THE EFFECT OF ULTRASOUND TIMES AND AMPLITUDES ON THE NANOEMULSIONS CHARACTERIZATION AND LIPID OXIDATION OF WHEY PROTEIN/CANOLA OIL NANOEMULSIONS DURING STORAGE

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***Abstract*— The current work was conducted in order to explore the influence of ultrasound times and amplitudes on the characterization (droplet size, turbidity, and emulsion stability) and lipid oxidation of whey protein nanoemulsions (WPCON). Ultrasound (US) application was employed using a VC-750 ultrasonic power equipment with the frequency of 20 kHz at various times (10, 20, and 30 minutes at 50% amplitude) and amplitudes (60%, 80%, and 100% for 5 min). The outcomes exhibited that the US process have a significant impact on both functional properties and lipid oxidation ($p<0.05$). The smallest droplet size was obtained for the samples exposed to 30 min US at 100% amplitude (608.3 nm). WPCON samples treated at 100% amplitude showed smaller droplet sizes compared to the other samples at 60% and 80% amplitudes. While the droplet size of WPCON samples treated with 10 min showed biggest droplet size (895.2 nm), samples treated with 30 min showed the smallest droplet size (795.1 nm) in terms of ultrasound times. There is a positive relationship between the WPCON of droplet size and turbidity. All US-treated WPCON samples exhibited less turbidity and smaller droplet size where the control WPCON samples demonstrated most turbid structure with the biggest droplet sizes. In addition, the best emulsifying properties (EAI: 255.08 m^2 /g and ESI: 43 min) was obtained for the samples exposed to 30 min US at 100% amplitude (US310). The same sample showed the least lipid oxidation during 7 days of storage.**

***Keywords*— Ultrasound, droplet size, emulsifying properties, lipid oxidation, whey protein/ canola oil nanoemulsion.**

I. INTRODUCTION

Whey protein is a crucial material of functional protein components for several conventional and novel food materials (Kumar *et al*., 2018). Whey proteins are recognized as complete proteins since they include all 9 essential amino acids. Lactose content is low in whey products. When the liquid whey is obtained as a by-product of cheese or yoghurt fabrication, it is subjected to different processes in order to make the protein content higher (Liu *et al*., 2014). After enough protein concentration is obtained, the liquid could be dried to develop whey protein concentrate (WPC) including nearly 80% protein. The major proteins found in whey can be listed as b-lactoglobulin, a-lactalbumin and bovine serum albumin (BSA), and these proteins are composed of almost seventy-percent of all whey proteins (Arzeni *et al*., 2012). These proteins are in charge of the functional features of WPC, such as solubility in water and propose various nutritional benefits to functionalized products (Krešicål *et al*., 2008).

Various methodologies have been promoted to alter the native protein structure for the purpose of improvement of the functionality. Modified whey proteins exhibit a very high level of functionality capacity. By molecular and physical alterations, it is achievable to reorganize protein compounds so that they develop into more practical and useful form. Ultrasound (US) technology is a cost effective and fast application which has been employed to alter both the structure and functional properties of protein molecules (Mason *et al*., 1996; Jambrak *et al*., 2008; Yıldız, 2018). The impact of US treatment is accomplished by the chemical, molecular, and physical consequences of acoustic cavitation. The cavitation mostly defined as a creation, development, and powerful breakdown of tiny droplets in solution. The cavitation could be the reason of protein structure modification thanks to hydrogen bonds and hydrophobic cooperation, and falling apart the protein molecules (Yildiz *et al*., 2017). By taking into account the benefits of US application such as being a cost-effective, non-toxic, fast and efficient process, it is anticipated to reach a goal of advanced whey protein/canola oil nanoemulsion functionality by using US application. For this reason, the purpose of the present work is to analyze the impact of US application on the droplet size, turbidity, emulsion stability, and lipid oxidation of WPCONs.

II. METHODS

A. Nanoemulsion preparation

Oil-in-water (O/W) WPCO nanoemulsion was prepared with canola oil (CO) and whey protein (WP). Canola oil (0.125 g) was mixed with 50 mL WP (10 mg/mL) and stirred powerfully for 5 min with a magnetic stirrer. For the achievement of better homogenization, WPCON specimens were sonicated at three different times (10, 20, and 30 minutes at 50% amplitude) and amplitudes (60%, 80, and 100% for 5 min) using a VC-750 ultrasound unit (20 kHz, Sonic & Materials, Inc., Newtown, CT, USA). The conditions for the US application were listed in Table 1.

