

## Impact of high-intensity thermosonication treatment on spinach juice: Bioactive compounds, rheological, microbial, and enzymatic activities

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### ABSTRACT

To study the impacts of thermosonication (TS), the spinach juice treated with TS (200 W, 400 W, and 600 W, 30 kHz, at  $60 \pm 1$  °C for 20 min) were investigated for bioactive compounds, antioxidant activities, color properties, particle size, rheological behavior, suspension stability, enzymatic and microbial loads. As a result, TS processing significantly improved the bioactive compounds (total flavonols, total flavonoids, total phenolic, carotenoids, chlorophyll, and anthocyanins), antioxidant activities (DPPH and FRAP assay) in spinach juice. Also, TS treatments had higher  $b^*$ ,  $L^*$ , hue angle ( $h^\circ$ ), and chroma (C) values, while minimum  $a^*$  value as compared to untreated and pasteurized samples. TS processing significantly reduced the particle size, improved the suspension stability and rheological properties (shear stress, apparent viscosity, and shear rate) of spinach juice as compared to the untreated and pasteurized sample. TS plays a synergistic part in microbial reduction and gained maximum microbial safety. Moreover, TS treatments inactivated the polyphenol oxidase and peroxidase from 0.97 and 0.034 Abs min<sup>-1</sup> (untreated) to 0.31 and 0.018 Abs min<sup>-1</sup>, respectively. The spinach juice sample treated at a high intensity (600 W, 30 kHz, at  $60 \pm 1$  °C for 20 min, TS3) exhibited complete inactivation of microbial loads (<1 log CFU/ml), the highest reduction in enzymatic activities, better suspension stability, color properties, and highest bioactive compounds. Collectively, the verdicts proposed that TS processing could be a worthwhile option to pasteurize the spinach juice to enhance the overall quality.

### 1. Introduction

Escalating market demand for natural products is replacing the synthetic ingredients in the food industry. Consumers successively demand minimally processed and additive-free juices which have a reasonable shelf life and antioxidant profile [1,2]. Owing to the perishable nature of certain vegetables, they are processed into a wide variety of products including juices. The nutritional profile of juices highlights their richness in vitamins, minerals, and antioxidants. Initially, thermal pasteurization was employed to reduce the microbial

and enzymatic load along with the extension in shelf life. However, several adverse effects including alteration in color, flavor, nutritional properties, and other quality parameters were observed. Therefore, an alternative to the conventional methods was researched to fulfill the existing demand along with improving the quality and safety for the end-users [3–6].

Thermosonication (TS) treatment, also known as ultrasonic-assisted heat treatment, is considered a good alternative to heat treatments for enzyme and microbial inactivation accompanied by minimal loss of quality parameters in juices. Other advantages associated with using

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sonoprocessing include high consistency level, lower consumption of energy, and enhanced throughput as compared to conventional methods [1,4,7–9]. Moreover, it is also known to improve the bioactive constituents while keeping the fruit juices safe from a microbial load, for instance, mango juice [10,11], carrot juice [4], hog plum [12], star-fruits [13], apple juice [14], orange juice [15], and pitaya juice [16]. Therefore, TS treatment is speculated to be a useful alternative method employed for fruit and vegetable juice production while maintaining nutritional quality and economic value.

Spinach (*Spinacia oleracea*) is a leafy green vegetable rich in glucosinolates, chlorophyll, vitamins, minerals, bioactive constituents including phenolic acids and carotenoids [17,18]. Epidemiological investigations highlighted the disease preventive role of these bioactive constituents against cardiovascular diseases, in improving immune health, and providing chemoprotection against different cancers [19,20]. Therefore, the consumption of spinach leads to an effective micronutrients' delivery inside the body. However, it is a highly perishable and seasonal crop, that is further accompanied by heavy losses owing to the non-availability of effective processing facilities, storage, and transport facility at the production centers [17,21]. Spinach being a heat-sensitive plant can get deteriorated if exposed to traditional pasteurization. Therefore, a pulsed electric field is considered suitable for preserving the bioactive constituents, minerals, and amino acid profile in spinach [17]. Additionally, ultrasound (US) (20 kHz, 100 W, and 15 min) also reportedly retained the nutrient composition in spinach juice better than other techniques. A combined treatment of US and ultraviolet radiations resulted in improved decontamination of juices (recommended 5 log reductions of microorganisms) while maintaining the nutritional content as compared to thermal methods [22,23]. These studies indicated that TS treatment could be an efficient alternative to preserve the antioxidants. This study was therefore designed to thoroughly investigate the effect of TS treatments on nutritional, particle size, rheological, microbial and enzymatic activities for enhancing the quality of spinach juice.

## 2. Materials and methods

### 2.1. Preparation of spinach juice, pasteurization, and thermosonication (TS) treatments

Fresh spinach leaves were purchased from a local market, after that stems were removed, and spinach leaves (1 kg) were washed to eliminate the undesirable particles. Later, the spinach leaves were crushed in a manual stainless-steel juice extractor to obtain juice. For uniform consistency extracted juice was filtered through a sterile muslin cloth. Then the juice was divided into five portions according to the study scheme, each portion contains 100 ml. For TS processing, 100 ml spinach juice was treated in an ultrasonic homogenizer (Scientz-IIID, Ningbo, China). TS was done with 30 kHz frequency, temperature  $60 \pm 1^\circ\text{C}$  for 20 min, and 50% duty cycle. The TS treatments were performed at 3 distinct ultrasonic powers 200 W (named as TS1), 400 W (TS2), and 600 W (TS3). For pasteurization, 100 ml of spinach juice was put into a thermostatic water bath (DK-2000, Tianjin, China) for 30 min at  $60 \pm 1^\circ\text{C}$ . After TS and pasteurization treatments juices were cooled on an ice bath and kept at  $4^\circ\text{C}$  until further analysis.

### 2.2. Determination of bioactive compounds

#### 2.2.1. Total phenolic contents (TPC)

TPC was determined using the Folin-Ciocalteu method described by Navajas-Porras, Pérez-Burillo, Morales-Pérez, Rufián-Henares and Pastoriza [24] with some minute alterations. Initially, 1 ml spinach juice samples, 1 ml of Folin-Ciocalteu reagent, and 3 ml of 75% (w/v) sodium carbonate solution was combined with the total volume adjusted to 10 ml using distilled water. In the dark environment, the reaction was carried out for 15 min after which the absorbance in the sample was

determined at 760 nm. A unit of gallic acid equivalent (GAE)  $\mu\text{g/g}$  was used to demonstrate the TPC of the tested samples.

