Role of carbohydrate conjugation on the emulsification and antioxidant properties of intact and hydrolysed whey protein concentrate

Maria Cermeño [a], Manuel Felix [a,b], Alan Connolly [a], Elaine Brennan [a], Bernadette Coffey [a], Edel Ryan [a], and Richard J. FitzGerald [a*]

[a] Department of Biological Sciences, University of Limerick, Limerick, Ireland

[b] Departamento de Ingeniería Química, Escuela Politécnica Superior, Universidad de Sevilla, Sevilla, Spain

Abstract

The conjugation of proteins with carbohydrates generates covalent bonds which may improve their techno- and biofunctional properties and therefore expand their applications in the food industry. In the present study, a design of experiments (DOE) approach was used to determine the effect of conjugation of whey protein concentrate (WPC) and WPC hydrolysates with carrageenan (CGN) on its emulsifying and antioxidant properties. The DOE was composed of 3 levels with 3 factors, i.e., WPC:CGN (1:1.0 1:3.5, 1:6.0), time of conjugation (6, 27 and 48 h) and degree of hydrolysis (DH; unhydrolysed, low DH and high DH). The conjugated samples were characterised for their oxygen radical absorbance capacity (ORAC) and emulsion activity (EA). Two samples, C3 (1:1.0, 6 h and unhydrolysed) and C4 (1:1.0, 6 h and low DH) with ORAC and EA values of 601.30 ± 14.71 and 709.32 ± 11.11 µmol Trolox equivalents g^{-1} freeze-dried powder and 0.51 ± 0.01 and 0.58 ± 0.01 absorbance units, respectively, were selected for further study. Emulsions were generated with WPC, non-conjugated (NC3 and NC4) and conjugated (C3 and C4) samples and their functional properties were compared. The NC3 and NC4 samples had higher viscoelastic moduli (G' and G'') than C3, C4 and the WPC samples which indicated that more stable emulsions may be formed with non-conjugated samples. However, the NC3, NC4 and WPC samples showed low emulsion stability (ES) after 28 days storage with values ranging from 78.6 ± 3.5 - 85.6 ± 3.6 % whereas the conjugated C3 and C4 samples had improved ES with significantly ($p < 0.05$) lower coalescence index values (64.2 ± 2.5 and 66.7 ± 3.7 %, respectively). However, conjugation of the hydrolysate (C4) had a significant ($p < 0.05$) negative effect on lipid oxidation (0.62 ± 0.04 equivalents of 1,1,3,3-tetraethoxypropane (TEP) /kg emulsion) compared to the unhydrolysed conjugate C3 (0.42 ± 0.04 eq TEP/kg emulsion).

Keywords: whey protein, carrageenan, conjugation, emulsion, hydrolysis, antioxidant.

1. Introduction

A range of synthetic emulsifier compounds are used to stabilise food emulsions. However, consumption of emulsifiers, such as carboxymethyl cellulose and polysorbate-80, has been associated with increased cardiovascular disease risk (Chassaing, et al., 2015). Dairy proteins represent natural alternative surface active agents due to their high emulsifying capacity. In an emulsion, proteins locate at the interface creating electrostatic and steric repulsion forces between the oil droplets thereby stabilising the system (Sarkar & Singh, 2016). These effects on emulsion stability can be further enhanced by the addition of high molecular weight polysaccharides, which leads to better separation between the oil droplets. Proteins and polysaccharides have diverse attributes in the creation of stable emulsions. The use of protein as an emulsifier allows a viscoelastic layer to form at the oil-water interface whereas inclusion of polysaccharides increases the viscosity of the interfacial layer (Dickinson, 2009; Krstonošić, Dokić, Dokić, & Dapčević, 2009). Furthermore, the conjugation of milk proteins and polysaccharides, by means of the Maillard reaction leading to the generation of glycoproteins, has been shown to improve their emulsifying properties (Mulcahy, Mulvihill, & O'Mahony, 2016; O'Mahony, Drapala, Mulcahy, & Mulvihill, 2017). Hydrolysis of proteins results in their breakdown into smaller molecular mass peptides. Protein hydrolysis has the ability to improve the EA (Connolly, Piggott, & FitzGerald, 2014b; Evangelho, et al., 2017). This is due to the higher amount of free amino groups resulting in reduced interfacial surface tension which helps to stabilise oil droplets leading to better and more consistent emulsions (Miñones Conde & Rodríguez Patino, 2007). Furthermore, the peptides released during hydrolysis can possess a wide range of bioactivities (Nongonierma, O'Keeffe, & FitzGerald, 2016). These bioactive properties are not only beneficial for consumer health but may also help prevent lipid oxidation, which is a significant issue for oil in water emulsions (Berton-Carabin, Ropers, & Genot, 2014). To the author's knowledge, few studies have been performed to determine the

contribution of conjugation of polysaccharides to whey protein hydrolysates to their emulsification and antioxidant properties. Therefore, the main objective of this study was to determine the role of polysaccharide conjugation on the emulsifying properties of whey proteins and their enzymatic hydrolysates and also to determine the optimum conditions for the generation of conjugates. A secondary objective was to determine the effect of conjugation on the antioxidant properties of the emulsions manufactured with the conjugates.

2. Material and methods

Materials

Whey protein concentrate (WPC) was obtained from Carbery Milk Products, (Ballineen, Ireland). According to the supplier specification sheet, the typical composition of their WPC80 was: 80 % protein min, 8.0 % lipid max, 4.0 % ash max and 3.0 % lactose max. Alcalase 2.4L®, Flavourzyme® and carrageenan (CGN), a mixture of κ and λ CGN, were purchased from Sigma (Dublin, Ireland). Corn oil was purchased in a local food store and it was directly used for the development of emulsions, avoiding any additional purification steps. Trinitrobenzenesulfonic acid (TNBS) was supplied by the Medical Supply Company (Dublin, Ireland). All other reagents were supplied by Sigma (Dublin, Ireland).

Experimental design

The statistical software Minitab (Version 17, Minitab Ltd, Coventry UK) was used to create a design of experiments (DOE). The DOE concentrated on three factors with three levels for each factor, which included DH (unhydrolysed, low DH and high DH), WPC:CGN (1:1.0, 1:3.5 and 1:6.0) and duration of conjugation (6, 27 and 48 h). Using these factors, a total of 15 experimental runs (C1-C15) were proposed using a Box-Behnken response surface design Non-conjugated (NC1-NC15) mixtures were also prepared as controls (Table 1). A response

surface model (RSM) was used to obtain the predictive values. The RSM was fitted to a full quadratic regression model:

$$Y= \beta_0 + \beta_1 \text{ x WPC:CGN} + \beta_2 \text{ x } t + \beta_3 \text{ x DH} + \beta_4 \text{ x WPC:CGN}^2 + \beta_5 \text{ x } t^2 + \beta_6 \text{ x DH}^2 + \beta_7 \text{ x WPC:CGN x } t + \beta_8 \text{ x } t \text{ x DH} + \beta_9 \text{ x WPC:CGN x DH}$$

Where Y was the dependent variable, β_0 was the constant coefficient, β_{1}- β_8 were the coefficients of the model, WPC:CGN was the ratio whey protein concentrate to carrageenan, DH was the degree of hydrolysis and t was time of conjugation.

Enzymatic hydrolysis of WPC

Enzymatic hydrolysis of a WPC solution (10 % (w protein/v)) was conducted using a pH stat (902 Titrando, Metrohm, Switzerland). The WPC solution was maintained at pH 7.0 using 0.5 N NaOH and at 50 ºC throughout the reaction. The proteolytic enzymes Alcalase 2.4L® and Flavourzyme® were added at an enzyme to substrate ratio (E:S) of 1.0 % (v/w protein) and 0.5 % (v/w protein), respectively. A control (unhydrolysed WPC) without enzyme addition was also generated. Aliquots of the solution were removed at different incubation times. The enzymatic reaction was stopped by incubating the sample for 20 min at 80 ºC. The degree of hydrolysis (DH) was determined using the TNBS method as described by Le Maux, Nongonierma, Barre and FitzGerald (2016).

Conjugation of protein to carbohydrate

Different ratios of protein (WPC) and carbohydrate (CGN) were combined in accordance with the DOE recommendations. Solutions of WPC:CGN at 1:1.0, 1:3.5 and 1:6.0 were prepared in distilled water and were vigorously stirred for approximately 1h at room temperature until homogenized. The mixtures were then freeze dried (Freezone 4.5, Labconco, Missouri, USA). The dried WPC:CGN samples were then placed in a desiccator at a relative humidity of 79

% using a saturated salt solution and were then heated to 60 ºC in an oven according to the method outlined by Al-Hakkak and Al-Hakkak (2010). The mixtures (C1-C15) were allowed conjugate in the desiccator for 6, 27 or 48 h. Non-conjugated (NC1-NC15) mixtures were also prepared as controls.

Characterisation of conjugated and non-conjugated samples

Gel permeation high performance liquid chromatography

GP-HPLC was carried out on samples (0.80 % (w/v) in distilled water, dH_2O) as previously described by Connolly, Piggott, and FitzGerald (2014a).

Measurement of colour

The effect of conjugation on the colour of the samples was measured using a colorimeter (Spectrophotometer CR-600d, Konica Minolta Inc., Japan). The colorimeter was placed over the powdered conjugated and non-conjugated samples and measurements were recorded in triplicate. The results were expressed with reference to the CIE lab model where L* represents lightness, a* represents the red-green axis and b* characterises the yellow-blue axis. All the measurements were performed in triplicate.

