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– Effect of pH, carrageenan and storage time

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Abstract

The different characteristics of many food products, such as aroma and texture, changes during storage time and become undesirable. The reason is instabilities within the product which become prominent as the product ages. These changes in characteristics are quality losses caused by changes in the product's intrinsic and extrinsic environment. The stability in UHT-milk and beverages similar to it have been studied in the past decades and a certain focus have been on age gelation which is a common defect in these type of products. Research concerning instabilities in milk-based UHT-beverages containing carrageenan are few. The main objective of the present study was to study the stability of an UHT-milk beverage fortified with protein and containing carrageenan. The specific aim was to study structural changes occurring during twelve weeks of storage and the following five products were analysed: T1 (low pH; low carrageenan), T2 (high pH; low carrageenan), T3 (low pH; high carrageenan), T4 (high pH; high carrageenan) and a reference (center point). The products consequently had two factors varied at three levels; high and low pH and high and low concentration of carrageenan; and a center point with values between high and low. The microstructures of the samples were studied using bright field microscopy, confocal laser scanning microscopy and transmission electron microscopy. In addition the particle size and particle concentration were measured using a NanoSight and the results were further analysed using factorial design and Student's t-test. The pH was analysed by an ANOVA and Student's t-test and visual sensory analysis was carried out to detect structural changes in the products during storage.

The results from bright field microscopy and visual sensory analysis confirmed that there were structural differences among samples which appeared after different times of storage. The instabilities were observed in the following order starting with the most unstable sample first: T4, Ref, T2, T3 and T1. The results from the pH-measurements showed that the pH increased significantly in T1 and decreased significantly in T4 and the reference between week 0 and week 12. The results from the particle size measurements showed that the particle size increased significantly in samples T3, T4 and reference and decreased significantly in T1 between week 0 and week 8. In the beginning of storage the size was affected by the amount of carrageenan in the sample, and after eight weeks of storage the size was affected by an interaction between pH and carrageenan. The results from the measurements of particle concentration showed that the number of particles significantly decreased between week 0 and week 8 in all samples. The amount of carrageenan, pH level and time affected the number of particles in the samples.

The complexity of the products made it hard to conclude what was causing the observed instabilities, resulting only in hypothetical suggestions. The conclusion from the study was however that pH was of primary importance for the stability of this product, where a higher pH seemed to result in less stable products. The effect of carrageenan in this product seemed to be only of secondary importance for product stability.

Keywords: UHT-milk, carrageenan, pH, casein micelles, bright field microscopy, confocal laser scanning microscopy, transmission electron microscopy, particle size, particle concentration

Sammanfattning

De olika egenskaperna hos livsmedel, så som arom och textur, förändras under lagringstiden och resulterar oftast i oönskade egenskaper. Förändringarna beror oftast på instabilitet i produkten som visar sig efter en viss lagringstid, där orsaken vanligtvis är förändringar i produktens inre eller yttre miljö. Forskare har i flera decennier undersökt stabilitet i UHT-mjölk och liknande produkter och fokus har oftast lagts på den så kallade ålders gelningen som är en vanlig kvalitetsdefekt i denna typ av produkt. Forskning gällande instabilitet i mjölkbaserade UHT-drycker som innehåller karragenan är däremot få. Huvudsyftet med denna studie var att undersöka stabiliteten i en mjölkbaserad UHT-dryck berikad med protein och kolhydrater samt innehållandes karragenan. Det specifika syftet var att studera strukturella förändringar som skedde under en tolv veckors lagringsperiod där följande fem produkter analyserades: T1 (lågt pH, låg karragenan), T2 (högt pH, låg karragenan), T3 (lågt pH, hög karragenan), T4 (högt pH, hög karragenan) och en referens (mittpunkt). Produkterna hade två faktorer som varierades på tre nivåer; högt och lågt pH samt hög och låg koncentration av karragenan; och en mittpunkt med värden mellan högt och lågt. Mikrostrukturerna hos proverna studerades med hjälp av ljusmikroskopi, konfokalmikroskopi och transmissions elektronmikroskopi. Partikelanalys användes för att bestämma partikelstorleken och partikelkoncentrationen i de olika proverna och mättes med hjälp av en partikelstorleksmätare, NanoSight. Resultaten från dessa mätningar analyserades med hjälp av faktoriell design och t-test. Mätningar av pH genomfördes kontinuerligt och analyserades med hjälp av ANOVA och t-test, dessutom genomfördes visuella sensoriska analyser för att studera strukturella skillnader hos produkterna under lagringen.

Resultaten från ljusmikroskopin samt den visuella sensoriska analysen bekräftade att det fanns strukturella skillnader mellan produkterna som framträdde efter olika lagringstider. Instabiliteten observerades i följande ordning, där den första var mest instabil: T4, Ref, T2, T3 och T1. Resultaten från pH-mätningarna visade att pH ökade signifikant i T1 och minskade signifikant i T4 och referensen. Partikelanalysen visade att partikelstorleken ökade signifikant i T3, T4 och referensen och minskade signifikant i T1 mellan vecka 0 och vecka 8. Den faktoriella designen visade partikelstorleken påverkades av koncentrationen av karragenan i produkten och efter åtta veckors lagring visade det sig att pH och karragenan tillsammans hade en interaktionseffekt på partikelstorleken. Resultaten från mätningarna av partikelkoncentrationen visade att antalet partiklar minskade signifikant mellan vecka 0 och vecka 8 i alla prover. Partikelkoncentrationen påverkades av interaktionen mellan samtliga faktorer som varierades i produkterna.

Produktens komplexitet gjorde det svårt att dra slutsatser kring vad som orsakade instabiliteten i de olika produkterna, vilket resulterade i endast hypotetiska orsaksförslag. Den generella slutsatsen från studien var att pH troligtvis hade en primär betydelse för stabiliteten i denna produkt, där ett högre pH verkade resultera i en mer ostabil produkt. Mängden karragenan verkade däremot endast ha en sekundär betydelse för produktstabiliteten.

Nyckelord: UHT-mjölk, karragenan, pH, kaseinmiceller, ljusmikroskopi, konfokalmikroskopi, transmissionselektronmikroskopi, partikelstorlek, partikelkoncentration

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Abbreviations

ANOVA	Analysis of Variance
BFM	Bright Field Microscopy
β-LG	β-lactoglobulin
CLSM	Confocal Laser Scanning Microscopy
DF	Degrees of Freedom
F	F-ratio
κ-CN	κ-casein
MS	Mean Square
NTA	Nanoparticle Tracking Analysis
P	p-value
SS	Sum of Squares
T_C	Critical temperature
TEM	Transmission Electron Microscopy
UHT	Ultra High Temperature

1 Introduction

The past decades, the trend in fortified drinks has increased on the market, and consumers today demand different kinds of fortified drinks. Protein enriched fresh milk based drinks are one type of a health drinks that recently came to the market and along with it came problems never seen before. Textural-quality defects such as sedimentation was problem that turned up after some time of storage. These issues are however not easily solved since factors such as processing, ingredients and storage conditions all affect the stability of these products. Knowledge of the composition and nano-, micro- and macrostructures are therefore crucial in order to be able to find a solution to these issues as well as knowledge about the interactions taking place between the components in the product. (Paquin, 2009)

Two important components contributing to the structural properties of UHT-milk beverages are proteins and polysaccharides. These components interact and show different properties depending on the environmental conditions in the product. In order to improve the properties of milk based UHT-beverages knowledge of their interactions needs to be understood. (Benichou et al., 2002) An understanding about the factors causing unwanted gelation over prolonged storage also need to be considered, as it is a common problem in UHT-milk. Researchers have explained the unwanted gelation in UHT-milk mainly as the result of enzymatic activity (Walstra et al., 1999), while studies explaining the age gelation of milk-carrageenan beverages are few.

The objective of this study was to study the structural differences in a milk based protein and carbohydrate enriched UHT-beverage during storage. Totally five different products were examined, with the factors pH and carrageenan varied at three different levels, all stored for twelve weeks. The study was inquired by Norrmejerier Ek. För. a Swedish dairy company, and performed at the Swedish University of Agricultural Sciences.

2 Objectives

The objective of this study was to twofold; the first objective was to develop methods for analysing the structure of a protein and carbohydrate enriched milk based UHT-beverage. The second objective was to analyse structural differences between five different beverages during storage for twelve weeks, with the two factors pH and carrageenan varied at three levels.

Specific aims of the study were the following:

- To develop methods for analysing structural changes in the product during twelve weeks of storage at room temperature.
- To relate the structural changes in the product to the pH-level, carrageenan concentration and storage time.

In order to develop the analytical methods previously published research analysing the structural composition of milk and milk-carrageenan gels were reviewed. For the microstructural analyses different staining techniques and variants of embedding were tested. The method for particle size analysis of the products was designed according to a general method applied for these types of analyses.

The methods were then used to analyse the structural changes and differences between the products. The methods used to analyse the structures in the different products were bright field microscopy (BFM), confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM). The particle size and particle concentration were measured using a NanoSight. In addition the pH of the products was also measured.

Key questions in the present study were:

- How do the products change during storage in regard to appearance, particle size, particle concentration and pH?
- How do the pH-level and carrageenan concentration affect the stability of the products during storage?

3 Background

Extended shelf life products are common on the market today which is due to an increased consumer and retailer demand for products with a longer shelf life. Fortified beverages such as sports drinks are examples of products which have an extended shelf life, typically sterilized via Ultra High Temperature (UHT) treatment. The development of fortified beverages such as protein enriched milk drinks originate from health trends often related to diets. These products often come with instabilities due to their complexity, which during storage result in loss of product quality. (Paquin, 2009) A common quality defect related to UHT-milk products is age-gelation which occurs during product storage, researchers have spent time investigating the cause of this unwanted defect and several explanations have been proposed. (Datta and Deeth, 2001) In order to improve structural stability of dairy products stabilizers such as carrageenan is added. There are however some issues encountered during recipe development needing to be considered, these include environmental conditions such as pH, temperature and storage time. In addition the amount of stabilizer need to be monitored in order to achieve optimal product stability. (Tziboula & Horne, 2000)

3.1 Milk composition

Milk is a colloidal suspension consisting mainly of water, fat, carbohydrates, proteins, mineral substances and organic acids. Milk fat is to a greater extent made up of triacylglycerides, however phospholipids, cholesterol, free fatty acids and diglycerides can also be found. The major carbohydrate found in milk is lactose. Milk consists of different kinds of proteins of which caseins make up about 80% of the total protein content. The serum proteins, also called whey proteins, make up the remaining part of the total protein content and consist of β-lactoglobulin (β-LG), α-lactalbumin, serum albumin, immunoglobulins and peptides. The most commonly found minerals in milk are K, Na, Ca, Mg, Cl and P and the most common organic acid is citrate. (Walstra et al., 1999)

3.2 Casein

Bovine milk consist of approximately 4% proteins of which caseins (mainly the amino acid chains a_{s1}-,a_{s2}-, β- and κ-casein) compromise 80% and whey proteins (mainly β-LG, α-lactalbumin and bovine serum albumin) 20%. Caseins exist mainly as aggregated spherical structures called casein micelles (95%), containing colloidal calcium phosphate, where κ-casein (κ-CN) creates the surrounding surface layer of the micelle. The hydrophilic glycosylated part of κ-CN keeps the micelle dissolved in the serum phase of the milk and provides steric and electrostatic stability. The rest of the caseins, which have hydrophobic features, are kept inside the micelle by van der Walls- and hydrophobic interactions as well as hydrogen bonding and calcium phosphate nanoclusters. The micelles have an average diameter of about 150-200 nm and contain four times more water than protein. The size of the κ-CN is 5-10 nm in diameter. (Walstra et al., 1999) The micelle is however not a completely enclosed structure, it is porous which enables migration of proteins such as β-casein and β-LG through the micelle. Enzymes and polyphenols also have access to the interior of the micelle. (Donato & Guyomarc'h, 2009)

3.2.1 Caseinate

Caseinate is casein which have been precipitated from milk using an acid. The food industry sometimes uses caseinate to increase the nutritive value of their products, to provide products with particular physical properties, displacing more expensive proteins or during the manufacture of novel products. The most common caseinates are K-caseinate, Na-caseinate and Ca-caseinate. The caseinates are precipitated using acid and dissolved using alkali; they are flavourless and soluble in water. (Walstra et al., 1999)

Caseinates produced using precipitation techniques do not resemble the casein micelles in milk, as the colloidal phosphate structure has been destroyed and aggregates formed. The properties of the caseins are dependent on the manufacturing method and also the properties of the food system in which they are employed. For example the sodium, potassium and ammonium forms are soluble above pH 5.5, whereas Ca-caseinates form colloidal dispersions (Modler, 1985)

3.3 UHT treatment of milk

UHT treatment is a sterilization process performed at temperatures above 140 °C for a few seconds. The main purpose of applying UHT-treatment to milk is to kill all microorganism, prolonging the shelf-life of milk. Milk subjected to UHT-processing is characterized by chemical and physical changes affecting the properties of the milk. These changes include protein denaturation, killing of microorganisms, nutritional changes and changes in colour. (Walstra et al., 1999) The sterilization process also lead to acidification of the milk, changes in aroma and enzyme inactivation (Hsu, 1970). UHT-treatment does not kill heat-resistant native- and bacterial proteases present in the milk, which means that enzymatic deterioration of the milk can occur after longer time of storage. Milk can also deteriorate due to non-enzymatic reactions caused by oxidation, influence of light and Maillard reactions. Maillard reactions result in the formation of brown-coloured pigments, polymers and acids and is affected by the severity of the heat treatment and also the storage time and temperature. (Dunkley and Stevenson, 1987) The positive effects of UHT-processing is that the milk can be stored at ambient temperatures for several months, however its down sides are browning of the milk, changes in aroma and vitamin losses. (Walstra et al., 1999)

3.3.1 Protein denaturation

Milk undergoing severe heat treatment is characterized by structural changes such as protein denaturation, the denaturation of β-LG is the most important of these changes. The dimeric structure of β-LG dissociates and the globular monomer unfolds resulting in exposure of –SH groups. These groups either interact with one another forming whey protein complexes or they interact with κ-CN forming β-LG–κ-CN complexes. (Griffiths, 2010) This association of whey proteins to the surface of the casein micelles prevents interactions between carrageenan and κ-CN enhancing the gel strength and stability of the carrageenan-milk system. (Tijssen, 2007) The pH, heating time and temperature during sterilization affect the amount of β-LG–κ-CN complexes formed during sterilization. A higher temperature, longer heating time and higher pH result in more complexes. (Elfagm and Wheelock, 1978)

3.4 Age gelation of UHT milk

Age gelation is a defect common in milk subjected to UHT treatment which occurs after a longer storage time and limits the shelf life of the product. It is a physical change in UHT-milk where the milk loses its fluidity and the viscosity increases due to the formation of a three dimensional protein network. (Datta and Deeth, 2001)

The mechanism of gelation is not fully understood, however both enzymatic and non-enzymatic reactions have been suggested as possible reasons. A two-stage process has been proposed as the cause of age gelation where the first stage involves structural changes and the second stage involves physico-chemical reactions resulting is loss of stability and gel formation. This theory suggests that gelation is related to aggregation of casein micelles, caused by the release of β-LG–κ-CN complexes which keep the micelles stable in the milk. (McMahon, 1996) Another theory however suggest that gel formation is not due to aggregation of micelles and studies using electron microscopy have showed that the casein particles are connected by hair-like protrusions which could be the β-LG–κ-CN complexes. (Andrews, 1977; Venkatachalam, 1993) The most accepted theory suggests that it is proteolytic activity that causes the release of the β-LG–κ-CN complexes. (Walstra et al., 1999)

3.4.1 Enzymatic age gelation

The unwanted gelation of UHT-milk has been related to proteolysis of the caseins present in the milk, probably caused by native milk proteinases (plasmin) and proteinases produced by psychrotrophic bacteria before or after heat treatment of the milk. (Walstra et al., 1999) The enzymes are thought to release the β-LG–κ-CN complex by cleaving the peptide bonds anchoring the κ-CN to the micelle. The complexes then aggregate forming a three dimensional protein network. (McMahon, 1996)

Plasmin

Plasmin is a native protease present in milk and is a part of the plasmin-plasminogen system which consists of plasmin, plasminogen (inactive form of plasmin), plasminogen activators and inhibitors as well as plasmin inhibitors. The enzymatic activity of plasmin is undesired in UHT-milk since it probably causes age gelation through its proteolytic activity. The plasmin-plasminogen system does not only interact with its own components but also with the components of the milk, these interactions either promote or inhibit proteolysis. Factors influencing the proteolysis is pH, salts, whey proteins, thermal processing and storage temperature. Plasmin dissociates from the casein micelles into the whey fraction and the dissociation is thought to be affected by pH, hydrolysis of casein by plasmin, ionic strength and storage temperature. A lower pH is thought to increase the disassociation. (Richardson and Elston, 1984; Grufferty and Fox, 1988) The temperature optimum for plasminogen activation is 37 °C which explains why the hydrolysis is more prone to take place at room temperature than cold storage. (Fox, 1981) The enzyme activators and inhibitors affect the activity of plasmin, the activators are not affected by sterilization. (Deharveng, & Nielsen, 1991) Plasmin show different affinities to the different caseins, with strongest affinity to β-casein and α_{S2}-casein and somewhat less for α_{S1}-casein and κ-CN. The affinity for κ-CN has shown to decrease if carbohydrates are present in the milk, since the attachment prevents the hydrolysis by plasmin. (Datta & Deeth, 2001)

Bacterial proteinases

Psychrotrophic bacteria are frequently found in milk and they are able to grow at temperatures below 7°C. The bacteria are killed by pasteurization, however they produce heat stable enzymes (proteases and lipases) that can survive UHT-treatment and later on cause deterioration of the milk. (Walstra et al., 1999) The heat stable bacterial proteinases are produced during refrigerated storage of raw milk, yet they can also be produced in UHT-milk after sterilization if the milk has been re-contaminated by these bacteria. (Cousin, 1982) The gel formation takes place when the proteases destabilizes the system by hydrolysing the κ-CN (Walstra et al., 1999). Bacterial proteinases show most affinity to κ-CN, however they also attack β-casein and α_{S1}-casein. (Datta & Deeth, 2001)

Factors affecting proteolysis

Many factors can affect the shelf life of UHT-milk and lead to gelation, these factors include: age of cow, stage of lactation, mastitis, season, microbiological quality of milk storage temperature, fat content and hydrolysis of lactose. The gelation of UHT-milk is more prone to happen if the cow is older since the plasmin activity increases with the age of the cow (Datta and Deeth, 2003). Milk produced at early lactation is also more prone to gelation which has been related to the extent of proteolysis (Auldist et al. 1996). A cow suffering from mastitis will produce milk with elevated levels of plasmin which promotes gelation as the proteolytic activity increases in the milk (Swartling, 1968). Milk produced during spring and late autumn have been related to a higher degree of gelation in UHT-milk, a possible explanation for this is the higher amount of minerals in the milk during these periods (Hardham and Auldist, 1996). Microorganisms present in the milk is perhaps the most important factor inducing gelation of milk, the reason is the heat-stable enzymes produced by the microorganisms affecting the structural stability. The storage temperature of the milk is crucial as it affects the time of gelation, the temperature affects the activity of enzymes present in the milk and gelation will be promoted at room temperature. (Walstra et al., 1999) The fat content has showed to affect the gelation of UHT-milk, a higher fat content hinders the enzymes from accessing the caseins prolonging the onset of gelation. The hydrolysis of lactose have shown to increase the degree of proteolysis in the milk compared to non-hydrolysed milk, this was the case regardless if the hydrolysis was performed before or after the heat treatment. (Tossavainen and Kallioinen, 2007)

3.4.2 Non enzymatic age gelation

A non-enzymatic cause of age gelation have been suggested since the relationship between proteolytic activity and gelation have not been fully explained. The loss of colloidal stability have been explained by a lowered surface potential among casein micelles due to spontaneous transformations. The lowered surface potential for some micelles creates differences in energy potential promoting aggregation. The amount of low energy micelles and contact between them will affect the time of gelation. (Harwalkar, 1982) A second suggestion for the cause of non-enzymatic gelation is polymerization of caseins and whey proteins caused by Maillard reactions, a study made by Andrews (1975) showed that 26% of the milk proteins existed as covalently bound polymers at 20°C and 40% 30°C. (Andrews, 1975)

3.5 Milk-carrageenan mixtures

The production of milk based UHT-beverages often requires the addition of stabilizers in order to enhance the stability and sensory qualities of the product. A common stabilizer used within the food industry is carrageenan which is known to improve the textural properties of milk beverages. The carrageenan creates a gel network in the product that surrounds both aggregates of proteins and fat droplets keeping them suspended. (Syrbe et al., 1998) The network contributes with stability to the product and reduces the risk of sedimentation and visual phase separation of proteins and polysaccharides, typical for UHT-products. (Walstra et al., 1999)

3.5.1 Carrageenan

There are three types of carrageenan, κ-, ι-, and λ-carrageenan, and all of them are used within the food industry to improve textural properties of food products. They are sulphated negatively charged linear polysaccharides found in red seaweeds (Rhodophyta) and consist of alternating β-1,3-and α-1-4-linked galactose residues. (Towle, 1973) The three types of carrageenan differ in their number and position of sulphate groups; one for κ-, two for ι-, three for λ-carrageenan per disaccharide repeating unit, which affects their gel forming ability. κ-and ι-carrageenan are the two types that can form gels, of which κ-carrageenan is the most commonly used in milk products. κ-carrageenan forms strong and brittle gels while ι-carrageenan forms more soft gels with elastic properties. (Yuguchi et al., 2002) λ-carrageenan is unable to form the three-dimensional network which can be formed by the other carrageenans, this is partly due to the extra sulphate group found in λ-carrageenan. This prevents the transition from a coiled form of carrageenan to the helical form crucial for the formation of a network. (Langendorff et al., 1997)

