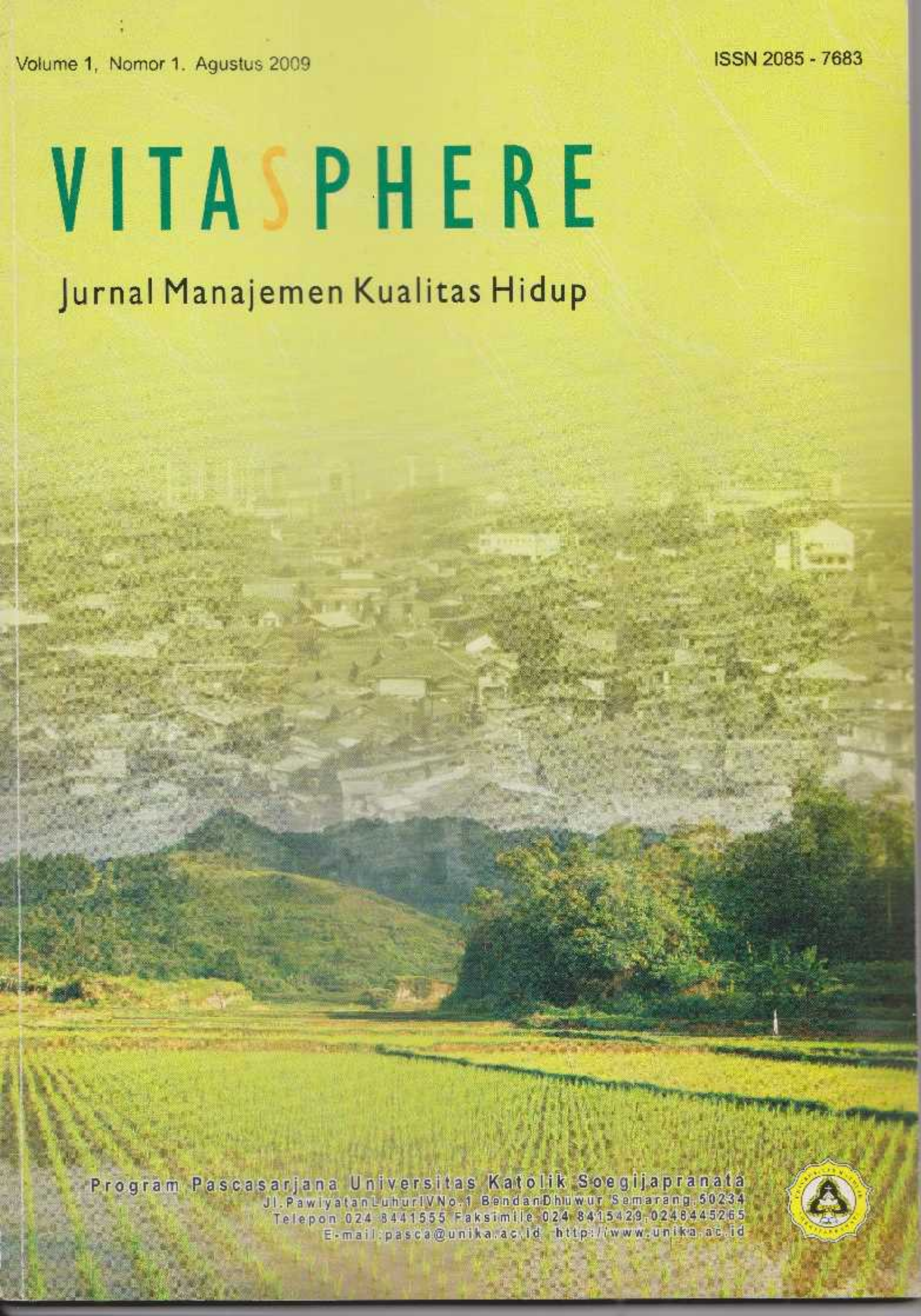
Volume 1, Nomor 1, Agustus 2009

ISSN 2085 - 7683

VITASPHERE

Jurnal Manajemen Kualitas Hidup



Program Pascasarjana Universitas Katolik Soegijapranata
Jl. Pawiyatan Luhur IV No. 1 Bendan Dhuwur Semarang 50234
Telepon 024 8441555 Faksimile 024 8415429, 02488445265
E-mail: pasca@unika.ac.id http://www.unika.ac.id

VITASPHERE, Volume I, Nomor. 1. Agustus 2009, hal. 38-52 ISSN 2085-7683

EFFECT OF DIFFERENT HEATING CONDITIONS CASEIN-SUGAR CONJUGATES ON THE EMULSION STABILITY

Inneke Hantoro [1], Tim J. Wooster [2]

ABSTRACT

Casein has been known as a good emulsifier. However, casein can be unstable in low pH and high salt conditions. Therefore, its utilization for commercial food application is limited. Modifying the interfacial structure of casein can increase their emulsification properties. The modification can be done by attaching various molecular weights of carbohydrates to casein. It is expected that the modification can increase the steric layer of casein, which results in the improvement of emulsion stability. The aim of this study was to observe the effect of Maillard conjugation produced using dry and wet heating on the emulsion stability of sodium caseinate. The dry reaction resulted in higher lysine modification than that of the wet reaction. This indicated the higher amount of carbohydrates which attached to casein on dry reaction. The flocculation stability of casein-Maillard conjugate emulsions was assessed by exposing the emulsions to $CaCl_2$ (0-20 mM) for 24 hours. An increase in emulsion particle size was observed for all emulsions stabilized by Maillard conjugates prepared using the wet reaction. Above 5 mM of $CaCl_2$ addition, the emulsions began to flocculate. In contrast, emulsions stabilized by Maillard conjugates prepared using the dry reaction remained stable. The different heating conditions were thought to be the main factor that influenced the emulsion stability of the conjugate emulsions. The distinctions may cause the difference attachment sites of sugar to casein, which influence the steric stabilization in the emulsions.

