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Ultra High Pressure Homogenization of Soy Milk: Effect on Quality Attributes during Storage

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Abstract: The present work analyzed soy milk prepared from whole dehulled soybeans. The traditional method of soy milk preparation leads to wastage of about 35% of soybean solids in the form of okara, which gets filtered out. In the current study, soy milk was prepared with practically 100% recovery of soybean solids and treated with continuous flow high pressure processing (207 and 276 MPa pressure, 121 and 145 °C exit temperatures, and 0.75 and 1.25 L/min flow rates), and the changes in the physical, chemical, microbial, and sensory properties during 28 days of storage at 4 °C were analyzed. The treated soy milk remained stable for 28 days. There was a significant reduction in the particle size of soybean solids which did not change during storage. The pH of the treated soy milk was significantly lower than the untreated soy milk and it reduced further upon storage. The soy milk was pasteurized with high pressure processing coupled with preheating. No lipoxygenase activity was detected. Compared to commercial samples, there was no significant difference in the astringency, bitterness, or chalkiness of soy milk prepared in the study.

Keywords: soy milk; continuous high pressure; throttling; yield; quality; sensory

1. Introduction

Food processing by heating, albeit economical and efficient, has damaging effects on heat labile compounds [1], affecting the color, flavor, and texture [2]. Non-thermal food processing methods are thus of special interest and high pressure processing (HPP) is one such method. Since HPP involves minimal heat, inactivates microbes, and maintains nutritional and sensory qualities, it has found favor in the food industry. HPP can be broadly classified into high hydrostatic pressure (HHP) processing and continuous high pressure (CHP) processing. HHP is a batch process in which the food is sealed in a flexible container, and the container put in a pressure vessel filled with pressure transmitting fluid such as water or oil. This vessel is then pressurized and the pressure transmitting fluid then in turn applies pressure to the food. An important aspect of HHP is that the pressure on the food is applied equally from all directions and, as a result, the foods maintain their shape. The pressure applied in HHP ranges from 100 to 900 MPa [3]. On the other hand, CHP, as the name implies, is a continuous process and is synonymous with high pressure homogenization (HPH). Conventional homogenization, such as that used in the dairy industry, operates at 20–60 MPa. HPH operates at higher pressures, up to 200 MPa. Ultra high pressure homogenization (UHPH) employs even greater pressures, up to 400 MPa. Specialized homogenization valves, which can withstand such high pressures, are required for CHP. CHP is being increasingly researched as a technique for reducing the size of particle in emulsions for making stable emulsions as well as modifying the viscosity [4].

The University of Georgia, GA, USA [5] developed a novel system called continuous flow high pressure throttling (CFHPT) by modifying the homogenizing valve to throttle instead of having a fixed opening as normally used by other researchers. This valve regulates the fluid flow under high

pressure via an adjustable orifice. The process of regulating the fluid flow under high pressure though an extremely narrow orifice has been referred to as throttling, and the homogenizing valve referred to as the throttling valve. Like homogenization, CFHPT employs turbulence, cavitation, and shear forces, which are generated as the food under pressure flows through a highly constricted opening of the throttling valve [6,7]. A noteworthy aspect of this process is a rise in temperature as the food throttles. The rise in temperature at the point of throttling is due to the frictional heat generated as a result of very high fluid velocities [8], and is directly proportional to the pressure. This temperature rise can be exploited for microbial reduction [9–11].

The conventional soy milk production method results in a loss of about 35% of the soybean solids in the form of okara [12], leading to a poor yield. This loss is a result of the removal of insoluble and coarse solids from soy milk during the filtration step. Additionally, soy milk produced in this way has a paint-like odor and flavor [13]. Particle size distribution (PSD), an important property of soy milk, is indicative of the changes that take place during processing [14] as well as of the formation of particle agglomerates [15]. Sivanandan *et al.* [6] used CFHPT attached with a micro-metering valve to process soy milk made from whole dehulled soybeans. Their method is special in that there is no filtration step and practically all the soybean solids are recovered in the final product.

Lipoxygenase (LOX) is an enzyme naturally present in soy milk, and catalyzes the oxidation of polyunsaturated fatty acids (chiefly linoleic acid), which soybeans are rich in. LOX is difficult to inactivate by pressure alone, and supplementing the treatment with heat greatly reduces LOX activity [16,17]. The pH of fresh soy milk, which usually ranges between 6.5 and 7.7, is lowered during storage [18,19]. Even though CHP reduces the initial bacterial counts in soy milk, the bacterial load tends to increase upon storage [11,15]. Bacterial spores are the most resilient microbial entity under pressure and usually require a combination of pressure and temperature for their inactivation [1,20]. The majority of soy milk manufacturers add flavors and mouth-feel improving agents such as sugar, cocoa powder, vanilla flavor, gums, *etc.*, to improve its overall appeal [21]. Very few studies have been conducted on the descriptive sensory analysis of continuous high pressure (CHP) processed soy milk [22].

In the current study, soy milk was prepared from whole dehulled soybeans leading to negligible wastage. The effect of UHPH on particle size distribution, lipoxygenase activity, pH, microbial, and sensory qualities of the soy milk was investigated, and the changes in these parameters during storage at 4 °C were monitored for four weeks.

2. Materials and Methods

2.1. Preparation of Soy Milk

The soybeans (Woodruff variety) were provided by the Georgia Seed Development Commission, Athens, GA, USA and stored at 4 °C and 20% relative humidity (RH). Soy milk was prepared according to the method developed by Sivanandan *et al.* [6] with some modifications. Soybeans were left overnight (16 h) in loosely covered HDPE (high density polyethylene) buckets to equilibrate them to room temperature (23–28 °C). The beans were put into perforated SS trays (1 kg per tray) and roasted at 154 °C for 5.5 min in an air impingement oven (Lincoln Impinger Model 1450, Lincoln Foodservice Products, Inc., Fort Wayne, IN, USA). They were cooled for 15–20 min and dehulled in a plate mill (Quaker City Mill Model 4-E, QCG Systems, LLC, Phoenixville, PA, USA). The cotyledons were separated from the hulls by air classification. Deionized water (DW) was used to blanch the soybeans (1:5 kg dehulled soybeans:kg DW) at 60 °C for 2.5 h, then rinsed three times with DW, covered, and stored overnight at 4 °C, 20% (RH). The following day, DW (three times the mass of blanched soybeans) was weighed and divided into two equal parts. The first part was used to grind the blanched soybeans in a food processor (Robot Coupe Model RSI 10 V, Robot Coupe UGA, Inc., Jackson, MS, USA) for 2.5 min at 3000 rpm followed by 2.5 min at 3500 rpm. The paste was then ground in a super mass-collider (Super Mass Collider Model MK CA6-3, Masuko Sangyo Co. Ltd., Kawaguchi-city,

Saitama-pref, Japan) using a sanitary stone pair (E6–46). To maintain consistent grinding speed, the electrical current to the equipment was kept between 2 and 3 amperes. The paste was passed through the mass collider eight times, after which the remaining water was mixed and the soy milk passed through the equipment four more times. To prevent clogging of the extremely small opening of the throttling valve during high-pressure processing, the soy milk was filtered using a 254 μm filter. Only 200–250 g residue was obtained from a batch of about 20 L of soy milk. Finally, a vacuum was applied to the soy milk for 20 min to remove the entrapped air. This was the control sample.

