at 25ºC by a Peltier plate system. The steady-state shear experiments were measured by shearing the samples at linearly increasing shear rates from 1 to 100 s^{-1} through 120 s. Product flow behavior was modeled using the Ostwaldde-Waele model.

$$\eta_{app} = K\hat{y}^{n\_1}$$

where η_{app} is apparent viscosity (Pa s), ŷ is the shear rate (s^{-1}), K the consistency index (Pa sn), and n the flow behavior index (dimensionless) (Zaaboul, F. et al., 2019; Atalar, I. et al., 2019).

Temperature effects on viscosity

For evaluating the effect of increasing temperature on the viscosity characteristics of hemp seed milk, the temperature of the samples was increased with a heating rate of 0.5°C/min from 10 to 80°C as described perviously by Iskakova & Smanalieva, (2021).

Statistical analysis

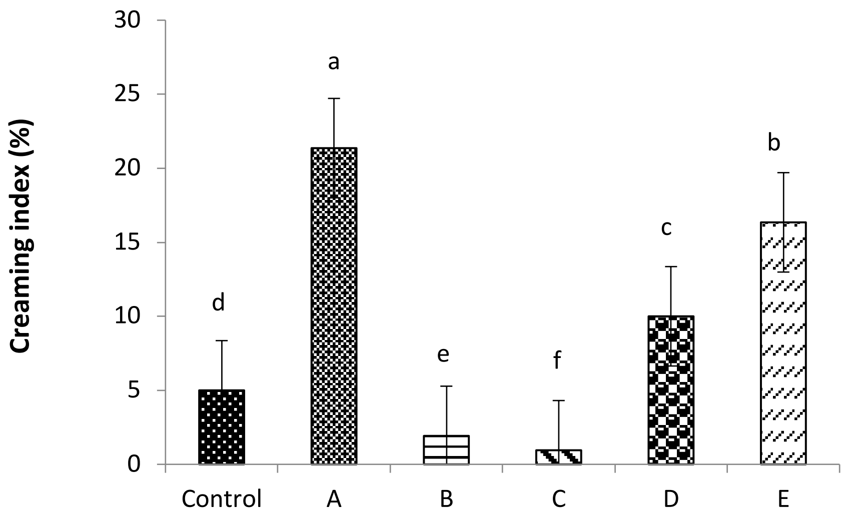
Statistical analysis of data was performed by one-way ANOVA analysis of variance using Minitab software. Thedata was expressed as the mean of triplicate estimation ± standard deviation and at $p<0.05$ level, the differences were considered statistically significant.

