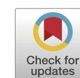
International Dairy Journal 137 (2023) 105512

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Stability of acidified milk drinks: Comparison of high hydrostatic pressure (HHP) and thermal treatments



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ARTICLE INFO

Article history:
Received 28 June 2022
Received in revised form
16 September 2022
Accepted 18 September 2022
Available online 23 September 2022

ABSTRACT

There is a search for effective stabiliser activity and alternative pasteurisation techniques for acidified milk drinks (AMD). In the present study, AMD were prepared at three different pH (4.0, 4.5 and 5.0) and high methoxyl pectin (HMP) levels (0.2, 0.5 and 0.8%) by thermal (75 °C, 15 min) and high hydrostatic pressure (HHP) (100, 300 and 500 MPa, 5 min) treatments. To achieve minimum steric stability, 0.5% HMP was required. HMP at 0.5%, pH 4.0 and 4.5, produced high protein solubility (60–70%), low storage sedimentation (10–15%) and mean particle size values in the range of 1.70–3.00 μm for all samples. Heat treatment induced lower particle size variation than HHP treatment. Heat-treated samples also showed lower Turbiscan stability index (TSI) values and smaller delta backscattering (ΔBS) variations. Nonetheless, HHP could replace heat treatment at 0.5% HMP concentration and pH 4.0–4.5.

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1. Introduction

Acidified milk drinks (AMD) are a class of dairy products including yoghurt drinks, fruit juice containing milk drinks, buttermilk and whey drinks (Guo, Wei, Cai, Hou, & Zhang, 2021). AMD are gaining popularity due to their nutritional aspects and refreshing taste. These drinks can be acidified either by direct acidification via addition of acidulants such as citric acid, glucono-δ-lactone and malic acid or microbial fermentation (Jensen, Bom Frøst, & Ipsen, 2010). However, these are low pH drinks (3.4–4.6) and instability of AMD is a common problem (Janhøj, Rolin, & Ipsen, 2008).

Acidic conditions reduce the stability of casein micelles since the electrostatic and steric repulsions that prevent aggregation of casein micelles are weakened below pH 6.7 (Fox & Brodkorb, 2008). Further decrease in pH to around isoelectric point (pI) of caseins (4.6) induces casein micelle aggregation due to lack of repulsive forces (Corredig, Sharafbafi, & Kristo, 2011). The main reason behind the depletion of repulsive forces and subsequent casein micelle aggregation is the function loss of κ-caseins located on the casein micellar surfaces below neutral pH (De Kruif, 1998). The negatively charged hydrophilic C-terminal regions of κ-caseins protrude from the surface and provide electrostatic and steric repulsions, keeping the casein micelles from aggregation (De Kruif & Zhulina, 1996). As the pH is lowered, these protruded hairy segments collapse and κ-caseins lose their stabilisation functionality (Tuinier & De Kruif, 2002). Another reason for destabilisation of AMD is the low viscosity of these products. Stokes' law dictates that a high continuous phase viscosity in a dispersion is likely to provide better physical stability by retarding the sedimentation process of particles (Wagoner & Foegeding, 2017). Thus, a combination of low pH and viscosity would most probably cause instability in AMD via casein micelle aggregation, wheying-off and finally phase separation.

Instability of AMD is often attempted to be resolved with anionic polysaccharide based stabilisers such as high methoxyl pectin (HMP) (Jensen et al., 2010), propylene glycol alginate (PGA) (Xia, Zong, Liu, Zhao, & Zhang, 2019), soybean soluble polysaccharides (SSPS) (Tian et al., 2021) and carboxymethylcellulose (CMC) (Du et al., 2007). Although all these stabilisers provide stability to AMD in a similar manner, HMP is used in most dairy systems due to its high efficiency and wide acceptance (Liu, Pedersen, Knarreborg, Ipsen, & Bredie, 2020b).

Besides providing stability to AMD, preservation of such products is also a crucial task. For this reason, milk-based products are subjected to heat treatments to ensure microbial safety and prolong their shelf-life (Serna-Hernandez, Escobedo-Avellaneda, García-García, Rostro-Alanis, & Welti-Chanes, 2021). Conventionally, thermal pasteurisation is still the core technology in dairy product processing but there are some drawbacks related to this technique

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https://doi.org/10.1016/j.idairyj.2022.105512
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including loss of flavour and nutritional value of the products and changes in appearance and texture (Huang, Wu, Lu, Shyu, & Wang, 2017). The reason for such effects is the physical and chemical modifications in the heat sensitive milk constituents by thermal treatment (Munir et al., 2019). Therefore, there is a search for alternative reliable pasteurisation technologies that would meet consumer demands in terms of fresh, appealing and safe products with preserved nutritional value (Serna-Hernandez et al., 2021).

Consequently, high hydrostatic pressure (HHP) has attracted a great attention as a non-thermal process that is capable of reducing the microbial load to a safe level at room temperature with a minimum loss in nutritional value and organoleptic properties of the milk and dairy products (Barba, Esteve, & Frígola, 2012). Independent from the size and shape of the material, HHP produces instant pressure acting isostatically and homogenously (Goyal, Sharma, Upadhyay, Sihag, & Kaushik, 2013). HHP also works through Le Chatelier principles shifting the equilibrium of the reactions such as chemical reactions, phase transitions and conformational changes to the side of reduced volume (Aganovic et al., 2021). In this way, reactions that would result in reduced volume are triggered (Balci & Wilbey, 1999). Despite its minimum effects on nutritional and sensory properties of AMD (Serna-Hernandez et al., 2021), HHP induces some physical changes in molecules at microscopic and/or macroscopic level (Serna-Hernandez et al., 2021; Yamamoto, 2017). Additionally, microscopic ordering may occur in the form of closer packing of molecules under HHP (Yamamoto, 2017). Nonetheless, HHP does not affect small molecules with no or little secondary, tertiary and quaternary structures such as vitamins, flavour-aroma components and amino acids (Balci & Wilbey, 1999).

In this study, our primary aim was to compare the effects of thermal pasteurisation and HHP on stability of the prepared AMD formulations. To the best of our knowledge, utilisation of HHP as a preservation technique for AMD has not been studied in detail. Herein, this study presents a comparative analysis of AMD stability as well as physicochemical changes in samples depending on the pasteurisation technique used. HMP was used as stabiliser and its concentration was altered to see the effect of concentration on stability. AMD were produced at three different pH values for detailed investigation of the efficiency of the stabiliser and the differences induced by thermal and HHP treatments at each pH. Three pressure levels were applied to understand the pressure effect on the samples. Stability assessments were conducted by soluble protein content, particle size, sedimentation (centrifuge-induced and storage) and Turbiscan measurements.

2. Materials and methods

2.1. Materials

HMP (Grindsted Pectin AMD 783, Danisco, Czechia) is a high methoxyl citrus pectin (DE: 70%) (see Supplementary material Fig. S1 for the FTIR spectrum of the HMP). Skimmed milk powder (Pinar Sut Inc., Izmir) (see Supplementary material Fig. S2 for the FTIR spectrum of the skimmed milk powder) produced from cow milk has protein and fat contents of 36% and 1.25% (w/w), respectively. Citric acid was provided by International Flavors and Fragrances Inc. (Kocaeli, Turkey). Peach concentrate (65 °Bx, 3.35% acidity as citric acid) was used for aroma purposes and kindly provided by Döhler (Karaman, Turkey). Sucrose (Gamsan Gida Imalat San. ve Dis Tic. Ltd., Sti., Istanbul) was also added to the samples.

2.2. Preparation of AMD

AMD were prepared with 4.5% (w/w) skimmed milk powder, different HMP concentrations (0.2, 0.5 and 0.8%, w/w), different pH values (4.0, 4.5 and 5.0) at the beverage laboratory of International Flavors and Fragrances R&D unit. pH values were adjusted with citric acid (50%, w/w, prepared in water) using a Mettler Toledo G20 Compact Titrator (Mettler Toledo, Switzerland). Sucrose and peach concentrate were also added to the samples at the concentrations of 6% and 0.48% (w/w), respectively. Preparation of AMD was based on the method given by Peterson, Rankin, and Ikeda (2019) with some modifications. Skimmed milk powder was reconstituted in water (1/10 ratio, w/w) at 60 °C for 2 h. HMP solution (5%, w/w) was also prepared in water at 90 °C. Then it was cooled down to room temperature. Prior to HMP solution addition, other ingredients were mixed and pH of the mixtures was adjusted to final values (4.0, 4.5 and 5.0) with citric acid solution. Then, HMP solutions were added and after hydration of HMP, samples were mixed by rotor-stator high-shear mixer at 8500 rpm (Silverson L4RT, USA).

