The effect of the crosslink density of whey protein isolate gels on their functional properties

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Abstract

The water binding capacity (WBC) of protein gels can be changed by altering their crosslink density. In micro gels or microparticles (MPs), the WBC depends on the swellability of the MPs and the amount of water between MPs. In larger particles, such as macro gels, the WBC only depends on the swellability of the particles. Therefore, in this study, the effect of altering the crosslink density of macro whey protein isolate (WPI) gels on their WBC and other functional properties was studied, so that it could be determined to which extent the change in WBC of WPI MPs due to altered crosslink density was dependent on a change in swellability of the MPs. To decrease the crosslink density of the macro WPI gels, the gels were incubated in dithiothreitol, which resulted in an increase in WBC from 1.6 g water/g protein to 5.9 g water/g protein. The crosslink density of the macro gels was increased by incubating them in a genipin, or a transglutaminase solution before or after mixing, or by making the gel at a pH of 5.8. These treatments resulted in a WBC of 1.7, 1.6, 1.2 or 0.5 g water/g protein respectively. Comparing these results with the results obtained for MPs showed that part of the WBC of the MPs could be ascribed to interstitial water between the MPs and that a larger part of the WBC could be ascribed to an increase in swelling of the proteins than was thought according to rough calculations by Peters (unpublished results).

Table of Contents

1 Introduction

Obesity and overweight are an increasing problem last decades. Therefore weight management becomes more and more important (Hossain, Kawar, & El Nahas, 2007) and currently is a hot topic. Industry is trying to respond to this by developing healthier products with a lower energy content and therefore wants to try to reduce the energy content of e.g. dairy products. Since fats and proteins are high energy dense nutrients, it can be tried to reduce the fat and protein content of dairy products in order to lower the overall energy content. This can be done by adding water. Simply adding water will, however, lead to an altered sensory perception and might cause syneresis and/or softening of the product (Childs, Yates, & Drake, 2007; Guinee, Auty, & Fenelon, 2000). A way has to be found to overcome those product defects, otherwise the consumer will not accept those low energy dairy products. Syneresis and/or softening of the product might be avoided by binding more water to the present dairy proteins. In literature it has been reported that there is a connection between the microstructure of a product, its water binding capacity (WBC) and its mechanical characteristics (Barbut, 1995a, 1995b; Bowland & Foegeding, 1995; Boye, Alli, Ramaswamy, & Raghavan, 1997; Chantrapornchai & McClements, 2002; Vardhanabhuti, Foegeding, McGuffey, Daubert, & Swaisgood, 2001). Micro-structuring might therefore be a solution for this problem. According to the Flory-Rehner theory (Flory & Rehner Jr, 1943a, 1943b), altering the crosslink density of a protein network will alter the elasticity of the network and thereby the swelling behaviour. Therefore, it is tried to alter the swelling behaviour of whey proteins by altering their crosslink density.

In previous research by Peters (unpublished results), the crosslink density of whey protein isolate (WPI) microparticles (MPs) was altered and the swelling behaviour of those MPs was studied. However, the size and the particulate nature of the MPs caused that it was not possible to get good insight in the swelling behaviour and mechanical characteristics of the MPs. Hence, in this research larger whey protein structures, macro whey gels, were used which were changed in crosslink density to investigate its effect on the functional properties of the gels. The aim of this research was to get more insight in the effect of changing the crosslink density of macro WPI gels and to relate this to mechanical and swelling characteristics, the WBC and the meso-structure of gels. Accordingly, the influence of changing the crosslink density of macro WPI gels will be compared with the effect found for whey micro-particles (MPs). The major goal is to obtain more information on the cause of the change in WBC of the MPs altered in crosslink density of Peters.

After production of the macro whey gels with different crosslink densities, they were tested on their swellability as well as on their water binding capacity (WBC). This was done for fresh as well as dried gels, to be able to compare the results as good as possible with the results found for the (dry) MPs. This information was used to see whether the results obtained for the MPs were due to an increased swelling of the MPs or due to an increased amount of interstitial water. Furthermore, the strength of the protein-water interactions was studied with NMR, to get insight in the swelling of the gels. Moreover, the swelling of protein gels was monitored over time by MRI. A deformation on large scale was applied with the texture analyser to get more insight in the mechanical characteristics of the gels and see whether there is a relation between gel strength and WBC, as described by the Flory-Rehner theory.

2 Theoretical background

2.1 Whey protein gels

Proteins are large biological molecules consisting of a certain sequence of amino acids. Depending on the sequence of these amino acids, proteins are usually folded in a three-dimensional structure. Proteins can form a gel when they denature. When proteins denature, they lose their original three-dimensional structure and can aggregate. In that way a gel is formed.

In previous research, whey protein isolate (WPI) was used to produce WPI microparticles (MPs). They were made with heat induced gelation which resulted in small protein gels, which are in fact microgels of about 70 µm (Purwanti, Moerkens, Van der Goot, & Boom, 2012). Whey protein is a mixture of globular proteins isolated from whey, the liquid material created as a by-product of cheese production. Whey protein can be obtained in the form of whey protein concentrate or isolate. Whey protein isolate contains the most whey protein and has a protein content of more than 90%. There are four main classes of proteins in whey: β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and the immunoglobulins (Ig)(Morr & Ha, 1993). Some of their characteristics can be seen in Table 1 (De Wit, 1981; DeWit & Klarenbeek, 1984; J. Kinsella & Whitehead, 1989). These proteins have a globular conformation and are susceptible to denaturation and aggregation induced by heat and high pressure.

Table 1 - Characteristics of the major whey proteins

Protein	pI	% in whey protein
β-Lactoglobulin	5.2	60
α-Lactalbumin	4.8-5.1	22
Bovine serum albumin	4.8-5.1	5.5
Immunoglobulins	5.5-6.8	9.1

Heat treatment of whey protein at neutral pH and low ionic strength causes denaturation due to weakening and breakage of hydrogen and disulphide bonds. The native conformational structures are disrupted which allows polymerization of the dissociated and denatured protein molecules. Due to the formation of intermolecular disulphide, ionic and hydrogen bonds, gels are formed (Hoffmann & van Mil, 1997). Most of the surrounding solvent is physically immobilized (bound) by the three dimensional network which is formed (Morr & Ha, 1993). WPI MPs can contain water, but they also have shown to be able to take op more water or swell (Purwanti, et al., 2012), so gels have a certain water binding capacity (WBC). Macro gels, which are used in this study, also swell with water or another solvent to an extent controlled by the crosslink density, the polymer/water compatibility, and the presence of electrical charges (Fernandez-Nieves, Wyss, Mattsson, & Weitz, 2010). It is important to note that micro gels have much larger interfacial areas than macro gels due to their respective sizes, so the speed of water uptake might not be proportional to the MPs.

2.2 WBC

In this study, a distinction between water uptake and WBC is made. The WBC is "the ability of food or its components to hold water under certain conditions" (Labuza & Busk, 1979). Water can be bound to food, or more specific a protein, via hydrogen bonds, but also by physical or surface forces of the protein matrix or can be captured in free space within the protein matrix. In this study, the water uptake is defined as the gain in weight of the gel after soaking in water. The WBC is defined as the amount of water that remains in the soaked gel after centrifugation. Water that interacts with a food or more specific a protein molecule exhibits different properties from those of "free" water. When water is hold by the protein, it may be so strongly immobilized that it cannot be squeezed out. This can be due to certain (long range) forces which are involved in holding the water within the protein system (Chou & Morr, 1979). "Bound" water behaves differently from "free" water. To see differences in the mobility of water molecules and to define the state in protein-water systems, nuclear magnetic resonance (NMR) can be used (Kuntz Jr, Kauzmann, C.B. Anfinsen, & Frederic, 1974).

In this study, the WBC of whey proteins is tried to be increased. According to the Flory-Rehner theory, the swelling of a protein matrix depends on the hydrophilicity and/or the elasticity (Flory & Rehner Jr, 1943b; J. E. Kinsella & Morr, 1984; Omidian & Park, 2008). The elasticity of a gel is determined by its network. By changing the gel morphology, the elasticity is varied. The main determinant of this parameter is the crosslink density (Van der Sman, 2012). The crosslink density is often used to characterize a network, in this case a whey protein network. The crosslink density is the number of moles of elastically effective network chains per cubic centimetre of sample (Flory, 1985), but is currently also defined as the number of crosslinks in a polymer (protein) network.

Increasing the crosslink density will increase material stiffness, as has been shown for, inter alia, elastin-like polymers (Trabbic-Carlson, Setton, & Chilkoti, 2003), β-hairpin peptide hydrogels (Hule et al., 2008), gelatin films (Bigi, Cojazzi, Panzavolta, Roveri, & Rubini, 2002) chitosan, bovine serum albumin (BSA) and gelatin (Butler, Ng, & Pudney, 2003). Therefore, the lower the crosslink density will be, the higher the gel elasticity and accordingly the greater the swelling of the protein matrix might be. It has been shown that swelling is inversely proportional with crosslink density for ovalbumin gels (Van Kleef, Boskamp, & Van Den Tempel, 1978) and different microgels (Eichenbaum, Kiser, Dobrynin, Simon, & Needham, 1999). Though, the effect might not be that big as expected, since due to rearrangements in the network, less water binding sites might become available and/or steric hinder might cause less water to bound (Kuntz Jr, et al., 1974). To alter the crosslink density of proteins, certain ingredients can be used (Damodaran & Parkin, 2008; Shewan & Stokes, 2013).

2.3 Altering crosslink density

2.3.1 Dithiothreitol

In this study, dithiothreitol (DTT) is used to decrease the crosslink density of the macro WPI gels. DTT is a small redox-molecule which is also known as Cleland's reagent (Cleland, 1964). It has strong reducing properties, since once it is in its oxidized form, it forms a stable six membered ring with an internal disulphide bond, as can be seen in Figure 1. In this figure can also be seen that to form this stable ring, disulphide bridges in protein are broken into free sulphide groups. Though, the remaining free sulphide groups are able to form new disulphide groups and therefore the overall effect of DTT is uncertain (Zirbel & Kinsella, 1988). Giroux et al. (Giroux, Houde, & Britten, 2010), however, have shown that DTT breaks down disulphide bonds in whey proteins.

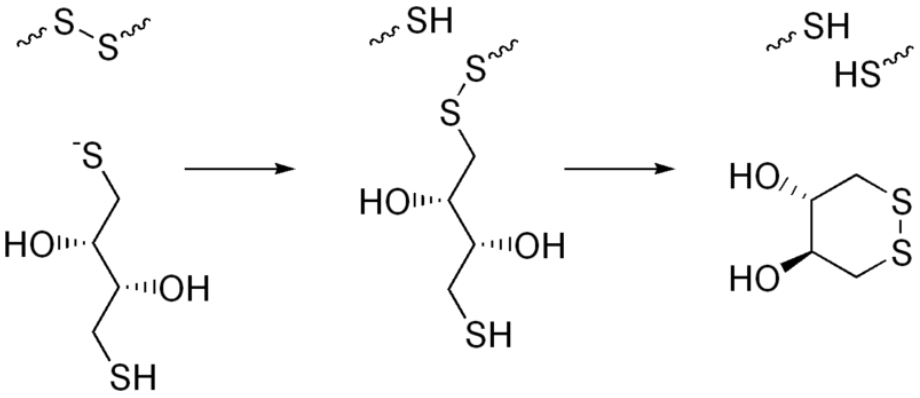


Figure 1 - Breaking of disulphide bridges by dithiothreitol

2.3.2 Transglutaminase

The crosslink density of the macro WPI gels was tried to be increased in several ways. This can be done by incubation in the chemical genipin or addition of or incubation in the enzyme transglutaminase (Tgase). Tgase is an enzyme that catalyses the formation of an isopeptide bond between a free amine group (e.g. lysine) and the acyl group at the end of the side chain of glutamine (Pisano, Finlayson, & Peyton, 1968), as can be seen in Figure 2. It has been shown in several studies that Tgase is able to react with whey protein (Aboumahmoud & Savello, 1990; Anuradha & Prakash, 2009; Truong, Clare, Catignani, & Swaisgood, 2004; Yildirim & Hettiarachchy, 1998).

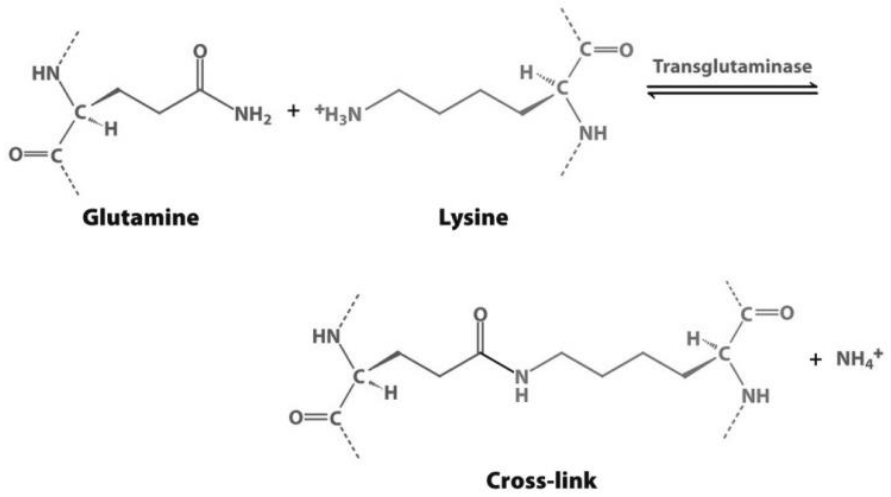


Figure 2 - Cross-linking of glutamine and lysine facilitated by transglutaminase

2.3.3 Genipin

Genipin is a chemical that is extracted from the fruit of *Gardenia jasminoides* (Endo, 1973). Genipin crosslinks proteins by a two-step reaction with free primary amine groups of proteins, which results in the formation of a blue colour (Butler, et al., 2003). The reaction mechanism can be seen in Figure 3. When both reactions took place, two free primary amine groups are crosslinked.