#### 2.2.2. Total flavonols

The method used for determining total flavonols is previously described by Manzoor, Zeng, Ahmad, Ahmed, Rehman, Aadil, Roobab, Siddique and Rahaman [25]. Initially, 2 ml of spinach juice sample was mixed in 2.0 ml of 2% aluminum chloride solution. It was then followed by adding a 3.0 ml solution of sodium acetate (50 g/L). This mixture was incubated for 150 min at  $20^\circ\text{C}$ , where the absorbance was measured at 440 nm against a blank. A unit of Quercetin equivalent (QCE)  $\mu\text{g/g}$  was used to demonstrate the total flavonols of the tested samples.

#### 2.2.3. Total flavonoids

The content of total flavonoids (TFC) was measured employing the method earlier adopted by Baba and Malik [26]. 1 ml spinach juice samples and 0.2 ml of 5% (w/v)  $\text{NaNO}_3$  were mixed to prepare the reaction mixture. Initially standing time of 6 min was provided for the reaction mixture that was followed by the addition of 0.2 ml 10% (w/v)  $\text{Al}(\text{NO}_3)_3$  solution followed by a second standing time of 6 min again. The mixture was then homogenized with 4.0 ml 4% (w/v) NaOH solution for 15 min. The spectrophotometric absorbance of the tested sample was measured at 500 nm. A unit of Catechin equivalent (CE)  $\mu\text{g/g}$  was used to demonstrate the TFC of the tested samples.

#### 2.2.4. Chlorophyll contents

Zhao, Wang, Liu, Dong, Huang, Xiong and Liao [27] explained the method for the measurement of chlorophyll content. Using that method, spinach juice (3 ml) was mixed with 3 ml of  $\text{C}_3\text{H}_6\text{O}$  (80% v/v). This solution was filtered with (3X) Whatman filter paper. The absorbance of the filtrate was measured at 664 and 647 nm. For the calculation of total chlorophyll contents following equation was used:

$$\text{Chlorophylla} = (11.85 \times A_{664}) - (1.54 \times A_{647}) \quad (1)$$

$$\text{Chlorophyllb} = (21.03 \times A_{664}) - (5.43 \times A_{647}) \quad (2)$$

$$\text{Totalchlorophyll} = (\text{chlorophylla}) - (\text{chlorophyllb}) \quad (3)$$

#### 2.2.5. Anthocyanin contents

A pH differential method was used to determine total anthocyanin, as described earlier by Aadil, Zeng, Wang, Liu, Han, Zhang, Hong and Jabbar [28].

#### 2.2.6. Carotenoid contents

The total carotenoid contents were determined as per the method explained by Liao, Sun, Ni, Liao, Hu, Wu and Chen [29]. A separation funnel containing an 80 ml solution of *n*-hexane/acetone (1:1, v/v) was filled with 25 ml of spinach juice. Using anhydrous  $\text{Na}_2\text{SO}_4$ , the organic phase was segregated and dehydrated. Lastly, the absorbance of test samples was estimated at 450 nm at room temperature. Using a standard solution of  $\beta$ -carotene, a standard curve was prepared.

### 2.3. Determination of antioxidant activities

#### 2.3.1. DPPH radical scavenging capacity

The DPPH capacity of TS and pasteurized spinach juice was determined according to the method described by Manzoor, Zeng, Rahaman, Siddeeg, Aadil, Ahmed, Li and Niu [30]. 2 ml of spinach juice and 2 ml of 0.2 mM of DPPH solution was mixed and then incubated in dark at  $27 \pm 2^\circ\text{C}$  for 30 min. The reduction in absorbance was estimated at 517 nm. The DPPH radical scavenging % was estimated according to the following equation (4);

$$\text{DPPH}\% = 1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \quad (4)$$

### 2.3.2. FRAP assay

FRAP assay of TS and pasteurized treated spinach juice samples were determined by the method of earlier described by Manzoor, Ahmad, Ahmed, Siddique, Mehmood, Usman and Zeng [31].

## 2.4. Determination of physical characteristics of spinach juice

### 2.4.1. Color

In the spinach juice, the color difference was determined using a CR-400 chroma meter (Konica Minolta Sensing, Japan) according to the method of Faisal Manzoor, Ahmed, Ahmad, Karrar, Rehman, Muhammad Aadil, Al-Farga, Waheed Iqbal, Rahaman and Zeng [32]. The  $a^*$ ,  $b^*$ , and  $L^*$  values were estimated, and Hue angle ( $h^\circ$ ) and chroma index ( $C^*$ ) was calculated according to the following equations:

$$\text{Hue}(h^\circ) = \tan^{-1} \left( \frac{b^*}{a^*} \right)^2 \quad (5)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (6)$$

### 2.4.2. Particle size distribution (PSD)

The PSD was measured using the BT-9300ST Mastersizer (Better Instrument Co. Ltd., Jinzhou, China) [33]. On the obscuration of samples to about 5%, measurement was started. The volume-mean diameter ( $D_{4,3}$ ) and area-mean diameter ( $D_{3,2}$ ) of all samples were determined by the device-recommended software. Three measurements per sample were taken and three readings per sample were recorded by the machine.

### 2.4.3. Rheological characteristics

Rheological measurements were conducted using a Discovery HR-1 (TA Instruments, America) as explained by Zhou, Guan, Bi, Liu, Yi, Chen, Wu and Zhou [34], and parallel plates (40-mm diameter) with a gap size of 1 mm were employed. A sample of 2 ml spinach juice was placed between the plates to maintain a constant temperature of 25 °C along with a shear rate ranging between 0.1 and 100 s<sup>-1</sup> for measuring apparent viscosity. Discovery HR-1 system was used for measuring the yield stress of the tested samples. Results indicate that the change of shear rate of the juice over time within 600 s was calculated at a constant temperature and shear rate. Moreover, for steady flow studies, the thixotropy of spinach juice was also measured. For this method, approximately 2 ml of spinach juice was placed between the plates, at 25 °C with the shear rate changing exponentially from 1 to 200 s<sup>-1</sup>, and then from 200 to 1 s<sup>-1</sup> the measurements were taken.

### 2.4.4. Centrifugal sedimentation and cloud stability (CS)

The centrifugal sedimentation was estimated by following the method of Cameron, Baker and Grohmann [35]. 15 ml of spinach juice was poured into the centrifugation tube and centrifuged for 15 min at 3500 × g. After that, the supernatant was removed and the sediment portion was weighed according to the following equation:

$$SR\% = (m_1/m_2) \times 100 \quad (8)$$

Where  $m_1$  and  $m_2$  are the precipitate weight after and before centrifugation, respectively.

The CS of spinach juice was estimated according to the method reported by Zhu, Shen, Wei, Xu, Cao, Liu and Li [36]. The spinach juice sample was centrifuged for 15 min at 4200×g and spectrophotometric absorbance of the supernatant was calculated at 625 nm. Following equation (7) was adopted for the calculation of CS:

$$CS\% = \left( \frac{C_A}{C_B} \right) \times 100 \quad (7)$$

where  $C_A$  and  $C_B$  are the absorbances after and before centrifugation, respectively.

