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EFFECT OF THERMAL PROCESSING AND INGREDIENTS ON EGG YOLK QUALITY

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The research aims to understand how heat and added ingredients influence the properties of egg yolk which in turn impact the stability of oil-in-water emulsions. Apparent viscosity and Zeta potential of egg yolk dispersion, interfacial tension between rice bran oil and water, oil droplet size and confocal microscope image of each emulsion were investigated. The confocal microscope images of emulsions clearly show that the adsorbed protein layer was thickest when the egg yolk was heated at 65°C for 9 min and a salt-sugar-vinegar mixture was used. Thus, this condition could stabilize an oil-in-water emulsion by preventing the coalescence of oil droplets.
Keywords: Egg yolk, emulsifier, hydrophobicity, interfacial tension, zeta potential, laser scanning microscopy

INTRODUCTION

The hen egg yolk mainly contains 31% lipids and 16% protein. The lipids in the egg yolk consist of 19.9% triglyceride, 8% phosphatidylcholine, 1.2% phosphatidylethanolamine, 0.2% lysophosphatidylcholine, 1.2% cholesterol and 0.2% sphingomyelin (Mine and Zhang, 2012; Sugino *et al*., 1996). All lipids form complexes with protein and are classified into low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). The proteins, comprise 6% apolipovitellenins in LDLs, 6.4% lipovitellinapoproteins in HDLs, 2.1% livetins (glycoprotein) and 2.1% phosvitin (phosphoprotein) (Mine and Zhang, 2012; Sugino *et al*., 1996). In general, egg yolk contains 34% LDLs and 8% HDLs (Mine and Zhang, 2012). Besides protein, egg yolk also contains another amphiphile substance called lecithin which is mainly composed of phospholipids. Subsequently, egg yolk has long been used as a natural emulsifier for oil-in-water emulsions such as salad dressings and mayonnaise (Ercelebi and Ibanoglu, 2010). All these products contain salt (NaCl), sugar and vinegar which affect the conformation and emulsifying properties of the egg yolk proteins. It was reported that the addition of either 2% NaCl, 7% sugar or 5% vinegar reduces the interfacial tension between oil and water when the whole egg yolk or its purified lipoproteins are used as emulsifiers. The reason for this is that the salt disrupts the egg yolk granules and results in the active sites being exposed to the interface (Taiwo *et al*., 1997). Sugar increases the hydrophobicity of the egg yolk proteins (Antipova *et al*., 1999), while vinegar increases the positive charge density on the protein surface leading to the intramolecular electrostatic repulsion between protein chains and results in the protein unfolding and the exposure of hydrophobic regions (Kiosseoglou and Sherman, 1983; Martinez *et al*., 2007). As a result, the proteins have a higher surface activity leading to the lowering of the interfacial tension between oil and water.

Nowadays, the production of industrial scale salad dressing and mayonnaise requires pasteurized egg yolk at 60-68°C for 3.5-4.5 min to eliminate microbial flora, especially *Salmonella* (Atilgan and Unluturk, 2008). It was reported that the emulsifying property of lecithin and egg yolk proteins is not affected by these pasteurization conditions (Atilgan and Unluturk, 2008; Kobayashi *et al*., 1996; Denmat *et al*., 1999). However, heating the whole egg yolk or its fractions at 65-67°C for a few minute results in a better emulsifying property than that of native whole egg yolk (Cotterill *et al*., 1976; Ibanoglu and Ercelebi, 2007). This is due to the fact that heating causes the exposure of protein hydrophobic groups (Manoi and Rizvi, 2009; Kato *et al*., 1983; Zayas, 1997). Thus, heat-denatured proteins can adsorb better at the oil-water interface resulting in the reduction of the interfacial tension and form a strong film around oil droplets (Ercelebi and Ibanoglu, 2010; Mustapha *et al*., 2012; Hatta *et al*., 1996; Tsutsui, 1988). These phenomena prevent the coalescence of oil droplets and enhance emulsion stability. Comparison with the absence of ingredients reveals that a negative or positive value of Zeta-potential (ψ_ζ) of the dispersion of the native egg yolk was found to increase in the presence of NaCl or vinegar, respectively (Kojima and Nakamura, 1985; Guilmineau and Kulozik, 2006b). A higher value of ψ_ζ indicates a higher surface charge density for protein. The higher surface charge density of the adsorbed proteins on the oil surface gives a higher repulsion force between the oil droplets. Thus, flocculation of oil droplets is prevented (Kojima and Nakamura, 1985; Gaonkar *et al*., 2010). However, it was also found that heat and NaCl do not affect ψ_ζ of egg yolk protein.

Lipoproteins, the supramolecular assemblies of lipids and proteins, present in egg yolk are responsible for determining the egg yolk properties (Anton, 2013). The protein structure and properties have been reported to be altered due to heat, ionic strength and pH (Anton, 2013). Therefore, a combination of heating and adding ingredients clearly affects the protein part of the egg yolk lipoproteins resulting in changes in the characteristics of the egg yolk.

The research on the effects of the combination of heating and the addition of ingredients on egg yolk properties is limited and the results are questionable. Therefore, better understanding is needed to understand that how the combination of heating and addition of ingredients influences the properties of egg yolk which in turn impacts its potential to act as an emulsifier for oil-in-water emulsions. Subsequently, this research aimed at determining the effects of heating and addition of ingredients (3.1% w/w NaCl solution, 28.5% w/w sucrose solution and 20.0% w/w acetic acid solution) on the properties of egg yolk, namely, apparent viscosity, ψ_ζ and interfacial tension between rice bran oil and water (γ). In order to understand what happened to both unheated and heated egg yolk, upon addition of ingredients in the oil-in-water emulsion, the volume weighted mean diameter of oil droplets (D[4,3]) of rice bran oil-in-water emulsions was determined and confocal laser scanning microscopy (CLSM) images of these emulsions were recorded.