3.5.2 Gel formation by carrageenan-mechanism

The mechanism by which carrageenan induces stabilization consists of two important events, gelation and complexation. Gelation is characterized by the formation of intermolecular bridges between carrageenan, which is preceded by a formation and alignment of double helixes into junction zones. (Towle, 1973) However only κ-carrageenan and ι-carrageenan can form these double helixes, λ-carrageenan is unable to do this as it contains a higher amount of sulphate groups (Spagnuolo et al., 2005). The presence of cations is also crucial for the gelation, κ-carrageenan contains K^+ and ι-carrageenan $Ca2^+$. (Syrbe et al., 1998) The gelation promoting cations are also present in the milk. (Drohan et al., 1997)

κ-carrageenan exists as a random coil at temperatures above 50°C and certain salt concentrations. The transformation from the coiled structure to a helical structure occurs when the temperature is lowered below this critical temperature (T_C). The formation of helices and hence the gelation is further affected by ions such as calcium and potassium (Bourriot et al., 1999), an increasing salt concentration lowers T_C. (Piculell, 2006)

The second event, complexation, is characterized by interactions between carrageenan and proteins. In milk the main interaction is taking place between the casein micelles and carrageenan, it is the κ-CN on the micelle surface that interacts with the κ-carrageenan. (Langendorff et al., 2000). More specifically it is an electrostatic interaction taking place between the sulphate groups of the κ-carrageenan

and a positively charged region on κ-CN. (Snoeren et al., 1975) Indirect interactions can also take place during complexation, where mainly whey proteins, also micelles, interacts with carrageenan via bridges created by cations. (Dickinson, 1998) Another theory regarding the complexation suggests that the stabilizing network is created by carrageenan-carrageenan interactions forming aggregates of neighbouring helices which facilitates the formation of a three-dimensional gel network. (Bourriot et al., 1999) The network is according to Bourriot et al. (1999) trapping the casein micelles and decreasing their mobility. Spagnuolo et al. (2005) however suggested that the network formation involves both the adsorption of κ-carrageenan to the surface of the micelles and the helix-helix aggregation. The gel formation and its strength is affected by the presence of Na^+, K^+, Ca^{2+} ions and the pH of the mixture. (Piculell, 1995) The ionic calcium neutralizes the polymers present in the mixture which decreases the distance between charged molecules promoting more hydrophobic interactions. (Caram-Lelham et al., 1997) Calcium ions have been suggested to facilitate interactions between a_{s1}-, a_{s2}- and β-casein and carrageenan by crosslinking. The cross-linking between κ-CN and carrageenan occurs in the absence of calcium ions. (Snoeren et al., 1975)

3.5.3 Interactions between carrageenan and milk proteins

The interactions between κ-carrageenan and casein micelles have been explained by two different theories. The first theory suggests that there is an electrostatic interaction between the negatively charged κ-carrageenan and the positively charged region between residues 97-112 on κ-CN. This positively charged region on the casein micelle is located inside the micelle and not in the hairy layer of the casein micelle. (Snoeren et al., 1976) This theory has been questioned since both the micelle and carrageenan is negatively charged and the micelle is already sterically stabilized by its outer hairy layer. The penetration is however not completely unlikely to happen since only one third of the casein micelle surface is covered with κ-CN, which makes the surface relatively bare. (Dalgleish, 1998) The second theory therefore suggests that the prevention of phase separation is due to the formation of a weak three-dimensional carrageenan gel (network) which keeps the casein micelles suspended and prevents them from diffusing freely. (Bourriot et al., 1999)

A study made my Snoeren (1976) showed that direct interactions occur between κ-CN and carrageenan, the interaction could not be seen with the other caseins which are only thought to interact in the presence of calcium. He assumed that this was due to the positive charge found between residue 97 and 122 in κ-CN, which can not be found in the other caseins (α_s- or β-casein). He further assumed that this interaction took place between casein micelles (containing κ-CN) and carrageenan even though the micelle was negatively charged. (Snoeren, 1976)

There are studies showing that interactions between carrageenan and casein micelles exist, these evidences are related to the size and charge density of the casein micelles. Studies using dynamic light scattering has showed that the particle size of the casein micelles increased when κ-carrageenan was added and the temperature of the solution was below the transition temperature, indicating that these polysaccharides were binding to the milk proteins. Temperatures above the transition temperature, i.e. when the carrageenan was still in coiled form, did not affect the micellar size which indicated the importance of the helical form for the interaction between the carrageenan and the casein micelles. (Langendorff et al., 2000) A study made by Dalgleish and Morris (1988) further showed that the

charge density of casein micelles, measured below transition temperature, became more negative when the concentration of κ-carrageenan increased. These results all indicate that the negatively charged κ-carrageenan was adsorbed to the casein micelles. Results from studies using scanning electron microscopy show direct evidence of carrageenan-casein networks. (Spagnuolo et al., 2005)

3.5.4 Factors affecting gel strength and stability

There are several factors that can affect the strength and stability of the carrageenan gel. Firstly the interactions between carrageenan and proteins are important in determining the stability of the gel. UHT-treatment of mixed milk systems result in the association of β-LG to the surface of the casein micelles and according to Odfield et al. (1998) the reaction is both pH- and temperature dependent. (Anema and Li, 2003a) A pH increase (pH 6.7) before heating leads to formation of whey protein aggregates, while a pH decrease (pH 6.5) leads to the formation of β-LG–κ-CN complexes (Anema and Li, 2003b). A pH below pH 7 gives a stronger and more stable gel (Galazka et al. 1999). The association of whey proteins to casein micelles prevents interactions between stabilizers such as carrageenan and milk proteins, which enhances gel strength and the stability of the mixed system. The increased stability is due to this displacement of carrageenan complexed to κ-CN with denatured β-LG. The displacement results in more carrageenan-carrageenan interactions which is crucial for the weak gel formation. (Tijssen et al., 2007) A higher amount of carrageenan-casein micelle interactions result in less carrageenan available for the formation of a gel network which weakens the gel. The whey proteins are important in reducing these interactions. (Tziboula and Horne, 1999)

Secondly the type and concentration of carrageenan is crucial for the stability of the product. κ-carrageenan forms weaker interactions with proteins than does ι-carrageenan, which resulted in a stronger gel. (Tijssen et al., 2007) A non-satisfactory concentration can lead to unwanted characteristics such as flocculation, coagulation and sedimentation. (Tziboula and Horne, 2000) A low concentration has showed to interfere with the gelation due to a more pronounced complexation. The critical lowest limit is a concentration of 0.018% carrageenan, below this concentration the interaction between protein (mainly the micelles) and carrageenan dominates interfering with the carrageenan-carrageenan interactions and hence the gelation. (Drohan et al., 1997). The lower dosages of carrageenan results in less flocculated casein micelles and a greater surface area available for interactions. (Arloft et al., 2006) A critical upper limit that has been suggested is a κ-carrageenan concentration of 0.03%, at this concentration the micelles are not firmly bound to the network which induces flocculation. (Dalgleish, 2007)

3.5.5 Phase separation and its prevention

Phase separation is a common event in dairy products containing polysaccharides. The reason for these quality changes is because many polysaccharides used in formulations within the dairy industry are thermodynamically incompatible with the proteins in the milk. (Schorsch et al., 2000). Phase separation usually originates from repulsive or attractive forces, where the attractive forces result in aggregation of oppositely charged molecules resulting in insoluble complexes (complex coacervation) while repulsive charges result in two separate phases due to the creation of segregates consisting of neutrally or equally charged molecules. (Vrij, 1976) Most studies conclude that the reason for segregative phase

separation is due to thermodynamic incompatibility or depletion flocculation. (Tolstoguzov, 2003) Thermodynamic incompatibility lead to the formation of a protein rich phase and a polysaccharide rich phase, while depletion flocculation give one particle rich phase and one phase with almost no particles. (Bourriot et al., 1999) Factors affecting the complexation of proteins and polysaccharides are the protein/polysaccharide ratio, pH, charge density, conformation, ionic strength and net charge of the polymers. High ionic strength often results in incompatibility. The net repulsion between a negatively charged protein and polysaccharide increases if the pH is higher, a lower pH decreases the repulsion since the charge of the protein is closer to its isoelectric point. (Benichou et al., 2002)

3.6 Microstructural analyses of milk-carrageenan mixtures

In order to study the structures of milk-carrageenan mixtures several methods are available. Microstructural methods such as BFM, CLSM and TEM give important information about the microstructures and how these structures changes over time. Particle analysis give valuable information of the particle size and concentration differences over time which facilitates a better understanding for what type of structural changes are taking place in the mixture.

3.6.1 Microscopy

Bright Field Microscopy (BFM)

In BFM light is collected by an objective from the specimen and the surrounding and an image is created. The light is either reflected off or passed through a specimen and the method is the simplest of all microscopic methods. The samples studied by BFM are often stained and placed on microscopic slides, the background of the image appears lighter than the specimen. (Nikon Instruments, 2014)

Confocal laser scanning microscopy (CLSM)

CLSM is a method giving valuable information of the microstructure in a sample. CLSM detects differences in fluorescence by collecting emitted light from different components. The method allows staining of several components simultaneously, assuming that the light is emitted at different wavelengths. The method allows visualization of the position of the different ingredients in relation to one another, hence giving information of the microstructure and the interactions taking place. (Dürrenberger et al., 2001)

Transmission electron microscopy (TEM)

TEM uses electrons instead of light when operating which is an advantage as it compared to BFM can get images with closer magnification. The electrons have a shorter wavelength which make it possible to see objects with sizes of 10^{-10} m. TEM emits electrons which travel through vacuum and is focused by electromagnetic lenses. The electrons are focused into a beam which travel through the sample. The electrons not scattered will hit a florescent screen creating the image characteristic of the microscope. The density of the specimens in the sample will affect the darkness in the image. (Nobelprize.org, 2014)

3.6.2 Particle analysis

Particle size and particle concentration measurements can be performed using a NanoSight instrument which characterises nanoparticles from 10nm-2000nm in a solution. The instrument utilizes Nanoparticle Tracking Analysis (NTA) when characterizing the nanoparticles and performs individual analyses of the particles. The analysis gives information about the particle size distribution and the number of particles. (Malvern Instruments Ltd, 2014)

4 Materials and methods

4.1 Experimental design

A full factorial design was applied in this study were two variables each set at three levels were studied. Totally five different products were examined of which one of the products was a center point. The interactions between the variables and their effect on unwanted gelation during twelve weeks of storage at room temperature were analysed. In addition, samples were stored at 30°C in order to allow visual comparison with samples stored at room temperature.

4.2 The milk based UHT product

The product studied in this project was a protein and carbohydrate enriched milk based UHT-product. The main components in the product were protein and carbohydrates, the stabilizer used in the product was carrageenan. The product was received with varying levels of pH and carrageenan. The pH and carrageenan was set as high and low, and a center point (reference) with a pH and carrageenan concentration between the high and low values was also received. The products are presented in Table 1.

Table 1. The five products that were analysed and their varying levels of pH and concentration of carrageenan.

Sample	pH	Carrageenan
T1	Low	Low
T2	High	Low
T3	Low	High
T4	High	High
Ref	Middle	Middle

The products were received three days after manufacturing and were stored at 21±1°C in the lab at the department of Food Science, Swedish University of Agricultural Sciences. The temperature in the lab increased slightly week 9 to 23±1°C. In addition, two samples of each product were stored at 30°C. The products contained the following ingredients: UHT-treated milk, milk protein, sugar (2.5%), lactase enzyme, aroma and carrageenan. The nutritive value of the product is shown in Appendix 1.

4.3 pH-measurements

The pH of the products were measured at 21±1°C using a pH meter (Radiometer, PHM 210) with a combination electrode (Orion, 420 A), calibrated at the measurements temperature right before use. The pH was measured every other week, starting week 0, until the end of the study week 12. The measurements were performed in triplicates at every occasion.

4.3.1 Data analysis

The data from the pH measurements were analysed using Minitab 17.0 statistical software (Minitab Inc., Chicago, USA, 2007) where an ANOVA was performed to calculate statistical differences among samples from different weeks. A Students't-test was carried out in order to study significant differ-

ences among individual samples between weeks. Differences were considered statistically significant at $p<0.05$.

4.4 Microstructural methods

To examine the microstructure of the different UHT products at different times of storage three microscopic methods were used: BFM, CLSM and TEM. BFM was used to obtain bright field and epifluorescent images in order to observe structural changes taking place each week in the product. CLSM was used to detect and confirm structural changes involving proteins, however the method did not work and epifluorescent was used instead. TEM was used to closer look at the structures characteristic for fresh and gelled samples.

4.4.1 Sample preparation

The samples were observed in two different ways: as fresh samples and embedded samples. The fresh samples were examined by BFM, while the embedded samples were examined by BFM, CLSM and TEM.

Staining

Different staining techniques were performed in order to find suitable stains for proteins and polysaccharides. The following stains were tested: Calcofluor, Safranin, Methylene Blue, Neutral Red, Neil Red, Iodine, Oxytetracykline, Rhodamine B, Light Green and Acid Fuchsin. For this study Light Green and Acid Fuchsin were chosen as these were the only stains that worked sufficiently enough.

Fresh sample preparation

Milk proteins were stained using Light Green 1% w/w (Sigma-Aldrich Co, st Louis, MO, USA), by adding 10 µl of the dye to 80 µl of the milk product (collected 1 cm down from the top of the package) in an Eppendorf tube. A vortex (Vortex Genie 2, Scientific industries, USA) was used to properly stir the solution and the mixture was incubated at room temperature (22±1°C), for 30 minutes in order to allow staining of the proteins. The staining was performed with all products: T_1, T_2, T_3, T_4 and the reference (center point). After the staining was completed, just before the observation in BFM, 6 µl of each sample was placed on glass slides (Superfrost Plus®, Fisher Scientific) and covered with a 18 mm square coverslips. The cover slips were sealed using transparent nail polish.

Embedding process and sectioning

1 ml of each sample was mixed with 50 µl Glutaraldehyde 25% in Eppendorf tubes in order to allow fixation of the proteins. After one hour, the Eppendorf tubes were placed in a water bath (40 °C) and 1 mL of agar (40 g/L, Agar Noble Difco laboratories, Detroit Michigan, USA) was added. The agar solution had been prepared by heating to 85 °C under continuous stirring and then cooled down to 50 °C before addition to the samples. The mixtures were then shaken to allow homogenization and transferred to a plastic plate with moulds. The plate was placed in the refrigerator for four hours in order to allow hardening of the samples. From each sample, 1 mm^3 pieces were obtained by triplicate, placed in plastic baskets and introduced in a Leica FM tissue processor for plastic embedding. The tissue processor was programmed according to the scheme in Appendix 2. The samples were fixed in glutar-

aldehyde and post-fixed in osmium tetraoxide, followed by dehydration in graded ethanol series (30, 50, 70, 95 and 99.5%). The samples were embedded in fresh resin, prepared according to the scheme in Appendix 3, in small plastic moulds and the blocks were left to harden for 24 hours at 60 °C in an oven. The samples were then sectioned under a Leica ultramicrotome. The sections (1.5 μm thick) were then placed on glass slides (Superfrost Plus®, Fisher Scientific), stained for proteins for 90 minutes with Light Green 1% w/w for bright field micrographs and with Acid Fuchsin 0.1 % w/w for epifluorescent micrographs and finally covered with a 24x50 mm coverslip.

4.4.2 Bright field microscopy (BFM)

BF) was applied to both the fresh and embedded samples. The samples were stained to increase contrast and enable identification of structures in the products. The fresh samples were only stained with Light Green while the embedded samples were stained with Light Green and Acid Fuchsin. A Nikon Eclipse Ni-U light microscope coupled to a HGFI mercury lamp and a DS-Fi2 camera (Nikon, Tokyo, Japan) was used for the observation of the samples. Bright field and epifluorescent micrographs were obtained using a CFI Plan Apo Lambda 60X objective. A red (Epi-FL Filterset Texas Red, excitation 540-580 nm, emission 600-660 nm) light fluorescence filter was used to observe the acid fuchsin fluorescence. Image acquisition and analysis was managed using NIS-Elements BR and images were collected for each sample.

4.4.3 Confocal laser scanning microscopy (CSLM)

CSLM was applied to the embedded samples and was performed utilizing a Carl Zeiss 780 Inverted Axio Observer with supersensitive GaASp detector (Jena, Germany) with a _60 objective. The sample was located using a LED illuminator for transmitted light mercury lamp and 3 filters (DAPI/GFP/RFP) for fluorescence signal. The excitation of Acid Fuchsin performed at 540 nm and the emission was recorded at 630 nm. Image acquisition and analysis was managed using Zeiss software ZEN2100, micrographs were collected for each sample and a corresponding bright field micrograph was collected for each fluorescent image.

4.4.4 Transmission electron microscopy (TEM)

TEM was performed on resin embedded samples from weeks 0, 8 and 12. Specimens were examined using a transmission electron microscope (Philips CM12 TEM from Philips, Amsterdam, Netherlands) at 80 kV. Micrographs were recorded a magnifications between 13000-45000X on Kodak Estar 4489 films. Negative TEM films were scanned using an Epson Perfection Pro 750 film scanner. Representative micrographs, to illustrate the structural differences between the samples were selected. Due to time limits micrographs were only collected for samples T3 and T4 from week 0 and week 8.

4.5 Particle analysis

4.5.1 NanoSight

Particle size analysis was performed using a Nanoparticle Size Analyzer (NanoSight NS300, Malvern Instruments, United Kingdom)) and analysed with NanoSight NTA 2.2 software at a fixed 90° angle and a wavelength of 658 nm. NanoSight provides sizing and count of protein aggregates in the region

of 10 – 2000 nm. The milk samples were collected from the received packages and diluted in 150mM NaCl, 20 mM NaPi, pH 7.2, 500 and 2000 times. The sample was placed into measurement chamber of the instrument. The particle analysis was performed at a constant temperature of 20°C, following an equilibration step. Measurements for each sample were performed three times and measurements were made week 0 and week 8. Due to time limits measurements were not made week 12.

4.5.2 Data analysis

The data from the particle analysis were analysed using Minitab 17.0 statistical software (Minitab Inc., Chicago, USA, 2007). Two factorial designs were performed and ANOVA was used to calculate statistical differences among samples from different weeks. The factorial design gave information about the main effects and interaction effects of the different factors on the responses i.e. what effect the pH, carrageenan and storage time had on the particle size and particle concentration. The second design also gave information of the curvature, telling if there were any significant differences in response between the center point and the other factors at their low and high settings. Differences were considered statistically significant at $p<0.05$.

4.6 Visual sensory analysis

A visual sensory analysis was performed four times in order to observe visual changes in the different products. The analysis was performed according to instructions given in an internal document obtained from Norrmejerier Ek. För. Grading of the following attributes was performed according to an 9-point scale system: colour, homogeneity when poured into the beaker and aggregates of particles.

5 Results

5.1 pH-measurements

Table 2 presents the results from a one-way ANOVA in which the pH of each individual sample during week 0 and week 12 were analysed and compared for significant differences. The results showed that the pH was significantly different in samples T1, T4 and the reference ($p<0.05$). A two-way ANOVA was also performed in which differences in pH between week 0 and 12 were analysed, the results showed that there was a significant difference in pH among samples between these weeks (see appendix 4).

Table 2. The p-values from a one-way ANOVA showing in red if there was a significant difference in pH within individual samples from week 0 and 12.

p-values	
Sample	**pH**
T1	0.001
T2	0.158
T3	0.116
T4	0.026
Ref	0.000

5.1.1 pH measurements

Figure 1 presents the results from the pH-measurements between week 0 and week 12. The results showed that T1 and T3 had a trend of an increasing pH whereas T2, T4 and the reference had a trend of a decreasing pH. In relation to the results from the one-way ANOVA the assumption can be made that the pH increases in T1 and decreases in T4 and the reference. The pH in T2 and T3 are assumingly unchanged.

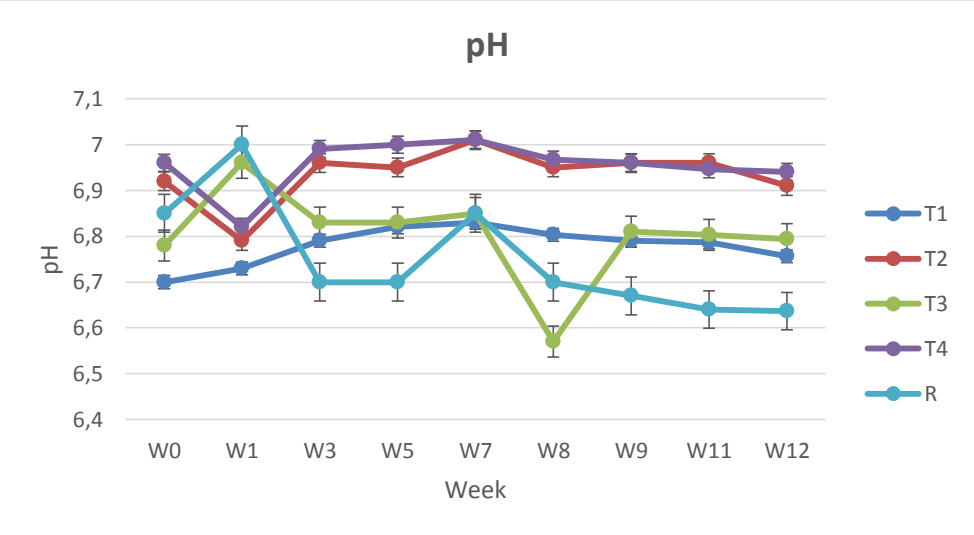

Figure 1. The measured pH in the different products during twelve weeks of storage.

5.2 Microstructural analysis

5.2.1 Bright field microscopy (BFM)

The samples studied during the twelve weeks of storage were examined for their degree and occurrence of flocculated micelles. Figure 2 presents an example of a sample containing visible strands of flocculated micelles (Figure 2a) and one sample without visible strands of flocculated micelles (Figure 2b). Figure 3 shows epifluorescent micrographs with characteristic structures found in samples T2, T3 and T4 during storage. The results from BFM (samples stored at room temperature) between week 0 and week 12 are presented in Figures 4-6, close-ups of these results are shown in Appendix 5-6.