Extent of conjugation

The free amino N group content was determined using the TNBS method as described by Le Maux et al. (2016). The extent of conjugation was estimated using the following equation:

$$Degree\ of\ Conjugation\ (\%) = \left(1 - \frac{amino\ groups\ conjugated\ sample}{amino\ groups\ non-conjugated\ sample}\right) x100$$

Oxygen radical absorbance capacity (ORAC)

The antioxidant activity of the non-conjugated and conjugated samples was determined using the ORAC assay following the method described by Cermeño, FitzGerald, and O'Brien (2016).

This measurement is based on the oxidative degradation of fluorescein in the presence of 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH). Fluorescence was measured over 2 h and the area under the curve was calculated. Trolox was used as standard, and the results were expressed as μmol Trolox equivalents (TE) g^{-1} freeze-dried powder (FDP). The experiments were performed in triplicate.

Emulsion Characterisation

Preparation of oil in water (o/w) emulsions

The method described by Akhtar and Dickinson (2007) was used to prepare o/w emulsions. Briefly, a 1 % solution (w/v) of the conjugated or non-conjugated protein/ hydrolysate sample was prepared in dH_2O (3.5 g per 350 ml). This solution was then gently stirred at room temperature until completely dissolved (approximately 1 h). The pH of the solution was adjusted to pH 4 using 1M HCl. This pH value is distant from the isoelectric point of WPC (~ pH 5.2), avoiding the formation of protein aggregates, which are not desirable for emulsion ability. Moreover, pH 4 is suitable for the development of whey protein stabilised emulsions exhibiting small droplet sizes (Demetriades, Coupland, & McClements, 1997). For the oil phase preparation, 0.04 g of Sudan Red III was added to 1 L of corn oil to visually check the stability of the emulsions generated (allowing the visual identification of phase separation). The antimicrobial agent sodium azide, at 0.02 % (w/v), was added to the oil before the phases were mixed. The oil (150 g) was then added to the protein solution to obtain a total mixture of 500 g. The solution was then sheared at 13000 rpm for 30 s using an Ultra Thurrax T-25 (IKA ® Werke Gmbh & Co. KG, Staufan, Germany) immediately before passing through a two-stage homogeniser (APV 1000 homogeniser, SPX FLOW, North Carolina, USA) at a pressure of 80 and 720 bar (for first and second stage pressures, respectively). All the process was carried out at room temperature.

Emulsifying activity (EA)

The absorbance of the emulsions was determined as described by Connolly et al. (2014b) with some modifications. Immediately after the emulsion was generated an aliquot of the emulsion was stabilized by diluting in 0.1 % (w/v) SDS at two predetermined ratios, 1:40 and 1:2000. The solutions were transferred to 1.5 ml cuvettes and their absorbance was recorded in triplicate at 500 nm using a UV-1800 spectrophotometer (Shimazu, Canby, USA). Emulsions were stored at 4 ºC for stability studies. The absorbance of the emulsions were recorded at day 0 (T0), day14 (T14) and day 28 (T28). Measurements were performed in triplicate.

Emulsion droplet size distribution

The droplet size distribution (DSD) of the emulsions, previously stabilised with SDS at a ratio 1:40, was evaluated using a Mastersizer 2000 (Malvern, Worcestershire, UK). The particle size of the emulsions was determined in triplicate at T0, T14 and T28. The Sauter mean diameter D[3,2] was calculated according to Eq. 1:

$$D[3,2] = \frac{n_i \cdot \sum d_v^3}{n_i \cdot \sum d_s^2} \quad (1)$$

Where d_s^2 and d_v^3 are surface and volume diameters for n_i particles obtained by laser diffraction measurements. Additionally, the coalescence and flocculation indices (CI and FI, respectively) were determined.

CI (%) was calculated according to Eq. 2:

$$CI\ (\%) = \left[\frac{D[3,2]_{14\ or\ 28}}{D[3,2]_0} - 1\right] x100 \quad (2)$$

Where $D[3,2]$ is the Sauter mean diameter calculated at day 0 ($D[3,2]_0$), 14 ($D[3,2]_{14}$) or 28 ($D[3,2]_{28}$).

The FI (%) was calculated according to Eq. 3:

$$FI\ (\%) = \frac{|D[3,2] - D[3,2]_{SDS}|}{D[3,2]} x\ 100 \quad (3)$$

Where $D[3,2]$ is the Sauter mean diameter and $D[3,2]_{SDS}$ is the Sauter mean when the sample was dispersed into 1 wt. % SDS. Measurements were performed in triplicate.

Optical microscopy of emulsions

Emulsion samples were diluted 1:10 in distilled water and the droplets were examined under a light microscope (Olympus CX31, Tokyo, Japan). The samples (20 µL) were analysed at a magnification of 100x following 0, 14 and 28 days storage at 4 °C.

Emulsion rheology

Small amplitude shear measurements were performed in a Discovery Hybrid Rheometer (DHR-2; TA Instruments, New Castle, USA) in order to obtain the mechanical spectra of emulsions by means of frequency sweep tests. Prior to these tests, the linear viscoelastic range (LVR) was determined by means of initial stress sweep tests at three frequency values. Subsequently, frequency tests were carried out at the selected stress within the LVR from 0.05 to 30 Hz. The rheometer geometry used consisted of 40 mm parallel plates. The G' (storage modulus), G'' (loss modulus) and tan δ (G'/G'') of the samples were determined. The experiment was performed in triplicate at 25 ºC.

Lipid oxidation

The thiobarbituric acid reactive substances (TBARS) assay was performed on the emulsions in order to quantify lipid oxidation. The method was performed as described by Fernandez-Avila and Trujillo (2016) with minor modifications. Emulsions (1 mL) were mixed with 5 mL of the TBA reagent (0.25 M HCl, 0.375 % TBA and 15 % trichloroacetic acid). This suspension was then heated into a water bath (70 ºC) for 15 min and centrifuged at room temperature for 10

min at 2700 g (Hettich Zentrifugen Universal 320R centrifuge, Andreas Heittich GmbH & Co., Tuttlingen, Germany). The absorbance of the supernatant was recorded at 532 nm. Results were expressed as equivalents of 1,1,3,3-tetraethoxypropane (TEP) per kg of emulsion. The experiment was performed in triplicate.

Statistical analysis

Data were reported as means ± standard deviation of at least three independent replicates. One way analysis of variance (ANOVA) with Tukey or Dunnett test was used to analyse the results using Graphpad Prism, version 6.00 for Windows. Significant levels were defined as $p < 0.05$.

3. Results and discussion

Analysis of WPC:CGN conjugates

Molecular mass distribution

Figure 1 shows the molecular mass distribution profiles of WPC, its low (5.74 ± 0.35 %) and high DH (11.72 ± 0.98 %) hydrolysates and the different test samples arising from the DOE (Table 1), i.e., the WPC/ WPC hydrolysates in the presence of different ratios of CGN with (C1-C15) and without conjugation (NC1-NC15). These profiles showed that WPC had a large proportion of material > 5 kDa, whereas the low and high DH hydrolysates had a greater proportion of material eluting < 5 kDa. All the conjugated samples (C1-15, Table 1) had a higher proportion of high molecular mass material (> 5 kDa) than the corresponding non-conjugated samples (NC1-15, Table 1) indicating that conjugates had been formed. Within the non-conjugated samples, those samples that were generated with either unhydrolysed WPC or at a high WPC to CGN ratio resulted in a higher molecular mass. Thus, the corresponding conjugates had no significant variation in their molecular mass distribution. For instance, NC3 generated with unhydrolysed WPC and at a WPC:CGN of 1:6.0 and its conjugate C3 had 52 and 57 % of material > 5 kDa, respectively. However, samples with low or high DH and with

lower WPC:CGN ratios had a large variation in the molecular masses of the corresponding conjugates. In the conjugated C1 sample, which had a WPC:CGN of 1:1.0 and was generated with the high DH WPC, 38 % of the material was > 5 kDa whereas in the corresponding non-conjugated sample NC1, 0.01 % of the material was > 5 kDa. The ratio of WPC:CGN also impacted the molecular mass distribution profiles for the conjugated samples generated with hydrolysed WPC. For example, in the conjugated C4 sample which had a WPC:CGN of 1:1.0, 13 % of the material was > 5 kDa whereas in the conjugated C15 sample which had a WPC:CGN of 1:6.0 approximately 43 % of the material was > 5 kDa. However, C3 and C8 generated from unhydrolysed WPC and incubated for 27 h, at a WPC:CGN of 1:6.0 and 1:1.0, respectively, showed minimal differences in their molecular mass distributions with approximately 55 % of the material > 5 kDa in both samples. Furthermore, the extent of hydrolysis of the WPC influenced to a lower extent the molecular mass profiles obtained. For example, in the C11 sample conjugated with high DH WPC for 48 h and at a WPC:CGN of 1:3.5, 37 % of the material was > 5 kDa whereas in the C12 sample, with the same conjugation time and ratio but generated with unhydrolysed WPC, 49 % of the material was > 5 kDa. In general, the duration of conjugation (within the experimental design) had no major effect on the molecular mass profiles obtained. For example, the C13 and C11 samples with similar composition (WPC:CGN of 1:3.5 and high DH WPC) but incubated for 6 and 48 h, respectively, both had similar profiles with approximately. 35 % of the material > 5 kDa.