RESULTS and DISCUSSION

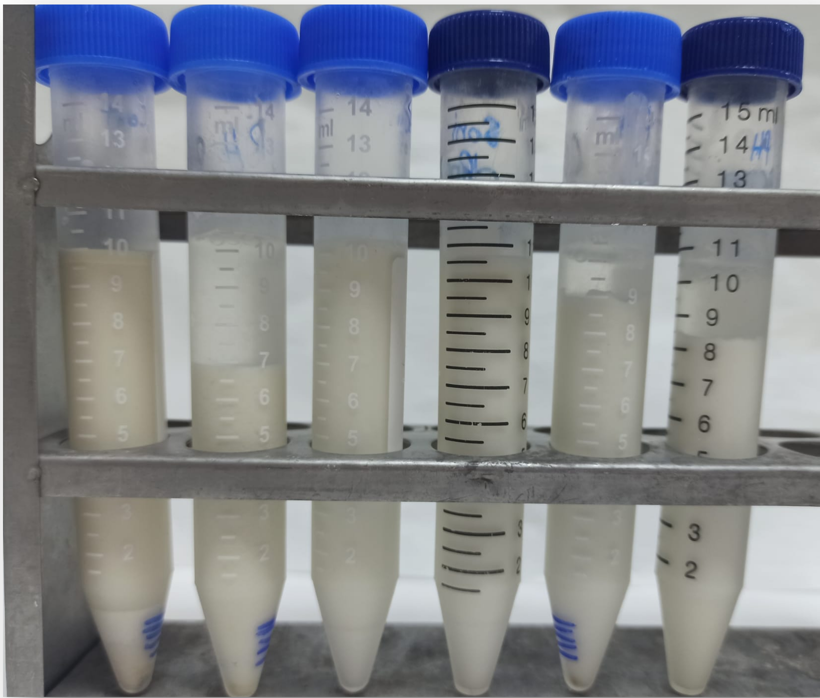
Creaming index

The creaming index is crucial to assess the stability of hemp and milk. The stability of hemp seed milk increases with a decrease in the creaming index %. As shown in Fig. (1A, 1B), the creaming index of hemp seed milk decreased when the duration of ultrasonication was increased. This could be caused by the cavitation effect, which reduces the size of fat or protein globules and prevents flocculation (Indu, C. et al., 2019). However, the pH-shift treatment showed a noticeable increase in the creaming index. The literature has reported that the pH-shift method, instead of directly changing the pH value to 7-8, could decline the protein solubility and emulsion stability due to the formation of insoluble aggregates (Jiang et al., 2018).

(A)



(B)



Control A B C D E

Fig. 1. Creaming index of hemp seed milk after 48 h of low temperature storage (4 ± 2 °C): **(A)** Creaming index of hempmilk, where a’, b’, c’ , d’, e’,and f’ represent statistical differences and **(B)** in sequence photos representing the separation of phases of all treatments. Where Control: without any treatment, A: pH-shift, B: 5 min of US, C: 10 min of US, D: pH-shift & 5 min US, E: pH- shift & 10 min US.

Color properties

The **Table 1.** shows the effect of pH shift and US processing on the colour parameters of hemp seed milk. On analyzing *L** value (lightness), samples treated with with the US statistically showed the highest L* value. Samples treated with pH-shift and US demonstrated high L* values with no statistical differences. As well as Control (untreated) and only pH-shift treated samples showed the lowest values with no significant differences. The increase in light scattering and greater lightness values of the samples can be attributed to the tendency of US treatment to cause the dispersed particles to increase by minimizing their size (Sarangapany, A. et al., 2022). The same outcomes have also been found for peanut milk; ultrasonication increased the color characteristics of the milk, particularly the lightness or L* value (Salve, S. et al., 2019). The* (redness) value showed no statistical differences among all samples except for the control sample, which demonstrated the highest value. For b* (yellowness) value, control and pH-shift treated samples showed the highest values.

Table 1. Effect of pH shift and US treatment on the colour of hemp seed milk

Treatments	*L**	*a**	*b**
Control	85.960 ± 0.18[c]	-0.5333 ± 0.12[a]	10.7133± 0.14[a]
pH-shift	86.497 ± 0.18[c]	-0.7600 ± 0.05[b]	9.9167 ± 0.10[b]
US-5 min	88.137 ± 0.26[b]	-0.7033± 0.06[ab]	8.8667 ± 0.05[c]
US-10 min	89.163± 0.22[a]	-0.8467 ± 0.04[b]	8.5233 ± 0.03[d]
pH-shift & US-5 min	88.313± 0.17[b]	-0.7633 ± 0.03[b]	8.27 ± 0.09[d]
pH-shift & US-10 min	88.117 ±0.17[b]	-0.7400 ± 0.02[b]	8.4233 ± 0.11[d]

Each value is represented by its mean and standard deviation (n=3). Values in the same column that are vertically present the same superscript letters do not differ statistically (p > 0.05).

Soluble solids (°Brix) and titratable acidity

The effects of treating hemp seed milk with pH- shift and/or US on total soluble solids (TSS) and titratable acidity of hemp seed milk are shown in Table 2. It has been observed that the sonicated samples have shown to have the highest TSS value 4.8 °Brix. Increasing the US time significantly affected the °Brix value of treated samples ($p<0.05$). However, similar results have been reported in previously published studies (Salve, A. et al., 2019; Maghsoudlou, Y. et al., 2016). Employing the power of sonication can enhance the breaking down of cell walls to speed up the release of their contents. The control, pH-shift, and pH and US-treated samples presented no significant difference. Additionally, it was shown that the titratable acidity of hemp seed milk decreased significantly as the US treatment duration increased. This may be caused by a change in the charge of particles by the sonication process (Salve, A. et al., 2019). However, pH-shift treatment alone or combined with US showed a statistically increased titratable acidity value.