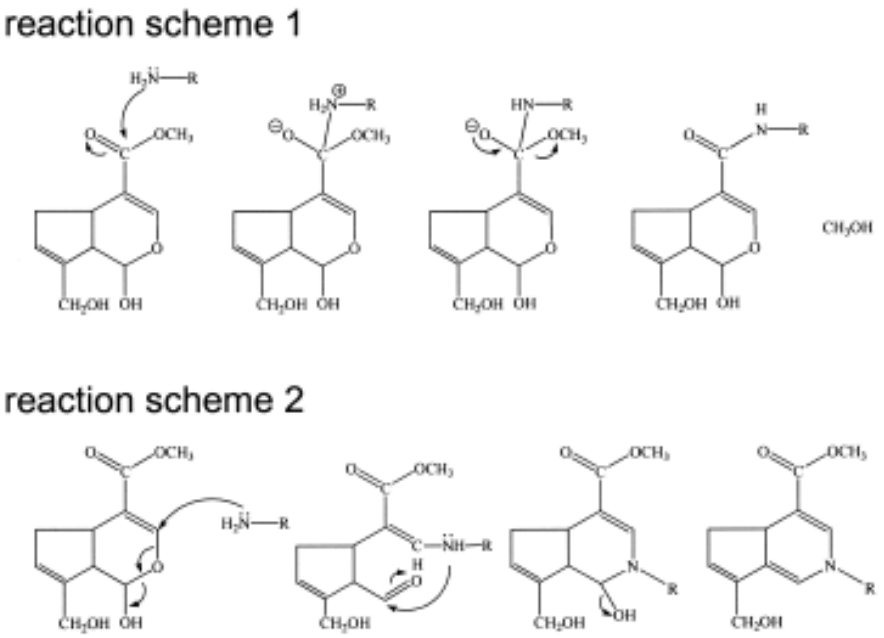


Figure 3 – Two-step reaction mechanism of genipin

2.3.4 pH

Lastly, it is tried to alter the crosslink density of the WPI macro gels by bringing their pH closer to the isoelectric point (pI) of whey protein. In Table 1 can be seen that the pI of whey proteins is 4.8-5.1. By bringing the pH close to the pI it is tried to reduce the electrostatic repulsions between the proteins and increase the electrostatic attractions. The interaction is repulsive when charges have the same sign and attractive when they have different signs (Israelachvili, 1992). The sign, magnitude and distribution of the charge on a protein are mainly determined by the pH. Above their pI proteins are negatively charged, below it they are positively charged and at the pI, they have no net charge (Bryant & McClements, 1998). The proteins contain both positive and negative charges close to the pI. Therefore, charges with opposite sign may be involved in aggregation, so more crosslinks might be formed (Nicolai, Britten, & Schmitt, 2011). A pH close to the pI was hence chosen to increase the crosslink density of the macro WPI gels. In this study a pH of 5.8 was used, to be able to compare the results from the MPs of the study by Peters (unpublished results). It has been shown that ionized amino acid groups bind more water than non-ionized groups (Kuntz, 1971). At the isoelectric point, where the net charge is zero, protein molecules exhibit minimal hydration and swelling (Chou & Morr, 1979). Therefore, it might be expected that the macro gels with a pH of 5.8 will swell less.

3 Materials and Methods

3.1 Materials

For production of the whey protein macro gels, whey protein isolate (WPI; BiPRO) with lot no. JE 034-7-440 (Davisco Food International Inc., Le Sueur, MN) with a protein content of 97.6% on dry basis was used. To alter the crosslink density, the gels were treated with dithiothreitol (DTT), genipin powder (both Sigma-Aldrich, Germany) or microbial Ca2+ independent transglutaminase (Tgase; Activa WM; activity of 81-135 units g-1 according to the manufacturer)(Barentz Raw Materials, the Netherlands). To decrease the pH of the WPI or a buffer solution, 1M HCl was used. For analysing the produced gels, several chemicals were used. In Ellman's method, to measure the amount of free sulphide groups, Bis-Tris and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) (both from Sigma-Aldrich, Germany) were used. In this method, cysteine hydrochloride monohydrate (Sigma-Aldrich, Germany) was used as a protein standard. In the OPA-method, to check for the amount of free lysine groups, sodium dodecyl sulphate (SDS), sodium tetraborate decahydrate (Borax), DTT (all from Sigma-Aldrich, Germany) and ethanol were used. L-lysine (Sigma-Aldrich, Germany) was used as a protein standard to obtain the standard curve. When water was used in an experiment, this always was Milli-Q water (resistivity of 18.2 MΩ cm at 25 °C, total oxidizable carbon <5 ppb, Merck Millipore, France).

3.2 Methods

3.2.1 Production of gels

3.2.1.1 Preparation of standard gels

WPI powder was mixed with Milli-Q water to obtain a 40% w/w WPI suspension. To dissolve the WPI powder completely, the suspension was stirred with a laboratory overhead mixer (IKA RW 20 digital) at 100 rpm at room temperature for at least 2 hours. Accordingly, the suspension was stirred at 4°C with a magnetic stirrer overnight. Before the heat induced gelation, the WPI suspension was centrifuged to remove air bubbles in 50 mL centrifuge tubes at 4700 rpm and 20°C for 20 min. After that, the remained foam at the surface was removed manually. Next, the WPI suspension was poured carefully into teflon tubes and the lids were gently turned on the tubes. The tubes containing the protein suspension were heated in a rotating water bath of 95 °C at 30 rpm for 50 min, after which the tubes were put in ice-water for 30 min to cool them. As a result, gel cylinders with a diameter of 2 cm were obtained which could be cut in the desired length. To be able to compare the standard gel as good as possible with gels with altered crosslink density, about 10g standard gel was soaked in 200 mL water for 24h. The standard gel was made in duplo.

3.2.1.2 Preparation of gels with a changed crosslink density

Besides the standard gels, gels with various crosslink densities were made by giving the gels different treatments. A 20% w/w Tgase solution was made, stirred for 2h at room temperature and kept in a fridge (4°C) for one week. Before the Tgase solution was used, it was filtered over a 0.45 µm filter. The effect of Tgase on the gels was investigated by either adding it to the WPI solution before mixing or by incubating the fresh gels in a Tgase solution. When the Tgase solution was added to the WPI solution before mixing (BM), the Tgase solution was added in an enzyme:protein ratio of 1:5 and water was added to obtain a 40% w/w WPI solution. The suspension was stirred with a laboratory overhead mixer (IKA RW 20 digital) at 100 rpm at room temperature for at least 2 hours. Accordingly, the suspension was stirred at 4°C with a magnetic stirrer overnight. Accordingly, the same steps as

for production of the standard gels were taken. To be able to compare the Tgase BM gel as good as possible with other gels, about 10g Tgase BM gel was soaked in 200 mL water for 24h. The Tgase BM gel was made in duplo.

The gel that reacted with the Tgase solution after mixing was made by incubating about 10 g gel in 50 mL Tgase solution, which is an enzyme:protein ratio of 1:1, for 24h at 50°C.

The gels that reacted with DTT or genipin were made by incubating about 10 g of fresh gel in a 50 mL solution that contained one of these chemicals and incubating this at 25°C for 24h. The gels treated with genipin were incubated in a 2.21 mM or a 0.39 mM genipin solution and the gels treated with DTT were incubated in a 40 mM DTT solution. To obtain the gel of pH 5.8, the pH of the 40% w/w WPI solution was decreased with 1 M HCl just before mixing. About 10g gel with pH 5.8 was also soaked in 200 mL water for 24h to be able to compare this gel with the other gels.

3.2.1.3 Preparation of dried gels

Besides fresh gels, dried gels were investigated on their functional properties. To obtain these dried gels, whey gel cylinders with a diameter of 20 mm and height of about 5 mm were put in an oven of 50°C for 48h. A dried version of the gel was made for all treatments, including a duplo for the standard and the Tgase BM gels.

3.2.2 Dry matter content of the gels

To check whether the 40% w/w WPI solution was made correctly and see how much water was taken up during incubation in a DTT/Tgase/Genipin solution, the dry matter content of all fresh gels was determined. Whey gel cylinders with a diameter of 20 mm and height of 3 mm were put in an oven of 105°C. After 24h the gels were weighed. The weight was compared with the fresh gels and was used to calculate the dry matter content.

3.2.3 Crosslink density of the gels

3.2.3.1 Amount of free sulphide groups

The crosslink density of the macro WPI gels was tried to decrease by treating them with DTT, which breaks disulphide bonds in proteins. The effect of this treatment was studied with Ellman's method (Ellman, 1959). The same method was used as described by Peters (unpublished results) with some adaptions. The amount of free sulphide groups was measured for the standard gel incubated 24h in water (in duplo), the gels incubated in a 40 mM DTT solution and the gels made at pH 5.8 incubated 24h in water. Both fresh gels, which were swollen for 24h, and dried gels were measured. For the measurement, a slice of gel from the middle of the gel cylinder soaked in one of the solutions was taken, so that the solution mainly had penetrated from the edge. The fresh or dried gels were first milled with an analytical mill (IKA A11 basic). Accordingly, a protein dispersion of 5 g protein per liter water was made with the milled gels. Then, 0.4 mL of the gel dispersion was added together with 5 mL of 50 mM Bis-Tris buffer (pH 7) and 0.5 mL DTNB solution (1 mg per mL Bis-Tris buffer) in 10 mL tubes. These tubes were wrapped in aluminium foil and rotated for 10 min. Subsequently, the tubes were centrifuged for 1 min at 4700 g (Sorvall Legend XFR, Thermo Scientific) and 3 mL of the supernatant was transferred to a 4.5 mL cuvette. After in total 15 min of incubation the absorbance was measured with a spectrophotometer (Genesys 2, Thermo Scientific) at 412 nm. For the standard series cysteine hydrochloride monohydrate was used. The gel samples were prepared and measured in quadruplicate, the standard was measured in triplicate.

3.2.3.2 Amount of free amino groups

The crosslink density of the macro WPI gels was tried to increase by treating them with Tgase, genipin or lower their pH to 5.8. To determine what effect these treatments had on the crosslink density, the gels were checked with the OPA-method (Nielsen, Petersen, & Dambmann, 2001). The same method was used as described by Peters (unpublished results) with some adaptions. The amount of free amino groups was measured for the standard gel incubated 24h in water (in duplo), the gels incubated in a 0.39 and 2.21 mM genipin solution, the gels incubated in a 20% w/w Tgase solution, the gels made with Tgase BM in a 1:5 enzyme:protein ratio (in duplo) and the gels made at pH 5.8 incubated 24h in water. Both fresh gels, which were soaked for 24h in a watery solution, and dried gels were measured. For the measurement, a slice of gel from the middle of the gel cylinder soaked in one of the solutions was taken, so that the solution mainly had penetrated from the edge. First, the fresh and dried gels were milled with an analytical mill (IKA A11 basic). Accordingly, 10 mg of the milled gel was mixed with 1 mL of a 10% w/w SDS solution and 4 mL of a 15.625 mM Borax solution for at least 1 h. Afterwards, the tubes were centrifuged for 15 min at 4500 rpm. Meanwhile, the OPA reagent was prepared. 9.53 g Borax and 2.5 g SDS were dissolved in 200 mL water. The reagents were completely dissolved before continuing. 200 mg OPA was dissolved in 5 mL ethanol and transferred to the before-mentioned solution. Then, 220 mg DTT was added to the solution and the solution was made up to 250 mL with water. The solution was filtered over a 0.45 µm filter and stored in a bottle wrapped in aluminium foil. When both the OPA reagent and the samples were prepared, the samples were subjected to the reagent. First, the absorbance of the OPA reagent was measured at 340 nm with a spectrophotometer (Genesys 20, Thermo Scientific) by transferring 1.5 mL of the reagent to a 4.5 mL cuvette. Then, 200 µL of the supernatant of the sample was put in an eppendorf tube and the OPA reagent was transferred from the cuvette to this eppendorf tube. The tube was vortexed and incubated for 5 min. Accordingly, the absorbance was measured again. For the standard series L-lysine was used instead of the sample. The gels were prepared and measured in quadruplicate, the standard was measured in triplicate.

3.2.4 Swelling of the gels in water

Fresh and dried whey gel cylinders with a diameter of about 20 mm and height of about 5 mm were cut out the middle of the original gel cylinders, so that influence of penetration of a (crosslinking) solution from the top and bottom of the gel cylinder was excluded as much as possible, and were put in 200 mL water in 250 mL beaker glasses at 25°C. For the dried gels, the weight of the gel cylinders was measured after 2, 4, 6 and 24h. The fresh gels were measured after 24h of soaking, which resulted for a total soaking time of 48h in a watery solution (incubation solution and water). The weight and size difference between the fresh or dry and hydrated gels was used to determine the water uptake. The measurement was done at least in triplicate.

3.2.5 Water binding capacity of the gels

The water binding capacity (WBC) of the whey gels was determined using a centrifugation procedure, called the net-test (Hermansson & Lucisano, 1982). Whey gel slices with a diameter of about 20 mm and height of about 5 mm were cut out the middle of the original gel cylinders, which had been incubated in a DTT, genipin or Tgase solution or in water, were hydrated in water at 25°C for 24h. A sample from the middle of the gel slice was taken, placed in a centrifuge tube containing a filter with a pore size of 0.2 µm, and centrifuged at 3000 and 14680 rpm (Eppendorf-Centrifuge 5424) at room temperature for 20 min. The results of centrifugation at 3000 rpm gave an indication for how strong the water was bound. The weight difference between the fresh gels which were soaked for in total 48h in a watery solution (incubation solution and/or water) and the hydrated gel after centrifugation at 14680 rpm was used to calculate the WBC. This method only gives relative WBC values, since the results heavily depend on the used method and circumstances. Therefore the obtained results can only be compared with those obtained using the same method under identical conditions (Trout, 1988). The measurement was done in quadruplicate.