Browning

Figures 2A and B show the variation in the CIELAB parameters, a*, b* and L* for red-blue, yellow-green and luminosity, respectively, between the conjugated and non-conjugated samples. The non-conjugated samples displayed very low b* values and high L* values. However, when samples were conjugated the b* parameter increased in the majority of the

samples along with a decrease in the L* value indicating that a brown colour was developed. During the Maillard reaction conjugation between WPC and CGN caused a yellow colour in the earlier stages while a brown colour was formed in the later stages of the reaction. This browning is attributed to the formation of coloured polymers termed melanoidins (Ames, 1998). The results obtained herein are in agreement with Mulcahy et al. (2016) who reported that the L* value declined following the conjugation of whey protein with maltodextrin following incubation at 90˚C for 8 h. This effect was also reported by Regan and Mulvihill (2013) during the conjugation of a sodium caseinate hydrolysate with maltodextrin.

Amino N content and extent of conjugation

The extent of conjugation was assessed using the TNBS assay. Figure 3a shows the amino N content, expressed as mg N g^{-1} FDP, for the non-conjugated (NC1-NC15) and conjugated samples (C1-C15). As expected, conjugated samples (C1-15) had lower free amino N content in comparison to their corresponding non-conjugated samples (NC1-NC15). For example, the conjugated C1 sample had a free amino N content of 2.38 ± 0.45 mg N g^{-1} sample whereas the corresponding non-conjugated NC1 sample had 3.67 ± 0.20 mg N g^{-1}. This confirms that the samples were conjugated and therefore, the amino N groups of the proteins were cross-linked with the carbonyl groups of the CGN. In general, extending the incubation time led to a greater extent of conjugation, e.g., sample C4 (6 h incubation) had an amino N content of 2.75 ± 0.16 mg N g^{-1} whereas the C7 (48 h incubation) had 1.44 ± 1.53 mg N g^{-1}. Interestingly, conjugation of intact WPC with CGN led to almost complete loss of amino groups, see C3, 8 and 12 following extended conjugation times (Fig 3a). On the other hand, the hydrolysed WPC samples still contained significant free amino N groups even after extended conjugation times, see C1, C4 and C13 (Fig 3a). This result was expected since, during enzymatic hydrolysis protein breakdown leads to the release of free amino groups (Cermeño, O'Brien, & FitzGerald,

2018). The WPC:CGN had a major impact on the amino N content of those samples obtained using hydrolysed WPC. For instance, C1 (WPC:GCN of 1:1.0) and C10 (WPC:GCN of 1:6.0) both generated with high DH WPC and incubated for 27 h had 2.38 ± 0.26 and 0.60 ± 0.02 mg N g^{-1} , respectively, similar trends were observed with sample sets C4 and C15, and C7 and C14. The highest degree of conjugation (DC) was for C7 (73.08 %) whereas the lowest was for C13 (4.32 %). In general, samples with low DH and extended conjugation times (48 and 27 h) had higher DC values, whereas samples generated with high DH and for shorter conjugation times (6 h) had lower DCs.

Antioxidant activity (ORAC)

The antioxidant activity of the non-conjugated and conjugated samples was determined using the ORAC assay (Figure 3b). The non-conjugated samples NC3, NC6, NC8 and NC12, all generated with unhydrolysed WPC, showed low or non-detectable antioxidant activities whereas those non–conjugated samples generated with low or high DH WPC had higher ORAC values (Fig. 3b). This is in agreement with O'Keeffe and FitzGerald (2014) who reported that WPC hydrolysates exhibited higher ORAC activity than intact WPC. Overall, the conjugated samples showed a significant ($p<0.05$) increase in antioxidant activity in comparison with the non-conjugated samples with the exception of C1. This indicates that antioxidant activity improved upon conjugation. The conjugated samples C4 and C7, both generated from low DH WPC and at a 1:1.0 ratio, showed the highest antioxidant values of 709 ± 11 and 710 ± 25 µmol TE g^{-1} FDP, respectively. These results were significantly higher ($p<0.05$) than that of the other conjugates. The effect of conjugation on whey protein antioxidant activity has been previously reported. According to Hiller and Lorenzen (2010), the level of antioxidant activity of whey protein isolate increased following conjugation with carbohydrates such as glucose (+31 %) or dextran (+30 %). Nooshkam & Madadlou, (2016) reported after 45 min conjugation of whey

protein isolate (WPI) and whey protein hydrolysates (WPH) with lactose and lactulose that the antioxidant activity (measured by the DPPH assay) of the WPH conjugates was significantly higher than that of the WPI samples. It has been suggested that the principal Maillard reaction products, melanoidins, could be responsible for the enhanced antioxidant activity of conjugates (Wang, Qian, & Yao, 2011). The antioxidant activity of melanoidins can be attributed to different mechanisms. These include the scavenging of hydroxyl groups which can disrupt the radical chain reaction by donation of hydrogen atoms and also by the scavenging of proxy radicals which function by chelating to pro-oxidant transition metal ions (Liang and Kitts, 2014). The results herein suggest that a combination of conjugation and hydrolysis treatments generating melanoidins as well as free amino N groups, respectively, may contribute to the enhancement of the antioxidant activity of WPC. However, it could be interesting to use other antioxidant assays, such as ferric reduction or DPPH radical scavenging assays, in order to determine the antioxidant mechanisms within the different samples.

Emulsification properties

The EA and Sauter mean diameter (D [3,2]) values for all emulsions generated using the conditions proposed in the DOE as a function of storage time are shown in Table 2. As previously reported by Cameron, Weber, Idziak, Neufeld, and Cooper (1991), a higher absorbance of the sample is indicative of smaller droplet size. Interestingly, at day 0 all the samples generated with unhydrolysed WPC (C3, 6, 8 and 12) had the highest EA and the lowest D [3,2] values (EA > 0.40, and D [3,2] < 10 μm) which indicates their good emulsifying activity. At day 0, the highest EA value (1.05 ± 0.04) and the lowest D [3,2] value (2.87 ± 0.59) was obtained for sample C8 generated with unhydrolysed WPC. From the conjugates generated from low DH WPC hydrolysates only C4 had good emulsifying properties (EA = 0.58 ± 0.01, and D [3,2] = 4.00 ± 0.03 μm at day 0) and was comparable with sample C8. It is interesting

that the duration of conjugation had an effect on emulsion activity. For instance, C4 and C7 represent the same composition but with different durations of conjugation time (i.e. 6h for C4 and 48 h for C7) and their EA results differed significantly. It is known that protein hydrolysis may improve the formation and stabilization of emulsions and foams (Miñones Conde, et al., 2007; Tamm, Herbst, Brodkorb, & Drusch, 2016). However, a high degree of hydrolysis generally yields a higher proportion of low molecular mass peptides, which are too small to stabilize o/w interfaces, resulting in decreased stability (Xu, et al., 2016). This was the case herein for those conjugates generated from high DH WPC hydrolysates (C1, 10, 11 and 13) which had relatively low EA and high D [3,2] values. Similar results were reported in sodium caseinate hydrolysates conjugated with maltodextrin, where low DH samples showed better emulsion properties, in regard to droplet size and distribution, than those generated with a higher extent of hydrolysis (Regan et al., 2013).

Response surface model and selected emulsions

The RSM curves generated with the results arising from the DOE are shown in Supplementary Figure S1. The results showed that the model developed with the EA values was significant ($p = 0.001$, Supplementary Table S1 and Figure S2A. The only parameter not having a significant effect on EA was the time of conjugation ($p = 0.171$). The model had an R^2 of 0.918 and the lack of fit was not significant ($p = 0.160$). However, no correlation was obtained between the model and the ORAC values obtained for the conjugates generated within the experimental design (p value model = 0.975, $R^2 = 0.285$, Supplementary Table S2 and Figure S2B). This indicated that the model may not be used to predict ORAC activity values. Therefore, two emulsions from the DOE were selected for further characterisation based on the preliminary analysis of their emulsifying and antioxidant properties. The highest ORAC values were obtained for samples C7, C4, C10 and C3 (710.93 ± 25.10, 709.32 ± 11.11, 613.18 ± 23.61 and

601.30 ± 14.71 μmol TE g^{-1} FDP, respectively; Figure 3). However, C7 and C10 displayed low EA values at time zero (0.26 ± 0.01 and 0.36 ± 0.01) whereas C3 and C4 had relatively high EA values (0.51 ± 0.01 and 0.58 ± 0.01) (Table 2). Therefore, samples C3 and C4 were selected. Emulsions were also generated with the corresponding non-conjugated (NC3 and NC4) samples and unhydrolysed WPC as controls.