2.2. Ultra High Pressure Homogenization (UHPH) of Soy Milk (Figure 1)

The soy milk was fed pneumatically into the Stansted high pressure equipment (Model nG7900, Stansted Fluid Power Ltd., Stansted, Essex, UK) at room temperature (23–28 °C) with an inlet pressure of 700 kPa. Soy milk was pressurized to 207 or 276 MPa using two alternately acting pressure intensifiers (Hydropax P60-03CXS, Stansted Fluid Power Ltd., Stansted, Essex, UK). A heat exchanger between the intensifiers and the throttling valve preheated the pressurized soy milk so as to achieve the target temperatures (121 °C and 145 °C) as measured at the end of the holding tube. The temperature of soy milk after preheating and after throttling was also monitored. The difference between the two was calculated as the temperature rise after throttling. A holding tube was placed after the throttling valve to allow time for microbial destruction. Two flow rates, 0.75 L/min and 1.25 L/min, were studied and they corresponded to a residence time of 20.8 s and 12.48 s, respectively, in the holding tube. Since the temperature of soy milk after throttling was above its boiling point, a back pressure valve (a minimum of 400 kPa) was placed at the end of the holding tube to prevent splashing. The soy milk was quickly cooled to room temperature in a heat exchanger prior to collection. Soy milk was collected in 15-mL sterile Polypropylene tubes (Corning, Inc., Corning, NY, USA) for physical, chemical, and microbial analyses, and in 946-mL HDPE jugs with lined caps for sensory analysis. The entire experiment was duplicated. The samples were stored at 4 °C until analyzed. All the analyses were done on day 1, and weeks 1, 2, 3, and 4 (except sensory analysis).

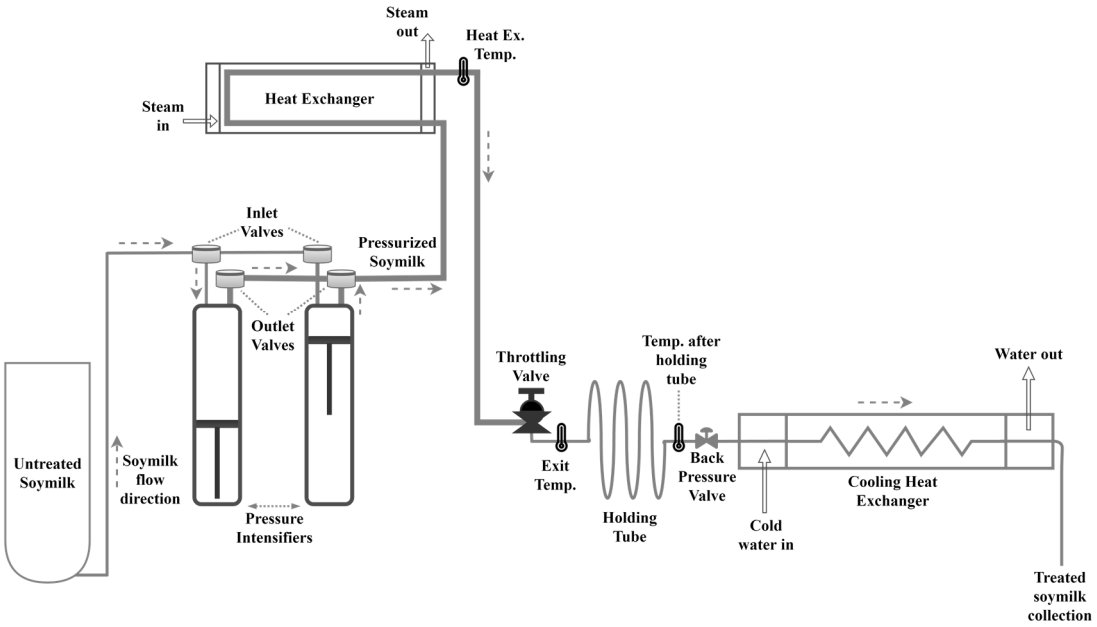

Figure 1. Ultra high pressure homogenization diagram.

2.3. Microbiology

Aerobic plate counts (APC) and total psychrotrophs were determined. The method of Smith *et al.* [19] was followed. All samples were analyzed in duplicated and the values averaged.

One milliliter soy milk samples were serially diluted in peptone water of the following concentration: 0.1 g peptone (BactoTM Peptone, Becton, Dickinson and Company, Sparks, MD, USA) per 100 mL DW for a dilution factor of 1/10 per dilution. Exactly 0.1-mL aliquots of appropriate dilutions were spread plated onto tryptic soy agar (Difco Tryptic Soy Agar, Becton, Dickinson and Company, Sparks, MD, USA) plates. If the number of colonies at these dilutions were too few to detect, 0.1-mL aliquots of soy milk samples were plated directly onto the plates. The plates were incubated at 30 ± 1 °C for 48 h (APC) and at 4 °C for 7–10 days (psychrotrophs). Results were recorded as colony forming units per milliliter (CFU/mL).

2.4. pH

Triplicate pH readings of samples were taken (Accumet Basic AB 15, Fisher Scientific Company L.L.C., Pittsburgh, PA, USA) and the averages recorded.

2.5. Dry Solids Content

As there was no loss of water or soybean solids during UHPH, the total solids remained unchanged. Thus, the control soy milk samples were analyzed for total solids using Halogen Moisture Analyzer (Model HR73, Mettler-Toledo, Inc., Columbus, OH, USA) at a temperature of 115 °C. The total solid content also served as an indicator of the control over soy milk preparation.

2.6. Particle Size Distribution (PSD)

The PSD was measured using a Malvern Laser Particle Size Analyzer, Mastersizer S with 300 mm lens (Malvern Instruments, Southborough, MA, USA). Soy milk samples were dispersed in 150 mL DW and the pump speed of the dispersion chamber was kept at 2100 rpm. The obscuration in the diffractometer cell was maintained at 16% ± 0.5%. The predicted scattering was calculated based on the following refractive index (RI) information fed into the software: real RI = 1.47; imaginary RI = 0.00; RI of water = 1.33. The software calculated the average volume-weighted diameter, D[4,3] = Σnidi4/Σnidi3 (where ni is the number of particles in a class of diameter di), the surface-weighted mean diameter; D[3,2] = Σnidi3/Σnidi2; and the D(v,0.9) value, which is the diameter below which 90% of the particles (by volume) are found [23,24]. All soy milk samples were analyzed in duplicate and averages were recorded.

2.7. Visible Layer Separation/Sedimentation

All samples were inspected twice a week for any visible layer separation.