3.2.6 Mechanical behaviour

To measure the strength and elasticity of the obtained fresh and dried whey gels, large deformation tests were used. Cylindrical samples with a diameter of about 20 mm and a height of about 20 mm for the fresh gels and about 6 mm for the dried gels were measured. Those samples were all soaked in a watery solution (incubation solution and/or water) for 24h and taken from the middle of the gel cylinder. The whey protein gel samples were subjected to uniaxial compression tests by a texture analyser (Instron 5564). A stainless steel cylindrical probe with a flat base diameter of 50 mm was attached to the moving crosshead. The gels were compressed at a constant speed of 1 mm/s up to a maximal applied force of 1 kN, with data acquisition every 6 ms. A load cell of 2000 N was used. Every gel was measured in triplicate. The main purpose of these measurements was to compare gels rather than allow an exact mechanistic interpretation of the data. From the obtained data, the Young's modulus at a true strain of 0.45-0.65 was calculated. Only for the fresh gel treated with DTT and the dried gel with a pH of 5.8 the Young's modulus was calculated at a different true strain, 0.15-0.3 and 0.15-0.25 respectively, since a true strain of 0.45 or higher was not reached for those samples.

3.2.7 Strength of protein-water bonds

To study the strength of the protein-water interactions in the fresh gels after 24h swelling in a watery solution (incubation solution and/or water) at 25°C, nuclear magnetic resonance (NMR), also called ^{1}H relaxometry, was used. This watery solution was 40 mM DTT, 2.21 mM genipin, a 20% w/w Tgase solution or water. NMR is based on the principle that atomic nuclei have a spin and therefore have its own small magnetic field. This magnetic field has a certain orientation, which differs per nucleus. After a 90° radio frequency (RF) pulse the nuclear spins are aligned in one direction, but this arrangement is gradually lost due to interactions between the spins. The rate of this decay is measured by the spin-spin relaxation time (T_2). Due to a difference in interaction with other spins, the T_2 of "bound" water is different from "free" water (Kuntz Jr, et al., 1974). The addition of a solute, such as a globular protein as whey proteins, to water causes a decrease in the relaxation time (Hills, 1998). This principle is used to study how strong water is bound within the different macro WPI gels.

^{1}H relaxometry was performed with a Maran Ultra NMR spectrometer (0.72 T magnetic field strength, 30.7 MHz proton resonance frequency), controlled by RINMR software (Resonance Instruments Ltd., Witney, United Kingdom). T_2 relaxation decay curves were recorded by means of a standard Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. The CPMG decay train consisted of 2048 echoes of five data points each. The sample time between the data points in each echo was 10 µs resulting in a spectral width of 100 kHz. The time between each echo was 1.0 ms. Experiments were averaged over 32 scans with a repetition time of 10 s resulting in a total measurement time of 6.5 minutes. Each CPMG echo train was carefully phase corrected and each echo was reduced to one averaged data point using the IDL package (ITT Visual Information Solution., Boulder, CO USA).

The Levenberg-Marquardt non-linear least squares algorithm implemented in SPLMOD (Provencher & Vogel, 1983) was used to analyse the T_2-experiments. The latter fits a sum of exponential decays to the echo train resulting in a relative amplitude and T_2 relaxation time constant per component.

3.2.8 Over time imaging of swelling

To see the influence of a denser crosslinked ring at the edge of the gel cylinder, the swelling of the standard gel and the gel treated with 2.21 mM genipin at 21.5°C was studied further by use of magnetic resonance imaging (MRI). For the gel treated with 2.21 mM genipin, a sample from the middle of the gel cylinder was taken, in order to exclude the influence of a more crosslinked top or bottom of the gel. Therefore, only the rand of the gel sample was more intensively crosslinked. Signal intensity and contrast in signal intensity in such an image are strong functions of relaxation times. There are two different relaxation times: spin-spin relaxation (T_2), as explained above, and spin-lattice relaxation (T_1). In the absence of an external magnetic field, the orientations of the magnetic field of the nuclei are at random and are of equal energy. When an external magnetic field is applied, these energy levels split into a higher and a lower energy level. Nuclei in the lower energy level are in line with the magnetic field and nuclei in the higher energy level oppose the applied field. When energy is applied in the form of RF, energy is absorbed by the nucleus and the small magnetic moment “flips” so that it opposes the applied magnetic field and now is in the higher energy state. When the RF is taken away, the nuclei want to turn back to the lower energy state. The time it takes the nucleus to turn back to the lower energy state is called the spin-lattice relaxation time (T_1) and also differs per “sort” of nucleus (Bloembergen, Purcell, & Pound, 1948). T_2 is more sensitive for protein-water interactions than T_1. Therefore the focus is here put on T_2 measurement. When monitoring the change in relaxation time over time, swelling of the gels can be monitored.

MRI was performed on a 3 T (128 MHz for protons) MRI system (Bruker, Karlsruhe, Germany), consisting of an Avance 200 console, a superconducting magnet with a vertical bore (Magnex, Oxford, UK), a 1 T/m gradient coil, and a birdcage RF coil with an inner diameter of 4 cm.

Swelling of gel cylinders was followed for 65 hours. During this time two sequences were alternated: a MSE sequence to obtain 2D proton density and R2 maps, and a TSE sequence to obtain 3D T_1-weighted images.

Two dimensional proton density and R2-maps where obtained using Multi Spin Echo (MSE) MRI sequence (Edzes, Van Dusschoten, & Van As, 1998; Scheenen, Van Dusschoten, De Jager, & Van As, 2000). A repetition time of 2 s, an echo time of 6 ms and a spectral width of 50 kHz was used. Per echo train 128 echoes were acquired; 4 acquisitions were averaged to improve image quality. The FOV was 40 x 35 mm^2 with a matrix size of 64 x 64 resulting in an in-plane resolution of 313 313 μm^2. The slice thickness was 3 mm. The experiments lasted 17 minutes.

Three dimensional images were obtained using a Turbo Spin Echo (TSE) MRI sequence (Scheenen, et al., 2000), with a repetition time of 2 s and a spectral bandwidth of 50 kHz. Because the water surrounding the gel cylinder had a longer T_1 compared to the T_1 of the gel itself, the short repetition time enhanced the contrast between gel and surrounding water. The images were measured with an effective echo time of 6 ms. Per scan 32 echoes were measured for image formation to speed up the measurements. Odd and even echoes were separately phase encoded forming two different images to avoid Nyquist ghost artifacts, so the speed up or turbo factor was 16. Four acquisitions were averaged to improve image quality. The Field-Of-View (FOV) for the 3D images was 40.0 x 40 x 40 mm^3 with a matrix size of 64 x 64 x 64 resulting in an in-plane spatial resolution of 313 x 313 x 313 μm^3. These experiments lasted 34 minutes and 8 seconds.

All data handling and analysis was performed with home-built software written in IDL (RSI, Boulder, CO).

4 Results

4.1 Crosslink density of the gels

4.1.1 Effect of DTT and pH on the amount of free sulphide groups

The effect of DTT and lowering the pH on the amount of free sulphide groups in a gel was checked with Ellman's method. Figure 4 shows that the treatment with DTT resulted in more free sulphide groups, so the crosslink density was decreased. The average amount of free sulphide groups was lower for dried gels than for fresh gels soaked 24h in a watery solution (40 mM DTT or water), though the difference was not significant. According to the manufacturer, the maximum amount of free sulphide groups in WPI is 248 µmol/g protein, so not all disulphide bonds present were broken during the DTT treatment. The amount of free sulphide groups in the fresh gels soaked 24h in water did not change significantly when the pH of the gel was brought to a pH closer to the pI of whey protein, but the amount of free sulphide groups decreased somewhat for the dried gels (Figure 4B).

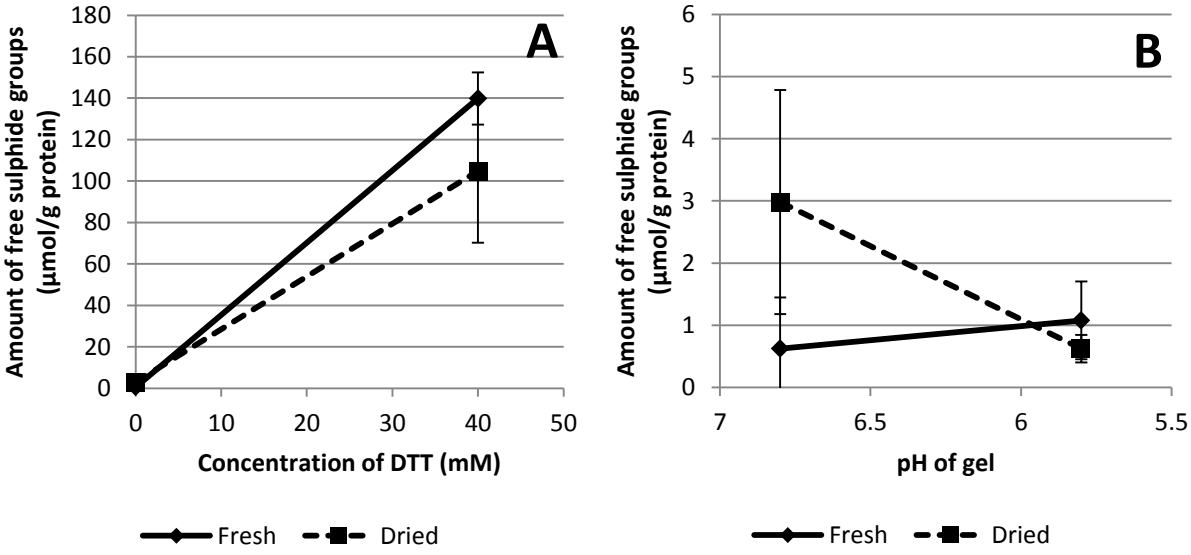


Figure 4 - Amount of free sulphide groups in fresh gels soaked 24h in a watery solution (40 mM DTT solution and/or water) and dried gels treated with A) DTT and B) made with a pH of 5.8

4.1.2 Effect of genipin, Tgase and pH on the amount of free amino groups

The effect of genipin, Tgase before (BM) and after (AM) mixing and lowering the pH closer to the isoelectric point of whey protein on the amount of free amino groups in a gel was checked with the OPA-method. These treatments should result in less free amino groups than for the standard gel, since more bonds with amino groups should be made. The resulting amount of free amino groups is shown in Figure 5 and Figure 7.

Figure 5 shows that the amount of amino groups decreased when the fresh gels were incubated in 2.21 mM genipin for 24h, while with a genipin concentration of 0.39 mM an increase in the amount of amino groups was found. Hence, according to the OPA-method, the fresh gel incubated in a 0.39 mM genipin solution had a decreased crosslink density compared to the standard gel, which is unexpected, and the fresh gel incubated in 2.21 mM genipin had an increased crosslink density compared to the standard gel. It has been reported that during the reaction with genipin a reaction

occurs resulting in a blue colour (Butler, et al., 2003), so the extent of the reaction can be seen visually. Figure 6 shows that at both concentrations of genipin a reaction had occurred, since the colour of the fresh gels was light brown (after incubation in 0.39 mM genipin) and dark blue (after incubation in2.21 mM genipin). The darker the colour, the less free amino groups were most likely present, so this confirmed that the fresh gel incubated in 2.21 mM genipin was more intensively crosslinked than the fresh gel incubated in 0.39 mM genipin. The dried gel incubated in 0.39 mM genipin before drying showed a decreased amount of free amino groups, so an increased crosslink density, and the dried gel incubated in 2.21 genipin before drying showed no significant difference from the standard dried gel.

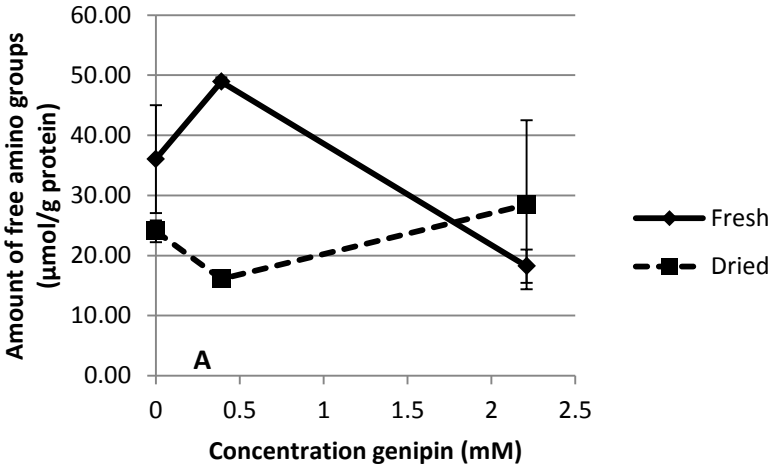


Figure 5 - Amount of free amino groups in the fresh gels which were soaked 24h in a watery solution (genipin solution and/or water) and dried gels treated with genipin

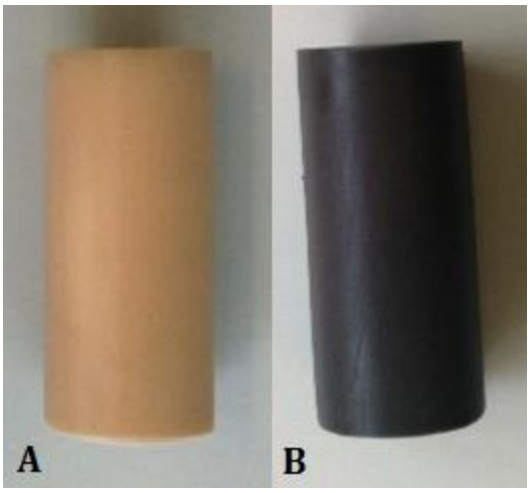


Figure 6 - 40% WPI gels treated with A) 0.39 mM genipin or B) 2.21 mM genipin

Figure 7 shows, as expected, that the amount of free amino groups in the fresh gel made with Tgase before mixing (BM) was lower than the fresh standard gel, both soaked for 24h in water. Also the amount of free amino groups of the dried gels made with Tgase BM and Tgase after mixing (AM) treatment was lower than dried standard gels. For the fresh gel treated with Tgase AM, the amount of free amino groups was not significantly lower than for the standard fresh gel soaked for 24h in water.

