EFFECT OF HIGH HYDROSTATIC PRESSURE (HHP) ON THE QUALITY PARAMETERS AND SHELF LIFE OF ACIDIFIED PROTEIN DRINK

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ABSTRACT

EFFECT OF HIGH HYDROSTATIC PRESSURE (HHP) ON THE QUALITY PARAMETERS AND SHELF LIFE OF ACIDIFIED PROTEIN DRINK

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Acidified milk drinks (AMD) has recently gained popularity due to their nutritional aspects. Most of these drinks suffer from sediment formation and wheying off during shelf life and in order to prevent it, hydrocolloids such as pectin is used as stabilizers. It is important to have a stable beverage without sacrificing milk protein content. Moreover, having an alternative pasteurization method such as High Hydrostatic Pressure (HHP) rather than a thermal treatment could also be a better choice to protect nutritional and sensorial properties. Considering all these and fragmentation effect of HHP on milk proteins, it was hypothesized that HHP may enhance the pectin adsorption onto caseins and help obtaining a physically stable beverage. Therefore, the aim of this study was to use HHP as a pasteurization method and observe its effects on the stability of the AMD in comparison with heat treatment. For this manner, HHP was applied at 100, 300 and 500 MPa for 5 minutes at 25 °C and sedimentation ratio, Brix^{o}, soluble protein amount were measured and particle size analysis was done in comparison with heat treated control samples which were pasteurized at 75 °C for 15 minutes. Finally, at a selected condition where the stability was maximum (0.5% pectin), Turbiscan analysis was carried out. It was

shown that either by thermal process or HHP, pectin concentration and pH are significant parameters for the stability of AMD. It was seen that a pectin dosage of 0.2% is not enough to stabilize the beverage. At pH 5, system was not stable either. The lowest brix values and protein solubility and the highest sediment ratios and serum separations were observed with 0.2% pectin at pH 5. The average particle size at pH 5 was significantly higher compared to the other samples and size distributions were in broader ranges. Protein aggregation by HHP became more visible at pH 5. However, at pH 4 and 4.5, HHP provided stable results. Therefore, it was seen that when there is sufficient and effective pectin support in the system, HHP could be utilized instead of heat treatment. Turbiscan stability assessment also justified that, at a certain pectin concentration and lower pH values, HHP could provide comparably stable products over heat treatment.

Keywords: Acidified Milk Drink, High Hydrostatic Pressure, Stability, Turbiscan

ÖZ

YÜKSEK HİDROSTATİK BASINCIN (YHB) ASİDİFİYE PROTEİN İÇECEĞİNİN KALİTE PARAMETRELERİ VE RAF ÖMRÜNE ETKİSİ

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Asidifiye sütlü içecekler, besin değeri yönüyle son zamanlarda populerlik kazanmıştır. Bu içeceklerde raf ömrü sürecinde çoğunlukla tortu ya da peynir altı suyu proteini ayrışması problemleriyle karşılaşılmaktadır, bunun önüne geçmek için pektin gibi stabilizörler kullanılmaktadır. Protein içeriğini koruyarak aynı zamanda da stabil bir içecek üretmek önemlidir. Dahası, Yüksek Hidrostatik Basınç (YHB) gibi ısıl işleme alternatif bir pastörizasyon yöntemi, besleyici ve duyusal özellikleri korumak açısından da daha iyi bir seçenek oluşturacaktır. Bütün bunlar ve YHB' nin protein fragmentasyonuna etkisi göz önüne alınarak şu hipotez ortaya konuldu: YHB pektinin proteinlere tutunmasını güçlendirebilir ve fiziksel olarak stabil bir içecek elde etmeye yardımcı olabilir. Böylelikle bu çalışmada, YHB'nin asidifiye sütlü bir içecekte pastörizasyon yöntemi olarak kullanılması ve stabilite parametrelerine etkisinin gözlemlenmesi amaçlanmıştır. Yüksek basınç değerleri 100, 300, 500 MPa olarak 25 °C'de, 5 dakikaya kadar uygulanmış, çökelme oranı, pH, Brix°, çözünür protein değerleri 75 °C'de 15 dakikalık ısıl işlemle pastörize olan kontrol numuneler ile kıyaslamalı olarak gözlemlenmiş ve parçacık boyutu analizi yapılmıştır. Son olarak, optimum koşullardaki HHP ve kontrol numuneleri karşılaştırmak için

turbiscan analizi yapılmıştır. Deneyler hem ısıl işlem hem de YHB uygulanan numuneler için pektin konsantrasyonu ve pH'ın stabilite için önemli parametreler olduğunu göstermiştir. 0.2%lik pektin oranının stabilite için yeterli olmadığı görülmüştür. pH 5'te de sistem stabil olmamıştır. En düşük protein çözünürlüğü ve brix değerleri ile en yüksek çökelme oranı ve serum ayrışması bu değerlerde görülmüştür. Ortalama parçacık boyutu pH 5'te en yüksek değerdedir ve parçacık dağılımı diğer numunelere göre daha geniş aralıklardadır. Basıncın protein yığışmasına etkisi pH 5'te en belirgindir. Fakat pH 4 ve 4.5'te stabil numuneler sağlamıştır. Görülmüştür ki, ortamda yeterli ve etkili pektin desteği olduğunda YHB ısıl işlem yerine kullanılabilir. Turbiscan stabilite değerlendirmesi de optimum pektin oranı ile düşük pH'ta YHB'nın ısıl işlem gibi stabil numuneler verdiğini doğrulamıştır.

Anahtar Kelimeler: Asidifiye Sütlü İçecek, Yüksek Hidrostatik Basınç, Stabilite, Turbiscan

To my Grandma

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LIST OF ABBREVIATIONS

ABBREVIATIONS

AMD: Acidified Milk Drinks

ANOVA: Analysis of Variance

BS: Backscattering

CMC: Carboxmethylcellulose

CN: Casein

DE: Degree of Esterification

GDL: Glucono-δ-lactone

HHP: High Hydrostatic Pressure

HMP: High Methoxyl Pectin

MSNF: Milk Solid Non-Fat

NMR: Nuclear Magnetic Resonance

PGA: Propyleneglycoalginate

SSPS: Soybean Soluble Polysaccharides

TSI: Turbiscan Stability Index

CHAPTER 1

INTRODUCTION

1.1 Acidified Milk Drinks (AMD)

Acidified Milk Drinks are currently gaining popularity due to their nutritional value and refreshing authentic taste. These drinks include drinkable yoghurts, milk drinks with juice, buttermilk and whey drinks. The pH range of these drinks is roughly between 3.4 - 4.6 (Liu et al., 2020). Acidification of these beverages are either done by direct addition of juices and/or acidulants like citric acid, malic acid or glucono-δ-lactone (GDL) or by microbial fermentation (Guo et al., 2021). Having low pH, along with having low viscosity is the main cause of the instability of AMDs. The instability in the AMDs can mainly be observed as wheying off and casein aggregation caused by low pH (Thi et al., 2009). Casein constitutes approximately 80% of the milk proteins and and at neutral pH of milk which is around 6.7, electrostatic and steric repulsions prevent casein from collapsing (Tian et al., 2021). When the pH is lowered around 4.6 which is the isoelectric point, κ-casein cleavage occurs and lack of repulsive forces leads to casein aggregation. In order to prevent this aggregation, anionic polysaccharides like pectin, propyleneglycoalginate (PGA), soybean soluble polysaccharides (SSPS) and carboxymethylcellulose (CMC) have been used as stabilizers in AMDs (Du et al., 2007). Having a stabilizer in the formula does not only help to provide stability but it is also a necessity to use a stabilizer for having the desired mouthfeel and taste, an acceptable viscosity and texture (Shirkhani et al., 2012). Pectin is the mostly used one among the other stabilizers.

1.2 Pectin

Pectins are very widely used in food industry due to their stabilizing, gelling or texturizing properties (Tromp et al., 2004). Pectins are the group of polysaccharides which exist in the structure of plant cell walls. They basically provide flexibility and give mechanical strength to the cell they exist. In industry, they are mainly extracted from citrus peels or apple pulps. They are approved and used as food additives in Europe with the codes E440a for low methoxyl and high methoxyl pectin and E440b for amidated pectin (Belkheiri et al., 2021). Although pectin can show some minor structural differences depending on its source or extraction method, it mainly consists of the D-galacturonic acid chains having α-(1–4)-linkages and 1,2-linked rhamnose unit interruptions. Both EU and FAO states that galacturonic acid should not be less than 65% for commercial applications (JECFA, 2017). As shown in Figure 1.1. (Belkheiri et al., 2021), in the backbone of the pectin, covalent linkages bind some complex polysaccharide units known as 'galacturonans' to each other. Homogalacturonan (HG) and rhamnogalacturonan I (RG-I) are the majority of these units. Xylogalacturonan (XGA), rhamnogalacturonan II (RG-II), apiogalacturonan (AGA) are the minority of these galacturonan units on the backbone. Even though this expression for the pectin structure is traditionally accepted, it is still being discussed as the new proposals come to find the exact microstructural characteristics of pectin.

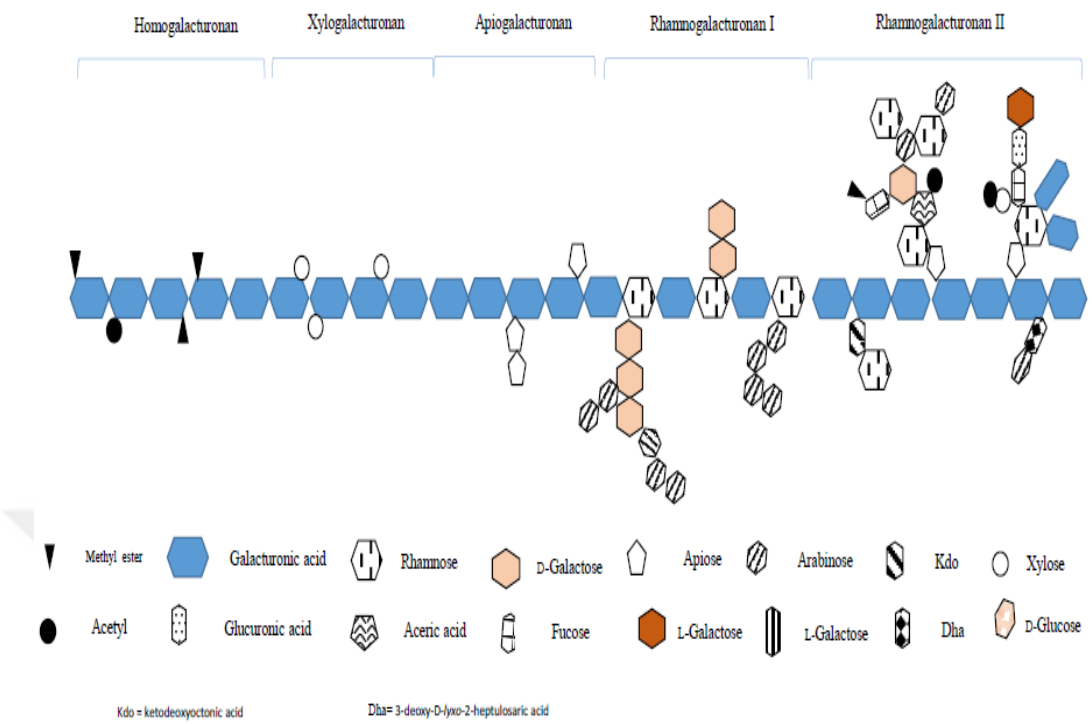

Figure 1.1. Pectin structure (Belkheiri et al., 2021)

There are two types of structural classifications for pectin (De Cindio et al., 2016). First one of these depends on the esterification level of carboxyl groups in galacturonic acid. Carboxyl groups are esterified by methanol as shown in Figure 1.2 (Belkheiri et al., 2021), therefore; this process is also called as '*methylation*' and degree of methylation (DM) or esterification determines the type of the pectin. DM is the percentage of esterified carboxylic groups in the main galacturonic acid chain. If more than half of the carboxyl groups are esterified, which means DM is more than 50%, it is a high methoxyl pectin (HMP). HMP is the native form of pectin and it can be turned into LMP by demethylation, having a DM lower than 50%.

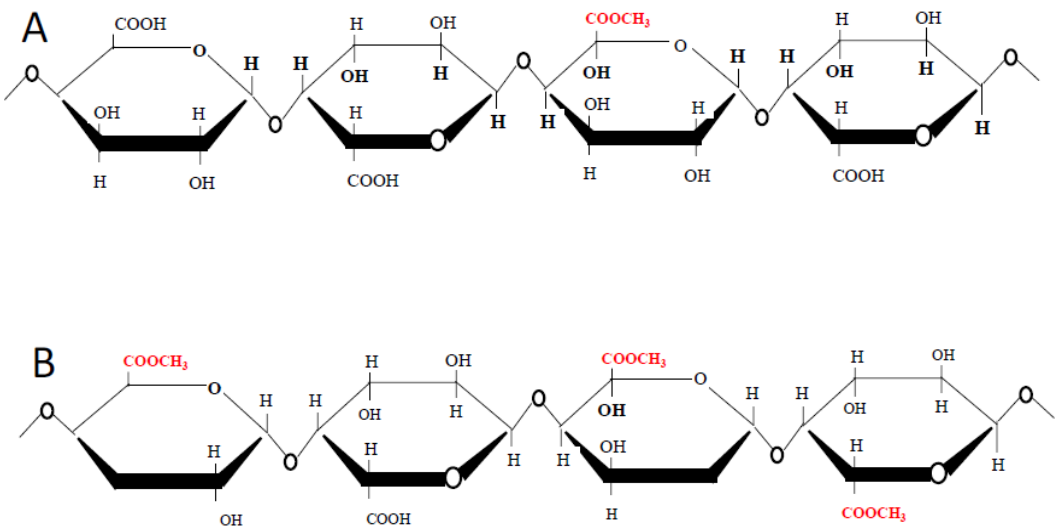

Figure 1.2. Partially methylated galacturonans (Belkheiri et al., 2021)
A – Low methoxyl and **B** – High methoxyl pectin structures

Metoxylation degree affects the water dispersibility and gelation properties of pectin. Low methoxyl pectins need calcium ions to form gels, whereas high methoxyl pectins need high amounts of cosolutes like sucrose and decrease of pH in order to come up with a gel network.

Other classifications are made based on the degree of acetylation (DAC) and amidation (DA). Degree of acetylation denotes the percent of galacturonosyl units that are esterified by acetyl on the hydroxyl group (Belkheiri et al., 2021). The importance of acetylation degree comes from the fact that as the number of acetylated groups increases, gelling ability decreases. On the other hand, large number of acetylated groups increases the surfactant ability of the pectin. Amidation is also done through the reaction of pectin with ammonia and degree of amidation denotes the % of carboxylic groups that exist in the amide form after amidation. Amidation process makes the pectin more hydrophilic. Moreover, it increases the thermoreversibility and provides an elevated calcium reactivity towards pectin and better gel properties.

1.2.1 Stabilization Mechanism of Milk Proteins by Pectin

The mechanism behind the stabilization of AMDs by pectin has been a topic for many research (Li et al., 2018; Maroziene, 2000; Tromp et al. 2004; Tuinier et al.,2002). Although it has not yet been identified how pectin involves in the suspension of milk proteins within the AMD system, it is proposed that stabilization depends on the steric forces between milk proteins and charged pectin molecules.

In neutral pH of milk which is around 6.7, negatively charged casein micelles are kept suspended by the repelling forces between one another and the steric hindrance resulted from the existence of κ-casein layer around them (Cai et al., 2007). As the pH decreases, cleavage of κ-casein occurs, calcium phosphate dissociate from the casein micelles and casein micelles start to lose their negative net charges. As the pH decrease continues and the isoelectric point, which is around 4.6, is reached, milk proteins start to coagulate due to having no net charge. In order to prevent micelle aggregation which is the result of weakened repulsive forces between caseins, pectin as an anionic polysaccharide is used in the acidified milk system (Peterson et al., 2019).

Before the acidification of milk, hairy layers of κ-casein are at extended form and they surround the micelle surfaces trying to reach the maximum entropy. Since the entropy of the chains has a tendency to reduce due to the coexisting interaction between the neighbouring micelles and κ-casein, casein micelles attain an intermicellar repulsive interaction (Li et al., 2018). This is the idea behind the steric stabilization mechanism of milk proteins in neutral pH. On the other hand, in acidified milk drinks, the pH is around 4 (Janhøj et al., 2008), at which most milk proteins are positively charged. When the pH is lower than the isoelectric point of milk proteins and higher than the pKa of carboxyl groups in pectin which is 3.5, negatively charged pectin molecules attain the same function what κ-casein does at the neutral pH. This replacement by pectin is represented in Figure 1.3.

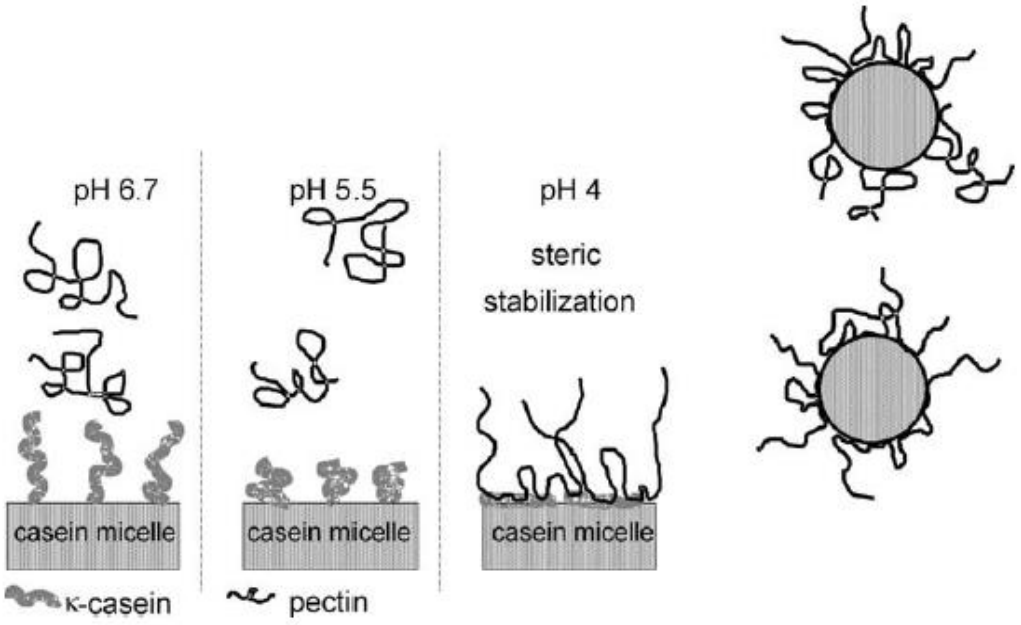

Figure 1.3. Replacement of κ-casein by pectin as the pH is lowered (Tromp et al. 2004)

According to the suggested stabilization mechanism by Tromp et al. (2004), negatively charged blocks of pectin is adsorbed on the casein surfaces through the electrostatic interactions and this adsorption starts to take place at pH values lower than 5. The uncharged chains extend into the solution forming some entropy rich regions and thus triggering the intermicellar repulsion, which compensates for the function of κ-casein.

1.3 High Hydrostatic Pressure (HHP)

Recently, consumer demands have a tendency towards nutritional, natural products with high quality. Market preferences head towards additive free, safe products with a taste of authenticity and freshness. In order to meet this arising demand, food industry has been seeking for alternative processing techniques for the treatment of foods. High Hydrostatic Pressure (HHP) is a novel processing technique which is

used as an alternative to conventional processes that is used to assure the safety of the food (Serna-Hernandez, 2021). Its effectiveness on microbial destruction and inactivation of certain enzymes without the need for extreme temperatures, makes HHP a promising technique for ensuring the safety of foods (Huang et al., 2017).

It is known that, thermal processing of foods induces chemical reactions like Maillard or causes caramelization, therefore; it triggers some physiological changes within the food system as color change or formation of new flavor components or loss of some functional ingredients (Yamamoto, 2017). HHP, however, instead of hastening the reactions, has the effect of inducing structural changes of foods at microscopic or macroscopic levels. This effect of HHP is explained with the Le Chatelier's principle (Goyal et al., 2013). According to this principle, when the pressure is applied to a system, system opposes to this stress with configurational changes in molecules or with reactions that favor the reduced volume. Restriction of molecular mobility, dissociation of hydrogen bonds, creating a compact structure of molecules or inducing the dispersion of air phase within a liquid system are the examples of how a food system responses HHP. Therefore, the most important superiority of HHP over thermal techniques is that the nutritional and sensorial characteristics of food products end up with minimal damage or change after the process. Moreover, the continuous thermal increases and decreases through the system in heat involving processes come up with high levels of energy requirements. In HHP, on the other hand, when the target pressure is reached, any additional energy is not needed to proceed at that pressure level, which makes HHP a more environmentally-friendly process compared to the thermal treatments (Yamamoto, 2017). Another advantage of HHP processing is that none of the shape or size of the food product has an effect on the transmittance of the applied pressure (Buzrul and Alpas, 2012). As a result of isostatic principle, applied pressure exerts on all parts of food evenly which could be problematic sometimes for the thermal processing of foods when massive productions are required.