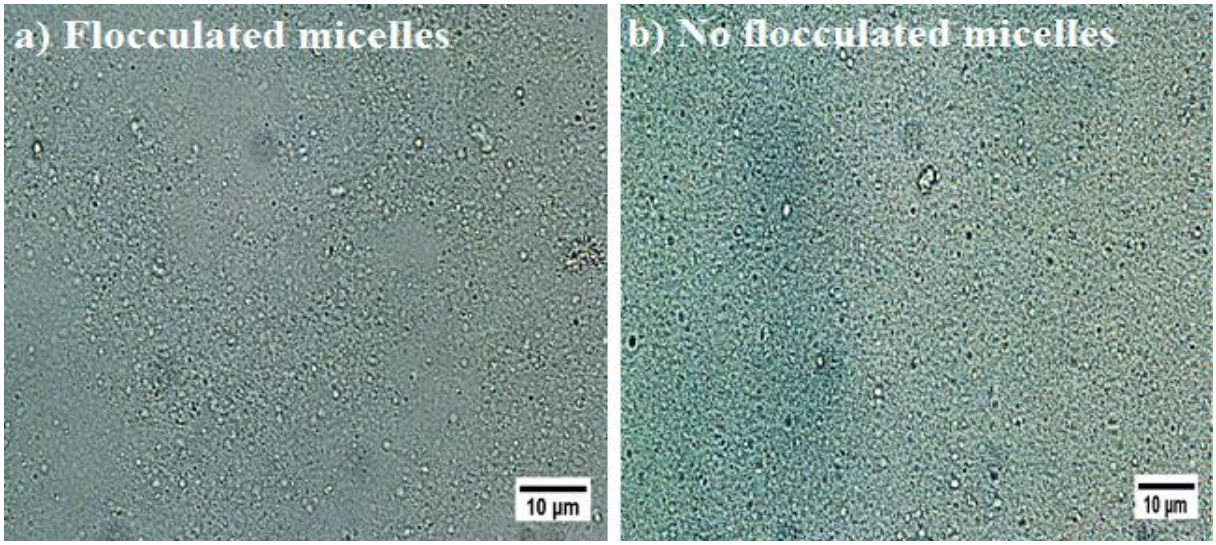

Figure 2. Micrograph a) shows an example of a sample (reference week 12) having visible strands of flocculated micelles and micrograph b) shows an example of a sample (reference week 0) having no visible strands of flocculated micelles.

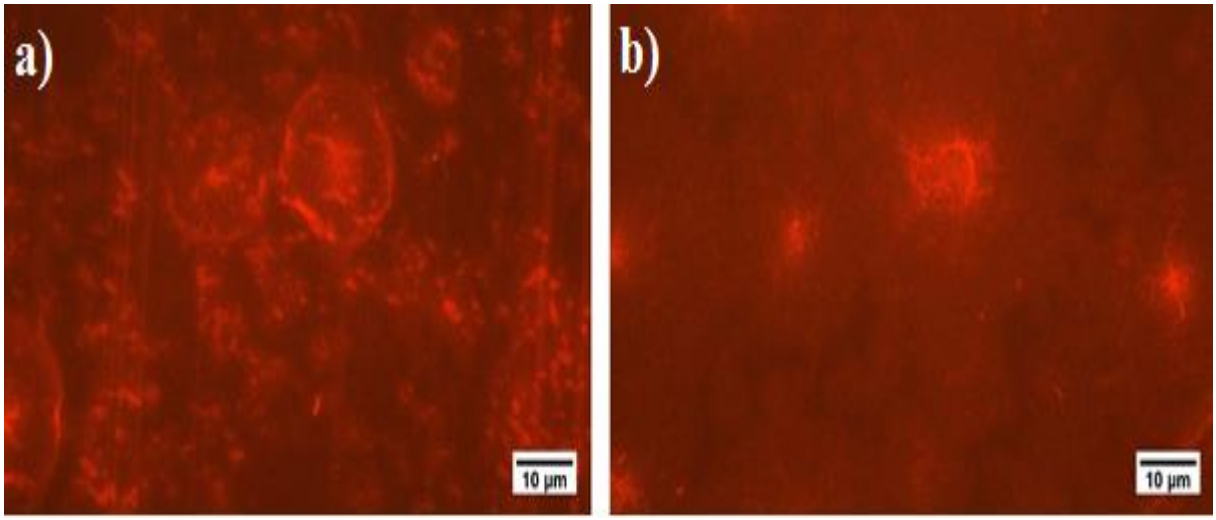

Figure 3. Epifluorescent micrographs showing characteristic structures found in samples T2, T3 and T4 during storage time. Figure a) shows T2 during week 0 and figure b) shows T3 week 0.

5.2.1.1Week 0

The micrographs from BFM week 0 are presented in Figure 4. The small spherical structure stained green in the samples are most likely the casein micelles. All samples also contain bigger spherical structures which are also stained green varying in size, these structures could be undissolved caseinate (see Figure 7). Samples T1, T2, T3 and the reference show similar appearances, they are more or less homogenous and contain the bigger spherical structures. These samples also contain parts were micelles are grouping together which could be a pre-stage of flocculation typical for a gelled sample. The sample that differs from the others is T4, it is less homogenous and have visible strands of flocculated micelles. The epifluorescent micrographs show different structures and there are also structural differences within individual samples, therefore it is hard to say what structures are characteristic for the different samples. The most similar structures are found in T2, T3 and T4.

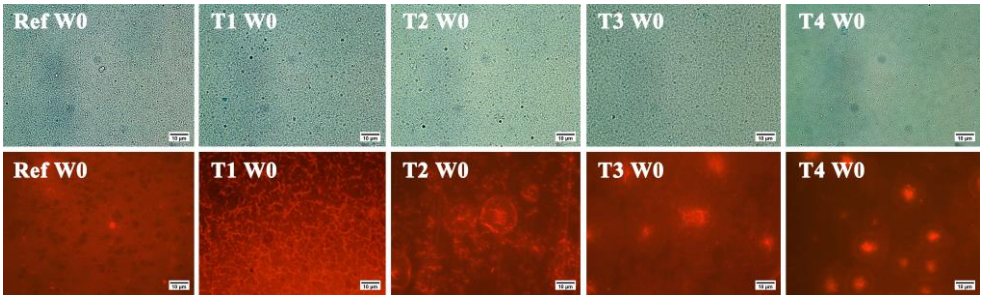

Figure 4. Micrographs from week 0 where the first row shows bright field micrographs of the fresh samples and the bottom row shows epifluorescent micrographs of the embedded samples.

5.2.1.2Week 8

The micrographs from week 8 are presented in Figure 5. The appearances of T1 and T3 have not obviously changed compared to week 0 and the samples are still relatively homogenous. Samples T2, T4 and reference however show distinct structural changes compared to week 0, of which T4 and the reference are least homogenous and contain more visible parts of flocculated micelles. These samples also contain crystalline structures which appeared week 4 in T2 and T4 and week 6 in the reference. The epifluorescent micrographs are still showing different structures, the fluorescent structures are most likely protein structure, yet it is hard to draw any conclusions from these micrographs.

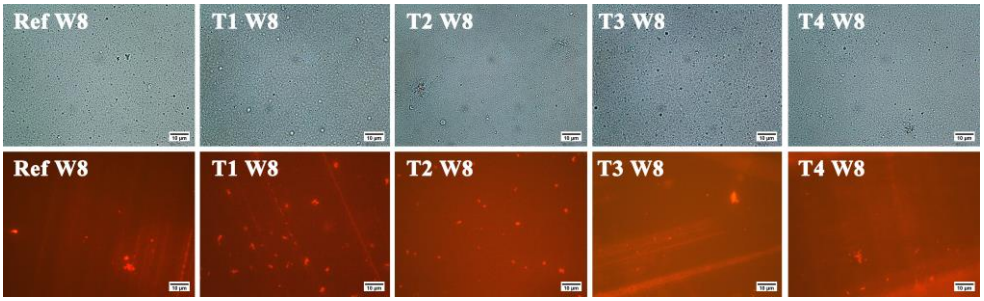

Figure 5. Micrographs from week 8 where the first row shows bright field micrographs of the fresh samples and the bottom row shows epifluorescent micrographs of the embedded samples.

5.2.1.3Week 12

The micrographs from week 12 are presented in Figure 6. The appearances of T1 and T3 are similar and they show visible strands of flocculated micelles, yet not to the same extent as in samples T2, T4

and reference week 8. Samples T2 and the reference have a similar appearance and are heavily flocculated. T4 differs from the rest of the samples by having a hazy appearance and floccules can hardly be distinguished. The crystalline structures still only appear in T2, T4 and the reference. The structures in the epifluorescent micrographs are still varying, however the spherical fluorescent structures in T2, T3 and T4 could be characteristic for these samples as the same structures were seen week 0.

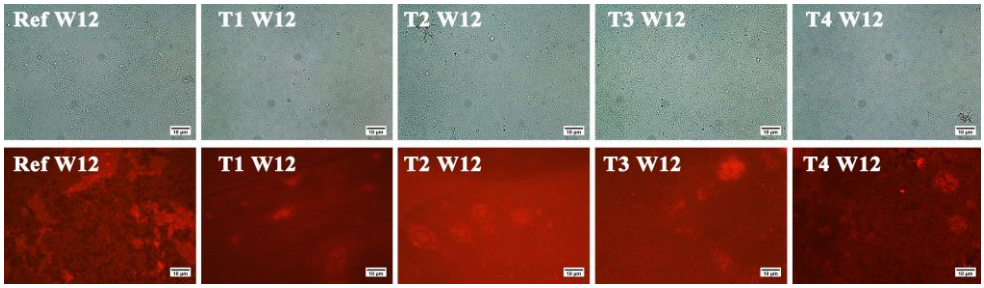

Figure 6. Micrographs from week 12 where the first row shows bright field micrographs of the fresh samples and the bottom row shows epifluorescent micrographs of the embedded samples.

5.2.1.4Samples stored at 30°C

Micrographs of samples stored at 30°C are presented in Figure 7 and close-ups of these results are presented in Appendix 7. Interesting results were gained from these samples, T1 was the sample which differed from the other samples by not having a hazy appearance characteristic for gelled products. Figure 8 presents an example of a sample with a hazy appearance and a sample without a hazy appearance. Another interesting observation was that none of the samples contained the crystalline structures which typically appear in the samples stored at room temperature, in all samples except for T1 this might have been caused by the hazy appearance masking these structures. The most similar appearances were found in T2 and T4, followed by T3 which had a few visible parts with flocculated casein micelles.

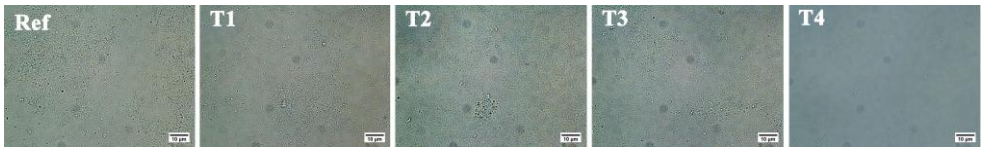

Figure 7. Micrographs of samples stored at 30°C for twelve weeks.

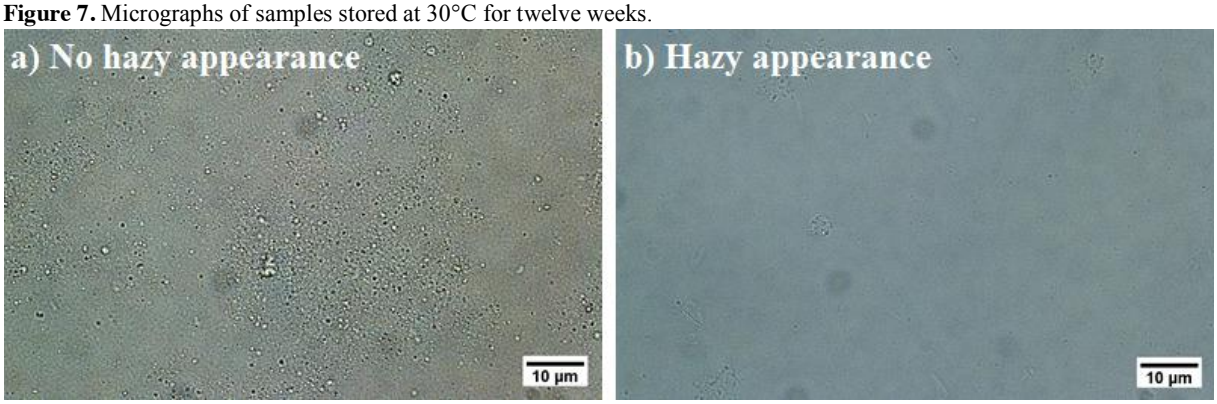

Figure 8. Micrograph a) shows an example of a sample (T1) without a hazy appearance and micrograph b) shows a sample (T4) with a hazy appearance.

5.2.1.5 Crystalline and spherical structures

Micrographs of the crystalline structures which appear in some of the samples after a certain storage time and the characteristic spherical structures found in all samples are presented in Figure 9. The approximate size of these structure were 10 µm in diameter, yet the size of both structure varied between 5-20 µm in diameter and grew bigger during the storage time.

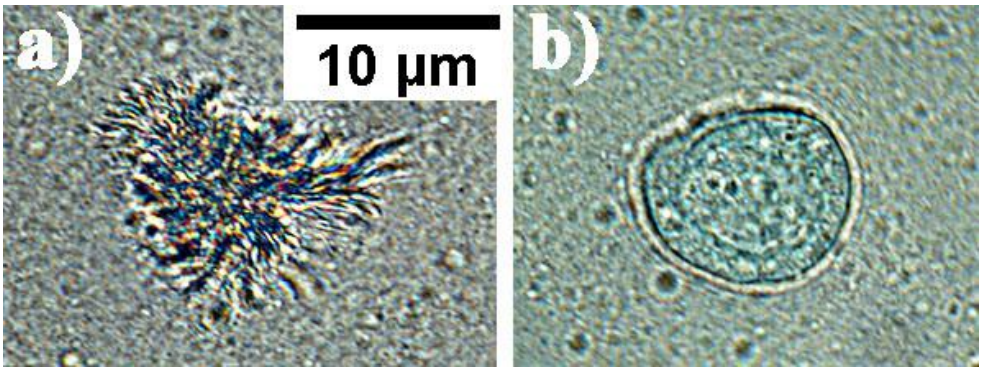

Figure 9. Micrograph (a) shows the crystalline structures which appear in T2, T4 and the reference after some time of storage. Micrograph (b) shows the round spherical structures present in all samples already week 0.

5.2.2 Confocal laser scanning microscopy (CSLM)

Figure 10 presents micrographs from CLSM showing the reference stained with Safranin and Acid Fuchsin. The micrographs can not be used in analysis of the sample structures as the staining did not work. Safranin should stain polysaccharides and Acid Fuchsin should stain proteins, yet both tunnels show that the same structures are stained which indicate that the staining did not work and that the structures are self-fluorescent.

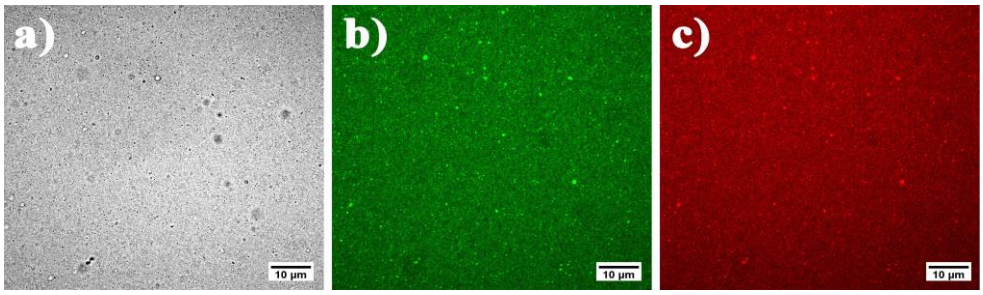

Figure 10. Micrographs from CLSM showing fresh reference stained with Safranin and Acid Fuchsin.

5.2.3 Transmission electron microscopy (TEM)

The micrographs from TEM are presented in Figure 11 and Figure 12. Figure 11 presents micrographs with a magnification of 13000-17000X. The black spheres in the micrographs are most likely the casein micelles and the fibrils found between the micelles could either be carrageenan or of β-LG–κ-CN complexes looking like hair protrusions as observed by McMahon (1996), possibly a mixture of both (Hood and Allen (1977). A study made by Hood and Allen (1977) concluded that the fibrils were carrageenan as similar networks have been observed in other milk-carrageenan gels. The same study also concluded that the interaction between the casein micelles and carrageenan took place on the surface of the casein micelles. The micrographs reveal that T3 consists of smaller areas with densely packed

casein micelles week 0 compared to T4 which has greater areas of densely packed casein micelles. Looking at the samples week 8 shows that these areas split up and the micelles separate from one another becoming more spread. The casein micelles seem to spread more in sample T4 compared to T3.

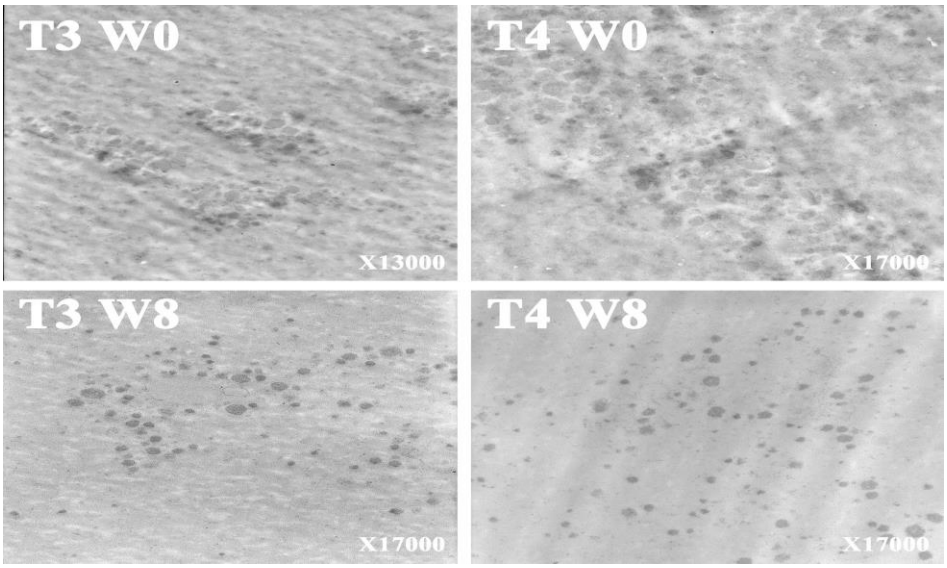

Figure 11. TEM micrographs of T3 and T4 from week 0 and week 8 with a magnification of 13000-17000X.

Figure 12 shows TEM micrographs with a magnification of 35000-45000X. The micrographs confirm the observations seen in Figure 11, namely that the storage time result in more spread casein micelles, T4 being characterized by greater distance between the spread micelles. The micrographs further show that different kinds of networks seem to exist in the samples week 0 and week 8. The strands creating the network seem to have detached during week 8 compared to week 0 where they seem to keep the micelles in place.

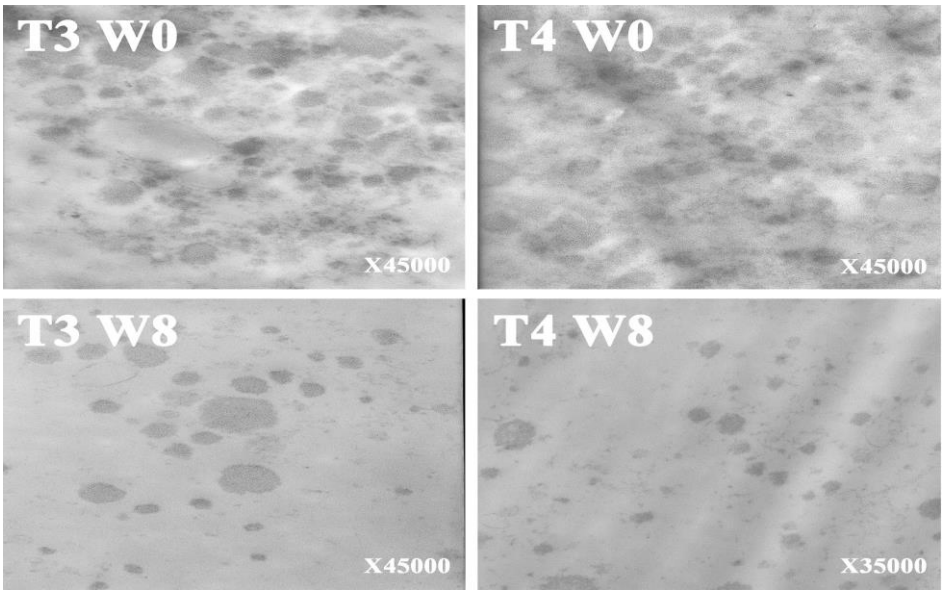

Figure 12. TEM micrographs of T3 and T4 from week 0 and week 8 with a magnification of 35000-45000X.

5.3 Particle analysis

Two factorial designs were analysed in order to understand the effect of the different factors on particle size and particle concentration. In the first design the effects of pH-level, carrageenan concentration and weeks of storage on particle size and particle concentrations were analysed, the limit of this design was that the center point (reference) could not be included. The second design analysed the effects pH-level and carrageenan concentration on the previously mentioned responses, this design included the center point (reference), however this design could only analyse the effects and responses on individual weeks, not between weeks. The second design also gave information about the curvature, when the average mean response at the center point is significantly greater or less than the average mean response of the factors at their low and high settings, then curvature is detected.

5.3.1 Particle size

Figure 13 presents the results from the particle size measurements performed between week 0 and week 8. Results showed that the particle size had an increasing trend in samples T3, T4 and reference and decreasing trend in samples T1 and T2.