2.8. Lipoxygenase Activity

The method of Wang *et al.* [17] was followed. First, a 0.2 M borate buffer of pH 9.0 was prepared using sodium borate (Sodium Borate, 10-Hydrate, Crystal, A.C.S. Reagent, J.T. Baker, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) and boric acid (granular, A.C.S. Reagent, J.T. Baker, Mallinckrodt Baker, Inc.). Next, the substrate solution was made by mixing 0.01 mL linoleic acid (TCI America, Portland, OR, USA), 0.01 mL Tween 20 (Fisher Scientific, Fair Lawn, NJ, USA) and 4.0 mL of the borate buffer at 25 °C. This was homogenized using a Pasteur pipette by repeatedly taking in the solution and pushing it out. For clarification, 0.55 mL of 0.5 N NaOH (pellets, F.C.C., J.T. Baker, Mallinckrodt Baker, Inc.) was added and the volume made up to 60 mL using the borate buffer. To obtain the enzyme solution, soy milk was centrifuged at 30,000× *g* for 30 min at 4 °C in a Sorvall RC6 Plus Centrifuge (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using a Sorvall SM-24 rotor. Both were allowed to equilibrate to 4 °C for 1 h prior to centrifugation. Since the supernatant was cloudy, it was filtered through a 0.1 µm syringe filter. Prior to the assay, the filtered supernatant was diluted 5 times with DW. This comprised the enzyme solution. If some samples could not be analyzed at the designated time interval, they were stored at −65 °C. The assay mixture consisted of 2.0 mL of substrate solution,

0.9 mL of borate buffer, and 0.1 mL of enzyme solution. This mixture was prepared in a quartz cuvette and the cuvette shaken to start the reaction. The increase in absorbance at 234 nm was monitored using an Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) for 5 min immediately after shaking the cuvette. The temperature of the lab and the reagents was maintained at 25 °C. The lipoxygenase activity was calculated from the linear portion of the absorbance-time curve as provided by the instrument software (UV-Visible Chem Station, Rev. B.04.01, Agilent Technologies). A blank (2.0 mL substrate solution and 1.0 mL borate buffer) was also prepared.

2.9. Sensory Analysis

Descriptive sensory analysis was conducted on two treatments: T6 (121 °C, 12.48 s, 276 MPa) and T8 (121 °C, 12.48 s, 207 MPa). Samples heated to 145 °C were found to have an excessive cooked flavor not favored by the panelists and were not evaluated. Data from 11 trained panelists were used for the analysis. All panelists were food science graduate students, experienced in sensory analysis, and were given seven 1-h training sessions. A 150-mm unstructured line scale, with indents marked at 12.5 mm from either end, was used for each attribute. The panelists were free to place a vertical mark anywhere on the line depending on their perception of that attribute's intensity, which was then converted into millimeters. Six attributes were evaluated and these, in the order evaluated by the panelists, were: beany aroma, beany flavor, astringency, cooked flavor, bitterness, and chalkiness.

For the first few training sessions, the panel was calibrated for low and high intensity concentrations of each attribute. The panel, as a whole, came up with the intensities for each of these two concentrations. After calculating these consensual intensities, 'x' marks were put on the scales at appropriate distances signifying low and high concentrations. Next, medium-intensity concentrations of the reference samples were prepared and the panel calibrated for this concentration (Table 1).

Table 1. Sensory attributes and reference samples.

Attribute	Reference Sample	Preparation Method	Intensity (mm)
Beany Aroma	Raw soybeans soaked in deionized water for 16 h (1:12 w/w)	Drained and ground with deionized water (1:4 w/w)	60
Beany Flavor	Same as Beany Aroma	Same as Beany Aroma	60
Astringency	Alum Powder [a]	0.01% solution in water [b]	20
Cooked Flavor	Evaporated Milk [c]	Diluted with water (1:6 w/w)	45
Bitterness	Caffeine [d]	0.03% solution in water	20
Chalkiness	Protein Juice [e]	Diluted with water (1:4 w/w)	55

[a] Alum, McCormick & Co., Inc., Hunt Valley, MD, USA; [b] Crystal Springs Natural Spring Water, DS Waters of America, Inc., Mableton, GA, USA; [c] Carnation Evaporated Milk with Vit. D, Nestle, Glendale, CA, USA; [d] Caffeine, Anhydrous, FCC, ScienceLab.com, Inc., Houston, TX, USA; [e] Naked Protein Juice Smoothie, Naked Juice Company, Monrovia, CA, USA.

The final scale had two indentation marks and a box with an 'x' mark corresponding to the medium intensity for each attribute. Sensory evaluation was conducted in fluorescent-lit, individual sensory booths. Refrigerated soy milk and reference samples were served in clear plastic cups (59.15 mL) with lids. The soy milk samples were coded with three-digit random numbers and the order of presentation was randomized. The panelists were asked to cleanse their palates with crackers and water before tasting each sample. The evaluation sessions were held on days 1, 6, 13, and 20. Three commercial soy milk samples were also evaluated on two separate occasions, but no storage study was performed. These were Silk Organic Unsweetened Soy milk (White Wave Foods, Broomfield, CO, USA), SoyCow Unsweetened Soy milk (Well Luck Co., Inc., Jersey City, NJ, USA), and Vita Soy Unsweetened Authentic Asian Fortified Soy Beverage (Vitasoy USA, Inc., Ayer, MA, USA).

2.10. Data Analysis

The data were analyzed with JMP Pro Version 10.0.0 (SAS Institute Inc., Cary, NC, USA) and the results were considered to be significantly different if $\alpha < 0.05$.

3. Results and Discussion

3.1. Effect of UHPH on Temperature Rise

The soy milk was fed into the equipment at 23–28 °C and its temperature rose during throttling (Table 2). For the pre-heated samples, only pressure had a significant effect on the temperature rise. The average temperature rise for pre-heated samples was 54.44 ± 4.656 °C (207 MPa) and 60.53 ± 5.174 °C (276 MPa). The temperature rise per unit applied pressure was calculated to be: 0.26 °C/MPa and 0.22 °C/MPa at 207 MPa and 276 MPa, respectively. However, for the treatments with no pre-heating, the average temperature rise per unit applied pressure was 0.22 °C/MPa, and this was not significantly different for either pressure level. Thus, when soy milk was pre-heated, the rise in temperature with increasing pressure did not follow a linear relation. However, at a constant pressure, as the soy milk was pre-heated to a higher temperature, the temperature of soy milk after throttling increased correspondingly. Thus, it is possible that the increase in temperature due to increased pre-heating occluded some of the temperature rise due to increased pressure. Other researchers have also reported a similar temperature rise [6,15,25]. In a related study, Floury *et al.* [26] subjected soy-protein stabilized emulsions to ultra-high pressure homogenization (UHPH) and found the same value for temperature rise. In milk, a linear temperature rise as a result of UHPH has been reported [27], though the temperature rise was lower (0.166 °C/MPa) than that in soy milk. This may be due to the higher viscosity of soy milk and the presence of coarser suspended particles. For high hydrostatic pressure processing (HHP), the rise in temperature is the result of adiabatic heating. This has been reported to be 0.028 to 0.085 °C/MPa depending on the product [28] and is a fraction of that observed for continuous high pressure (CHP) processing. Thus, to achieve similar temperatures during HHP, much greater pressure has to be applied, which leads to greater energy input. Additionally, the temperature rise is gradual in HHP while in CHP, the temperature rise is sudden (0.7 s as reported by Poliseli-Scopel *et al.* [15]), which could be beneficial from a microbial inactivation as well as a quality standpoint.