2.3. Heat treatment

All AMD were bottled in glass containers (200 mL glass tubes with metal covers) and heat-treated at 75 °C for 15 min in a laboratory scale in-bottle pasteuriser (Miele G 7835, Germany). Samples were pre-cooled down to around 40 °C for about 15 min in the pasteuriser after pasteurisation took place. Then, heat-treated samples were kept in refrigerator at 4 °C for 24 h before analysis.

2.4. HHP treatment

HHP treatment was performed by 760.0118 type pressure equipment (SITEC-Sieber Engineering AG, Zurich, Switzerland) having a built-in heating – cooling system (Huber Circulation Thermostat, Offenburg, Germany). Pressure increase and release times were not included in the HHP treatment time. For the HHP experiments, AMD were filled into 25 mL sterile polyethylene cryotubes (LP Italiana SPA, Milano, Italy) and pressurised at 100, 300 and 500 MPa for 5 min at 25 °C. Pressurised samples were kept in the refrigerator at 4 °C for 24 h before analysis.

2.5. Particle size analysis

Particle size distribution and mean particle size of the samples were determined by laser diffraction using a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, United Kingdom). To set the required parameters for the measurements, refractive index (RI) and density of the samples were measured by Anton Paar Refractometer (Abbemat 3200, Germany) and density meter (Anton Paar DMA 4500, Germany), respectively. Particle RI, dispersant (water) RI, particle absorption index and laser obscuration values were 1.35, 1.33, 0.00 and 3.00–8.01, respectively.

2.6. Soluble protein content determination

Samples were initially centrifuged at 2862×*g* for 20 min (Hanil MF80 Benchtop Centrifuge, Hanil Science Industrial Co., Ltd., Incheon, Korea). Then, the protein solubilities of AMD were determined by Lowry method. The procedure given by Waterborg (2009) was used for this purpose.

2.7. Instant sedimentation

Instant (centrifuge-induced) sedimentation test was conducted by the method provided by Cai, Wei, Guo, Ma, and Zhang (2020) with some modifications. Firstly, samples were weighed and centrifuged at 2862×*g* for 20 min in a benchtop centrifuge device (Hanil MF80 Benchtop Centrifuge, Hanil Science Industrial Co., Ltd., Incheon, Korea). Then, the tubes were turned upside-down for

10 min to drain all supernatant. Sedimentation ratios (SR) were expressed as the percent ratio of wet sediment weight over the whole sample weight as follows:

$$SR(\%) = \frac{Wet\ Sediment\ Weight(g)}{Whole\ Sample\ Weight(g)} \times 100 \quad (1)$$

2.8. Storage experiments

2.8.1. Phase separation

Samples were placed in 2 mL sterile cryogenic cylindrical tubes and kept in refrigerator at 4 °C for one month. The amount of sample that was placed in a cryogenic tube was 1.8 mL. At the first day (day 1), end of second week (day 15) and end of the storage (one month – day 30), visual observations were done. Additionally, at the end of one month, phase separations were determined as final sedimentation ratios. Separations were expressed as the percent ratio of the sedimented phase height from the bottom to the total sample height.

2.8.2. Turbiscan stability analysis

Storage stability of AMD prepared at 0.5% HMP was also analysed by delta backscattering (ΔBS) profiles and Turbiscan stability index (TSI) values. This HMP level was chosen based on the results of previous storage phase separation experiment. Analyses were performed according to the method of Wu, Guo, and Lin (2020) with modifications. Samples were scanned every 5 min for 5 h at 40 °C with Turbiscan™ LAB Stability Analyser (Formulaction, Toulouse, France). Stability was also expressed as TSI which gives an estimation of the stability and/or instability of samples. TSI expresses all the data collected from the analysed sample as a single number. The following equation is used for the calculation of TSI (Zheng et al., 2018):

$$TSI = \sqrt{\frac{\sum_{i=1}^{n} (x_i - x_{BS})^2}{n-1}} \quad (2)$$

where n, x_i and x_{BS} denote number of scans, average backscattering for each specific time and average of x_i values, respectively.

2.9. Statistical analysis

Statistical analysis of the data was performed by MINITAB (Version 16.1.1., Minitab Inc., Coventry, United Kingdom). Analysis of variance (ANOVA) was conducted using general linear model by Tukey's test with 95% confidence level and the results were considered as significantly different at $p \leq 0.05$ level. Each sample was produced as at least three replicates and from each of these samples, three replicates were analysed.

3. Results and discussion

3.1. Particle size and distribution

Analysis of mean particles size and distribution of all samples indicated that both HMP concentration, treatment type/level and pH had distinct effects. Table 1 shows the mean particle size values of each sample. Firstly, mean particle sizes of HHP and heat-treated samples were in the range 1.70–3.78 μm and did not differ significantly for all concentrations at pH levels of 4.0 and 4.5. However, increasing the pH of AMD to 5.0 resulted in significantly higher mean particle size values for most of the samples, and HHP-treated samples attained even bigger mean sizes than the heat-treated ones ($p \leq 0.05$). At this pH, pressure level also became significant on the mean particle size. Significant variations on the particle size were created at different pressures as well. This could also be observed by the volume-based particle size distribution profiles of the samples as shown in Fig. 1.

Table 1
Mean particle size D [4, 3] values.[a]

HMP	pH	Thermal	100 MPa	300 MPa	500 MPa
0.2%	4.0	2.06 ± 0.00^{h}	3.78 ± 0.05^{h}	2.40 ± 0.02^{h}	2.24 ± 0.02^{h}
	4.5	1.94 ± 0.00^{h}	2.73 ± 0.02^{h}	2.06 ± 0.03^{h}	2.68 ± 0.11^{h}
	5.0	9.62 ± 0.52^{fg}	17.90 ± 1.94^{ef}	62.40 ± 4.28^{a}	57.11 ± 1.06^{a}
0.5%	4.0	2.99 ± 0.00^{h}	1.98 ± 0.05^{h}	1.70 ± 0.01^{h}	1.77 ± 0.01^{h}
	4.5	3.00 ± 0.00^{h}	1.73 ± 0.01^{h}	1.79 ± 0.02^{h}	2.06 ± 0.03^{h}
	5.0	2.92 ± 0.00^{h}	6.32 ± 0.88^{gh}	33.77 ± 5.20^{c}	13.19 ± 2.49^{efg}
0.8%	4.0	2.51 ± 0.00^{h}	2.44 ± 0.06^{h}	1.90 ± 0.01^{h}	2.30 ± 0.01^{h}
	4.5	2.47 ± 0.00^{h}	2.33 ± 0.03^{h}	2.25 ± 0.03^{h}	2.13 ± 0.01^{h}
	5.0	3.14 ± 0.00^{h}	28.16 ± 14.52^{cd}	44.06 ± 1.29^{b}	21.99 ± 1.47^{de}

[a] Abbreviation: HMP, high methoxyl pectin. Particle size D [4, 3] values (in μm) with different superscript letters are statistically different at $p \leq 0.05$; errors are represented as standard deviations.

At 0.2% HMP, all samples had similar particle size values at pH 4.0 and 4.5 but HHP-treated samples showed broader particle size distribution with tail at bigger size region (>10 μm). There was no such a bimodal size distribution pattern for heat-treated samples. Interestingly, those treated with HHP had an increased population of small size particles compared with heat-treated samples. The reason of such a small-size population was the casein fragmentation effect of HHP. Application of pressure disrupted the hydrophobic and electrostatic forces of the casein micelles and then calcium phosphates within the micelles were solubilised (Huppertz, Fox, de Kruif, & Kelly, 2006). Pressures, especially above 300 MPa, induce severe casein dissociation and fragmentation leading to considerably small particle size (Harte, Gurram, Luedecke, Swanson, & Barbosa-Cánovas, 2007). However, HHP may also favor hydrophobic bonding over hydrophobic solvation and induce reassociation of micellar casein particles up to 300 MPa (Huppertz et al., 2006). This could be one of the reasons for the bigger size population observed, especially for samples treated at 300 MPa.

Another reason for such a distinct bigger-size population could be the associations between denatured β-Lg and casein micelles (Huppertz, Fox, & Kelly, 2004; Scollard, Beresford, Needs, Murphy, & Kelly, 2000). Denatured whey proteins may react with κ-caseins located on micelle surfaces and form aggregates with bigger size (Needs et al., 2000). Additionally, tighter molecular packing promoted by HHP may facilitate the interactions between the exposed free thiol groups of denatured β-Lg and κ-caseins (Liu et al., 2020a). Therefore, a combination of complexation of denatured β-Lg with casein micelles and formation of casein aggregates under HHP may be the reason for the broader size distribution of HHP-treated samples than that of heat treatment.