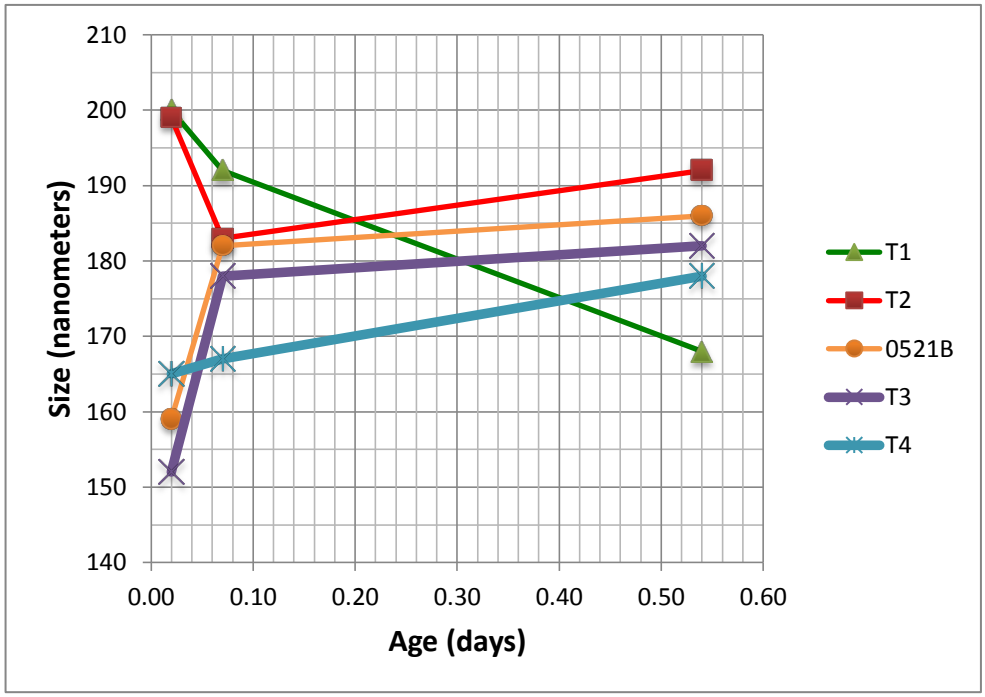

Figure 13. The results from the particle size measurements showed that the particle size had a trend of decreasing in samples T1 and T2, and increasing in samples T3, T4 and the reference (0521B).

A student's t-test was carried out to study individual samples differences between week 0 and week 8 and the results are presented in Table 3. The results from the t-test showed that there was a significant difference in particle size among all individual samples except for T2. Samples T3, T4 and the references can therefore be assumed to increase in size, whereas T1 decreases in size and T2 is unchanged. A second t-test was also carried out to compare samples against each other and the results are presented in Appendix 8. The results from the second t-test showed that T1 was significantly different in particle size compared to the rest of the samples after eight weeks of storage.

Table 3. The p-values from the t-test performed on each individual sample comparing particle size week 0 and week 8, showing in red if there was a significant difference.

Sample	p-value
T1	6.66E-03
T2	0.100684
T3	3.90E-04
T4	3.77E-02
R	0.002103

1st Factorial design

Figure 14 presents the results from the first factorial design, analysing the effect of pH-level, carrageenan concentration and weeks of storage, on particle size in the products (T1, T2, T3 and T4). The results showed that an interaction existed between the amount of carrageenan and weeks of storage on particle size. The size difference among particles was bigger in the beginning of storage, where a low carrageenan concentration resulted in a greater particles size and a high concentration resulted in a smaller particle size. The particle size became more similar after eight weeks of storage. The results also showed that there was an interaction between the pH-level, carrageenan concentration and weeks of storage on particle size. The ANOVA, Normal Probability Plot and Histogram can be seen in Appendix 9.

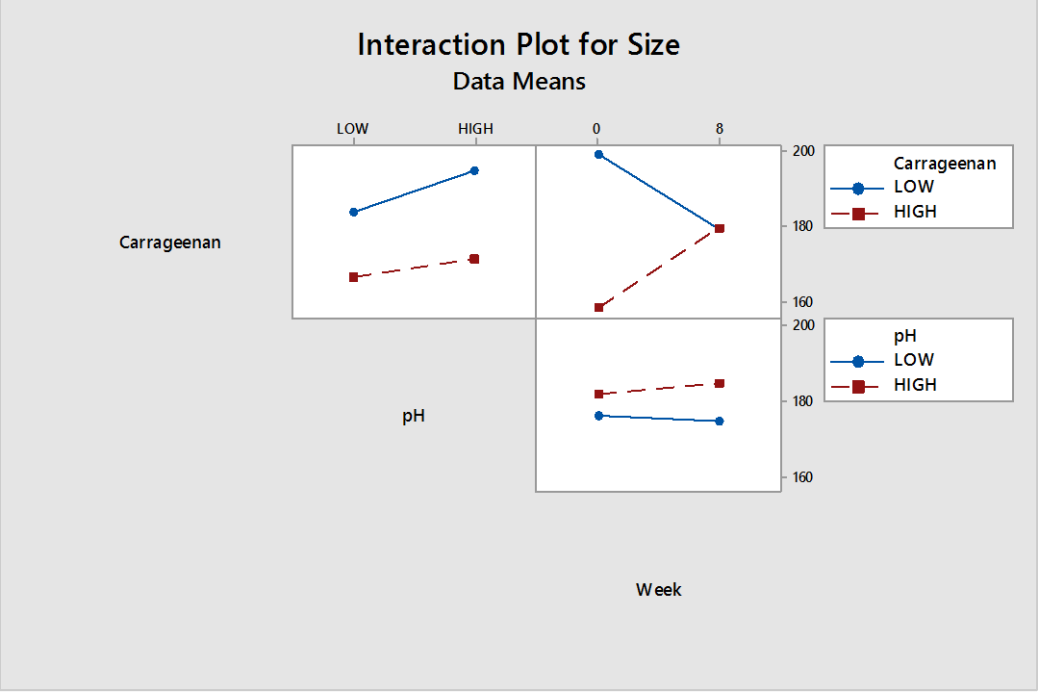

Figure 14. The plot shows the effect of the weeks of storage, pH-level and carrageenan concentration on particle size.

2nd Factorial design

Figure 15 presents the results from the second factorial design week 0 which showed a main effect of carrageenan concentration on particle size. No interactions between pH-level and carrageenan existed however curvature was observed, showing that the center point was significantly greater in size compared to samples with low concentration of carrageenan. The ANOVA, Normal Probability Plot and Histogram are presented in Appendix 10.

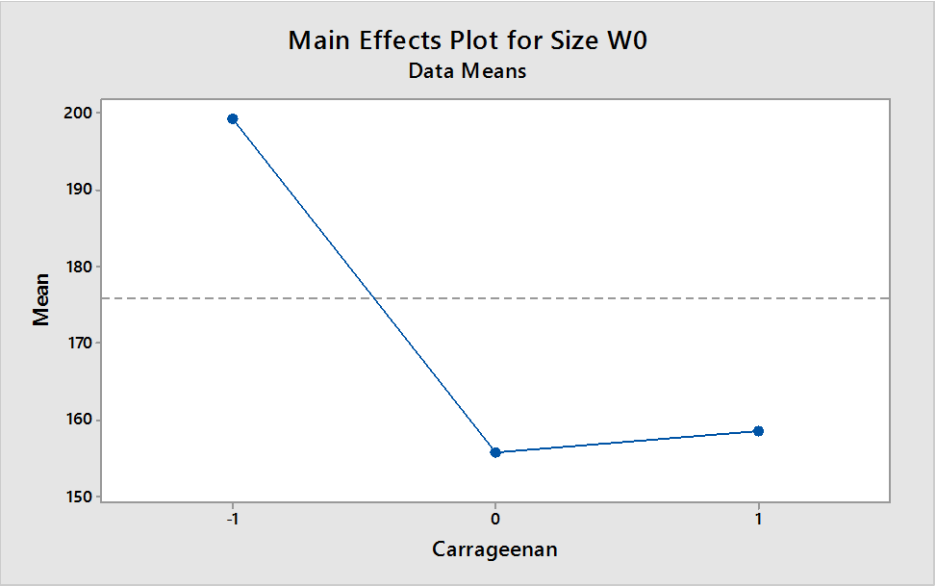

Figure 15. The plot shows that the carrageenan concentration has a main effect on the particle size week 0.

Figure 16 presents the results from the second factorial design week 8 which showed that there was an interaction between the pH-level and carrageenan concentration on particle size. No curvature was observed, showing that the center point was not significantly different in particle size compared to the other samples. The ANOVA, Normal Probability Plot and Histogram are presented in Appendix 11.

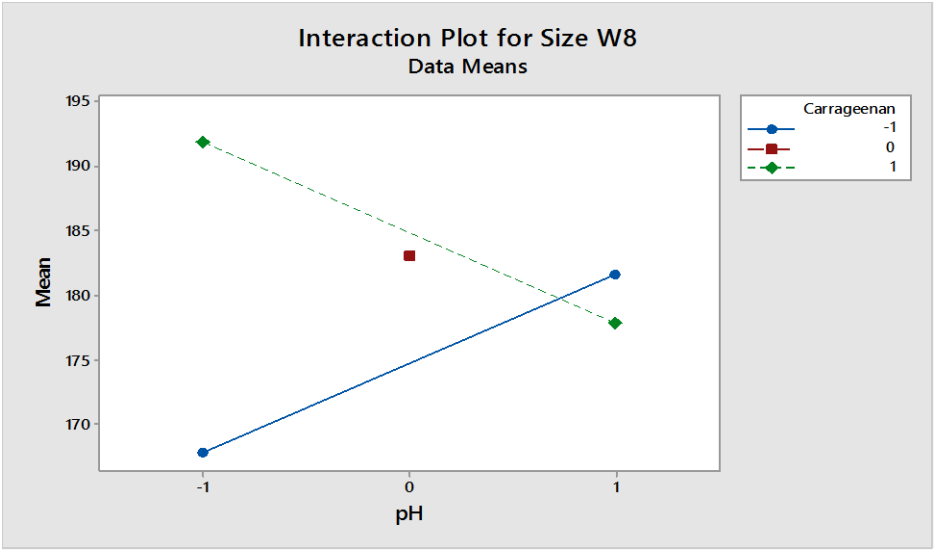

Figure 16. The plot shows the interaction between the pH-level and carrageenan concentration on particle size week 8.

5.3.2 Particle concentration

Figure 17 presents the results from the measurements of particle concentration between week 0 and week 8. Results show that the particle concentration has a decreasing trend in all samples.

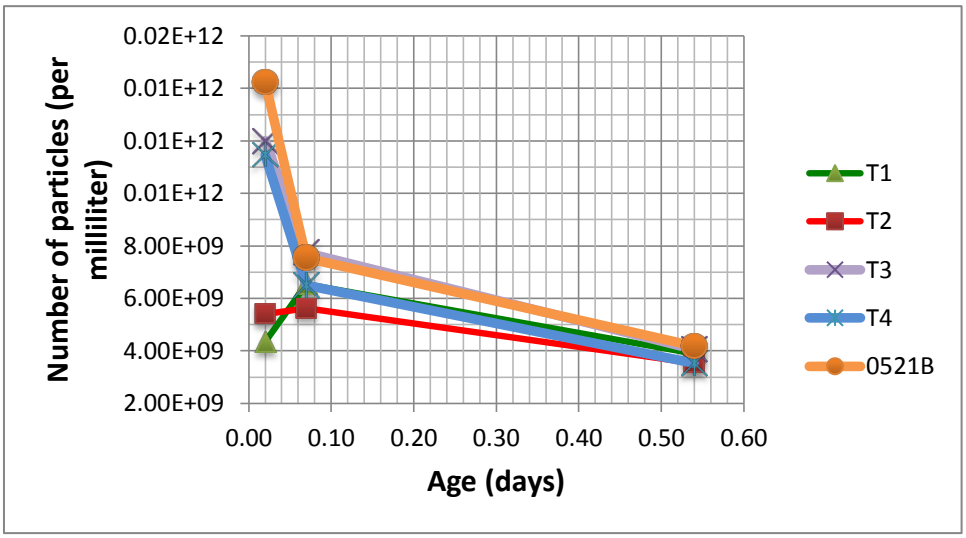

Figure 17. Number of particles (per mL) of the milk products during storage. Results showed that the particle concentration had a decreasing trend in T1, T2, T3, T4 and the reference (0521B).

A Student's t-test was carried out to study individual sample differences between week 0 and week 8 and the results are presented in Table 4. The results from the t-test performed on the individual samples showed that there was a significant difference among all individual samples. The decrease in particle concentration was therefore significant. A t-test was also carried out to compare samples against each other and the results can be seen in Appendix 12. The results from this ANOVA showed that all samples were significantly different in number of particles after eight weeks of storage except for T2 and T4, respectively, as well as T3 and the reference.

Table 4. The p-values from the t-test performed on each individual sample comparing particle concentration week 0 and week 8, showing in red if there was a significant difference

Sample	p-value
T1	6.30E-07
T2	0.00192
T3	9.64E-05
T4	9.64E-05
R	0.00012

1st Factorial deign

Figure 18 presents the results from the first factorial design, analysing the effect of pH-level, carrageenan concentration and weeks of storage, on the particle concentration in the products. The results showed that the particle concentration was affected by interactions between the amount of carrageenan and weeks of storage; pH-level and weeks of storage; pH-level and carrageenan concentration; and an interaction between all factors combined. In the beginning of storage a high amount of carrageenan resulted in a greater particle concentration whereas a lower concentration resulted in a smaller particle concentration. After eight weeks of storage the trend changed and an opposite effect could be seen for

the level of carrageenan. In the beginning of sample storage the particle concentration was approximately the same regardless of pH-level. After eight weeks of storage the concentration was affected by the pH and differences between concentrations could be observed. A high pH had a bigger effect on the particle concentration when combined with carrageenan, the particle concentration decreased with a smaller carrageenan concentration and increased with a higher concentration. Appendix 13 shows the ANOVA, Normal Probability Plot and Histogram from the first factorial design.

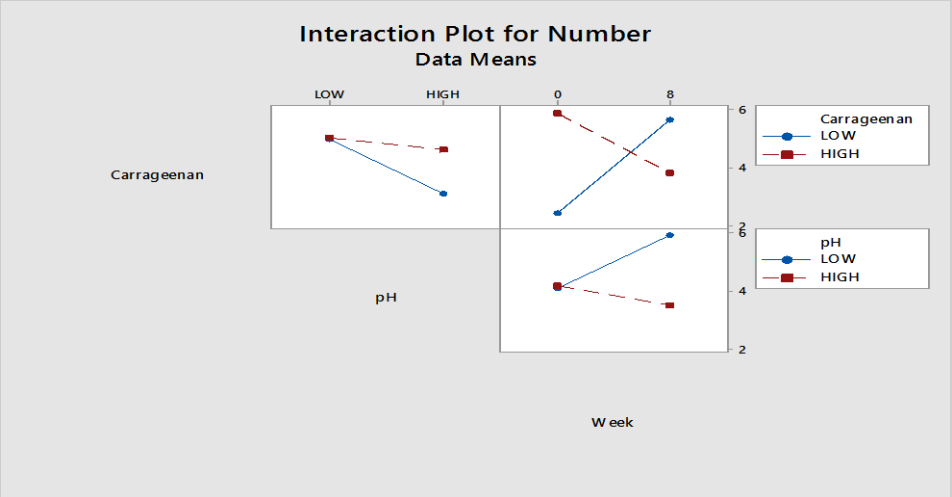

Figure 18. The interaction plot showing the effect of amount of carrageenan concentration, pH-level and weeks of storage on particle concentration.

2nd Factorial design

Figure 19 presents the results from the second factorial design week 0, showing that there was an interaction between the pH-level and carrageenan concentration on the particle concentration. Curvature was observed, showing that the center point was significantly different in particle concentration compared to samples with low concentration of carrageenan. The ANOVA, Normal Probability Plot and Histogram from the second factorial design are presented in Appendix 14.

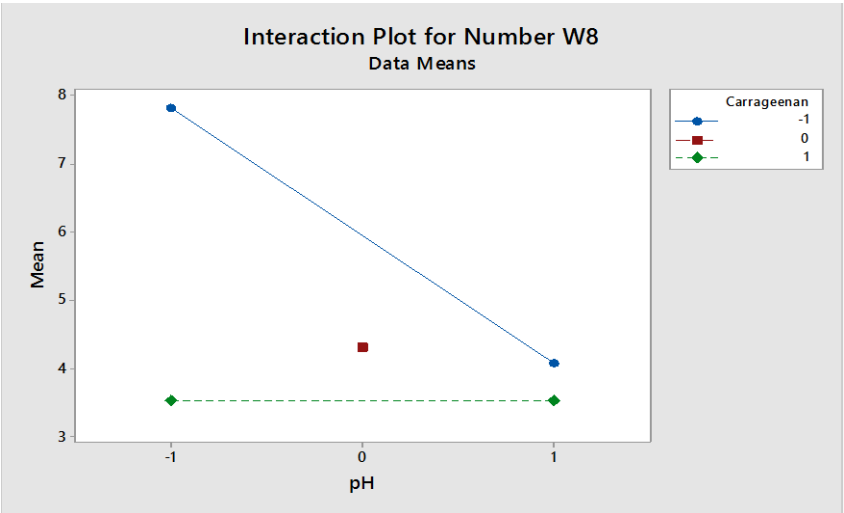

Figure 19. The plot shows the interaction between the pH-level and carrageenan concentration on particle concentration week 0.

Figure 20 presents the results from the second factorial design week 8 which showed that there was an interaction between the pH-level and carrageenan concentration on the particle concentration. Curvature was observed, showing that the center point was significantly different in particle concentration compared to the sample with low pH and low concentration of carrageenan. The ANOVA, Normal Probability Plot and Histogram from the second factorial design are presented in Appendix 15.

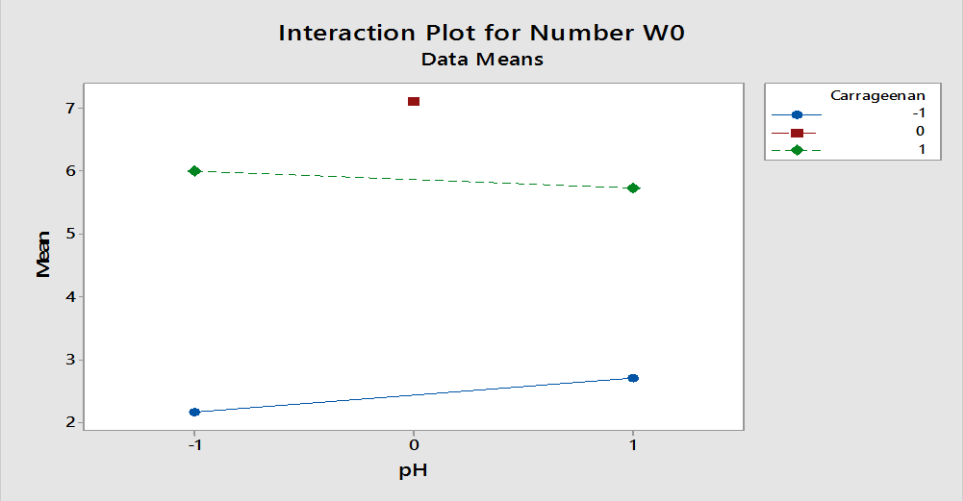

Figure 20. The plot shows the interaction between the pH-level and carrageenan concentration on particle concentration week 8.

5.4 Visual sensory analysis

Visual sensory analysis was carried out in order to observe visual changes taking place during storage of the samples. Figure 21 image a) shows the appearance of a gelled sample (T4 stored at 30 °C) after it has been poured into a beaker and stirred gently. Image b) shows the appearance of a non-gelled sample (T1 stored at room temperature) and image c) shows the appearance of a gelled sample (T4 stored at 30 °C).

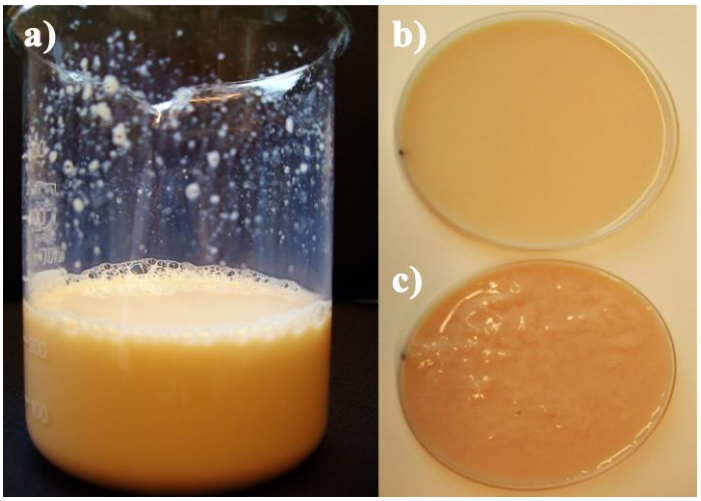

Figure 21. Image a) shows how a gelled sample looks like when it is poured into a beaker and stirred gently. Image b) shows a sample which has not gelled and c) a sample that has gelled.

5.4.1 Storage at room temperature

Week 1: The results from the first visual sensory analysis of the samples did not show any obvious quality changes in the samples. A slight difference in colour was however observed in T2 and T4 which had a darker tone than T1 and T3. No visually aggregated particles could be observed on the beaker.

Week 5: The results from the sensory analysis showed no visual changes in samples T1 and T3. However a few small particles could be anticipated on the beaker wall when observing sample T2 and few small distinct particles was observed in sample T4. Sample T2 and T4 were not homogenous when poured into the beaker, these samples also had darker tone than T1 and T3.

Week 10: The results from the sensory analysis showed no visual changes in sample T1. In T3 a few small particles could be observed on the beaker wall, yet it was still homogenous when poured into the beaker. In T2 and T4 many particles was observed on the beaker wall, the particles were bigger in T4, and both samples were non-homogenous when poured into the beaker. These samples still had a darker tone than T1 and T3.

Week 13: The results from the sensory analysis showed no visual changes in sample T1 and it was homogenous when poured into the beaker. In T3 no small particles could be observed on the beaker wall, which was the case week 10, and it was still homogenous when poured into the beaker. In T2 and T4 many small and big particles was observed on the beaker wall, the particles were slightly bigger in T4, both samples were non-homogenous when poured into the beaker and still had a darker tone than T1 and T3.