Keywords: *casein-sugar conjugates, emulsion, stability, flocculation*

Introduction

The quality of life can be determined from many factors. One of the influential factors is food. The quality of food products is influenced by intrinsic and extrinsic factors. Intrinsic factors affecting the quality of food products include organoleptic factors (appearance, color, taste, flavor, and so on), nutritional value, functionality, safety performance, packaging and the product's shelf-life (Peri, 2006).

Food comprises of many organic and inorganic components, including water, carbohydrate, proteins, lipids or fat, and other minor substances. In most cases, some of those components can not mix each other. For example fat and water or aqueous phases, which are two keys components in food system, present as immiscible components and exist as different phases within a food matrix. Therefore, many food products are emulsion based in which one substance (dispersed phase) is dispersed in the other (continuous phase).

Over time emulsions become unstable and the dispersed and the continuous phases tend to separate. Emulsion instability brings

[1] Fakultas Teknologi Pertanian Unika Soegijapranata Semarang

[2] Food Science Australia, Werribee, Victoria 3030

undesired consequences for shelf-life of food products. The phase separation phenomenon profoundly affects organoleptic properties of food products, particularly the textural and mouth-feel (Damodaran, 2005). The occurrence of organoleptic changes can lessen the shelf-life of food products. A long shelf-life is often desirable for emulsion based-food products, therefore a high emulsion stability is required.

Physical separations in food emulsions must be avoided in order to obtain longer shelf-life of food products and maintain the consumer acceptance. Preventing physical separation phenomenon can be done by adding emulsifiers - substances which can stabilize an emulsion.

Emulsifiers are surface-active substances that are capable of adsorbing to an oil-water interface and reduce the interfacial tension between the dispersed and aqueous phases. Emulsifiers has very important role to stabilize the structure of dairy-based emulsion such as acidified milk drinks, yoghurt, and other fermented dairy products (Dickinson, 2001). Nowadays emulsifiers are also applied as materials for microencapsulating specific compounds. These capsules are used to deliver nutrients (calcium, proteins, lipids, etc.), flavors and nutraceuticals. The capsules may be incorporated in beverages, dairy products, bakery, and other food products (Semo et al., 2007; Given, 2008).

Surfactants, biopolymers (e.g. proteins and polysaccharides), and other small molecular weight of emulsifiers (e.g. phospholipids) are widely used in food emulsions (Damodaran, 2005; McClemments, 2005). Macro-molecular emulsifiers, such as proteins have been known for the emulsifying and the stabilizing roles. Unlike surfactant, proteins are naturally present in food and are generally considered to be safer. Compared to polysaccharides, proteins have a strong tendency to adsorb to oil-water interfaces, to form stabilizing layers around oil droplets (Dickinson, 2003). Proteins have been widely used as emulsifying agents in many industries including food, pharmaceutical and cosmetic industries. Proteins, particularly milk proteins are good emulsifiers due to their interfacial structure, which contain hydrophilic and hydrophobic regions (McClements, 1999; Damodaran, 1996).

Caseins are the predominant components in milk and widely available. Caseins consist of four constituents, namely αs_1-, αs_2-, β- and κ-casein in proportions of approximately 3:0.8:3:1 respectively by weight in cow milk and has ranges molecular weight between 19,000 - 25,000 Dalton. In addition, caseins have significant fractions of non-polar regions along their polypeptide chains, which promote self association through hydrophobic interactions (Dickinson, 1999; McClemments, 2005).

In food emulsions, caseins are used as thickening and gelling agents. Sodium caseinate, a soluble mixture of αs_1-, αs_2-, β- and κ-casein, is one of the most popular milk ingredients that are widely used as emulsion stabilizing agent in foods due to its good solubility, surface activity, heat resistance and water holding properties (Dickinson, 1999).

Caseins form a thick fluid interfacial layer approximately 13 - 15 nm (Dalgleish 2004). Furthermore, caseins can form a stable emulsion due to combination of electrostatic and steric stabilization (Dickinson, et al., 2003; Dalgleish et al., 2004). The steric

stabilizing casein layer protects fine droplets against immediate recoalescence, and it confers long-term stability during processing and storage (Dickinson & Stainsby, 1982). The application of casein as a food ingredient has been in bakery products (bread, biscuits, cookies, breakfast cereals, cake mixes, pastries, frozen cakes, pastry glaze), dairy type foods (coffee creamers, cultured milk products, milk beverages), beverages, desserts, confectionary, and comminuted meat products (Fennema, 1996).

However, the stability of caseins as emulsifying agents decreases due to several factors. Lowering pH or the presence of ethanol in the system and proteolysis can lead to the collapse of steric layer and cause the casein emulsions become unstable (Dalgleish et al., 2004). Proteins are relatively ineffective emulsifiers close to their acidic isoelectric points, where charge and solubility are minimal. Several previous studies found that the decrease of pH towards the isoelectric point (pH 5) of the caseins can lead to the lost of the charge of droplets (Shepherd et al., 2000). As a result, the activity of emulsion and protein solubility is considerably decreased. Therefore, casein is not effective as emulsifier at low pH. Another factor such as the presence of high concentrations of sodium chloride (NaCl) also can reduce the effectiveness of their emulsifying properties as a result of charge shielding effects. Hence, the applications of caseins are limited near their isoelectric points and in high salt environments (Dalgleish, 1997; Damodaran, 1996; Dickinson, 2001).

Improving the emulsifying properties of proteins, particularly related to emulsion stabilization can be reached using several methods. One of the common methods is conjugating polysaccharides to proteins. Attaching polysaccharides to protein can increase steric layer thickness. The conjugation of proteins and polysaccharides can improve their functional properties, such as solubility, heat stability, and emulsion stabilizing ability (Chevalier et al., 2001; Hattori et al., 2000).