AMD prepared at pH 5.0 (0.2% HMP) demonstrated even larger size variations and bigger mean size values ($p \leq 0.05$) than their coounterparts at pH 4.0 and 4.5. This could be related to the low HMP concentration (0.2%). Several studies have reported that there is a minimum pectin concentration required to cover the surfaces of casein micelles (Laurent & Boulenguer, 2003; Sun et al., 2020). If this minimum concentration is not provided, particles may undergo bridging flocculation and aggregates with larger sizes are formed (Syrbe, Bauer, & Klostermeyer, 1998). This bridging effect is induced by the sharing of a polysaccharide chain by two or more casein micelles at low polysaccharide (stabiliser) concentrations (Corredig et al., 2011). It is very likely that this was the case in AMD prepared with 0.2% HMP at pH 5.0 where electrosorption efficiency of HMP was much lower than it was at pH 4.0 and 4.5. Effect of

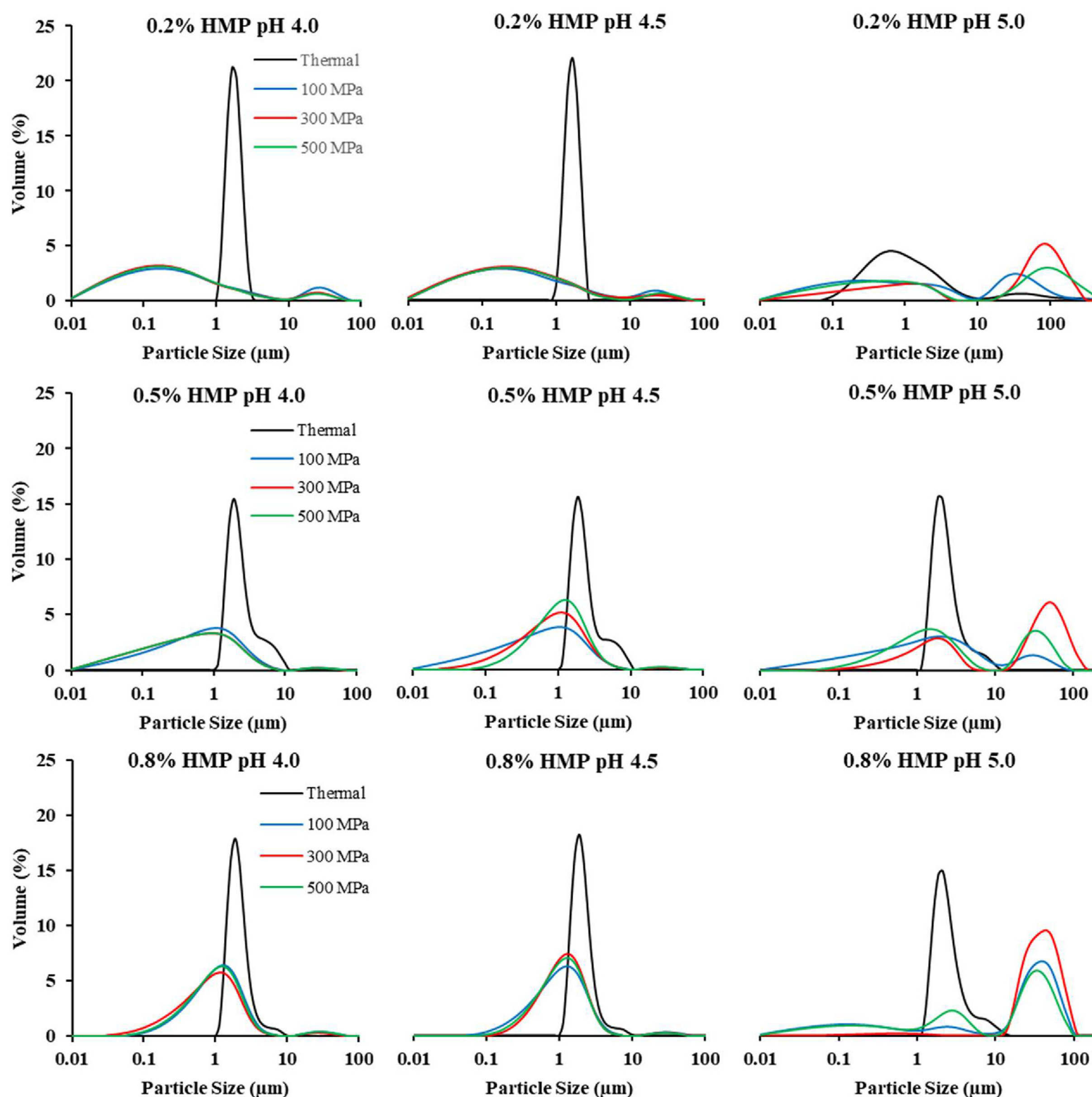

Fig. 1. Volume-based particle size distributions.

pressure level also became significant at pH 5.0. There was a significant increase in the mean particle size values of the samples at 300 MPa when compared with 100 MPa ($p \leq 0.05$). It was previously reported that mild pressure treatments around 250 MPa may increase the mean particle size of the dairy systems (Serna-Hernandez et al., 2021). Since casein micelle fragmentation does not predominate the system below 300 MPa, such associations increase the mean particle size.

There were some differences in the particle size and variations at higher HMP concentrations (0.5 and 0.8%). Firstly, all heat-treated samples showed statistically the same mean particle size values (2.47–3.14 µm) at both HMP concentrations (0.5 and 0.8%) and the entire pH range (4.0–5.0) as demonstrated in Table 1. Heat-treated samples also showed narrower size distributions when compared with HHP-treated ones, even at pH 5.0 at higher HMP concentrations (Fig. 1). At sufficient HMP concentrations, heat-treated systems remained fairly stable in terms of particle size and distribution. On the contrary, HHP-treated samples maintained their larger particle size and bimodal size distribution patterns at pH 5.0. Again, 300 MPa created larger mean particle size values at pH 5.0 (0.5 and 0.8% HMP). However, the same samples indicated significant decreases ($p \leq 0.05$) in their mean particle size values from 300 MPa to 500 MPa due to the casein fragmentation effect of HHP (Bravo, Felipe, López-Fandiño, & Molina, 2015).

At the pH levels of 4.0 and 4.5, HHP-treated samples showed similar mean particle size values to heat-treated samples (0.5 and 0.8% HMP). Some distinctions were also present. For instance, HHP-treated AMD had broader particle size distribution than heat-treated ones and they also showed very small secondary peaks above 10 µm (Fig. 1). These secondary peaks of HHP-treated samples enhanced greatly especially at pH 5.0 for both 0.5 and 0.8% HMP concentrations. These bigger-size populations were not observed in heat-treated samples due to sufficient adsorption levels of HMP at 0.5 and 0.8% concentrations. However, particle aggregation and complexation may still take place at high stabiliser concentrations (Kruif & Tuinier, 2001).

It is known that more than 80% (w/w) of HMP in AMD formulations does not adsorb onto the casein micelles and remains in the continuous phase (Tromp, De Kruif, Van Eijk, & Rolin, 2004). Therefore, increasing the HMP concentration above the levels required for complete coverage of casein particles may excessively increase the bulk viscosity (Laurent & Boulenguer, 2003). Consequently, depletion flocculation of the casein micelles may emerge. In this mechanism, presence of excess non-adsorbed polysaccharide (stabiliser) in the system increases the osmotic pressure of the bulk phase (Guo et al., 2021). This may lead to the exclusion of water along with polymers from the interparticle region into the bulk phase. As a result, protein particles aggregate and bigger-size complexes are formed (Kruif & Tuinier, 2001). Since the concentration of HMP was above the required level (0.5 and 0.8%) for full micelle surface coverage and a higher fraction of HMP was in non-adsorbed form at pH 5.0, HHP may have induced depletion flocculation of casein micelles via inducing closer molecular packing arrangements (Aganovic et al., 2021). Furthermore, HHP was reported to increase the dissolution of HMP (Zhong et al., 2021). If this is the case, HMP may have contributed to the osmotic pressure created by the high-viscosity bulk phase and accelerated the depletion flocculation of the particles. Therefore, thermal treatment did not lead to depletion flocculation and broad particle size distribution at pH 5.0, despite the presence of a considerable amount of non-adsorbed HMP.