Table 2. Treatment combinations and temperature rise [a].

T. No. [b]	Pressure (MPa)	Heat Ex. [c] Temp. (°C)	Temp. Rise (°C)	Exit [d] Temp. (°C)	Rise Per Unit Applied Pressure (°C/MPa)	Residence Time (s)	Temp. After Holding Tube (°C)
T1 [e]	207	26.28 (0.025)	46.23 (0.325)	72.51 (0.300)	0.22 (0.002)	20.80	68.68 (0.525)
T2 [e]	207	27.25 (0.750)	44.90 (2.300)	72.15 (1.550)	0.22 (0.011)	12.48	70.08 (0.725)
T3 [e]	276	26.98 (0.375)	59.35 (0.550)	86.33 (0.925)	0.22 (0.002)	20.80	82.28 (0.225)
T4 [e]	276	29.10 (1.500)	58.05 (0.950)	87.15 (0.550)	0.21 (0.003)	12.48	85.30 (0.300)
T5 [f]	276	72.20 (4.700)	53.50 (7.800)	125.70 (3.100)	0.19 (0.028)	20.80	121.15 (0.300)
T6 [f]	276	59.85 (8.350)	64.85 (13.050)	124.70 (4.700)	0.23 (0.047)	12.48	121.38 (1.375)
T7 [f]	207	75.50 (2.200)	53.35 (3.350)	128.85 (5.550)	0.26 (0.016)	20.80	120.98 (0.875)
T8 [f]	207	72.25 (3.750)	61.25 (6.250)	133.50 (2.500)	0.30 (0.030)	12.48	122.30 (0.400)
T9 [g]	276	84.40 (1.400)	63.95 (2.750)	148.35 (1.350)	0.23 0.010)	20.80	144.15 (1.550)
T10 [g]	276	84.60 (5.600)	59.80 (7.000)	144.40 (1.400)	0.22 (0.025)	12.48	146.65 (1.250)
T11 [g]	207	95.45 (1.250)	50.85 (1.550)	146.30 (0.300)	0.25 (0.008)	20.80	141.88 (0.075)
T12 [g,h]	207	93.40 (0.400)	52.30 (1.900)	145.70 (2.300)	0.25 (0.009)	12.48	143.80 (1.300)
UT [h]	-	-	-	-	-	-	-

[a] The values are mean and SD (in parentheses) from two independent experiments. Temperature rise was calculated by subtracting the temperature of pre-heated soy milk from the temperature of soy milk measured at the exit point of throttling valve; [b] Treatment number; [c] Temperature of the pre-heated soy milk, measured at the end of the heat exchanger; [d] Exit Temperature, measured at the exit of throttling valve; [e] Treatments 1–4 received no preheating; [f] Treatments 5–8 were preheated for a target temperature of 121 °C at the end of holding tube; [g] Treatments 9–12 were preheated for a target temperature of 145 °C at the end of holding tube; [h] Control Sample.

3.2. Effect of UHPH on the Microbiological Quality of Soy Milk

The initial total microbial counts (Figures 2 and 3) in the untreated (UT) control sample were 4.98 ± 0.153 log· CFU/mL (APC) and 3.12 ± 0.697 log· CFU/mL (psychrotrophs). All the treated samples had significantly lower counts. Pre-heating the soy milk (T5–T12) resulted in significantly lower APC counts, although there was no significant effect of the exit temperature. The average count for the preheated samples was 0.51 ± 0.288 log· CFU/mL. Similarly, there was no significant difference in the APC counts amongst the soy milk samples that were not pre-heated (T1–T4) and the average was 2.30 ± 0.349 log· CFU/mL. For some of the treatment combinations (T6 and T8), no microbial growth was detected. Similarly, for psychrotrophs there was no significant difference between the four non-preheated samples and the eight preheated samples. However, these two sets of treatments (preheated samples and non-preheated samples) differed significantly. The average psychrotrophs count for the samples receiving no preheating was 0.27 ± 0.544 log· CFU/mL, while no psychrotrophs were detected in any of the preheated samples. It can be inferred that CHP, by itself, causes an almost 3 log reduction in APC and at least a 3 log reduction in psychrotrophs. Supplementing pressure with high temperature for short durations can lead to further reduction. It should be mentioned that the collection of samples was not done aseptically and this may have led to some contamination at the point of sample collection. Poliseli-Scopel *et al.* [15] treated soy milk with CHP (200–300 MPa) at 105–135 °C. In contrast to our results, they concluded that the reduction in microbial population was pressure dependent and reported a higher reduction at 300 MPa. Cruz *et al.* [29] also performed CHP of soy milk at 200–300 MPa but at lower temperature: 88–100 °C. This might explain why they observed lower log reductions: 2.42 log· CFU/mL (200 MPa) and 4.24 log· CFU/mL (300 MPa). On the other hand, Smith *et al.* [19] studied the effect of HHP on soy milk at a pressure range of 400–600 MPa at different initial temperatures and dwell times ranging from 1 to 5 min. They did not find the log reduction to be pressure dependent but to be significantly affected by the temperature. At the higher initial temperature of 75 °C, they observed a 4.5 log reduction. This reduction is comparable to the one that was achieved in this study. This shows that, as compared to HHP, lower pressures combined with brief high temperatures in CHP processing leads to similar or higher microbial reduction.

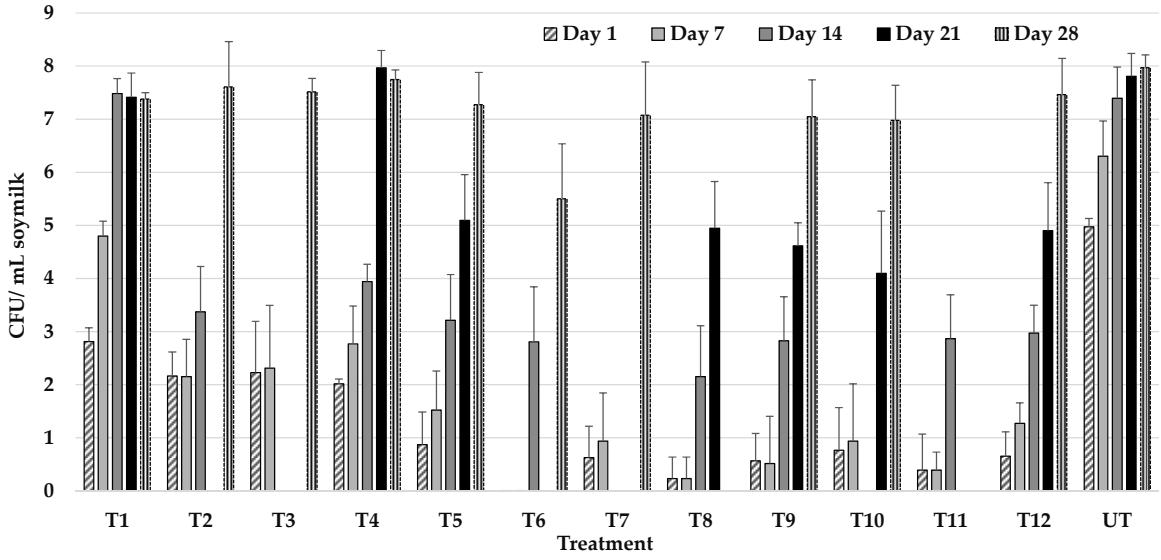

Figure 2. Changes in the aerobic plate counts over four weeks of storage at 30 °C.