MATERIALS AND METHODS

Fresh egg yolk was purchased from KCF Distribution Company, Nakhonpathom, Thailand and rice bran oil from Thai Edible Oil Company, Bangkok Thailand. Analytical grade sodium chloride (NaCl) sucrose and acetic acid (vinegar) were obtained from Merck, Germany. Analytical grade sodium dodecyl sulfate, Nile Blue, Nile Red and 1,2-propanediol were obtained from Sigma Chemical Company, St. Louis, MO, USA. The water used in this study was the reverse osmosis treated water.

***Sample preparation*:** Egg yolk (80 g) and 1% w/w sodium chloride (20 g) were mixed. Some of this egg yolk mixture was heated and held in a water bath (DaihanLabtech Co., Ltd, Korea) at 60 and 65°C, while stirring at 100 rpm (IKA Works Sdn. Bhd., Malaysia) for 3, 6, 9 min. Then the heated egg yolk mixture was cooled down to 30°C by storing it at room temperature (27±2°C) for 2 h. Both unheated and heated egg yolk mixtures were kept at 7°C for 12 h. Then egg yolk dispersions or oil-in-water emulsions using egg yolk as emulsifier, were prepared from these unheated and heated egg yolk mixtures.

***Preparation of egg yolk dispersion*:** Egg yolk dispersions were prepared by dispersing either unheated or heated egg yolk mixture in five different aqueous solutions as described by Guilmineau and Kulozik (2006a). The aqueous solutions were 1) water, 2) 3.1% w/w NaCl solution, 3) 28.5% w/w sucrose solution, 4) 20.0% w/w acetic acid solution, and 5) combinations of 3.1% w/w NaCl–28.5% w/w sucrose–20.0% w/w acetic acid solution. The final concentration of egg yolk in each dispersion was 22.8% w/w. Apparent viscosity (η_{app}), Zeta-potential (ψ_ζ) and pH of these dispersions as well as interfacial tension (γ) between these dispersions and rice bran oil were determined.

***Preparation of oil-in-water emulsion*:** Either unheated or heated egg yolk mixture (18.1 g) was mixed with each of the aqueous solutions (45.5 g) separately, and then rice bran oil (36.4 g) was added. The oil-in-water emulsion was formed by stirring with a high shear mixer (L5M, Silverson, UK) at 7500 rpm for 4 min, and then homogenized by passing it through a Homogenizer (APV Systems, UK) at 200 bar for 1 cycle. The volume weighted mean diameter of oil droplets in emulsion was determined, and confocal microscopy images of emulsions were taken.

Physicochemical Property Determination and Image Analysis:

***Apparent viscosity (η_{app})*:** The η_{app} was determined with a Brookfield viscometer (model DV-II, Brookfield Engineering Laboratories Inc., USA) using the disk spindle number 2 at 24°C and 12 rpm (the calculated shear rate = 2.54 sec^{-1}). The η_{app} was recorded at a constant shearing time of 10 s.

***Zeta-potential (ψ_ζ)*:** The egg yolk dispersion was diluted 1,000 fold (v/v) with reverse osmosis treated water prior to the ψ_ζ measurement with the Malvern Zetasizer (Zetasizer 3000 HSA, Malvern Instruments Inc., UK) at 25±1°C as described by Guilmineau and Kulozik (2006a).

***pH*:** The pH values of egg yolk dispersions were measured at 25±1°C using a pH meter (HANNA instruments, USA) calibrated with buffer solutions having pH 4 and 7 (HANNA Instruments, Bangkok, Thailand).

***Interfacial tension (γ)*:** The γ between rice bran oil and water was determined with a Goniometer (FTA-200, First Ten Angstroms, Bangkok, Thailand) using the pendant drop shape analysis method as described by Shogren and Biresaw (2007). A disposable syringe (3 ml) with a blunt needle (22 gauge, 0.711 mm. OD) was used to drop either water or egg yolk dispersion into rice bran oil contained in a glass cuvette. The software fta32 V2.0 (FTA-200, First Ten Angstroms) was used for calculation of γ. All measurements were taken 24 h after preparation of the egg yolk mixture and at 24±2°C.

***Volume weighted mean diameter (D[4,3]) of oil droplet*:** The oil-in-water emulsions were diluted 100 fold (v/v) with 0.01% w/v sodium dodecyl sulfate solution, then D[4,3] of the oil droplets was determined using the laser diffraction particle size analyzer (Horiba LA-950V2, Horiba Instruments, Japan) according to the method of Guilmineau and Kulozik (2006a). The refractive index was 1.33 for water and 1.47 for oil.

***Confocal laser scanning microcopy*:** The continuous phase and oil phase were fluorescently labeled with Nile Blue

(0.01% w/w in water) and Nile Red (0.01% w/w in 1,2-propandiol), respectively. The images of the emulsions were taken with a confocal laser scanning microscope (CLSM; Olympus Fluoview FV1000, Tokyo, Japan) at the excited wavelengths of 473 nm and 635 nm with 60X objective lens, 2.2 zoom and 512x512 pixel resolution. The color image was obtained using the Olympus FV10-ASW 3.0 viewer software (Olympus Corporation, Japan) as described by Langton *et al.* (1999) and Yusoff and Murray (2011).