## 2.5. Peroxidase and polyphenol oxidase activity

As per the proposed method of Ali, Popović, Koutchma, Warriner and Zhu [37], peroxidase (POD) and polyphenol oxidase (PPO) activities in spinach juice were determined. After the centrifugation of the spinach juice sample for 5 min at 5,000 g, the supernatant was diluted in distilled water (100-fold For POD activity measurement). Then, 1 ml of diluted supernatant was added in a test tube containing K<sub>3</sub>PO<sub>4</sub> buffer 0.32 ml with pH 6, 0.1 M, and C<sub>6</sub>H<sub>6</sub>O<sub>3</sub> 0.16 ml of 5% (w/v). The addition of H<sub>2</sub>O<sub>2</sub> (0.5% w/w) initiated the reaction and an absorbance increase was recorded at 485 nm for up to 5 min. Similarly, for PPO activity, 1 ml of diluted supernatant was mixed with 1 ml of C<sub>6</sub>H<sub>6</sub>O<sub>2</sub> (0.07 M) in Na<sub>3</sub>PO<sub>4</sub> buffer with pH 6.5, 0.05 M. An increased absorbance was recorded at 420 nm up to 10 min.

## 2.6. Microbiological analysis

Microbiological assessment in spinach juice was done according to the method earlier described by Manzoor, Ahmad, Ahmed, Siddique, Mehmood, Usman and Zeng [31]. This method was adopted to determine the number of total plate count (TPC), *E. coli/coliforms*, and yeasts and mold (Y&M), in the untreated, pasteurized and TS treated samples. The heterotrophic counts in untreated, pasteurized, and TS treated samples were taken as CFU/ml, the analytical detection limit was adjusted at 1 log CFU/ml. All samples were examined in duplicate.

## 2.7. Statistical analysis

The obtained results were analyzed by using SPSS Statistics 18.0 through one-way ANOVA, and graphs were plotted by using OriginPro 2017. The results were presented as mean ± SD and the significant differences were considered to be significant at  $p$ -value < 0.05.

## 3. Results and discussion

### 3.1. Impact of TS, and pasteurization on TPC, TFC, and total flavonols

Bioactive compounds play a vital part in human well-being by suppressing the threat of various diseases and also significantly contribute towards color and flavor development. In the present study, TPC, TFC, and total flavonols in the USJ sample were 753.87 GAE µg/g, 614.12 CE µg/g and 4.12 QCT µg/g, respectively, as presented in Table 1, while significantly increased ( $p < 0.05$ ) in TS1, TS2, and TS3 samples. But in the case of the PSJ sample a significant decrease ( $p < 0.05$ ) in TPC (738.35 GAE µg/g) and TFC (602.09 CE µg/g) was observed as compared to USJ and TS treated samples. It was noted that an increase in bioactive compounds was associated with increasing ultrasonic intensity. The increase in TPC and TFC in TS1, TS2, and TS3 as compared to the PSJ sample could be associated with the extended-release of bound polyphenols through high-intensity US treatments produced cell disruption, and these unbound polyphenols readily viable for the assay [38]. Moreover, during TS treatment increased phenolic compounds might be associated with the production of micro-cavities that enhance the mass transfer rate [39–41]. Comparable impacts of TS processing on TPC and TFC were recorded in blueberry wine [42], blueberry juice [16], and cloudy apple juice [43]. The effects of TS processing on TFC and TPC showed that pasteurization combined with the US was a more trustworthy option as compared to individual pasteurization. In earlier investigations, conventional heat approaches have been bestowed to decrease the TFC and TPC in apple juice [44]. Earlier, Abid, Jabbar, Hu, Hashim, Wu, Lei, Khan and Zeng [14] have stated that the apple juice samples processed at 60 °C had the maximum retention of TFC and TPC as compared to those samples processed at 20 and 40 °C. In the present study, the high power ultrasound 600 W had a greater impact on the TFC, and TPC than lower power ultrasound (200 W and 400 W) and presented better efficiency than pasteurization in enhancing the TFC and

**Table 1**

Impact of thermosonication on TPC (GAE  $\mu\text{g/g}$ ), TFC (CE  $\mu\text{g/g}$ ), total flavonols (QCE  $\mu\text{g/g}$ ), chlorophyll ( $\mu\text{g/ml}$ ), carotenoids ( $\mu\text{g/ml}$ ), and anthocyanin ( $\mu\text{g/ml}$ ) contents of spinach juice.

Parameters	USJ	TS1	TS2	TS3	PSJ
TPC	753.87 $\pm$ 0.13 <sup>c</sup>	827.08 $\pm$ 0.12 <sup>b</sup>	838.12 $\pm$ 0.15 <sup>b</sup>	902.15 $\pm$ 0.07 <sup>a</sup>	738.35 $\pm$ 0.14 <sup>d</sup>
Total flavonols	4.12 $\pm$ 0.08 <sup>e</sup>	4.85 $\pm$ 0.06 <sup>bc</sup>	4.93 $\pm$ 0.13 <sup>b</sup>	5.25 $\pm$ 0.11 <sup>a</sup>	4.29 $\pm$ 0.09 <sup>d</sup>
TFC	614.12 $\pm$ 0.09 <sup>e</sup>	654.29 $\pm$ 0.12 <sup>bc</sup>	664.28 $\pm$ 0.16 <sup>b</sup>	692.35 $\pm$ 0.13 <sup>a</sup>	602.09 $\pm$ 0.11 <sup>d</sup>
Chlorophyll a	37.26 $\pm$ 0.03 <sup>e</sup>	41.46 $\pm$ 0.04 <sup>b</sup>	39.64 $\pm$ 0.02 <sup>c</sup>	44.21 $\pm$ 0.04 <sup>a</sup>	38.26 $\pm$ 0.05 <sup>d</sup>
Chlorophyll b	13.78 $\pm$ 0.02 <sup>c</sup>	15.72 $\pm$ 0.04 <sup>bc</sup>	15.98 $\pm$ 0.01 <sup>b</sup>	17.22 $\pm$ 0.06 <sup>a</sup>	13.98 $\pm$ 0.01 <sup>c</sup>
Chlorophyll a + b	51.04 $\pm$ 0.02 <sup>e</sup>	57.18 $\pm$ 0.03 <sup>b</sup>	55.62 $\pm$ 0.05 <sup>c</sup>	61.43 $\pm$ 0.04 <sup>a</sup>	52.24 $\pm$ 0.06 <sup>d</sup>
Carotenoids	3.56 $\pm$ 0.03 <sup>d</sup>	3.78 $\pm$ 0.02 <sup>c</sup>	3.95 $\pm$ 0.05 <sup>b</sup>	4.22 $\pm$ 0.02 <sup>a</sup>	3.46 $\pm$ 0.04 <sup>d</sup>
Anthocyanin	37.32 $\pm$ 0.04 <sup>e</sup>	40.38 $\pm$ 0.08 <sup>c</sup>	41.62 $\pm$ 0.05 <sup>b</sup>	43.65 $\pm$ 0.08 <sup>a</sup>	35.33 $\pm$ 0.06 <sup>d</sup>