In industrial applications of HHP, applied pressure values range between 200 to 600 MPa and the temperature is preferably either at room or chilling temperatures with holding time of 5 minutes mostly. (Aganovic, 2021).

HHP is generally used as a batch system where foods are pressurized in a chamber around which a pressure transmitting medium exists (Buzrul and Alpas, 2012). This pressure transmitting liquid is generally water and it allows pressurized food to be processed without any edge effects. Other than the vessel and the pressure transmitting medium, other parts include the closures which seal the vessel and a device to hold these closures, a high pressure intensifier pump, a pressure and temperature monitoring system, and the system for the removal or replacement of the product (Figure 1.4).

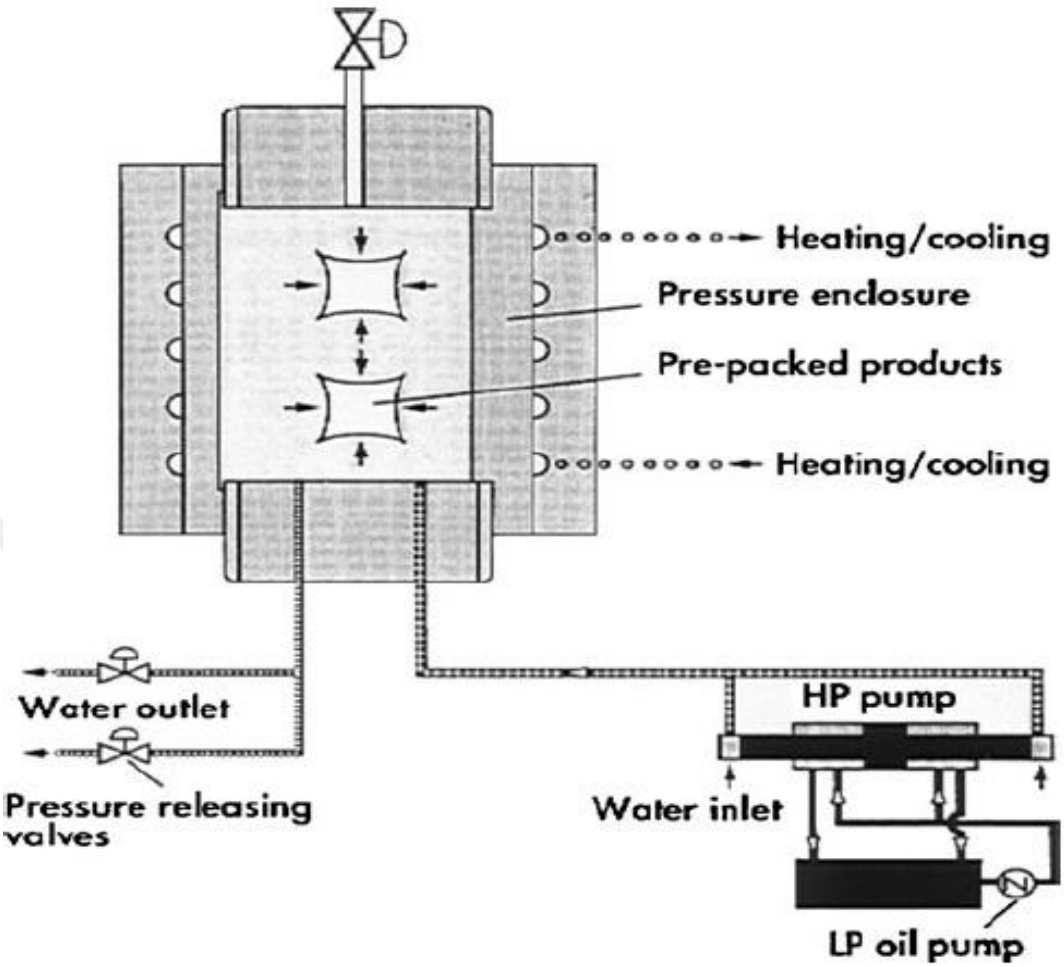

Figure 1.4. Represantation of a basic HHP system (Chawla et al., 2011)

Basically, batch operation starts with the replacement of product in the sealed vessel. It continues with the pumping of liquid medium to until all filled volume is occupied by air. Then the pressure valve is closed and the desired pressure is set all through the system. The pressure is maintained for a specific time on the system and then the valve is opened and the system is depressurized through the expansion of liquid medium until the atmospheric pressure is reached. Finally, the vessel is opened and the food is removed (Chawla et al., 2011).

Nowadays, there are many commercial applications of HHP in the market (Table 1.1.). In particular, some dairy applications of HHP form market such as fresh milk, colostrum or whey derived formulas, fruit smoothies blended with milk or yoghurt, dairy including coffee products like latte or mocha are also given in Figure 1.5. demonstrates some HHP - treated dairy products from the market. In addition to the commercial applications of HHP, its effects on microbial inactivation, modification of functional properties of food components, improving functional properties of food ingredients or the protection of nutritional or sensorial aspects have been the topics for so many researches for many years (Yamamoto, 2017).

Table 1.1. HHP treated food examples from the market (HPP Applications, 2020)

Category	**End-uses**	**Advantages**
Juices and beverages	Fruit and vegetable juices, smoothies and milk alternatives, probiotic enhanced drinks, functional drinks, ready-to-eat (RTE) vegetable soups, cold brew coffee, tea infusions, fermented beverages	Preserved color, flavor, and fresh attributes, microbial safety, extended shelf life, retained functionality, clean label, and possibility of different packaging choices.
Guacamole and avocado Products	Avocado pulp, halves, avocado based beverages	Maintenance of natural green color, flavor, and freshness, extension of shelf life, enabling opportunities for new products, providing different packaging options
Meat Products	Cured and sliced ham, roasted chicken and turkey cuts, hand-cut pork, cured sausages, raw and marinated meats, duck and goose delicacies	Enhanced food safety, premium quality, extension of shelf life, clean label, prevention of cross-contamination, providing application variety and packaging options, allowing innovative product development and market expansion opportunities
Seafood products	Oysters, clams, mussels, lobster, crab, shrimp, salmon, cod, and ready-to-eat (RTE) seafood meals	Efficient extraction of meat from Mollusks and Crustacean, prevention of cross-contamination, microbial safety

Salsas, plant based and fruit products	Wet salads, hummus, sauces, plant-based dips, and spreads, fruit purees, fruit cups in fruit juice with yoghurt or cereal	Shelf life extension, inactivation of pathogens, maintenance of color, flavor, nutrition, allowing free label and innovative products, enabling variety of packaging
Baby Food	Ready-to-eat (RTE) baby foods or fruit purees	Development of natural, healthy products with clean label, minimal processing and premium quality with retained functional ingredients, allowing new product development and wider range of packaging
Ready-to-Eat Meals	RTE meals with meat, fish, vegetables, rice, and pasta	Providing clean label and premium quality, minimized previous cooking, extended shelf life, microbial safety, variety of products and packaging formats
Dairy Products	Fresh cheese, aged cheese, cheese-based sandwich fillings, yoghurts, smoothies, sauces, and spreads with milk base	Modifying maturation in aged cheese, ensuring food safety, clean label, and extended shelf life, preserving nutritional value, allowing development of nutritional dairy products with variety of packaging, providing product innovations with fresh characteristics

Figure 1.5. Examples of HHP treated dairy beverages (HPP Applications, 2020)

1.3.1 Effect of HHP on Milk Proteins

Dairy applications of HHP used to focus mostly on the microbial inactivation in milk, at the beginning. Research has shown that the milk treated with HHP showed comparable results to pasteurized milk in terms of microbial aspects and shelf life (Trujillo et al., 2016). However; in time, there has been also many interests and investigations on physicochemical and functional changes in milk components such as lipids, caseins, whey proteins, minerals by HHP, including various dairy products like cheese, cream, buttermilk etc. (Al Nabulsi et al., 2012).

Milk proteins comprises of caseins and whey proteins which are almost 80% and 20% of the total protein respectively (Serna-Hernandez, 2021). The amino acids in the structure of these proteins have some interactions which keeps them in their native form such as covalent bonds, electrostatic interactions, hydrogen bonds, disulfide bonds, and hydrophobic interactions. Among these interactions, the most

resistant one is the covalent bonds, in terms of HHP – susceptibility (Munir et al., 2019). This is why the primary structure of proteins remains un-disordered after HHP whereas the quaternary, tertiary and the secondary structures which possess the weaker interactions get damaged significantly. When the caseins are considered, these proteins consist of 4 different categories, α_{S1}-, α_{S2}-, β-, and κ- caseins (CN) (Trujillo et al., 2016). Their stability in the milk system is attained by the electrostatic repelling forces in between the micelles and the micellar calcium phosphate keeping them together. The mechanism behind the HHP induced alterations in milk proteins lay behind the structural rearrangements in water molecules (Huppertz et al., 2006). Upon pressure application, water molecules are compressed and they tend to pack themselves around charged groups. This results in ionization and increased mineral solubility. Therefore, solubilization of micellar calcium phosphate accompanied by the water penetration into the micelle structure end up with micelle fragmentation. Hydrophobic and hydrogen bonding and electrostatic interactions are also disrupted at the same time. Consequently, alterations in size, structure, and functionality of the proteins occur. These changes are also affected from the process conditions such as temperature, pH, or treatment time. Studies done with the milk proteins showed that, pressures greater than 300 MPa cause irreversible changes in the size of casein micelles which is related to micelle fragmentation during the treatment (Trujillo et al., 2016). Mild pressures below 300 MPa, however, may cause an increase in micelle size which can be reversed during storage. This size increase is associated with the coaction of whey protein denaturation and its association with κ- casein (Huppertz et al., 2004).

The other important constituent of milk proteins is the whey protein. The most well known whey proteins are β – lactoglobulin (β – lg), α – lactalbumin (α – la), and comparably lower amounts of bovine serum albumin (BSA), immunoglobulins (IG) (Huppertz et al., 2004). Whey proteins have globular structures and they are soluble in water in their native state. The denaturation of whey protein by HHP again occurs due to the attack of water into the protein structure. As the pressure is applied, the native form of whey protein is unfolded and it is hydrated by water. When the

hydrophobic areas are in contact with water, the quaternary structure of proteins are disrupted and they start to aggregate (Munir et al., 2019). Among the most abundant whey proteins, β – lactoglobulin is more susceptible to HHP induced alterations than α – lactalbumin. The rigidity of α – la is associated with the disulfide bridges in between the molecule and having no thiol groups in its structure. β – lg, on the other hand, having less disulfide bonds and one free thiol group in its structure, becomes more sensitive to HHP. The order of sensitivity towards HHP among the whey proteins could be given as β – lg, IG, BSA, and α – la. The effects of applied pressure intensity on the extend of protein denaturation are given in Table 1.2 in detail (Trujillo et al., 2016).

Table 1.2 Effects of HHP on whey proteins at different pressure levels (Trujillo et al., 2016).

Protein Fraction	**Pressure (MPa)**	**Effect**
α – la	600	10 % denaturation
	800	50 % denaturation
β – lg	100	Denaturation begins
	400	70 - 80 % denaturation
	800	90 % denaturation
IG	300	Denaturation begins
	500	35 % denaturation
BSA	400	Resistant; no denaturation
	600	Denaturation begins

α – la: α – lactalbumin; β – lg: β – lactoglobulin; IG: immunoglobulin; BSA: bovine serum albumin

1.4 Effect of HHP on Pectin

Literature review shows that most of the HHP studies involving pectin has focused on the inactivation of pectin methyl esterase (Zhong et al., 2021). There are studies which explored the effects of HHP on the molecular weight, degree of esterification (DE), β-elimination or methoxylation, or improvement of the functional properties of pectin. Pectin is one of the main polysaccharides in the plant cell wall which provides rigidity to the fruits and vegetables. Besides, the changes occur during the ripening process, heat treatments applied in processing also triggers non-enzymatic conversions resulting in loss of the rigid structure (Roeck et al., 2009). β-eliminative depolymerization is the main reason of rigidity loss in the fruits after heat treatments and is supported by the demethoxylation of pectin which occurs simultaneously during thermal treatment. In order to observe the effect of HHP to prevent or limit these undesirable reactions, Roeck et al. (2009) applied combined high pressure - high temperature treatments and observed retarded β-elimination besides stimulated demethoxylation.

In addition to its role in providing rigidity to the plant cells, pectin has so many demands in food industry due to its functional properties. It is used in various foods and beverages for its thickening or stabilizing ability (Peng et al., 2016). Therefore, viscosity is the main concern when it comes to investigate and improve these functional properties of pectin by utilizing HHP. Moreover, physicochemical properties such as degree of esterification and molecular weight which have a strong relation with viscosity are also investigated. Peng et al. (2016) and Xie et al. (2018) observed decreases in the molar mass and degree of esterification (DE) of sugar beet pectin and potato peel pectins respectively with the application HHP. In addition, Xie et al. (2018) reported increased viscosity upon the application of HHP in their study. Former studies by Michel et al. (1998) and Kato et al. (1997) also claimed increased viscosity after HHP treatment. These increases in viscosity of pectin solutions were related to the modifications of the protein groups by stretching, degrading, or associating under HHP. In addition to modifications on viscosity,

effect of HHP on pectin solubility was also investigated. De la Pe˜na Armada et al. (2020) showed that the solubility of pectin from apple peel was enhanced with an HHP application of 200 MPa at 15 minutes.

1.5 Stability analysis by Turbiscan

Turbiscan™ LAB is a device which is employed for stability assessment of products through the analysis of size or concentration variations or the physical instability phenomenon. The technology behind the analysis is based on multiple light scattering technique (*Formulaction, n.d.*). The operation principle of Turbiscan is shown in Figure 1.6. The product is filled into a cylindrical glass cell and replaced inside the device. Near-infrared light (850 nm) is sent all through the sample and light transmission and the backscattering intensities are measured and recorded from bottom to top of sample height. The measurements are done in every 40 µm which ensures the vertical resolution and the detection of instability beforehand. Data are displayed as delta backscattering (ΔBS) and delta transmission (ΔT) along the sample height (Zalewska et al., 2019). Backscattering and transmission intensities coming out of the sample strongly depend on the transport length of photons (*the penetration of light*) in the sample. This transport length, indeed, is proportional to the diameter and concentration of the particles. This means that, the size variations due to coalescence or aggregation throughout the system or the local concentration changes as a result of molecular migration such as creaming or sedimentation affect the transport length of light which in turn causes variations in backscattering and transmission intensities along the sample.

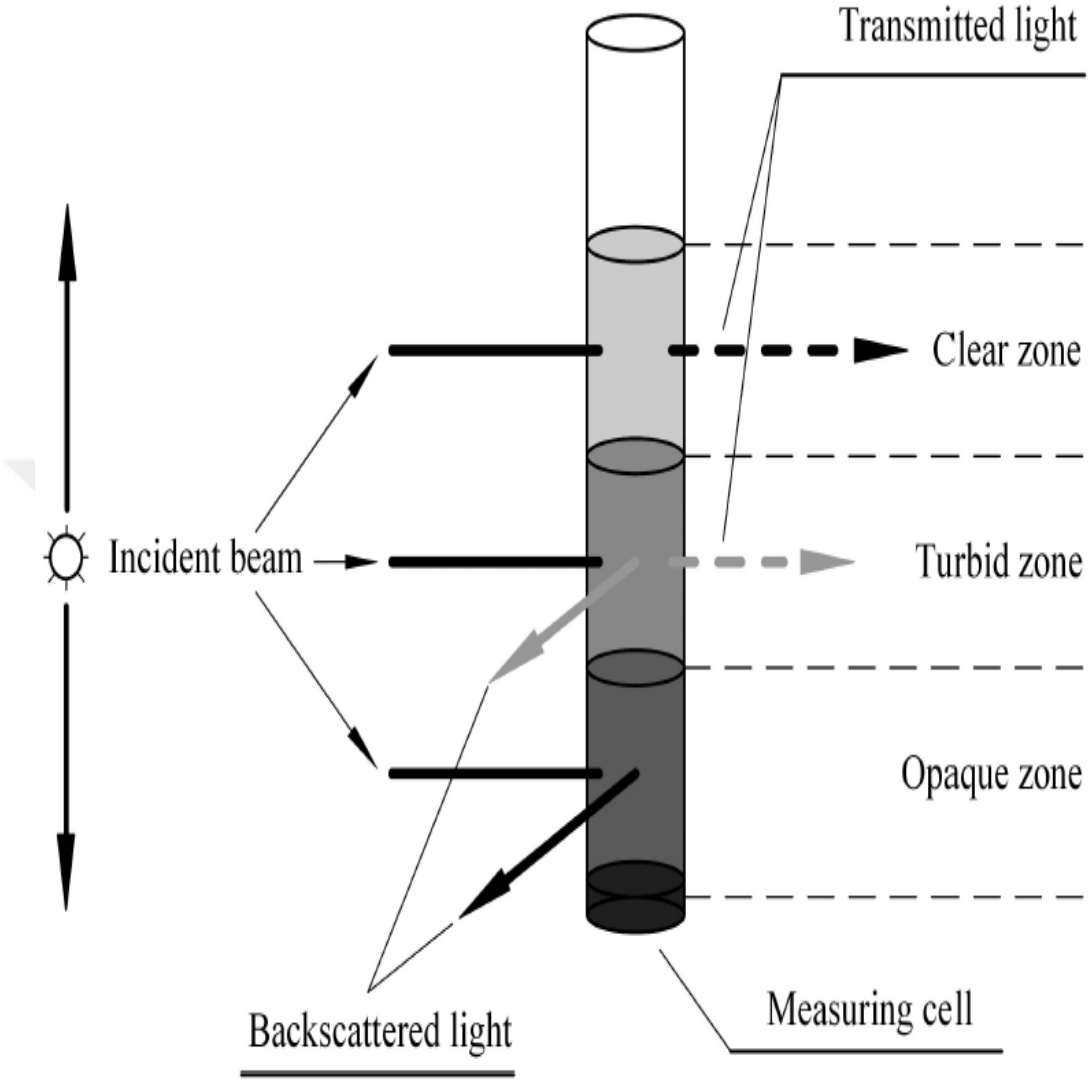

Figure 1.6. Represantation for Turbiscan operation principle (Zalewska et al., 2019)

Another expression of stability assesed by Turbiscan is TSI (Turbiscan Stability Inex) which could be regarded as an estimation for the instability of samples. (Zheng et al., 2018) TSI is an expreesion of all the data signals gathered from the sample as a single number and it is calculated with the following equation :

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

Where;

n: The number of scans,

x_i*: The average backscattering for each specific time,*

x_{BS}*: The average of* x_i *values*

Higher TSI values mean the stability of the sample is weaker compared to the samples with lower TSI values.

1.6 Objective of the Study

There are many studies in literature investigating the stability of acidified milk drinks. However, to the best of knowlegde, there is no study utilizing HHP as a preservation method on AMDs. The aim of this study is to apply the HHP as an preservation technique for an acidified milk drink (AMD) and to evaluate the stability of products comparatively with heat treated reference samples. It is hypothesized that HHP would possibly strengthen the interaction between milk proteins and pectin due to its fragmentation effect on caseins. In order to see how HHP affect the stability, different pressure levels were applied in combination with constant temperature and time. In addition to pressure levels, effects of different pH values and pectin concentrations were also investigated. Stability assessment was done through the measurements of Brix^o, sediment ratio, soluble protein content, and particle size distribution, together with Turbiscan analysis and storage observations.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The pectin used in this study was GRINDSTED® Pectin AMD 783 provided by Danisco (Czech Republic). It was a high methoxyl citrus pectin. Milk powder was sourced from Pınar Süt Inc. (İzmir, Turkey). Milk powder was obtained from cow milk. The protein content of milk powder was 36% (w/w) and the fat content was 1.25% (w/w). Citric acid was obtained from International Flavors and Fragrances Inc. (Gebze, Turkey). Peach concentrate (65 Bx and 3.35% acidity as citric acid) was provided by Dohler (Karaman, Turkey).

2.2 Preparation of Acidified Milk Drink

Acidified milk drinks with 4.5% milk solid non-fat (MSNF) with different concentrations of pectin (0.2% w/w, 0.5% w/w and 0.8% w/w) were prepared at Beverage laboratory in International Flavors and Fragrances R&D, at pH 4, pH 4.5 and pH 5 respectively. pH values were adjusted with citric acid (50% w/w, prepared in water) by using Mettler Toledo G20 Compact Titrator (Mettler Toledo, Switzerland). Formulations used in the study and related coding for the samples used throughout the study are given in Table 2.1. These samples were used for the stability investigation by brix analysis, sediment test, soluble protein content by Lowry method, mean particle size, Turbiscan, and storage observations.