***Statistical analysis*:** The experimental design was a completely randomized design with two replicates. All properties were determined twice for each sample and reported as mean ± standard deviation. Statistical analyses of the data were performed using the analysis of variance (ANOVA) and significant differences between samples were analyzed by the Duncan's new multiple range test technique at 95% confidence levels using SPSS version 16.0 (SPSS, Inc., USA).

RESULTS AND DISCUSSION

***Temperature and time effecting apparent viscosity, zeta potential and ph of yolk dispersion*:** According to Guilmineau and Kulozik (2006a), both unheated and heated (76°C up to 12 min) egg yolk suspension of 20% w/w in 0.17 M NaCl (1% w/w) had flow behavior index of 1 in the shear rate range between 0-1000 sec^{-1}. Their results indicated that η_{app} of the unheated and the heated egg yolk suspensions used in this study would be independent of shear rate. Therefore, η_{app} of the unheated and the heated egg yolk suspensions could be compared at a single shear rate of 2.54 sec^{-1}. Compared to the unheated egg yolk dispersion, heating at 60°C and 65°C for 6 and 9 minutes significantly increased the η_{app} by 48, 118, 418 and 976%, respectively (Table 1). The ψ_ζ was negative for all egg yolk dispersions indicating the egg yolk had net negative charges. The value of ψ_ζ also suggested that the net negative charges increased after heating the egg yolk at 60°C and 65°C. The higher heating temperature resulted in higher net negative charges. However, the ψ_ζ was not significantly affected by the heating time at constant temperature. All egg yolk dispersions had pH of 6.2 indicating that heating had no effect on pH. The results indicated that heating at 60 or 65°C for both 6 and 9 min significantly denatured the egg yolk protein by unfolding the egg yolk protein resulting in increased protein size, and thus increasing η_{app} (Hatta *et al*., 1996; Guilmineau and Kulozik, 2006b). Egg yolk contains about 16% protein and 31% lipids (Mine and Zhang, 2012). All of the lipids of egg yolk are associated with proteins to form lipoproteins. Egg yolk can be separated into two phases: plasma (80%) and granules (20%) (Guilmineau and Kulozik, 2006a). The majority of egg yolk plasma proteins are low density lipoproteins (LDLs: 72%) and livetins (10%) which are easier to denature by heating than egg yolk granule proteins (Denmat *et al*., 1999). Both LDLs and livetins undergo irreversible denaturation at temperatures above 60°C (Tsutsui, 1988). Heating the egg yolk at around 64-65°C causes the protein to unfold resulting in an increase in viscosity, and heating at around 70°C causes an intermolecular interaction between protein chains resulting in protein gelation or coagulation (Mine and Zhang, 2012). Egg yolk granule proteins (HDL and phosvitins) are bound together by a phosphocalcic bridge between their seryl residues to form a nonsoluble complex (Denmat*et al*., 1999; Tsutsui, 1988). These complexes could protect the proteins against thermal denaturation. Therefore, upon heating above 76°C HDL and phosvitins are not affected. However, phosvitins become denatured at around 79.7°C at neutral pH when measured using differential scanning calorimetry (Chung and Ferrier, 1991). An increase in the apparent viscosity of egg yolk dispersion with temperature and heating time indicates that the degree of denaturation/unfolding of LDLs and livetin proteins increases as temperature and heating time increases (Guilmineau and Kulozik, 2007). Previously, the unfolding of globular proteins (such as LDLs and livitins) was reported to bring about the exposure of hydrophobic groups to the protein surface, so the hydrophobicity of the protein surface increases (Kojima and Nakamura, 1985). Recently, with the measurement of ψ_ζ, any changes in the net surface charge can be monitored. Benitez and Lozano (2006) reported that unheated whey protein at neutral pH had a net negative charge (with ψ_ζ of -24 mV) but

Table 1. Influence of temperature and heating time on apparent viscosity (at 24°C and 2.54 sec^{-1}), Zeta-potential and pH of aqueous egg yolk dispersion (22.8% w/w).

Egg Yolk	Heating at Temperature (°C)	Heating at Time (min)	Apparent Viscosity (mPa.S)	Zeta Potential (mV)	pH^{ns}
Unheated	-	-	7.05^{e}±0.00	-9.65^{c}±0.07	6.22±0.01
Heated	60	6	10.45^{d}±0.07	-10.80^{b}±0.14	6.26±0.01
		9	15.40^{c}±0.57	-11.45^{b}±0.78	6.25±0.01
	65	6	36.55^{b}±1.48	-11.65^{ab}±0.35	6.26±0.00
		9	75.85^{a}±1.20	-12.90^{a}±0.71	6.29±0.01

Values above are mean ± SD; Means within a column with the same letter are not significantly different at the 5% level of probability; ^{ns} indicates no significant difference in pH among the samples at the 5% level of probability.

Table 2. Influence of ingredients on apparent viscosity, Zeta potential and pH of the unheated and heated egg yolk dispersions.