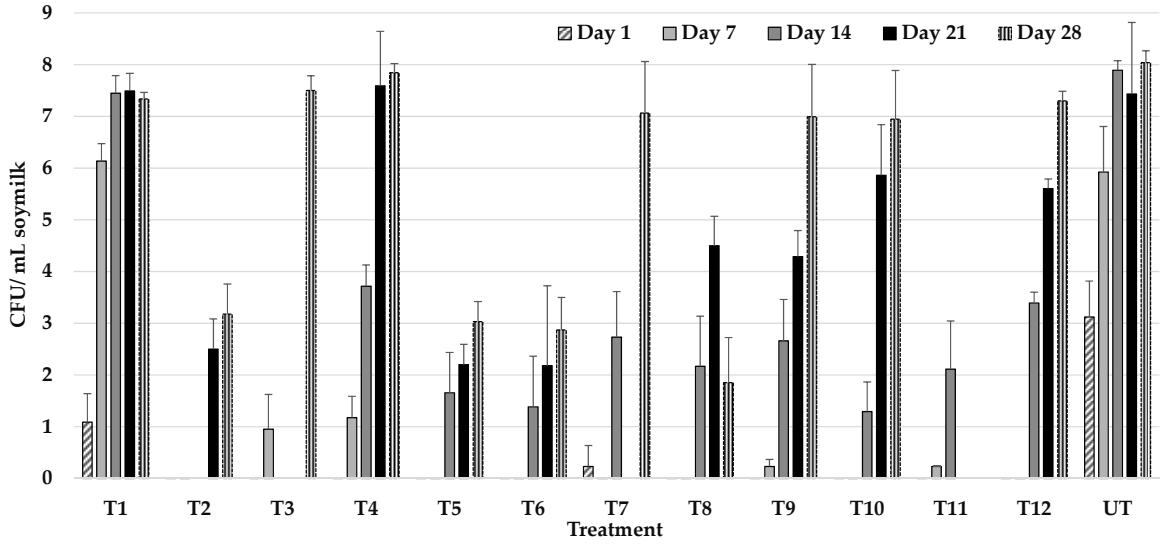

Figure 3. Changes in the psychrotrophs counts over four weeks of storage at 4 °C.

During storage (Figures 2 and 3), only time (D1, W1, W2, W3, and W4) had a significant effect. For the control, there was a significant increase in microbial counts after one week; the counts were 6.43 log· CFU/mL and 6.17 log· CFU/mL for APC and psychrotrophs, respectively. By the end of four weeks, these had risen to 7.97 log· CFU/mL and 8.01 log· CFU/mL, respectively, and there was no significant difference between the two counts. For treatments 1 to 4 (no pre-heating), there was no significant increase in the two counts for the first week. After one week, there was a continuous rise in counts that reached 7.56 log· CFU/mL (APC) and 6.46 log· CFU/mL (psychrotrophs) at the end of four weeks. Treatments 5–12 (pre-heated to achieve 121 °C or 145 °C at exit) also did not show a significant increase in APC until week 1, while the psychrotrophs remained non-detectable. After one week, however, both the counts began to rise, reaching 6.89 log· CFU/mL (APC) and 5.15 log· CFU/mL (psychrotrophs) after four weeks. Smith *et al.* [19] have determined the spoilage detection level to be 7 log· CFU/mL, and several treatments did not reach this level, even at the end of the storage period. Other authors have also noted an increase in microbial counts upon storage of HPP milk. This signifies that HPP causes injury to many cells, especially at lower pressures [11]. Poliseli-Scopel *et al.* [15] observed an increase in microbial counts of soy milk samples upon storage, even though no counts were detected immediately after high pressure processing. They stored the samples at 30 °C rather than at 4 °C and in the case of samples treated at 300 MPa and 135 °C there was no growth even after 20 days of storage. However, their sample collection method (laminar flow) ensured no contamination at the point of sample collection. The same authors, in a more recent study [22], treated soy milk at a pressure of 300 MPa and 144 °C, and collected and packed the soy milk aseptically. The soy milk showed no microbial spoilage when stored at room temperate for six months. Wang *et al.* [21] pasteurized soy milk (82 °C, 1 min) and observed no microbial growth after four weeks of storage at 3 °C. However, their soy milk contained flavoring, gum, and sugar, which may have acted as preservatives. In a similar study [30] on bovine milk with initial APC of 5 log· CFU/mL, no microbial counts could be detected after high pressure homogenization. However, the counts increased to 8 log· CFU/mL after 14 days of storage at 5 °C. Thus, the effectiveness of high pressure processing on microbial inactivation varies widely.

3.3. Effect of UHPH on pH

The UT sample was the control and had a pH 7.10 ± 0.046 and UHPH caused a reduction in pH (Figure 4). The pH of all the treated soy milk samples was significantly lower than the control, but the pH values did not differ across treatments and the average pH was 6.90 ± 0.052. The application

of high pressure and temperature possibly changed the conformation of certain proteins affecting their charge and/or solubility, leading to a change in pH. As the soy milk is throttled, there is a tremendous reduction in particle size. This reduction means that there is a large increase in the surface area of soybean solids, thereby exposing more surfaces. If there are charged molecules on the exposed areas, the pH could be affected. A study on heating of soy milk containing okara found increased interactions between protein and liberated lipids, which caused lower pH values [31]. Malaki Nik *et al.* [14] pasteurized soy milk at 95–100 °C for 7 min followed by homogenization, albeit at a lower pressure of 69 MPa. No change in the pH value of 6.7 was found. Hayes and Kelly [32] studied the HPH of bovine milk and found that the pH level reduced with increasing pressure. Pereda *et al.* [33] performed HPH on milk and again found a small reduction in pH from 6.74 to 6.72 at 200 MPa. The drop in pH with pressure has been highlighted by Farkas and Hoover [34] as well. Interestingly, no change in pH is generally observed with high hydrostatic pressure processing [19,35,36] even when soy milk is only thermally treated without the application of pressure. However, in all these studies, the okara was filtered out, changing the composition of suspended matter in soy milk.