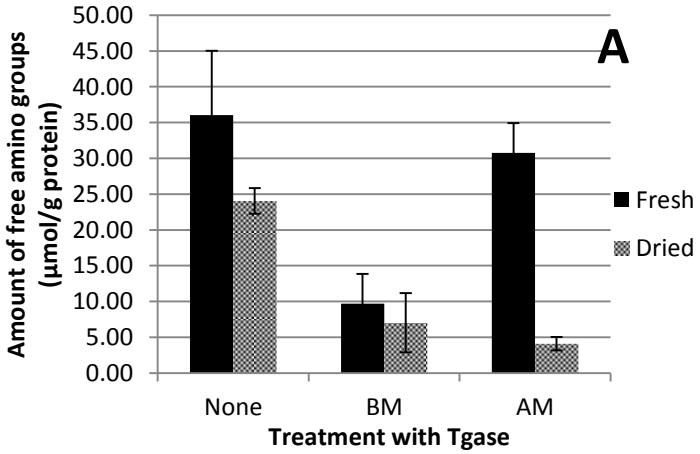


Figure 7 - Amount of free amino groups in the fresh gels which were soaked for 24h in a watery solution (Tgase solution and/or water) and dried gels treated with Tgase

The amount of free amino groups was higher for the gels with a pH of 5.8 for both the fresh gel soaked for 24h in water and the dried gel (Figure 8), so those gels had a decreased crosslink density according to the OPA-method, while a stronger cross-linked network was expected.

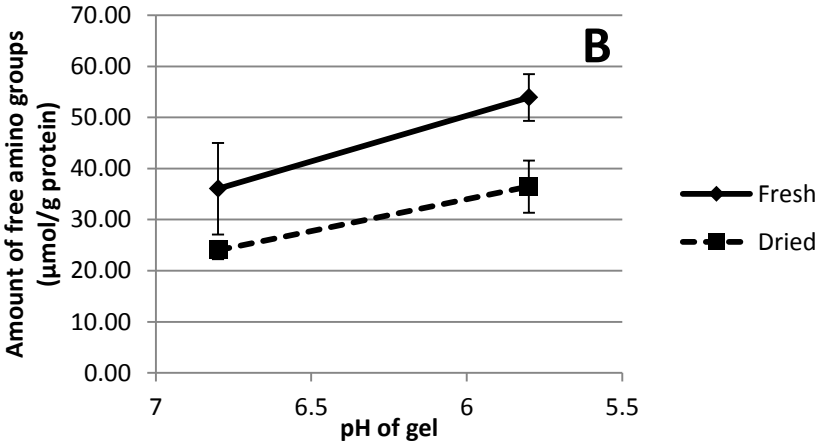


Figure 8 - Amount of free amino groups in the fresh gels made at pH 5.8 which were soaked for 24h in water and dried gels made at pH 5.8

4.2 Swelling of the gels in water

According to the Flory-Rehner theory a decrease in the crosslink density will result in an increase in the water uptake of the gels, while an increased crosslink density will result in a decrease in the water uptake. The water uptake as a function of the crosslink density can be found in Figure 9. As expected, a decreased crosslink density induced with DTT resulted in an increased water uptake after in total 48h of swelling in a watery solution (incubation solution and/or water) for fresh gels and 24h in water for dried gels (Figure 9). Figure 9B shows there was no trend between the samples with an increased crosslink density and their water uptake. The fresh Tgase BM and 2.21 mM genipin gels had a lower amount of free amino groups, but took up more water than the standard gel. The gel with a pH of 5.8 had more free amino groups, but it took up less water than the standard gel. The water uptake of the dried gels was lower than the uptake of the fresh gels, which can also be seen per treatment in Figure 9 and Figure 11. The swelling of dried gels over time can be found in Appendix 1.

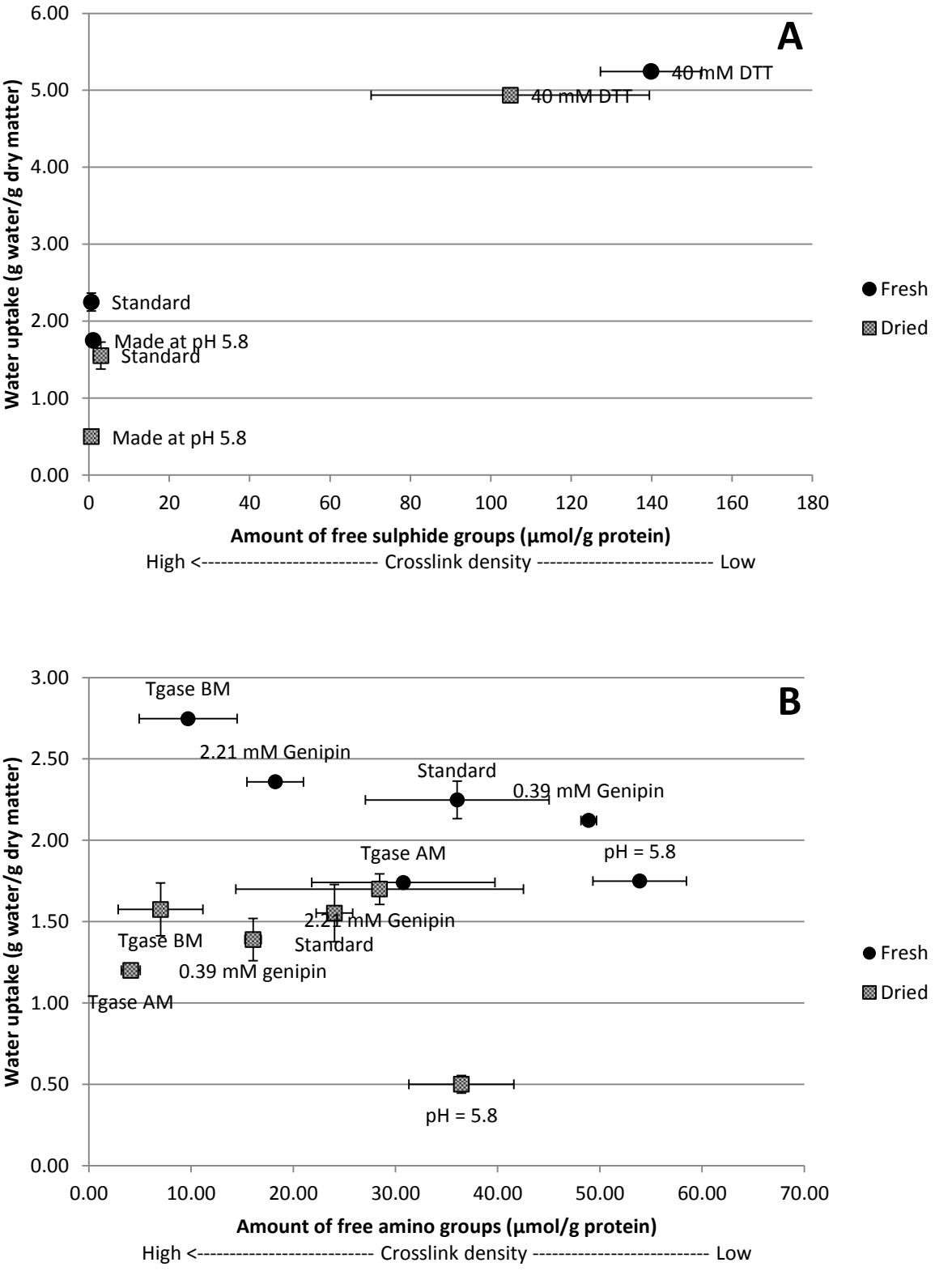


Figure 9 - Relation between crosslink density and water uptake after in total 48h of swelling in a watery solution (incubation solution and/or water) for A) gels treated with DTT and B) gels treated with genipin, Tgase or made at pH 5.8

4.3 Water binding capacity of the gels

By use of centrifugation at 14680 rpm it was investigated how strong and how much absorbed water was bound in the gels which were used for determination of the water uptake. Figure 10 shows which part of the water in the gel after 48h of soaking is “bound” en which part is “free”. The water in the fresh gel made at a pH of 5.8 consisted for almost half of “free” water and also the water in the fresh gel treated with 40 mM DTT consisted considerably of “free” water. The water in the other gels consisted only of about ten percent or less of “free” water. The part of “free” water was smaller for the dried gels after 24h of soaking than for the fresh gels after in total 48h of soaking.

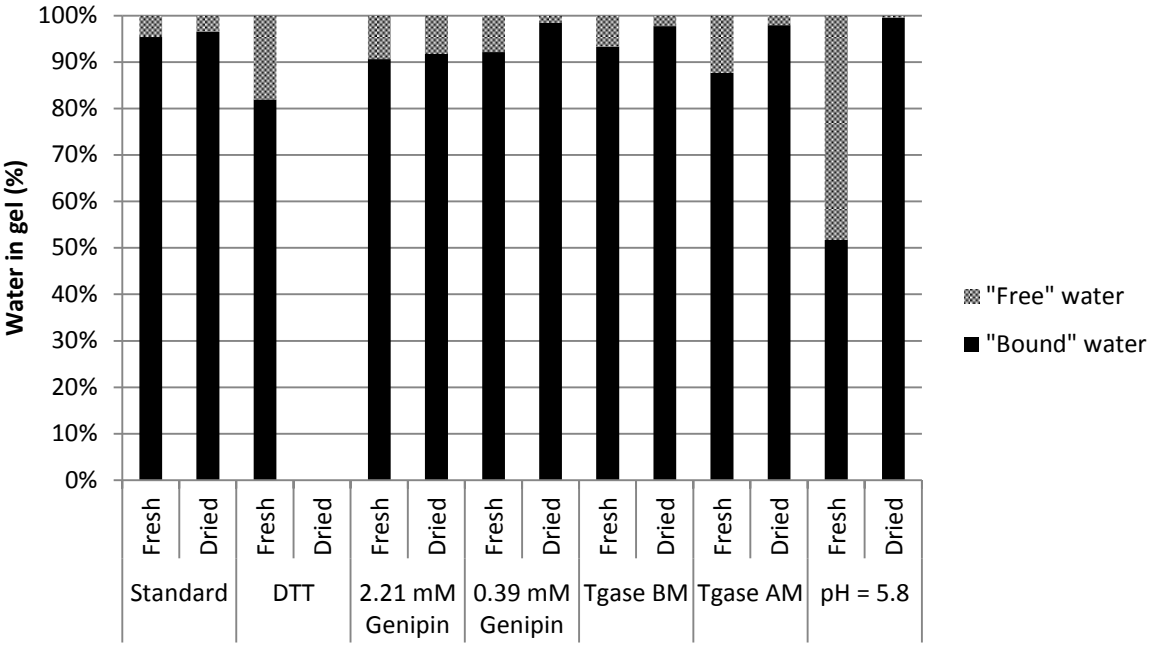


Figure 10 - Percentage of "free" and "bound" water of total amount of water per gel after 48h soaking in a watery solution (incubation solution and/or water) for fresh gels and 24h soaking in water for dried gels and centrifugation at 14680 rpm

In Figure 11 the difference between total water uptake and “bound” water (WBC) per gram of dry matter can be found quantitatively. The water uptake and WBC was higher for fresh gels than for dried gels. To all gels applied that less water was taken up after drying than water was “bound” in fresh gels, except for the gel treated with 40 mM DTT (Figure 11A).

In Figure 11B is shown that fresh gels made with Tgase before mixing took up and bound more water than standard gels. Figure 11C shows that incubation in 2.21 mM genipin also increased the water uptake and the WBC of as well the fresh as the dried gels. Decreasing the pH of the gel to 5.8 led to a lower water uptake and WBC, as can be seen in Figure 11D.

The results of the centrifugation of the soaked gel samples at 3000 and 14680 rpm can be found in Appendix 2.