The covalent linkage between the polysaccharide and protein component creates a stable conjugate. By covalent linkage, the solubility of protein-polysaccharide conjugate over a wide range of solution conditions can be maintained (Dickinson, 1999).

The conjugation of polysaccharides to proteins has been investigated by several researchers (Shepherd et al., 2000; Morris et al., 2004; Dunlap & Cote, 2005; Wooster & Augustin 2006, 2007[a], 2000[b]). All the studies reveal that conjugating these biopolymers can enhance their emulsifying properties through steric stabilization. Polysaccharides introduce a bulky polymeric layer to the droplet surface which prevents flocculation and coalescence.

The conjugates are typically formed either by the Maillard reaction using dry heating (Shepherd et al., 2000) or via carboiimide attachment of carbohydrate (Hattori, 2002) and enzymatic methods, for instance transglutaminase cross-linking and enzymatic hydrolysis (van Derven et al., 2001).

Maillard reaction occurs naturally in food processing. Moreover compared to other methods, conjugating protein and carbohydrates via Maillard reaction is safer. Unlike other methods, no toxic substances that expose health risks are created in Maillard reaction. However, the Maillard reactions are complex

and influenced by many factors that can affect the stabilization of protein-polysaccharide conjugates.

Maillard reaction is categorized as one of non-enzymatic browning mechanisms. The Amadori rearrangement is a step in Maillard reaction, where the amines in protein (terminal and intrastitial) are linked to the reducing end of polysaccharide and it is not a reversible reaction (BeMiller & Whistler, 1996; Kato et al., 1992; Nursten, 2002).

Some studies showed that dry reaction is quite effective for producing casein-Maillard conjugates (Shepherd et al., 2000; Dunlap & Cote, 2005; Wooster & Augustin 2006, 2007[a], 2000[b]). But, no study has been done in comparing dry reaction and wet reaction methods for producing casein-Maillard conjugates.

The aim of the study is to observe the effect of Maillard conjugation produced using different heating methods on the emulsion stability of sodium caseinate.

Materials and Methods

Materials

Sodium caseinate (Alanate 180) was obtained from New Zealand Milk Products (Rowville, Victoria). Maltodextrins were obtained from The Grain Processing Corporation, United States. The trade names of the maltodextrins are Maltrin®M100 and Maltrin®M040, which have an average molecular weight 1,900 Dalton and 3,600 Dalton respectively. Dextran 35 was purchased from Sigma Aldrich and has an average molecular weight 21,000 Dalton. All of buffer and salt used for the study were analytical grade and were purchased from Sigma.

Methods

Casein conjugates preparation

Casein-carbohydrate conjugates were prepared using approximately 1:1 mole ratio of available NH_2 to reducing sugar for maltodextrins and 1:5 for dextran. The concentration of sodium caseinate solutions were 2 %w/v. Sodium caseinate was dissolved in either milli – Q water or 0.1 M sodium dihydrogen phosphate buffer solution (pH 6.8) or 0.1 M NaCl solution at 60°C. The pH of casein solution was adjusted to pH 7.5 using NaOH 1 M before sugars were added into the solution. Three different types of carbohydrates which have different molecular weights were used in this study, i.e. Maltodextrins (Maltrin®M100 and Maltrin®M040) and Dextran 35.

The casein-carbohydrate conjugates were produced using two methods, i.e. dry and wet reactions. The dry reaction only was done for Maltrin 040 and Dextran 35. The dry reaction for conjugation was performed by heating the freeze dried casein-dextran 35 mixture at 60°C and under controlled humidity (76%) for 4-5 days. While, the wet reaction was done by heating casein-carbohydrate solutions at 100°C for 4 hours using reflux. The conjugates then were freeze dried for 1-2 days. The nitrogen content analysis and OPA assay were determined for all conjugate powder to determine the amount of conjugate samples required for emulsion stability analysis.

Measurement of the extent of conjugation

The degree of modification was described as the average number of lysine residues conjugated with s carbohydrates during Maillard reaction. A colorimetric assay was used to assess the extent of conjugation. The

assay is based on the reaction between the free primary amine groups of the protein and ortho-pthaldyaldehyde (OPA) reagent (Brands & van Boekel 2001; Chevalier et al., 2001; Morris et al., 2004; Wooster & Augustin, 2006).

OPA reagent was prepared daily by dissolving 90 mg OPA in 2 ml ethanol 100%. Then the OPA is mixed with 50 ml of 0.1M sodium tetraborate buffer solution (pH 9.7-10), 5 ml of 20w/v% sodium dodecyl sulphate (SDS) and 0.2 ml of 2-mercaptoethanol. The mercapthoethanol could be replaced with dithiothretitol (DDT) (100mg/100mL total OPA solution). The volume solution was then made up to 100 ml with deionized water.

Sample solutions (containing about 50-500 µg/ml casein equivalent) were measured by placing 80 µL of sample solution in a cuvette and mixing with 3 ml of OPA reagent. The solution was stirred briefly and incubated for 3 minutes at room temperature. The absorbance was determined at wavelength 340 nm using Shimadzu UV-vis Spectrophotometer UV-1700 PharmaSpec.

Lysine as a standard solution was used to calibrate the amount of available NH_2 in each sample. The lysine calibration solution was prepared by dissolving lysine of varying concentrations (0.1-1 mg/mL) in pH 9 sodium tetraborate buffer. The measurement was done in triplicate for each sample.

Emulsion preparation

The emulsion preparation was done based on Wooster & Augustin (2006). Emulsions were prepared by mixing 20 wt% oil and 80 wt% aqueous casein-carbohydrate conjugate emulsifier solutions at room temperature. As a comparison, emulsions prepared from 20 wt% oil and 80% wt% caseins were used. The expected concentration of casein in the aqueous emulsifier solution was approximately 1 wt% for all samples.