Table 2. Effects of pH shift and US processing on physic chemical properties of hemp seed milk

Treatments	°Brix (%)	Titratable acidity	Sedimentation index
Control	1.95 ± 0.212[c]	0.043 ± 0.002[d]	37.16 ± 0.1[e]
pH-shift	1.95 ± 0.283 [c]	0.166 ± 0.007[b]	42.47 ± 0.2[c]
US-5 min	3.95 ± 0.141[b]	0.058 ± 0.004[C]	37.38 ± 0.1[d]
US-10 min	4.8 ± 0.07[a]	0.048 ± 0.006[cd]	34.04 ± 0.2[f]
pH-shift & US-5 min	1.95 ± 0.141[c]	0.207± 0.002[a]	46.95 ± 0.08[a]
pH-shift & US-10 min	2.0 ± 0.07 [c]	0.154 ± 0.005[b]	45.97± 0.2[b]

Each value is represented by its mean and standard deviation (n=3). Values in the same column that are vertically present the same superscript letters do not differ statistically (p > 0.05).

Sedimentation index

The higher sedimentation value is associated with lower product stability. The results showed that ultrasonication significantly ($p< 0.05$) lower sedimentation values, Fig.2 In another word, treated hemp seed milk without pH- shift resulted in a more stable emulsion. The same result has been reported by

Wang, Q. et al. when they studied the effect of pH shift and homogenization pressure on hemp seed milk stability (Wang, Q. et al., 2018). Whereas the application of pH-shift or the combined application of pH-shift and the US unexpectedly statistically ($p<0.05$) increased the sedimentation values. The improvement in the sedimentation index in the US-treated samples is driven by some factors, including the reduction in particle size that facilitated intermolecular interactions. Additionally, denaturing hemp proteins aided in their unfolding, revealing their active sites and raising the hydrophobicity of their surfaces (Salve, A. et al., 2019). On another hand, it has been discussed in the literature that the pH-shift process could have resulted in undesirable molten globule conformation in some cases. It is known that the pH-shift process increases the solubility of proteins, but after an excessive pH treatment, solubilized proteins must immediately go through refolding; otherwise, the pH-shift process results in a decreased protein solubility due to the formation of insoluble aggregates; this was observed in the literature on soy globulins (Jiang, J. et al., 2018), a similar result was found out during our study.

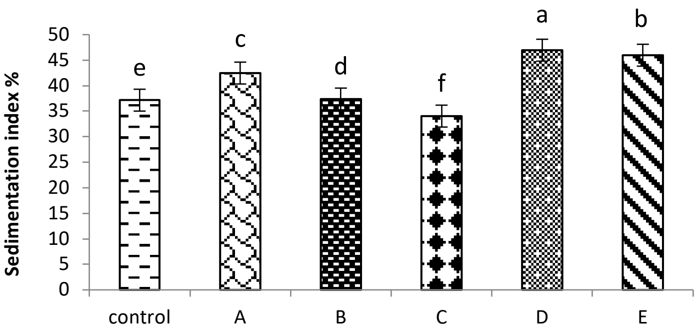


Fig. 2. Sedimentation index of hemp seed milk, where a', b', c' , d', e'and f' represent statistical differences. Control: without any treatment, A: pH-shift, B: 5 min of US, C: 10 min of US, D: pH-shift & 5 min US, E: pH- shift & 10 min US.

Rheological properties

Effect of pH shift and US on rheological parameters

The pH shift and US treatments considerably impacted the viscosity of the hemp seed milk sample **Table. 3** Compared to the untreated (control) milk sample (42.4 mPa.s). For all samples, hemp seed milk had non-Newtonian fluid flow characteristics - have varying relationships with shear stress and non-constant viscosity-. Viscosity was estimated with a Herschel–Bulkley equation. According to the results, only pH-shift treated samples showed significantly reduced viscosity values (12,68 mPa.s) compared to the control samples. As previously discussed, the pH-shift process causes the protein structure to unfold, and when the pH is returned to neutral, where intramolecular charge repulsions are significantly reduced, some degree of refolding occurs. During this study, we noticed phase separation and aggregation, which could be associated with the formation of undesired molten structures during the pH-shift process. However, in their previous published research, Nicoud, L. et al. (2015) claimed that the aggregate distribution's polydispersity is why an aggregated protein solution has a lower viscosity than a monomeric protein solution (Nicoud et al., 2015). Likewise, the viscosity of pH-shift treated samples was statistically lower than those treated with pH shift and US (5 and 10 min). It is worth mentioning that according to the literature, US treatment (400, 600, and 200 W) alone has decreased the viscosity of peanut milk (Salve, A. et al., 2019), and this is consistent with our results for only US-treated samples (5 and 10 min) which had similar results with no satistical differences. The US impacts the milk by changing the balance and reducing the larger particles into smaller ones. The cavitation treatment caused rheological changes in milk samples that reduced their viscosity, increased their fluidity, and decreased their tendency to behave in a pseudoplastic manner (Salve, A. et al., 2019) Table 3.