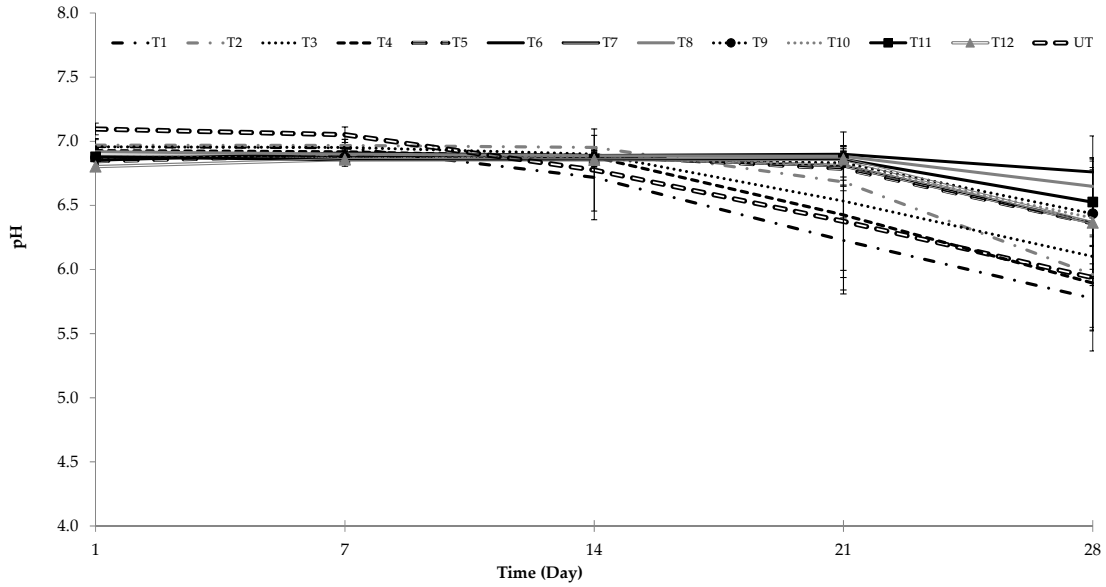

Figure 4. Change in the pH of soy milk over four weeks of storage at 4 °C.

The change in pH over four weeks of refrigerated (4 °C) is shown in Figure 4. There was a significant drop in the pH of the control sample (UT) after two weeks, when it fell to 6.78 ± 0.320 signifying acidification. After four weeks, the pH dropped to 5.94 ± 0.410. The pH of non-preheated soy milk samples (T1–T4) changed significantly only at the three-week mark, although by the end of four weeks their pH was around that of the control. The samples that were preheated (T5–T12) to achieve a final temperature of 121 °C or 145 °C remained stable for three weeks and there was a drop in their pH value only at the four-week mark. Their final pH nonetheless was still higher than that of all other samples. Neither pressure nor the exit temperature affected the change in pH upon storage for these samples. Poliseli-Scopel *et al.* [18] also observed a decrease in pH CHP soy milk and the pH dropped to 6.7–6.8 after four weeks of storage at 4 °C. Smith *et al.* [19], using a high hydrostatic pressure system for processing soy milk, reported similar results, although the pH of their control sample dropped to 4.8 after four weeks. Lakshmanan *et al.* [35] stored homogenized and pasteurized soy milk for two days and did not observe any change in pH. Achouri *et al.* [37] used only thermal treatment (116 °C for 6 min) to process soy milk and found that the pH did not change much during the first three weeks, but dropped by 0.6–0.7 units at the end of four weeks. Referring to their results, even though the soy milk samples in this study were treated at high temperatures

(121–145 °C), the pH stability was almost identical, and the maximum pH drop in the preheated samples was about 0.5 units. The change in pH is probably due to the interaction between protein and lipids, protein aggregation, or the growth of microbes.

3.4. Total Solids Content (%) and Comparisons with Commercial Samples

The samples prepared in the lab had the highest total solids content, while the SoyCow sample had the lowest (Table 3). Thus, there is some margin to further dilute the soy milk produced in this study to make the dry solids content comparable to that of commercial samples. The opportunity for further dilution means a yet higher yield. Also, the average pH of SoyCow samples was 6.7 ± 0.01 and that of Vita Soy samples was 6.4 ± 0.02. These values are comparable to the pH of the T6 and T8 samples (Figure 4). The Silk soy milk was alkaline; this may be due to the addition of calcium carbonate, which is alkaline when in solution.

Table 3. Dry solids content [a].

Sample	Mean (%)	SD (%)
T6 [b]	8.71	0.045
T8 [c]	8.78	0.170
Silk	7.03	0.085
SoyCow	3.77	0.030
Vita Soy	6.89	0.045

[a] The values are from two independent experiments; [b] 121 °C, 12.48 s, 276 MPa; [c] 121 °C, 12.48 s, 207 MPa.

3.5. Effect of UHPH on the Particle Size Distribution (PSD) of Soy Milk

There was a significant reduction the particle size of all the treated samples (Table 4). However, there was no significant effect of pressure, temperature, or residence time on the D[4,3] values, although the D[4,3] values were generally lower for the higher pressure level. Interestingly it was seen that this value was generally higher for higher temperatures. The D[3,2] values were significantly affected by pressure and temperature. The average value at 207 MPa was 13.46 ± 2.751 μm, while at 276 MPa it was 12.19 ± 1.783 μm. Increasing the exit temperature caused a significant increase in this value and the averages were: 10.38 ± 0.564 μm (no preheating), 12.80 ± 0.748 μm (121 °C) and 15.29 ± 1.604 μm (145 °C). Thus, increasing the pressure significantly reduced the particle size of finer soybean solids present in the soy milk, while the size of coarser particles was reduced to a level that did not differ significantly between the two pressure levels. The D(v,0.9) value did not differ significantly between any of the treatment combinations.

Sivanandan *et al.* [6] observed a significant reduction in both, D[4,3] and D[3,2] values as well as narrowing of the distribution with increasing pressure. They attributed the reduction in particle size to the weakening of membranes of the particles as the pressure was increased, causing the particles to easily disintegrate during throttling. Cruz *et al.* [29] observed an opposite effect and found that the soy milk that was homogenized at 300 MPa had higher values of D[4,3] and D[3,2] as compared to the soy milk treated at 200 MPa. They attributed this to the coalescence of soybean particles. The results of Poliseli-Scopel *et al.* [15] agree with the result of our study in that they also did not find a significant effect of either pressure or temperature on the mean diameters. The diameter of majority of the particles in their study was below 1 μm, which is much lower than the particle size of the soy milk processed in the current study. This could be because they filtered out the okara. The okara being hard may have caused the average mean diameter to be higher in this study. Also, the chalkiness (discussed in the following sections) of soy milk was not objectionable, and was comparable to that of market samples. Thus, even though the average particle size of soy milk particles was higher in our study compared to soy milk made by filtration, it did not affect its sensory qualities. A lot of researchers have performed HPH on bovine milk and the particle size of milk fat globules after high pressure homogenization is much smaller, in the nanometer range [31,33,38]. This is probably

due to the absence of hard, plant cell well type materials in bovine milk. Additionally, the average particle size of non-homogenized milk is generally reported to be around 3 μm, which is smaller than that of non-homogenized soy milk. No significant changes in any of these parameters were observed during storage. The stable particle size means that no aggregation or flocculation of soybean solids suspended in the soy milk occurred and all the samples remained physically stable during storage. Poliseli-Scopel *et al.* [22] found the CHP-processed soy milk to be stable even after six months of storage.