There were two steps for making the emulsions. First, the coarse emulsions were blended using an Ultra-turrax mixer at 13,500 rpm for 3 minutes. Then, the mixtures were passed through a valve homogenizer (3 times passes at 70-80 bar) to produce a fine emulsion. The fresh emulsion was directly used for stability tests, which included the particle size distribution and the Turbi Scan measurements.

Emulsion stability measurement

The emulsion instability was measured by adding salt ($CaCl_2$) to the freshly prepared emulsions. Several calcium concentrations (0-20 mM at the final solutions) were used. 5 ml of emulsion sample was placed on the plastic tube. Then, 5 ml of $CaCl_2$ was added into the sample and mixed thoroughly. The stability of the emulsions was evaluated by measuring the emulsion size after exposure to $CaCl_2$ for 18-24 hours. Sodium azide was added at 0.02 wt% to the samples after formation to prevent microbial contamination (Wooster & Augustin, 2006).

Laser light scattering using a Malvern Mastersizer 2000 was used to measure the particle size of emulsion. About 0.002 wt% (approximately 1-2 drops) of samples were diluted with distilled water to avoid multiple scattering effects. Particle size distributions were determined using the Mastersizer 2000 (Malvern Instruments Worcestershire, UK). The results of particle size of emulsions were obtained from three replications of each sample.

Besides measuring the particle size of the emulsions samples, the flocculation rate was also observed using Turbi Scan. The measurement was used due to its ability to describe the rate of flocculation in emulsion more accurately rather than just visual observation. 2.5 ml of $CaCl_2$ solutions at various concentrations (0 – 20 mM) was added into the 2.5 ml emulsion samples and mixed well. The measurement was done for all samples at time 0 and after 18-24 hours.

Results and Discussion

The extent reaction of casein-Maillard conjugates

The improvement of casein-based emulsions can be obtained by attaching steric layer through the attachment of polysaccharide to casein via Maillard reaction. The extent of Maillard reaction, which shows the degree of casein and polysaccharide conjugation, was measured using OPA assay. Carbohydrates with different molecular weight were used. The larger molecular weight of carbohydrate is expected to exhibit an increase in emulsion stability. Optimizing the reaction conditions for producing protein-Maillard conjugates using the wet reaction has been shown to improve the formation of Maillard conjugates (Morris et al., 2004). However, the effect of Maillard conjugation prepared using the wet reaction on the flocculation stability of casein has not been investigated. On the other hand, many studies had shown that attachment of carbohydrates to globular proteins through the dry reaction increases emulsion stability (Dunlap & Cote, 2005; Wooster & Augustin, 2006 and 2007[b]). Different heating conditions may affect the Maillard conjugates structure and their emulsion stability. The purpose of using wet and dry reaction was to evaluate the effect of different heating conditions on the emulsion stability of casein-carbohydrate conjugate emulsions.

Table 1 shows that the dry reaction results in higher extent of conjugation amount of casein-carbohydrate conjugates than the wet reaction. The higher extent of conjugation indicated the more sugar molecules that attach to casein. The average number of sugar molecules attached to casein molecule were 6.73 and 2.54 in casein-Maltrin 040 and casein-Dextran 35 prepared by wet reaction, respectively. Dry reaction conjugate samples produced 7.15 and 3.65 modified lysine per casein molecule in casein-Maltrin 040 and casein-Dextran 35, respectively. The percentages of modified lysine of casein-Dextran 35 conjugates are lower than casein-Maltrin 040 because the difference of mole ratio used in the reaction. Moreover, dextran 35 has larger molecular weight (21,000 Da) than Maltrin 040 (3,800 Da), and requires longer time for dextran to react with casein (Pan et al., 2006).

The dry reaction is effective for forming casein-carbohydrate Maillard conjugates (Shepperd et al., 2000; Mu et al., 2006). The heating conditions in the dry reaction are more controllable than in the wet reaction (reflux), particularly related to the temperature and water activity. The temperature of the dry reaction is milder than in the reflux, so the protein is less likely to be denatured by the heat. On the other hand, the temperature of reflux reaction can reach more than 100°C, which can degrade reactants during heating. Protein and sugar hydrolysis are some examples of the degradation of the reactants due to the high temperature.

Table 1. The extent of reaction and number of carbohydrate molecules attached to casein – comparison between wet and dry reactions.

	Casein-				
	Maltrin 100 [a]	Maltrin 040 [a]	Maltrin 040 [b]	Dextran 35 [a]	Dextran 35 [b]
NH_2 to reducing sugar mole ratio	1:1	1:1	1:1	1:5 [c]	1:5 [c]
Extent of reaction [d]	36.97% (n = 3, SD=0.008)	47.20% (n = 3, SD=0.008)	50.15% (n = 3, SD=0.006)	17.83% (n = 3, SD=0.008)	25.60% (n = 3 SD=0.004)
Average number of modified amino acid per casein molecule	5.27	6.73	7.15	2.54	3.65

[a] Conjugation performed using wet reaction (reflux with temperature > 100°C) for 4 hours.

[b] Conjugation performed using dry reaction (heating at 60°C with 76% of relative humidity) for 5-6 days.

[c] For casein – dextran 35 conjugate, the reaction based on casein to reducing sugar mole ratio.

[d] As determined by o-pthalydialdehyde (OPA) available NH2 assay.

The stability of casein-conjugate emulsions induced by $CaCl_2$

The purpose of conjugation of casein to carbohydrates was to improve the emulsion stability. Electrostatic and steric interactions significantly contribute to the colloidal stability of casein emulsions (Dickinson, 1999). According to Hunter (1986), compared to electrostatic stabilization, steric stabilization exhibits some advantages, such as being insensitive to pH and electrolyte, and weak flocculation behaviour. Moreover, improving the steric layer can overcome electrostatic interaction problem by the presence of high concentration of electrolytes and the problem with solubility of conjugates over a wide range of solution conditions (Dickinson, 1999).