3.2. Soluble protein content

Soluble protein measurements showed that HMP concentration is quite important for protein solubility. There was an increasing trend for protein solubility with the increasing HMP concentrations as shown in Fig. 2. Protein solubility values were in the ranges 10–23%, 16–74% and 31–76% for 0.2, 0.5 and 0.8% HMP, respectively. There was a dramatic increase in the protein solubilities from 0.2 to 0.5% HMP and a subtle increase from 0.5 to 0.8% HMP. The reason was the inadequacy of HMP action on casein micelles at 0.2% concentration. This HMP amount was lower than the minimum amount needed to achieve the full coverage of casein micelles as previously discussed in particle size section (Laurent & Boulenguer, 2003). Without full coverage of casein micelles with HMP, the system could not keep these particles suspended in the solution. Therefore, poor protein solubility values with a maximum around 23% were recorded at 0.2% HMP for all pH values studied. However, HHP treatment induced slightly higher protein solubility than heat treatment at 0.2% HMP and all pH values, especially at 500 MPa ($p \leq 0.05$). High casein fragmentation effect of HHP produced a group of smaller-size particles (see particle size section) that were able to sustain their suspension in the system where there was no sufficient amount of HMP (Chawla, Patil, & Singh, 2011). Solubilisation of sub-micellar casein particles under high pressures may also have contributed to the higher protein solubility results of HHP-treated samples at 0.2% HMP (Orlien, Boserup, & Olsen, 2010).

When HMP concentration was increased to 0.5%, electrosorption efficiency of HMP onto casein micelles increased and it became easier to keep the proteins at suspended position in the system (Tuinier et al., 2002). At full coverage of casein micelle surfaces, HMP created an acceptable steric and electrostatic stability within the system, particularly at pH 4.0 and 4.5. Further increase of pH to 5.0 resulted in less HMP adsorption onto casein micelles since this pH is above the pI of caseins (~4.6) (Corredig et al., 2011). At pH 5.0, caseins lost their positive charge that they possessed at pH 4.0–4.5 and electrosorption of negatively charged HMP molecules onto the micellar surfaces was severely impaired (Sedlmeyer, Brack, Rademacher, & Kulozik, 2004). Consequently, protein solubility levels declined to 16–28% range which was close to the overall levels observed at 0.2% HMP.

Although the general trend depended mostly on HMP concentration and pH levels, treatment types also demonstrated some distinctions. For instance, heat treatment resulted in higher protein solubility ($p \leq 0.05$) as compared with all HHP-treated samples at pH 4.0 for 0.5% HMP. Since HMP adsorption onto casein micelles was enhanced as the pH was lowered, heat-treated samples attained a decent stability (Jensen et al., 2010). Despite its casein hydration and dissociation effects (Goyal et al., 2013), HHP treatment could not provide a better stability than heat treatment at sufficient HMP concentration and low pH. Therefore, it could be concluded that heat-treatment can provide a good stability without any casein micelle dissociation effect at the right HMP concentration and pH combination. However, this superior behaviour of heat treatment disappeared at pH 4.5 since the heat treated samples experienced a lower HMP action on their casein micelles due to the slight increase in pH. Here, 500 MPa treatment revealed a comparable protein solubility level with heat treatment. Pressure treatment at 100 MPa, on the other hand, produced the lowest protein solubility at both pH 4.0 and 4.5 ($p \leq 0.05$). Pressure of 100 MPa cannot significantly affect casein micelles and can only initiate mild β-Lg denaturation (Anema, Lowe, & Stockmann, 2005). Thus, HHP treatment at 100 MPa did not reveal higher protein solubility.

Effects of 0.8% HMP on protein solubility were similar to those of 0.5% HMP. Increase of pH from 4.5 to 5.0 reduced the protein

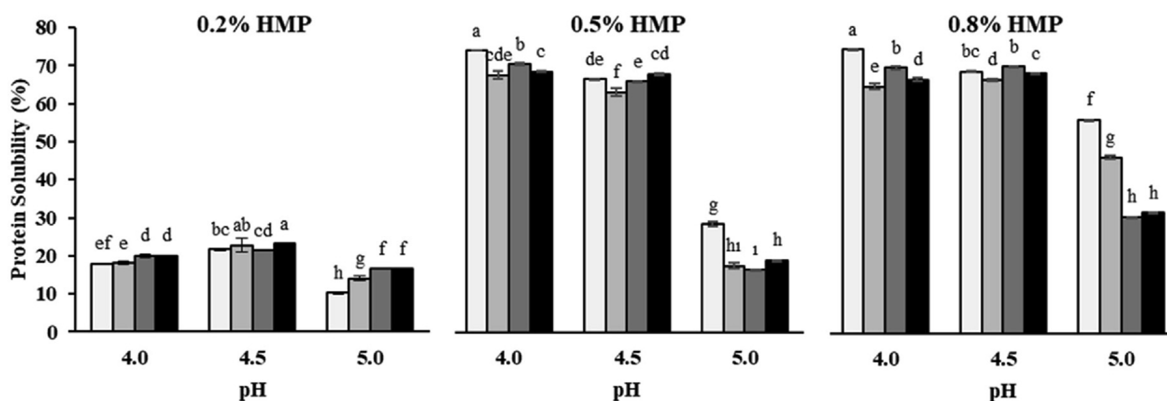

Fig. 2. Soluble protein contents: □, thermal; ■, 100 Mpa; ■, 300 MPa; ■, 500 MPa. Significant differences ($p \leq 0.05$) are indicated by small letters; lettering is done for each HMP concentration, separately. Errors are represented as standard errors.

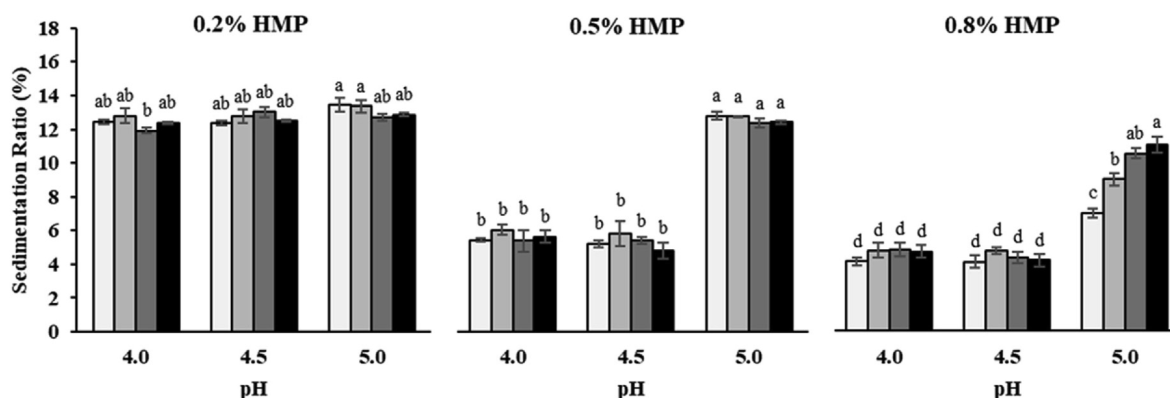

Fig. 3. Instant sedimentation ratios: □, thermal; ■, 100 Mpa; ■, 300 MPa; ■, 500 MPa. Significant differences ($p \leq 0.05$) are indicated by small letters; lettering is done for each HMP concentration, separately. Errors are represented as standard errors.

solubility of all samples due to lower adsorption of HMP onto casein micelles and a possible depletion flocculation process (Maroziene & De Kruif, 2000). However, samples had higher overall protein solubility values at 0.8% HMP, pH 5.0 (47–32%) than those at 0.5% HMP, pH 5.0 (28–19%). This could be due to the decrease in whey protein denaturation level caused by the higher amount of HMP in the bulk phase at 0.8% concentration. Michel et al. (2001) stated that whey protein denaturation decreased in the presence of HMP for both heat and pressure-treated samples.

Presence of sucrose in such systems could also contribute to this whey protein denaturation protection effect due to the effects of sucrose on solvent properties of water. Consequently, solubility behaviour of whey proteins may be influenced (Dumay, Kalichevsky, & Cheftel, 1994). Since our samples also included sucrose along with HMP, the degree of whey protein denaturation may have been reduced at higher bulk HMP concentration (0.8% HMP samples), thereby increasing the overall protein solubility at 0.8% HMP as compared with that of 0.5% HMP. However, increasing pressures resulted in lower protein solubility at 0.8% HMP, pH 5.0 ($p \leq 0.05$). It means, independent from bulk HMP concentration effect observed on whey protein denaturation, HHP-induced whey protein denaturation was promoted at higher pressures in the presence of high non-adsorbed HMP concentration (Huppertz et al., 2004; Zhong et al., 2021). Therefore, lower protein solubility was observed at 0.8% HMP, pH 5.0 with increasing pressures.