Table 2.1 Acidified Milk Drink Formulation

Ingredients (Percent in formulations)	*A*	*B*	*C*
5% w/w Pectin Solution	*0.2%*	*0.5%*	*0.8%*
Base of the AMD: 4.5 gr skim milk powder reconstituted in 45.5 gr water	50	50	50
Sucrose	6	6	6
Peach juice concentrate (65 Bx & 3.35% c.a.)	0.48	0.48	0.48
Water	39.5	33.5	27.5
Citric acid solution (50% w/w in water) *to adjust pH to 4.0, 4.5, and 5.0*			

Acidified Milk Drink (AMD) samples were prepared based on the method given by Peterson et al. (2019) with some modifications. Milk powder was reconstituted in water at 60°C for 2 hours in 1/10 (w/w) ratio. A 5% w/w pectin solution was prepared with water at 90°C using rotor-stator mixer (Silverson L4RT, USA). Then it was cooled to room temperature. All ingredients were mixed to have AMDs with 3 different pectin concentrations. Citric acid solution (50% w/w) was used to adjust the pH of the drinks to 4, 4.5 and 5.

2.3 Heat Treatment

All control samples were bottled in glass containers and heat treated at 75 °C for 15 minutes in a laboratory scale pasteurizer (Miele G 7835). Samples were kept in refrigerator at 4 °C for 24 hours before analysis.

2.4 High Hydrostatic Pressure (HHP) Treatment

HHP treatment was performed by using 760.0118 type pressure equipment (SITEC-Sieber Engineering AG, Zurich, Switzerland). It has a built-in heating–cooling system (Huber Circulation Thermostat, Offenburg, Germany) which maintains the required temperature measured by a thermocouple type K in the vessel. The vessel has 100 mL volume with 24 mm internal diameter and length of 153 mm. As the pressure transmitting medium distilled water is used which fills the vessel. The system has a pressure increase rate of 340 MPa/min for 400 MPa and pressure release takes place in less than 5 seconds. This pressure release and increase times were not included in the pressurization time reported in this study. Previously prepared AMD samples were filled into 25 ml sterile polyethylene cryotubes (LP Italiana SPA) and pressurized at 25°C for 5 minutes at 100, 300 and 500 MPa respectively. Pressurized samples were kept in the refrigerator at 4°C overnight to cool down before the experiments. Two replicates were done for HHP experiment.

2.5 Soluble Protein Content Determination

Samples were initially centrifuged at 4,000 rpm for 20 minutes. Then, for the evaluation of solubility, Lowry method was conducted by following the procedure of Waterborg (2009).

The reagents used in Lowry method are as follows:

- Reagent 1: 2% $Cu_2SO_45H_2O$
- Reagent 2: 2% Na-K Tartarate
- Reagent A: 2% Na_2CO_3 in 0.1 N NaOH
- Lowry ACR Reagent: Mix of reagents A: 1:2 in a ratio of 100:1:1
- Folin – Phenol Reagent: Diluted 2N stock solution (commercially available) with distilled water in 1:1 ratio

After the preparation of reagents in the given ratios, a calibration curve was drawn using the series dilutions of Bovine Serum Albumin (BSA) stock solution starting with 1 mg /ml and up to 0.03125 mg/ml concentrations (Figure A.1.).

Following centrifugation, supernatant was diluted with distilled water at a ratio of 1:20 and 0.5 ml of this diluted sample was mixed with 2.5 ml Lowry ACR reagent using vortex mixer (VM-10, Witeg Labortechnik GmbH, Germany). After waiting for 10 minutes, 0.25 ml Folin reagent was added and again mixed in vortex mixer. After waiting for 30 minutes in room temperature, absorbance values were recorded at 750 nm using Nano-Bio UV Spectrophotometer (OPTIZEN POP; Mecasys, Daejeon, Korea). As a reference, a blank sample was also put into the same procedure by using distilled water instead of the sample. Three replicates were done for each sample.

2.6 Brix°

At the end of the 1st month of storage, samples were taken out of refrigerator and the brix values of the serum layers (top layers) of the samples were recorded using Anton Paar Refractometer (Abbemat 200, Germany). Measurements were recorded once the samples are equilibrated to room temperature. Three replicates were done for each sample.

2.7 Sediment Ratio

Sediment test was conducted with some modifications of the method given by Cai et al. (2020). Samples were weighed and centrifuged at 4,000 rpm for 20 minutes at Universal 320R Benchtop Centrifuge Device (Hettich 1406, Canada). The tubes were turned upside down for 10 minutes in order to drain all of the supernatant. The results were expressed as the percent ratio of wet sediment weight over the whole sample weight. Experiment was performed in three replicates.

2.8 Particle Size Analysis

Mean particle size analysis of the AMD samples was done using Malvern Mastersizer 3000 (Malvern Instruments, United Kingdom). Refractive index and density of each sample was measured using Anton Paar Refractometer (Abbemat 3200, Germany) and Density Meter (Anton Paar DMA 4500, Germany) to set the values for the measurements. Refractive index of dispersant was set to 1.330. Measurements were done in three replicates.

2.9 Storage Experiments

Samples were kept in refrigerator at 4 °C for 30 days. Observations were done at the 1st day, 2nd week, 1st month of the and photos were taken.

At the end of the 30 days, serum separation in AMDs were measured and it was expressed as the percent ratio of separated serum phase (ml) over whole sample (ml) referring the formulation given by Sun et al. (2020). Experiment was done in three replicates.

2.10 Turbiscan Analysis

In order to analyze the stabilities of the AMD samples, ΔBS (Delta Backscattering) profiles and TSI (Turbiscan Stability Index) values were recorded. Analysis was done with a modification on the method given by Wu et al. (2020). Samples were scanned every 5 minutes for 5 hours at 40 °C with Turbiscan™ LAB Stability Analyzer (Formulaction, France). Turbiscan stability analysis were done for the samples with 0.5%(w/w) pectin concentration only. In addition, skimmed milk (Pınar Milk Co.) was analyzed as a reference which would provide a basis for the comparison of the stabilities. For the analysis, a cylindrical glass cell was cleaned with non-abrasive tissues and it was filled with the sample which was shaken gently beforehand and the cell was placed in the provided holder. Sample level in the cell

was kept up to the height of the holder which is around 42 mm and the surface was cleared from the air bubbles and foams.

2.11 Statistical Analysis

The data obtained in this study were analyzed using MINITAB (Version 16.1.1, Minitab Inc., Coventry, UK). 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.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Soluble Protein Content

The solubility of milk proteins inside the AMD system is very important for the stability. As the solubilization gets lower, proteins start to form aggregates and deposit at the bottom. Soluble protein contents at pectin concentrations of 0.2, 0.5, and 0.8% were shown in Figure 3.1, Figure 3.2, and Figure 3.3. respectively. First of all, it was seen that, both pectin concentration and pH had significant effect on the soluble proteins in the AMD ($p \leq 0.05$).

At 0.2% pectin concentration, the solubilities were significantly lower compared to 0.5 and 0.8% ($p \leq 0.05$). When it comes to pH 4 and 4.5 higher solubility results were observed ($p \leq 0.05$) compared to pH 5. This was also the case for for all pectin concentrations. The highest protein solubility was seen for the heat-treated sample with 0.8% pectin at pH 4 and the heat-treated sample with 0.2% pectin at pH 5 showed the lowest solubility.

When the solubility results are examined, it is seen that regardless of process type or pH, 0.2% pectin samples always had poor solubility. Studies showed that, there is a minimum pectin dosage for each acidified milk system depending on the milk solid content, pectin type, or processing conditions (Thi et al., 2009). Below this value, full coverage of proteins is not achieved and system does not keep the proteins suspended. Therefore, results implied that this minimum pectin concentration must be higher than 0.2% for this AMD system, according to solubility results.

Moreover, as seen in all of the figures, at pH 5, pectin activation was not enough to keep the beverage stable. From these results it can be clearly deduced that soluble

protein amount is strictly related to the activity of pectin. According to general consensus, pectin's stabilization mechanism basically depends on three different facts: *electrostatic and steric repulsions*, *viscosity enhancing effect*, and a *weak gel formation* (Guo et al., 2021). Suspension of the protein particles in the system is ensured by means of these facts. Among these, electrostatic and steric forces come into play at pH 5 or lower. This is why, lower solubility results are observed at pH 5. Tuinier et al. (2002) investigated the electrostatic adsorption of pectin onto casein micelles between pH 3.5 and 4.8. They reported that the adsorption increases as the pH decreases and suggested this as the result of the increase in positive charges of caseins. Therefore, more pectin is expected to be adsorbed to the surface charge of caseins. As suggested in the electrostatic adsorption theory explained by Tromp et Al. (2004), increased pectin – casein interaction may be the explanation of observed high protein solubilities at lower pHs compared to pH 5.

When the system has 0.2% pectin (shown in Figure 3.1.), HHP treated samples had slightly higher solubility values than heat treated samples, especially at pH 5. There is not any study so far as we know comparing HHP with heat treatment in this manner. Nevertheless, it is well known that, HHP increases the protein solubility and causes hydration of the molecules (Goyal et al., 2013). This elevated solubility without adsorbed pectin layer on the micelle surface may have resulted in better suspension of proteins within the system which may explain the slightly higher solubility results. Moreover, as in the HHP treatment, heat induced denaturation of whey proteins also results in aggregates. Denatured whey proteins interact with caseins and trigger more aggregation in the milk system (Thi and Ipsen, 2009). In this regard, in a dilute system with 0.2% pectin, heat induced denaturation may have overrode the denaturation by HHP.

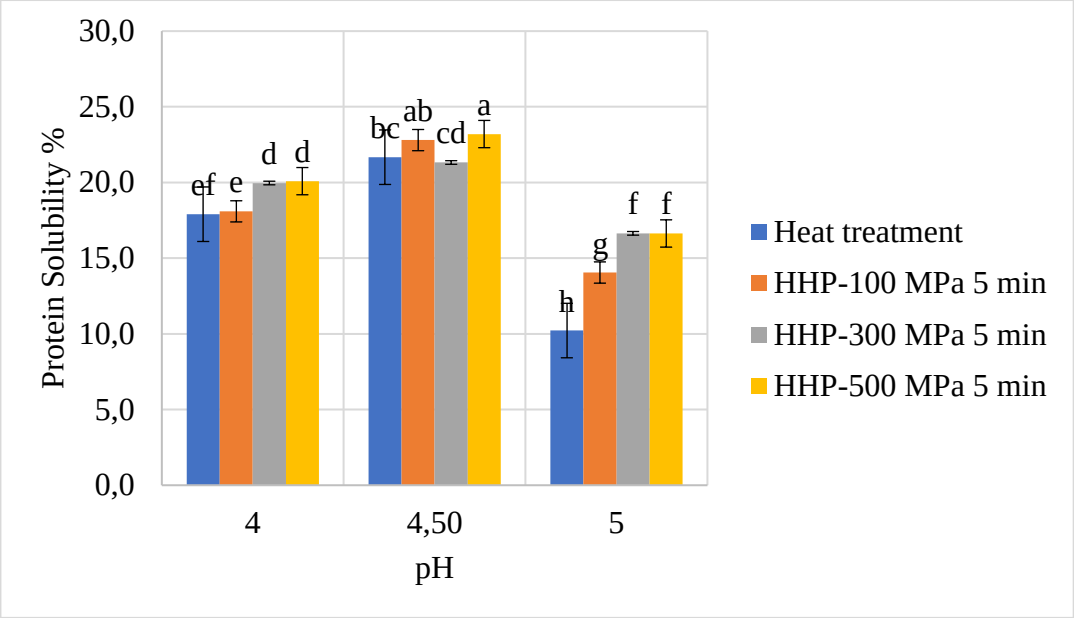

Figure 3.1. Soluble Protein Content for the sample with 0.2 % w/w pectin

At pectin concentrations with 0.5% and 0.8% (shown in Figure 3.2. and Figure 3.3.) heat treated samples at pH 4 showed the highest solubility. Except this sample, no certain pattern or a significant difference was observed between heat treatment and HHP samples at lower pH values. A research on the protein distribution of skim milk by Bravo et al. (2015) showed that HHP increased the casein solubility above 250 MPa enhancing the whey protein denaturation at the same time. Fluctuations in the protein solubility of pressurized samples were explained by these two overlapping effects. This showed that, once an effective adsorption of pectin onto proteins is ensured, processing conditions does not affect the solubility in either positive or negative way. However, at pH 5, there is a significant difference between heat and HHP treatments ($p \leq 0.05$). As explained above, assuming there is low pectin adsorption at high pH, HHP induced denaturation may have decreased the protein solubility compared to the applied heat treatment. Moreover, when pectin amount was increased up to 0.8% (Figure 3.3.), at pH 5, the effect of pressurization level was visible. Solubilities at 300 and 500 MPa were significantly lower than those at 100 MPa ($p \leq 0.05$). This could be a result of increased denaturation of whey protein with elevated pressures. It is known that, as the applied pressure increases extend of whey

denaturation increases (Huppertz, 2004). In this regard, association of denatured whey with caseins may have increased the aggregation as the pressure gets higher and results in lower solubility values.

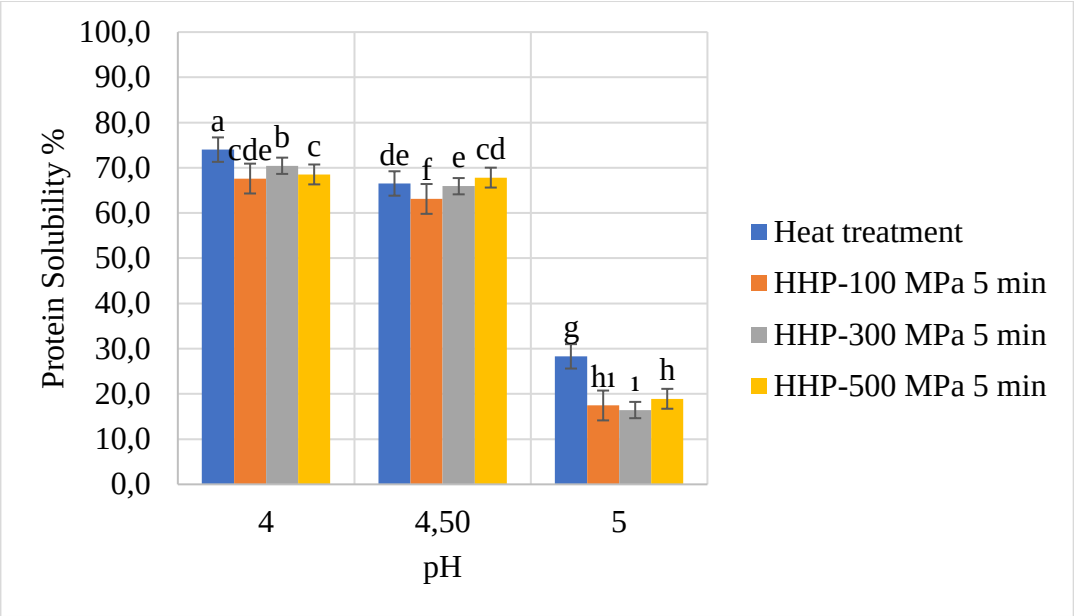

Figure 3.2. Soluble Protein Content for the sample with 0.5 % w/w pectin

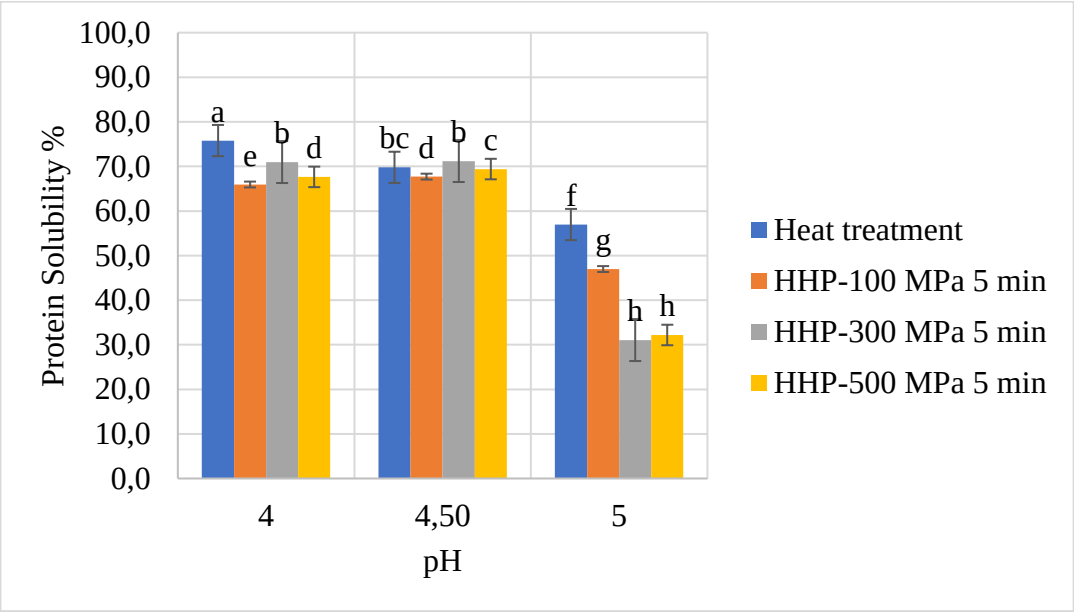

Figure 3.3. Soluble Protein Content for the sample with 0.8 % w/w pectin

3.2 Brixº

Brixº is the expression of soluble solids obtained by a calibration based on the refractive index of sucrose solution (Considine, 2014). It is generally used for characterizing the fruits in terms of their maturity depending on their sugar content. In dairy industry, Brixº values or refractive index are used to determine the milk quality in terms of soluble solids or detecting the adulteration (Jääskeläinen, 2001). Moreover, there also literature studies which obtain correlations between the milk fat or casein content and refractive index (Stocker et al., 2016). In this study, Brixº values of serum part of AMD samples were compared. Considine (2014) stated that the Brixº value, being a function of refractive index, is directly proportional to the amount of solids dissolved and how tight they bind to the water in the system. Therefore, hydration behavior of the solids in the solution affect the Brixº value positively. It was inevitable to obtain higher Brixº values at higher pectin levels. Therefore, in order to avoid the interference of this pronounced effect, the results are examined for three different pectin concentrations separately.

For the sample with 0.2 % pectin (as shown in Figure 3.4.), no significant difference between HHP and heat treatment was observed ($p>0.05$). However, in general, both heat treatment and HHP treatment showed a decreasing pattern in terms of pH. However, from pH 4.5 to 5 there was no significant decrease for HHP treated samples. Yang et al. (2020) reported significant pH decreases in skimmed milk after HHP treatment. This pH decrease may have added more positive charges to caseins, resulting in more interaction with pectin compared to heat treated samples. Therefore, the results for pH 5 did not show a significant decrease ($p>0.05$) as in the heat-treated samples. Since Brixº is affected from the dissolved solids in the system, it was an expected result to have the lowest Brixº at pH 5 which has the highest serum separation and sediment during the storage.

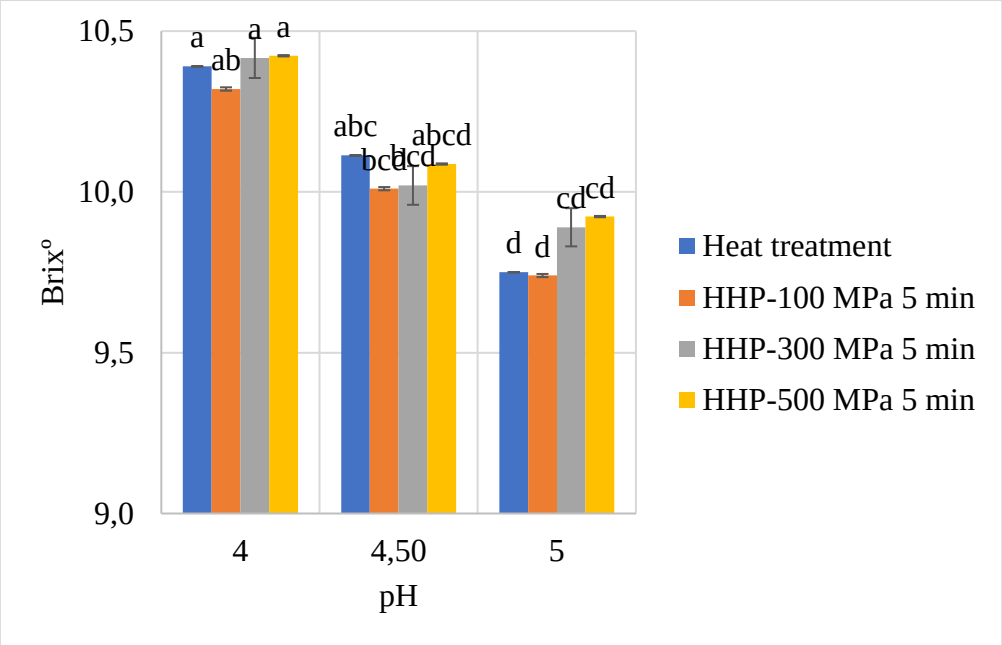

Figure 3.4. Brixº values for the sample with 0.2 % w/w pectin

At 0.5% pectin concentration (Figure 3.5.), the same rule for pH holds for both heat and HHP treatments. Brixº values decreased with the elevation in pH. At pH 4 and 4.5, there was no significant difference between thermally processed and pressurized samples ($p>0.05$). At pH 5, however; heat treated samples had significantly higher Brixº values than HHP samples ($p\leq0.05$). From this result, it could be assumed that when pectin has lost its effectiveness at high pH, denaturation effect of pressure becomes more visible. However, when pectin was effective at lower pH values, HHP effect was suppressed.