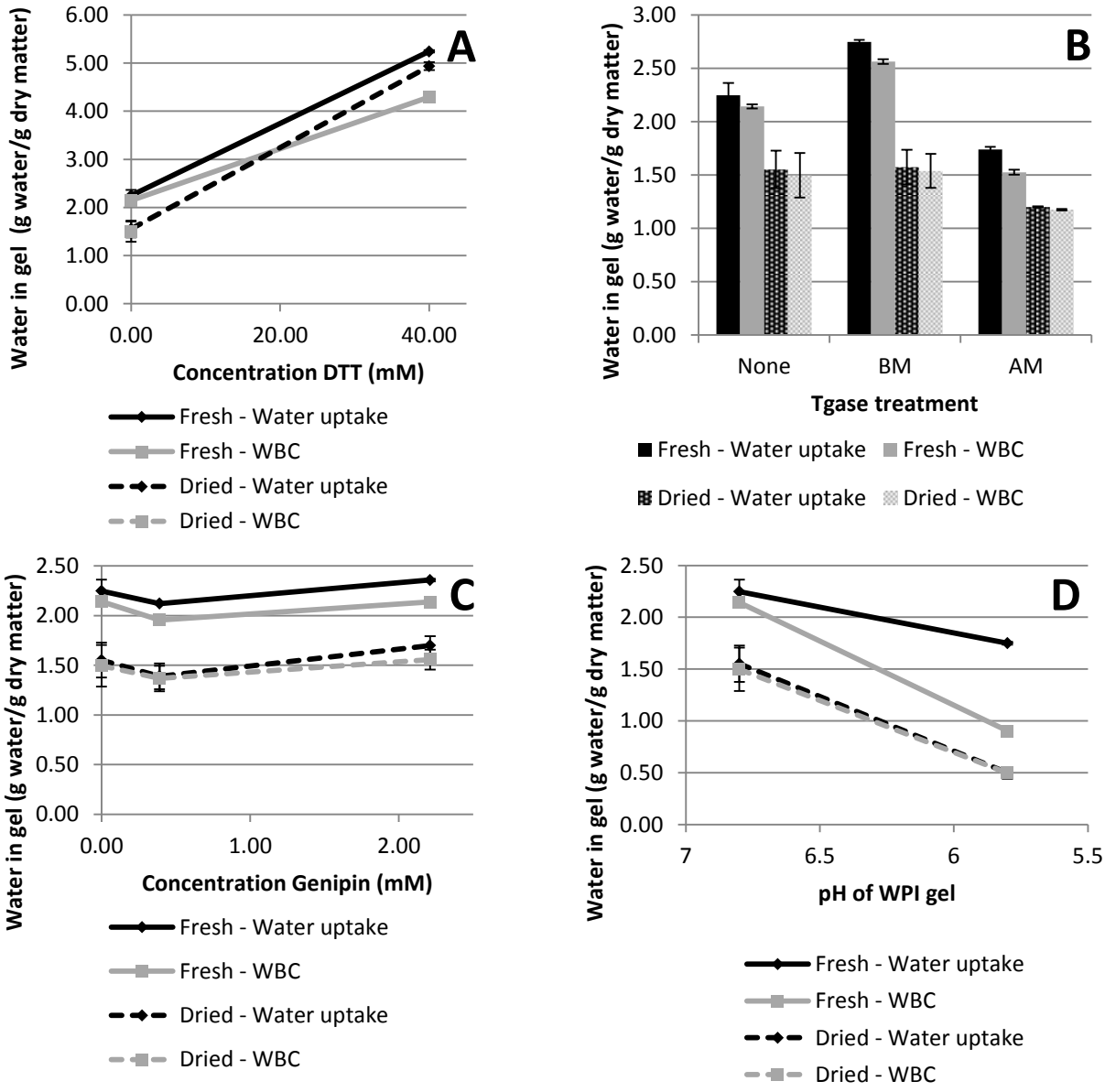


Figure 11 - Water uptake and WBC, measured after centrifugation at 14680 rpm for 20 min, of fresh gels soaked 48h in a watery solution and dried gels soaked 24h in water for A) gels treated with DTT B) gels treated with Tgase C) gels treated with genipin or D) with a lowered pH

4.4 Strength of protein-water bonds

By using NMR the protein-water interactions in the gels were investigated. With NMR the relaxation rate (R_2) of the ^1H protons was determined, and via a modelcurve (Peters, unpublished results) this rate was converted to the corresponding water concentration in the gel.

The measured T_2 and corresponding R_2 values can be found in Appendix 3. The higher the water concentration, the more the gel has swollen, since all gels started as 40% WPI gels. For all gels can be seen that the gels had swollen more at the edge than at the middle (Figure 12), so probably the swelling process was not fully performed yet after 24h in a watery solution (incubation solution and/or water). The gels treated with DTT had the highest water concentration at the edge of the gel, but in the middle the water concentration was the same as the standard gel. The calculated water

concentrations from the obtained T_2 values were compared with the water concentration of the gels measured by oven drying. All water concentrations obtained by oven drying were lower than the values obtained by NMR. The gels treated with crosslinking agents to increase the crosslink density all swelled less than the standard gel, as expected, except the gel treated with 2.21 mM genipin.

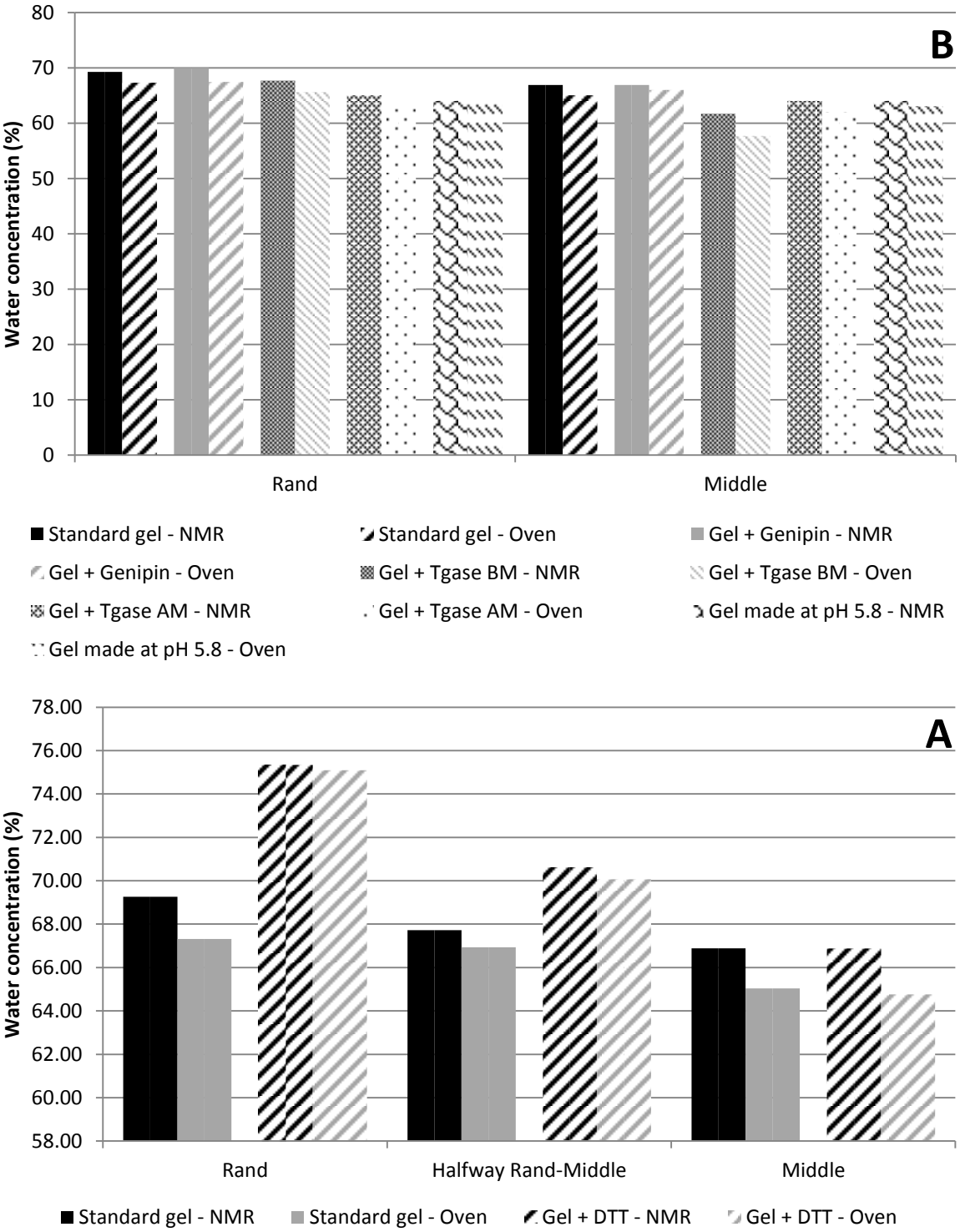


Figure 12 - Calculated WPI concentrations (%) from T_2 values (NMR) and measured by oven drying of A) gels with a decreased crosslink density and B) gels treated with crosslinking agents, which had been 24h in a watery solution (incubation solution and/or water)

4.5 Over time imaging of swelling

The swelling of the standard gel and the gel treated with 2.21 mM genipin was monitored during 65h by use of MRI. In Figure 13 the longitudal relaxation rate (R2) as a function of the distance from the centre of the gel is presented. The lower the R2, the more the gel swelled. It can be seen that the R2 decreased over time for both the standard gel and the gel treated with genipin. The R2 of the gel treated with genipin was lower at the start throughout the whole gel than the standard gel. This was because the gel treated with genipin had been in a watery solution for 24h, while the standard gel had not. Therefore, the results cannot be compared straightforward. The lower R2 closer to the edge of the gel treated with 2.21 mM genipin at the start of the measurement indicates a higher water concentration at the edge, which is a result of the "pre-soaking" in the genipin solution. In the end, the swelling of the standard gel became more equal to the gel treated with genipin, but the gel treated with genipin had still taken up more water. It can be seen that the R2 values were about the same throughout the whole diameter of the gel at the end of the measurement, so the gels were swollen (almost) maximally and therefore more homogenous in concentration again. Lastly, in Figure 13 can be seen that gels increased in size upon taking up water, since the maximal distance from centre is larger after 65h in water for both gels.

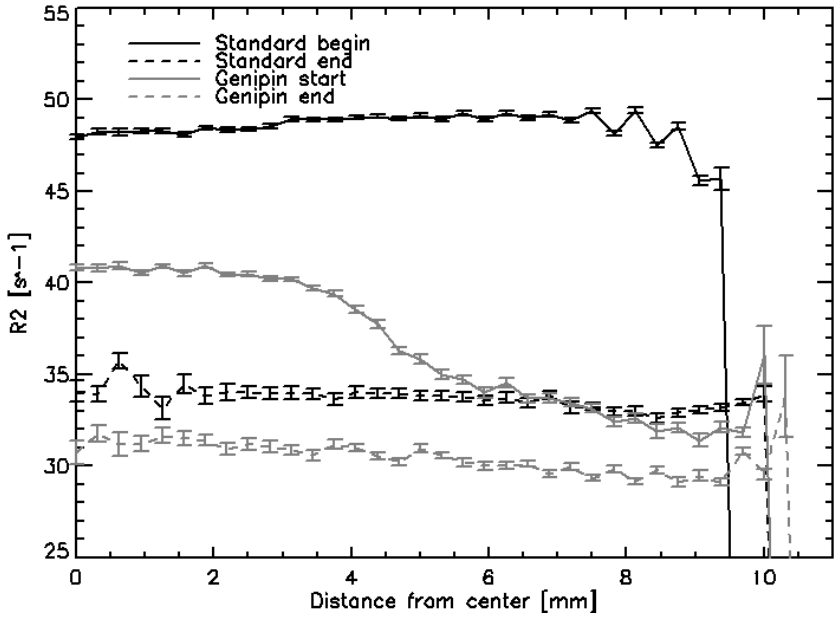
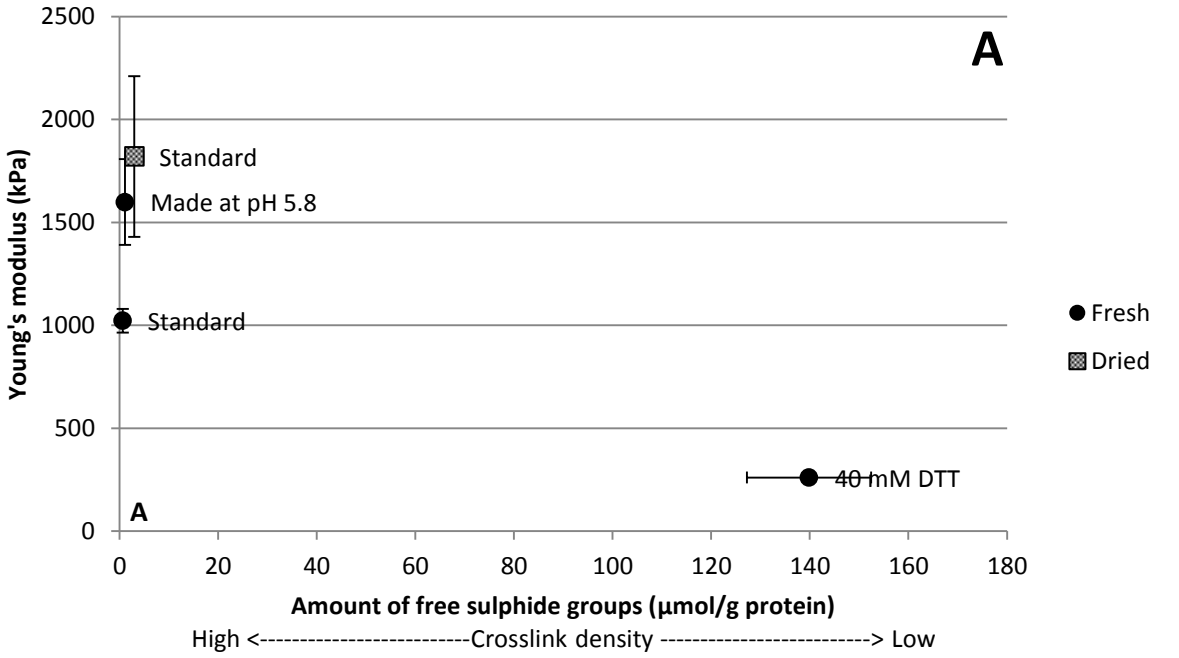


Figure 13 - The longitudinal relaxation rate (R2) as a function of distance from centre of the standard gel and the gel treated with genipin at t=0 ("start") and t=65 ("end")

4.6 Mechanical behaviour

Changing the crosslink density should influence the elasticity of the gels, according to the Flory-Rehner theory. An increase in the crosslink density should lead to a decrease in elasticity of the protein network and accordingly a decrease in WBC and vice versa. In Figure 14, it can be seen that no relationship between crosslink density and gel elasticity was found. What can be seen in Figure 14, is that dried gels had a higher Young's modulus, so a lower elasticity, than fresh gels. Especially the dried gel made at pH 5.8 after 24h of soaking was very strong (high Young's modulus). Furthermore, the gels treated with 40 mM DTT were considerably weaker than the standard gels (Figure 14A) and the gels made at pH 5.8 were increased in gel strength (Figure 14B), so altering the crosslink density can have an influence on gel strength.