Egg yolk	Ingredients(%w/w)			Apparent viscosity (mPa.S)	Zeta potential (mV)	pH
	Salt	Sugar	Vinegar			
Unheated	-	-	-	7.50^{Db}±0.00	-9.65^{Db}±0.07	6.2±0.0
	3.1	-	-	7.50^{Db}±0.00	-11.50^{Ens}±0.14	6.3±0.0
	-	28.5	-	10.00^{Cb}±0.00	-7.85^{Cb}±0.21	6.2±0.0
	-	-	20.0	5.00^{Eb}±0.00	24.20^{Ans}±0.71	3.8±0.0
	3.1	28.5	-	7.50^{Db}±0.00	-11.00^{Ens}±0.42	6.2±0.0
	3.1	-	20.0	10.00^{Cb}±0.00	25.50^{Ab}±0.99	3.7±0.0
	-	28.5	20.0	12.50^{Bb}±0.00	24.80^{Ans}±0.57	3.8±0.0
	3.1	28.5	20.0	15.45^{Ab}±0.64	23.10^{Ba}±0.57	3.7±0.0
Heated	-	-	-	75.85^{Ca}±1.20	-12.90^{Ea}±0.71	6.2±0.0
(65°C,	3.1	-	-	10.00^{Ea}±0.00	-12.50^{Ens}±0.28	6.3±0.0
9min)	-	28.5	-	17.70^{Ea}±0.28	-9.85^{Da}±0.21	6.2±0.0
	-	-	20.0	8.75^{Ea}±1.77	25.50^{Bns}±0.14	3.8±0.0
	3.1	28.5	-	13.80^{Ea}±1.77	-12.20^{Ens}±0.07	6.2±0.0
	3.1	-	20.0	32.80^{Da}±0.14	28.50^{Aa}±0.99	3.8±0.0
	-	28.5	20.0	117.00^{Ba}±2.83	25.40^{Bns}±0.57	3.8±0.0
	3.1	28.5	20.0	348.50^{Aa}±10.60	21.50^{Cb}±0.14	3.7±0.0

Values above are mean ± SD; Means within a column with same uppercase letter are not significantly different at the 5% level of probability; Different lowercase letters represent statistically significant differences for pairwise comparisons between the unheated and heated egg yolks; ^{ns} indicates no significant difference in pH among the samples at the 5% level of probability.

heating at 80°C for 15 minutes generated a higher net negative charge (with ψ_ζ of -36.9 mV). Thermal denaturation is thus the major factor in changing the distribution of the charged surface amino acids (Zhong *et al*., 2013). In terms of the results of pH values measurement, there was no prominent effect of the heating on the pH values of egg yolk dispersion.

***Heating and ingredients that effect apparent viscosity of yolk dispersion*:** Table 2 shows the effect of heating and ingredients on the apparent viscosity of egg yolk dispersions. The concentrations of salt, sugar and/or vinegar used in the egg yolk dispersions were 3.1, 28.5 and 20.0% w/w, respectively. For the unheated egg yolk dispersion, the presence of salt and salt-sugar did not significantly affect the apparent viscosity (η_{app}) at 24°C and 2.54 sec^{-1} shear rate, but the presence of sugar increased η_{app} compared to the absence of the ingredient. Vinegar decreased η_{app}. However, this property increased by adding salt and/or sugar together with vinegar. Without added ingredients, heating the egg yolk at 65°C for 9 min resulted in an increase in η_{app} in the egg yolk dispersion. For the heated egg yolk dispersions, the presence of salt, sugar, vinegar, salt-sugar, and salt-vinegar decreased the η_{app} by 86, 76, 88, 81 and 56%, respectively. The decrease in the η_{app} of the heated egg yolk dispersion in the presence of salt indicated that surface charges of heated protein were shielded by their counter ions from salt causing a more compact conformation (Fink *et al*., 1994). From Huggins equation (Eq 1), viscosity of polymer solution is a function of viscosity of solvent, concentration (C) of polymer and the intrinsic viscosity of polymer in solvent ([η]). In this case, polymers were egg yolk proteins.

$$[(\eta_{sol}/\eta_0)\text{-}1]/C = [\eta] + k_H[\eta]^2\, C \qquad [1]$$

All aqueous solutions used in this research were good solvents for protein. Therefore, a Huggins constant (k_H) of about 0.3 can be assumed for all solvents (Pamies *et al*., 2008). The [η] of protein depends on its size and shape. If protein size and shape do not change by adding salt, sugar and/or vinegar, [η] becomes constant. If k_h, C and n were constant that results in $[\eta] + k_H[\eta]^2C$ being constant as well. Therefore, change in the viscosity of protein was solely due to a change in the viscosity of solvent following Eq 2.

$$\eta_{sol_v}/\eta_{sol_w} = \eta_{o_v}/\eta_{o_w} \qquad [2]$$

Subscripts w and v indicate the properties in water and aqueous solution with salt, sugar and/or vinegar, respectively. Table 3 shows the comparison between $\eta_{sol_v}/\eta_{sol_w}$ (η') and η_{o_v}/η_{o_w} (η"). If the change in the viscosity of protein solution is solely due to the change in solvent viscosity, η'/η" = 1. Deviation of η'/η" from 1 indicates change in protein size and shape as affected by adding ingredients (salt, sugar and/or vinegar). The larger size of protein and/or shape deviating from the sphere gives η'/η" >1. Change in the protein to a more compact size gives η'/η"<1. The analysis indicated that the heated egg yolk proteins had larger size and/or shape deviating from the sphere after adding the mixture of salt, sugar and vinegar. The mixture of salt and vinegar did not affect the unheated egg yolk protein, while the other solutions

Table 3. Assessment of the effect of ingredients on egg yolk protein conformation.