3.3. Instant sedimentation

Instant sedimentation was induced by centrifugation of the samples after one day storage at 4 °C. The idea was to accelerate phase separation process that would be observed during long-term storage. In this way, initial stability of the samples was determined. Fig. 3 shows the centrifuge-induced sedimentation ratios of all samples at each HMP concentration and pH. Firstly, all samples showed high sedimentation ratios (11.9–13.4%) at 0.2% HMP. Moreover, all samples had statistically the same sedimentation level regardless of the treatment type and pH. Apparently, the effects of treatment type and pH are negligible below the critical HMP content to provide steric stabilisation. Additionally, the sedimentation ratios at 0.2% HMP were higher compared with the 0.5 and 0.8% HMP at pH 4.0 and 4.5 ($p \leq 0.05$). Thus, firstly a minimum HMP concentration for steric stability must be provided. Jensen et al. (2010) experienced a similar situation where HMP concentrations below the critical level (~0.2%) increased the sedimentation of their AMD up to 30%. Their sedimentation ratio dramatically decreased down to negligible levels around 5% following the increase of HMP concentration above the critical value. Bridging flocculation was probably the dominant mechanism taking place at 0.2% HMP, leading to easy phase separation (Pereyra, Schmidt, & Wicker, 1997).

Increasing the HMP concentration to 0.5% resulted in lower sedimentation (4.8–6.0%) for the samples at pH 4.0 and 4.5, with no significant difference between the treatment types. At sufficient HMP concentration, steric stabilisation created by HMP adsorbed onto the casein micelles prevented the excessive sedimentation of the samples. However, this was not the case at pH 5.0. All samples reached high sedimentation ratios (12.3–12.8%) at pH 5.0, close to the values obtained at 0.2% HMP. The reason behind the poor instant stability of all samples at pH 5.0 was again the insufficient HMP adsorption onto casein micelles (Sedlmeyer et al., 2004). The same trend was also observed for the samples prepared at 0.8% HMP. Similar to 0.5% HMP, all samples attained the same low sedimentation (4.1–4.8%) at pH 4.0 and 4.5 where HMP was active on casein micelles.

The slightly lower sedimentation ratios of the samples at 0.8% HMP (pH 4.0 and 4.5) than those at 0.5% HMP could be due to the multilayer adsorption behavior of HMP onto micelles (Tuinier, Rolin, & de Kruif, 2002). The inner part of the adsorption layer is composed of firmly adsorbed HMP molecules so that the outer layer can contribute to the steric stabilisation of the system by protruding into the serum phase (Jensen et al., 2010). At 0.8% HMP, which is above the minimum micelle surface coverage concentration, the amount of HMP may have increased on the outer layer of the adsorbed HMP and provided more steric stabilisation that would keep a higher number of particles at suspended position (Koksoy & Kilic, 2004). The samples again had higher sedimentation ratios (7.0–11.0%) for 0.8% HMP at pH 5.0, but the pattern was different this time.

Heat-treated samples experienced a lower sedimentation (7.0%) whereas HHP treatment induced higher sedimentation (9.0–11%) ($p \leq 0.05$). Additionally, increasing pressures promoted more sedimentation. The reason for the sedimentation-promoting behaviour of HHP could be the HHP-induced depletion flocculation process at high non-adsorbed HMP concentrations (Elamin, Endan, Yosuf, Shamsudin, & Ahmedov, 2015), which was discussed in the particle size and distribution section. In addition, there was an apparent inverse proportionality between the protein solubility and sedimentation results of the samples at all HMP and pH levels. For

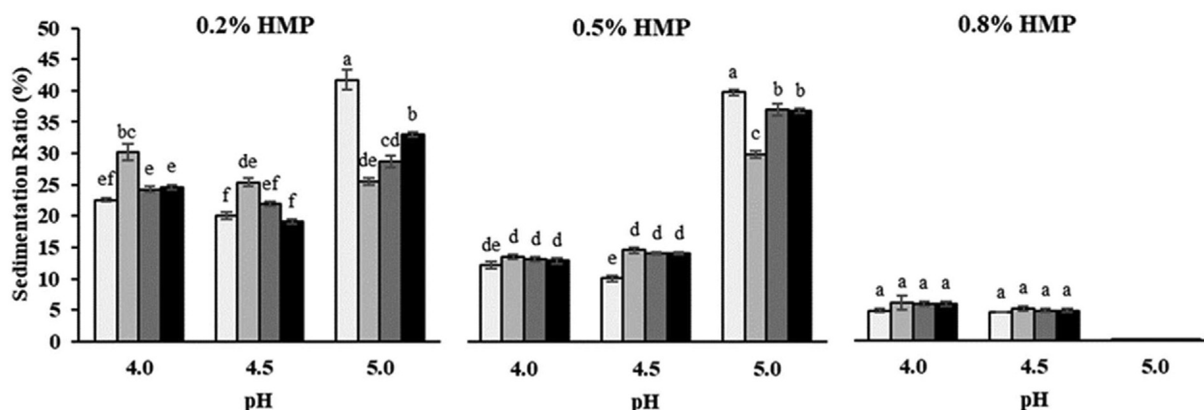

Fig. 4. Sedimentation ratios after one-month storage: □, thermal; ■, 100 Mpa; ■, 300 MPa; ■, 500 MPa. Significant differences ($p \leq 0.05$) are indicated by small letters; lettering is done for each HMP concentration, separately. Errors are represented as standard errors.

instance, all samples generally attained very low protein solubility at 0.2% HMP (Fig. 2) and the same set of samples had the highest sedimentation among the other HMP concentrations.

Effect of pH also reflected itself on this relation. The high protein solubility of samples at pH 4.0 and 4.5 (0.5 and 0.8% HMP) was accompanied by lower sedimentation at the same conditions. Moreover, the decrease in protein solubility of HHP-treated samples at 0.8% HMP − pH 5.0 with increasing pressures resulted in increased sedimentation of the same samples. Consequently, it is clear that high protein solubility along with sufficient HMP adsorption activity are needed for short-term stability of AMD. Generally, presence of some non-adsorbed HMP highly retards serum separation and sedimentation by increasing the continuous phase viscosity (Acero-Lopez, Alexander, & Corredig, 2010), but this was not the case in our samples (0.8% HMP) since the sedimentations were induced deliberately by centrifugation. The role of non-adsorbed HMP on long-term AMD stability will be considered in storage experiments in the next section.

3.4. Storage experiments

3.4.1. Phase separation

Long-term storage stability of AMD was visually observed and the final sedimentation ratios were calculated after as demonstrated in Fig. 4. Insufficient HMP concentration (0.2% HMP) could not provide a good storage stability to the samples even at pH 4.0 and 4.5 (19.1–30.2% sedimentation) as expected. Sufficient HMP concentration (0.5% HMP), on the other hand, improved the stability of the samples at pH 4.0 and 4.5 by decreasing the final sedimentations (10.2–14.6%).

Further increase in the concentration of HMP to 0.8% produced the lowest sedimentations at pH 4.0 and 4.5 (4.4–6.1%). When pH was increased to 5.0 at 0.8% HMP, samples did not show any measurable phase separation and/or sedimentation, despite the higher mean particle size and broader size distributions observed for the majority of these samples (Table 1; Fig. 1). This could be attributed to the high viscosity provided by excessive amount of non-adsorbed HMP at 0.8% HMP, pH 5.0 (Sun et al., 2020). For instance, Li et al. (2018) reported an almost three-fold increase in viscosity of fermented milk beverages when HMP dosage was increased from 0.3 to 0.6% (w/w).

In general, higher bulk phase viscosity is associated with better stability as particle diffusions are restricted and hence sedimentation of the particles are retarded (Wagoner & Foegeding, 2017). However, extreme viscosity in AMD may deteriorate the sensory properties and eliminate the acceptability of the product. Therefore, a good stability should be provided to AMD by high steric stability and minimum viscosity. Such stable systems are generally produced by small-size particles that are uniformly distributed (Du et al., 2007). The visual and sensorial observations of the products that showed no sedimentation (0.8% HMP, pH 5.0) after the storage also confirmed the high viscosity character of these samples.

Storage sedimentation results indicated higher sedimentations at pH 5.0 similar to the centrifuge-induced sedimentation results. The only exception was the 0.8% HMP, pH 5.0 samples that showed no sedimentation due to the viscosity effect. However, such a situation was not observed for the same samples after centrifuge-induced sedimentation indicating that the viscosity effect provided by non-adsorbed HMP molecules was only effective during long-term storage. Samples prepared at 0.2% HMP also had high final sedimentations. Additionally, almost all samples of 0.2% HMP reached their final sedimentation levels after just one day except for the samples treated with 500 MPa at pH 4.0 and 4.5. This fast sedimentation was also an indication of poor stability at insufficient HMP concentration. The slightly higher protein solubility ($p \leq 0.05$) of 500 MPa-treated (pH 4.0 and 4.5) samples may have been the reason for their slower sedimentation, but they achieved similar final sedimentation levels to heat-treated samples after one-month storage. In addition, 100 MPa was not effective at all in terms of providing long-term stability at 0.2% HMP.