5.4.2 Storage at 30°C for 12 weeks

In the samples stored at 30°C for twelve weeks interesting visual changes had taken place compared to the samples stored at room temperature. The sample showing least quality changes was T1 although many small particles could be observed on the beaker wall. This sample had also the lightest colour of all the samples. The other samples had clearly gelled, of which T2 and T4 was more gelled than T3. In these three samples big particles could be noticed on the beaker wall and they were all non-homogenous when poured into the beaker. T4 had the darkest colour of these samples which can probably be linked to a higher degree of Maillard reaction in this sample.

6 Discussion

The objective of this study was to develop methods for analysing structural changes during twelve weeks of storage in five protein and carbohydrate enriched milk-based UHT-beverages with varying levels of pH and carrageenan. The second objective was to observe when structural changes appeared, note the appearance of these structural changes and relate them to the different levels of pH and concentrations of carrageenan in the products.

6.1.1 Discussion of method

The methods used in this study were suitable in order to get a better understanding of the structural changes taking place in the products. The analyses gave valuable information of when structural changes started appearing and how these changes appeared. The methods also gave information of interactions between factors and their effects on the responses. The time limit of this study unfortunately restricted the number of measurements made and also the use of other methods which could have given valuable information of the changes taking place in the products. The methods in this study hence gave interesting information yet could be further extended and developed in order to achieve even more information which would make it easier to draw conclusion of the causative events of the product instabilities.

6.1.2 Discussion of results

The examination of the samples using BFM and visual sensory analysis confirmed that there were structural differences among products appearing after different storage times. The results from BFM, showing visible flocculation of casein micelles in T4 already the first week of storage, implicated that T4 with high pH and high carrageenan was unstable already from the beginning of storage. These observations were further confirmed by small particles appearing in the product at an earlier stage compared to the other samples. The microstructural analysis further revealed that T2 and the reference showed similar changes during storage time. The flocculation of casein micelles appeared the same week in these two samples, yet the flocculation was more pronounced in the reference. The visible particles confirming the observed instabilities appeared in these samples after T4. These results indicated that the high pH and low carrageenan concentration in T2 resulted in a product which developed instabilities at an early stage. The same accounted for the reference, which had a middle pH and a middle carrageenan concentration. The samples that differed from the rest were T1 and T3, both behaving similarly during the store time. The sample showing more instabilities was T3 in which small visible particles appeared before T1, these particles were not observed in T1 during the entire storage time. The samples stored at 30°C for twelve weeks confirmed the above results and a more distinct difference between T1 and T3 was observed, in T1 only small particles could be observed and it was still homogenous compared to T3 which contained bigger particles and was not homogenous when poured into the beaker. These results indicated that T1 with its low carrageenan and a low pH gave a more stable product than T3 with a low pH and high concentration of carrageenan.

The more pronounced instabilities observed in T2 and T4 could possibly be explained by their higher pH as this is believed to affect the gel strength in the product. Firstly a higher pH (6.7) during steriliza-

tion result in the formation of more whey protein aggregates, while a lower pH (6.5) leads to the formation of more β-LG–κ-CN complexes. (Anema and Li, 2003b) The β-LG–κ-CN complexes are important for gel stability as it results in more carrageenan-carrageenan interactions, instead of carrageenan-casein interactions, which has been linked to a greater gel stability. The β-LG–κ-CN complexes block the carrageenan from interacting with casein resulting in more carrageenan-carrageenan interactions (Tijssen et al., 2007) and since the storage stability is affected by the conditions during sterilization this could explain why the reference show instabilities even though it week 12 has the lowest pH. Secondly the pH does not just affect the type of interactions within the products, it also effects the enzyme activity. A low pH (4.6-4.7) increases the dissociation of plasmin into the whey fraction, increasing the amount of enzymes available for proteolysis. (Richardson and Elston, 1984; Grufferty and Fox, 1988) The results showed a drop in pH for T2 and T4 the first week and it is possible that this might have contributed to a higher dissociation of plasmin in these samples, yet when the dissociation has its maximum peak is not clear making these theories only speculations. Secondly the plasmin activity is highest at 37°C and around pH 7.5-8.0 (Fox, 1981), which is closer to the pH of T2 and T4. The enzyme activity was not measured during this experiment and therefore we can not know how it looks in the different samples, yet there is a possibility that the higher pH in samples T2 and T4 might have contributed to a greater enzyme activity in these samples further promoting gelation. This theory is however not consistent with the fact that the reference have the lowest pH and still showed greater instabilities than T1 and T3. The lowered pH in samples T2, T4 and the reference could be the result of proteolysis resulting in a decreased pH. Another possible explanation for the instabilities of the samples are protein and carrageenan rearrangements resulting in gelation. Tijssen et al (2007) concluded that the gelation of milk drinks containing carrageenan was not affected by proteolysis but of the rearrangements taking place. (Tijssen et al., 2007) In this study this theory is only a speculative suggestions of a possible cause of gelation.

Trying to relate the instabilities observed in the samples to the results from the particle size and concentration measurements is complicated. The particle size increased in samples T3, T4 and reference and decreased in samples T1 and T2, this decrease was however not significant in T2. The increase in size in T3 and T4 was also supported by TEM micrographs. The factorial design showed that the amount of carrageenan had an effect on particle size during week 0, implicating that different structures existed in the samples in the beginning. The reference behaved like T3 and T4 the first week of storage. After eight weeks of storage the particle size became more similar and was affected by an interaction between pH and carrageenan and there were no longer a significant difference between the reference and the other samples. According to earlier research made on semi-skimmed UHT-milk the particle size decreased in storage at room temperature. The change in particle size has by Walstra and Jenness (1984) been explained by a decrease in pH and by Crudden et al. (2005) as the cause of proteolysis, both resulting in a decreased micellar size. The theory suggesting that a decreasing pH will reduce size is not consistent with the results in this study, nor can the theory concerning proteolysis be confirmed since no measurements of this have been performed. The particle sizes were more different among samples in the beginning of storage compared to the end where they were more similar and since the carrageenan had an effect on particle size one could think that different structures (networks) exist in the beginning due to the amount of carrageenan. The results indicate that T1 and T2 have similar structures whereas T3, T4 and the reference behave alike. The concentrations of carrageenan is not

known in the samples, however studies have shown that a too high concentration of carrageenan results in aggregation of casein micelles (Dalgleish, 2007). Therefore it would be possible that if β-LG–κ-CN complexes are released and repulsion between particles decreases, the increased particle size in T3, T4 and the reference could be due to aggregation of casein micelles or complexation of these with other components, perhaps pushed by the higher carrageenan concentration. The decrease in particle size in T1 and T2 could be due to the loss of β-LG–κ-CN complexes and aggregation not pushed due to the lower concentration of carrageenan. Tijssen et al. (2007) explained the increase in particle size by the formation of weak aggregates which could consist of proteins and/ carrageenan. The greater particle size in T1 and T2 in the beginning of storage time compared to the other samples, could be result of bridging between micelles. The higher carrageenan concentration in T3, T4 and reference could possibly keep the micelles separated. The crystalline structures appearing in the samples could be precipitated salts. A study made by Gaucher et al. (2007) found crystalline structures in skim-milk in which salt had been added and they concluded that these structures were precipitated salts. These structures are however too big to be detected by the particle analyser, ruling out the possibility of them contributing to the increased particle size in T3 and T4.

The decrease in number of particles between week 0 and week 8 in all samples was affected by the amount of carrageenan, pH-level and time. The first week of storage the reference was significantly different in particle size compared to the samples with low carrageenan (T1 and T2), which indicated that it behaved more like T3 and T4. Interesting behaviours in particle concentration was seen week 8, when T1 differed from the rest of the samples by having a greater particle concentration. The number of particles week 0 and week 8 was affected by an interaction between pH and concentration of carrageenan. These results showed that the particle concentration could be crucial for the stability of the product since T1 is much more stable than the rest of the products. Finally the results from the concentration measurements evokes the question of how it is possible that the particle size decreases in T1 and T2 and at the same time also the concentration of these particles decreases. The reason for the decrease in particle concentration could be explained by complexation of whey proteins, casein micelles or carrageenan. (Tijssen et al., 2007) The decrease in particle size and particle concentration in T1 and T2, could be explained by release of β-LG–κ-CN complexes binding to undissolved caseinate in the samples which would not be detected by the particle analyser hence showing a decreased particle concentration. The pattern can however not be stated since no analyses have been made on the particle composition in the samples.

7 Conclusion

The complexity of the product made it hard to conclude exactly what was causing the instabilities in the products. The lack of additional information concerning complexes formed and the extent of proteolysis, made it impossible to prove if these events were causing the changes seen in the products. The suggestive cause of the instabilities seen could be proteolysis or physiochemical changes resulting in structural rearrangements, however these are only hypothetical suggestions of what is happening and further studies with other analytical methods are needed for a deeper understanding.

The pH seemed to have an important effect on product stability as the least stable products carried a high pH, the sample being the center point was however an exception. Different structures existed in the beginning of storage contributing to differences in particle size and particle concentration. The particle size was is in the beginning of storage effected by the concentration of carrageenan, where T1 and T2 behaved alike and T3, T4 and the reference had more similar particle sizes. After eight weeks of storage the interaction between pH and carrageenan became important for particle size and T2, T4 and the reference were more alike. The particle concentration was in the beginning of storage affected by the interactions between pH and carrageenan, where the reference showed similar characteristics as T3 and T4 (high carrageenan). After eight weeks of storage all samples showed similar concentrations except for T1, having a higher particle concentration. These results points out the importance of carrageenan in the beginning of sample storage, however after eight weeks of storage the pH in combination with carrageenan seem to affect the structural behaviour of the samples.

The conclusion from the study was therefore that pH was of primary importance for the stability of this product, where a higher pH seemed to result in less stable products. The effect of carrageenan was also important, however in this case only of secondary importance.

8 Future research

In order to better understand the structural changes taking place in the product it would be interesting to perform analyses of the proteolytic activity. The analyses could include identification of peptides resulting from proteolytic breakdown of proteins and could be analysed utilizing mass spectrometry. The breakdown products could give information of the milk proteins being hydrolysed in the product. Furthermore the actors of proteolysis could be classified in order to better understand the cause of gelation. The results from such analyses could give information about the enzymatic involvement in gelation of milk based UHT-beverages.

To understand the structural differences between the different products it would be interesting to perform TEM on all the samples. The limitations of this study did not make this possible, however this could give valuable information of how the structural differences appear in the samples which would allow comparison. Finally it would be interesting to perform an analysis of the compounds formed in the product upon storage, this could give valuable information of markers for instabilities and be performed by implementation of principal component analysis (PCA).

9 Acknowledgements

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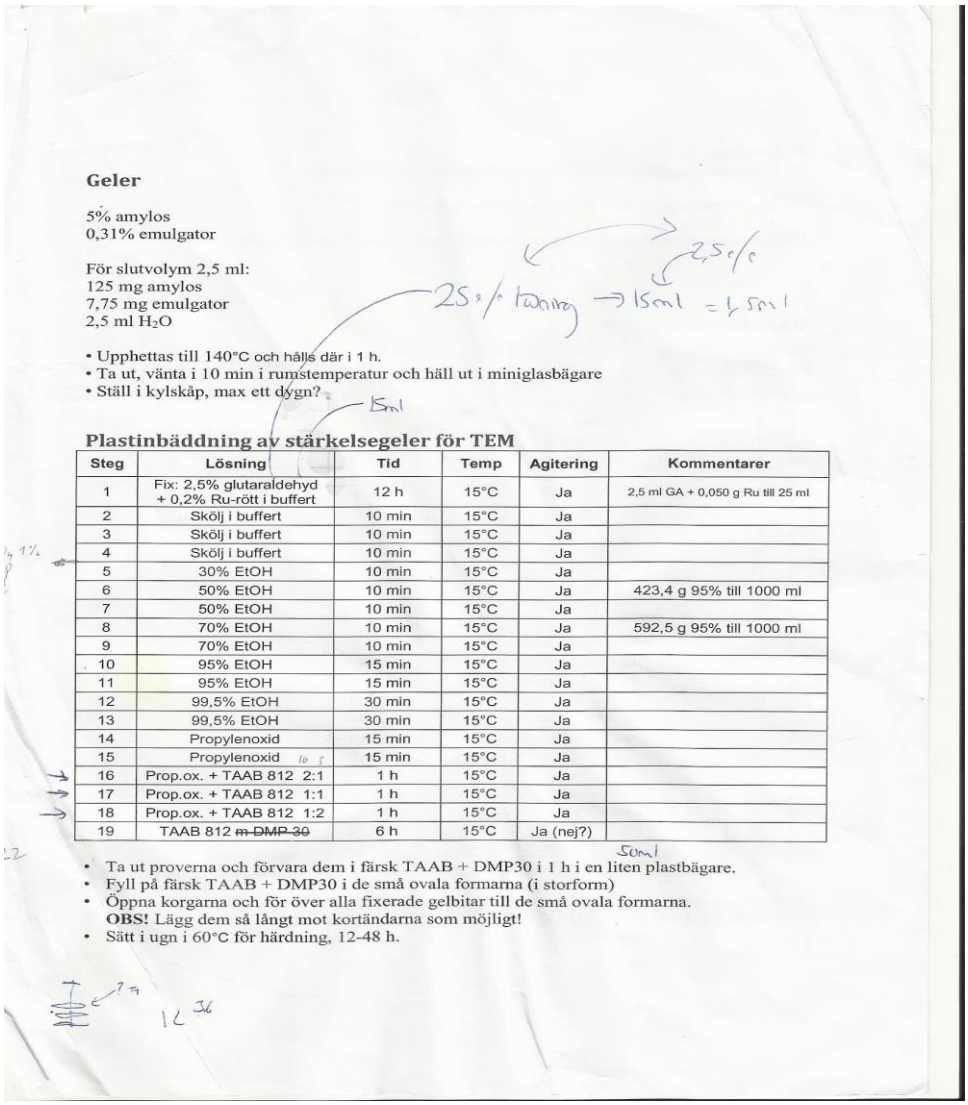
11 Appendix

Appendix 1. The nutritive value of the products analysed in this study.

Nutritive value	per 100 ml
Energy	290 kJ/70 kcal
Fat	0.5 g
Carbohydrates	7.4 g
– sugars	7.4 g
Protein	8.3 g
Salt	0.1 g
Calcium	202 mg (25 % av DRI)

Appendix 2. Preparation scheme for the tissue processor.



Geler

5% amylos
0,31% emulgator

För slutvolym 2,5 ml:
125 mg amylos
7,75 mg emulgator
2,5 ml H_2O

- Upphettas till 140°C och hålls där i 1 h.
- Ta ut, vänta i 10 min i rumstemperatur och häll ut i miniglasbägare
- Ställ i kylskåp, max ett dygn?

Plastinbäddning av stärkelsegeler för TEM

Steg	Lösning	Tid	Temp	Agitering	Kommentarer
1	Fix: 2,5% glutaraldehyd + 0,2% Ru-rött i buffert	12 h	15°C	Ja	2,5 ml GA + 0,050 g Ru till 25 ml
2	Skölj i buffert	10 min	15°C	Ja	
3	Skölj i buffert	10 min	15°C	Ja	
4	Skölj i buffert	10 min	15°C	Ja	
5	30% EtOH	10 min	15°C	Ja	
6	50% EtOH	10 min	15°C	Ja	423,4 g 95% till 1000 ml
7	50% EtOH	10 min	15°C	Ja	
8	70% EtOH	10 min	15°C	Ja	592,5 g 95% till 1000 ml
9	70% EtOH	10 min	15°C	Ja	
10	95% EtOH	15 min	15°C	Ja	
11	95% EtOH	15 min	15°C	Ja	
12	99,5% EtOH	30 min	15°C	Ja	
13	99,5% EtOH	30 min	15°C	Ja	
14	Propylenoxid	15 min	15°C	Ja	
15	Propylenoxid	15 min	15°C	Ja	
16	Prop.ox. + TAAB 812 2:1	1 h	15°C	Ja	
17	Prop.ox. + TAAB 812 1:1	1 h	15°C	Ja	
18	Prop.ox. + TAAB 812 1:2	1 h	15°C	Ja	
19	TAAB 812 ~~m DMP 30~~	6 h	15°C	Ja (nej?)	

- Ta ut proverna och förvara dem i färsk TAAB + DMP30 i 1 h i en liten plastbägare.
- Fyll på färsk TAAB + DMP30 i de små ovala formarna (i storform)
- Öppna korgarna och för över alla fixerade gelbitar till de små ovala formarna.
  OBS! Lägg dem så långt mot kortändarna som möjligt!
- Sätt i ugn i 60°C för härdning, 12-48 h.

Appendix 3. The recipe for the preparation of fresh embedding resin.

DATA SHEET 3

TAAB

TAAB LABORATORIES EQUIPMENT LIMITED

3 MINERVA HOUSE, CALLEVA INDUSTRIAL PARK
ALDERMASTON, BERKSHIRE, ENGLAND RG7 4QW
TEL: 0734 817775 FAX: 0734 817881 TELEX: 847838

TAAB EMBEDDING RESIN

TAAB EMBEDDING RESIN is a specially developed epoxy-resin for embedding biological specimens for electron microscopy.

The resin has the following valuable properties:

Relatively low viscosity
Freedom from background
Good cutting characteristics
Good contrast

A wide range of hardnesses can be obtained by using different proportions of the hardeners dodecenylsuccinic anhydride and methyl nadic anhydride. DMP-30 or BDMA is used as the accelerator.

Tissues may be fixed and dehydrated by any of the standard procedures and infiltrated in the mixed resin before blocking up in fresh resin.

Typical formulations are as follows:

	(A) Soft Blocks	(B) Hard Blocks	(C)
TAAB EMBEDDING RESIN	50ml	50ml	50ml
DDSA	50ml	25ml	45ml
MNA	-	25ml	5ml

Shake well,add DMP-30 (2ml) or BDMA (3ml) and shake again until the mixture is homogeneous.

Curing is carried out at 60 C for at least 24hrs. Mixture (A) cured for 24hrs.produces blocks which,while quite soft,give excellent sections of soft tissues.Mixture (B) is brittle during trimming,but gives good sections and is particularly suitable for hard botanical specimens.Mixture (C) gives blocks of intermediate hardness suitable for most applications.Other mixes may be used provided that the total volume of hardeners equals the volume of resin.

STORAGE:

It is not necessary to refrigerate any of the components during storage,but all components should be securely capped after use. DMP30 in particular should be kept dry.

HANDLING PRECAUTIONS:

All epoxy resins **may** cause dermatitis and therefore care **should** be taken to avoid skin or eye contact.Skin may be washed with soap and plenty of water.Eyes should be irrigated with copious quantities of water.Contaminated clothing should be changed immediately.Cured resins are inert although resin dust should be avoided.Appropriate caution should always be exercised when using the chemicals and users should be restricted to competent technicians only.Always wear gloves and if possible use a fume hood.

Catalogue reference

T004 Taab embedding resin kit - with **DMP-30**
T004/1 Taab embedding resin kit - with **BDMA**
T027 Taab embedding resin Premix kit, ***hard***
T028 Taab embedding resin Premix kit, ***medium***
T029 Taab embedding resin Premix kit, ***soft***

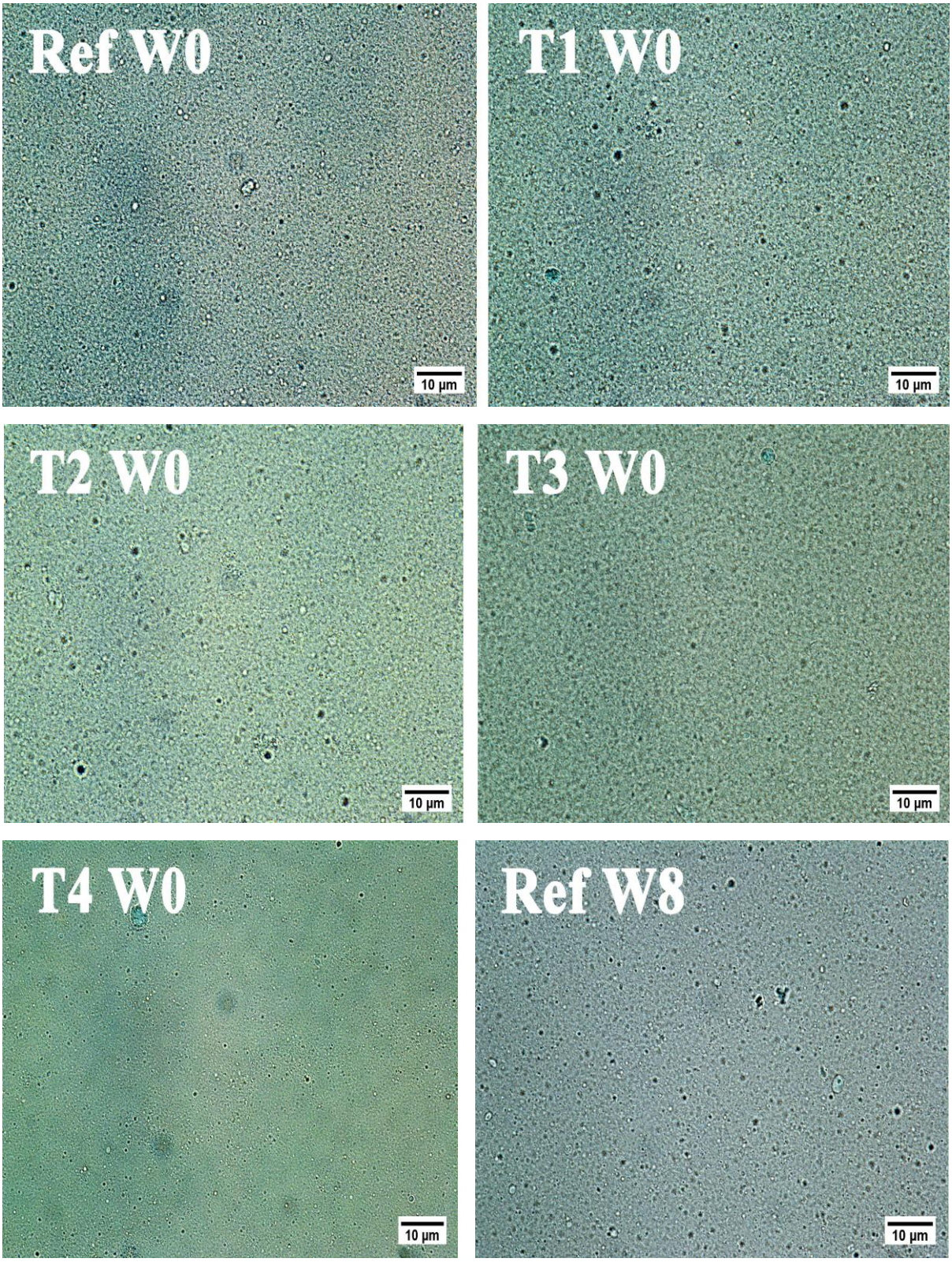
All components are available separately and in varying quantities.