The effectiveness of steric stabilization is a function of the thickness and density of the steric layer (Wooster & Augustin, 2007[b]). The attachment of carbohydrates to protein increases the steric stabilization (Kato, 2002; Wooster & Augustin, 2006 & 2007[b]). The increase of steric stabilization has been shown to be a function of carbohydrates length (Wooster & Augustin, 2007[b]). Thus, emulsion stability was assessed using Maillard conjugates with different molecular weight of carbohydrates.

The stability of emulsions was assessed by monitoring emulsion flocculation after electrolyte additions. Several concentrations of $CaCl_2$ (0, 2.5, 5, 10, 15, and 20 mM) were added into conjugate emulsions, which contained 1% (w/w) of protein equivalent and 20% (w/w) canola oil. The effect of calcium chloride on particle size of casein and casein-carbohydrate Maillard conjugate emulsions after 24 hours exposure is presented in Figure1.

Figure 1 shows that all emulsions stored for 24 hours had similar sizes in the absence of calcium chloride. The addition of calcium

resulted in an increase in emulsion size. The changes in particle size distribution with time imply the emulsion is unstable (Dalgleish, 2004). Destabilizing effect of calcium on casein emulsions is caused by the binding of calcium to the phosphoserine residues of the casein emulsions (Dalgleish, 1997). The sodium caseinate contains as$_1$-casein and â-casein as the major components, and each has 8 and 5 cluster of phosphoserine residues, respectively (Dickinson et al., 1998). The calcium binding can collapse the adsorbed layers of casein that cover the emulsion and decrease steric stabilization. Eventually, the emulsion begins to aggregate. The concentration of calcium ions significantly affects the destabilization reactions, a higher calcium concentration leads to coalescence (Dalgleish, 1997).

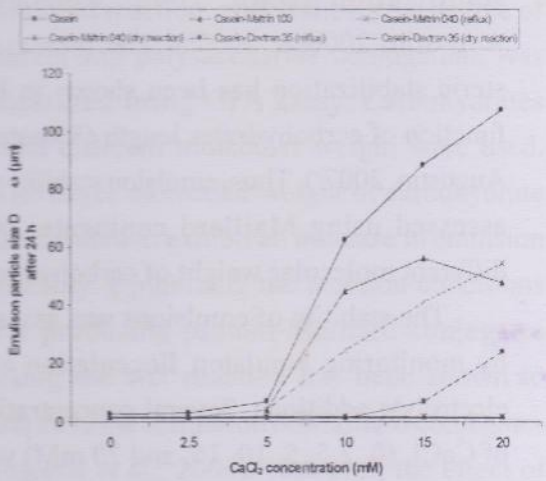


Figure 1. The effect of calcium addition on particle size of casein and casein-sugar Maillard conjugate emulsions after 24 hours. The comparison between conjugates prepared by wet reaction and dry reaction (for casein-Maltrin 040 and casein-Dextran 35).

Figure 1 demonstrates that casein-Maillard conjugate emulsions produced by wet reaction were more unstable and more susceptible to calcium chloride compared to casein emulsion. Above 2.5 mM of calcium chloride, particle size ($D_{4,3}$) of casein-sugar Maillard conjugate emulsions increased, which denoted the occurrence of flocculation. On the other hand, instability of casein emulsion was observed by the addition of calcium chloride above 10 mM. It appears that the attachment of carbohydrates to casein via wet reaction using reflux heating can not improve steric stabilization of the emulsions.

Flocculation has been observed in emulsions made from casein – maltodexrin conjugates (1900 Da and 3800 Da) using wet reaction. In contrast, conjugating carbohydrates to globular protein (β-lactoglobulin) has shown an increase in emulsion stability (Dunlap & Cote, 2005; Wooster & Augustin, 2006 & 2007[b]).

The attachment of β-lactoglobulin to dextran with different molecular weights (150, 500 and 2,000 kDa respectively) shows excellent emulsion stability (Dunlap & Cote, 2005). Wooster & Augustin (2006) found the increase of steric layer thickness of emulsion ranging from 5 nm to 20 nm by conjugating β-lactoglobulin with 18.5 – 440 kDa of dextrans. The emulsion stability assessment shows no changes on to emulsion size after exposing the emulsion to calcium chloride for 24 hours. Conjugating β-lactoglobulin with lower molecular weight carbohydrates such as maltodextrins also demonstrates the increase of emulsion stability (Wooster & Augustin, 2007[b]). Conjugation of β-lactoglobulin with a 900 Da, 1,900 Da and 3,800 Da maltodextrins (with the observed steric layer thickness are approximately 1.1, 2.5, and 7.3 nm, respectively) can give sufficient steric stabilization in high salt environment and heat induction. The

attachment of thin steric layer to β-lactoglobulin seems enough to stabilize the emulsions against flocculation.

In contrast to globular proteins, a thin steric layer does not seem to be sufficient for preventing flocculation in casein-Maillard conjugate emulsions. There are two possible explanations. First, casein and β-lactoglobulin have different interfacial structures. β-lactoglobulin has a thin and dense interfacial layer (2-3 nm in neutral pH), which give excellent electrostatic stability (Dalgleish, 2004). Unlike globular proteins, casein form extended layers about 10-13 nm thick into the aqueous phase (Dalgleish, 2004). The differences of interfacial structure of casein and globular proteins can be seen in Figure 2. The extended layers of casein come from the two major monomeric caseins, i.e. α_{s1}-casein and α-casein. The adsorbed layer of α-casein protrudes further from the interface than α_{s1}-casein (Dalgleish, 2004). The excellent emulsifying properties of sodium caseinate are related to the large parts of those caseins. However, both of caseins are sensitive to precipitation by calcium ions (Dickinson, 1989; Dalgleish, 2004). Hence, when the steric layer of carbohydrates is not thick enough, calcium ion can easily create a binding with the caseins and lead to the instability of emulsion.