The most promising results for long-term storage stability belong to samples prepared at 0.5% HMP and pH levels of 4.0 and 4.5. Besides their low final sedimentation values, the pace of the sedimentation process was also considerably slower for these samples. They reached 20–25% of the final sedimentation after one day storage and maintained this sedimentation ratio for two more weeks. This slow and low sedimentation was also consistent with the high protein solubility of these samples under the same conditions (Fig. 2). HHP-treated samples provided similar final sedimentations to heat-treated ones after one-month storage, especially at pH 4.0 (0.5% HMP). The same trend was also applicable to the same set of samples at 0.8% HMP. Therefore, it can be concluded that the HHP treatment provides long-term stability to AMD similar to heat treatment at effective HMP concentrations (0.5–0.8%) and pH values (4.0–4.5).

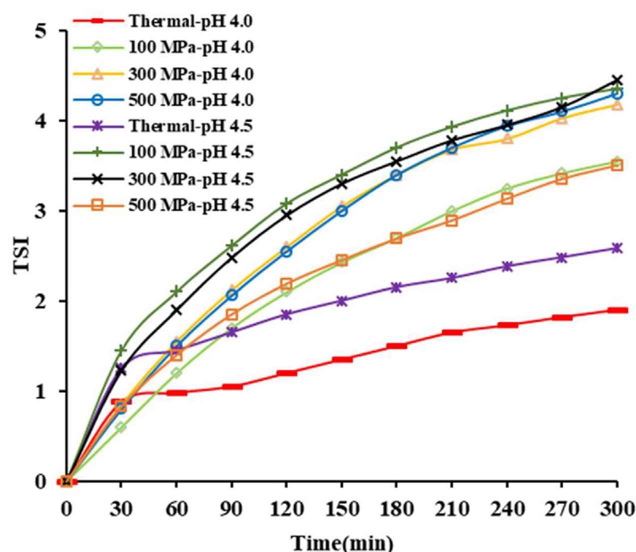

Fig. 5. Comparison of TSI profiles of the samples prepared at 0.5% HMP and pH 4.0–4.5.

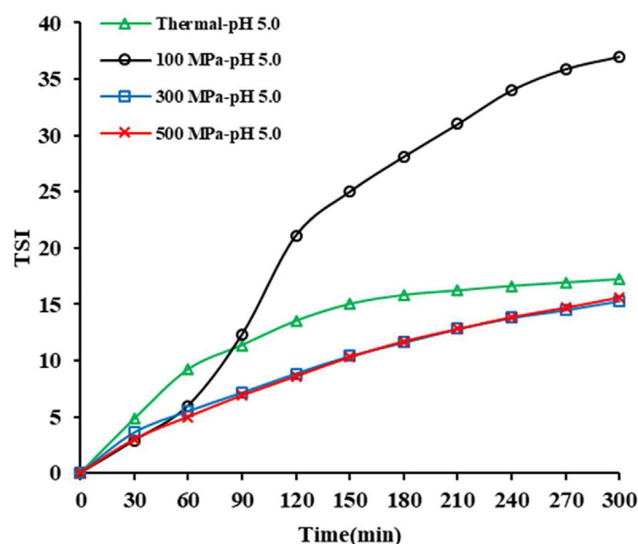

Fig. 6. Comparison of TSI profiles of the samples prepared at 0.5% HMP and pH 5.0.

3.4.2. Turbiscan stability analysis

ΔBS measurements were conducted only for samples prepared with 0.5% HMP, because 0.2% HMP concentration was not sufficient for effective steric stabilisation, whereas 0.8% HMP concentration can cause sensory problems and reduce product acceptability (see storage phase separation section). ΔBS spectra should be examined to obtain detailed information on stability of samples but TSI values can also be used to easily compare the stabilities. TSI provides a first look at the stability by summarising all ΔBS variations within a sample at specific times and producing a single number that reflects the extent of destabilisation (Zalewska, Kowalik, & Grubecki, 2019). Turbiscan technique could also be considered as an accelerated shelf-life analysis (Mengual, Meunier, Cayre, Puech, & Snabre, 1999).

Fig. 5 shows the TSI profiles of the samples (0.5% HMP, pH 4.0 and 4.5) for 5 h. Samples prepared at pH 4.0 and 4.5 attained final TSI values less than 5.0. Heat treatment at pH 4.0 and 4.5 resulted in the final lowest TSI values, 1.9 and 2.58, respectively. On the other hand, TSI profiles of the samples prepared at pH 5.0 (Fig. 6) had considerably higher TSI than the ones produced at pH 4.0 and 4.5. While 100 MPa, pH 5.0 sample reached a TSI of 37, other pH 5.0 samples achieved final TSI values between 15.3 and 17.2. These samples also experienced a very high TSI variation within the experiment since they had much lower TSI in the beginning. Since a lower TSI is associated with higher stability (Zheng et al., 2018), pH lower than 5.0 provided better stability for all samples. This trend was consistent with the storage experiments.

A more detailed analysis of stability of AMD was performed by investigation of ΔBS profiles (Figs. 7–10). These profiles present the distribution of ΔBS data throughout the sample height in the cylindrical cell. The bottom (0–10 mm), middle (10–30 mm) and top (30–42 mm) segments represent the sample height from left to right of the profiles. It is possible to detect both real instant dispersion state throughout the sample and time-dependent variation at the same point by ΔBS profiles (García, Alfaro, Calero, & Muñoz, 2014). Inhomogeneous variations in ΔBS over time are due to particle migration causing sedimentation at the bottom and creaming/clarification at the top (Mengual et al., 1999). Besides, changes of ΔBS in the middle segment could be a sign of particle size variation (coalescence, flocculation) (Zheng et al., 2018). While increase in ΔBS at the bottom segment in time corresponds to sedimentation, the decrease in ΔBS at the top segment is due to clarification (serum separation). According to Fig. 7, heat-treated samples at pH 4.0 and 4.5 formed sediment at the bottom and a clarified layer at the top. The same pattern was also observed for the same samples at pH 5.0, but there was a greater variation in the ΔBS profile. The sedimentation peak of pH 5.0 demonstrated a broader distribution indicating a higher sedimentation. Additionally, variation of ΔBS throughout the sample (pH 5.0) was much higher indicating a high variation in particle size in agreement with the particle size distribution results (Fig. 1). Turbiscan results of heat-treated samples reflected the storage results where a better stability was also observed at pH 4.0 and 4.5 (Fig. 4).

HHP treatment also resulted in some sediment formation and serum separation (Figs. 8–10). At pH 4.0 and 4.5, HHP treatment showed ΔBS profiles similar to heat treatment. However, ΔBS of HHP-treated samples showed a time-dependent decreasing trend throughout the sample height. ΔBS variations that created this trend were also much more evident than those observed in heat-treated samples. When the initial size of the particles within a system is larger than the used light wavelength, increase in particle size leads to decrease in backscattering flux (Snabre & Arhaliass, 1998). Since the mean size values of all samples (at least 1.70 μm) were above the wavelength of the light (850 nm) produced by the light source, increasing particle size resulted in lower ΔBS (Zheng et al., 2018). Therefore, ΔBS measurements suggested that HHP-treated samples experienced broader size distribution and greater particle diffusion during sedimentation.

HHP also induced significantly higher sedimentation ($p \leq 0.05$) for all pressures than heat treatment at pH 4.5 (0.5% HMP; storage experiments, Fig. 4). This was in agreement with the ΔBS profiles obtained by Turbiscan analysis since a higher variation in the particle size induces more instability in the AMD systems (Du et al., 2007). Increasing pH to 5.0 also produced unstable ΔBS profiles for HHP-treated samples (Figs. 8–10). Moreover, the ΔBS variations expanded to larger areas compared with the ones observed in heat-treated samples at the same pH. This was not unexpected since HHP treatment produced bimodal size distribution at 0.5% HMP, pH 5.0. There was a large secondary peak located at bigger size population for each pressure level. In contrast, heat-treated samples did not exert such a broad size distribution at 0.5% HMP, pH 5.0 (Fig. 1).