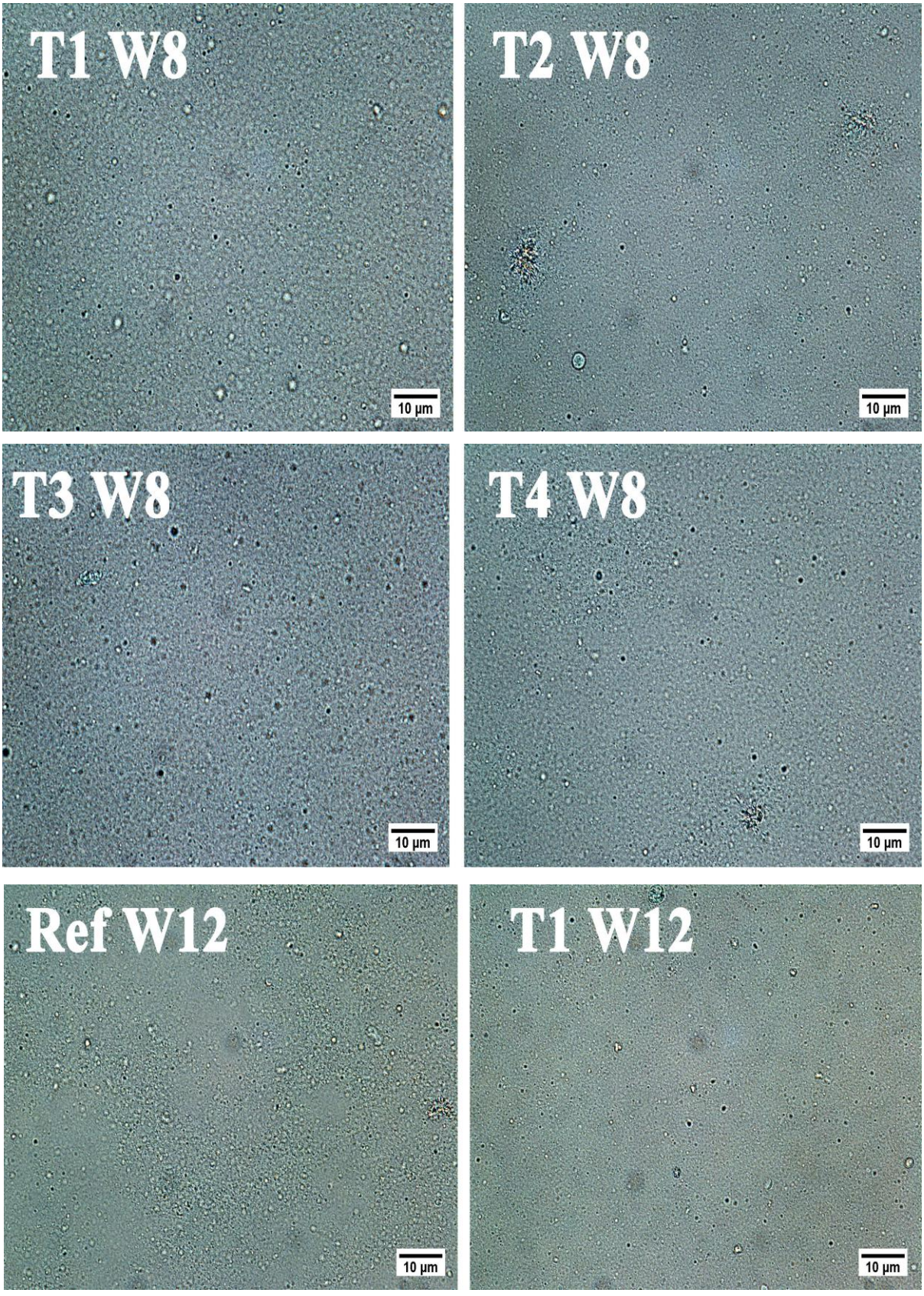
A full range of embedding moulds and capsules,together with safety and protection equipment,chemicals and clothing are listed in TAAB's full 232 page catalogue No.4

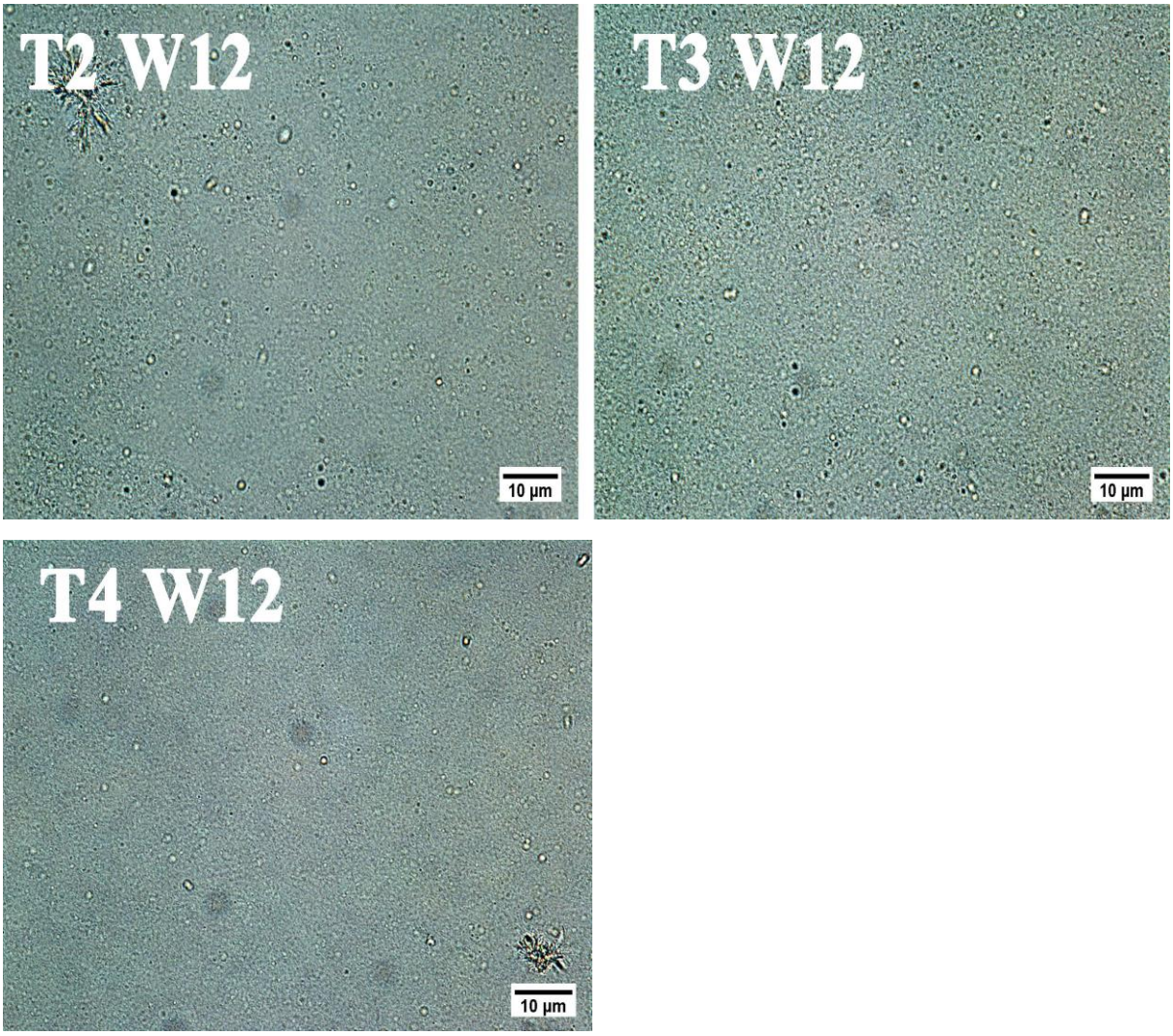
Appendix 4. The results from the two-way ANOVA in which differences in pH between all samples was analysed. The results show that there is a significant difference in pH among all samples between week 0 and week 12. The abbreviations in the table have the following meaning: DF (Degrees of freedom), SS (Sum of squares), MS (Mean square), F (F-ratio) and P (p-value).

Source	DF	SS	MS	F	P
Week	3	0.016	0.008	0.730	0.492
Sample	4	0.482	0.121	10.840	0.000
Interaction	8	0.199	0.021	1.910	0.095
Error	30	0.333	0.011		
Total	44	1.001			

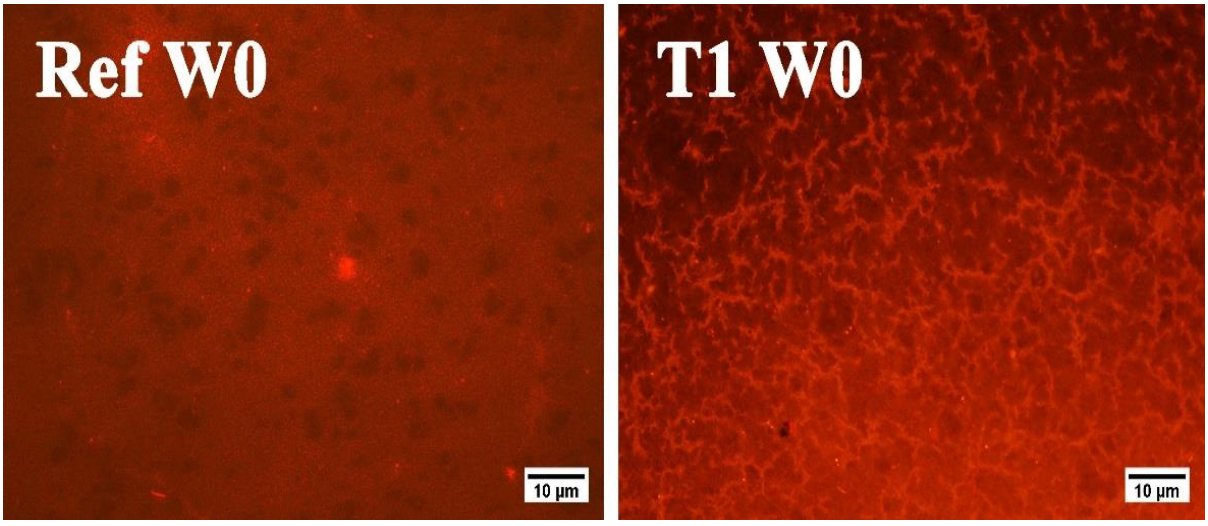
Appendix 5. Micrographs from week 0, week 8 and week 12 of samples stored at room temperature.

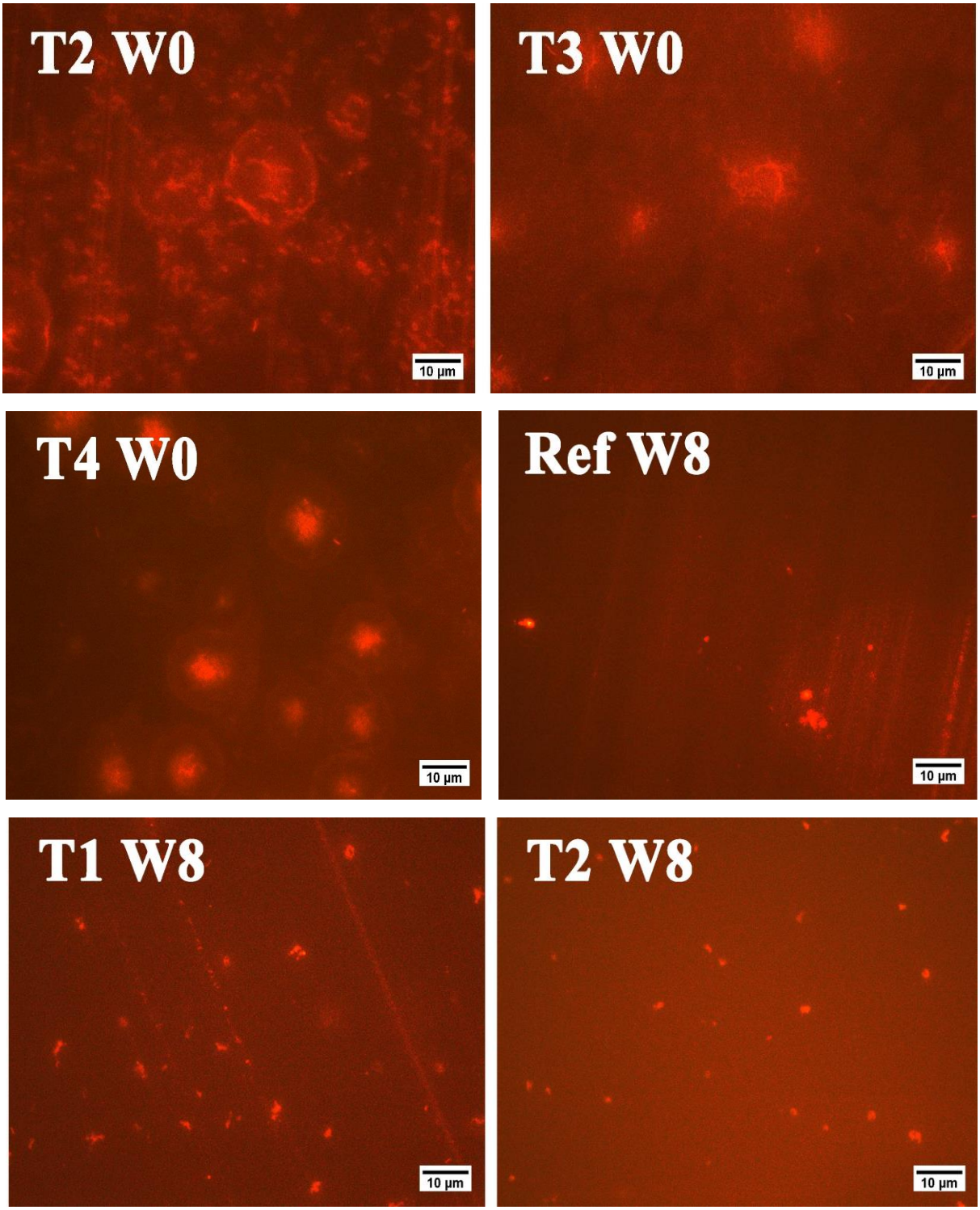


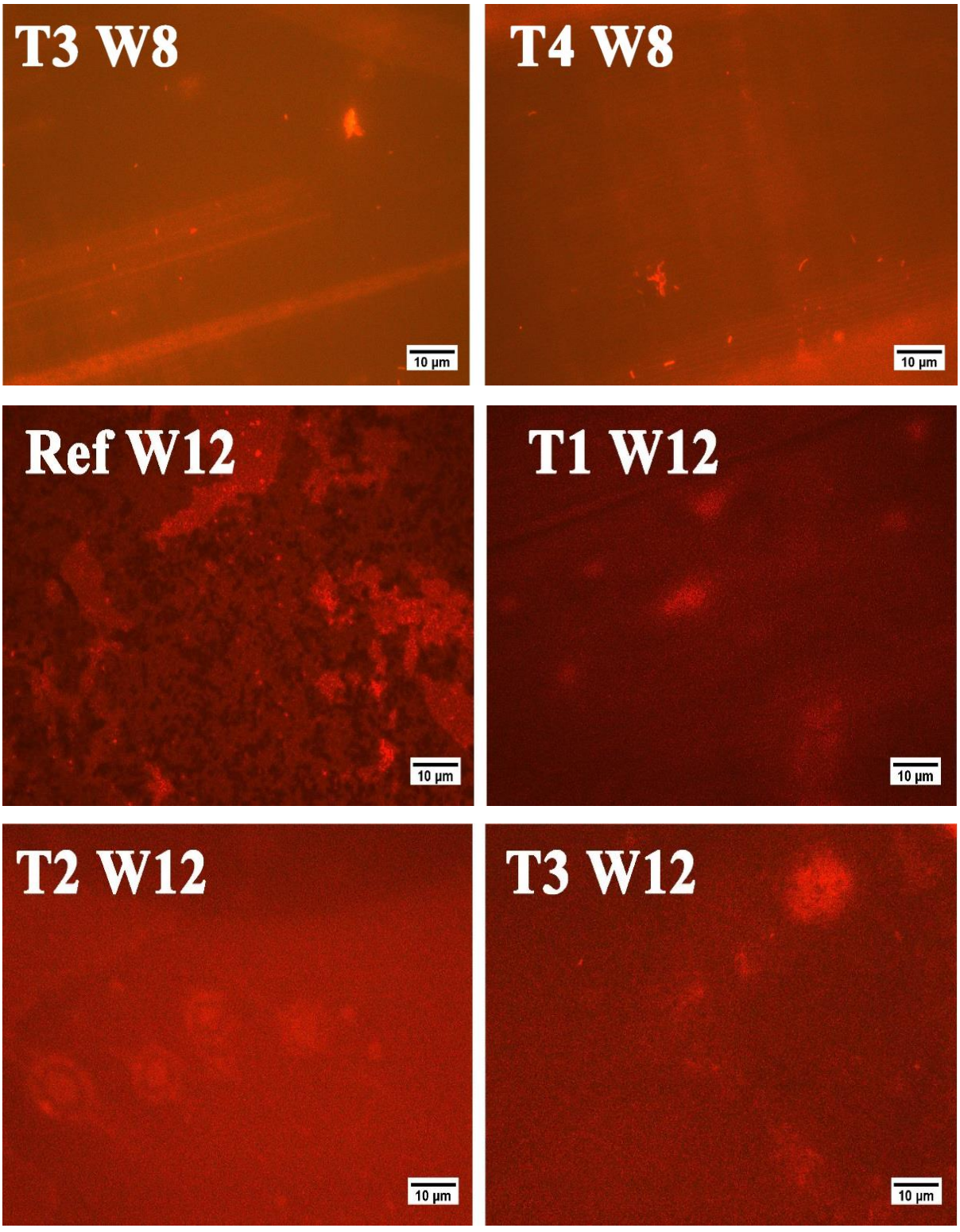

T1 W8
10 µm
T2 W8
10 µm
T3 W8
10 µm
T4 W8
10 µm
Ref W12
10 µm
T1 W12
10 µm

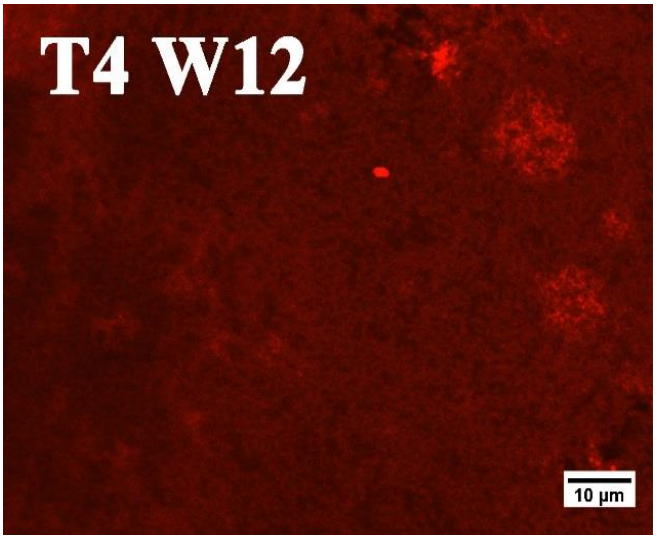


Appendix 6. Epifluorescent micrographs from week 0, week 8 and week 12 of samples stored at room temperature.