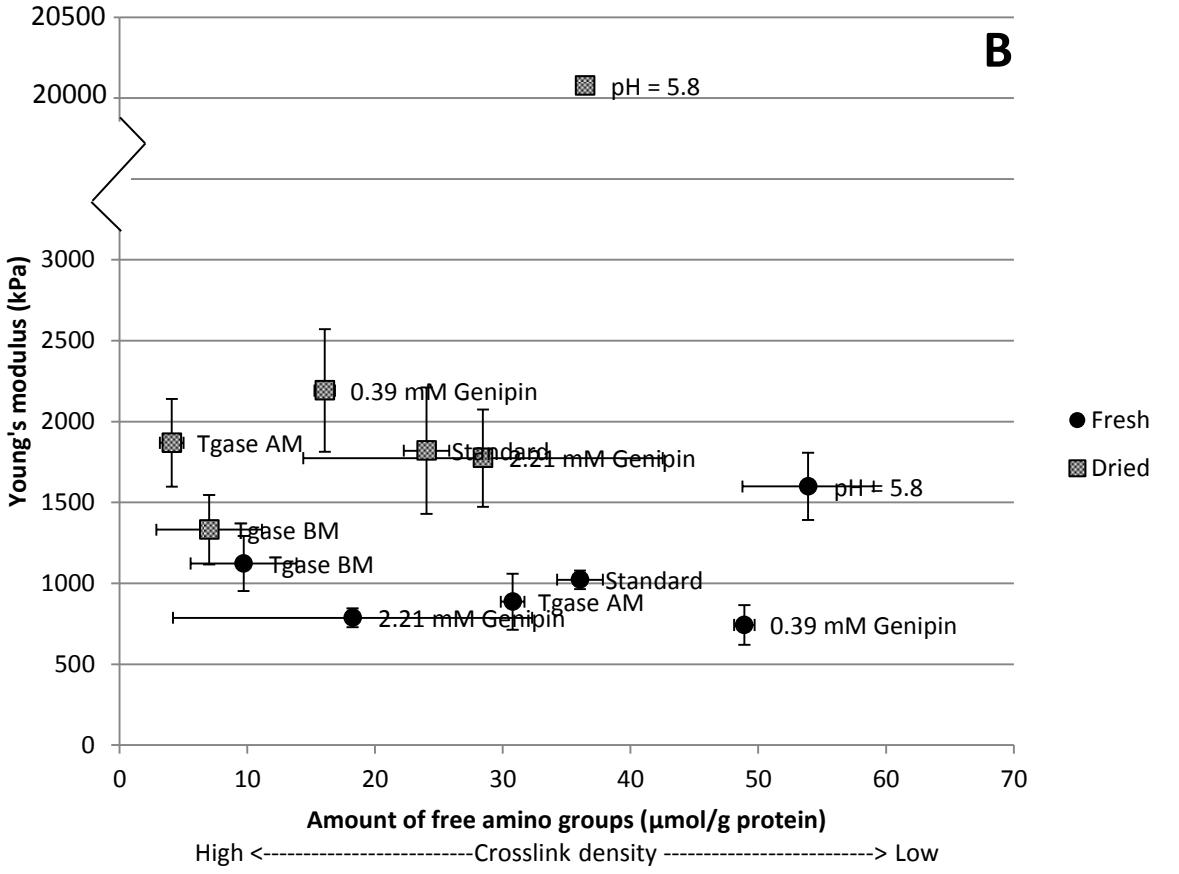


Figure 14 - Gel strength dependency of crosslink density for gels treated with A) DTT and B) Genipin, Tgase and a lower pH compared with standard gels for fresh gels soaked 48h in a watery solution (incubation solution and/or water) and dried gels soaked 24h in water

5 Discussion

5.1 DTT

DTT was used to decrease the crosslink density of the macro WPI gels and thereby increase their WBC. In previous studies, it was found that using DTT to break up disulphide bonds resulted in an increase in solubility of the protein gel (Peters (unpublished results);(Shimada & Cheftel, 1988). Also here was found that part of the gel solubilized during soaking in a 40 mM DTT solution. The amount of gel getting solubilized was dependent on the gel/DTT solution ratio. At a gel/DTT solution ratio of about 0.15 g gel/g DTT solution 1.24% (±0.38) of the gel dissolved into the DTT solution. For 0.5 g gel/g DTT solution this percentage went up to 4.24% (±0.09). For the experiments performed with WPI gels incubated in 40 mM DTT, a 0.2 g gel/g DTT solution ratio was used. As could be seen in Figure 4, DTT increased the amount of free sulphide groups in the gels, so their crosslink density was indeed decreased. According to the Flory-Rehner theory, this would lead to an increased elasticity of the gel and accordingly an increased WBC. Figure 14A showed that the Young's modulus of the fresh gel treated with DTT indeed decreased, as has been shown before (Zirbel & Kinsella, 1988), and Figure 11A showed that this also led to an increase in water uptake and WBC. The Young's modulus of the dried gel treated with DTT could not be determined, since this gel fell apart in little pieces upon drying. The increased swelling towards the standard gel was also found in the determination of the water concentration by NMR, which increased the most of all gels for the gel treated with DTT. The water concentration of all gel samples was also determined by oven drying to check the model of Peters (unpublished results). For all samples, the water concentration determined by oven drying was lower than the water concentration determined by NMR. This can be due to incomplete evaporation of water after 24h at 105°C or an inconsistency in the model. The overall trend of the result obtained by oven drying, however, was the same as the trend of the result obtained by NMR. In the results for the fresh gel incubated in 40 mM for 24h of the NMR experiment, a small T2 peak which corresponds to "free" water was found (Appendix 4), which indicates some "free" water was present in the fresh gel incubated in 40 mM DTT for 24h. The large amount of water loss during centrifugation of the fresh gel incubated in 40 mM DTT and accordingly soaked in water for 24h at 14680 rpm (Appendix 2) supports the presence of "free" water in this gel.

5.2 Transglutaminase

The crosslink density was tried to increase by treatment with Tgase before (BM) and after (AM) mixing. The aim of increasing the crosslink density was decreasing the WBC. Treatment with Tgase BM resulted in an increase in crosslink density for both the fresh and dried gels, while for treatment with Tgase AM the crosslink density only seemed to increase significantly for the dried gels according to the OPA-method. This can be due to rearrangements of bonds during drying, but more likely due to a decreased solubility, since both fresh and dried gels had the same treatment, so before drying the amount of free lysine groups will be equal. Protein gels are reported before to become less soluble when dried (Chou & Morr, 1979; Koury & Spinelli, 1975). When gels are less soluble, less free lysine groups can be detected and gels seem to have a higher crosslink density, while in fact they had not. Besides that, the crosslink density of the gel incubated in Tgase AM for 24h probably mainly increased at the edge of the gel and less in the middle of the gel, because Tgase was not able to penetrate the whole gel. This was also visible as a more opaque ring in the dried gels treated with Tgase AM. Therefore, the crosslink density of the largest part of the gel was not altered by incubation

in Tgase AM. Though, after drying, the crosslink density was relatively altered more due to shrinkage of the gel.

The water uptake and WBC after 48h soaking in a watery solution (Tgase solution and/or water) of fresh gels incubated with Tgase BM increased with about 22%, while the water uptake and WBC of the dried version of those gels did not change significantly according to swelling experiments. The calculated water concentrations from the NMR experiment seem to indicate that fresh gels treated with Tgase BM swelled less than standard gels, since they had a lower water concentration at as well the middle as the rand. It has to be noted that these gels had been in water for only 24h, so it is possible that after 48h of soaking the fresh gel treated with Tgase BM would have had a higher water concentration than the fresh standard gel, just like was seen for the WBC experiment. Furthermore, the water concentrations in the 24h soaked fresh gels treated with Tgase BM might have been lower than in fresh standard gel soaked for 24h in water, because the present Tgase, which is a protein itself, also contributed to the protein/dry matter content. Therefore, the measured water concentrations from the gel treated with Tgase BM cannot be compared straightforward with the standard gel. The difference between the fresh and dried gels can again be ascribed to rearrangements of bonds during drying or a difference in solubility. The increased water uptake for the fresh gels can be explained by the fact that crosslinking, as done by Tgase, can result in very large polymers who therefore are unfavourable for thermally induced unfolding (Truong, et al., 2004). Truong et al. (2004) showed that crosslinking by Tgase BM increased the heat stability of whey proteins. It was suggested that crosslinking of proteins by Tgase resulted in intramolecular bonds, which impeded thermally induced unfolding of the proteins (Tanimoto, 1988). In that case, the proteins might denature to a lesser extent and a weaker network is possibly formed. This results in a more elastic gel network, which hypothetically binds more water. According to Figure 14, the Young's modulus, however, did not change significantly, but the protein concentration of the gel treated with Tgase BM is higher than of the standard gel, since Tgase also is a protein. When this is kept in mind, the gel treated with Tgase BM might have become relatively more elastic. Another explanation for the increased water uptake could be that the proteins are ordered in the network in such a way that more water can be captured due to less steric hinder or more reachable polar amino acid groups, at which protein-water interactions primarily take place (Chou & Morr, 1979). Furthermore, Tgase, as mentioned before, is a protein itself and might therefore also bound water, which increases the water uptake further. It was shown before that the water uptake of gels made from Tgase-treated skimmed milk powder was increased (Imm, Lian, & Lee, 2000).

The WBC of the WPI gels treated with Tgase AM decreased as shown by swelling experiments and NMR measurement of water concentrations, while the elasticity of that gel did not change significantly compared to the standard gel. This was most likely due to a crosslink density which was only increased in a small ring on the outside of the gel cylinder. Since this ring was denser crosslinked, the swelling of the gel was partly impeded. When compressing the gel to measure the elasticity, only a small ring on the outside probably had a lower elasticity. Therefore, the Young's modulus of the whole gel probably was not different from the standard gel. When the gel would have been more intensively crosslinked completely by Tgase AM, the gel strength probably would have been increased.

5.3 Genipin

The crosslink density was also tried to increase by treatment of the macro WPI gels with genipin. Two different concentrations of genipin were used, to see the difference in effect. Strangely, the amount of crosslinks was increased for the treatment with 0.39 mM genipin, while a decrease was expected based on the function of genipin. How this is possible cannot be explained with the available results yet. Just like with the gels treated with Tgase AM, the crosslink density of the macro gels treated with genipin was only changed on the outer border, since the genipin did not penetrate that much into the gel. The ring with a changed crosslink density could be seen very clearly, as can be seen in Figure 15, since bond formation with genipin results in the formation of a blue colour. In this figure can also be seen that when the crosslinking reaction had taken place to a lesser extent due to contact with the beaker glass or less genipin available, the gel became light brown. Accordingly, an inhomogeneous denser crosslinked ring rimmed the WPI macro gel, which could have influenced the results for water uptake and gel strength, due to weaker spots in the denser crosslinked ring.

Though the crosslink density of the gel treated with 0.39 mM genipin decreased according to the OPA-test, the WBC of this gel decreased slightly. Surprisingly, though the crosslink density of the gels treated with 2.21 mM increased, their WBC was equal to the standard gels. This correlates with the elasticity of the gel treated with 2.21 mM genipin, since the elasticity of the standard gel and the gels treated with genipin was about the same. As said before, genipin only increased the crosslink density in the outer layer of the macro gels and therefore, the largest part of the gel stayed the same as the standard gel. According to the NMR experiments, the gels treated with genipin swelled even somewhat more than the standard gel. Therefore, and to investigate the influence of a denser crosslinked ring at the edge of the gel cylinder, the swelling of the gel treated with genipin was studied further with MRI. For this experiment, a cylindrical sample from the middle of the gel incubated in 2.21 mM genipin for 24h was taken and compared with a cylindrical sample from the middle of a standard gel. As could be seen in Figure 13, the gels treated with genipin were more swollen at the start of the measurement than the standard gels, which is due to the fact that they have been incubated in the genipin solution for 24h already and therefore already swelled, while the standard gels had not been in a watery solution for 24h. Therefore, both gels cannot be compared straightforward. After 65h of swelling, the gel treated with genipin was still more swollen than the standard gel. From MRI results could be seen that the standard gel, however, was not completely swollen after 65h of soaking, while the gel treated with genipin was completely swollen after 41h of soaking. Therefore, it cannot be said whether the gel treated with genipin would swell more after all than the standard gel.

In Figure 13 could also be seen that the gel treated with genipin had a little peak in R2 at the edge of the gel at as well the start as the end of the measurement. The crosslink density of these gels had only been increased at the edge of the gels by genipin (Figure 15) and therefore the edge bound less water than the middle of the gel, even though the water still passed the edge of the gel to reach the middle. Another reason for the about equal water uptake of the fresh gel treated with 2.21 mM genipin as the fresh standard gel could be that the water penetrated this gel mainly from the top or bottom of the gel cylinder, at which the crosslink density was not increased.