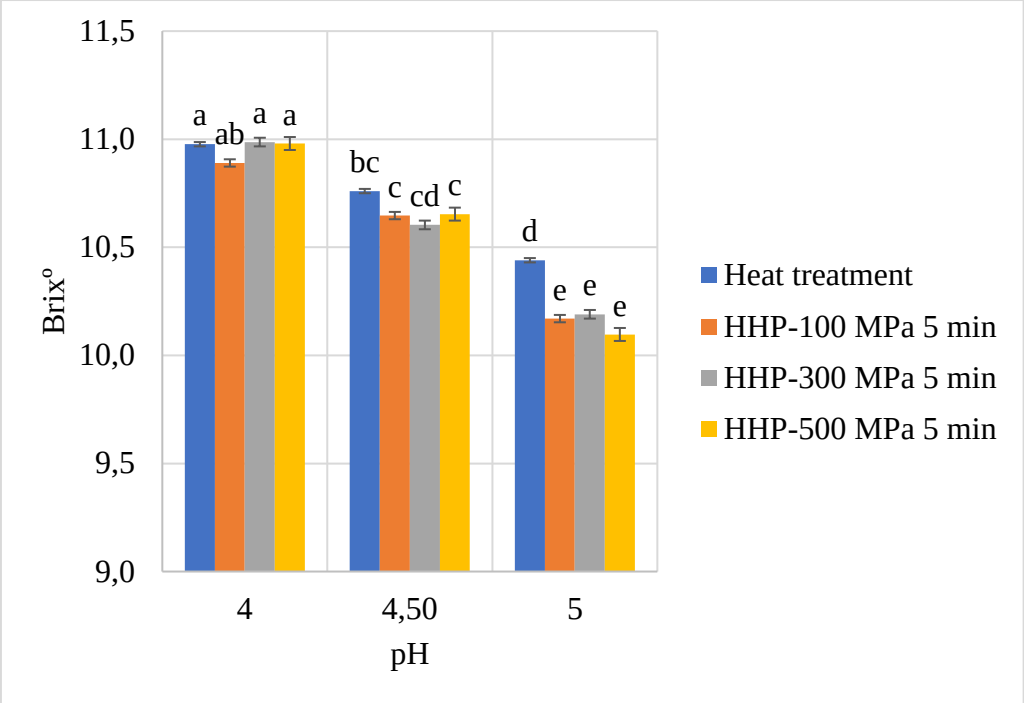

Figure 3.5. Brixº values for the sample with 0.5 % w/w pectin

For the sample with 0.8 % pectin content (as shown in Figure 3.6.), at pH 5, HHP effect became more visible and even pressure intensity has a significant effect on brix value from 100 to 300 MPa ($p \leq 0.05$). In addition to pH5, there was also significant differences ($p \leq 0.05$) between the heat-treated samples and HHP samples at pH 4 and pH 4.5. The reason for observing these differences between HHP and heat processes at a very high pectin level (at 0.8 %) could be depletion flocculation. Even though, high levels of pectin contribute the stability by viscosity enhancing effect, unbound pectin in the serum may result in depletion flocculation. This depletion effect of excess pectin is explained by the water release from intermolecular region to bulk phase due to increased osmotic pressure (Guo et al., 2021). Therefore, pectin is discarded from the region between the particles and then protein aggregation occurs. It is also possible that HHP may increase the depletion flocculation at 0.8 % and cause more aggregation. Consequently, clarified beverage may have lower Brixº values.

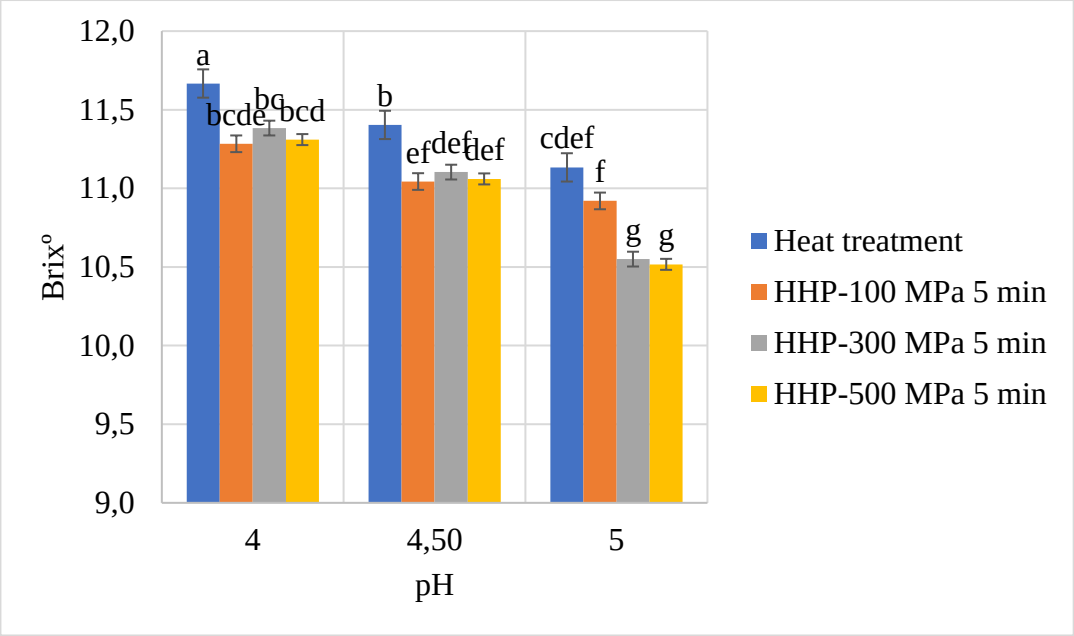

Figure 3.6. Brixº values for the sample with 0.8 % w/w pectin

3.3 Sediment Ratio

Sediment measurements obtained by centrifugation process is used by many researchers for the analysis of stability in acidified dairy products (Cai et al., 2020; Jensen et al., 2010; Sedlmeyer et al., 2004). Centrifugation is a way of accelerating the phase separation process that is observed in storage. Therefore, it gives an opinion for the product stability in shelf life. In general, for all pectin concentrations, if pH 5 is set aside, at lower pH values for both heat and HHP treatments; sediment ratio followed the same pattern as reported by Cai et al. (2020), Jensen et al. (2010), and Sedlmeyer et al. (2004). These studies revealed that, when there is no pectin in the system, sediment amount is relatively lower than the system with low amount of pectin. This increase in sediment ratio with little pectin addition is explained by the bridging flocculation phenomena (Guo, et al., 2021). If there is not sufficient pectin to cover caseins, one pectin molecule is shared by multiple casein micelles. This results in the aggregation of particles at lower pectin dosages. As the pectin amount

in the system increased to sufficient levels to cover caseins, sediment ratio decreases sharply and remains constant upon more pectin addition.

At 0.2% pectin (Figure 3.7.), there was not a significant difference (p>0.05) between the sediment results. As explained above, samples have very high levels of sediments due to pectin insufficiency in the system.

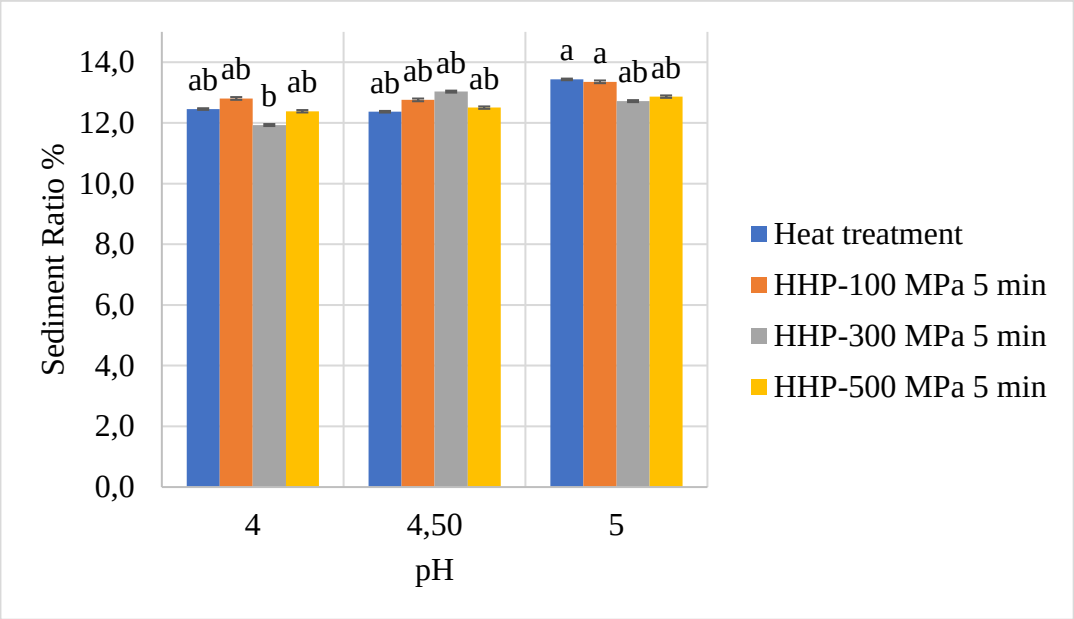

Figure 3.7. Sediment ratios for the sample with 0.2 % w/w pectin

At 0.5 % pectin (Figure 3.8.), there was a significant difference (p≤0.05) between pH 5 and lower pH values which agree with the soluble protein and brix results. This big differences at pectin level of 0.5 % justifies that the pectin used in this study is effective under pH 5. Moreover, having no significant differences (p>0.05) between heat treatment and HHP at lower pH values implies that, at the effective pectin concentration in the system, HHP induced aggregation of milk proteins could be prevented.

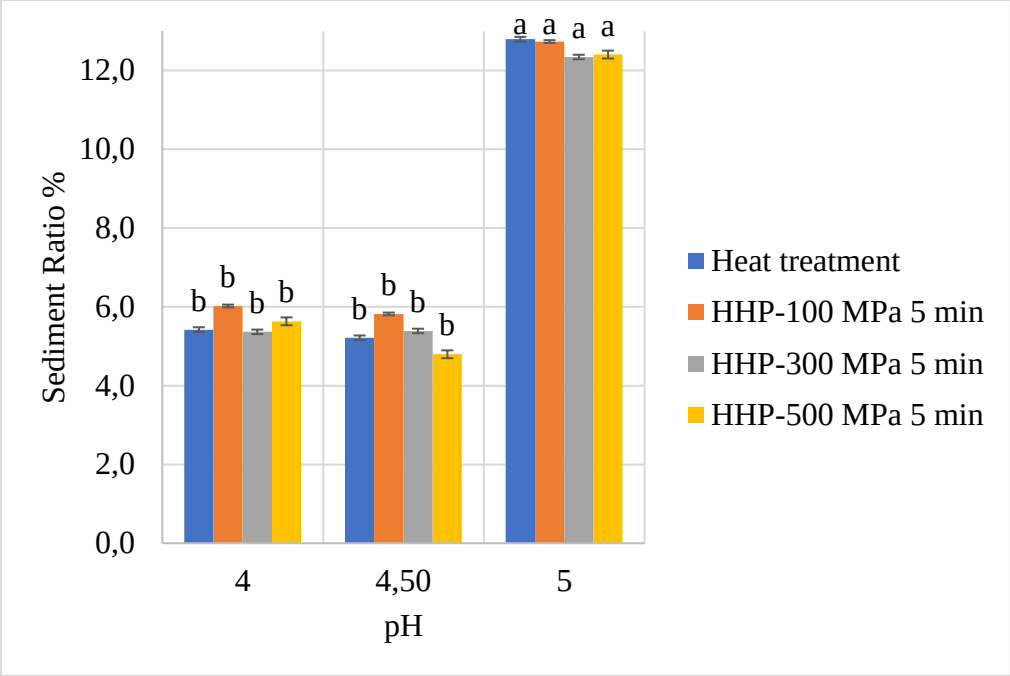

Figure 3.8. Sediment ratios for the sample with 0.5 % w/w pectin

For 0.8 % pectin dosage (Figure 3.9.), pH 4 and 4.5 showed similar pattern with 0.5% pectin in terms of sediment level. In addition, there is not a significant difference ($p>0.05$) between the sediment ratios of 0.5 % and 0.8 % pectin levels at these pH values. However, at pH 5, there is almost 3 % decrease in sediment ratios when pectin level is increased to 0.8 %. This result may be attributed to the viscosity enhancing effect of pectin. As the stabilizer concentration increased, increased viscosity makes the migration of particles more difficult (Guo et al., 2021). Moreover, at pH 5 effect of HHP became evident as in the brix results and increased pressures resulted in higher sediment ratios. From 100 MPa to 500 MPa, there was 2 % increase in sediment ratio.

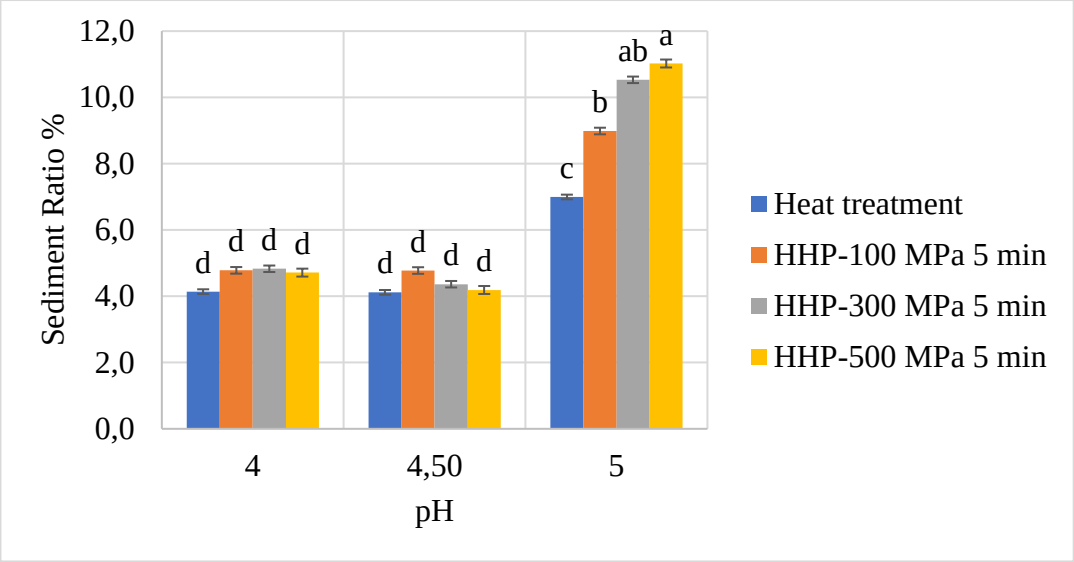

Figure 3.9. Sediment ratios for the sample with 0.8 % w/w pectin

In general, considering pH 4 and 4.5 and for sufficient pectin levels (0.5 % and 0.8 %) HHP treated samples showed no significant difference ($p>0.05$) with heat treated samples in terms of sediment levels. It is known that HHP disrupts the electrostatic interactions (Goyal et al., 2013). In our case, either HHP didn't disrupt the electrostatic interactions for observed conditions or stabilization of HHP treated samples occurred due to another process. It is also possible that HHP may have altered the pectin characteristics such as molecular weight (MW), degree of acylation (DA), degree of esterification (DE), or degree of substituents (DS) which are known to affect the interaction of pectin with caseins (Guo et al., 2021). It was shown that, high MW polysaccharides are more successful in stabilizing due to complex forming ability (Tian et al., 2021). Zhong et al. (2021) observed the effect of HHP on MW of pectin solution and reached increased MW results with increased pressures between 0-400 MPa. If the electrostatic binding is not completely disrupted by HHP, such changes may even strengthen this interaction between pectin and caseins. On the other hand, Peng et al. (2016) observed increased viscosity with elevated pressures after HHP treatment of pectin. Therefore, even though the electrostatic interactions are disrupted by HHP, increased viscosifying effect may be responsible from the small differences between HHP samples and heat-treated ones.

3.4 Mean Particle Size and Particle Size Distribution

When the results for average particle size are examined, the most effective parameter on the size is pH. Table 3.1. shows the average particle sizes as D [4;3] µm. For all pectin concentrations, particle sizes at pH 5 are significantly ($p \leq 0.05$) higher than pH 4 and 4.5. Peterson et al. (2019) also found similar results with using high methoxyl pectin (HMP) and low methoxy pectin (LMP) at pH 5.5. They explained the result by the lack of effective electrostatic forces between the caseins and pectin molecules at higher pH. Therefore, proteins form aggregate and result in big clusters. However, the samples are very heterogenous beverages with large span of particle distribution so; it is not accurate to evaluate particle size individually. Particle size distributions are shown in Figures 3.10 – 3.18. Distribution data showed that, even though there is no significant difference ($p > 0.05$) between the average particle sizes of heat treated and HHP treated samples; there is a larger span of smaller particles for HHP treated samples. Scanning electron microscopy (SEM) images of HHP treated milk proteins previously showed the disintegration of caseins after HHP results in smaller micelles compared to untreated samples (Serna Hernandez et al., 2021). Moreover, Liu et al. (2020) showed that, above 300 MPa HHP treated samples had significantly smaller micelles compared to thermal treatments of low-temperature low-time (LTLT), high-temperature short-time processes (HTST) ($p \leq 0.05$). According to general rule, micelle size increases up to the pressures of 250 MPa. The reason behind this change is the interaction of denaturated whey with casein at these pressures (Serna Hernandez et al., 2021). However, all HHP samples including the ones treated at 100 MPa had larger peaks at smaller particles compared to heat treated samples. Therefore, it can be suggested that pectin – casein interaction may prohibit the association of denaturated whey protein with casein at some extent.

Table 3.1 Average particle sizes as D[4;3]µm.

Sample	Heat treatment	100 MPa, 5min	300 MPa, 5min	500 MPa, 5min
A4	2.06372 ± 0.00015[h]	3.781428 ± 0.04827[h]	2.396903 ±0.01859[h]	2.236178 ±0.01832[h]
A4.5	1.938244 ± 0.00022[h]	2.727122 ± 0.01679[h]	2.062117 ±0.03054[h]	2.678352 ±0.109[h]
A5	9.615011 ± 0.51647[fgh]	17.90133 ± 1.93996[ef]	62.40318 ±4.27648[a]	57.10997 ±1.06293[a]
B4	2.994887 ± 0.00201[h]	1.984879 ± 0.04826[h]	1.697296 ±0.01448[h]	1.768493 ±0.0109[h]
B4.5	2.995376 ± 0.00129[h]	1.729787 ± 0.00959[h]	1.790998 ±0.02481[h]	2.063482 ±0.02517[h]
B5	2.919904 ± 0.00093[h]	6.3241 ± 0.88463[gh]	33.76959 ±5.20435[c]	13.18634 ±2.49405[efg]
C4	2.508221 ± 0.00069[h]	2.440275 ± 0.05751[h]	1.904147 ±0.01327[h]	2.302576 ±0.00723[h]
C4.5	2.469287 ± 0.00107[h]	2.323842 ± 0.02502[h]	2.248774 ±0.02669[h]	2.131167 ±0.0085[h]
C5	3.136229 ± 0.00044[h]	28.15396 ± 14.5192[cd]	44.05609 ±1.29391[b]	21.99042 ±1.4705[de]

- A, B, and C correspond to the pectin concentrations of 0.2 %, 0.5 %, 0.8 % respectively. 4, 4.5, and 5 are the pH values.

For the samples with 0.2% pectin, for both heat treated and HHP samples, at pH 4 (Figure 3.10) and pH 4.5 (Figure 3.11.) relatively homogenous results were obtained compared to pH 5 (Figure 3.12.). Even though experiments showed unstable results for 0.2 % pectin, particle distributions at pH 4 and pH 4.5 may be a sign of pectin aided homogeneity of the particles in the serum phase. This shows that, vigorous shaking of samples made the loose sediment layer incorporated back into the solution. However, the relative homogeneity observed at pH 4 and 4.5 for 0.2 % pectin did not imply the stability in these samples.

For 0.2 % pectin, HHP resulted in a bigger span compared to heat treatment due to micelle fragmentation. HHP samples have the major peaks at smaller particles, except pH5. At pH 5, major peaks for HHP treated samples were around 10-500 µm. Moreover, these peaks were higher than the peak for heat treated sample. This may imply that, with low pectin dosage at pH 5, HHP enhanced the bridging effect and resulted in bigger particles.