Rheology

Figure 4 shows the mechanical spectra for the selected emulsions. The emulsions exhibited a gel-like behaviour, since G' was higher than G" within the overall frequency interval studied. Moreover, both viscoelastic moduli exhibited a low frequency dependence. This type of response has previously been reported for protein-stabilized emulsions (Bengoechea, Puppo, Romero, Cordobés, & Guerrero, 2008; Felix, Romero, & Guerrero, 2017b; Pal, 2000). This has been related to the plateau region of the overall mechanical spectrum due to the formation of protein-polysaccharide entanglements, which finally result in the formation of a protein-polysaccharide network (Tasneem, Siddique, Ahmad, & Farooq, 2014). According to this premise, the inclusion of CGN would involve an increase in both viscoelastic moduli when compared to WPC alone. In the absence of conjugation (NC3 and NC4), the increase in both viscoelastic moduli is higher, due to the capacity of the polysaccharide itself to form a gel-like structure. However, on conjugation WPC and CGN were cross-linked and the polysaccharide chains had less availability for interaction at the interface, which resulted in weaker gel-like structures in C3 and C4. Table 3 summarises the evolution of the elastic modulus at 1 Hz (G'_1) over storage time (0, 14 and 28 days) for all emulsions tested (WPC, NC3, C3, NC4 and C4). In general, low values of tan δ were obtained from the emulsions denoting the solid character of the gel obtained. The results are comparable to those obtained for other proteins stabilized emulsions (Felix, Romero, & Guerrero, 2017a; Fischer & Erni, 2007; Franco, Partal, Ruiz-

Marquez, Conde, & Gallegos, 2000; Puppo, et al., 2008). Additionally, the highest moduli were obtained for the non-conjugated sample emulsions (NC3 and NC4). During emulsion storage, the G'_1 values decreased for NC3 and NC4 resulting in a weakening of the structure (Ağar, Gençcelep, Saricaoğlu, & Turhan, 2016). On the contrary, the C3 and C4 emulsions displayed an increase of G'_1 with increasing storage time, being more evident in C3. This has been previously linked to flocculation of the emulsion over time (Ladjal Ettoumi, Chibane, & Romero, 2016).

Emulsion microstructure

Figure 5 shows the DSD obtained using laser diffraction together with the corresponding light microscopy photographs of the emulsions over 28 days storage. The emulsions stabilized with WPC showed a polydisperse profile. This polydispersity is reflected in a larger droplet size distribution after 28 days (Figure 5a) which corresponds with a high FI and CI index (6.1 ± 0.5 and 78.6 ± 3.5 %, respectively; Table 4). On the other hand, the emulsions stabilized using the conjugated samples C3 (Figure 5c) and C4 (Figure 5d) exhibited greater stability against droplet coalescence. These results were expected since glycoconjugates have been shown to have better dynamics of adsorption to the interface which results in better interfacial properties (Corzo-Martínez, Carrera Sánchez, Moreno, Rodríguez Patino, & Villamiel, 2012). In particular, C3 (generated with unhydrolysed WPC) had the smallest particle size distribution at time 0 in comparison with the rest of the emulsions (Figure 5c). This is accompanied by the lowest mean CI value after 28 days of storage. On the contrary, C3 showed the highest FI value (7.7 ± 0.1 %) at 28 days indicating that the sample had a tendency to flocculate. The C4 emulsion had a less notable change over time with FI and CI values of 3.3 ± 0.1 and 66.7 ± 3.7 %, respectively, 28 days after emulsion manufacture. The higher CI values were obtained for emulsions containing the non-conjugated samples (NC3 and NC4) and WPC, which indicated

their poor stability. The destabilization of these emulsions may be as a result of depletion flocculation, which is related to an excess of unabsorbed polymer in the continuous phase (Dickinson & Golding, 1997). However, this requires further study.

Lipid oxidation

Figure 6 shows the lipid oxidation values for the C3, C4, NC3, NC4 and WPC emulsions as a function of storage time. The TBARS assay is used to determine the interaction of lipids with oxygen-active species. Initially, all the emulsions generated produced similar quantities of secondary oxidation products. At day 28, the results differed significantly between samples, where the highest extent of lipid oxidation was obtained for the emulsions containing hydrolysed WPC NC4 and C4, (0.63 ±0.00 and 0.62 ± 0.04 Eq. TEP/kg emulsion, respectively). Emulsions C3 and NC3 also displayed lipid oxidation products followed by WPC with the lowest value (0.25 ± 0.03 Eq. TEP/kg emulsion). The increase in lipid oxidation values, at day 28, when CGN was present could be related to the small droplet sizes generated in these emulsions. It has been previously shown that a large interfacial area is a key factor favouring the promotion of lipid oxidation (Hebishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015). Interestingly, the conjugation process itself did not appear to have an effect on the generation of secondary oxidation products.

Conclusion

This study demonstrates that the combination of a low DH WPC hydrolysate and conjugation with CGN generates conjugates with improved antioxidant (ORAC) and emulsifying activities compared to unhydrolysed WPC conjugated with CGN. Two WPC:CGN conjugates (generated from low DH and unhydrolysed WPC) were selected for further study from the DOE used in this research, on the basis of the smallest mean droplet size obtained (D [3,2]) and the highest antioxidant activity as measured by the ORAC assay. Emulsions were

generated from the conjugates, their correspondent non-conjugated samples and intact WPC. The emulsion stabilized only with WPC showed the highest extent of flocculation and coalescence. Non-conjugated emulsions, NC3 and NC4, exhibited higher rheological properties with higher viscoelastic moduli than C3, C4 and WPC. However, after 28 days storage, NC3 and NC4 demonstrated the appearance of emulsion destabilization due to depletion flocculation. By contrast, the conjugated emulsions, C3 and C4, were more stable within the storage period studied (28 days). Additionally, C3 and C4 also seemed to exhibit less polydisperse droplet size distributions as well as smaller lipid droplets compared to the non-conjugated equivalent emulsions. However, conjugation in combination with WPC hydrolysates surprisingly seemed to have a negative effect on lipid oxidation. Limited information has been reported in relation to the combination of hydrolysed proteins and conjugation. The specificity of the proteolytic enzyme is a key factor in the generation of the final peptide profile. Therefore, it is conceivable that hydrolysis of WPC with other proteolytic enzymes followed by conjugation may result in different antioxidant and emulsification properties of the subsequent conjugates. On the other hand, the polysaccharide used for conjugation may also have an effect on the final properties of the conjugates. Therefore, additional investigations are necessary to elucidate the potential application of carbohydrate conjugation with protein hydrolysates to enhance the techno- and biofunctional properties of the conjugates following their incorporation into food products as natural emulsifiers.

Acknowledgements

Funding for this research was provided under the National Development Plan 2007–2013, through the Food Institutional Research Measure (FIRM), administered by the Department of Agriculture, Food and Marine, Ireland under grant issue 11/F/064. The authors gratefully

acknowledge the University of Seville (VPP, grant II.5) for the financial support given to Manuel Felix.

References

Ağar, B., Gençcelep, H., Saricaoğlu, F. T., & Turhan, S. (2016). Effect of sugar beet fiber concentrations on rheological properties of meat emulsions and their correlation with texture profile analysis. *Food and Bioproducts Processing, 100*, 118-131.

Akhtar, M., & Dickinson, E. (2007). Whey protein-maltodextrin conjugates as emulsifying agents: An alternative to gum arabic. *Food Hydrocolloids, 21*, 607-616.

Al-Hakkak, J., & Al-Hakkak, F. (2010). Functional egg white-pectin conjugates prepared by controlled Maillard reaction. *Journal of Food Engineering, 100*, 152-159.

Ames, J. M. (1998). Applications of the Maillard reaction in the food industry. *Food Chemistry, 62*, 431-439.

Bengoechea, C., Puppo, M. C., Romero, A., Cordobés, F., & Guerrero, A. (2008). Linear and non-linear viscoelasticity of emulsions containing carob protein as emulsifier. *Journal of Food Engineering, 87*, 124-135.

Berton-Carabin, C. C., Ropers, M. H., & Genot, C. (2014). Lipid oxidation in oil-in-water emulsions: involvement of the interfacial layer. *Comprehensive Reviews in Food Science and Food Safety, 13*, 945-977.

Cameron, D. R., Weber, M. E., Idziak, E. S., Neufeld, R. J., & Cooper, D. G. (1991). Determination of interfacial areas in emulsions using turbidimetric and droplet size data: correction of the formula for emulsifying activity index. *Journal of Agricultural and Food Chemistry, 39*, 655-659.

Cermeño, M., FitzGerald, R. J., & O'Brien, N. M. (2016). In vitro antioxidant and immunomodulatory activity of transglutaminase-treated sodium caseinate hydrolysates. *International Dairy Journal, 63*, 107-114.

Cermeño, M., O'Brien, N., & FitzGerald, R. J. (2018). Angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory activities of transglutaminase treated sodium caseinate hydrolysates. *International Dairy Journal, 78*, 85-91.

Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E., & Gewirtz, A. T. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature, 519*, 92-96.

Connolly, A., Piggott, C. O., & FitzGerald, R. J. (2014a). In vitro α-glucosidase, angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory properties of brewers' spent grain protein hydrolysates. *Food Research International, 56*, 100-107.

Connolly, A., Piggott, C. O., & Fitzgerald, R. J. (2014b). Technofunctional properties of a brewers' spent grain protein-enriched isolate and its associated enzymatic hydrolysates. *LWT - Food Science and Technology, 59*, 1061-1067.

Corzo-Martínez, M., Carrera Sánchez, C., Moreno, F. J., Rodríguez Patino, J. M., & Villamiel, M. (2012). Interfacial and foaming properties of bovine β-lactoglobulin: Galactose Maillard conjugates. *Food Hydrocolloids, 27*, 438-447.

Demetriades, K., Coupland, J. N., & McClements, D. J. (1997). Physical properties of whey protein stabilized emulsions as related to pH and NaCl. *Journal of Food Science, 62*, 342-347.

Dickinson, E. (2009). Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocolloids, 23*, 1473-1482.

Dickinson, E., & Golding, M. (1997). Depletion flocculation of emulsions containing unadsorbed sodium caseinate. *Food Hydrocolloids, 11*, 13-18.