USJ: untreated spinach juice, TS1: ultrasound treated at 30 kHz, power 200 W, 50% duty cycle, 60  $\pm$  1  $^{\circ}\text{C}$  for 20 mint, TS2: ultrasound treated at 30 kHz, power 400 W, 50% duty cycle, 60  $\pm$  1  $^{\circ}\text{C}$  for 20 mint, TS3: ultrasound treated at 30 kHz, power 600 W, 50% duty cycle, 60  $\pm$  1  $^{\circ}\text{C}$  for 20 mint, and PSJ: pasteurized spinach juice (60  $\pm$  1  $^{\circ}\text{C}$  for 30 mint).

All results are specified as mean  $\pm$  SD. Results with different alphabets in the same row are significantly different ( $p < 0.05$ ) from each other. GAE: Gallic acid equivalent, QCE: Quercetin equivalent CE: Catechin equivalent.

TPC.

### 3.2. Impact of TS and pasteurization on anthocyanins, carotenoids, and chlorophyll contents

Anthocyanin contents are the most valuable bioactive compounds presented in spinach juice. As presented in Table 1, the anthocyanins of USJ, and PSJ, were 37.32  $\mu\text{g/ml}$  and 35.33  $\mu\text{g/ml}$ , respectively. TS processing significantly ( $p < 0.05$ ) enhanced the anthocyanins than USJ and PSJ samples, while the TS3 sample showed the highest anthocyanins value (43.65  $\mu\text{g/ml}$ ). The adverse impact of the US was also observed in some earlier studies. For example, in strawberry and mango juice, anthocyanins reduction was recorded after the US processing [45,46]. Taken together, these observations propose that TS improves the overall antioxidant contents, such as TFC, TPC, and anthocyanins in spinach juice, which could be advantageous for human wellness. Some researchers have described that US-assisted extraction possibly improves the anthocyanins extraction yield. These investigations showed that US processing did not break down the anthocyanins. But, higher intensity US treatment, might induce critical cell wall disruption, leading to anthocyanins' migration into the juice. Furthermore, high-intensity US has been associated with significant destruction of the activity of PPO and POD, which are most possibly accountable for the browning of juice and enzymatic degradation of anthocyanins [47].

Chlorophyll contents were increased in TS3 (61.43  $\mu\text{g/ml}$ ), TS1 (57.18  $\mu\text{g/ml}$ ), and TS2 (55.62  $\mu\text{g/ml}$ ) as compared to the PSJ (52.24  $\mu\text{g/ml}$ ) and USJ (51.04  $\mu\text{g/ml}$ ). As presented in Table 1, carotenoid contents were significantly increased ( $p < 0.05$ ) during TS treatments than PSJ and USJ samples. Moreover, TS3 (4.22  $\mu\text{g/ml}$ ) showed the highest increase in carotenoid contents than all other samples. Carotenoid contents increased during TS treatments might be associated with the cavitation process that damage the cell walls and unbound the carotenoids into the juice [14]. The TS treatment could stimulate hydration, swelling, and resulted in cell wall pores enlargement; it happened in greater extraction of chlorophyll contents during treatment [32]. Earlier, a significant increase in carotenoids was observed in TS treated hog plum juice [12]. During pasteurization, elevated temperature leading towards the isomerization might be responsible for carotenoids

reduction [10].

### 3.3. Impact of TS and pasteurization on antioxidant activities

For the estimation of the antioxidant potential of spinach juice, DPPH and FRAP assays were considered to be reliable methods and exhibited a reliable picture of the antioxidant properties of food products. The results of FRAP and DPPH capacity of all treated samples are presented in Fig. 1. The DPPH % inhibition of USJ and PSJ was 60.40% and 58.30%, respectively, but the maximum DPPH activity was recorded in TS3 (79.7%), which was significantly higher ( $p < 0.05$ ) USJ and PSJ. Earlier, Wang, Ni, et al., (2019) also observed similar results in TS-treated strawberry juice. As presented in Fig. 1, the FRAP assay (%) values significantly increased ( $p < 0.05$ ) in all TS treated samples, while significant reduction ( $p < 0.05$ ) was observed in PSJ (65.4%) sample. Earlier, Adiamo, Ghafoor, Al-Juhaimi, Babiker and Ahmed [48] also reported the increased retention of polyphenols and elevated antioxidant activities of TS treated carrot juice. The increment may be associated with maximum polyphenols in high-power US processing of juice, induced by cavitation which improved the availability and extraction of these valuable compounds [49]. Some authors correlated an improvement in antioxidant capacity with the minimized generation of free  $^{\circ}\text{OH}$  during TS treatment of juice [50]. On the other hand, a high level of free  $^{\circ}\text{OH}$  for an extended period has been described to harm the antioxidant potential [10].

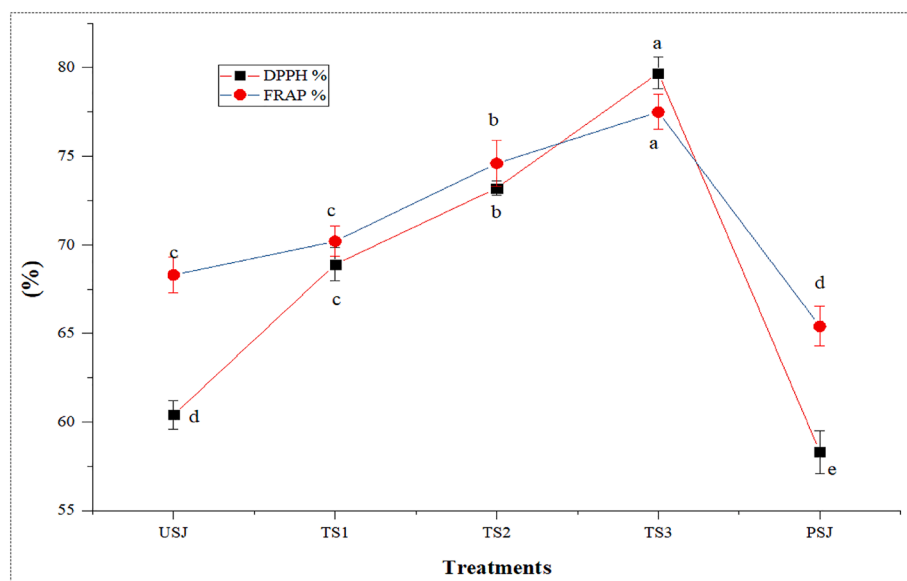
### 3.4. Impact of TS and pasteurization on a physical characteristic