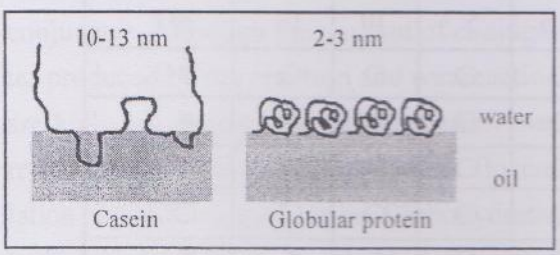


Figure 2. The differences of interfacial structure of casein and globular protein (McClement, 2005).

An alternative explanation for the destabilization of casein-Maillard conjugate emulsions is the effects of heating conditions on the conjugates produced. Heating using a high temperature can lead to hydrolysis of the reactants, i.e. protein and carbohydrate hydrolysis. The effect on heating conditions on the protein and carbohydrates in the system was assessed by OPA assay and reducing end sugar measurement (Somogyi-Nelson method).

The stability of casein- Maillard conjugates (casein-Maltrin 040 and casein-Dextran 35) made using wet and dry reaction was assessed. Figure 1 shows the increase in particle size of casein-Dextran 35 emulsion prepared by wet reaction after exposing the emulsion to calcium chloride for 24 hours. The aggregation of casein-Dextran 35 conjugate emulsion has been observed by the presence of calcium chloride at concentration above 2.5 mM. The particle size of casein-dextran 35 conjugate emulsion prepared by wet reaction, were bigger than that of casein-Maltrin 040 prepared using the same method. It can be explained by the lower number of dextran attached to casein. The numbers of attached dextran molecules in casein-dextran conjugate emulsion were only 2.54 per molecule casein. Hence, it may create weak steric interactions between droplets and cause the aggregation of the droplets more easily by calcium chloride addition.

Unlike conjugates heated using the wet reaction, emulsions made from casein-Maltrin 040 and casein-Dextran 35 conjugates heated using the dry reaction were remained stable after exposing the emulsions to calcium chloride for 24 hours. There were no significant particle size changes in both

emulsions. Casein-Maillard conjugates prepared using the dry reaction show excellent emulsion stability. In casein-Maltrin 040 conjugate emulsion, flocculation can be prevented by attaching 7.15 maltodextrin molecules to lysine. While in casein-Dextran 35 conjugate emulsion, the attachment of 3.65 dextran molecules is enough to stabilize the emulsion. It can be assumed that steric interactions have important contribution in stability of conjugate emulsions. When one droplet approaches another droplet, the overlap of carbohydrate steric layer can prevent the aggregation of droplets. Although calcium chloride can bind a s1-casein and α-casein, the attached carbohydrates steric layer is able to prevent flocculation. The thickness of steric layer of the conjugates was not measured in this study.

Table 2 shows the flocculation rate based on back scattering measurements in emulsion samples, which were prepared using wet and dry reaction. The back scattering assessments were done after the addition of calcium for 19-22 hours. All emulsions are stable in low salt environment (0 – 5 mM $CaCl_2$). Above 5 mM of calcium, flocculation occurred in all emulsions prepared using wet reaction, while emulsions prepared by the dry reaction remain stable. It was expected that carbohydrates with longer chain, such as Maltrin 040 and Dextran 35 would be able to stabilize the emulsions. However, the result demonstrates that emulsion of casein-Maltrin 040 and casein-Dextran 35 conjugates produced using wet reaction have poor stability against the flocculation. Therefore, these casein-carbohydrate conjugates should be observed further (see Table 3).

Possible explanations for the difference in emulsion stability of conjugates prepared under different heating conditions are assumed to be the reason of the emulsion instability. There was no significant increase of available lysine in casein heated in the absence of sugar (data is not shown). This indicates no protein hydrolysis during the reaction. Since protein hydrolysis has not been observed in this study, the other possible reason is the occurrence of carbohydrates hydrolysis during heating using reflux.

Table 2. The instability map of casein and casein-conjugate emulsions after the addition of $CaCl_2$ for 19-22 hours.

Emulsions	$CaCl_2$ concentrations (mM)					
	0	2.5	5	10	15	20
Casein	○	○	○	○	○	●
Casein-Maltrin 100[a]	○	○	○	●	●	●
Casein-Maltrin 040[a]	○	○	○	●	●	●
Casein-Maltrin 040[b]	○	○	○	○	○	○
Casein-Dextran 35[a]	○	○	○	●	●	●
Casein-Dextran 35[b]	○	○	○	○	○	○

[a] made using wet reaction, [b] made using dry reaction, ○ stable; ● flocculation

Table 3. The reducing end sugar absorbance of Maltrin 040 and Dextran 35

Carbohydrates	Absorbance (nm)		% change in absorbance
	Unheated	Heated (4 hours) using reflux	
Maltrin 040	0.658	1.115	40.99
Dextran 35	0.268	0.352	23.86

The reducing end of heated Maltrin 040 and Dextran 35 (without casein) was observed using Somogyi-Nelson method (Wood & Bhat, 1988). Table 3 illustrates the increase absorbance of heated Maltrin 040 and Dextran 35, which signs carbohydrates hydrolysis during conjugation process using reflux. The assessment indicates that hydrolysis rate in Maltrin 040 is higher than Dextran 35 because the absorbance of heated Maltrin 040 was almost double than the unheated one, while the increase of Dextran 35 was about 1.3 times. A high temperature can stimulate hydrolysis reaction. Therefore, it can be assumed that exposing carbohydrates to the high temperature for 4 hours can cause hydrolysis reaction. A high temperature can break carbohydrate with large molecular weight into shorter chains, such as monosaccharide. Carbohydrate with shorter chains can not provide sufficient steric layer thickness to against the flocculation. This can cause emulsion instability.