Table 3. Rheological parameters of pH-shift and US treated hemp seed milk.

	K (Pa sn)	n (-)	R^2	η_{50} (mPa s)
Control	0.0016 ± 0.000^b	0.885 ± 0.044^a	0.757 ± 0.069^b	42.48 ± 1.92^a
pH-shift	0.053 ± 0.002^b	0.636 ± 0.008^b	0.994 ± 0.000^a	12.68 ± 1.39^c
US-5 min	0.006 ± 0.004^b	0.643 ± 0069^b	0.732 ± 0.053^b	1.332 ± 0.57^d
US-10 min	0.002 ± 0.008^b	0.881 ± 0.105^a	0.964 ± 0.008^a	1.368 ± 0.04^d
pH-shift & US-5 min	0.475 ± 0.002^a	0.253 ± 0.035^c	0.992 ± 0.003^a	24.88 ± 2.72^b
pH-shift & US-10 min	0.369 ± 0.05^a	0.298 ± 0.02^c	0.994 ± 0.003^a	23.46 ± 1.53^b

Each value is represented by its mean and standard deviation (n=3). Values in the same column that are vertically present the same superscript letters do not differ statistically (p>0.05).

Temperature effects on viscosity

Food flow behavior can be affected by temperature variations (Forster and Ferrier, 1979). Likewise, Simuang et al., (2004), in their study, showed that the viscosity of coconut milk was significantly affected by heat treatment. Thus, the flow behavior of hemp seed milk at various temperatures was investigated. The temperature influence on rheological parameters is shown Fig. 3.

Data showed a reduction in the viscosity over continuous heating until it reached a certain extent, the apparent viscosity changed slightly and started to increase at higher temperatures. The literature has reported that increasing temperatures cause liquid viscosity to decrease. The result of increasing a liquid's temperature is a decrease in cohesive forces and an increase in the rate of molecular interchange. In other words, the increase in temperature causes kinetic or thermal energy and the molecules become more mobile (Iskakova, J. 2021). On the other hand, increasing viscosity with continuous heating is suggested to be due to protein denaturation and subsequent association/polymerization. However, Liu and Chang, (2007) reported the same result: soy milk's viscosity increased as the heating time increased. They claimed that heating caused both 11S and 7S proteins to dissociate. The dissociated polypeptides and subunits of the 7S and 11S proteins then interacted (Liu & and Chang, 2007). Hence, the maximum denaturation of 7S protein, which caused a sharp increase of soymilk viscosity, was observed at 70ºC.

Likewise, according to the literature, hemp proteins are sensitive and have a lower denaturation temperature, therefore, it is suggested to keep heat treatment below 80°C in order to retain their heat stability and solubility (Besir et al., 2022). The maximum denaturation temperature of hemp protein was not observed during the current study, but it has been noted that the maximum decline and the start of an increase in hemp seed milk viscosity of all samples occurred between 50 and 70ºC. We propose that an increase in viscosity at these levels indicates the beginning of protein structural changes, however it may not be a complete denaturation or coagulation. For investigating the effect of pH-shift and US treatment on hemp seed milk viscosity during the application of various temperatures, after a steady and moderate drop in the viscosity of samples treated with pH-shift alone or with US (5 and 10 min), we observed a sharp increase in the viscosity, which indicates the highly sensitivity and heat instability of those samples Fig.3. These results are highly compatible with the results we discussed previously for measuring sedimentation and creaming indexes for these samples. Whereas, control and only US (5 and 10 min) treated hemp seed milk samples showed a less severe increase in the viscosity, which indicates a more cohesive structure and consequently more heat stability.