Egg yolk	Ingredients (%w/w)			η'' (η_{o_v}/η_{o_w})	η_{sol_v}	η' ($\eta_{sol_v}/\eta_{sol_w}$)	η'/η''
	Salt	**Sugar**	**Vinegar**				
Unheated	-	-	-	1.00	7.50	1.00	1.00
	3.1	-	-	1.35	7.50	1.00	0.74
	-	28.5	-	2.20	10.00	1.33	0.61
	-	-	20.0	1.16	5.00	0.67	0.57
	3.1	28.5	-	2.41	7.50	1.00	0.42
	3.1	-	20.0	1.30	10.00	1.33	1.03
	-	28.5	20.0	2.30	12.50	1.67	0.72
	3.1	28.5	20.0	2.63	15.45	2.06	0.78
Heated	-	-	-	1.00	75.85	1.00	1.00
(65°C, 9 min)	3.1	-	-	1.35	10.00	0.13	0.10
	-	28.5	-	2.20	17.70	0.23	0.11
	-	-	20.0	1.16	8.75	0.12	0.10
	3.1	28.5	-	2.41	13.80	0.18	0.08
	3.1	-	20.0	1.30	32.80	0.43	0.33
	-	28.5	20.0	2.30	117.00	1.54	0.67
	3.1	28.5	20.0	2.63	348.50	4.59	1.75

η_{o_v}, η_{o_w} : Viscosity of solvent, water; η_{sol_v}, η_{sol_w}: Viscosity of polymer solution, water.

caused both unheated and heated egg yolk proteins to be more compact.

This might be due to the fact that salt shields the protein surface charge, sugar enhances the pair-wise hydrophobic interactions of proteins and protein becomes the molten globule at pH ≤ 4 (Kurt and Zorba, 2009).

***Zeta potential*:** The Zeta potential reported in Table 2 indicates that both unheated and heated egg yolk proteins had a net negative surface charge and heating caused an increase in surface charge density. The results also indicate that the net surface charge remained negative after adding salt and/or sugar, but inverted to a positive charge in the presence of vinegar. The reason for vinegar altering the zeta potential of the egg yolk dispersions from negative to positive for both the unheated and heated egg yolk proteins was due to the pH of the system (pH = 4), which was lower than the isoelectric point (pI, which is the pH that proteins have a net zero charge) of the major protein of the egg yolk, lipoproteins: pH ~ 6.3-7.5 (Kojima and Nakamura, 1985; Gaonkar *et al*., 2010). Thus, protein had a net positive surface charge. Despite the increase in screening by sodium ions from the dissolved salt, the negative Zeta-potential ($-\psi_\zeta$) of the unheated egg yolk proteins increased by adding salt. This suggested that salt not only caused the screening effect, but also increased the surface charge density of the egg yolk proteins. However, salt did not affect the surface charge density of the heated egg yolk protein. Sugar decreased the surface charge density of both the unheated and heated egg yolk. This may be explained by the expanding location of the slipping plane as the sugars may form a viscous hydration layer on the surface of the proteins (Matsumoto, 1994). In the presence of vinegar, protein surface charge density did not change by adding sugar, but it decreased after adding the combination of salt and sugar for both unheated and heated egg yolk. The presence of salt together with vinegar did not affect the surface charge density of the unheated egg yolk, but increased the surface charge density of the heated egg yolk. This result suggested that the same phenomena occurred as for addition of salt alone to the unheated egg yolk.

***pH*:** For both unheated and heated egg yolk dispersion, adding salt and/or sugar did not change the pH, but adding vinegar decreased the pH from 6.2-6.3 to 3.7-3.8 (Table 2), due to the acidic characteristic of vinegar.

***Effect of heating and ingredients on the interfacial tension between rice bran oil and water*:** The heated egg yolk gave lower γ between rice bran oil and water, and thus was a better emulsifier than the unheated egg yolk. The conformational modification of proteins as a result of heat treatment leads to partial protein unfolding and the generation of additional exposed hydrophobic regions previously buried inside the native structure (Manoi and Rizvi, 2009). For both unheated and heated egg yolk, only the salt increased the interfacial tension, while the presence of sugar and/or vinegar decreased γ between rice bran oil and water (Table 4). The best ingredient for lowering γ was found to be sugar. The effect of salt on γ between rice bran oil and water might be because salt, as a water-soluble electrolyte, raised the surface tension between water and air. The electrolyte ions are repelled from the air-water interface due to the electrostatic repulsive interaction between electrolyte ions and air (Hoofar *et al*., 2006).

Table 4. Influence of heating and ingredients on the interfacial tension between rice bran oil and water using unheated and heated egg yolk as emulsifiers.

Egg yolk	Ingredients (% w/w)			Interfacial tension (mN/m)
	Salt	Sugar	Vinegar	
Unheated	-	-	-	9.38^{Ba}± 0.02
	3.1	-	-	10.93^{Aa}± 0.03
	-	28.5	-	4.39^{Fa}± 0.02
	-	-	20.0	7.59^{Ca}± 0.06
	3.1	28.5	-	5.33^{Ea}± 0.03
	3.1	-	20.0	5.60^{Da}± 0.04
	-	28.5	20.0	3.87^{Ga}± 0.03
	3.1	28.5	20.0	3.95^{Ga}± 0.02
Heated	-	-	-	8.62^{Bb}± 0.02
	3.1	-	-	8.84^{Ab}± 0.01
	-	28.5	-	3.18^{Fb}± 0.09
	-	-	20.0	6.78^{Cb}± 0.00
	3.1	28.5	-	4.57^{Eb}± 0.01
	3.1	-	20.0	5.16^{Db}± 0.06
	-	28.5	20.0	3.02^{Gb}± 0.09
	3.1	28.5	20.0	3.03^{Gb}± 0.04

Values above are mean ± SD; Means within a column with same uppercase letter are not significantly different at the 5% level of probability; Different lowercase letters represent statistically significant differences for pairwise comparisons between the unheated and heated egg yolks.