The emulsion stability and the reducing end sugar analysis indicate that different heating conditions can affect the structure of conjugates. Although the amount of conjugates produced by dry reaction and wet reaction are high, the conjugates produced using wet reaction show poor stability against flocculation in emulsion application. Carbohydrates hydrolysis is one reason of the destabilization of emulsion. However, based on the reducing end sugar analysis, apparently carbohydrates hydrolysis is not enough to cause the destabilization effect. The result shows that 23.86% and 40.99% of Maltrin 040 and dextran 35, respectively, were hydrolyzed during heating. There were some amount of Maltrin 040 and dextran 35 attached to casein, which shall provide steric stabilization. But the emulsion stability assessments demonstrated that the calcium addition caused flocculation in these conjugate emulsions.

Another possible explanation is that carbohydrates might be attached to the difference sites of casein in dry and wet reaction. In conjugation using dry reaction, carbohydrates might be attached to lysine at different backbone compared to conjugation done using wet reaction. Unfortunately, there is no supporting data to prove the assumption. Therefore, further assessment and analysis for casein-sugar conjugates is required, particularly focusing on the different structure of the Maillard conjugates which prepared using wet and dry reaction.

Further research on interfacial structure of casein-sugar conjugates should be conducted as they can be beneficial for beverages as acidified milk drinks, which have low pH. The pH of these products ranges from 3.4 to 4.6, therefore they need emulsifier which can resist in acidic environment (Nakamura et al., 2006). In beverages, especially those which containing flavor oil blends, a stable emulsifier is required (Given, 2008). The casein-sugar conjugates may valuable to be applied in

beverages since they have good emulsifying capacity and demonstrate the ability against flocculation in extreme conditions.

In addition, the Maillard conjugates can also be used as wall materials for microencapsulation or nanoencapsulation of functional compounds, flavor, lipids, essential oils, nutraceuticals, etc. Solubility, good properties of emulsification and high stability are some important characteristics required for wall materials for microencapsulation (Gharsallaoui et al., 2007). Proteins, carbohydrates or their blend are commonly used as wall materials (Gharsallaoui et al., 2007; Drusch, et al., 2009). Although carbohydrates are good encapsulating agents, they have poor interfacial properties. On the other hand, proteins, especially milk proteins have good encapsulation properties. Modifying the physico-chemical properties of the combining of those compounds can be beneficial for creating efficient encapsulating blends.

The application emulsifier for encapsulating specific substances has showed by Semo et al. (2007). They observed that casein micelle can be used as natural nano-capsular vehicle for nutraceutical substances for enrichment food products. They proved that encapsulation of vitamin D_2 can protect the vitamin from photochemical degradation. The effect of Maillard conjugates on the stabilization of microencapsulated fish oil also has been investigated by Drusch, et al. (2009). Both fundamental and applied research on Maillard conjugates, including casein-sugar conjugates may indirectly address consumer interest in healthy foods and beverages. Resistance to low pH and targeted delivery in the body are functional features of nutrient emulsions.

Conclusion

This work investigated the impact of the conjugation of casein to various molecular weights of carbohydrates via the Maillard reaction under different heating conditions on emulsion stability. The extension rate of Maillard reaction is not always indicating the increase of emulsion stability. The methods used for producing the Maillard conjugates significantly influence the emulsion stability. The Maillard conjugates prepared using dry reaction, are successful in increasing casein emulsion stability. The attachment of Maltrin 040 and dextran 35 to casein seems to increase the steric stabilization of the emulsions. Conjugation of maltodextrin and dextran through dry reaction can prevent flocculation in high salts environment. On the other hand, the casein-carbohydrates conjugates produced using wet reaction can not enhance the emulsion stability. The high temperature during reaction leads to carbohydrate hydrolysis that partially causes the weak steric stabilization in casein-conjugate emulsions. Moreover, the heating conditions of wet reaction may result in attachment of carbohydrate to protein backbones at different sites compared to dry reaction. Different attachment sites of sugar to casein may affect the strength of steric stabilization. However the different of the attachment sites at different heating conditions are still not clear. Therefore a further study focusing on the influence of heating conditions in Maillard conjugates production on the emulsion stability must be considered. Moreover, the limitation of information of interfacial structure of casein based emulsions open the opportunity for further study focusing on the interfacial design of casein-Maillard conjugate emulsions. The

research on modifying the interfacial of Maillard conjugates can be useful for delivery system of functional compounds via food products.

REFERENCES

BeMiller, J. M. & Whistler, R.L. (1996). Carbohydrates. In Fennema (Ed.), *Food Chemistry* (3rd Ed.) (pp. 157-224). New York: Marcel Deckker.

Brands, C. M. J. & van Boekel, M. A. J. S. (2001). Reactions of monosaccharides during heating of sugar-casein systems: Building of a reaction network model. *Journal of Agricultural and Food Chemistry 49*, 4667-4675.

Chevalier, F., Chobert, J.M., Popineau, Y., Nicolas, M.G. & Haertle, T. (2001). Improvement of functional properties of beta-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *International Dairy Journal 11*, 145-152.

Dalgleish, D. G. (1997). Adsorption of protein and the stability of emulsions. *Trends in Food Science & Technology 8*, 1-6.

Dalgleish, D. G. (2004). Food Emulsions: Their structures and properties. In S. E. Friberg, K. Larsson & J. Sjoblom (Eds.), *Food Emulsions* (4th Ed., Revised and Expanded). New York: Marcel Dekker, Inc.