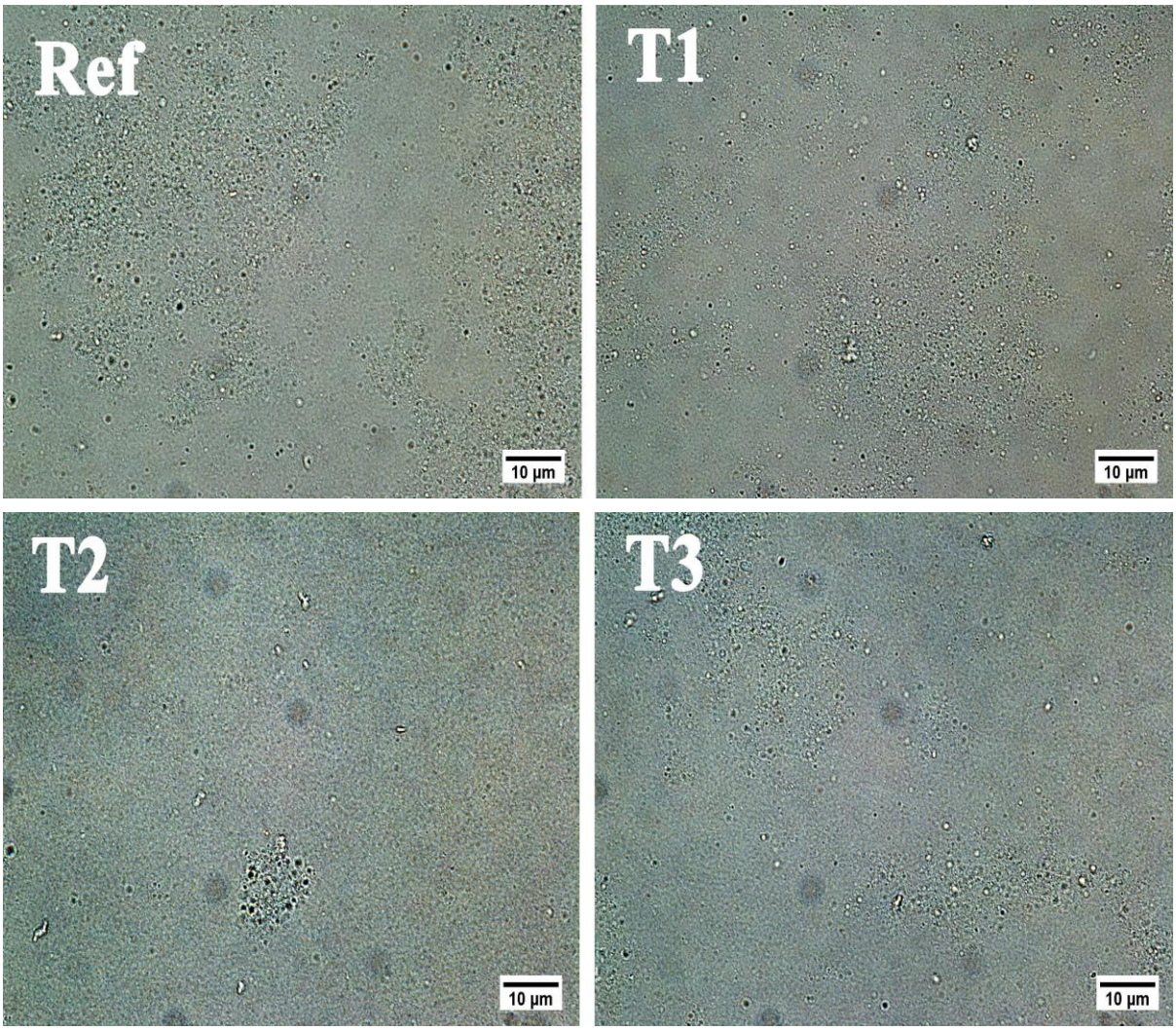


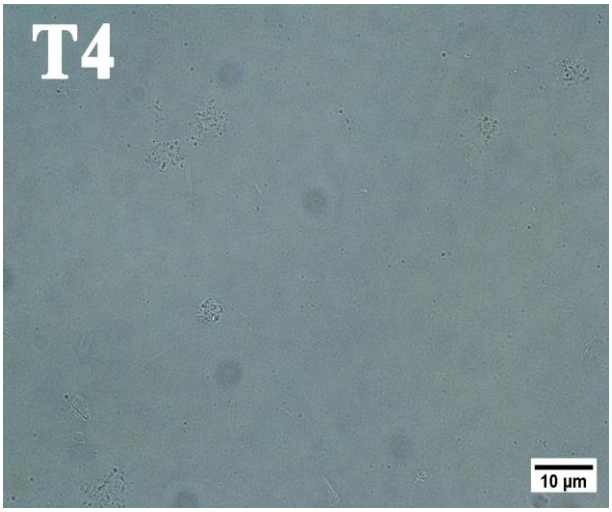

T2 W0
10 µm
T3 W0
10 µm
T4 W0
10 µm
Ref W8
10 µm
T1 W8
10 µm
T2 W8
10 µm


T3 W8
10 µm
T4 W8
10 µm
Ref W12
10 µm
T1 W12
10 µm
T2 W12
10 µm
T3 W12
10 µm



Appendix 7. Micrographs from week 12 of samples stored at 30°C.





Appendix 8. The p-values from the t-test comparing different samples week 0 and week 8, the values marked red show significant differences in particle size.

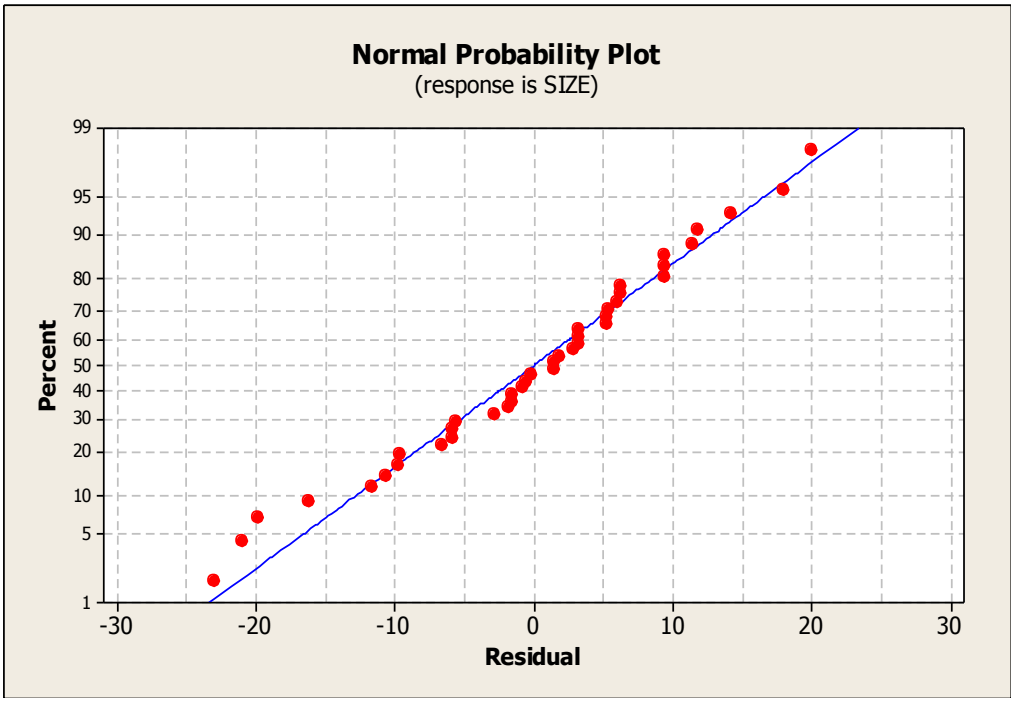
t-test	p-value (week 0)	p-value (week 8)
T1 vs T2	9,18E-01	2,66E-02
T1 vs T3	3,40E-03	2,44E-02
T1 vs T4	4,01E-02	2,65E-02
T2 vs T3	7,14E-04	3,57E-01
T2 vs T4	1,20E-03	1,38E-01
T3 vs T4	5,17E-02	3,37E-01
Ref vs T1	1,12E-02	3,60E-02
Ref vs T2	2,87E-03	5,61E-01
Ref vs T3	1,93E-01	6,00E-01
Ref vs T4	3,20E-01	2,15E-01

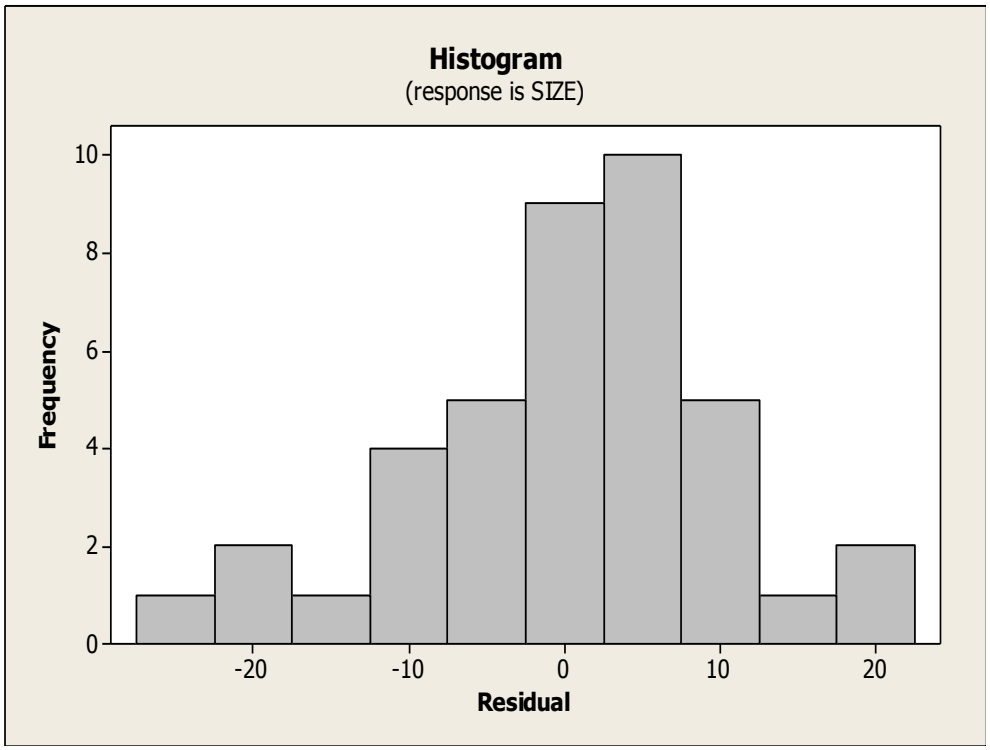
Appendix 9. The ANOVA from the first factorial design showing the effects of different factors on particle size. The Normal Probability Plot showing that the data is normally distributed. A histogram showing the distribution of data.

Analysis of Variance for SIZE, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	656,1	656,1	656,1	5,35	0,027
Carrag	1	4202,5	4202,5	4202,5	34,30	0,000
Week	1	8,1	8,1	8,1	0,07	0,799
pH*Carrag	1	102,4	102,4	102,4	0,84	0,367
pH*Week	1	40,0	40,0	40,0	0,33	0,572
K Carrag*Week	1	4161,6	4161,6	4161,6	33,97	0,000
pH*Carrag*Week	1	1144,9	1144,9	1144,9	9,34	0,004
Error	32	3920,8	3920,8	122,5		
Total	39	14236,4				

S = 11,0691 R-Sq = 72,46% R-Sq(adj) = 66,43%



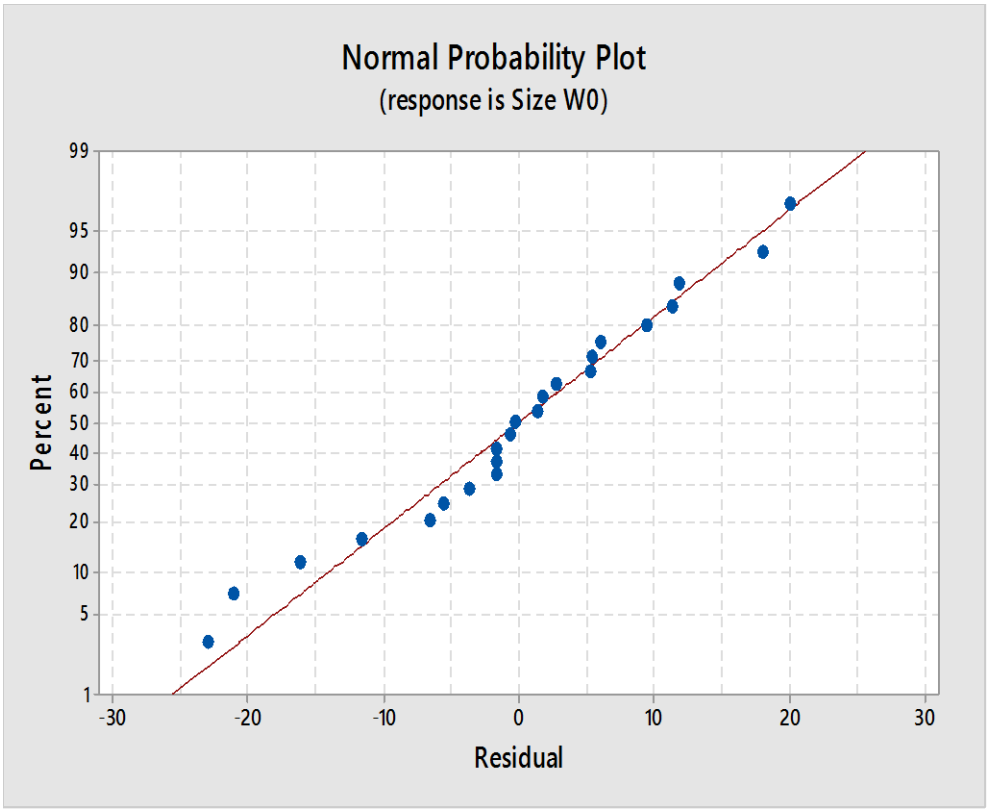


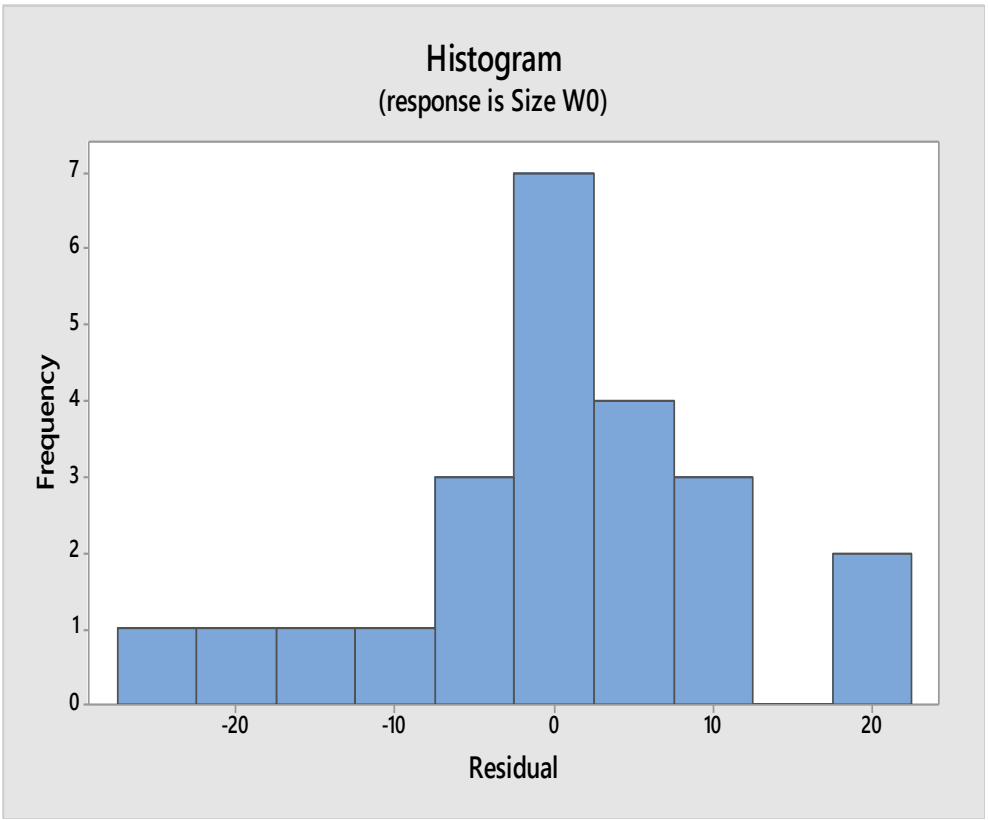
Appendix 10. The ANOVA from the second factorial design showing the effects of different factors on particle size week 0. The Normal Probability Plot showing that the data is normally distributed. A histogram showing the distribution of data.

Factorial Regression: Size W0 versus pH; Carrageenan; CenterPt

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	4	10233,4	2558,4	17,37	0,000
Linear	2	8550,1	4275,1	29,02	0,000
pH	1	186,0	186,0	1,26	0,276
Carrageenan	1	8364,1	8364,1	56,77	0,000
2-Way Interactions	1	281,3	281,3	1,91	0,184
pH*Carrageenan	1	281,3	281,3	1,91	0,184
Curvature	1	1402,1	1402,1	9,52	0,006
Error	18	2651,9	147,3		
Total	22	12885,3			


Normal Probability Plot
(response is Size W0)
Percent
99
95
90
80
70
60
50
40
30
20
10
5
1
-30
-20
-10
0
10
20
30
Residual

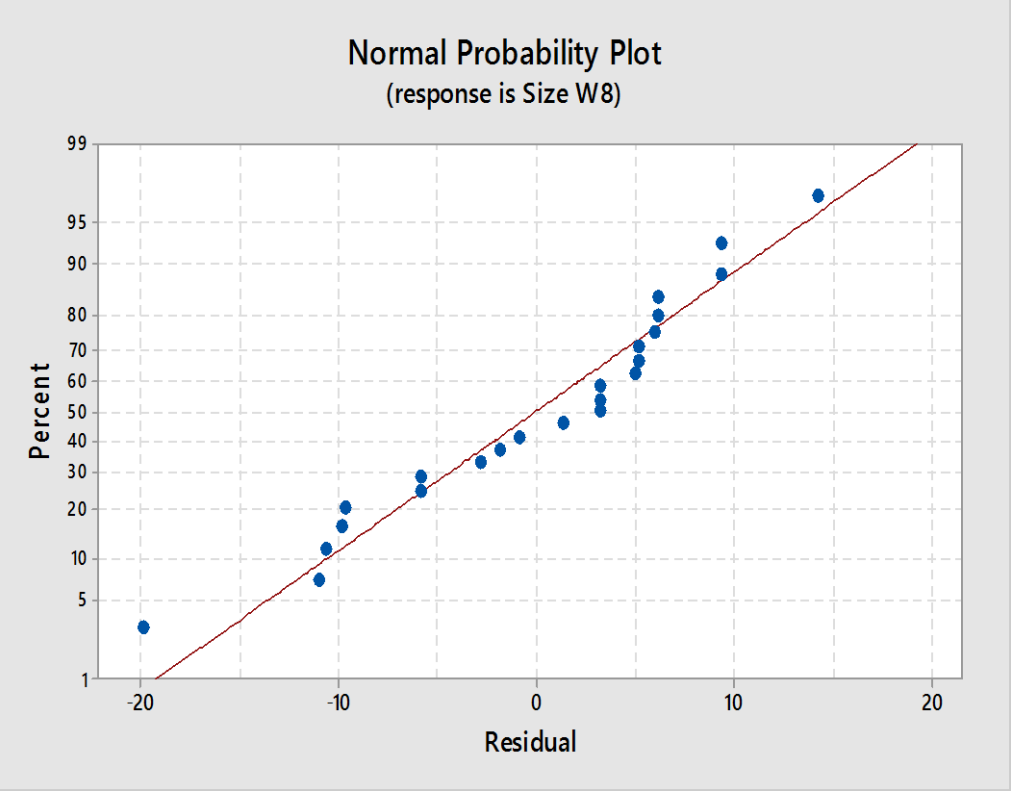

Histogram
(response is Size W0)
Frequency
7
6
5
4
3
2
1
0
-20
-10
0
10
20
Residual

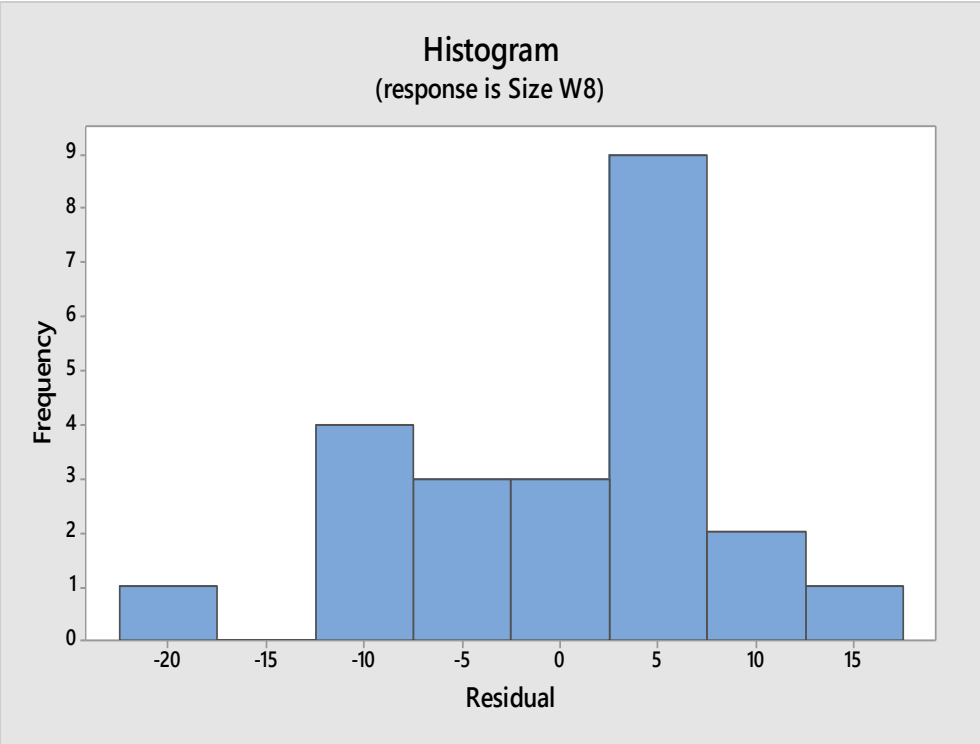
Appendix 11. The ANOVA from the second factorial design showing the effects of different factors on particle size week 8. The Normal Probability Plot showing that the data is normally distributed. A histogram showing the distribution of data.

Factorial Regression: Size W8 versus pH; Carrageenan; CenterPt

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	4	1503,70	375,926	4,52	0,011
Linear	2	510,10	255,050	3,07	0,071
pH	1	0,05	0,050	0,00	0,981
Carrageenan	1	510,05	510,050	6,14	0,023
2-Way Interactions	1	966,05	966,050	11,63	0,003
pH*Carrageenan	1	966,05	966,050	11,63	0,003
Curvature	1	27,55	27,554	0,33	0,572
Error	18	1495,60	83,089		
Total	22	2999,30			





Appendix 12. The p-values from the t-test comparing different samples week 0 and week 8, the values marked red show significant differences in particle concentration.