Addition of less than 1M sucrose has been found earlier to increase the interfacial adsorption rate of protein due to the presence of sugar protein having a compact form and reduced protein aggregation resulting in a fast diffusion rate and allowing more protein to be involved in film formation at interfaces (Wilde *et al*., 1997). The reasons for a decrease in interfacial tension by acid were: 1) the molecular concentration adsorbed was lower than at the pH close to the pI; consequently, protein spread more readily along the interface, and 2) the higher positive surface charge density might have promoted spreading by weakening those bonds which normally stabilize the micelle structure.

***Volume weighted mean diameter of oil-in-water emulsions effected by heating and ingredients*:** Table 5 shows that using the heated egg yolk as an emulsifier showed a larger D[4,3] when compared to the unheated egg yolk except in the presence of only sugar. D[4,3] was hardly affected by the addition of salt and salt-sugar, but decreased after the addition of sugar alone when either unheated or heated egg yolk was used as the emulsifier. D[4,3] became larger in the presence of vinegar. Adding salt in the presence of vinegar resulted in slightly lower D[4,3] for vinegar only; on the other hand, adding sugar or salt-sugar significantly enlarged D[4,3]. The largest D[4,3] was found when the three ingredients were added together. The D[4,3] reflected the combination of oil and adsorbed protein layers. Lowering the γ between rice bran oil and water using heated egg yolk and adding ingredients suggested an increase in the adsorbed protein on the surface

Table 5. Influences of heating and ingredients on the volume weighted mean diameter of oil droplets for oil-in-water emulsion using the egg yolk dispersion as an emulsifier.

Egg yolk	Ingredients (% w/w)			Volume weighted mean diameter (μm)	Size ranges (μm)
	Salt	Sugar	Vinegar		
Unheated	-	-	-	1.36^{Db}±0.02	0.29-4.47
	3.1	-	-	1.46^{Db}±0.05	0.22-5.86
	-	28.5	-	0.96^{Ens}±0.01	0.22-2.97
	-	-	20.0	9.62^{Cb}±0.21	2.26-17.84
	3.1	28.5	-	1.09^{DEb}±0.01	0.25-17.42
	3.1	-	20.0	9.14^{Cb}±0.10	2.26-15.21
	-	28.5	20.0	10.32^{Bb}±0.26	1.98-17.33
	3.1	28.5	20.0	16.27^{Ab}±0.31	2.59-28.52
Heated	-	-	-	2.99^{Da}±0.02	1.00-8.81
	3.1	-	-	2.57^{Da}±0.03	0.66-7.69
	-	28.5	-	1.16^{Ens}±0.00	0.25-3.95
	-	-	20.0	11.15^{Ca}±0.73	2.26-16.14
	3.1	28.5	-	2.65^{Da}±0.01	0.87-11.81
	3.1	-	20.0	10.50^{Ca}±0.21	2.59-16.75
	-	28.5	20.0	13.98^{Ba}±0.49	0.33-19.66
	3.1	28.5	20.0	19.44^{Aa}±0.70	2.97-33.82

Values above are mean ± SD; Means within a column with same uppercase letter are not significantly different at the 5% level of probability; Different lowercase letters represent statistically significant differences for pairwise comparisons between the unheated and heated egg yolks.

of oil droplets. Therefore, to verify whether an increase in D[4,3] was due to an increase in oil droplet size, adsorbed protein layer, or both, the confocal image of each emulsion was taken and is reported in the following section.

***Confocal Laser Scanning Microscope (CLSM) Images*:** Figure 1 shows the images of emulsions formed by either unheated (u) or heated (h) egg yolk taken by confocal laser scanning microscope. For each emulsion, three pictures were