Damodaran, S. (1996). Amino Acids, Peptides, and Proteins. In Fennema (Ed.), *Food Chemistry* (3rd Ed.) (pp. 321-430). New York: Marcell Dekker.

Dickinson, E. (1999). Caseins in emulsions: Interfacial properties and interactions. *International Dairy Journal 9*, 305-312.

Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B 20*, 197-210.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed system. *Food Hydrocolloids 17*, 25-39.

Dickinson, E., Flint, F.O. & Hunt, J.A. (1989). Bridging flocculation in binary protein stabilized emulsions. *Food Hydrocolloids 3*, 389-397.

Dickinson, E., Radford, S.J. & Golding, M. (2003). Stability and rheology of emulsions containing sodium caseinate: combined effects of ionic calcium and non-ionic surfactant. *Food Hydrocolloids 17*, 211-220.

Dickinson, E. (1999). Caseins in emulsions: interfacial properties and interactions. *International Dairy Journal 9*, 305-312.

Drusch, S., Berg, S., Scampicchio, M., Serfert, Y., Somoza, V., Mannino, S. & Schwarz, K. (2009). Role of glycated caseinate in stabilization of microencapsulated lipophilic functional ingredients. *Food Hydrocolloids 23*, 942-948.

Dunlap, C. A. & Cote, G.L. (2005). Beta-lactoglobulin-dextran conjugates: Effect of polysaccharide size on emulsion stability. *Journal of Agricultural and Food Chemistry 53*, 419-423.

Fennema, O. R. (Ed.). (1996). *Food Chemistry* (3rd Ed.). New York: Marcel Dekker.

Gharsallaoui, A., Roudaut, G. Chambin, O., Voilley, A. & Saurel, R. (2007). Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Research International 40*, 1107-1121.

Given, P.S. (2008). Encapsulation of flavors in emulsions for beverages. *Current Opinion in Colloid and Interface Science.* (doi:10.1016/j.cocis.2008.01.007).

Hattori, M. (2002). Functional improvements in food proteins in multiple aspects by conjugation with saccharides: Case studies of beta-lactoglobulin-acidic polysac-

charides conjugates. *Food Science and Technology Research 8,* 291-299.

Hattori, M., Okada, Y. & Takahashi, K. (2000). Functional changes in beta-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *International Dairy Journal 11,* 145-152.

Kato, A. (2002). Industrial applications of Maillard-type protein-polysaccharide conjugates. *Food Science and Technology Research 8,* 193-199.

Kato, A., Mifuru, R., Matsudomi, N. & Kobayashi, K. (1992). Functional casein-polysaccharide conjugates prepared by controlled dry Heating. *Bioscience Biotechnology and Biochemistry 56,* 567-571.

McClements, D. J. (1999). *Food Emulsions: Principles, Practice and Techniques.* Boca Raton: CRC Press.

McClements, D. J. (2005). *Food Emulsions: Principles, Practices, and Techniques* (2nd Ed.). Boca Raton: CRC Press.

Morris, G. A., Sims, I.M., Robertson, A.J. & Furneaux, R.H.. (2004). Investigation into the physical and chemical properties of sodium caseinate-maltodextrin glyco-conjugates. *Food Hydrocolloids 18,* 1007-1014.

Mu, M., Pan, X., Yao, P. & Jiang, M. (2006). Acidic solution properties of â-casein-graft-dextran copolymer prepared through Maillard reaction. *Journal of Colloid and Interface Science 301,* 98-106.

Nakamura, A., Yoshida, R., Maeda, H. & Corredig, M. (2006). The stabilizing behavior of soybean polysaccharide and pectin in acidified milk beverages. *International Dairy Journal 16,* 361-369.

Nursten, H. (2004). Maillard Reactions. In H. Roginski, J. W. Fuquay & P. F. Fox (Eds.), *Encyclopedia of Dairy Sciences, Vol.1* (pp. 1657-1672). New York: Elsevier.

Shepherd, R., Robertson, A. & Ofman, D. (2000). Dairy conjugate emulsifiers: casein-maltodextrins. *Food Hydrocolloids 14,* 281-286.

Pan, X., Mu, M., Hu, B., Yao, P. & Jiang, M. (2006). Micellization of casein-graft-dextran polymer prepared through Maillard reaction. *Biopolymers 81,* 29-38.

Peri, C. (2006). The universe of food quality. *Food Quality and Preference 17,* 3-8.

Semo, E., Kesselman, E., Danino, D. & Livney, Y.D. (2007). Casein micelle as a natural nano-capsular vehicle for nutraceuticals. *Food Hydrocolloids 21,* 936-942.

van Derven, C., Gruppen, H., DeBont, D.B.A. & Voragen, A.G.J. (2001). Emulsion properties of casein and whey protein hydrolysates and the relation with other hydrolysate characteristics. *Journal of Agricultural and Food Chemistry 49,* 5005-5012.

Wood, T. M. & Bhat, K.M. (1988). Methods Enzymol. 160: 87-112. In: Wooster, T. J. and M. A. Augustin (2007[b]). The emulsion flocculation stability of protein-carbohydrate diblock copolymers. *Journal of Colloid and Interface of Science* (doi:10.1016/j.jcis.2007.04.054).

Wooster, T. J. & Augustin, M.A. (2006). Beta Lactoglobulin - dextran Maillard conjugates: Their effect on interfacial thickness and emulsion stability. *Journal of Colloid and Interface Science 303,* 564-572.

Wooster, T. J. & Augustin, M.A. (2007[a]). Rheology of whey protein-dextran conjugate films at the air/water interface. *Food Hydrocolloids 21,* 1072-1080.

Wooster, T. J. & Augustin, M.A. (2007[b]). The

emulsion flocculation stability of protein-carbohydrate diblock copolymers. *Journal of Colloid and Interface of Science* (doi:10.1016/j.jcis.2007.04.054)..