Table 4. Particle size distribution [a].

T. No. [b]	Temp. [c] (°C)	Residence Time (s)	Pressure (kPa)	D[4,3] [d] (μm)	D[3,2] [e] (μm)	D(v,0.9) [f] (μm)
UT [g]	-	-	-	129.86 (10.659)	17.04 (0.690)	335.90 (30.196)
T1	No heating	20.80	207	23.34 (3.903)	10.60 (0.940)	46.90 (8.560)
T2	No heating	12.48	207	25.21 (3.076)	10.63 (0.820)	51.13 (6.247)
T3	No heating	20.80	276	20.91 (1.131)	10.75 (0.757)	40.90 (2.885)
T4	No heating	12.48	276	19.74 (1.294)	9.54 (0.350)	39.21 (3.002)
T5	121	20.80	276	22.44 (2.517)	12.63 (0.841)	43.70 (5.469)
T6	121	12.48	276	23.47 (0.240)	12.31 (1.039)	46.70 (0.106)
T7	121	20.80	207	22.60 (1.584)	12.38 (0.686)	43.51 (3.140)
T8	121	12.48	207	26.24 (2.058)	13.91 (1.336)	51.98 (4.179)
T9	145	20.80	276	23.63 (6.039)	13.59 (1.937)	46.53 (12.459)
T10	145	12.48	276	28.25 (8.775)	14.34 (1.648)	56.39 (19.958)
T11	145	20.80	207	30.19 (3.543)	17.08 (1.478)	58.35 (7.266)
T12	145	12.48	207	30.10 (7.6374)	16.14 (0.778)	58.65 (14.711)

[a] The values are mean and SD (in parentheses) from two independent experiments; [b] Treatment number; [c] Exit Temperature, measured at the exit of throttling valve; [d] D[4,3] = average volume-weighted diameter ($\Sigma n_i d_i 4/\Sigma n_i d_i 3$); [e] D[3,2] = surface-weighted mean diameter ($\Sigma n_i d_i 3/\Sigma n_i d_i 2$), where, n_i is the number of particles in a size class of diameter d_i; [f] D(v,0.9) = the diameter below which 90% of the particles (based on volume) are found; [g] Untreated/Control Sample.

3.6. Visible Layer Separation

The control sample separated into two layers with a well-defined boundary within 1–2 days. Also, even though in the present method of soy milk processing, there is no filtration of okara and there is a greater amount of solids suspended in the soy milk as compared to the soy milk in other studies, none of the treated samples showed any separation even after four weeks of storage. Other researchers [29] have reported similar results.

3.7. Effect of UHPH on the Lipoxygenase (LOX) Activity in Soy Milk

No LOX activity in the control sample was detected. Since all the treatments involved further heating and application of pressure, it was deduced that no other treatment combination would have any LOX activity and the LOX activity in these samples was not analyzed. The absence of LOX activity is in agreement with the results of Poliseli-Scopel *et al.* [15]. They ground the soybeans at 80 °C for 20 min. In our study, the soybeans were blanched at 60 °C for 2.5 h; this explains the lack of any LOX activity.

3.8. Effect of UHPH on the Sensory Attributes of Soy Milk

SoyCow and Vita Soy soy milks are made in Asia, while Silk is made in the USA. It is important to note that no additives, sweeteners, flavors, viscosity modifiers, *etc.* were used in the processing of soy milk in the current study. However, all three commercial samples had the following added to them (based on the ingredients statement):

- Silk: Calcium Carbonate, Sea Salt, Flavors, Gum, Vitamins
- SoyCow: Emulsifier
- Vita Soy: Tricalcium Phosphate, Salt, Zinc Oxide, Vitamins

These additives could improve the flavor perception and mouthfeel of soy milk.

Silk had an average beany aroma intensity of 16.9, which was significantly lower than the other four samples (Figure 5). The beany aroma intensities for these samples ranged from 32.5 for SoyCow to 40.3 for T8, but were not significantly different from one another. Silk had the lowest intensity of beany flavor (26.7) while Vita Soy had the highest (50.7). The beany flavor of other samples ranged from 40 to 45. Even though no lipoxygenase activity was detected in the soy milk, the presence of beany aroma and flavor indicates that some non-enzymatic reactions gave rise to the beany notes. There was no significant difference in the astringency of the samples and it ranged from 14.1 for Silk, to 20.9 for T8. The samples prepared in the current study had significantly higher cooked flavor intensities as compared to the commercial samples. Sample T6 had the highest bitterness intensity (21.2), which was significantly different from Silk, which had the lowest (12.3). There was no significant difference in the chalkiness of the samples; it ranged from 20 to 23 for all the samples. Thus, even though no gum or emulsifier was added to the processed soy milk to improve the mouthfeel, the UHPH process made the chalkiness of samples highly comparable to commercial samples. Thus, a processor can incorporate all the soybean solids into soy milk without the issue of chalkiness, and this translates into a higher yield. In addition, it is imperative to mention that in general, the astringency, bitterness, and chalkiness were quite low in intensity (less than 25) for all the samples, especially considering that a 150 mm scale was used.