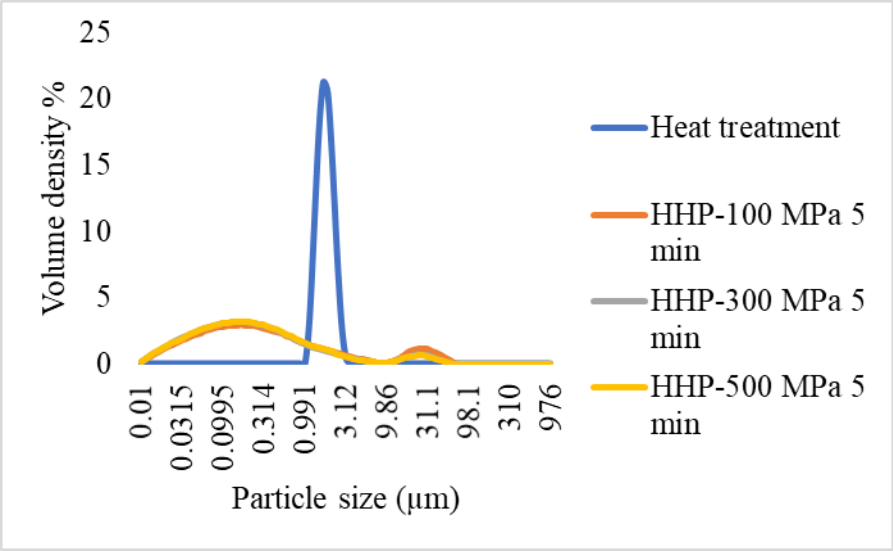

Figure 3.10. Particle size distribution for the sample with 0.2 % w/w pectin at pH 4

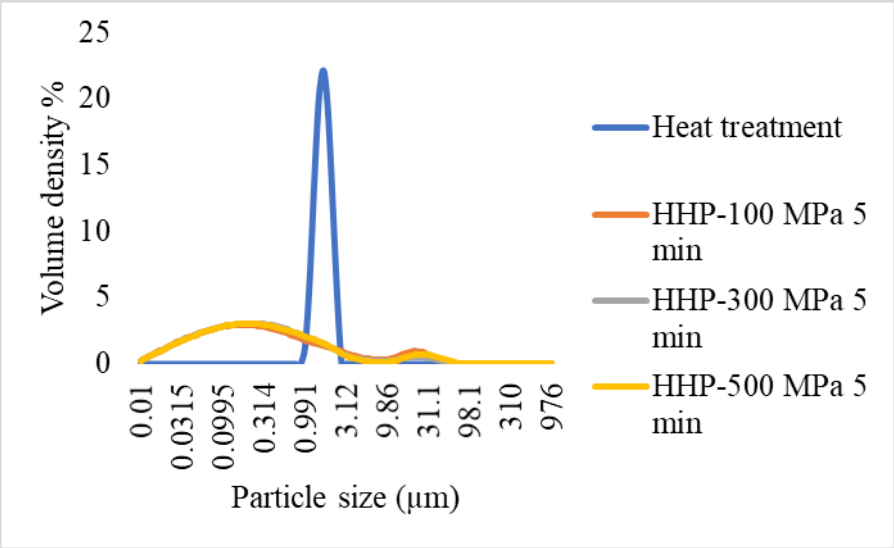

Figure 3.11. Particle size distribution for the sample with 0.2 % w/w pectin at pH 4.5

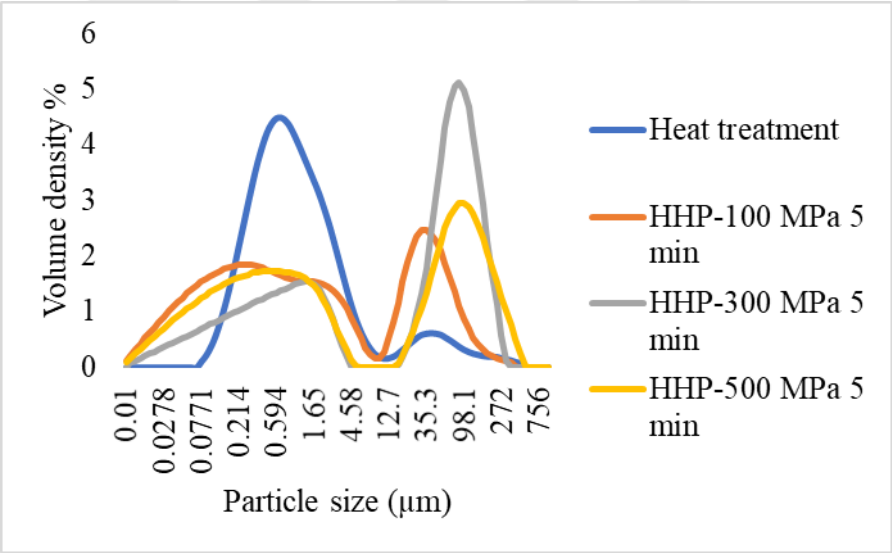

Figure 3.12. Particle size distribution for the sample with 0.2 % w/w pectin at pH 5

The most homogenous distributions for HHP samples were obtained at pectin levels of 0.5% (Figures 3.13, 3.14., and 3.15.) and 0.8% (figures 3.16, 3.17., and 3.18) at pH 4 and 4.5. Even there are broader areas of homogenous small particles in these samples, existence of second peaks around 10-50 µm shows there are aggregated regions in the system as well. This may prove that, increased adsorption efficiency

provided by HHP was accompanied by the bridging effect of HHP. Nevertheless, having stable results for these samples showed that the first effect prevailed the latter. Tromp et al. (2004) previously investigated the effect of homogenization pressure on AMD stabilized by pectin. They found out that increase in homogenization pressure increased the adsorption of pectin molecules and increased the pectin use efficiency. In their study, it was proposed that adsorbed pectin plays key role in the stabilization of AMD matrix. On the other hand, free network of pectin is not needed for the stability. Moreover, Peterson et al. (2019) also suggested that, non-adsorbed free pectin restricts the mobility of caseins and cause protein-protein aggregations. As a result, pressure aided pectin adsorption could be responsible for the stability of the samples treated with HHP at pH 4 and 4.5 at 0.5% and 0.8% pectin.

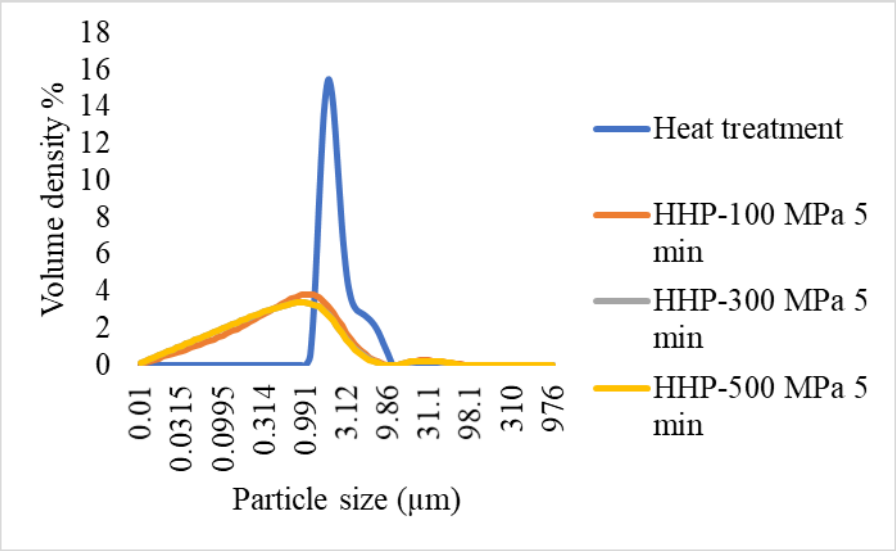

Figure 3.13. Particle size distribution for the sample with 0.5 % w/w pectin at pH 4

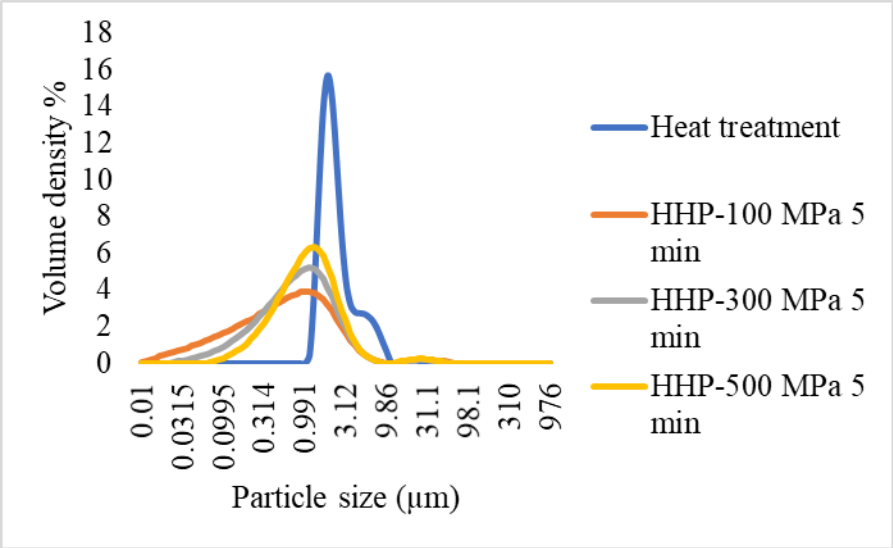

Figure 3.14. Particle size distribution for the sample with 0.5 % w/w pectin at pH 4.5

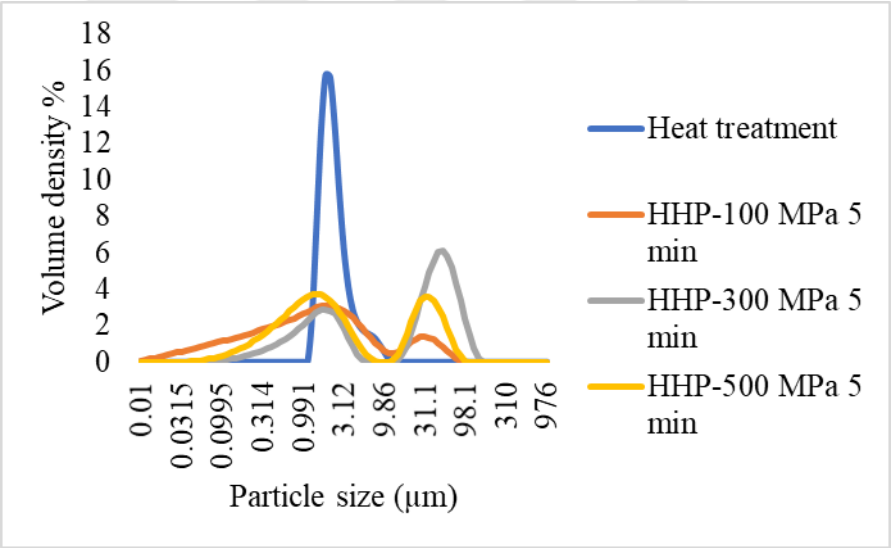

Figure 3.15. Particle size distribution for the sample with 0.5 % w/w pectin at pH 5

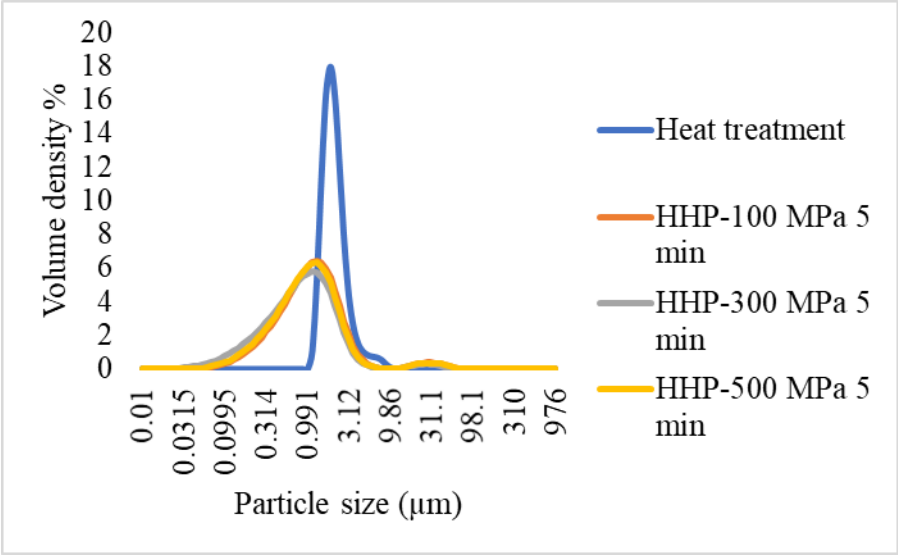

Figure 3.16. Particle size distribution for the sample with 0.8 % w/w pectin at pH 4

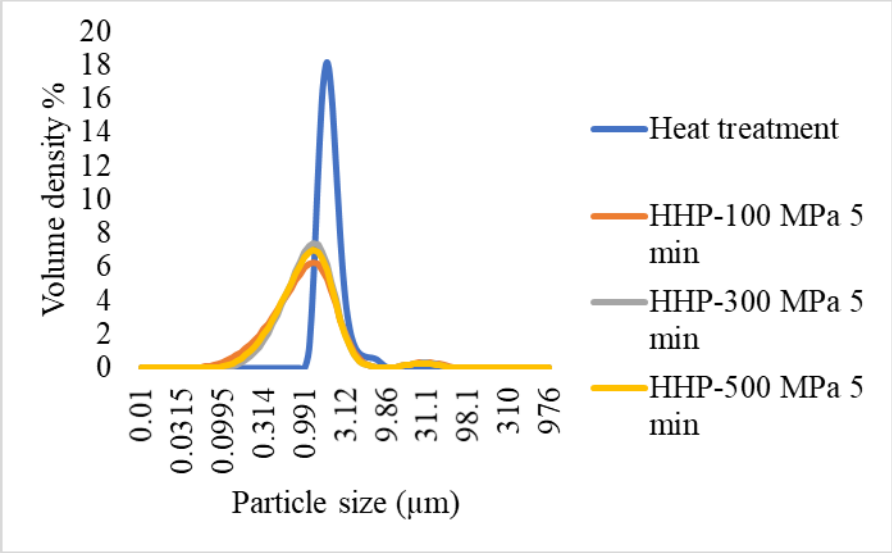

Figure 3.17. Particle size distribution for the sample with 0.8 % w/w pectin at pH 4.5

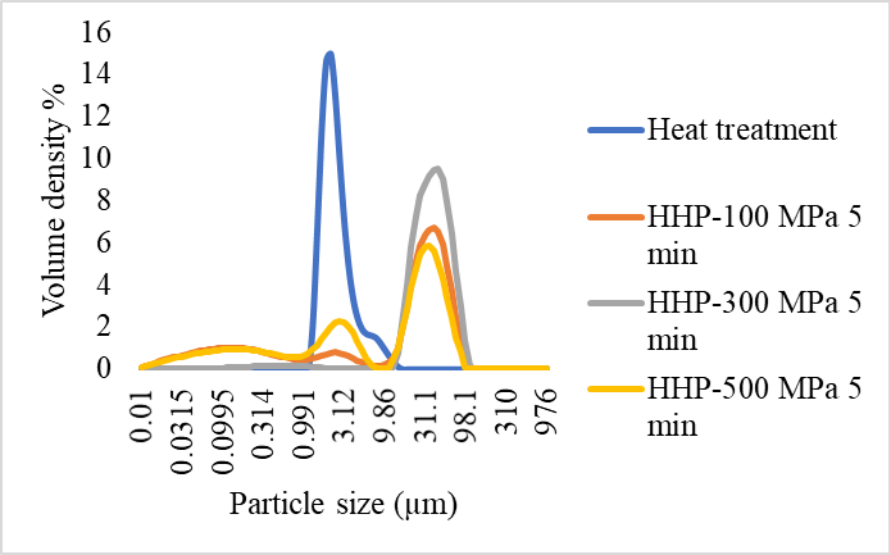

Figure 3.18. Particle size distribution for the sample with 0.8 % w/w pectin at pH 5

3.5 Storage Experiment

Physical stability is one of the main concerns for the stability assessment of a product. Therefore, this study focused on the phase behavior and the extent of sedimentation through the visual observation of products. Photos of the samples at the end of 1st day, 2nd week, and 1st month of storage were given all together in Figure A.1. in Appendix A. To compare the final conditions of samples, serum separations were given for the final day of storage and the photos for 0.2 % (Figure A.2.), 0.5 % (Figure A.3.), and 0.8 % (Figure A.4.) pectin were also recorded. Separations were expressed as the percent ratio of the separated phase height from the bottom to the total sample height.

For 0.2 % pectin, at the first day of storage, except for the sample treated at 500 MPa, serum separations were almost the same as the final separations (given in Figure 3.19.). For 500 MPa, sediment layers were almost 80 % and 50% of the final values respectively for pH 4 and pH 4.5. In the second week, these ratios were 90 % and 75 % of the final values. This result suggests that the phase separation occurs more

slowly for the sample treated at 500 MPa than other samples. At pH 5, both heat treated samples and HHP samples were completely clarified and the sedimentation was complete at the first day.

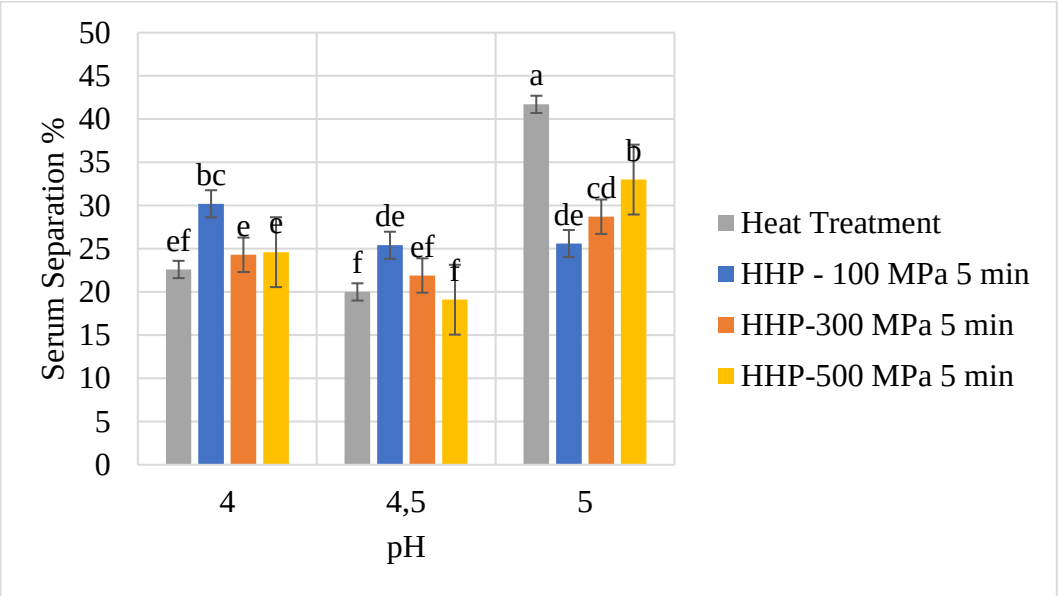

Figure 3.19. Serum separations at the end of storage for the samples with 0.2 % w/w pectin

For 0.5% pectin, at pH 4 and 4.5 samples had approximately the same amount of sediments at the first day. The sediments are 25 and 20% of the final separations (given in Figure 3.20.) respectively. Even at second week, they remained the same as in the first day, then reached at the final values. This shows that, separation of serum phase and casein aggregates takes time in these samples. At pH 5, the sedimentation behaviors were different for heat treatment and HHP. At the first day and second week of storage, heat treated sample reached the 75 % of final sedimentation. However, for HHP treated samples, separation started very slowly from a whey layer emerged very close to top. At the first day, there were 90, 72, and 72 % initial separations with a clarified layer at the top for 100, 300, and 500 MPa respectively. These levels decreased down to 55, 47, and 44% respectively in second week. At this stage the separated bottom layers were still loose and could be

suspended back into the system by shaking. At the end of storage, separations were complete and rigid casein sediments were set at the levels of 30, 37, and 37% respectively.