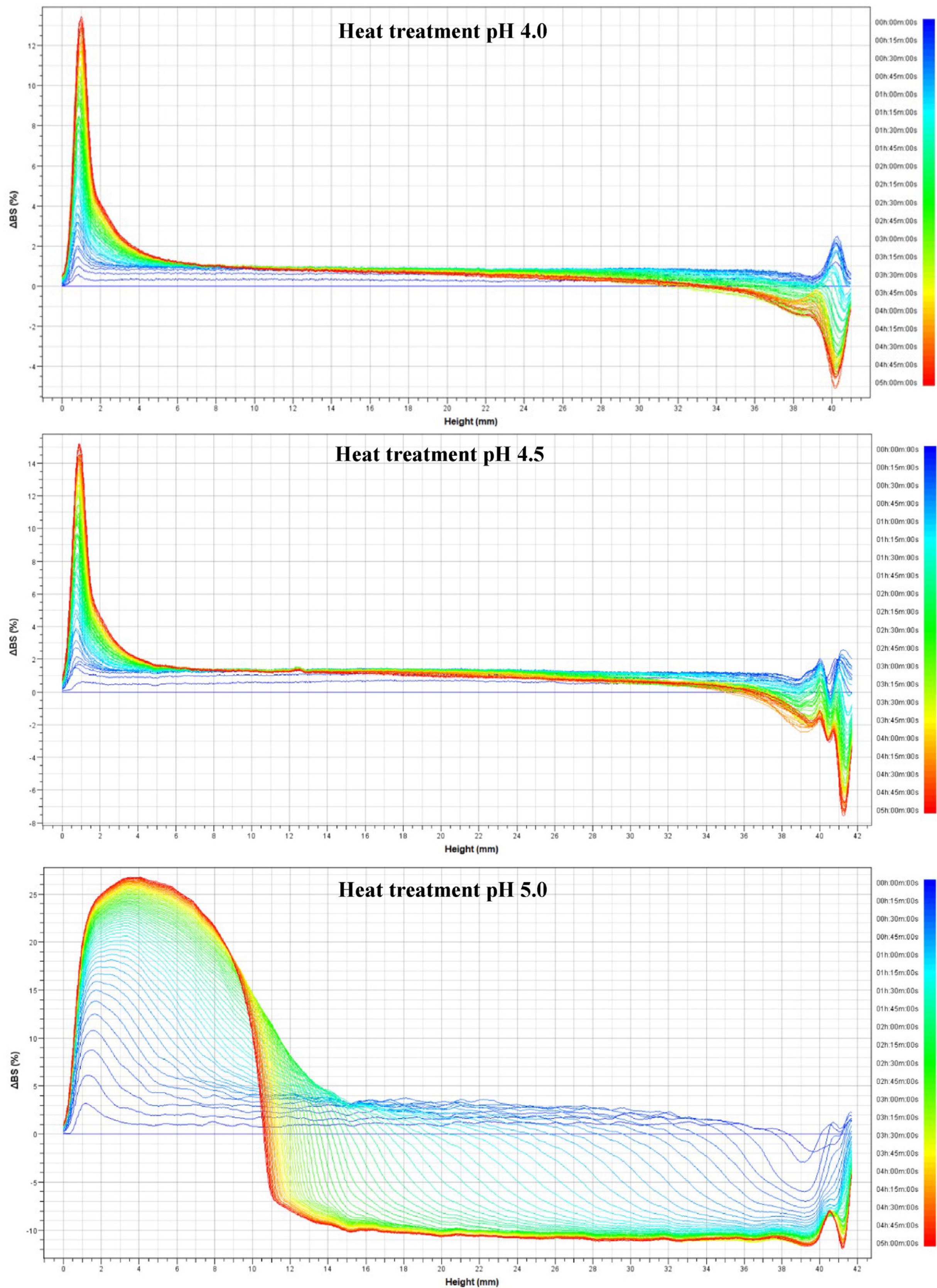

Fig. 7. Delta backscattering profiles of heat-treated samples prepared with 0.5% HMP at pH 4.0 (top), 4.5 (middle) and 5.0 (bottom).

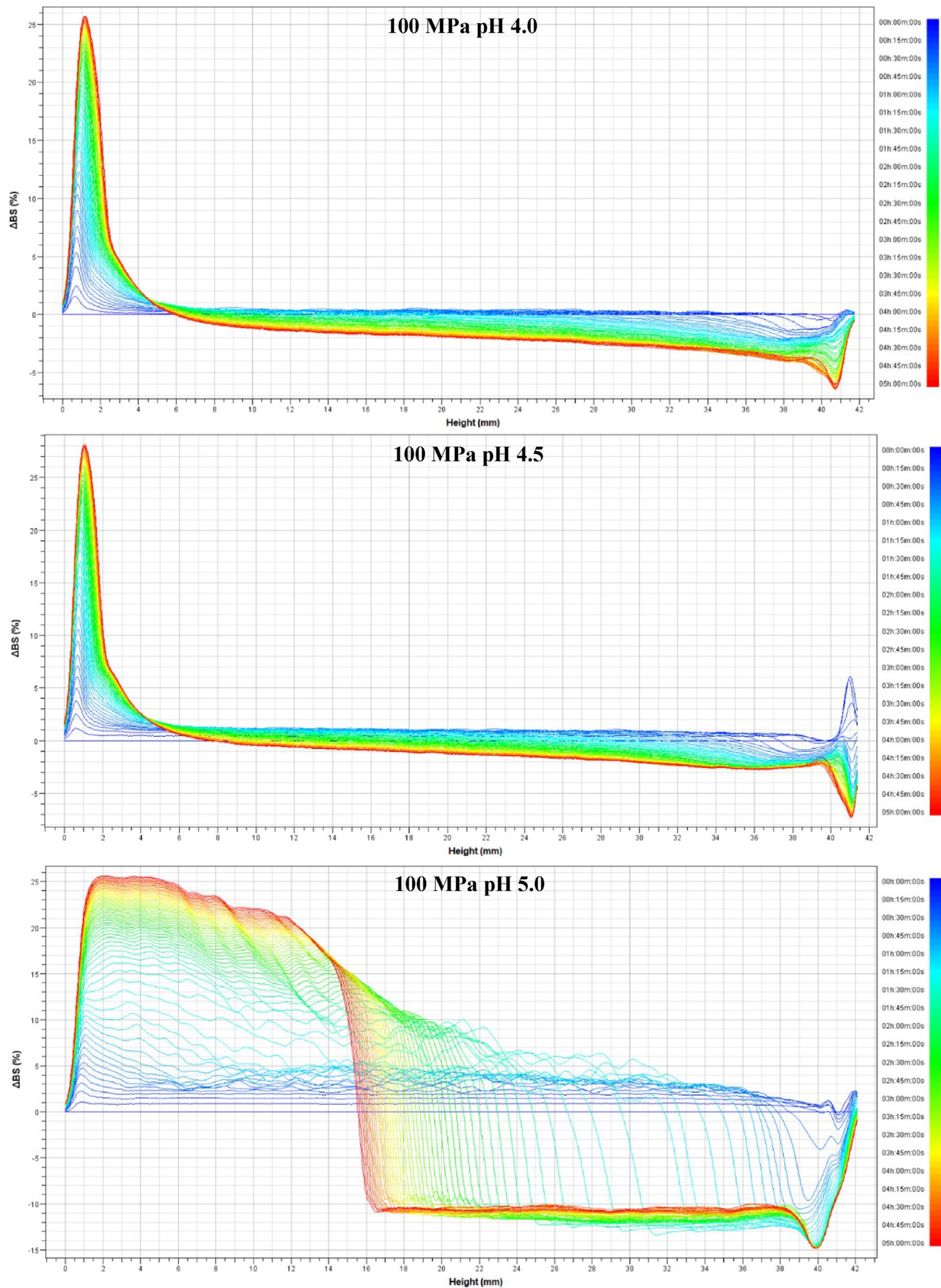

Fig. 8. Delta backscattering profiles of HHP-treated (100 MPa) samples prepared with 0.5% HMP at pH 4.0 (top), 4.5 (middle) and 5.0 (bottom).