#### 3.4.1. Color

Table 2 presents the variations in the color of TS and pasteurized treated spinach juice samples. After TS treatment,  $L^*$  (lightness) and  $+b^*$  (yellowness) values increased, while  $-a^*$  (greenness) was decreased as compared to the USJ. This kind of color variation was mainly associated with the higher treatment temperature, leading to lower saturation and dense color of the spinach juice. The highest  $L^*$  (52.32) abs  $b^*$  (6.18) values were recorded in TS3 samples, and the lowest  $a^*$  values were also observed in TS3. The color variations in TS treated samples are associated with the cavitation process, which generated various physical, chemical, and biological reactions, improving the diffusion rate and breakdown of sensitive particles. Earlier, Wang, Wang, Ye, Vanga and Raghavan [51] also observed an increase in  $L^*$  values in TS treated strawberry juice. During the pasteurization, PPO and POD were inactivated and the reduction of coloring pigments (anthocyanins and carotenoids) might be the main reason for the discoloration of spinach juice [52].

#### 3.4.2. PSD

The variations of PSD, volume-mean diameter ( $D_{4,3}$ ), and area-mean diameter ( $D_{3,2}$ ) of pasteurized and TS treated samples are presented in Table 3. The span value,  $D_{4,3}$ , and  $D_{3,2}$  of PSJ were significantly ( $p < 0.05$ ) higher as compared to TS treated and USJ samples. Also, the  $D_{3,2}$  values of pasteurized and TS treated samples were lower than the  $D_{4,3}$ . Fenoglio, Ferrario, García Carrillo, Schenk and Guerrero [53] have stated that  $D_{4,3}$  and  $D_{3,2}$  are more influenced by larger particles and smaller particles, respectively. In this study, the increase in the intensity of US PSD more reduces, and the lowest PSD was observed in TS3. The PSD trend in Fig. 2 verified this result. The TS3 sample had more particles size ranging between 0.2 and 4  $\mu\text{m}$  and showed monomodal PSD. Furthermore, TS1, TS2, and PSJ had more particles size ranging between 10 and 100  $\mu\text{m}$  and showed bimodal PSD. Our results show that TS treatment at high intensity could dramatically decrease the size of suspended particles in juice, due to the high shear influence happening during TS treatment. Moreover, cavitation limits the polysaccharide's agglomeration and splits them into small sizes [39]. In the case of pasteurization, an increase in PSD was observed due to higher temperature causing small particles to aggregate into larger particles.



**Fig. 1.** Impact of thermosonication on antioxidant activities of spinach juice. Results are illustrated in % retention of FRAP and DPPH assay. **USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).

**Table 2**

Effect of thermosonication on the color properties of spinach juice.

Parameters	USJ	TS1	TS2	TS3	PSJ
<i>L</i>	49.24 ± 0.47 <sup>d</sup>	51.59 ± 0.74 <sup>bc</sup>	51.83 ± 0.28 <sup>b</sup>	52.32 ± 0.23 <sup>a</sup>	49.12 ± 0.41 <sup>d</sup>
<i>a</i> *	-5.38 ± 0.13 <sup>a</sup>	-5.70 ± 0.11 <sup>c</sup>	-5.48 ± 0.08 <sup>b</sup>	-6.05 ± 0.32 <sup>d</sup>	-5.46 ± 0.21 <sup>b</sup>
<i>b</i> *	5.39 ± 0.13 <sup>d</sup>	5.75 ± 0.14 <sup>bc</sup>	5.85 ± 0.18 <sup>b</sup>	6.18 ± 0.09 <sup>a</sup>	5.29 ± 0.27 <sup>d</sup>
Hue	45.11 ± 0.21 <sup>c</sup>	45.45 ± 0.13 <sup>c</sup>	48.74 ± 0.18 <sup>a</sup>	46.17 ± 0.18 <sup>b</sup>	43.18 ± 0.12 <sup>d</sup>
<i>C</i>	7.62 ± 0.12 <sup>c</sup>	8.10 ± 0.11 <sup>b</sup>	8.02 ± 0.09 <sup>b</sup>	8.64 ± 0.13 <sup>a</sup>	7.60 ± 0.08 <sup>c</sup>

**USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).

All results are specified as mean ± SD. Results with different alphabets in the same row are significantly different ( $p < 0.05$ ) from each other.

**Table 3**

Effect of thermosonication on the particle size of spinach juice.

Parameters	USJ	TS1	TS2	TS3	PSJ
$D_{(v,0.10)} \mu\text{m}$	3.27 ± 0.08 <sup>b</sup>	2.98 ± 0.19 <sup>c</sup>	2.93 ± 0.11 <sup>c</sup>	2.85 ± 0.03 <sup>d</sup>	3.38 ± 0.07 <sup>a</sup>
$D_{(v,0.50)} \mu\text{m}$	6.78 ± 0.13 <sup>a</sup>	5.18 ± 0.15 <sup>b</sup>	4.34 ± 0.13 <sup>b</sup>	3.92 ± 0.14 <sup>c</sup>	6.88 ± 0.12 <sup>a</sup>
$D_{(v,0.90)} \mu\text{m}$	12.82 ± 0.18 <sup>a</sup>	8.34 ± 0.12 <sup>b</sup>	7.12 ± 0.08 <sup>c</sup>	6.26 ± 0.11 <sup>d</sup>	12.93 ± 0.19 <sup>a</sup>
$D_{(3,2)} \mu\text{m}$	5.87 ± 0.14 <sup>b</sup>	4.52 ± 0.17 <sup>c</sup>	3.98 ± 0.09 <sup>c</sup>	3.43 ± 0.08 <sup>d</sup>	6.01 ± 0.15 <sup>a</sup>
$D_{(4,3)} \mu\text{m}$	7.62 ± 0.11 <sup>b</sup>	5.92 ± 0.11 <sup>c</sup>	4.95 ± 0.07 <sup>c</sup>	3.98 ± 0.12 <sup>cd</sup>	7.76 ± 0.10 <sup>a</sup>
Span value	1.22 ± 0.05 <sup>a</sup>	1.15 ± 0.08 <sup>b</sup>	1.08 ± 0.05 <sup>c</sup>	1.02 ± 0.05 <sup>d</sup>	1.26 ± 0.06 <sup>a</sup>

**USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).

All results are specified as mean ± SD. Results with different alphabets in the same row are significantly different ( $p < 0.05$ ) from each other.