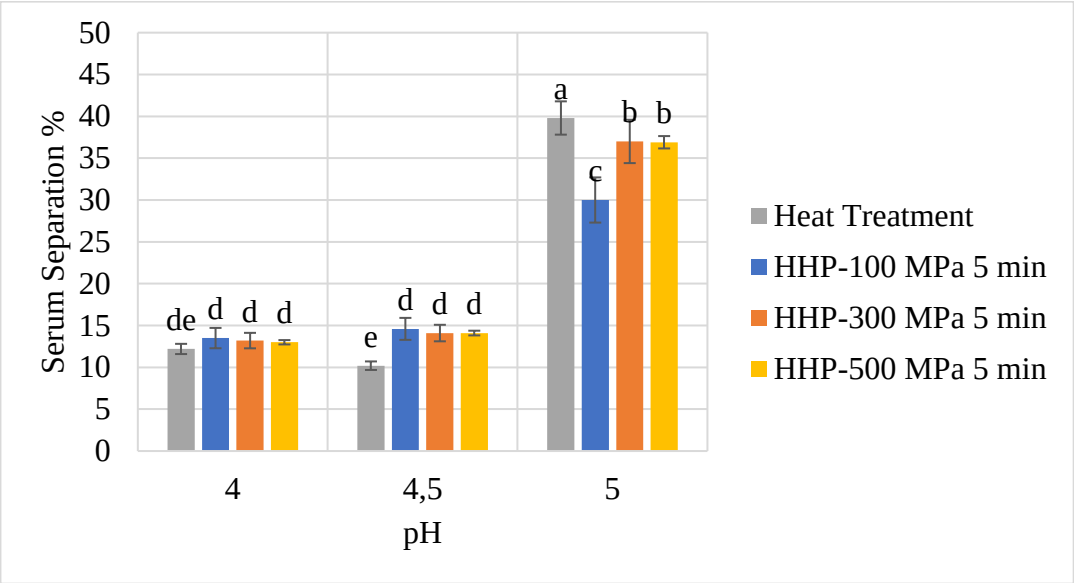

Figure 3.20. Serum separations at the end of storage for the samples with 0.5 % w/w pectin

Looking for the results at pH 5 for 0.2% and 0.5%, it could be stated that phase separation in the HHP treated samples took place more slowly than heat treated samples. This could be explained by having different ranges of particle sizes. Smaller particles created by HHP may take time to migrate to bottom. It can also be seen that the speed of particle migration increased as the pressure increased from 100 to 500 MPa. In general, despite having smaller particles and increased adsorption area, HHP samples did not attain a better stability than heat treated samples 0.2% and for 0.5% pectin. Same results were obtained by Wu et al. (2014), Tuinier et al. (2002), and Tromp et al. (2004) upon their observations on the effect of homogenization pressure. As homogenization pressures increased up to certain level, particle sizes decreased significantly accompanied by increased adsorption efficiency. However, increasing adsorption surface by creating smaller micelles arises more positive

charges to cover. Therefore, more pectin is needed to stabilize the system. The reason behind observing poorer stability than expected in HHP samples despite having large adsorption area could be explained by this mechanism.

At 0.8%, in the first day and the second week of storage, no sign of sedimentation was observed in the samples. At the end of first month, for pH 4 and 4.5, separation levels (given in figure 3.21) in the samples was approximately 5% with no significant difference between the samples ($p \leq 0.05$). For the samples prepared at pH 5, no observable sediment existed at the end of first month either. Li et al. (2018) observed that, increasing HMP dosage from 0.3% to 0.6% caused 3-fold increase in viscosity. However, at high pectin level, results showed a decreasing trend in viscosity as pH decreases. This was explained by the increased adsorption of pectin into caseins at low pH. Since adsorption decreased the serum pectin, viscosity decreased at lower pH values. Therefore, better stability at pH 5 could be explained by the viscosity increase due to non-adsorbed pectin in these samples. Moreover, only for these samples (samples with 0.8% pectin, at pH5) HHP treated samples had quite transparent color compared to heat treated sample despite having no sediment at all. It is known that, HHP decreases the particle size and results in transparency in milk color (Serna Hernandez et al., 2021). However, this color difference was not observed at pH 4 and pH 5. This may be explained by the fact that pectin was not adsorbed to micelles due to high pH and remained in the serum phase. Therefore, the stability of these samples may only be attained by the viscosity enhancing affect. Since this may show that stability could be attained without the electrostatic forces using HHP, this suggestion may be important for understanding the stability of HHP induced acidified milk drinks.

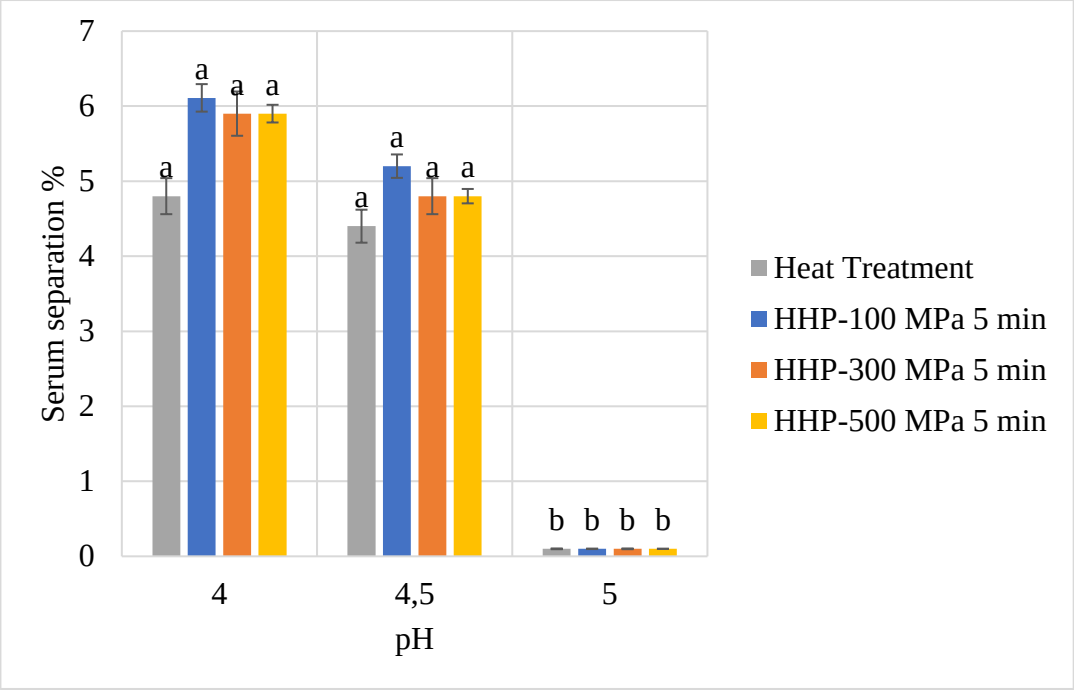

Figure 3.21. Serum separations at the end of storage for the samples with 0.8 % w/w pectin

3.6 Turbiscan Results

Although the storage observations or the results from the other experiments provide an insight on the stability of the AMDs, it is possible to observe the destabilization mechanism all through the sample via Turbiscan analysis. For this manner, Turbiscan analysis was employed with different purposes such as shelf life comparison, analysis of sedimentation or phase separation, or particle variations (Du et al., 2009; Sedlmeyer et al., 2004; Wu et al., 2014). Stability analysis using Turbiscan Lab was done by comparison of turbiscan stability indexes (TSI) and the observation of delta backscattering graphs. According to previous results, 0.2% pectin concentration was obviously not enough to ensure stability. 0.8% pectin showed stable results. However; this amount of pectin resulted in a remarkable increase in the product viscosity which would in turn affect the sensorial attributes of the product (Liu et al., 2020). Therefore, Turbiscan analysis was done with the

pectin concentration of 0.5%. In order to provide a basis as a homogenous, stable product, skimmed milk was used as reference.

The results showed that the importance of pH is undeniable for an AMD system. Both heat treatment and HHP treatments showed the same pattern in terms of pH. At pH values 4 and 4.5, the stability indexes of AMDs (shown in Figure 3.22.) were very close to that of skimmed milk. However, at pH 5, TSI values are considerably higher. Therefore, considering TSI values, it could be concluded that the stabilities are much more dependent on the pH than the process.

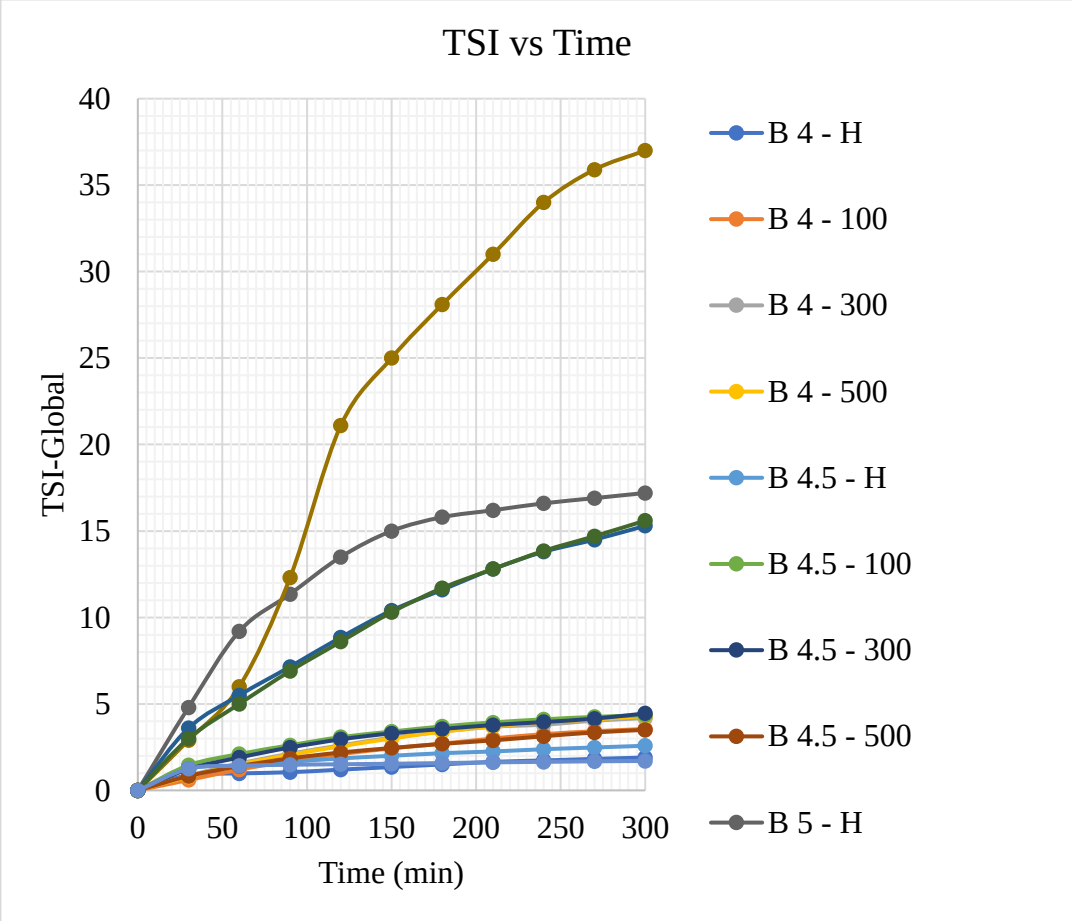

Figure 3.22. TSI Graph for Sample B

(B denotes the sample name: pectin concentration of 0.5% w/w. 4, 4.5, and 5 represent the pH values. 100, 300, and 500 show the pressure levels and H is for Heat treatment).

Since the samples are exposed to an extreme temperature in a short time period, Turbiscan analysis, in fact, enables to observe the samples under an accelerated shelf life. In this regard, delta backscattering data (given in figures 3.23.-3.35.) could be examined to understand the instability dynamics. First of all, analysis of the reference sample (skimmed milk) showed ΔBS increase of 2%, 1.5%, and 2% respectively at the bottom (between 0-10 mm height), middle (10-30 mm height), and top (30-42 mm height) segments (Figure 3.35.). The very small increase in the ΔBS intensity at the top of the reference sample could be explained by a hint of creaming due to the tiny amount of fat it contains. Since the AMD samples had almost half of the fat the skimmed milk has, there is not a ΔBS increase i.e. any sign of creaming at top the samples. Apart from that, the main observation from backscattering data was also the effect of pH. Samples at pH 5 (Figures 3.25, 3.28, 3.31, and 3.34.) had very complicated, diverse changes for both heat treated and HHP samples. This diversity indicates variations in signals coming out of sample i.e. in particle size, which validates the Mastersize results. Besides having bigger ΔBS variations, ΔBS increases and decreases at pH 5 are expanded in larger areas separated by a cross point which is an evidence of phase separation taking place. Thi et al. (2009) also used these points to estimate the sedimentation levels in AMDs. In this study, scanning period was not enough to see the sedimentation fully, so; these points were regarded as phase separation points. Putting these together, it could be inferred that pH 5 resulted in a very poor stability regardless of the process type. Out of the 12 samples examined, heat treated samples at pH 4 (Figure 3.23) and pH 4.5 (Figure 3.24.) showed the closest profiles to the reference sample. ΔBS changes of B4-H and B4.5-H are around 13-15%, 1%, and 5-7% at the bottom, middle and top. These 2 samples especially have smaller ΔBS increases at the bottom part. This means that the sedimentation in heat treated samples occurs more slowly compared to the HHP treated ones at pH 4 and pH 4.5. Nevertheless, during the scanning period, which could also be regarded as the simulation of accelerated shelf life, no sign of sedimentation or phase separation was observed at pH 4 and pH 4.5. Overall for

Turbiscan stability analysis, we can conclude that the stability of AMD either treated thermally or by HHP directly depends on the pH.

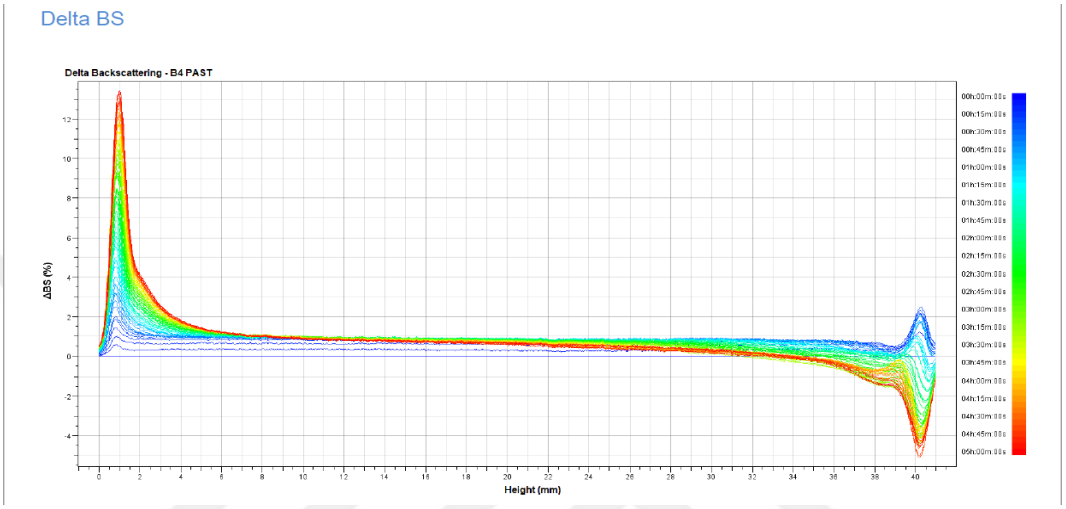

Figure 3.23. Delta Backscattering Graph for the Heat-treated Sample B at pH 4

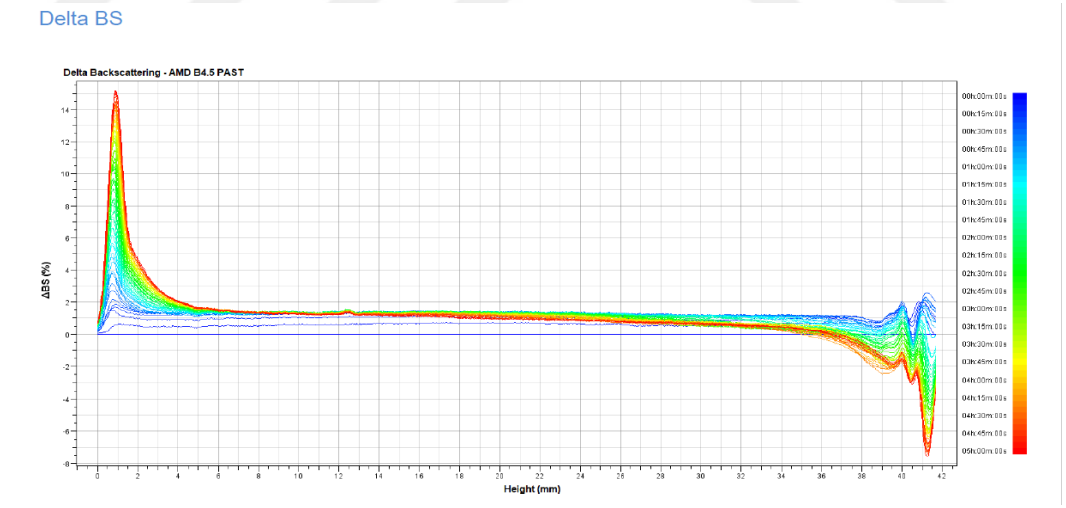

Figure 3.24. Delta Backscattering Graph for the Heat-treated Sample B at pH 4.5

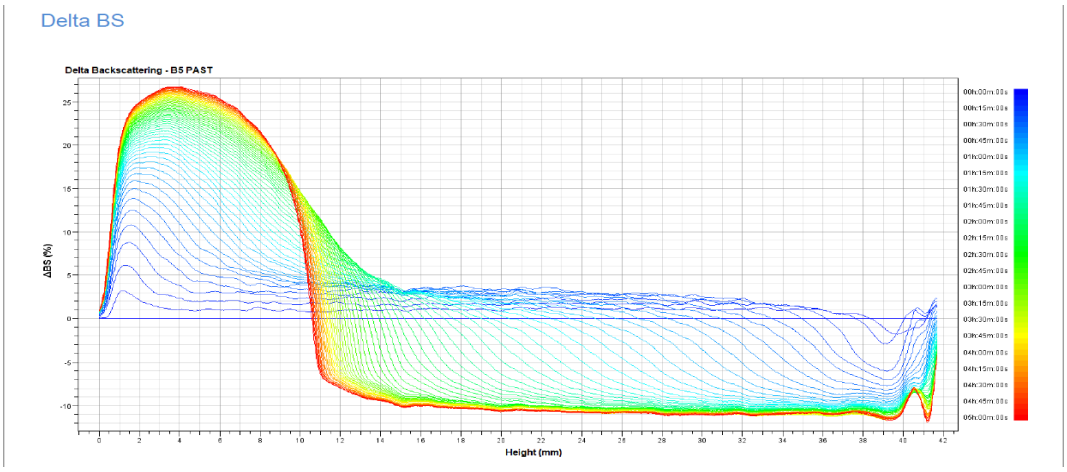

Figure 3.25. Delta Backscattering Graph for the Heat-treated Sample B at pH 5

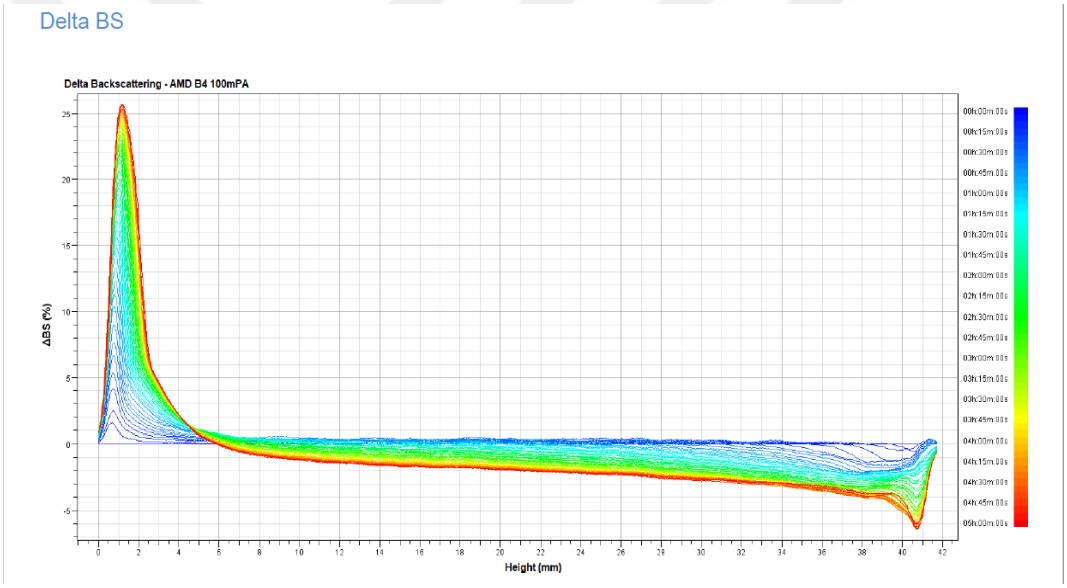

Figure 3.26. Delta Backscattering Graph for the HHP treated (100 MPa, 5min) Sample B at pH 4

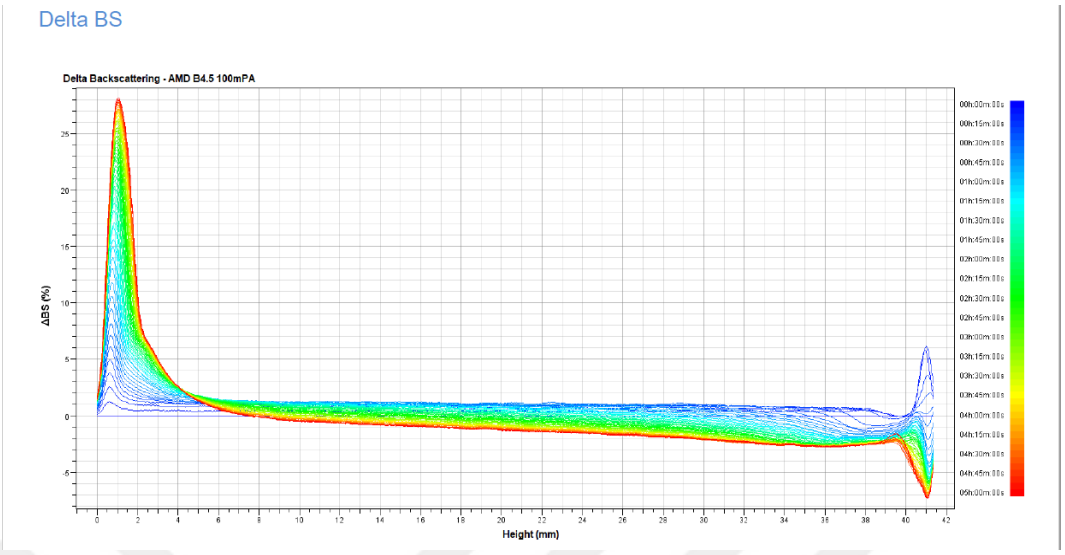

Figure 3.27. Delta Backscattering Graph for the HHP treated (100 MPa, 5min) Sample B at pH 4.5