Table 1. The description of the WPCONs and treatments

Sample names	Treatments
Control	Untreated WPCON, no ultrasound
US1	Ultrasound treatment with 10 min (50% amp.)
US2	Ultrasound treatment with 20 min (50% amp.)
US3	Ultrasound treatment with 30 min (50% amp.)
US6	US treatment at 60% amplitude (5 min)
US8	US treatment at 80% amplitude (5 min)
US10	US treatment at 100% amplitude (5 min)
US16	US treatment with 10 min at 60% amp.
US18	US treatment with 10 min at 80% amp.
US110	US treatment with 10 min at 100% amp.
US26	US treatment with 20 min at 60% amp.
US28	US treatment with 20 min at 80% amp.
US210	US treatment with 20 min at 100% amp.
US36	US treatment with 30 min at 60% amp.
US38	US treatment with 30 min at 80% amp.
US310	US treatment with 30 min at 100% amp.

B. Droplet size and turbidity

The droplet sizes of WPCON were measured following the methodology figured out by Yildiz *et al*. (2017) via dynamic light scattering (DLS) with the assist of NICOP 38 DSL instrument (Santa Barbara, CA, USA). WPCONs were diluted 500-fold with deionized H_2O before achieving DSL analysis. All experiments were conducted at a stable scattering angle of 90º along with the wavelengths of 658 at room environment. The average of droplet sizes was achieved as the mean of 3 measurements where each measurement was performed for about a minute.

Turbidity of the WPCON dispersions was figured out by a spectrophotometer according to the methodology proposed by Yildiz *et al.* (2017). DI water was used as the blank, and the absorbance at 600 nm was obtained.

C. Emulsifying characteristics

Emulsifying feature of WPCON samples was achieved by the approach of Yildiz *et al*. (2018). Oil in H_2O suspensions were made with the addition of a mL of canola oil in three mL of the whey protein. The ratios of oil quantity were 0.25% (w/w) in this measurement. The combined oil and whey protein were stirred harshly for around 5 min and then sonicated at three different times (10, 20, and 30 minutes at 50% amplitude) and amplitudes (60%, 80, and 100% for 5 min). After suspension occurrence, the absorbances of the WPCON were determined at 500 nm at 0 (A_0) and 10 min (A_{10}), subsequently. Both emulsifying activity index (EAI) and emulsion stability index (ESI) were determined by using the below formulas:

$$\textbf{EAI } (m^2/g) = 2T\ A_0 \times \text{dilution factor} / c \times Q \times L \times 10.000$$

$$\textbf{ESI (minute)} = A_0 / (A_0 - A_{10}) \times 10 \text{ (minute)}$$

where T: 2.303, dilution element: 100, c: protein weight per unit volume (g/mL), L: width of the optical pathway (0.01 m), and Q: volumetric oil concentration (0.25).

D. Lipid oxidation

Lipid hydroperoxide values formed at storage of WPN were measured as stated in Min *et al.* (2003). WPN samples (around 5 mL) were added in a test tubes and let the oxidation under 25 ºC in the dark. Lipid hydroperoxide value was determined after mixing 0.3 mL of whey emulsions with 1.5 mL of isooctane/2-propanol (3:1, v/v) via vortexing (around 10 s and 3 times) and isolation of organic solvent parts subsequent to centrifugation at 1000 g for about 2 min. The organic solvent part (200 μL) was mixed into 2.8 mL of methanol/1-butanol (2:1, v/v), and followed by 15 μL of 4 M ammonium thiocyanate and 15 μL of ferrous iron solution (created via blending 0.15 M $BaCl_2$ & 0.144 M FeSO4). Subsequent to 20 minutes time period, the absorbances of the whey protein solutions were determined at the wavelengths of 510 nm. Lipid hydroperoxide values of the WP nanoemulsion was determined at 1, 2, 3, 4, 5, 6, and 7th days.

E. Statistical Analysis

The differences were achieved with the General Linear Model process in SAS (version 9.3, SAS Institute, Inc., Cary, North Carolina, USA). A significant difference among the mean values was defined by Fisher's least significant difference (LSD) test at alpha = 0.05.

III. RESULTS

A. Droplet size and Turbidity

Table 2 displays the findings related to droplet sizes of the WPCON samples exposed to different US treatments. All US treated WPCON samples displayed significantly smaller droplet size in comparison with the control WPCONs. Moreover, the smallest droplet size was obtained for the WPCON samples exposed to 30 min US at 100% amplitude (608.3 nm). WPCON samples treated at 100% amplitude showed smaller particle size compared to the other WPCON samples at 60% and 80% amplitudes (Table 2). An inverse relationship between the droplet size and ultrasound amplitude was determined. The higher the amplitudes, the smaller the particle sizes. While the amplitude was lowest (60%), the droplet size was the highest (797.3 nm). On the other hand, while the amplitude highest (100%), the particle sizes was the smallest (693.5 nm). It was obviously seen that rising US amplitudes advances the droplet size of whey protein/canola oil nanoemulsions. WPCON samples treated with 30 min showed the smallest particle size (795.1 nm) compared to the WPCON samples treated with 10 and 20 min (Table 2). Similar to the amplitude, ultrasound times have also inverse relationship with droplet size. Increasing ultrasound time from 10 to 30 min leads to smaller droplet size. While the droplet size of WPCON samples treated with 10 min showed the biggest particle size (895.2 nm), WPCON samples treated with 30 min showed the smallest particle size (795.1 nm). The unfolding process especially by ultrasound process may cause WPCON samples to become more susceptible to breakdown. The decline in the droplet sizes of plant protein sources (i.e., soy protein, and pea protein) were reported in previous works (Lee *et al*., 2016; Yildiz *et al*., 2017; Yildiz *et al*., 2018; Jiang *et al.*, 2019). In the study of Jambrak *et al*. (2014) following application with an ultrasonic probe (20 kHz), high intensity ultrasound treatment