Evangelho, J. A., Vanier, N. L., Pinto, V. Z., Berrios, J. J., Dias, A. R. G., & Zavareze, E. R. (2017). Black bean *(Phaseolus vulgaris L.)* protein hydrolysates: Physicochemical and functional properties. *Food Chemistry, 214*, 460-467.

Felix, M., Romero, A., & Guerrero, A. (2017a). Influence of pH and xanthan gum on long-term stability of crayfish-based emulsions. *Food Hydrocolloids, 72*, 372-380.

Felix, M., Romero, A., & Guerrero, A. (2017b). Viscoelastic properties, microstructure and stability of high-oleic O/W emulsions stabilised by crayfish protein concentrate and xanthan gum. *Food Hydrocolloids, 64*, 9-17.

Fernandez-Avila, C., & Trujillo, A. J. (2016). Ultra-High Pressure Homogenization improves oxidative stability and interfacial properties of soy protein isolate-stabilized emulsions. *Food Chemistry, 209*, 104-113.

Fischer, P., & Erni, P. (2007). Emulsion drops in external flow fields — The role of liquid interfaces. *Current Opinion in Colloid & Interface Science, 12*, 196-205.

Franco, J. M., Partal, P., Ruiz-M rquez, D., Conde, B., & Gallegos, C. (2000). Influence of pH and protei thermal treatment on the rheology of pea protein-stabilized oil-in-water emulsions. *Journal of the American Oil Chemists' Society, 77*, 975-984.

Hebishy, E., Buffa, M., Guamis, B., Blasco-Moreno, A., & Trujillo, A.-J. (2015). Physical and oxidative stability of whey protein oil-in-water emulsions produced by conventional and ultra high-pressure homogenization: Effects of pressure and protein concentration on emulsion characteristics. *Innovative Food Science & Emerging Technologies, 32*, 79-90.

Hiller, B., & Lorenzen, P. C. (2010). Functional properties of milk proteins as affected by Maillard reaction induced oligomerisation. *Food Research International, 43*, 1155-1166.

Krstonošić, V., Dokić, L., Dokić, P., & Dapčević, T. (2009). Effects of xanthan gum on physicochemical properties and stability of corn oil-in-water emulsions stabilized by polyoxyethylene (20) sorbitan monooleate. *Food Hydrocolloids, 23*, 2212-2218.

Ladjal Ettoumi, Y., Chibane, M., & Romero, A. (2016). Emulsifying properties of legume proteins at acidic conditions: Effect of protein concentration and ionic strength. *LWT - Food Science and Technology, 66*, 260-266.

Le Maux, S., Nongonierma, A. B., Barre, C., & FitzGerald, R. J. (2016). Enzymatic generation of whey protein hydrolysates under pH-controlled and non pH-controlled conditions: Impact on physicochemical and bioactive properties. *Food Chemistry, 199*, 246-251.

Liang, N., & Kitts, DD. (2014). Antioxidant Property of Coffee Components: Assessment of Methods that Define Mechanisms of Action. *Molecules, 19(11)*, 19180-19208.

Miñones Conde, J., & Rodríguez Patino, J. M. (2007). Phospholipids and hydrolysates from a sunflower protein isolate adsorbed films at the air–water interface. *Food Hydrocolloids, 21*, 212-220.

Mulcahy, E. M., Mulvihill, D. M., & O'Mahony, J. A. (2016). Physicochemical properties of whey protein conjugated with starch hydrolysis products of different dextrose equivalent values. *International Dairy Journal, 53*, 20-28.

Nongonierma, A. B., O’Keeffe, M. B., & FitzGerald, R. J. (2016). Milk protein hydrolysates and bioactive peptides. In *Advanced Dairy Chemistry* (pp. 417-482): Springer.

Nooshkam, M., & Madadlou, A. (2016). Maillard conjugation of lactulose with potentially bioactive peptides. *Food Chemistry, 192*, 831-836.

O'Keeffe, M. B., & FitzGerald, R. J. (2014). Antioxidant effects of enzymatic hydrolysates of whey protein concentrate on cultured human endothelial cells. *International Dairy Journal, 36*, 128-135.

O'Mahony, J. A., Drapala, K. P., Mulcahy, E. M., & Mulvihill, D. M. (2017). Controlled glycation of milk proteins and peptides: Functional properties. *International Dairy Journal, 67*, 16-34.

Pal, R. (2000). Shear viscosity behavior of emulsions of two immiscible liquids. *Journal of Colloid and Interface Science, 225*, 359-366.

Puppo, M. C., Beaumal, V., Chapleau, N., Speroni, F., de Lamballerie, M., Añón, M. C., & Anton, M. (2008). Physicochemical and rheological properties of soybean protein emulsions processed with a combined temperature/high-pressure treatment. *Food Hydrocolloids, 22*, 1079-1089.

Regan, J. O., & Mulvihill, D. M. (2013). Preparation, characterisation and selected functional properties of hydrolysed sodium caseinate–maltodextrin conjugate. *International Journal of Dairy Technology, 66*, 333-345.

Sarkar, A., & Singh, H. (2016). Emulsions and foams stabilised by milk proteins. In *Advanced Dairy Chemistry: Volume 1B: Proteins: Applied Aspects: Fourth Edition* (pp. 133-153).

Tamm, F., Herbst, S., Brodkorb, A., & Drusch, S. (2016). Functional properties of pea protein hydrolysates in emulsions and spray-dried microcapsules. *Food Hydrocolloids, 58*, 204-214.

Tasneem, M., Siddique, F., Ahmad, A., & Farooq, U. (2014). Stabilizers: indispensable substances in dairy products of high rheology. *Critical Reviews in Food Science and Nutrition, 54*, 869-879.

Wang, H.-Y., Qian, H., & Yao, W.-R. (2011). Melanoidins produced by the Maillard reaction: Structure and biological activity. *Food Chemistry, 128*, 573-584.

Xu, X., Zhong, J., Chen, J., Liu, C., Luo, L., Luo, S., Wu, L., & McClements, D. J. (2016). Effectiveness of partially hydrolyzed rice glutelin as a food emulsifier: Comparison to whey protein. *Food Chemistry, 213*, 700-707.

Figure captions

Figure 1: Molecular mass distribution of carrageenan (GCN), whey protein concentrate (WPC), WPC hydrolysates and their mixtures as specified from the design of experiments with (C1-C15) and without conjugation (NC1-NC15). DH: degree of hydrolysis. Numbers in black: percentage of molecular mass <5 kDa, numbers in white: percentage of molecular mass >5 kDa.

Figure 2: Colour parameters a*, b* (A) and L* (B) of the non-conjugated (NC1-15, ■) and the conjugated mixtures (C1-15, ○) of the whey protein concentrate (WPC)/ WPC hydrolysates carrageenan mixtures as specified from the design of experiments.

Figure 3: Graph of A) free amino nitrogen content and percentage degree of conjugation and B) oxygen radical absorbance capacity (ORAC) of the whey protein concentrate (WPC) carrageenan (CGN) mixtures from the design of experiments with (C1-C15, ■) and without a

conjugation treatment (NC1-NC15, ■). Data represent mean ± SD of three independent experiments. FDP: freeze dried powder. nd: non-detectable.

Figure 4: Elastic and viscous moduli (G' and G") as a function of oscillation frequency obtained for the emulsions generated with conjugates from the experimental design coded samples (C3, C4, NC3, NC4 and WPC) on day 1 of emulsion preparation.

Figure 5: Light microscopy and droplet size distribution (DSD) profiles of the emulsions generated with (a) WPC, (b) NC3, (c) C3, (d) NC4 and (e) C4 at day 0 (—),14 (---) and 28 (···) post emulsion manufacture.

Figure 6: Lipid oxidation values of emulsions obtained with whey protein concentrate (WPC), and the WPC:carrageenan (CGN) samples, non conjugated: NC3, NC4 and conjugated C3, C4, at day 0 (black), 14 (light gray) and 28 (dark gray) post emulsion manufacture. Different letters for each storage time represent significant differences ($p < 0.05$).