### 3.4.3. Rheological behavior

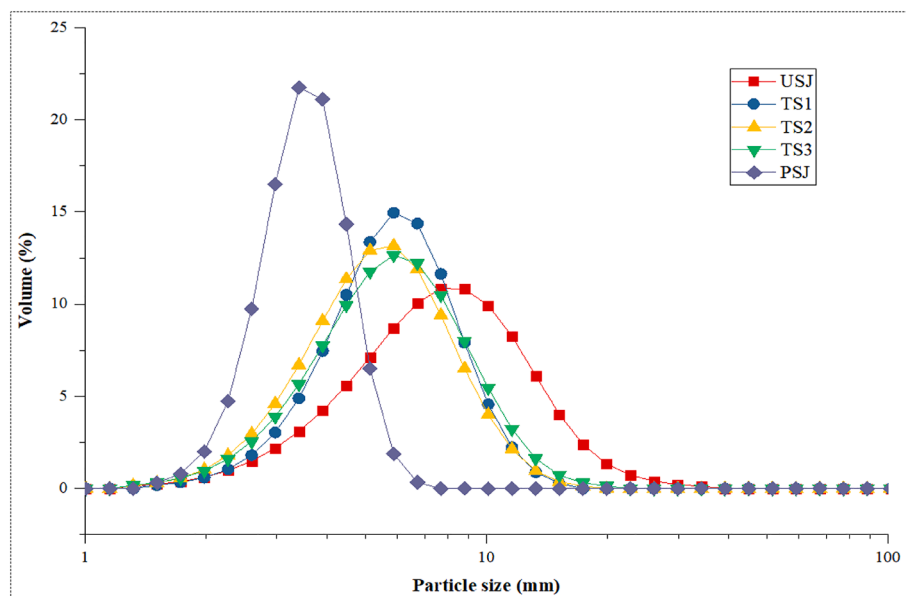
The impact of pasteurization and TS treatments on rheological parameters ( $K$ ,  $n$ ,  $R^2$ ,  $\eta$ , and  $\sigma_y$ ) with the Herschel-Bulkley model at a shear rate of  $100 \text{ s}^{-1}$  are shown in Table 4. All TS-treated samples are due to the smaller PSD and weaker resistance toward shear flocculation show a non-Newtonian behavior ( $n < 1$ ). TS processing normally leads to a significant decrease ( $p < 0.05$ ) in yield stress. TS processing ruptured the cell membrane may be related to improvement in yield stress and liquid indicates close to a Newtonian behavior ( $n \approx 1$ ) in TS2 sample. The reduction in PSD showed a decrease in apparent viscosity (Fig. 3) and a lower consistency index (Table 4). The decrease in PSD leads towards a greater interfacial area and reduction in the mean distance, which causes nearly more effective interparticle interactions [54]. Earlier, [55] reported similar results in US-treated apple juice. To measure the flow behavior ( $n$ ), shear stress (Pa) at the y-axis of all samples was plotted versus shear rate ( $\text{s}^{-1}$ ) at the x-axis. It was noted that TS2 and TS3 treatments had a larger influence on shear stress (Fig. 4). During TS processing, the apparent viscosity was significantly ( $p < 0.05$ ) decreased by a raise of shear rate ( $0\text{--}100 \text{ s}^{-1}$ ) and directed to a prompt decline of initial shearing.

### 3.4.4. Centrifugal sedimentation and cloud stability

According to Fig. 5 (A and B), TS treatment significantly reduce ( $p < 0.05$ ) the centrifugal sedimentation, while significantly increased ( $p < 0.05$ ) cloud stability of spinach juice as compared to USJ and PSJ samples. The cloudy stability of TS1 and TS3 was better than that of other samples, while the centrifugal sedimentation of TS1 and TS3 was lesser than others. But the PSJ sample exhibited significantly lower ( $p < 0.05$ ) cloud stability and higher centrifugal sedimentation. The improved cloud stability of spinach juice is possibly due to the decrease in particle size and TS processing also improved the particle interactions. The cloud particle smaller and uniform dispersion (Fig. 3) of spinach juice at TS1 to TS3 treatments resulted in a more stable suspension. Earlier, Shen, Zhu, Xi, Cai, Cao, Liu and Li [43] also observed the higher cloud stability and lower centrifugal sedimentation in TS treated cloudy apple juice.

### 3.5. Impact of TS and pasteurization on enzymes activity

The results about the impacts of TS processing on POD and PPO spoilage enzymes in spinach juice are exhibited in Table 5. The POD and PPO enzymes activity in the PSJ sample were  $0.26$  and  $0.014 \text{ Abs min}^{-1}$ , respectively. TS processing significantly ( $p < 0.05$ ) reduced the POD



**Fig. 2.** The particle size of spinach juice in USJ, PSJ, and thermosonicated spinach juice. **USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).

**Table 4**

Effect of thermosonication on rheological behavior of spinach juice (Herschel-Bulkley model).

Parameters	USJ	TS1	TS2	TS3	PSJ
K (Pa.s <sup>n</sup> )	5.432 ± 0.8 <sup>a</sup>	3.234 ± 0.2 <sup>c</sup>	2.234 ± 0.6 <sup>d</sup>	1.123 ± 0.2 <sup>e</sup>	4.013 ± 0.4 <sup>b</sup>
n	0.734 ± 0.001 <sup>d</sup>	0.876 ± 0.03 <sup>c</sup>	0.901 ± 0.06 <sup>a</sup>	0.891 ± 0.12 <sup>b</sup>	0.688 ± 0.003 <sup>e</sup>
$\sigma_y$ (Pa)	3.234 ± 0.07 <sup>e</sup>	5.341 ± 0.04 <sup>c</sup>	7.127 ± 0.05 <sup>b</sup>	4.207 ± 0.10 <sup>d</sup>	7.972 ± 0.04 <sup>a</sup>
R <sup>2</sup>	0.992	0.998	0.996	0.998	0.887
$\eta_{90}$ s <sup>-1</sup>	5.2 ±	4.8 ±	4.1 ±	3.6 ±	5.8 ±
(mPa.s)	0.03 <sup>b</sup>	0.02 <sup>c</sup>	0.04 <sup>d</sup>	0.03 <sup>e</sup>	0.02 <sup>a</sup>

**USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).

K = consistency index; n = flow behavior; ( $\sigma_y$ ) = yield stress ( $10^{-3}$ ). All results are specified as mean ± SD. Results with different alphabets in the same row are significantly different ( $p < 0.05$ ) from each other.