led to a decrease in particle size as well as narrowed their distribution, and significantly raise specific free surface ($p<0.05$) in whey protein specimens. When the use in protein suspensions, ultrasound treatment was expressed to significantly lower the droplet sizes of whey protein samples (Jambrak *et al*., 2008). Moreover, Karki *et al*. (2010) determined that the droplet sizes of defatted soy flakes samples were decreased approximately 10-fold after ultrasound application. It was figured out that the cavitation may be the explanation of the breakage of protein aggregates, and decline in the droplet size (Arzeni *et al*., 2012; Yildiz, 2019; Yildiz and Aadil, 2020). Gordon and Pilosof (2010) accomplished to control particle size via high intensity ultrasound by merging several treatment periods, temperatures and ratios of whey protein dispersions. Ultrasound process develops a new surface and makes lower the size of the aggregates (Yildiz and Feng, 2019). In this case, the protein droplet sizes are decreased due to the cavitation phenomena. This involves the degradation of protein aggregates and agglomerates. Ultrasonic cavitation is very efficient to break up protein substances and smaller particle aggregates the van der Walls's forces (Jambrak *et al*., 2014).

The turbidity findings of WPCON specimens are demonstrated in Table 2. Both the solubility and particle sizes of soluble protein aggregates determine the turbidity of a protein dispersions (Lee *et al*., 2016). Martini *et al*. (2010) handled with a power ultrasonic sound waves to lessen the turbidity of whey solutions. It was concluded that around 90% decrease was observed in the turbidity of samples treated with ultrasound processing. The highest decline in turbidity values was determined for the samples treated with 30 min at 100% amplitude (US310 samples). While the highest turbidity was obtained for the untreated WPCON (4.88), the lowest turbidity was observed for the US310 samples (3.26). There is a positive relationship between the variables of droplet sizes and turbidities. All US-treated WPCON specimens showed less turbidity and smaller droplet size where the control WPCON samples exhibited most turbid appearance and the biggest droplet size (Table 2). In overall, US treatment with 30-minutes at 100 % amplitude showed the smallest droplet size (608.3 nm) and least turbidity (3.26) compared to the other sonication times and amplitudes. Both the number of soluble protein components in the dispersion figured out by solubility and the sizes of the soluble protein components determine the turbidities of a whey protein dispersion (Gregory,1998). Employing the US at 20 kHz raised the clearness and transparency of whey protein suspensions mostly because of the decrease in the size of the suspended insoluble protein components (Zisu *et al.*, 2011).

Table 2. Droplet size (nm) & turbidity of WPCON samples

Treatments	Droplet size (nm)	Turbidity
Control	952.3 ± 0.3^{a}	4.88 ± 0.8^{a}
US1	895.2 ± 0.5^{c}	3.79 ± 0.9^{b}
US2	822.1 ± 0.3^{e}	3.74 ± 0.3^{bc}
US3	795.1 ± 0.2^{f}	3.66 ± 0.2^{c}
US6	905.5 ± 0.1^{b}	3.79 ± 0.2^{b}
US8	842.2 ± 0.9^{d}	3.75 ± 0.5^{bc}
US10	819.4 ± 0.1^{e}	3.74 ± 0.6^{bc}
US16	797.3 ± 0.1^{f}	3.65 ± 0.5^{c}
US18	749.4 ± 0.8^{g}	3.63 ± 0.3^{c}
US110	693.5 ± 0.4^{j}	3.44 ± 0.8^{e}
US26	726.5 ± 0.6^{h}	3.55 ± 0.7^{d}
US28	711.6 ± 0.1^{i}	3.51 ± 0.2^{de}
US210	693.1 ± 0.5^{j}	3.37 ± 0.5^{f}
US36	698.2 ± 1.7^{j}	3.45 ± 0.1^{e}
US38	661.7 ± 0.8^{k}	3.38 ± 0.3^{f}
US310	608.3 ± 0.4^{l}	3.26 ± 0.8^{g}

[a-l] Mean ± standard deviation (n=3) of features with the same letter are not different significantly ($p < 0.05$)

*All statistical analysis was achieved separately for all and each parameter (droplet size, turbidity, etc.)