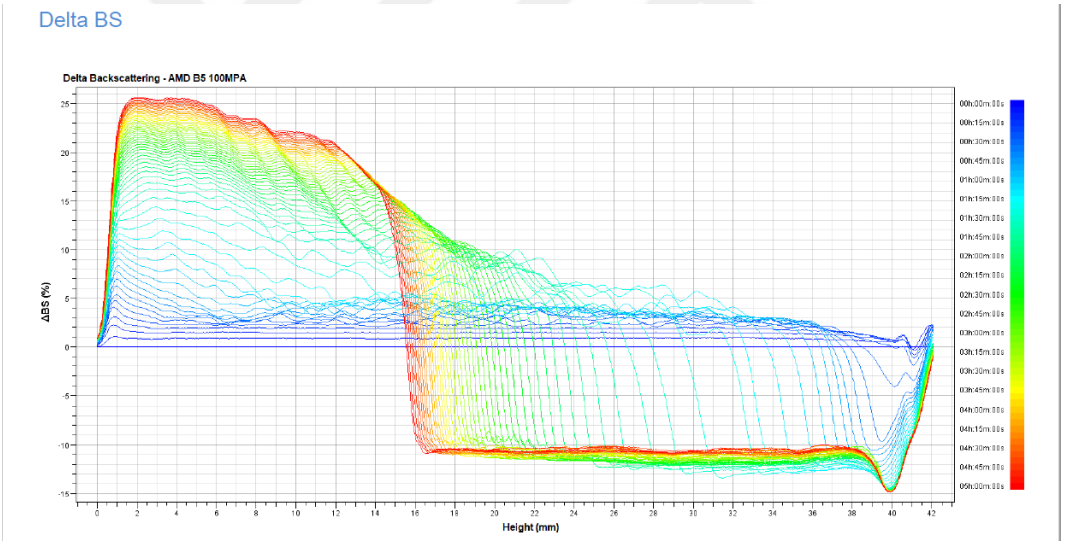

Figure 3.28. Delta Backscattering Graph for the HHP treated (100 MPa, 5min) Sample B at pH 5

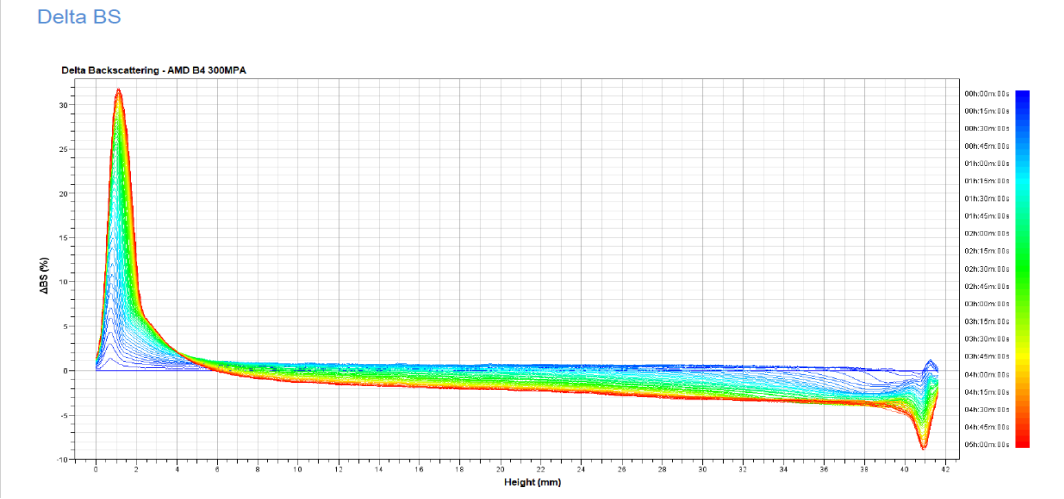

Figure 3.29. Delta Backscattering Graph for the HHP treated (300 MPa, 5min) Sample B at pH 4

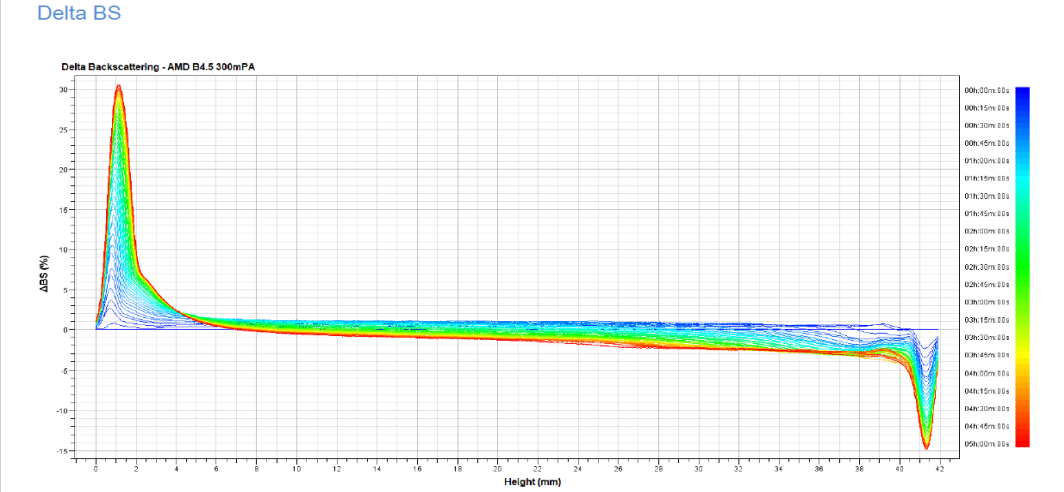

Figure 3.30. Delta Backscattering Graph for the HHP treated (300 MPa, 5min) Sample B at pH 4.5

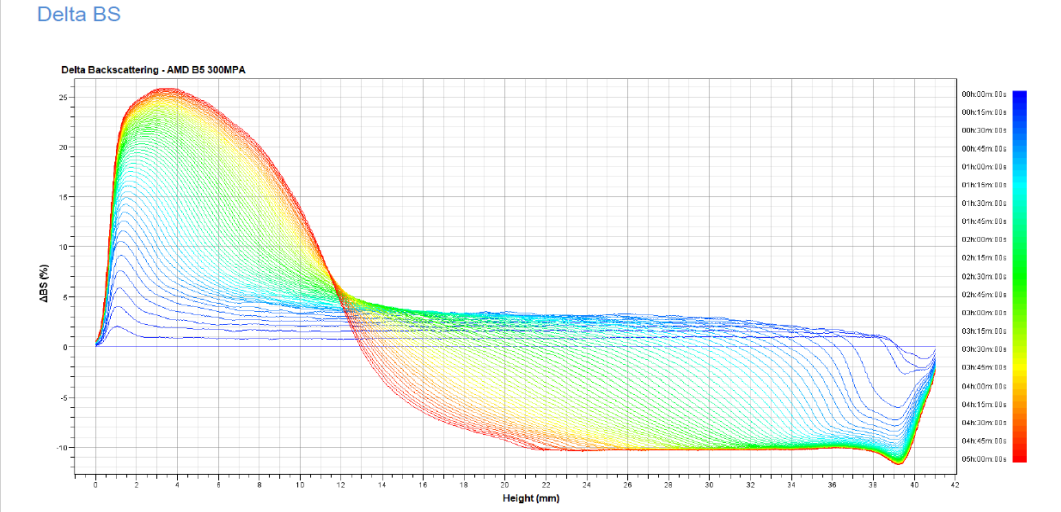

Figure 3.31. Delta Backscattering Graph for the HHP treated (300 MPa, 5min) Sample B at pH 5

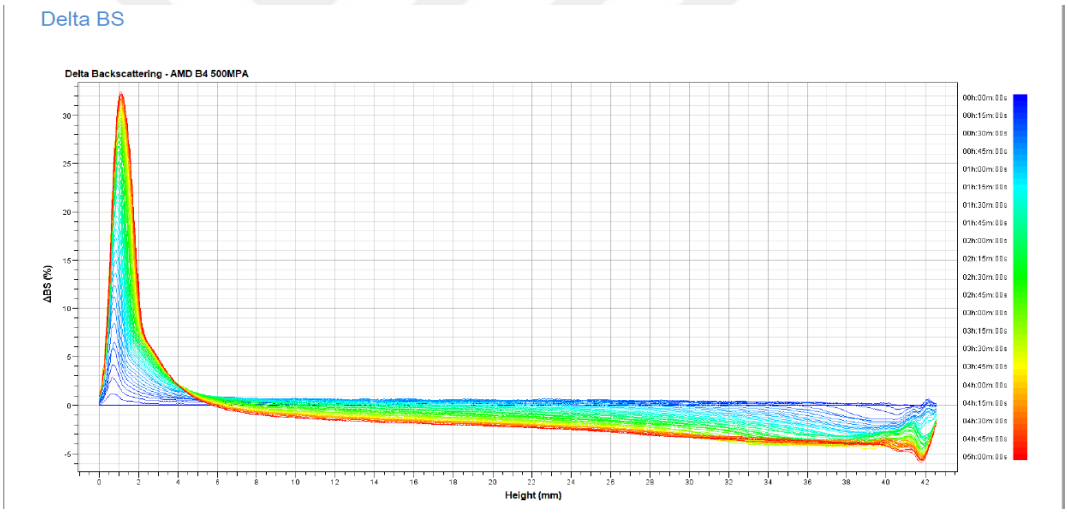

Figure 3.32. Delta Backscattering Graph for the HHP treated (500 MPa, 5min) Sample B at pH 4

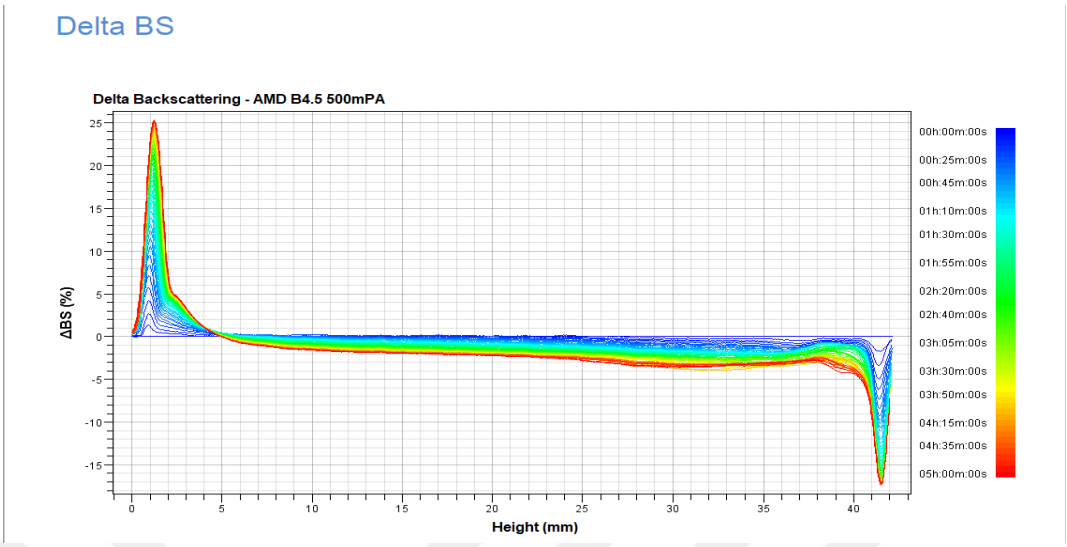

Figure 3.33. Delta Backscattering Graph for the HHP treated (500 MPa, 5min) Sample B at pH 4.5

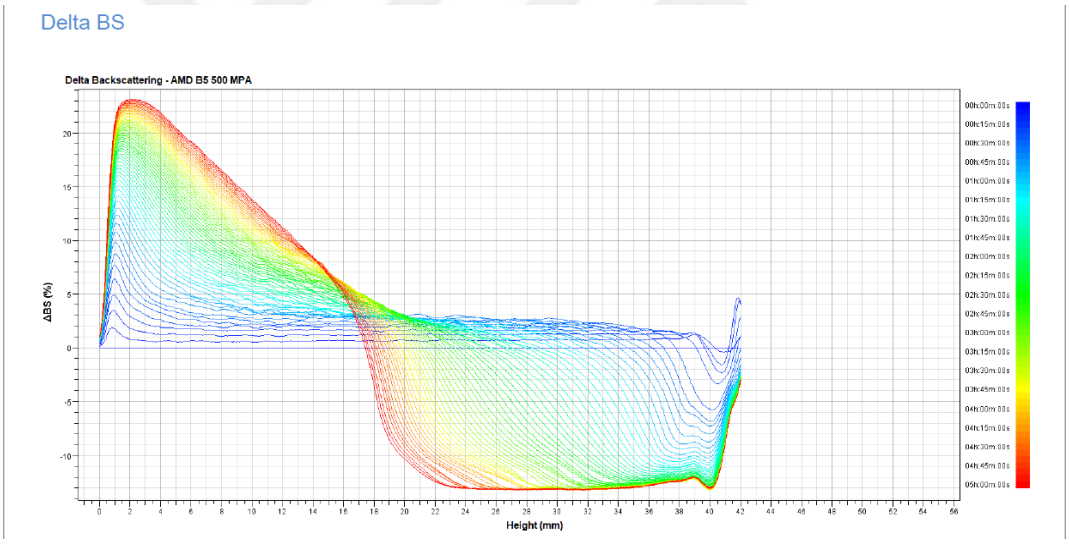

Figure 3.34. Delta Backscattering Graph for the HHP treated (500 MPa, 5min) Sample B at pH 5

Delta BS

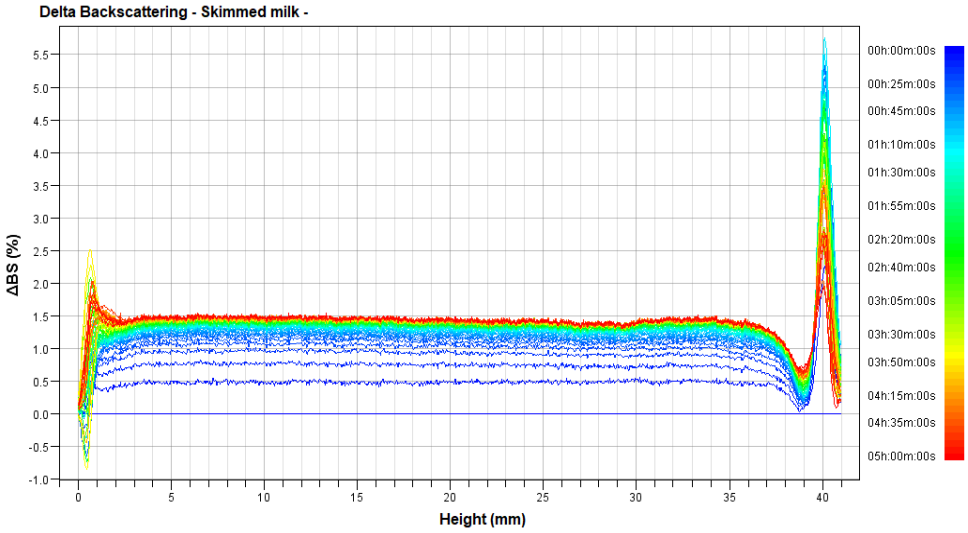

Figure 3.35. Delta Backscattering Graph for the Reference (Skimmed milk)

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

In this study, effect of HHP on the stability of an acidified milk drik was investigated at different pH values and pectin concentrations, in comparison with heat treatment. Results showed that pH and pectin concentration have significant effects on the stability for both heat treatment and HHP ($p \leq 0.05$). Samples had significantly higher Brix^o and protein solubility at pH 4 and 4.5 compared to pH 5 ($p \leq 0.05$). At 0.2 % pectin, both Brix^o and soluble protein content were significantly low ($p \leq 0.05$). Effect of the processes on solubility and Brix^o was seen at pH 5 and results were lower compared to heat treatment. Sediment ratio was the highest at pH 5 for all pectin concentrations and pH 4 and 4.5 did not show any significant difference ($p \leq 0.05$). Increasing pectin concentration from 0.2 to 0.5% showed a significant decrease in sediment ratio ($p \leq 0.05$). Particle size analysis indicated that, pH 5 resulted in a significant aggregation of proteins ($p \leq 0.05$). Size distributions were more homogenous for heat treated samples, however; HHP shifted the main peak towards smaller particles. Observations in storage experiment pointed out that HHP slows down the sedimentation during 1 month of storage at 4°C. Serum separation at the end of storage period was significantly higher at 0.2% pectin ($p \leq 0.05$). Except for 0.8% pectin, separations were the highest at pH 5. The lowest serum separation was achieved with 0.8% pectin at pH 5. Stability assessment for 0.5% pectin by Turbiscan demonstrated very stable results regarding the ground on reference skimmed milk with for both heat treated and HHP samples at pH 4 and 4.5. Overall for Turbiscan stability analysis, we can conclude that the stability of AMD either treated thermally or by HHP directly depends on the pH.

All in all, this study proved that, with sufficient and effective pectin cover, HHP treated AMD demonstrated comparable results with heat treated samples. For better

understanding the mechanism behind the HHP induced stabilization, viscosity analysis and confocal microscopy should be performed in future studies.

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APPENDICES

A. Calibration Curve for Lowry Method

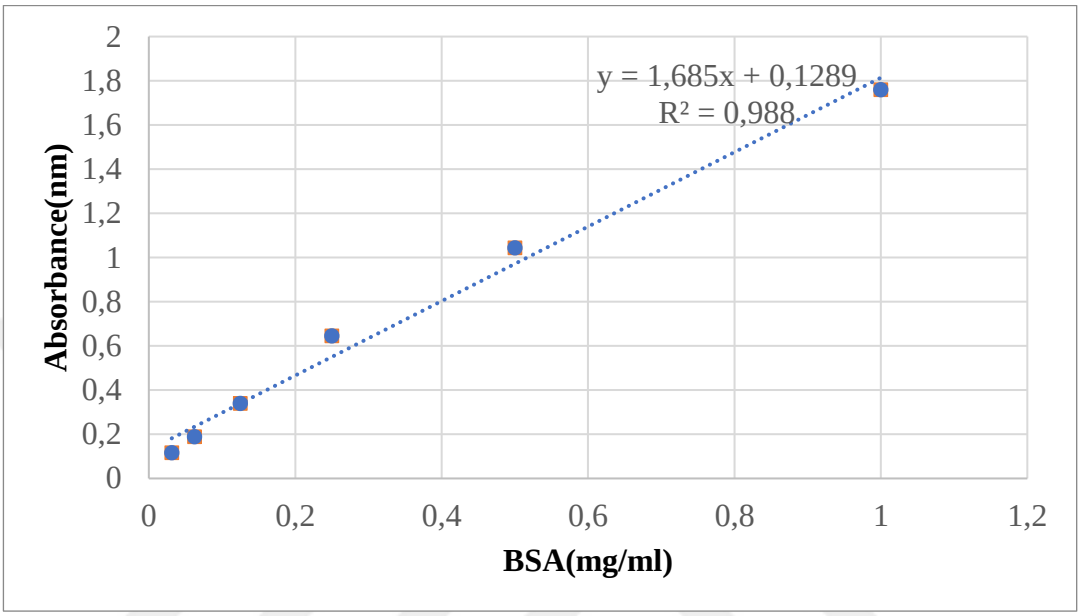

Figure A.1. Calibration curve for Lowry method prepared with the absorbances at 750 nm

B. Storage Experiment Photos

Heat Treated Samples

HHP Treated Samples – 100 MPa, 5 min

HHP Treated Samples – 300 MPa, 5 min

HHP Treated Samples – 500 MPa, 5 min

Figure B.2. Photos of the samples at the end of first day, second week and first month of storage.

Samples are in the order of; A4 & B4 & C4, A4.5 & B4.5 & C4.5, A5 & B5 & C5 from left to right. A, B, and C represent the pection concentrations of 0.2%, 0.5%, and 0.8% respectively and 4, 4.5, and 5 are pH values.

Figure B.3. Photos of the samples with 0.2%w/w pectin, at the end of storage.

Samples are in the order of; heat treated, HHP treated at 100MPa, 300MPa, and 500MPa for 5 mins respectively at pH 4, 4.5, and 5 from left to right.