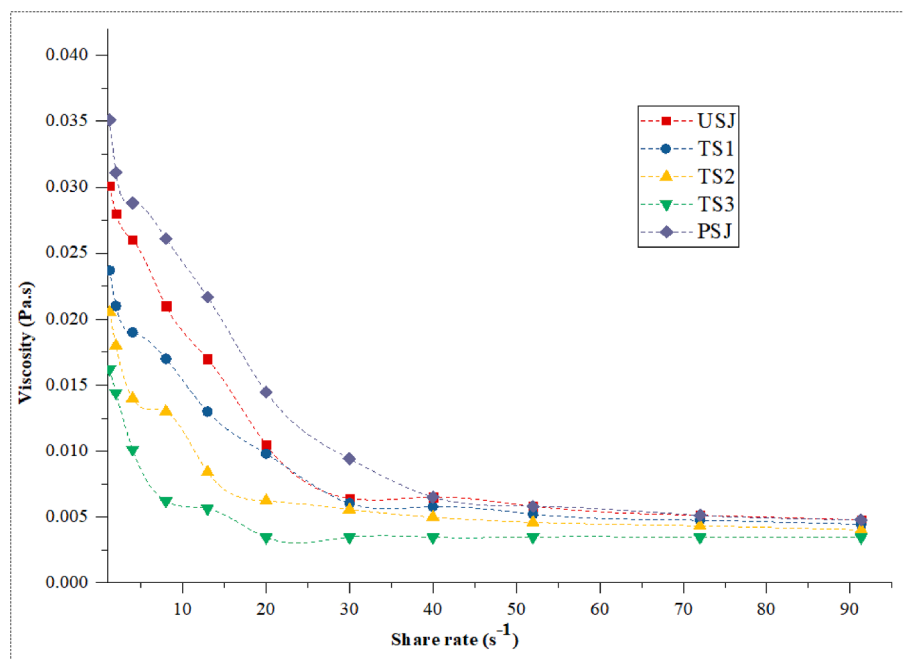
and PPO activities from 0.97 to 0.31 Abs min<sup>-1</sup> and 0.034 to 0.018 Abs min<sup>-1</sup>, respectively. The POD and PPO activities of spinach juice were decreased by rising the US intensity from 200 to 600 W. Earlier, reduction in POD activity in the TS-treated apple juice (at 60 °C, 30 min, 20 kHz, and 500 W) was observed by Abid, Jabbar, Hu, Hashim, Wu, Lei, Khan and Zeng [14]. Earlier, Cao, Cai, Wang and Zheng [56] described that the seventeen times more reduction of POD activity in US-treated and pasteurized bayberry juice at 60 °C. Mechanical shock with heat due to micro streaming is possibly the reason for the inactivation of enzymes generated by thermosonication. Moreover, these factors individually or in combination destroyed the enzyme's protein structure that reduces the enzyme activity [57]. Sonication with the acoustic field produced cavitations and bubbles that exerted mechanical force due to their collapse, which is responsible for enzymes inactivation [58]. During TS, processing generation of free radicals due to sonoprocessing has also been described to cause catalase and POD enzymes inactivation [59]. Besides, several extrinsic and intrinsic control parameters are accountable for the effectiveness of US treatment [60].

### 3.6. Impact of TS and pasteurization microbial loads

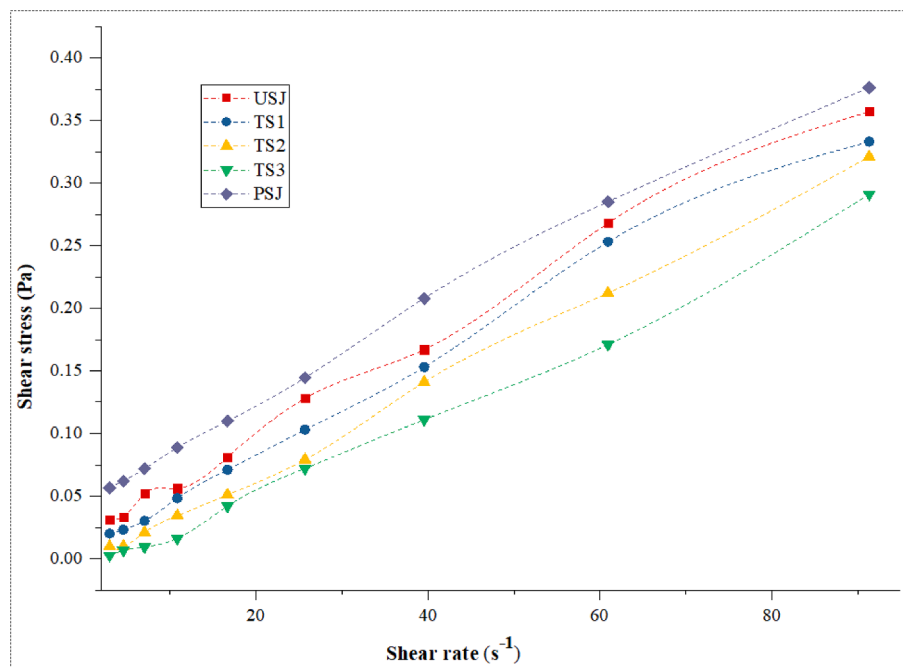
US processing can keep the unique quality of juices while accomplishing the object of a 5-log decline in pathogens or microbial loads as approved by the FDA [45]. The impact of TS treatments on *E. coli/coli-form*, yeast & mold (Y&M), and total plate counts (TPC) of spinach juice is presented in Table 5. After pasteurization and TS treatments significant decrease ( $p < 0.05$ ) was noted in total microbial loads than USJ sample. In the PSJ sample, complete microbial inactivation may be associated with heat treatment that causes the cell membrane and nuclear components rupturing which leads to cell damage [61]. Moreover, it was observed that microbial loads reduced significantly ( $p < 0.05$ ) with increasing US intensity. The Y&M, *E.coli/coli-form*, and TPC in the TS3 sample were reduced up to 4 log CFU/ml, due to the rise in local heating induced by cavitation and the formation of hydroxyl radicals, which ultimately inactivated the microbial loads [14]. The spinach juice acidity synchronically generated osmotic pressure along with some other reactions and probably enhance the cavitation impact on the structure of microbes directed to the release of nuclear compounds protein, and lipids [17,31]. Earlier, the reduction in microbial loads was reported in TS-treated hog plum juice [12], and carrot juice [62]. The increase in US intensity resulted in complete inactivation of *E. coli/coli form*, Y&M, and TPC was recorded which could meet the required regulatory obligations. The inactivation mechanism of microorganisms through TS is the result of many complex physical processes. The US processing to microorganisms resulted in puncturing their cell membranes and extrusion of the intracellular matrix and generated free radicals finally eliminating the microorganisms [63].

## 4. Conclusion

The present study describes that TS processing at a higher intensity 600 W, significantly increases the TPC, TFC, total flavonols, chlorophyll, carotenoids, and anthocyanin. It also increased the DPPH and FRAP assay, as well as enhanced color properties in spinach juice than USJ and PSJ. TS treatment caused a notable decline in microbial loads and enzymatic activities. The quality of TS treated spinach juice also increased as compared to pasteurized juice. TS improved the cloudiness while reducing the centrifugal sedimentation and particle size



**Fig. 3.** Apparent viscosity of spinach juice in USJ, PSJ, and thermosonicated spinach juice. **USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).