Table 3. Particle size, EAI and ESI of WPCON specimens

Treatments	EAI (m^2 /g)	ESI (min)
Control	61 ± 1.7^{l}	11.0^{i}
US1	89 ± 0.2^{j}	15.0^{h}
US2	104 ± 1.3^{i}	20.0^{g}
US3	146 ± 2.1^{g}	24.0^{f}
US6	75 ± 3.6^{k}	14.0^{h}
US8	107 ± 1.5^{i}	20.0^{g}
US10	123 ± 0.7^{h}	21.0^{g}
US16	147 ± 0.4^{g}	25.0^{f}
US18	162 ± 1.3^{f}	29.0^{e}
US110	194 ± 1.9^{d}	37.0^{c}
US26	161 ± 0.7^{f}	34.0^{d}
US28	175 ± 2.2^{e}	33.0^{d}
US210	204 ± 0.5^{c}	41.0^{b}
US36	193 ± 1.1^{d}	37.0^{c}
US38	222 ± 0.3^{b}	41.0^{b}
US310	255 ± 0.8^{a}	43.0^{a}

[a-l] Mean ± standard deviation (n=3) of feature with the same letter are not different significantly ($p < 0.05$)

*All statistical analysis was achieved separately for all and each variable (EAI, ESI, etc.)

B. Emulsifying properties

Table 3 displays the EAI and ESI of WPCON samples exposed to the US. It was found that the WPCON samples treated with 30 min US at 100% amplitude resulted with the highest EAI (255 m^2g^{-1}) and ESI (43.0 minutes), while the WPCONs with no US treatment exhibit the lowest EAI (61 m^2g^{-1}) and ESI (11.0 min). Similar progression in the emulsion characteristics of proteins with US process was pointed out in the works of Yildiz *et al*. (2017). It is possible to see an inverse relationship between droplet size and emulsifying properties (Table 3). Basically, the WPCON samples with smallest droplet size, namely WPCON samples treated with 30 min US at 100% amplitude, also had high ESI and EAI. In overall, the use of ultrasound treatment, especially with higher amplitudes and times, was resulted with an improvement in emulsifying properties (ESI and EAI).

C. Lipid oxidation

Lipid hydroperoxide value of the whey protein nanoemulsions with oil concentration of 0.25% for 7-days (168 h in total) of storage period under 25 ^{o}C is demonstrated in Fig. 1. No oxidized lipid particles were measured at day 0 for all WPCON samples including control samples. Starting from the second day, the lipid oxidation was started to defined. Significant increases in

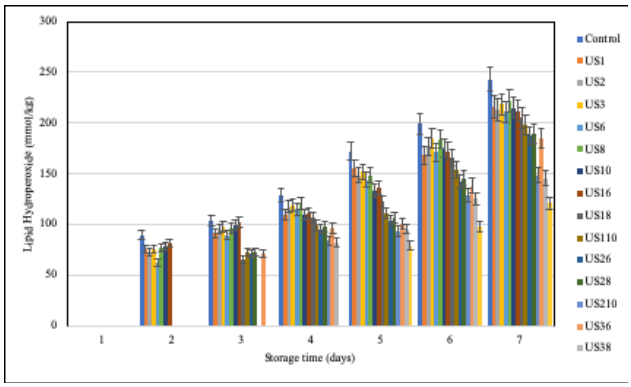

Figure 1. Lipid hydroperoxide values of WPCON during storage at 25 ^{O}C for 7 days

lipid oxidation beginning from second days to seventh days for control WPCON specimens were determined. The tendency of whey protein's role as the chemical stopper in order to postpone lipid oxidation is proved at the first 5 days of storage period. For the WPCON samples treated with 30 min US at 100% amplitude (US310), no oxidized lipids were observed till the day of 5 (Fig. 1). It can be concluded that the encapsulation of secondary components with the whey protein/canola oil nanoemulsions might be conducted within 120 hours (5 days) subsequent to preparation of WP nanoemulsions, right before oxidation stage of oil used in the nanoemulsion.

IV. CONCLUSIONS

An ultrasound treatment was examined for the purpose of modification and enhancement of the WPCON functionality. Compared with other US-treatments, a significant improvement in the droplet sizes, turbidities, emulsion stabilities, and lipid oxidations of WPCON samples was achieved with a US310 treatment. Overall, US310 is a promising treatment to strengthen the functional characteristics of WPCON as indicated within the present study by its ability to smaller droplet size, less turbidity, improved emulsion stability, and less lipid oxidation right after ultrasonication. The findings of current research proved the potential of the US310 treatment as an effective method for protein modification. The functionalized WPCON produced by US310 treatment can be used in a liquid food with less precipitation.

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