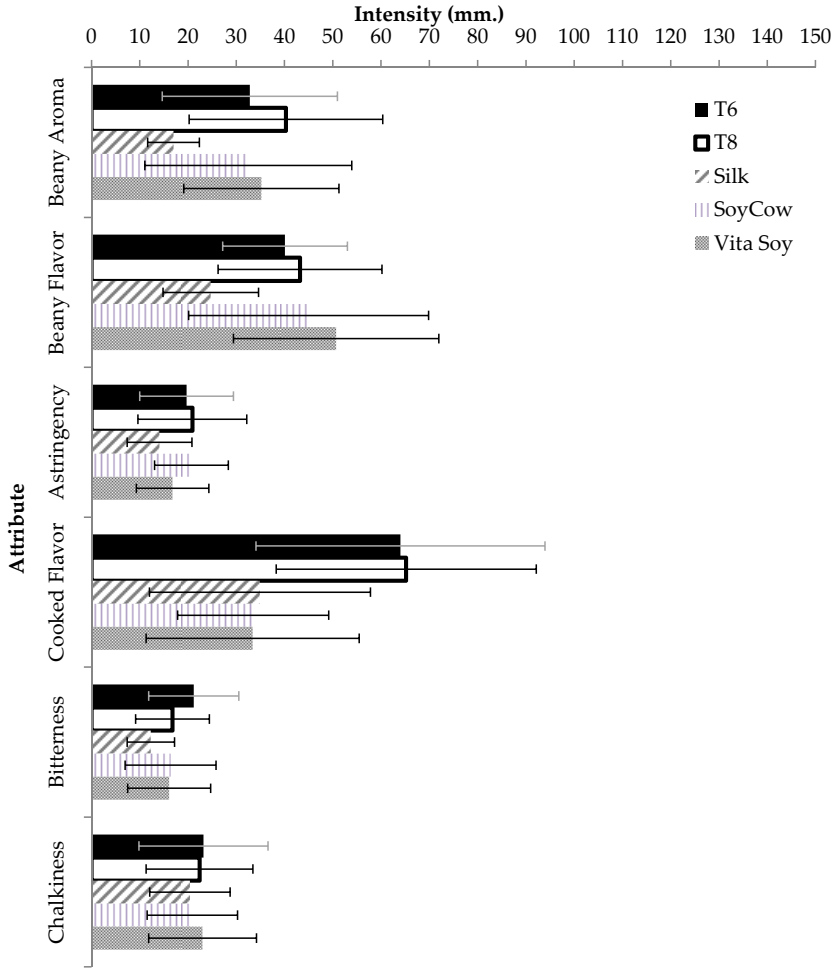

Figure 5. Intensities of various sensory attributes (measured on a 150 mm scale) for five different samples, as evaluated by 11 panelists. T6 (121 °C, 12.48 s, 276 MPa) and T8 (121 °C, 12.48 s, 207 MPa) samples were processed in the lab, while the remaining samples were bought from the market. Evaluation was done on day 1.

Only the samples processed in the lab were used for storage study. There was no significant difference between the two samples in any attribute throughout the storage (Figure 6). However, there was a significant change in the intensity of only the beany flavor on day 20, which reduced from 41.6 on day 1 to 30.5 on day 20. This value is close to the beany flavor intensity of the Silk soy milk sample. Achouri *et al.* [37] noticed a general decrease in the total volatile content of soy milk after storage at 4 °C. If some of these volatiles contribute to a beany flavor, then the intensity of beany flavor will also be reduced over a period of time. Poliseli-Scopel *et al.* [22] also did not observe any change in the sensory perception of beany flavor, grassy flavor, oxidized flavor, astringency, and thickness over a period of six months.

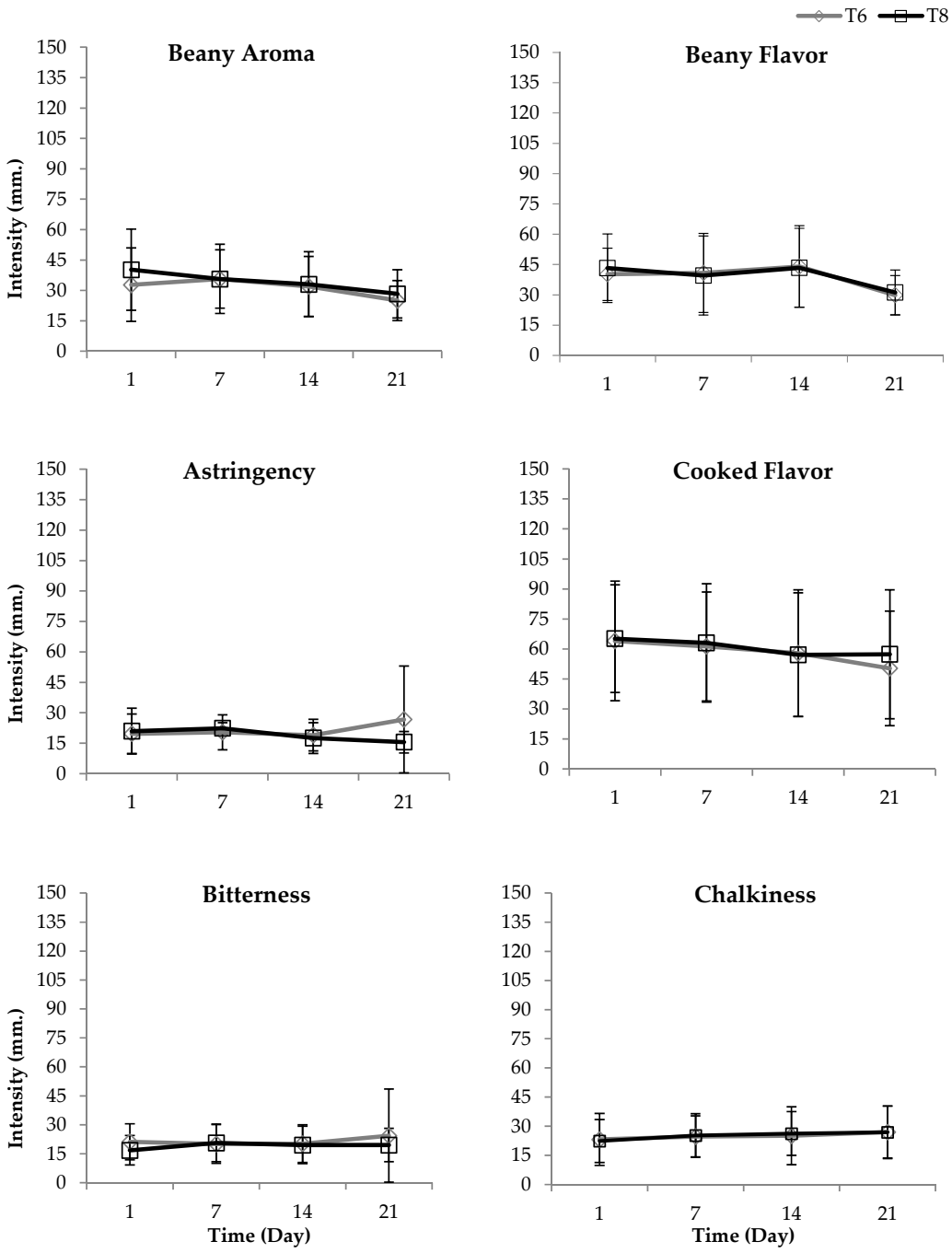

Figure 6. Changes in the intensities of sensory attributes over 20 days of storage at 4 °C.

4. Conclusions

Soy milk from whole dehulled soybeans was prepared in the current study without any substantial wastage of soybean solids, signifying greater yields for a processor. The soy milk was processed with continuous high pressure and minimal heating to obtain a pasteurized and physically stable product. The sensory characteristics of the soy milk were not very different from commercial samples. No lipoxygenase activity was detected in the soy milk. Preheating the soy milk had a significant effect on the stability of the soy milk. For further research, the nutritional characteristics as well as the consumer acceptability of UHPH soy milk could be evaluated.

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Author Contributions: Rakesh Singh conceived the project; Rakesh Singh and Jaideep Sidhu designed the experiments; Jaideep Sidhu performed the experiments, analyzed the data, and wrote the draft of the paper, which was revised by Rakesh Singh.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

APC	Aerobic Plate Counts
CFU	Colony Forming Units
CHP	Continuous High Pressure
CFHPT	Continuous Flow High Pressure Throttling
DW	Deionized Water
HDPE	High Density Polyethylene
HHP	High Hydrostatic Pressure
HPP	High Pressure Processing
LOX	Lipoxygenase
PSD	Particle Size Distribution
RH	Relative Humidity
RI	Refractive Index
UHPH	Ultra High Pressure Homogenization
UT	Untreated

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