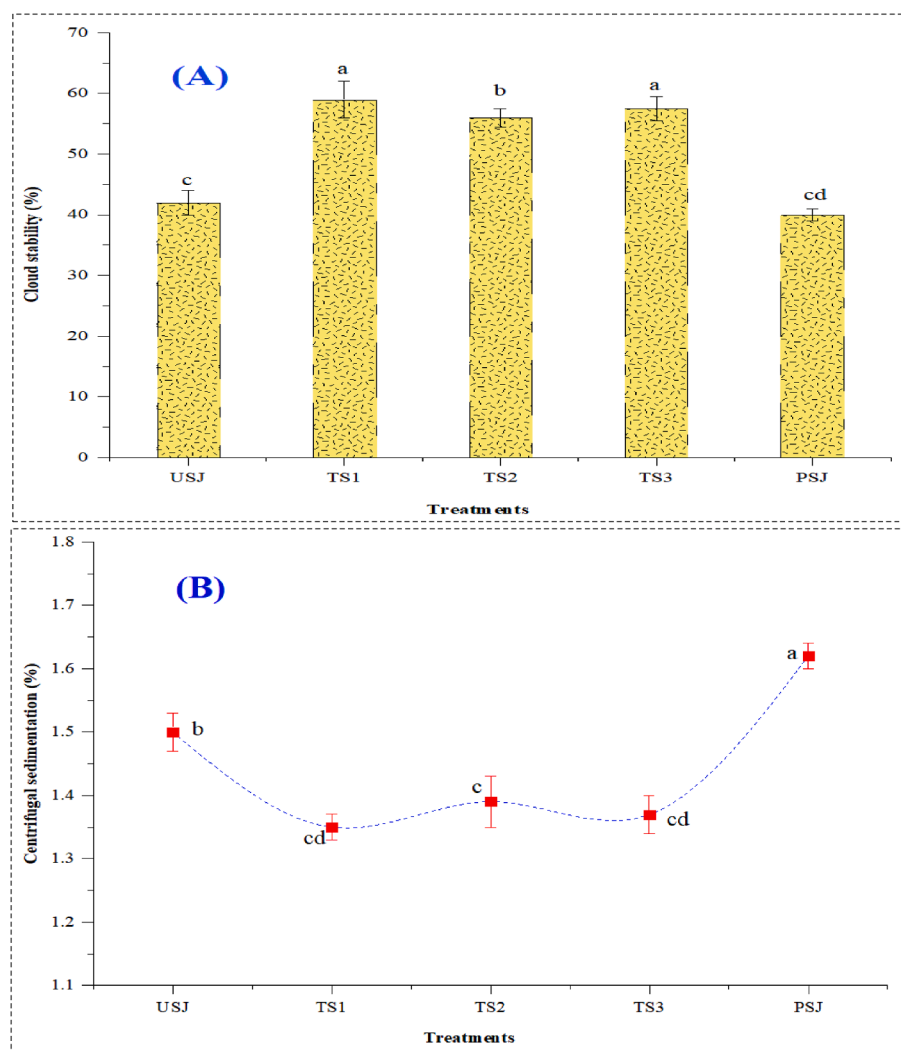
**Fig. 4.** Flow curve of spinach juice in USJ, PSJ, and thermosonicated spinach juice. **USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).

distribution. Besides, TS significantly altered the rheological characteristics including shear stress, apparent viscosity, and shear rate, along with increasing the suspension stability of spinach juice. The spinach juice sample treated at a high intensity (600 W) exhibited complete inactivation of microbial loads ( $<1$  log CFU/ml), better suspension stability, and the highest bioactive compounds. The antioxidant activities and suspension stability were enhanced mainly due to tissue breakdown and interplay between particles and juice components

caused by the ultrasound.

#### CRediT authorship contribution statement

**Muhammad Faisal Manzoor:** Conceptualization, Methodology, Formal analysis, Validation, Writing – original draft, Writing - review & editing. **Bin Xu:** Data curation, Funding acquisition. **Sipper Khan:** Writing – original draft, Writing - review & editing. **Rizwan Shukat:**



**Fig. 5.** (A) Cloud stability and (B) Centrifugal sedimentation in USJ, PSJ, and thermosonicated spinach juice. **USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).

**Table 5**  
Impact of thermosonication on enzymes activities and microbial loads of spinach juice.

Parameters	USJ	TS1	TS2	TS3	PSJ
*POD (Abs min <sup>-1</sup> )	0.97 ± 0.03 <sup>a</sup>	0.69 ± 0.04 <sup>b</sup>	0.60 ± 0.01 <sup>c</sup>	0.31 ± 0.02 <sup>d</sup>	0.26 ± 0.04 <sup>e</sup>
*PPO (Abs min <sup>-1</sup> )	0.034 ± 0.002 <sup>a</sup>	0.026 ± 0.002 <sup>b</sup>	0.023 ± 0.001 <sup>c</sup>	0.018 ± 0.002 <sup>d</sup>	0.014 ± 0.002 <sup>e</sup>
<i>E. coli/Coliform</i> (log CFU/ml)	1.75 ± 0.05 <sup>a</sup>	0.98 ± 0.04 <sup>b</sup>	ND <sup>c</sup>	ND <sup>c</sup>	ND <sup>c</sup>
Total plate count (log CFU/ml)	4.10 ± 0.13 <sup>a</sup>	2.12 ± 0.09 <sup>b</sup>	0.58 ± 0.11 <sup>c</sup>	ND <sup>d</sup>	ND <sup>d</sup>
Yeast & Mold (log CFU/ml)	3.63 ± 0.08 <sup>a</sup>	1.92 ± 0.03 <sup>b</sup>	0.38 ± 0.08 <sup>c</sup>	ND <sup>d</sup>	ND <sup>d</sup>

**USJ:** untreated spinach juice, **TS1:** ultrasound treated at 30 kHz, power 200 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS2:** ultrasound treated at 30 kHz, power 400 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, **TS3:** ultrasound treated at 30 kHz, power 600 W, 50% duty cycle,  $60 \pm 1$  °C for 20 mint, and **PSJ:** pasteurized spinach juice ( $60 \pm 1$  °C for 30 mint).

ND: not detected, All results are specified as mean ± SD. Results with different alphabets in the same row are significantly different ( $p < 0.05$ ) from each other.

Resources, Visualization. **Nazir Ahmad:** Writing – original draft, Writing - review & editing. **Muhammad Imran:** Resources, Visualization. **Abdur Rehman:** Data curation, Software. **Emad Karrar:** Data curation, Software. **Rana Muhammad Aadil:** Data curation, Software. **Sameh A. Korma:** Data curation, Software.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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