Figures

Figure 1

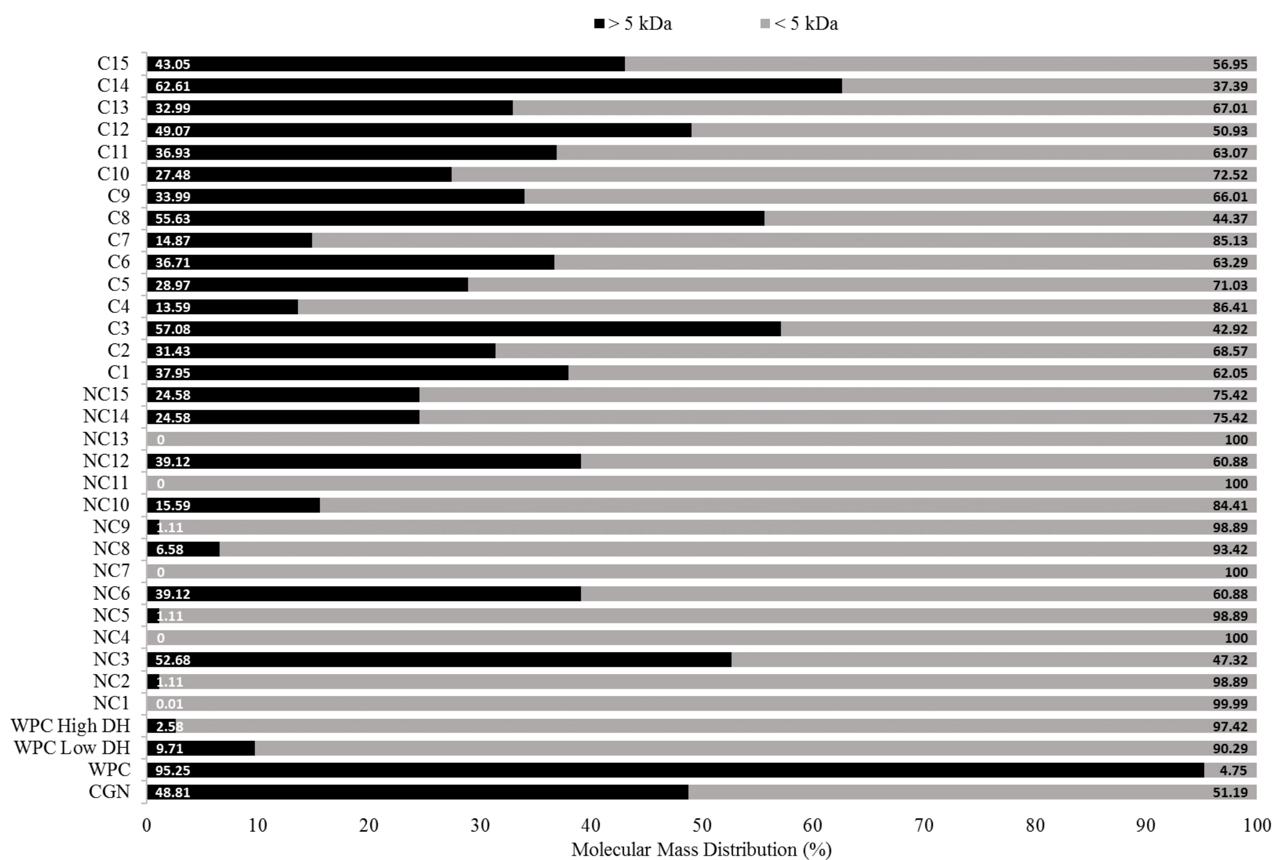


Figure 2

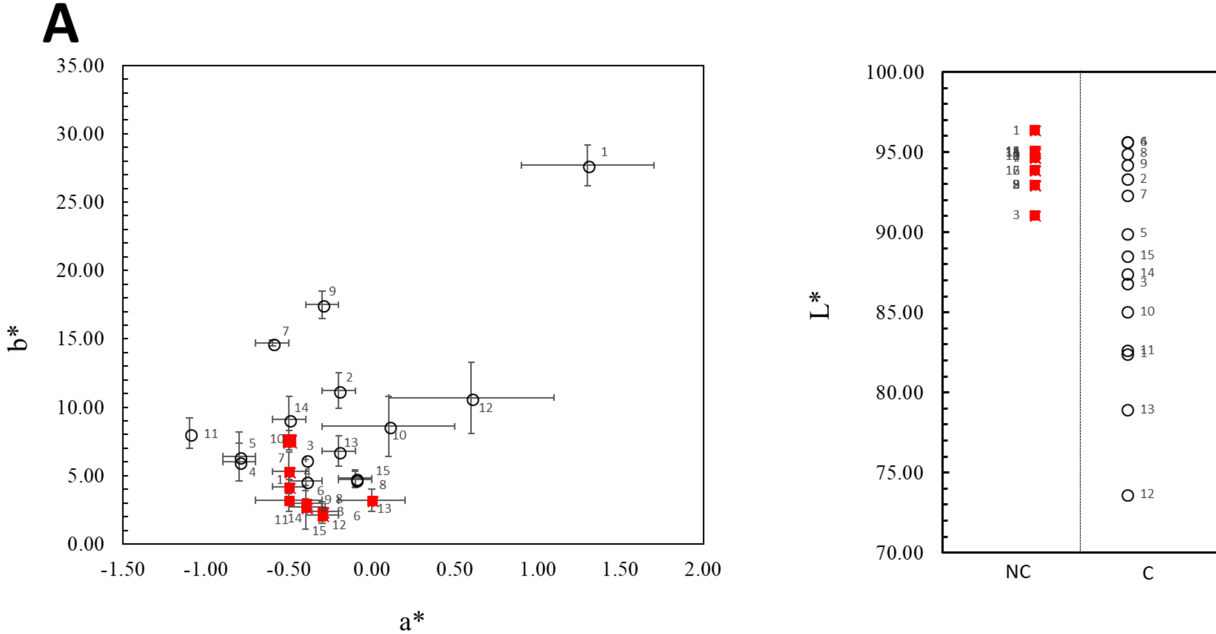
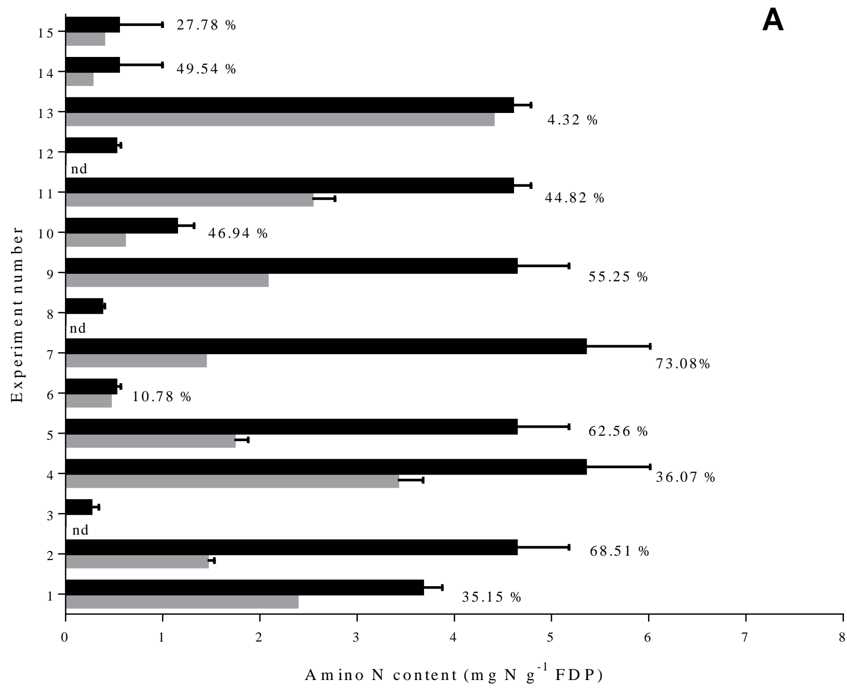