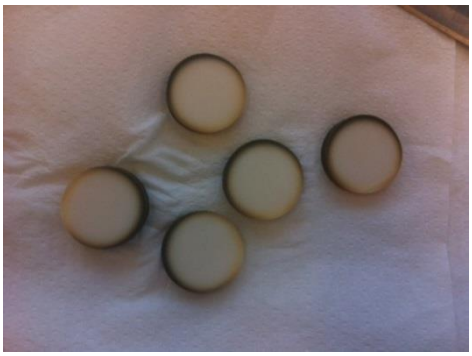


Figure 15 - Slices of macro WPI gels treated with 2.21 mM genipin

5.4 pH

The last used way to alter the crosslink density of the WPI macro gels was lowering the pH closer to the pI of whey protein, to a pH of 5.8. Due to less electrostatic repulsion between amino groups, the crosslink density of the gel was expected to increase. This was studied by determining the amount of free amino and sulphide groups. The amount of free amino groups was increased for the lower pH gel, as could be seen in Figure 7C, while a stronger cross-linked network was expected. This has also been found for WPI MPs with a pH of 5.8 by Peters (unpublished results). There could still be a stronger cross-linked network in the pH=5.8 gels, since it is possible that the crosslinks were formed between other groups than amino groups. An explanation could be that the lysine groups, with which the OPA-method has a large affinity with, were positively charged (IEP = 9.74 (Stoker, 2012)) and caused repulsion between the lysine groups when the pH was 5.8 during the gel formation. This might have caused that more amino groups were free. Therefore, the gels with pH 5.8 were also checked for the free sulphide groups. The amount of free sulphide groups stayed equal to the amount in the standard gel, so also this indicates no more crosslinks. Most likely, the increase in crosslink density for gels with a pH closer to their pI was due to an increase in ion bonds instead of an increase in covalent bonds, which was not measurable by Ellman's and the OPA test. Furthermore, in the gel made at pH 5.8 a particulate network is formed instead of a fine-stranded network (Langton & Hermansson, 1992), which might have resulted in a low availability of water binding spots. The low gel elasticity of the gel with pH 5.8 which was measured indicated that a strong protein network existed within the gel. Despite the increased amount of free amino groups, the gels with pH 5.8 showed less water uptake than the standard and due to a lot of water loss during centrifugation at 14680 rpm (30.8% (±0.28)) of sample weight against 3.36% (±0.31) for the standard gel) the WBC was a lot lower than for the standard. This decrease in WBC has also been shown for microgels (Eichenbaum, et al., 1999) and whey protein gels with a pH of 4-6 (Stading & Hermansson, 1990). The large water loss during centrifugation showed that water is not very strongly bound at all in the gel with pH 5.8. This was also visible during the measurement of the elasticity of the gels, since a lot of water came out of the gel during compression and even when no force is exerted on the gel, syneresis took place, which was also observed before (Sağlam, Venema, de Vries, van Aelst, & van der Linden, 2012). NMR showed an equal water concentration at the middle of the gel as at the edge of the gel. Possibly, this was because water was bound to a smaller extent than for other gels, so water could penetrate more freely into the gel and therefore reached the centre of the gel sooner than in other gels. Though, no peak for free water was visible in NMR results, so an exact explanation for the homogenous water concentration in the gel made at pH 5.8 which was soaked for 24h in water cannot be given.

5.5 Comparison with microparticles

In previous research done with whey microparticles (Peters (unpublished results)), the water uptake (called WBC by Peters) of whey protein particles (MPs) with various crosslink densities was determined. This was done with a centrifugation method, which resulted in the formation of a pellet of swollen MPs with interstitial water. Though, from those experiments it was not clear if a change in the water was mainly caused by a change in the swelling of the MPs or due to a change in the amount of interstitial water. Therefore, here large "particles" in the form of macro gels were made and checked for their water uptake. Since there is no possibility for water to be between these macro gels, the water uptake depends only on the water within the gels.

The water uptake of the MPs treated with DTT was increased compared to the standard MPs, while the water uptake of the MPs made at pH 5.8 was decreased (Table 2). With the macro gels it was found that a treatment with DTT increased the water uptake. Gels made at pH 5.8 showed a significantly lower water uptake than the standard gels, so the macro gels behaved in the same way as the MPs, though the water uptake of the macro gels was less than of the MPs.

Table 2 - The effect of altering the crosslink density of whey protein microparticles (MPs) (Peters (unpublished results)) and dried macro gels on their water uptake (g water/g protein). For the macro gels, the water uptake was determined after 24h soaking in water

	Water uptake MPs (g water/g protein)	**Water bound in MPs** (g water/g protein)	**Water uptake macro gels** (g water/g protein)
Standard	4.3	1.0	1.6
40 mM DTT	8.0	3.0	5.9
2 mM Genipin	5.1		1.7
Tgase AM 24h	5.0		1.2
Tgase BM 24h	4.3		1.6
pH = 5.8	2.8	1.2	0.5

The macro gels cannot contain interstitial water, so the whole water uptake can be ascribed to swelling of the particle (macro gel). The found water uptake of the standard 40% WPI macro gels and of the macro gels treated with 40 mM DTT were higher than the theoretical amount of water bound in MPs based on rough calculations based on the water uptake and size of the MPs by Peters. This can probably be ascribed to the fact that during size measurement of the MPs the smallest MPs were not measurable, with the mastersizer. In addition, the mastersizer, assumed the MPs to be round, while they are not. Accordingly, the average $D_{4,3}$ could be measured wrongly, which could have resulted in a too low estimation of the volume of the MPs. Therefore, the results from the mastersizer are suspected not to be very reliable.

The water uptake of the macro gels was lower for all gels than the water uptake of the pellets of MPs. This confirms the idea that part of the water in the pellet of MPs was interstitial water (Peters (unpublished results)). The water uptake of the MPs consists partly of interstitial water, but whether the amount of interstitial water depends on the degree of crosslinking within the MPs and/or the given treatment to the MPs is not clear yet. It has to be taken into account that the results cannot be compared straightforward, since up scaling might have had an effect. It is expected that this mainly might have influenced the results of the gels with a certain treatment, since the effect of the treatment may differ due to the difference in size and contact surface.

6 Conclusion

As has been shown before, it was possible to alter the crosslink density of whey protein gels and thereby alter their water binding capacity (WBC). By decreasing the crosslink density by means of dithiothreitol, the WBC of macro WPI gels could be increased substantially, by an increase in the elasticity of the gels. For the gels of which the crosslink density was tried to increase with transglutaminase, genipin and by reducing the pH to 5.8, only the gel made at a pH of 5.8 showed a substantial decrease in WBC. This probably was caused by the measured decrease in gel elasticity. The gel treated with transglutaminase before mixing and gelation had an increased crosslink density, but its WBC was increased towards the standard gel, which was probably due to formation of a different protein network, in which water molecules could be taken up better.

Since macro gels cannot contain interstitial water, the water uptake of the macro gels can be ascribed to only the swelling of the “particles” (macro gels). The WBCs of all macro gels were lower than the WBCs of the pellets of MPs. This confirmed the idea that part of the water in the pellet of MPs was interstitial water. Though, the swelling of the standard gel and the gel treated with 40 mM DTT appeared to be larger than what was thought based on rough calculations on the size of the MPs. The water uptake of the MPs consists partly of interstitial water, but whether the amount of interstitial water depends on the degree of crosslinking within the MPs and/or the given treatment to the MPs is not clear yet.

7 Recommendations

It is recommended to study the influence of the relative contact surface further, especially for the macro gels incubated in transglutaminase or genipin. The amount of crosslinks made by use of these treatments probably partially depends on the contact surface of the gel with the solution. When the volume/contact surface ratio is smaller, the crosslink density of a larger part of the gel or the whole gel can be increased. When studying the swelling of those gels, the influence of an increased crosslink density on the WBC can be seen better.

Furthermore, the strength of the produced macro gels should be investigated further and compared with a standard curve, so that it can be calculated as which WPI percentage the produced macro gels behave according to gel strength. To be able to compare this commensurate, standard gels with different WPI concentrations should be incubated in water for 24h before measurement of the gel strength. Besides that, the influence of other concentrations of crosslinking agents or DTT should be investigated, since e.g. a high concentration of dithiothreitol as 40 mM lowers the gel strength too much to keep the sensory perception equal to the standard.

Finally, after this research it is clear that the water uptake of the MPs consists partly of interstitial water, but whether the amount of interstitial water depends on the degree of crosslinking within the MPs and/or the given treatment to the MPs is not clear yet. Therefore, it would be interesting to investigate the effect of the treatment and/or crosslink density of WPI MPs on the amount of interstitial water between the MPs.

8 Ethics part

8.1 Introduction into the research subject

More and more people are being overweight these days and therefore weight management gets lots of attention. (Hossain, et al., 2007)Industries task herein is to develop healthier products with a lower energy content to help people control their weight. FrieslandCampina is looking for a way to develop dairy products with a lower energy content. To do this, the fat and/or protein content can be lowered and this can be done by adding water. Simply adding water will, however, cause syneresis and/or softening of the product, which results in an altered sensory perception (Childs, et al., 2007; Guinee, et al., 2000). Since the consumer will only accept healthier products if they are equally attractive, a way has to be found to overcome those problems. This can possibly be done by enhancing the water binding capacity of dairy proteins, so that more water can be incorporated into the product without altering the sensory perception.

From previous research, it appears that there is a connection between microstructure of a product, its water binding capacity and its mechanical characteristics (Barbut, 1995a, 1995b; Bowland & Foegeding, 1995; Boye, et al., 1997; Chantrapornchai & McClements, 2002; Vardhanabhuti, et al., 2001). It is therefore thought that micro-structuring can be an interesting route in this situation. When a milk protein solution is heated, a gel is formed which is able to bind water (Purwanti, et al., 2012). This 'standard' water binding is, however, not sufficient to decrease the protein and fat content without altering the sensory perception. It is thought that when the protein network in the gel is made more elastic, the gel is able to swell more and can thereby bind more water. The elasticity of the protein network can be altered by altering the crosslink density of the protein network. In this research it is investigated whether altering the crosslink density has influence on the water binding capacity of those milk proteins and on its textural properties. The crosslink density of the protein gels is altered by adding certain ingredients, namely dithiothreitol, transglutaminase, genipin or by lowering the pH (Damodaran & Parkin, 2008; Shewan & Stokes, 2013). Diothiothreitol breaks disulphide bounds and will therefore decrease the crosslink density and possibly increase the water binding capacity. Transglutaminase, genipin and lowering the pH are thought to decrease the water binding capacity since they are likely to increase the crosslink density of the protein network.

8.2 Explication

In the explication stage, the exact moral problem is defined and explained. Accordingly, possible action options are given and it is identified which factual information is missing.

8.2.1 Moral problem

The final aim of this research is to develop dairy products with a lower energy content, which can help people in their weight management. Weight management is gaining a lot of attention lately, since more and more people are overweight or even obese. Therefore, at first sight, the outcome of this research is in favour of the human population. People can have the same eating/drinking experience with less calories and will therefore gain less weight or even lose weight. However, the caloric content of the products is lowered by adding water. Adding water to food products is something that commonly occurs nowadays and is a hot topic in the food industry. Water is added to poultry, ham and milk and even special machines, called tumblers, are developed to be able to add more water to meat. Adding water to a food product is allowed – as long as it is correctly labelled.

But consumers will not notice this most of the time. Consumers are, so to say, fooled and pay the price of milk or meat for water. The industry, in this way, makes larger profit on the same amount of meat.

When looking in another way to the aim of this research, it can be said that the final goal is to add more water to dairy products without the consumer noticing it. The moral problem here is; should it be allowed to add water to food products or should the consumer be protected against it?

8.2.2 Other moral problems

Another thing to consider shortly is that this research is partly financed by FrieslandCampina, a large dairy company. That brings up the question whether this research is independent or not. Is FrieslandCampina able to influence the outcomes of the research by the way they finance it?

Furthermore, it has to be considered whether modifying the milk proteins to let them bind more water is a 'good' thing to do. The long-term health effects of modified foods are not clear yet. Besides that, part of the world population thinks that food should stay natural and (heavily) processed foods are unhealthy. On the other hand, maybe it is necessary to process foods to take advantage of them optimally, so that there still will be enough food for the whole world population in the future.

Lastly, the question arises whether the fight against being overweight is something the consumer is responsible for itself. The big amount of money that is spent on research on this topic could have been spent to other, maybe more important research if people just would not eat that much and would have exercised more. Besides that, there are already a lot of 'diet' products on the market of which the consumer can choose from. Would it have been better to spend these big amounts of money on other purposes?

8.2.3 Action options

The most obvious action here is to forbid legally to add water to food products such as chicken, ham and milk. The consumer would be protected to be fooled with buying water. This option is only not as easy as it seems. The regulations should be adapted worldwide or at least in the whole of Europe. Adapting these regulations involves a lot of administrative fuss and costs a lot of money. Besides that, adding water to food products is not always a bad thing and a trick to earn more money, but could also be in favour of the consumer.

Another option is not perform this research and use the available money for other things. Consumers can already choose from a lot of diet/light products, so another one is not necessarily needed. Besides that, people should be able to stay healthy without all the new, over-processed food, just like in earlier days.

A third option is to make strict regulations on the amount of water which can be added to food legally to still call the food by its name. If for example more than 10% of water is added, the milk is not allowed to be called milk anymore, but should be called diluted milk or something like that.

Another option is to conduct the research and if the outcome is useful, dairy products containing less calories can be developed. These less calorie dense products are healthier for the consumer and can help in weight control. Besides that, adding water lowers the cost price of dairy products and

therefore, the dairy farmers could be paid a fairer price for their milk, instead of the low milk price they get nowadays.

8.2.4 Lacking factual information

There is no data available on which percentage of the consumers actually cares about the water being added to food products, so an estimation of the severity of the problem is hard to make. Besides that, added water has to be declared on the label of the food product and it is not known whether the consumer takes notice of this or not.