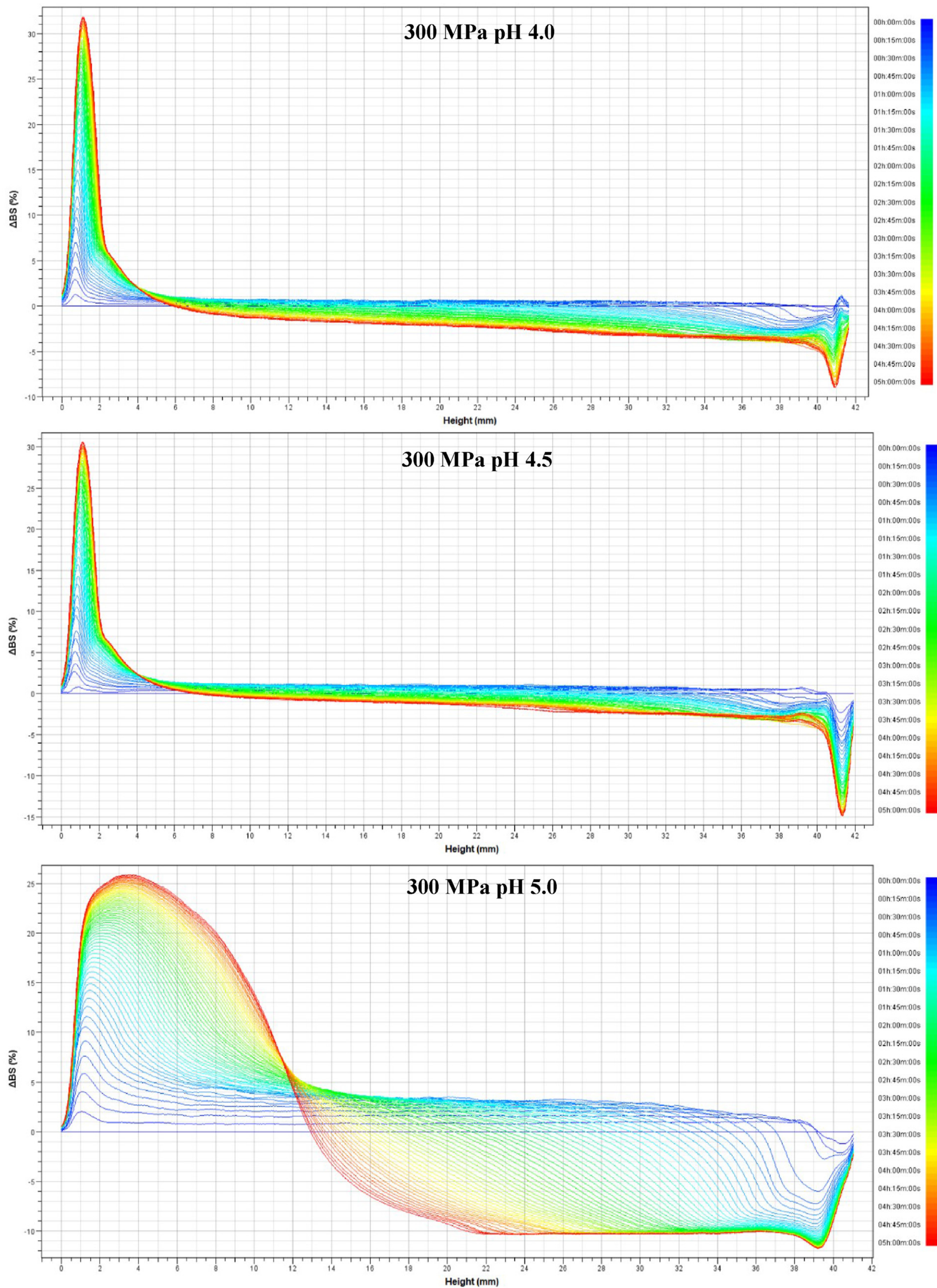

Fig. 9. Delta backscattering profiles of HHP-treated (300 MPa) samples prepared with 0.5% HMP at pH 4.0 (top), 4.5 (middle) and 5.0 (bottom).

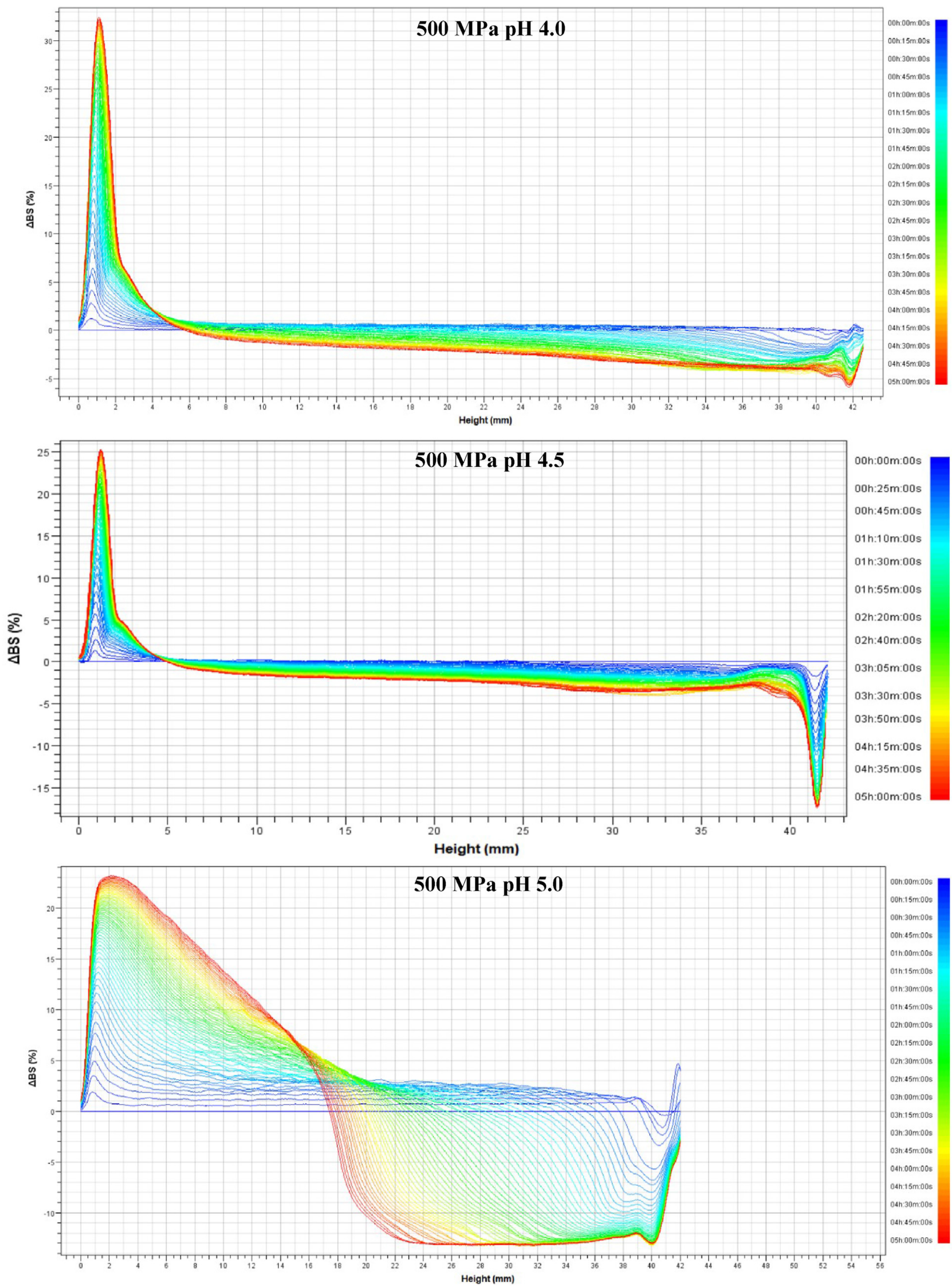

Fig. 10. Delta backscattering profiles of HHP-treated (500 MPa) samples prepared with 0.5% HMP at pH 4.0 (top), 4.5 (middle) and 5.0 (bottom).

4. Conclusions

There is an increasing trend for replacing thermal pasteurisation of dairy products with other techniques such as HHP due to nutritional concerns. For this reason, effects of HHP and heat treatments on the short and long-term stability of AMD were compared in this study. HMP was used as stabiliser. Results showed that HHP treatment was able to provide stability to AMD similar to heat treatment at 0.5% HMP and pH levels of 4.0 and 4.5. Samples having high protein solubility showed better storage stability. In general, both treatments failed to provide a decent stability at pH 5.0. ΔBS profiles and TSI values of the samples were mostly in agreement with the particle size, protein solubility and sedimentation results. However, all these experiments indicated that HHP treatment induced specific changes in the AMD constituents different than heat treatment, such as casein fragmentation (>250–300 MPa), modification of HMP solubility and broader particle size distribution. Nonetheless, HHP exerted a promising performance at the right HMP concentration (0.5%) and pH (4.0 and 4.5). Therefore, it is possible to replace thermal pasteurisation with HHP under certain conditions. In this way, AMD with prolonged shelf life and preserved nutritional value could be produced in an energy-efficient manner.

CRediT authorship contribution statement

Bige Tirpanci: Investigation, Formal analysis, Resources, Writing – original draft. Baris Ozel: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. Mecit Halil Oztop: Conceptualization, Methodology, Supervision, Writing – review & editing. Hami Alpas: Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2022.105512.

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