Figure 3



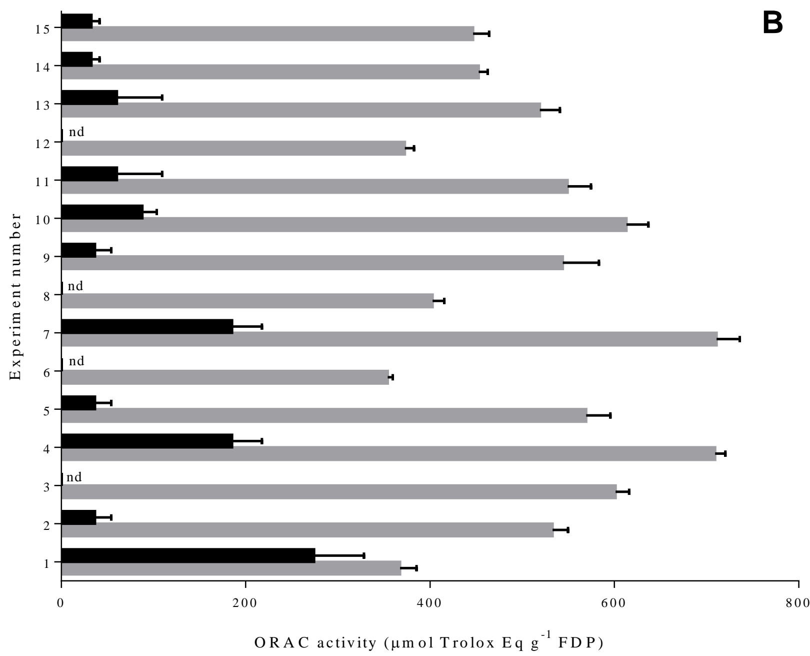


Figure 4

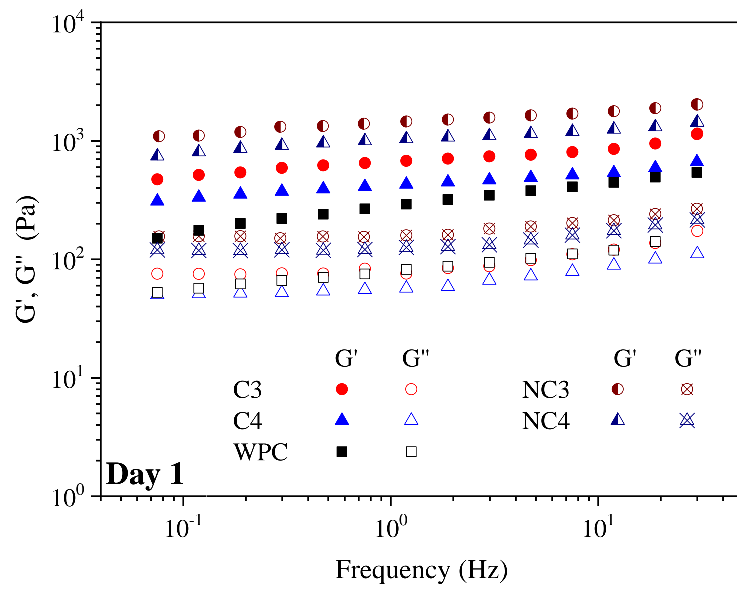


Figure 5

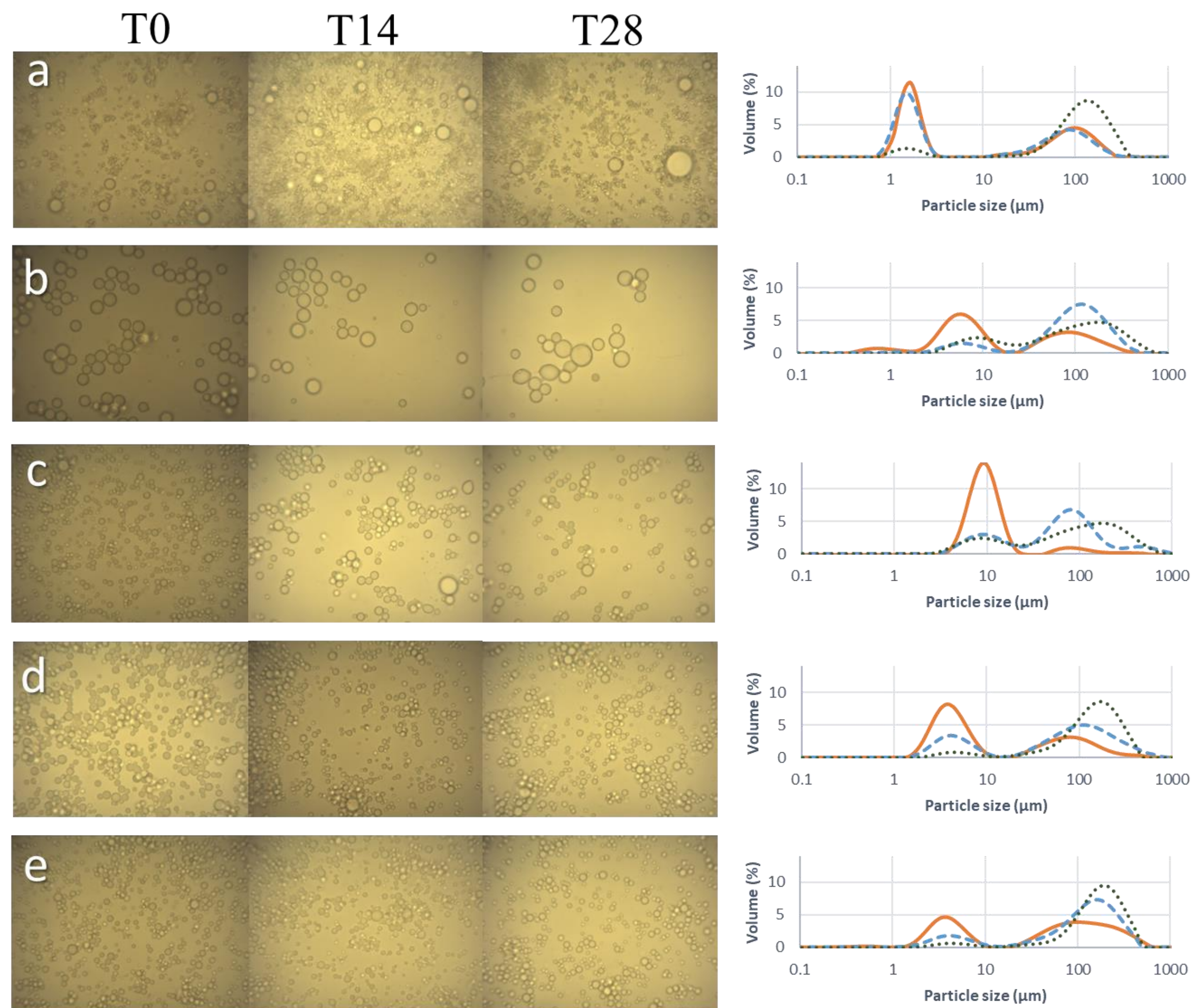
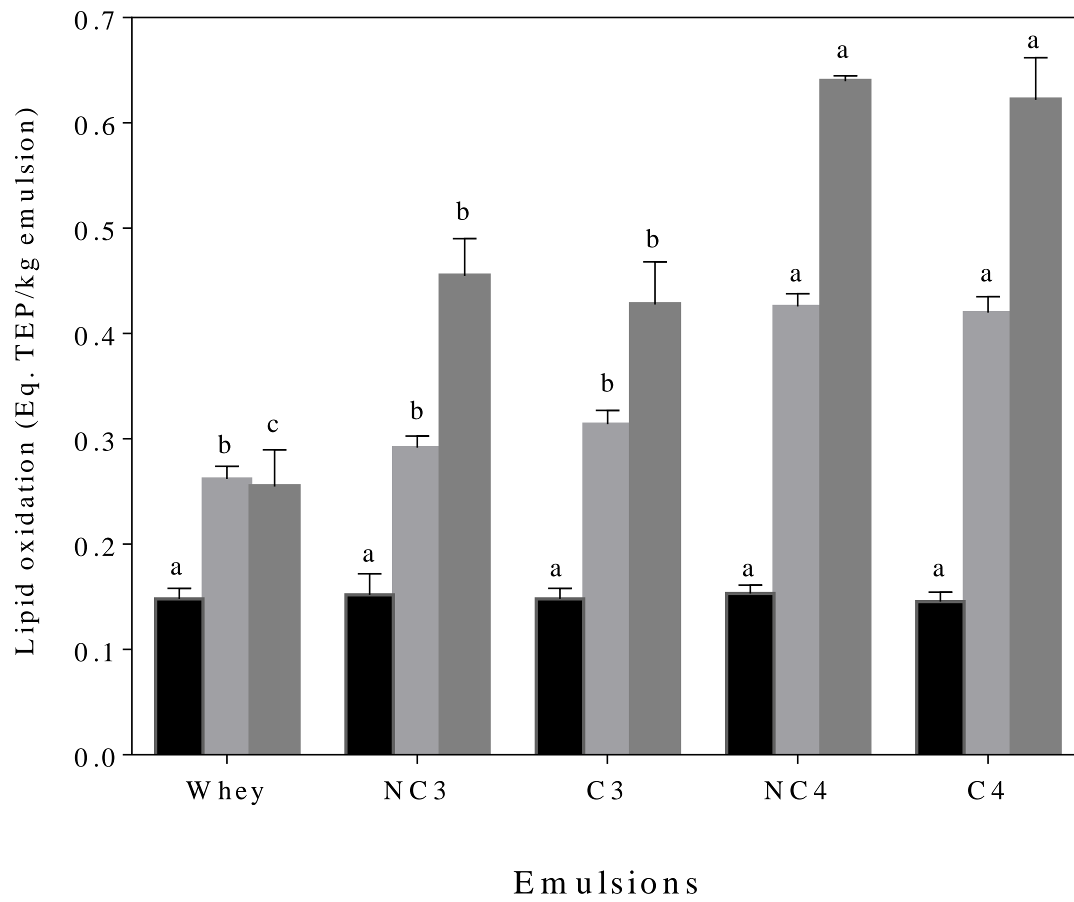


Figure 6



Tables

Table 1: Experimental design parameters for the generation of whey protein concentrate (WPC) and carrageenan (CGN) samples

Conjugate #	WPC:CGN	Time (h)	DH
C1	1:1.0	27	High DH
NC1	1:1.0	0	High DH
C2*	1:3.5	27	Low DH
NC2	1:3.5	0	Low DH
C3	1:6.0	27	Unhydrolyzed
NC3	1:6.0	0	Unhydrolyzed
C4	1:1.0	6	Low DH
NC4	1:1.0	0	Low DH
C5*	1:3.5	27	Low DH
NC5	1:3.5	0	Low DH
C6	1:3.5	6	Unhydrolyzed
NC6	1:3.5	0	Unhydrolyzed
C7	1:1.0	48	Low DH
NC7	1:1.0	0	Low DH
C8	1:1.0	27	Unhydrolyzed
NC8	1:1.0	0	Unhydrolyzed
C9*	1:3.5	27	Low DH
NC9	1:3.5	0	Low DH
C10	1:6.0	27	High DH
NC10	1:6.0	0	High DH
C11	1:3.5	48	High DH
NC11	1:3.5	0	High DH
C12	1:3.5	48	Unhydrolyzed
NC12	1:3.5	0	Unhydrolyzed
C13	1:3.5	6	High DH
NC13	1:3.5	0	High DH
C14	1:6.0	48	Low DH
NC14	1:6.0	0	Low DH
C15	1:6.0	6	Low DH
NC15	1:6.0	0	Low DH

DH: degree of hydrolysis
*Samples corresponding to the central point of the model

Table 2: Emulsion activity (EA) and Sauter mean droplet size (D[3,2]) of the conjugates as a function of storage time (0,14 and 28 days) post emulsion manufacture.