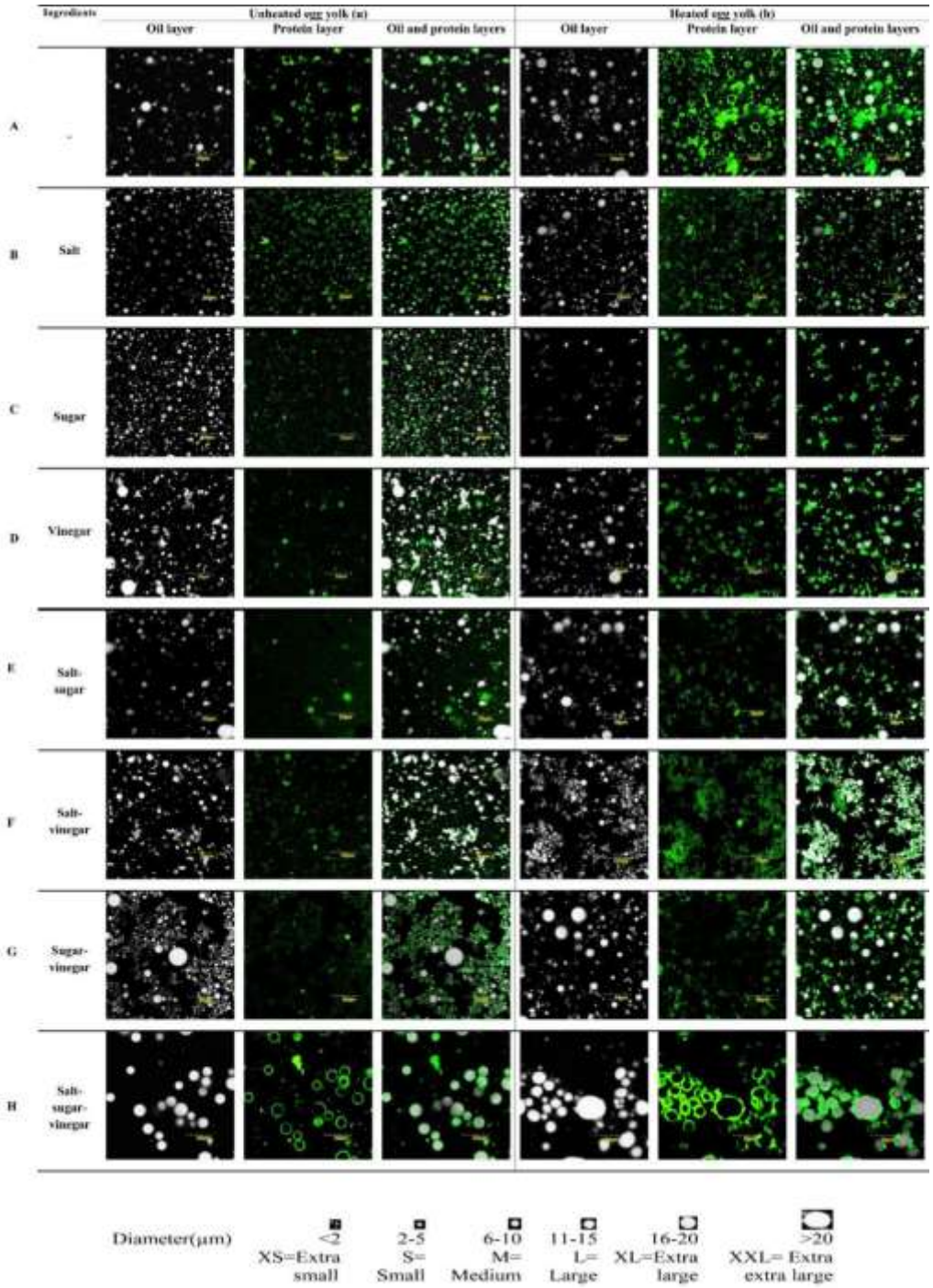

Figure 1. CLSM images of oil-in-water emulsion using the unheated egg yolk and the heated egg yolk (65°C, 9 min) in the absence of and in the presence of three ingredients; the white labelled oil droplet and the protein layer labelled green color.

processed from the same CLSM image. To differentiate between egg yolk proteins adsorbed and non-adsorbed on the surface of oil droplets, the first picture shows the oil (white) labelled layer, the second the protein (green) labelled layer, and the third the oil (white) and protein (green) labelled layers. Additionally, the O, P and OP letters represent the oil layer, protein layer and oil-protein layers, accordingly. The sizes of oil droplets were defined by their diameters as extra small (XS), small (S), medium (M), large (L), extra large (XL) and extra-extra large (XXL) for < 2, 2-5, 6-10, 11-15, 16-20, >20 µm, respectively.

***Unheated egg yolk*:** In the absence of ingredients (Figure 1A-u), oil droplets with a size range from XS to M were found. The number of XS oil droplets constituted more than 50% while M droplets constituted less than 10%. Egg yolk proteins were mostly adsorbed on the oil surface. Some non-adsorbed egg yolk proteins were also found. The XS droplets were fully covered by proteins, whereas proteins were only partially adsorbed on the surface of the medium sized droplets. No protein was adsorbed on the largest droplets. Figure 1A-u-OP also shows the bridging flocculation of oil droplets by proteins. Compared to the emulsion without any added ingredients, the size of the oil droplets in the presence of salt solution (Figure 1B-u) was more homogeneous. About the same amounts of XS and S oil droplets were found. Almost all of oil droplets were covered with proteins, except for a few S droplets on which proteins were only partially adsorbed. Bridging flocculation of oil droplets by proteins was also found.

In the sugar solution (Fig. 1C-u), only oil droplets of sizes XS and S were found similar to those found in the salt solution, though the number of S droplets in the sugar solution was lower than that in the salt solution. However, the oil droplets fully covered by proteins were fewer than those in the salt solution. Furthermore, the bridging flocculation of oil droplets by proteins was not as clear as in the salt solution. The homogeneity of oil droplet size in vinegar solution (Fig. 1D-u) was less than that in the absence of ingredients. All sizes of oil droplets were found, although the majority of oil droplets were of XS or S sizes. Only one oil droplet was XL size. Proteins only partially adsorbed on the surface of most of the oil droplets. Coalescence of oil droplets was clearly visible.

Compared to the emulsions in either the salt solution or sugar solution, the salt-sugar solution (Fig. 1E-u) had greater heterogeneity in the size of oil droplets. Oil droplets ranging in size from XS to L were detected. In addition, the adsorbed proteins on the surface of oil droplets were fewer than in the emulsions with either the salt or sugar solutions. For the emulsion in the salt-vinegar solution (Fig. 1F-u), oil droplets had wider range of size and the adsorbed proteins were fewer than those in salt solution. The opposite phenomena occurred when comparing the emulsion in the salt-vinegar solution to that in the vinegar solution. The bridging flocculation of oil droplets by proteins and the coalescence of oil droplets in the salt-vinegar solution were less than those in the vinegar solution and vice versa in the salt solution. Similar to the emulsion in vinegar only solution, the emulsion in sugar-vinegar solution (Fig. 1G-u) had oil droplets ranging in size from XS to XL. The number of adsorbed proteins on XS and S droplets in the sugar-vinegar solution was much higher than those in the vinegar only solution. As in the vinegar solution, the L and XL droplets were not covered by proteins. The bridging flocculation of oil droplets by proteins in the sugar-vinegar solution was more pronounced than that in either the sugar solution or vinegar solution. In the salt-sugar-vinegar solution (Fig. 1H-u), the size of almost all of the oil droplets was in the range from M to L and they were fully covered by the adsorbed proteins. Aggregation of proteins was also found (Fig. 1H-P). Neither the bridging flocculation of oil droplets by proteins nor the coalescence of oil droplets was evident.