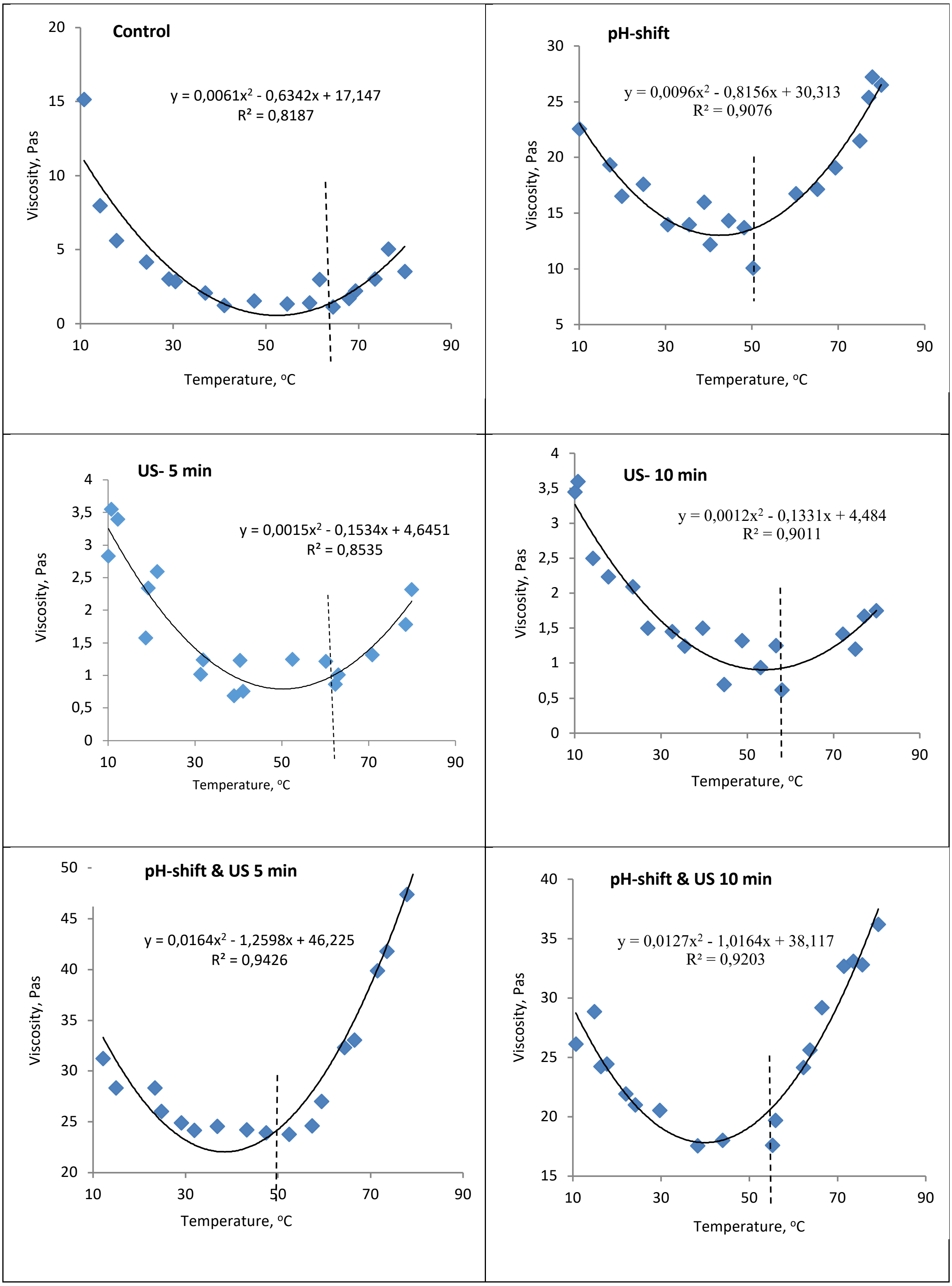


Fig. 3. Temperature effect on the viscosity characteristics of hemp seed milk samples.

Conclusion

Even though each of the pH shifts and US processes had a different stabilizing impact on the hemp seed milk emulsion, their combination did not yield the best results. The pH shift treatment encouraged protein interactions that eventually resulted in large clusters and aggregates, which negatively impact the stabilization of the emulsion. Hemp seed milk treated only with US showed such interactive structures that were stable against coalescence coagulation and showed better temperature-related viscosity characteristics. Since the pH-shift process showed unstable emulsion, it is suggested for the future to apply the direct change of pH to 7-8 (without incubation period), which is expected to be more effective. Finally, last but not least, using US or combining pH shift with US with various modifications may present new potential for producing non-thermally hemp seed milk without stabilizers or emulsifiers.

Data Availability

The datasets of their study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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