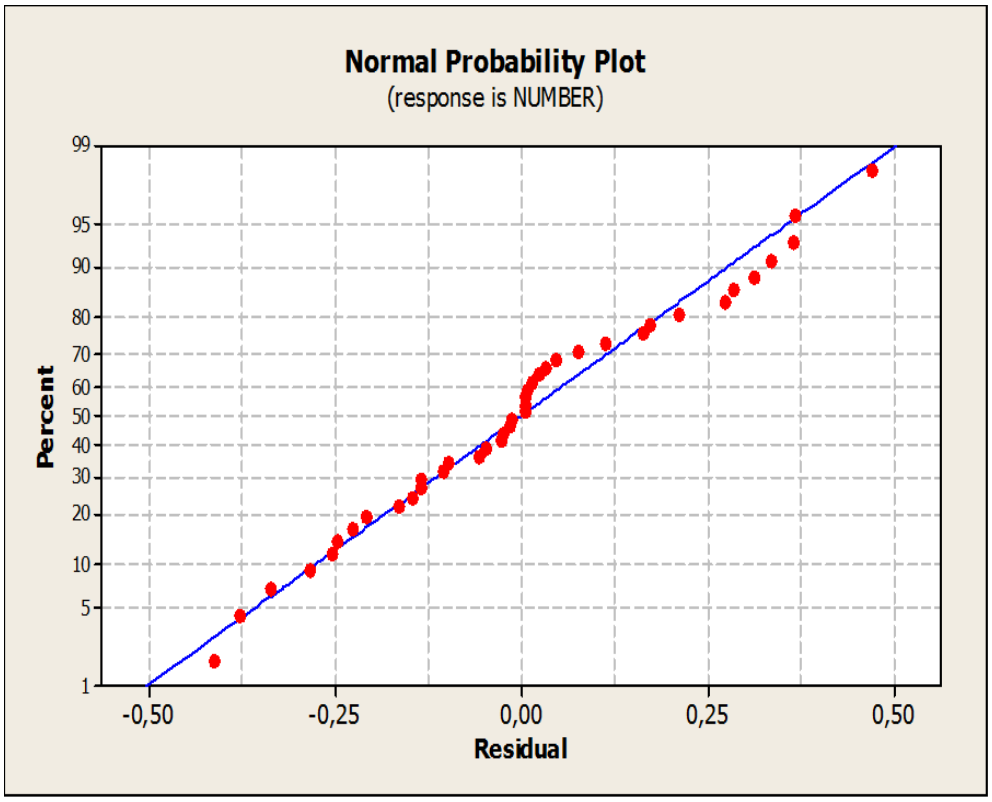
t-test	p-value (week 0)	p-value (week 8)
T1 vs T2	6,34E-03	1,99E-05
T1 vs T3	1,82E-05	8,79E-07
T1 vs T4	4,14E-05	1,72E-06
T2 vs T3	4,21E-06	4,67E-02
T2 vs T4	3,23E-05	9,87E-01
T3 vs T4	2,21E-01	8,62E-04
Ref vs T1	5,61E-06	2,93E-05
Ref vs T2	3,05E-07	1,16E-02
Ref vs T3	9,88E-04	4,21E-01
Ref vs T4	7,01E-04	1,65E-02

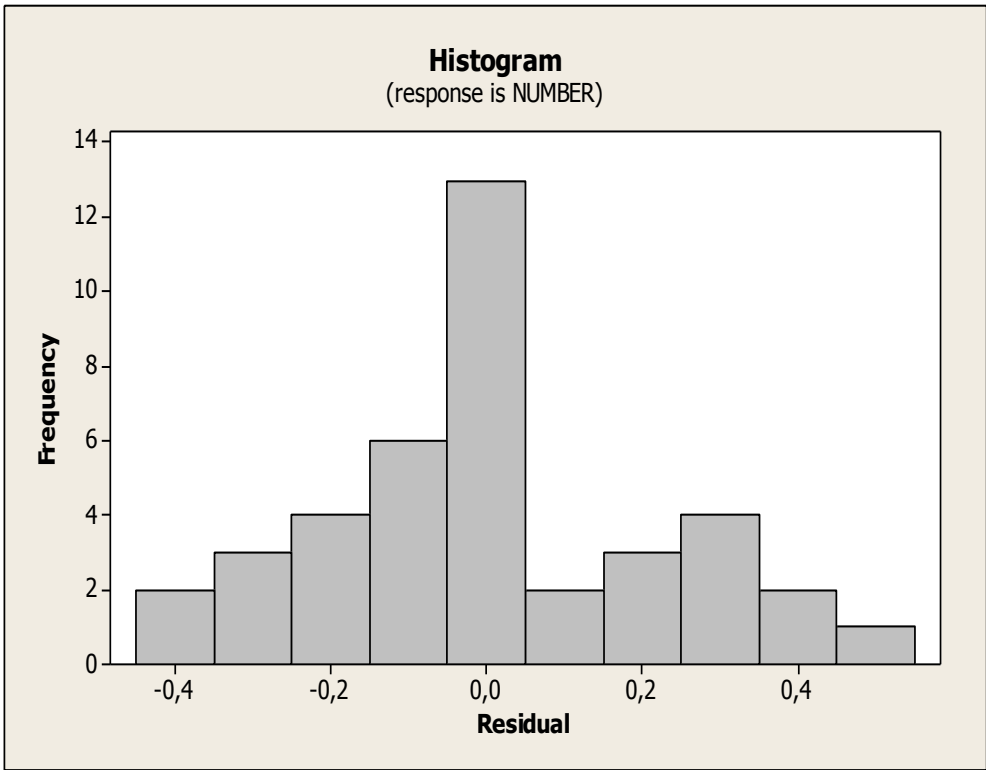
Appendix 13. The ANOVA from the first factorial design showing the effects of different factors on particle concentration. The Normal Probability Plot showing that the data is normally distributed. A histogram showing the distribution of data.

Analysis of Variance for NUMBER, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	13,007	13,007	13,007	228,34	0,000
Carrag	1	6,092	6,092	6,092	106,94	0,000
Week	1	3,393	3,393	3,393	59,56	0,000
pH*Carrag	1	5,366	5,366	5,366	94,19	0,000
pH*Week	1	16,066	16,066	16,066	282,03	0,000
Carrag*Week	1	70,358	70,358	70,358	1235,10	0,000
pH*Carrag*Week	1	12,894	12,894	12,894	226,34	0,000
Error	32	1,823	1,823	0,057		
Total	39	128,997				

S = 0,238673 R-Sq = 98,59% R-Sq(adj) = 98,28%



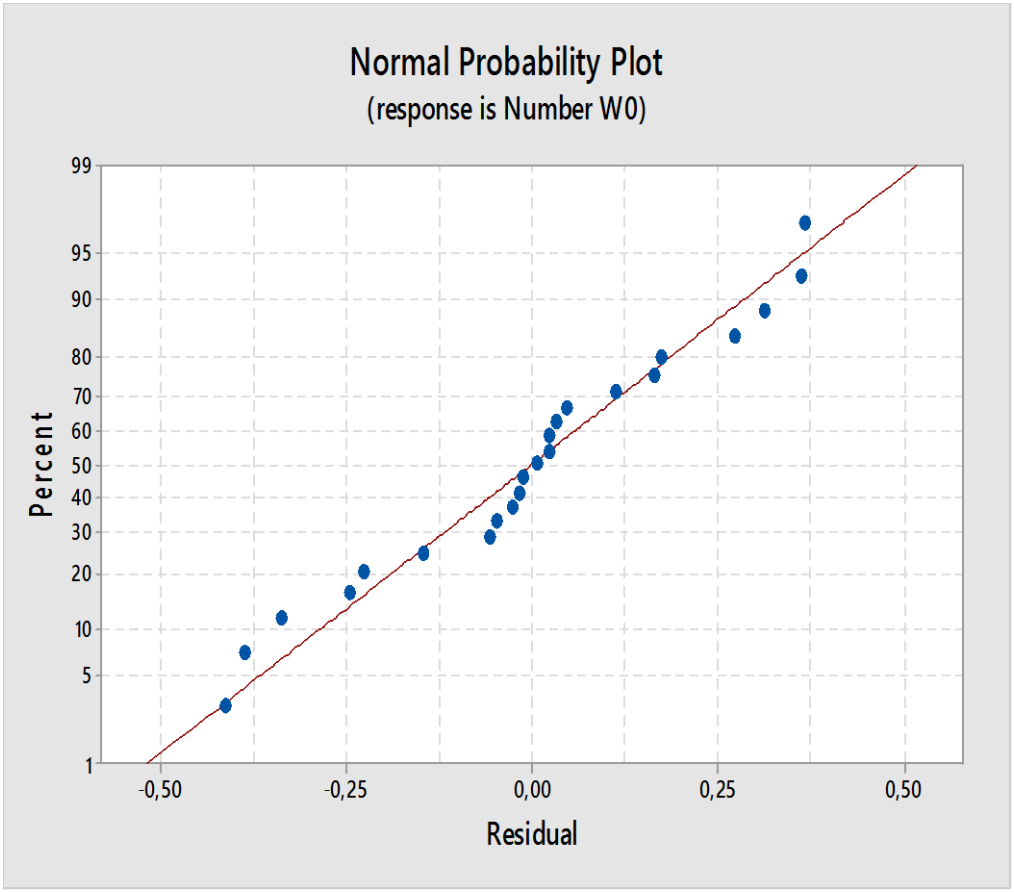


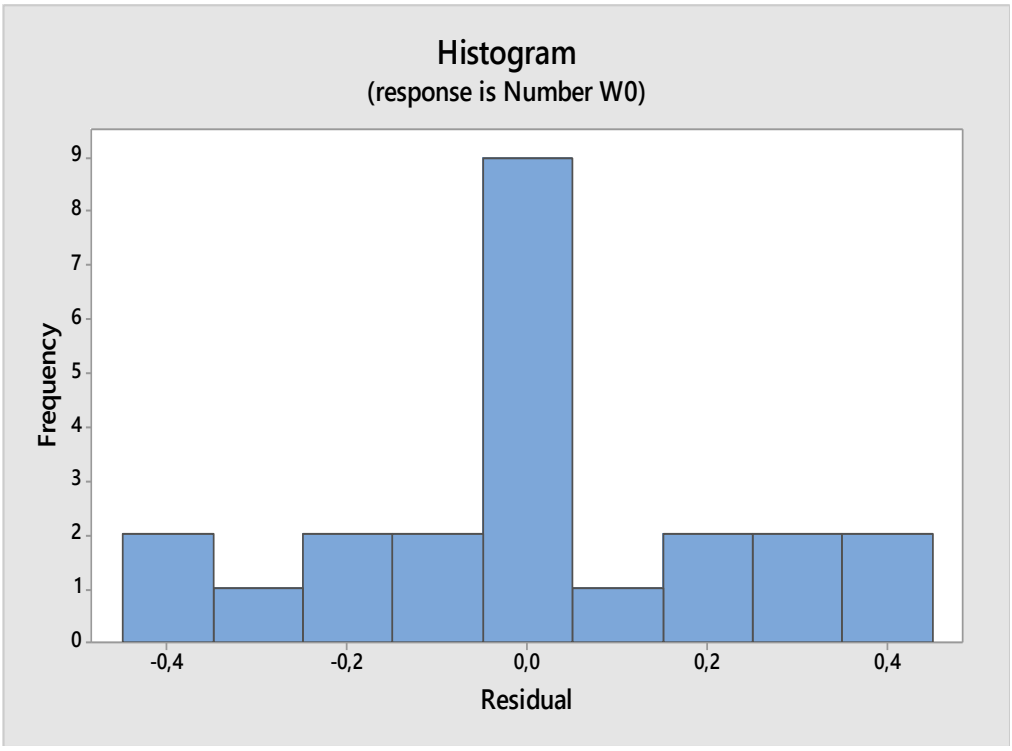
Appendix 14. The ANOVA from the second factorial design showing the effects of different factors on particle concentration week 0. The Normal Probability Plot showing that the data is normally distributed. A histogram showing the distribution of data.

Factorial Regression: Number W0 versus pH; Carrageenan; CenterPt

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	4	82,6636	20,6659	342,12	0,000
Linear	2	59,0081	29,5040	488,43	0,000
pH	1	0,0806	0,0806	1,34	0,263
Carrageenan	1	58,9274	58,9274	975,52	0,000
2-Way Interactions	1	0,8120	0,8120	13,44	0,002
pH*Carrageenan	1	0,8120	0,8120	13,44	0,002
Curvature	1	22,8435	22,8435	378,17	0,000
Error	18	1,0873	0,0604		
Total	22	83,7509			


Normal Probability Plot
(response is Number W0)
Percent
99
95
90
80
70
60
50
40
30
20
10
5
1
-0,50
-0,25
0,00
0,25
0,50
Residual

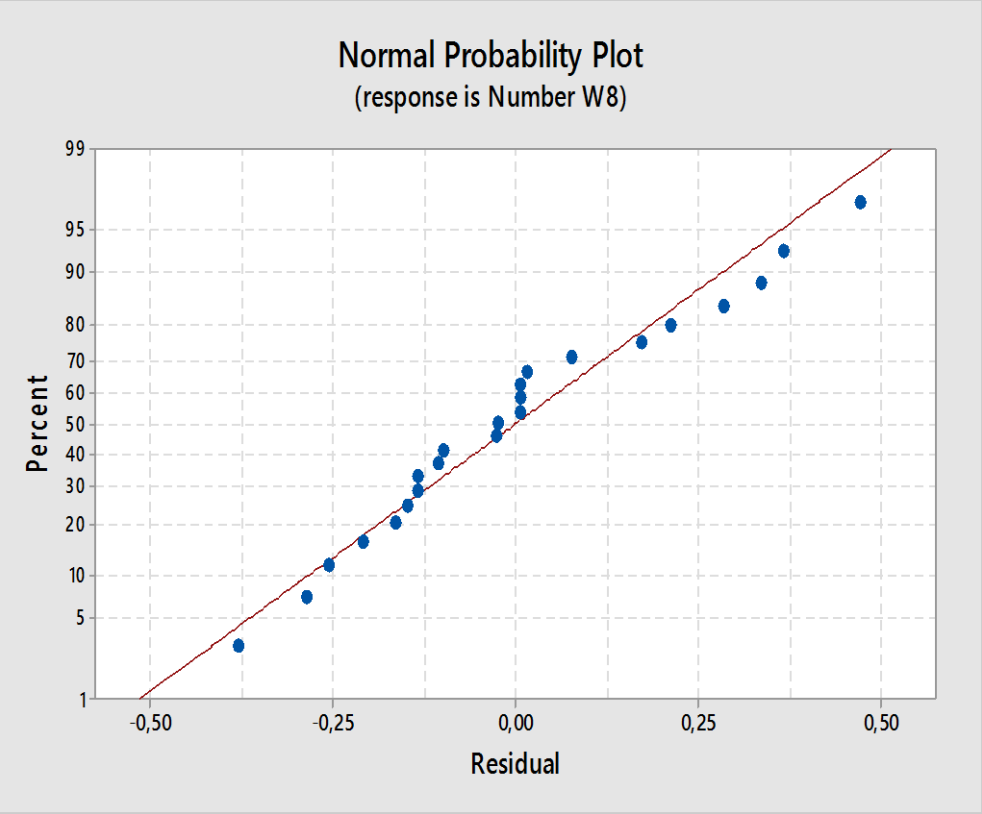

Histogram
(response is Number W0)
Frequency
9
8
7
6
5
4
3
2
1
0
-0,4
-0,2
0,0
0,2
0,4
Residual

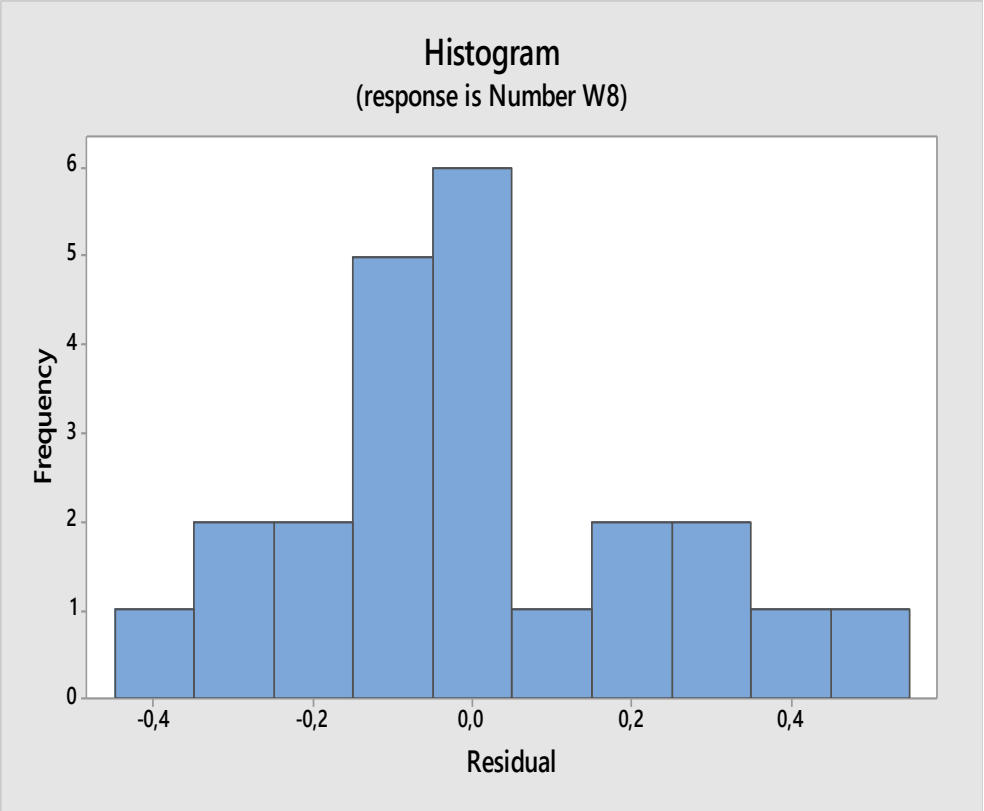
Appendix 15. The ANOVA from the second factorial design showing the effects of different factors on particle size week 8. The Normal Probability Plot showing that the data is normally distributed. A histogram showing the distribution of data.

Factorial Regression: Number W8 versus pH; Carrageenan; CenterPt

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	4	64,4289	16,1072	270,99	0,000
Linear	2	46,5142	23,2571	391,28	0,000
pH	1	17,5219	17,5219	294,79	0,000
Carrageenan	1	28,9923	28,9923	487,76	0,000
2-Way Interactions	1	17,4471	17,4471	293,53	0,000
pH*Carrageenan	1	17,4471	17,4471	293,53	0,000
Curvature	1	0,4675	0,4675	7,87	0,012
Error	18	1,0699	0,0594		
Total	22	65,4988			





Appendix 16: Popular summary

This study focused on the effects of pH and carrageenan on the storage stability of a protein and carbohydrate enriched milk-based beverage sterilized through ultra-high temperature treatment (UHT). The past decades a trend for fortified drinks have increased on the market and a certain demand for health drinks have been evident. Protein enriched milk based drinks is a category of beverages which recently came to the market, along with them came storage instabilities never seen before. A common problem appearing in these types of drinks were gel lumps appearing after some time of storage. These issues are however not easily solved by food producers since the beverages are often very complex and several factors such as processing, ingredients and storage conditions can affect the quality during storage time.

During formulation of a recipes for milk based UHT-beverages it is common to add stabilizers in order to prevent quality defects such as formation of gel lumps. A typical stabilizer is carrageenan extracted from red seaweeds. Carrageenan ensures that all components in the beverage are kept in place and that product stability is ensured for a longer time. Carrageenan creates a network in the product by interacting with itself and with milk proteins. The stability of the network created is affected environmental conditions such as pH. The UHT-treatment of fortified drinks is performed in order to prolong storage time and avoid deterioration. Heat treatment is typically performed for a few seconds at 140 °C resulting in killing of unwanted microorganisms and the formation of stabile protein components in the milk. Despite the fact that stabilizer is added and that the product is heat treated to enhance storage

stability, the product will show quality defects after some storage time. This quality defect is a problem which also occurs in regular UHT-milk after a certain storage time, the defect is called age gelation. The cause of this gelation has by many researcher been explained as the result of activity from enzymes always present in the milk or from bacteria producing similar enzymes. Questions that food producers ask include, how could we formulate the recipe in order to increase the storage stability of the milk based UHT-beverages? How can a low versus high pH, or a high versus a low concentration of carrageenan, affect the storage stability? How can the cause of the instabilities be explained? These are some of the questions this study has tried to answer.

The objective of this study was to study the quality changes in a milk based protein and carbohydrate enriched UHT-beverage and try to explain these changes. In this study five protein and carbohydrate enriched milk based UHT-beverages were obtained, all stored at room temperature for twelve weeks. The products varied with respect to pH and concentrations of carrageenan. The pH-levels and carrageenan concentrations were either low or high and one of the products had a pH and carrageenan concentrations corresponding to a value in the middle of the high and low values. The changes in the products were analysed every week by a microstructural method called BFM, this made it possible to observe when changes in microstructures started occurring in the products. The pH of the products were measured every second week and analysed in relation to the visual changes taking place in the products. In order to understand these structural changes better, high magnification images were collected of two of the products, performed by another microstructural technique called transmission electron microscopy. The micrographs obtained through this technique made it possible to observe structures such as milk proteins and carrageenan. Finally, particle analysis was made, providing additional information about the particle size and particle concentration in the products. All samples were also examined for visual changes during storage time, mainly focusing on finding signs of gelation in the form of visible aggregated particles.

The current study showed that the two products having a high pH, and also the product having a middle pH, were the ones showing quality defects earlier than the two products having low pHs. These products showed obvious changes in the microscope but also during visual observation of the products. The measured pH strengthened the theory that high pHs were related to colloidal instabilities, however the product with middle values could not be explained by this theory. The particle size showed different behaviours in the different products, it decreased in the samples having a low concentration of carrageenan and increased in the samples having a high or middle concentration of carrageenan. The increase in particle size might have been due to complexation of milk proteins while the decrease in size might have been caused by the release of certain components attached to the milk proteins. The change in particle concentration on the other hand behaved similarly in all samples and decreased over time, this was possibly caused by aggregation of some components in the milk.

The complexity of the product made it hard to conclude exactly what was causing the instabilities, resulting in only hypothetical suggestions. The conclusion of the study was however that the pH was of primary importance for the stability of this product, where a higher pH seemed to result in less stable products. The effect of carrageenan was also important, however in this case only of secondary importance.