Figure B.4. Photos of the samples with 0.5%w/w pectin, at the end of storage.

Samples are in the order of; heat treated, HHP treated at 100MPa, 300MPa, and 500MPa for 5 mins respectively at pH 4, 4.5, and 5 from left to right.

Figure B.5. Photos of the samples with 0.8%w/w pectin, at the end of storage.

Samples are in the order of; heat treated, HHP treated at 100MPa, 300MPa, and 500MPa for 5 mins respectively at pH 4, 4.5, and 5 from left to right.

C. Statistical Analysis

Lowry

Comparisons for Soluble Protein Content%

Tukey Pairwise Comparisons: Pectin concentration

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration	N	Mean	Grouping		
0.8	36	60.4693	A		
0.5	36	52.0901		B	
0.2	36	18.5479			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	N	Mean	Grouping		
4.0	36	53.0826	A		
4.5	36	52.5396		B	
5.0	36	25.4851			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Process

Grouping Information Using the Tukey Method and 95% Confidence

Process	N	Mean	Grouping	
0	27	46.7978	A	
500	27	42.7141		B
300	27	42.6610		B
100	27	42.6368		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH	N	Mean	Grouping					
0.5 4.0	12	70.1474	A					
0.8 4.0	12	70.0890	A					
0.8 4.5	12	69.5247	A					
0.5 4.5	12	65.8474		B				
0.8 5.0	12	41.7943			C			
0.2 4.5	12	22.2468				D		
0.5 5.0	12	20.2754					E	
0.2 4.0	12	19.0115						F

0.2 5.0	12	14.3855	G

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*Process	**N**	**Mean**	**Grouping**
0.8 0	9	67.5145	A
0.8 100	9	60.2086	B
0.8 300	9	57.7402	C
0.8 500	9	56.4141	D
0.5 0	9	56.2823	D
0.5 500	9	51.7561	E
0.5 300	9	50.9340	E
0.5 100	9	49.3879	F
0.2 500	9	19.9722	G
0.2 300	9	19.3087	G
0.2 100	9	18.3140	H

0.2 0	9	16.5968	I

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

pH*Process	**N**	**Mean**	**Grouping**								
4.0 0	9	55.9025	A								
4.0 300	9	53.7935		B							
4.5 500	9	53.4749		B	C						
4.5 300	9	52.8154			C	D					
4.5 0	9	52.6616			C	D					
4.0 500	9	52.0882				D					
4.5 100	9	51.2065					E				
4.0 100	9	50.5464					E				
5.0 0	9	31.8294						F			
5.0 100	9	26.1576							G		
5.0 500	9	22.5793								H	
5.0 300	9	21.3740									I

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH*Process	**N**	**Mean**	**Grouping**			
0.8 4.0 0	3	75.7946	A			
0.5 4.0 0	3	74.0102		B		
0.8 4.5 300	3	71.1994			C	
0.8 4.0 300	3	70.9734			C	D
0.5 4.0 300	3	70.4424			C	D
0.8 4.5 0	3	69.7870			C	D E

0.8 4.5 500	3	69.40 28	D E F
0.5 4.0 500	3	68.52 52	E F G
0.5 4.5 500	3	67.82 36	F G H
0.8 4.5 100	3	67.70 97	F G H
0.8 4.0 500	3	67.65 23	F G H I
0.5 4.0 100	3	67.61 18	G H I
0.5 4.5 0	3	66.52 89	H I

0.8	4.0	3	65.93	I		
100			57			
0.5	4.5	3	65.92	I		
300			62			
0.5	4.5	3	63.11		J	
100			07			
0.8	5.0	0 3	56.96			K
			18			
0.8	5.0	3	46.98			L
100			04			
0.8	5.0	3	32.18			M
500			72			
0.8	5.0	3	31.04			M
300			78			

0.5	5.0	0	3	28.3076	N
0.2	4.5500		3	23.1983	O
0.2	4.5100		3	22.7990	O P
0.2	4.5	0	3	21.6691	O P Q
0.2	4.5300		3	21.3207	P Q
0.2	4.0500		3	20.0871	Q R
0.2	4.0300		3	19.9647	Q R

0.5 5.0 500	3	18.9195	R	S
0.2 4.0 100	3	18.0918	S	T
0.2 4.0 0	3	17.9025	S	T
0.5 5.0 100	3	17.4411	S	T
0.2 5.0 300	3	16.6407		T
0.2 5.0 500	3	16.6313		T
0.5 5.0 300	3	16.4335		T

0.2	5.0 100	3	14.05 12	U
0.2	5.0 0	3	10.21 87	V

Means that do not share a letter are significantly different.

Brix

Comparisons for Brix

Tukey Pairwise Comparisons: Pectin concentration

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration	**N**	**Mean**	**Grouping**		
0.8	36	11.1144	A		
0.5	36	10.6161		B	
0.2	36	10.0903			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	**N**	**Mean**	**Grouping**

4.0	36	10.9189	A		
4.5	36	10.6253		B	
5.0	36	10.2767			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Process

Grouping Information Using the Tukey Method and 95% Confidence

Process	**N**	**Mean**	**Grouping**		
0	27	10.7370	A		
300	27	10.5715		B	
500	27	10.5611		B	
100	27	10.5581		B	

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH	**N**	**Mean**	**Grouping**	
0.8 4.0	12	11.4108	A	
0.8 4.5	12	11.1525		B

0.5 4.0	12	10.9583		C				
0.8 5.0	12	10.7800			D			
0.5 4.5	12	10.6658			D			
0.2 4.0	12	10.3875				E		
0.5 5.0	12	10.2242					F	
0.2 4.5	12	10.0575						G
0.2 5.0	12	9.8258							H

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*Process	**N**	**Mean**	**Grouping**				
0.8 0	9	11.4011	A				
0.8 100	9	11.0822		B			
0.8 300	9	11.0122		B			
0.8 500	9	10.9622		B			
0.5 0	9	10.7256			C		

0.5 300	9	10.5933	C	D	
0.5 500	9	10.5767		D	
0.5 100	9	10.5689		D	
0.2 500	9	10.1444			E
0.2 300	9	10.1089			E
0.2 0	9	10.0844			E
0.2 100	9	10.0233			E

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

pH*Process	**N**	**Mean**	**Grouping**			
4.0 0	9	11.0111	A			
4.0 300	9	10.9289	A	B		
4.0 500	9	10.9044	A	B	C	
4.0 100	9	10.8311		B	C	
4.5 0	9	10.7589			C	
4.5 500	9	10.6000				D

4.5 300	9	10.5756	D	E	
4.5 100	9	10.5667	D	E	
5.0 0	9	10.4411		E	
5.0 100	9	10.2767			F
5.0 300	9	10.2100			F
5.0 500	9	10.1789			F

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration* pH*Process	**N**	**Mean**	**Grouping**		
0.8 4.0 0	3	11.6667	A		
0.8 4.5 0	3	11.4033	A	B	
0.8 4.0 300	3	11.3833	A	B	
0.8 4.0 500	3	11.3100		B	C

0.8 4.0 100	3	11.2833	B	C	D			
0.8 5.0 0	3	11.1333	B	C	D	E		
0.8 4.5 300	3	11.1033	B	C	D	E		
0.8 4.5 500	3	11.0600		C	D	E	F	
0.8 4.5 100	3	11.0433		C	D	E	F	
0.5 4.0 300	3	10.9867			D	E	F	
0.5 4.0 500	3	10.9800			D	E	F	
0.5 4.0 0	3	10.9767				E	F	
0.8 5.0 100	3	10.9200				E	F	G
0.5 4.0 100	3	10.8900				E	F	G H

0.5 4.5 0	3	10.7600		F	G	H	I				
0.5 4.5 500	3	10.6533			G	H	I	J			
0.5 4.5 100	3	10.6467			G	H	I	J			
0.5 4.5 300	3	10.6033				H	I	J	K		
0.8 5.0 300	3	10.5500					I	J	K		
0.8 5.0 500	3	10.5167					I	J	K		
0.5 5.0 0	3	10.4400						J	K	L	
0.2 4.0 500	3	10.4233						J	K	L	
0.2 4.0 300	3	10.4167						J	K	L	M
0.2 4.0 0	3	10.3900						J	K	L	M N

0.2 4.0 100	3	10.3200			K	L	M	N	O	
0.5 5.0 300	3	10.1900				L	M	N	O	P
0.5 5.0 100	3	10.1700				L	M	N	O	P
0.2 4.5 0	3	10.1133					M	N	O	P
0.5 5.0 500	3	10.0967						N	O	P
0.2 4.5 500	3	10.0867						N	O	P
0.2 4.5 300	3	10.0200							O	P	Q
0.2 4.5 100	3	10.0100								P	Q
0.2 5.0 500	3	9.9233								P	Q
0.2 5.0 300	3	9.8900								P	Q

0.2 5.0 0	3	9.7500	Q
0.2 5.0 100	3	9.7400	Q

Means that do not share a letter are significantly different.

Sediment Ratio

Comparisons for Sediment Ratio%

Tukey Pairwise Comparisons: Pectin concentration

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration	**N**	**Mean**	**Grouping**		
0.2	36	12.7174	A		
0.5	36	7.8284		B	
0.8	36	6.1201			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	**N**	**Mean**	**Grouping**	
5.0	36	11.6817	A	
4.0	36	7.5403		B

4.5	36	7.4439	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Process

Grouping Information Using the Tukey Method and 95% Confidence

Process	**N**	**Mean**	**Grouping**	
100	27	9.11540	A	
500	27	8.94594	A	B
300	27	8.94419	A	B
0	27	8.54902		B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH	**N**	**Mean**	**Grouping**	
0.2 5.0	12	13.0928	A	
0.2 4.5	12	12.6653	A	
0.5 5.0	12	12.5690	A	
0.2 4.0	12	12.3941	A	
0.8 5.0	12	9.3833		B

0.5 4.0	12	5.6111	C		
0.5 4.5	12	5.3053	C	D	
0.8 4.0	12	4.6157		D	E
0.8 4.5	12	4.3613			E

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*Process	**N**	**Mean**	**Grouping**	
0.2 100	9	12.9728	A	
0.2 0	9	12.7529	A	
0.2 500	9	12.5844	A	
0.2 300	9	12.5593	A	
0.5 100	9	8.1927		B
0.5 0	9	7.8099		B
0.5 300	9	7.6993		B
0.5 500	9	7.6119		B

0.8 500	9	6.6415	C	
0.8 300	9	6.5740	C	
0.8 100	9	6.1807	C	
0.8 0	9	5.0843		D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

pH*Process	**N**	**Mean**	**Grouping**		
5.0 500	9	12.0978	A		
5.0 300	9	11.8634	A	B	
5.0 100	9	11.6914	A	B	
5.0 0	9	11.0741		B	
4.0 100	9	7.8699			C
4.5 100	9	7.7848			C
4.5 300	9	7.5941			C
4.0 500	9	7.5769			C
4.0 300	9	7.3750			C

4.0 0	9	7.3393	C
4.5 0	9	7.2336	C
4.5 500	9	7.1631	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH*Process	**N**	**Mean**	**Grouping**	
0.2 5.0 0	3	13.4336	A	
0.2 5.0 100	3	13.3540	A	
0.2 4.5 300	3	13.0308	A	
0.2 5.0 500	3	12.8657	A	B
0.2 4.0 100	3	12.8060	A	B
0.5 5.0 0	3	12.7932	A	B
0.2 4.5 100	3	12.7585	A	B
0.5 5.0 100	3	12.7352	A	B
0.2 5.0 300	3	12.7178	A	B

0.2 4.5 500	3	12.5033	A	B				
0.2 4.0 0	3	12.4567	A	B				
0.5 5.0 500	3	12.4050	A	B	C			
0.2 4.0 500	3	12.3843	A	B	C			
0.2 4.5 0	3	12.3684	A	B	C			
0.5 5.0 300	3	12.3425	A	B	C			
0.2 4.0 300	3	11.9293	A	B	C			
0.8 5.0 500	3	11.0227		B	C			
0.8 5.0 300	3	10.5299			C	D		
0.8 5.0 100	3	8.9851				D		
0.8 5.0 0	3	6.9955					E	
0.5 4.0 100	3	6.0223					E	F
0.5 4.5 100	3	5.8204					E	F G
0.5 4.0 500	3	5.6333					E	F G
0.5 4.0 0	3	5.4220					E	F G
0.5 4.5 300	3	5.3888					E	F G

0.5 4.0 300	3	5.3667	E	F	G
0.5 4.5 0	3	5.2145	E	F	G
0.8 4.0 300	3	4.8291		F	G
0.5 4.5 500	3	4.7973		F	G
0.8 4.0 100	3	4.7814		F	G
0.8 4.5 100	3	4.7756		F	G
0.8 4.0 500	3	4.7130		F	G
0.8 4.5 300	3	4.3629		F	G
0.8 4.5 500	3	4.1887		F	G
0.8 4.0 0	3	4.1394		F	G
0.8 4.5 0	3	4.1179			G

Means that do not share a letter are significantly different.

Particle size

Comparisons for Particle Size

Tukey Pairwise Comparisons: Pectin concentration

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration	**N**	**Mean**	**Grouping**

0.2	36	13.9095	A		
0.8	36	9.6387		B	
0.5	36	6.1021			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	**N**	**Mean**	**Grouping**		
5.0	36	25.0472	A		
4.0	36	2.3399		B	
4.5	36	2.2632		B	

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Process

Grouping Information Using the Tukey Method and 95% Confidence

Process	**N**	**Mean**	**Grouping**			
300	27	16.9255	A			
500	27	11.7186		B		
100	27	7.4852			C	
0	27	3.4045				D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH	N	Mean	Grouping			
0.2 5.0	12	36.7574	A			
0.8 5.0	12	24.3342		B		
0.5 5.0	12	14.0500			C	
0.2 4.0	12	2.6196				D
0.2 4.5	12	2.3515				D
0.8 4.5	12	2.2933				D
0.8 4.0	12	2.2888				D
0.5 4.5	12	2.1449				D
0.5 4.0	12	2.1114				D

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*Process	N	Mean	Grouping

0.2 300	9	22.2874	A				
0.2 500	9	20.6748	A				
0.8 300	9	16.0697		B			
0.5 300	9	12.4193		B	C		
0.8 100	9	10.9727			C		
0.8 500	9	8.8081			C	D	
0.2 100	9	8.1366			C	D	
0.5 500	9	5.6728				D	E
0.2 0	9	4.5390				D	E
0.5 100	9	3.3463					E
0.5 0	9	2.9701					E
0.8 0	9	2.7046					E

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

pH*Process	**N**	**Mean**	**Grouping**
5.0 300	9	46.7430	A

5.0 500	9	30.7622	B			
5.0 100	9	17.4598		C		
5.0 0	9	5.2237			D	
4.0 100	9	2.7355			D	
4.0 0	9	2.5223			D	
4.5 0	9	2.4676			D	
4.5 500	9	2.2910			D	
4.5 100	9	2.2603			D	
4.0 500	9	2.1024			D	
4.5 300	9	2.0340			D	
4.0 300	9	1.9994			D	

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH*Process	**N**	**Mean**	**Grouping**
0.2 5.0 300	3	62.4032	A

0.2 5.0 500	3	57.1100	A						
0.8 5.0 300	3	44.0561		B					
0.5 5.0 300	3	33.7696			C				
0.8 5.0 100	3	28.1540			C	D			
0.8 5.0 500	3	21.9904				D	E		
0.2 5.0 100	3	17.9013					E	F	
0.5 5.0 500	3	13.1863					E	F	G	
0.2 5.0 0	3	9.6150						F	G	H
0.5 5.0 100	3	6.3241							G	H
0.2 4.0 100	3	3.7814								H
0.8 5.0 0	3	3.1362								H
0.5 4.5 0	3	2.9954								H
0.5 4.0 0	3	2.9949								H
0.5 5.0 0	3	2.9199								H
0.2 4.5 100	3	2.7271								H
0.2 4.5 500	3	2.6784								H

0.8 4.0 0	3	2.5082	H
0.8 4.5 0	3	2.4693	H
0.8 4.0 100	3	2.4403	H
0.2 4.0 300	3	2.3969	H
0.8 4.5 100	3	2.3238	H
0.8 4.0 500	3	2.3026	H
0.8 4.5 300	3	2.2488	H
0.2 4.0 500	3	2.2362	H
0.8 4.5 500	3	2.1312	H
0.2 4.0 0	3	2.0637	H
0.5 4.5 500	3	2.0635	H
0.2 4.5 300	3	2.0621	H
0.5 4.0 100	3	1.9849	H
0.2 4.5 0	3	1.9382	H
0.8 4.0 300	3	1.9041	H
0.5 4.5 300	3	1.7910	H

0.5 4.0 500	3	1.7685	H
0.5 4.5 100	3	1.7298	H
0.5 4.0 300	3	1.6973	H

Means that do not share a letter are significantly different.

Storage experiment

Comparisons for Serum separation

Tukey Pairwise Comparisons: Pectin concentration

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration	**N**	**Mean**	**Grouping**		
0.2	36	26.4043	A		
0.5	36	20.7099		B	
0.8	36	3.5364			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH

Grouping Information Using the Tukey Method and 95% Confidence

pH	**N**	**Mean**	**Grouping**	
5.0	36	22.7494	A	
4.0	36	14.6914		B

4.5	36	13.2099	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Process

Grouping Information Using the Tukey Method and 95% Confidence

Process	**N**	**Mean**	**Grouping**
0	27	17.3156	A
500	27	16.8218	A
100	27	16.7395	A
300	27	16.6572	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH	**N**	**Mean**	**Grouping**				
0.5 5.0	12	35.9259	A				
0.2 5.0	12	32.2222		B			
0.2 4.0	12	25.4167			C		
0.2 4.5	12	21.5741				D	
0.5 4.5	12	13.2407					E

0.5 4.0	12	12.9630			E	
0.8 4.0	12	5.6944				F
0.8 4.5	12	4.8148				F
0.8 5.0	12	0.1000					G

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*Process	**N**	**Mean**	**Grouping**				
0.2 0	9	28.0864	A				
0.2 100	9	27.0370	A	B			
0.2 500	9	25.5556		B	C		
0.2 300	9	24.9383			C		
0.5 300	9	21.4198				D	
0.5 500	9	21.2963				D	
0.5 0	9	20.7407				D	E
0.5 100	9	19.3827					E

0.8 100	9	3.7988	F
0.8 300	9	3.6136	F
0.8 500	9	3.6136	F
0.8 0	9	3.1198	F

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

pH*Process	**N**	**Mean**	**Grouping**					
5.0 0	9	27.1938	A					
5.0 500	9	23.3049		B				
5.0 300	9	21.9469		B				
5.0 100	9	18.5519			C			
4.0 100	9	16.6049				D		
4.5 100	9	15.0617				D	E	
4.0 500	9	14.5062					E	F
4.0 300	9	14.4444					E	F
4.5 300	9	13.5802					E	F G

4.0 0	9	13.2099	F	G	
4.5 500	9	12.6543		G	H
4.5 0	9	11.5432			H

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Pectin concentration*pH*Process

Grouping Information Using the Tukey Method and 95% Confidence

Pectin concentration*pH*Process	**N**	**Mean**	**Grouping**			
0.2 5.0 0	3	41.6667	A			
0.5 5.0 0	3	39.8148	A	B		
0.5 5.0 300	3	37.0370		B		
0.5 5.0 500	3	36.8519		B		
0.2 5.0 500	3	32.9630			C	
0.2 4.0 100	3	30.1852			C	D

0.5 5.0 100	3	30.0000	C	D				
0.2 5.0 300	3	28.7037		D	E			
0.2 5.0 100	3	25.5556			E	F		
0.2 4.5 100	3	25.3704				F		
0.2 4.0 500	3	24.6296				F	G	
0.2 4.0 300	3	24.2593				F	G	
0.2 4.0 0	3	22.5926				F	G	H
0.2 4.5 300	3	21.8519					G	H	I
0.2 4.5 0	3	20.0000						H	I
0.2 4.5 500	3	19.0741							I

0.5 4.5 100	3	14.6296	J		
0.5 4.5 300	3	14.0741	J		
0.5 4.5 500	3	14.0741	J		
0.5 4.0 100	3	13.5185	J		
0.5 4.0 300	3	13.1481	J	K	
0.5 4.0 500	3	12.9630	J	K	
0.5 4.0 0	3	12.2222	J	K	
0.5 4.5 0	3	10.1852		K	
0.8 4.0 100	3	6.1111			L
0.8 4.0 300	3	5.9259			L

0.8 4.0 500	3	5.9259	L	
0.8 4.5 100	3	5.1852	L	
0.8 4.0 0	3	4.8148	L	
0.8 4.5 300	3	4.8148	L	
0.8 4.5 500	3	4.8148	L	
0.8 4.5 0	3	4.4444	L	
0.8 5.0 0	3	0.1000		M
0.8 5.0 100	3	0.1000		M
0.8 5.0 300	3	0.1000		M
0.8 5.0 500	3	0.1000		M

Means that do not share a letter are significantly different.