8.3 Analysis

During the analysis stage, the involved stakeholders are identified. Accordingly, the relevant norms for the dilemma are stated.

8.3.1 Stakeholders

In this moral dilemma, a number of stakeholders can be identified:

- The scientists that conduct the research and are responsible for setting up the experimental design and the independency of the outcomes.
- The industry, who is the initiator of the research. The industry hopes to finally be able to develop dairy products with a lower energy content. At first, the research being conducted costs the industry a lot of money, but they hope to earn this back by increased sales in the future. Besides that, being able to add more water to dairy products will increase the profit that can be made, due to lower raw material costs.
- The consumer, who will finally have the option to buy less calorie dense dairy products. On the other hand, buying these products is buying partly water. Besides that, they can be against modifying natural products.
- Health institutions, because products with lower energy contents help people to lose weight, which results in an healthier population.
- The government, because they have to take care of the regulations, but they are also involved in the health care in a country. Less overweight people is in favour of a countries government.
- Dairy farmers, since they might receive higher milk prices if industries profit grows due to the lower raw material prices.
- Universities, since they are dependent on financing by industry to be able to perform research. Universities have to do research in order to gain knowledge and to publish.

8.3.2 Norms

The moral issue that plays a role in this question is to what extent it is allowed to add water to dairy products when the consumer thinks he buys natural dairy products. It is government's duty to protect the consumer against the domination of large food companies, who often try to mislead the consumer to make more profit. One way of doing this is selling water under the name of meat or milk. The industry lives for making profit and use all methods possible in order to increase their income. The consumer believes what is being told to them and that is often not the whole truth, so it is easy to fool the consumer. The consumer expects to be protected from this by the government. Therefore the government has set up certain rules and expects the industry to handle in line with this.

Above all, it is everyone's duty to take care of your own and others health. Government and health institutions should keep this in mind and strive for a healthy population. Therefore, dairy products which help losing weight and thereby improve population's health should be encouraged by the government and health institutions.

According to their website, FrieslandCampina, the sponsor of this research, has different values. They are a commercial company, so they want to maximize their profit, but on the other hand they also want to act responsible and sustainable. To reach this, they have to put the customer and the environment on the first place. Therefore, they probably do not want to fool the consumer, since in that case they can obtain a bad image. Quality of their products is also an important value for them and therefore it can be expected that FrieslandCampina will only sell products which are good for their consumer. Besides that, FrieslandCampina is a cooperation of farmers, so they stand close to their consumer.

8.4 Deliberation

8.4.1 Review

This ethical problem is a difficult one, since, like a lot of ethical problems, it has two sides. The question remains: which side is higher valued? Is it more important to protect the consumer against buying water? Or is it more important that the consumer is able to buy low calorie products?

Looking from the deontology point of view, in which the morality of an action is judged based on the action's adherence to a rule, I would say the best option is to forbid adding water to food products, since the government's duty is to protect its population, even though that is not a hard rule.

The utilitarianism is an ethical theory in which the greatest good counts or with other words the total 'profit' should be maximized. From an utilitarian point of view, I would say the dairy product with an low energy content should be investigated, produced and sold, since in these times of ubiquitous overweight, the consumer should be encouraged more and given the option to choose for the healthier variant. However, this stays a matter of opinion and an objective solution for the problem can never be found, just like in every ethical problem. The chosen action option always depends more or less on the way you look at the problem and what your stake is in the problem.

8.4.2 Preferred action options

Since the aim of adding water to dairy products in this manner is not to make more profit by selling water, but lowering the energy content and thereby increasing the health of the consumer, it is favourable to actually produce the product. Even though, it is necessary to make it very clear to the consumer that the product contains added water, so the consumer should be informed well about the contents and that it is a modified product. In that case, the consumer will be able to weigh the advantages against the disadvantages and can make a reasoned choice whether to buy the product or not. This is possible to the fact that 'natural' milk should still be available to buy.

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Appendix

Appendix 1

Water uptake of dried gels over time

In Figure 16 the water uptake of dried gels with different treatments can be found. All gels showed a slower water uptake from 6h of soaking to 24h of soaking compared to the first 6h of soaking. The gel incubated in 40 mM DTT for 24h and accordingly dried showed a considerably larger water uptake than the other dried gels. The dried gel made at a pH of 5.8 showed a considerably lower water uptake than the other gels.

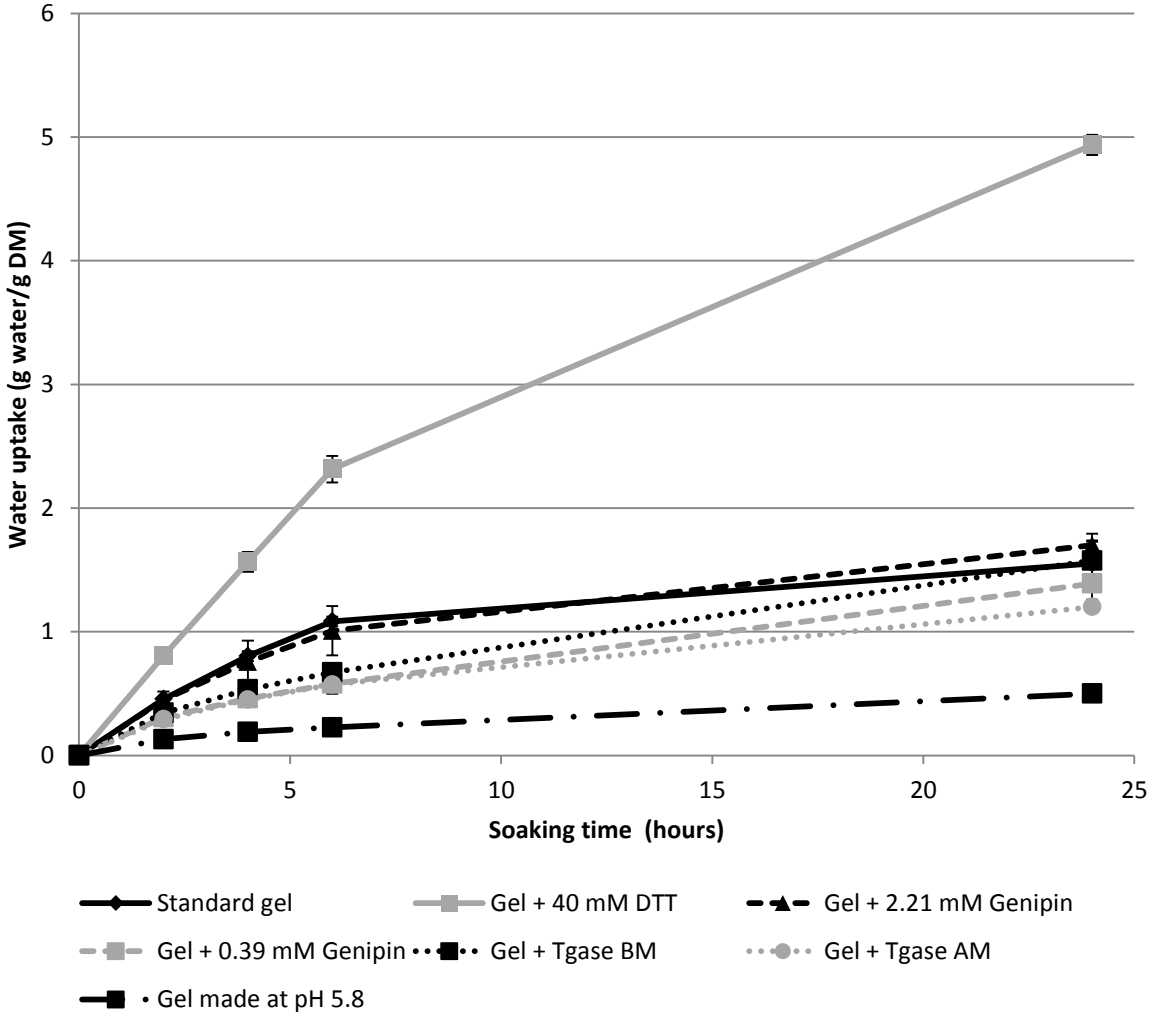

Figure 16 - Water uptake of dried gels with different treatments over time

Appendix 2

Water loss during centrifugation at 3000 and 14680 rpm

Gel		Water loss centrifugation 3000 rpm (% of sample weight)	Water loss centrifugation 14680 rpm (% of sample weight)
Standard	Fresh	0.18 (± 0.1959)	3.36 (± 0.3145)
	Dried	0.17 (± 0.2060)	3.27 (± 1.6252)
DTT	Fresh	3.44 (± 3.3903)	15.16 (± 7.9078)
	Dried	-	-
2.21 mM Genipin	Fresh	0.10 (± 0.1331)	6.59 (± 0.3833)
	Dried	-0.17 (± 0.1135)	5.23 (± 1.8791)
0.39 mM Genipin	Fresh	0.16 (± 0.0853)	5.34 (± 0.4569)
	Dried	0.13 (± 0.1755)	0.93 (± 0.2117)
Tgase BM	Fresh	0.66 (± 0.5992)	6.53 (± 1.6892)
	Dried	0.12 (± 0.1530)	1.41 (± 0.8475)
Tgase AM	Fresh	0.25 (± 0.1114)	7.84 (± 2.1304)
	Dried	-0.02 (± 0.0140)	1.16 (± 0.4233)
pH = 5.8	Fresh	0.2 (± 0.1265)	30.78 (± 0.2783)
	Dried	0.11 (± 0.0897)	0.18 (± 0.1183)

Appendix 3

Results of NMR experiment

Table 3 - Measured T2 values during NMR of several gel samples with their corresponding R2 values and accordingly calculated WPI concentrations by use of a model of Peters (unpublished results)

		WPI concentration (%)	T2 (s)	R2 (s-1)
Standard gel	Middle	33.11	0.024	41.66667
	Halfway rand-middle	32.27	0.025	40.00000
	Rand	30.74	0.027	37.03704
Gel + 2.21 mM genipin	Middle	33.11	0.024	41.66667
	Rand	30.04	0.028	35.71429
Gel + 40 mM DTT	Middle	33.11	0.024	41.66667
	Halfway rand-middle	29.38	0.029	34.48276
	Rand	24.65	0.038	26.31579
Gel made at pH 5.8	Middle	35.98	0.021	47.61905
	Rand	35.98	0.021	47.61905
Gel + Tgase AM	Middle	35.98	0.021	47.61905
	Rand	34.96	0.022	45.45455
Gel + Tgase BM	Middle	38.26	0.019	52.63158
	Rand	32.27	0.025	40.00000

Appendix 4

T_2 graph of the rand of the gel + 40 mM DTT

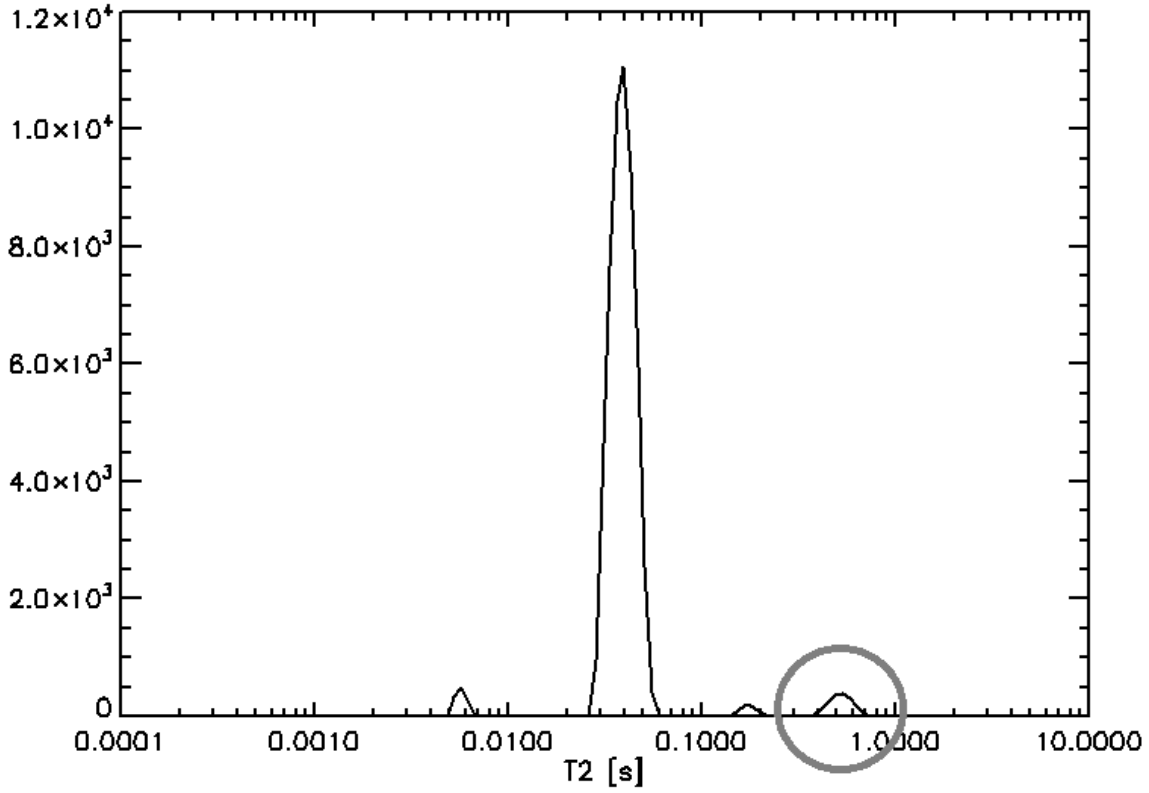

Figure 17 - T2 graph obtained by NMR of a gel sample from the rand of a gel incubated in 40 mM DTT for 24h. The T2 peak for "free" water lays within the gray circle