Sample	Day 0		Day 14		Day 28	
	EA	D[3,2] (μm)	EA	D[3,2] (μm)	EA	D[3,2] (μm)
C1	0.25 ± 0.01^{d,e}	14.05 ± 0.86^{c,d}	0.27 ± 0.01^{e,f}	42.18 ± 4.20^{e}	0.04 ± 0.01^{h}	47.47 ± 8.15^{e}
C2*	0.16 ± 0.01^{e}	6.11 ± 2.01^{a,b}	0.19 ± 0.01^{g}	11.74 ± 5.09^{b,c}	0.17 ± 0.01^{f,g}	9.45 ± 2.62^{a,b,c}
C3	0.51 ± 0.01^{b,c}	4.01 ±0.26^{a,b}	0.47 ± 0.01^{c}	4.27 ± 0.24^{a}	0.45 ± 0.01^{b}	4.14 ± 1.70^{a}
C4	0.58 ± 0.01^{b}	4.00 ± 0.03^{a,b}	0.47 ± 0.01^{c}	4.45 ± 1.13^{a}	0.35 ± 0.05^{c}	6.08 ± 1.23^{a}
C5*	0.12 ± 0.01^{e}	4.38 ± 0.47^{a,b}	0.13 ± 0.03^{h}	5.49 ± 0.60^{a,b}	0.14 ± 0.01^{f,g}	6.11 ± 0.55^{a}
C6	0.55 ± 0.01^{b}	5.93 ± 0.73^{a,b}	0.60 ± 0.03^{b}	5.33 ± 1.66^{a,b}	0.47 ± 0.01^{b}	7.00 ± 1.36^{a,b}
C7	0.26 ± 0.01^{d}	8.12 ± 1.14^{a,b,c}	0.30 ± 0.02^{e}	15.27 ± 6.42^{c}	0.24 ± 0.01^{d,e}	14.55 ± 1.96^{c}
C8	1.05 ± 0.04^{a}	2.87 ± 0.59^{a}	1.20 ± 0.01^{a}	5.62 ± 0.56^{a,b}	1.08 ± 0.01^{a}	8.15 ± 0.55^{a,b}
C9*	0.10 ± 0.01^{e}	4.05 ± 0.93^{a,b}	0.13 ± 0.01^{h}	7.06 ± 0.17^{a,b}	0.13 ± 0.01^{g}	6.99 ± 1.34^{a,b}
C10	0.36 ± 0.03^{c,d}	3.28 ± 0.81^{a}	0.24 ± 0.01^{f,g}	8.13 ± 0.28^{a,b}	0.19 ± 0.01^{e,f}	15.52 ± 3.62^{b,c}
C11	0.06 ± 0.01^{e}	8.65 ± 1.90^{a,c}	0.09 ± 0.01^{h,i}	13.91± 2.18^{c}	0.06 ± 0.01^{h}	17.39 ± 1.29^{c}
C12	0.47 ± 0.04^{b}	6.18 ± 0.97^{a}	0.41 ± 0.02^{d}	6.81 ± 0.11^{a,b}	0.29 ± 0.07^{d}	6.40 ± 0.28^{a}
C13	0.05 ± 0.01^{e}	18.11 ± 1.28^{d}	0.09 ± 0.01^{h,i}	23.77 ± 2.59^{d}	0.06 ± 0.01^{h}	13.03 ± 2.23^{b,c}
C14	0.08 ± 0.02^{e}	9.97 ± 0.52^{b,c,d}	0.08 ± 0.02^{h,i}	10.32 ± 0.58^{a,b,c}	0.05 ± 0.01^{h}	14.70 ± 0.49^{b,c}
C15	0.04 ± 0.01^{e}	54.81 ± 3.15^{e}	0.05 ± 0.01^{i}	58.02 ± 0.48^{f}	0.04 ± 0.01^{h}	68.88 ± 2.40^{f}

Values represent mean ± SD of at least 3 independent experiments. Different letters within columns denote significant differences ($p<0.05$)
EA: emulsion activity, [D3,2]: Sauter mean diameter.
*Conjugates corresponding to the central point of the model

Table 3: Elastic modulus (G'_1) and loss tangent ($Tan\ \delta_1$) over storage time (0, 14 and 28 days) of selected emulsions generated with conjugates from the experimental design.

Sample	Day 0		Day 14		Day 28	
	G'_1 (Pa)	$Tan\ \delta_1$	G'_1 (Pa)	$Tan\ \delta_1$	G'_1 (Pa)	$Tan\ \delta_1$
WPC	292.4 ± 13.2 [e]	0.28 ± 0.02 [a]	305.9 ± 8.7 [e]	0.26 ± 0.02 [a]	348.7 ± 11.4 [c]	0.30 ± 0.02 [a]
NC3	1456.1 ± 1.7 [a]	0.11 ± 0.01 [b]	1351.2 ± 5.6 [a]	0.12 ± 0.01 [b]	1350.4 ± 8.6 [a]	0.12 ± 0.01 [b]
C3	678.6 ± 4.6 [c]	0.11 ± 0.01 [b]	717.8 ± 21.7 [c]	0.12 ± 0.01 [b]	989.6 ± 31.2 [b]	0.12 ± 0.01 [b]
NC4	1231.5 ± 8.4 [b]	0.12 ± 0.01 [b]	1038.9 ± 1.9 [b]	0.12 ± 0.01 [b]	852.1 ± 14.8 [b,c]	0.12 ± 0.01 [b]
C4	429.9 ± 9.2 [d]	0.13 ± 0.01 [b]	532.9 ± 9.1 [d]	0.12 ± 0.01 [b]	704.6 ± 12.1 [c]	0.14 ± 0.01 [b]

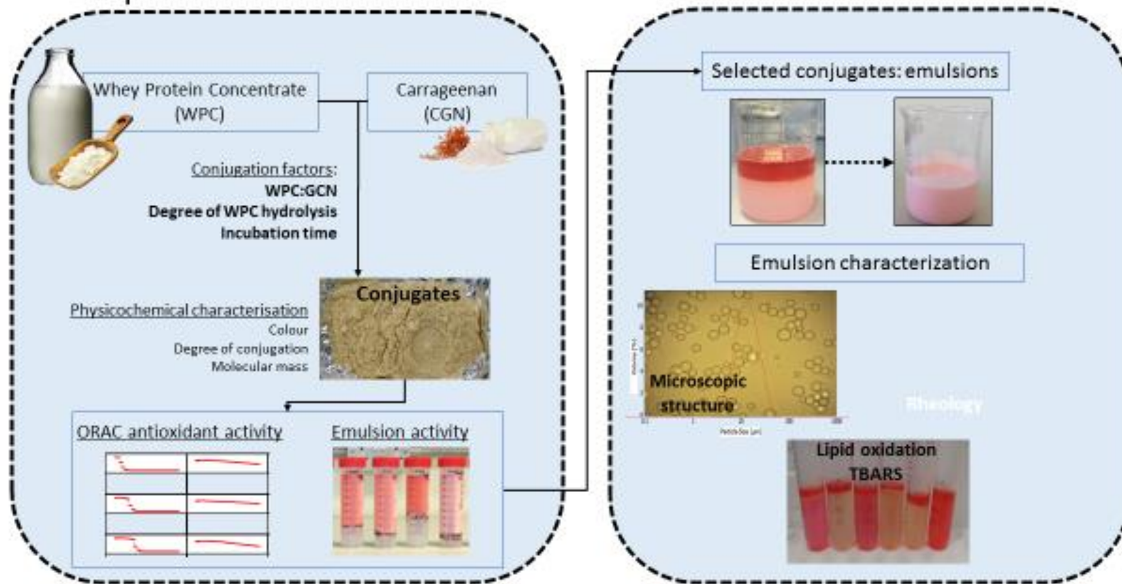
NC3 and NC4= non conjugated samples
C3 and C4= conjugated samples
WPC= whey protein concentrate

Table 4: Floculation (FI) and coalescence index (CI) over storage time (0, 14 and 28 days) of selected emulsions generated with conjugates from the experimental design.

Sample	FI (%)			CI (%)		
	T0	T14	T28	T0	T14	T28
WPC	3.4 ± 0.2 [b]	4.6 ± 0.3 [c]	6.1 ± 0.5[c]	6.6 ± 1.4 [a]	17.4 ± 2.4 [a]	78.6 ± 3.5 [b]
NC3	0.5 ± 0.1 [a]	0.9± 0.2 [a,b]	1.7± 0.2 [a]	34.4 ± 1.9 [b]	72.5 ± 3.4 [b]	80.9 ± 4.1 [b]
C3	0.3 ± 0.1 [a]	4.5 ± 0.1 [c]	7.7 ± 0.1 [d]	33.7 ± 2.1 [b]	62.0 ± 2.6 [b]	64.2 ± 2.5 [a]
NC4	0.2 ± 0.1 [a]	0.5 ± 0.1 [a]	1.2 ± 0.1 [a]	20.8 ± 1.6 [b]	28.6 ± 4.1 [a]	85.6 ± 3.6 [b]
C4	0.1 ± 0.1 [a]	1.2 ± 0.1 [b]	3.3 ± 0.1 [b]	66.6± 2.9 [c]	69.1 ± 1.9 [b]	66.7 ± 3.7 [a]

NC3 and NC4= non conjugated samples
C3 and C4= conjugated samples
WPC= whey protein concentrate

Graphical abstract



Highlights:

- Whey protein concentrate and its hydrolysates were conjugated with carrageenan.
- Conjugates had antioxidant and emulsifying activity.
- Emulsions from selected conjugates were stable over time.
- Conjugation had a negative effect on lipid oxidation.