***Heated egg yolk*:** Compared to the unheated egg yolk in the absence of ingredients (Figure 1A-u), the heated egg yolk (Fig. 1A-h) gave a wider size range of oil droplets from XS to L. Moreover, the oil droplets were almost entirely covered by proteins and the bridging flocculation of small oil droplets was clear-cut. In the heated egg yolk emulsion, no L size oil droplets were found in the presence of salt (Fig. 1B-h). The adsorbed proteins covered the surface of S and M droplets and the bridging flocculation in the presence of salt was less than in emulsions without added ingredients (Fig. 1A-h). For the emulsion of the oil-in-salt solution, the heated egg yolk (Fig. 1B-h) had a greater number of M size droplets than the unheated egg yolk (Fig. 1B-u). Oil droplets of sizes XS and S fully covered by proteins were found in the emulsion of the oil-in-sugar solution emulsified by the heated egg yolk (Fig. 1C-h). The bridging flocculation in this emulsion was less than in the emulsion without ingredients (Fig. 1C-h). The bridging flocculation was easily seen compared to the emulsion of the oil-in-sugar solution emulsified by the unheated egg yolk (Fig. 1C-u). Using heated egg yolk as emulsifier, the major differences between oil-in-water (Fig. 1A-h) and oil-in-vinegar (Fig. 1D-h) emulsions were the higher numbers of M size droplets and the larger bridging flocculation in oil-in-water emulsion. For the oil-in-vinegar emulsion, emulsifying with the heated egg yolk clearly produced oil droplets of smaller size more heavily covered and flocculated by proteins than emulsifying with the unheated egg yolk (Fig. 1D-u). No coalescence of oil droplets was found in the emulsion formed by the heated egg yolk. Using the heated egg yolk as emulsifier, the droplet size and distribution of the emulsion of oil-in-solution of salt and sugar (Fig. 1E-h) was similar to that of oil-in-water emulsion (Fig. 1 A-h). However, the L size droplets were not fully covered with proteins and bridge flocculation occurred to a lesser extent than in the emulsion of the oil-in-water emulsion. In emulsion with salt-sugar solution, the heated egg yolk

covered and flocculated oil droplets better than the unheated egg yolk (Fig. 1E-u).

With heated egg yolk, droplets larger than M size were not found in the emulsion in salt-vinegar solution (Fig. 1F-h). This emulsion had greater numbers of XS and S size droplets than both the oil-in-water emulsion emulsified by the heated egg yolk (Fig. 1A-h) and the emulsion in salt-vinegar solution emulsified by unheated egg (Fig. 1F-u). The bridge flocculation in this emulsion had substantially thinner protein threads than in the oil-in-water emulsion formed by the heated egg yolk. In contrast, this emulsion had more extended bridge flocculation than the emulsion in the salt-vinegar solution emulsified by unheated egg yolk. Compared to the oil-in-water emulsion, oil droplets were larger (up to XL), but were covered with a thinner protein layer in the emulsion of the oil-in-solution of sugar and vinegar when the heated egg yolk was used in both cases. The bridge flocculation in the emulsion of oil-in-solution of sugar and vinegar with the heated egg yolk was less compared to that in both the oil-in-water emulsion emulsified by the heated egg yolk (Fig. 1A-h) and the emulsion in sugar-vinegar solution emulsified by unheated egg (Fig. 1G-u). Furthermore, in the sugar-vinegar solution, the unheated egg yolk delivered a significantly greater number of XS size droplets than the heated egg yolk (Fig. 1G-h). Among all emulsions, that containing salt, sugar and vinegar, emulsified by the heated egg yolk, had the largest number of oil droplets covered and flocculated by the thickest layer of adsorbed proteins (Fig. 1H-h).

The results of confocal laser scanning microscope images of O/W emulsion confirmed that the presence of the mixture of salt, sugar and vinegar promoted the unfolding and adsorbing at the oil surface of the heated egg yolk proteins. Consequently, a thicker layer of adsorbed proteins around oil droplets prevented the coalescence of oil droplets.

***Conclusions*:** The apparent viscosity and negative zeta potential of the egg yolk dispersion increased with temperature and heating time. Mostly, the presence of ingredients resulted in more compact egg yolk proteins (for both unheated and heated) as compared to the absence of ingredients, except for the mixture of salt-sugar-vinegar which enlarged the heated egg yolk proteins. Without vinegar, egg yolk proteins had a net negative surface charge, while with vinegar, egg yolk proteins had a net positive surface charge with about twice the surface charge density. For both heated and unheated egg yolk, sugar lowered the net negative surface charge density, while the mixture of salt-sugar-vinegar decreased the net positive surface charge density. Compared to the unheated egg yolk proteins, the heated egg yolk proteins were better surface active agents for the rice bran oil and water interface. In general for both the heated and unheated egg yolk proteins used as emulsifier, the γ between rice bran oil and water was lower in the presence of ingredients than in the absence of ingredients. Vinegar resulted in larger oil droplet size and thicker adsorbed protein layer for both unheated and heated egg yolk. The heated egg yolk proteins and the mixture of salt-sugar-vinegar gave the thickest adsorbed protein layer which was the best for preventing the coalescence of oil droplets. According to these results, using heated egg yolk in oil-in-water emulsion could stabilize the product by inhibiting coalescence. Heat stable oil-in-water products might be produced using heated egg yolk as emulsifier.

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