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Whey Protein Concentrate and Egg White Powder as Structuring Agents of Double Emulsions for Food Applications

--Manuscript Draft--

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Abstract:	This work aims at studying the effects of whey protein concentrate (WP) and egg white powder (EW) as structuring agents in double emulsions (W1/O/W2). A D-optimal design was developed considering the following factors: type (WP, EW) and concentration (0, 5, 10 g/100 mL) of protein used to gel the inner water phase (W1), W1 volume percentage (20, 30, and 40%) in primary emulsion (W1/O), and W1/O volume percentage (40, 50, 60%) in W1/O/W2. The 21 samples were investigated by FT-IR spectroscopy, which revealed different protein conformations depending on W1 and W1/O fractions, and a better interaction with oil of WP rather than EW. Highly significant ($p < 0.001$) multivariate models were computed for yield, rheological properties and creaming stability of W1/O/W2, being W1 and W1/O the most influent factors. Protein type significantly affected W1/O/W2 rheology, revealing a better structuring ability of EW with respect to WP, resulting in higher apparent viscosity and consistency coefficient values. A W1/O/W2 optimized for maximum values of apparent viscosity, yield, and creaming stability was developed, composed of 10 g/100 mL EW in W1, 29% W1, and 60% W1/O, with an oil content of 42.6 mL/100 mL. The optimized emulsion gave results in good agreement with the predicted values, thus confirming the validity of the developed multivariate models for the design of double emulsions with desired features.

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Whey Protein Concentrate and Egg White Powder as Structuring Agents of Double Emulsions for Food Applications

Maria Eletta Moriano, Cristina Alamprese

ANSWERS TO REVIEWER#1

Reviewer #1: The manuscript is well written and has a strong food technology aspect. The results of double emulsion yield and creaming stability show the same value independent of the formulation proposed. The emulsion formulation developed should be applied to encapsulate some bioactive component that shows the performance.

We would like to thank the Reviewer for the positive evaluation of our work. The possibility to use the formulated double emulsion for encapsulation of bioactive compounds has been now mentioned in the Conclusions section.

ANSWERS TO REVIEWER#2

Reviewer #2: The manuscript "Whey Protein Concentrate and Egg White Powder as Structuring Agents of Double Emulsions for Food Applications" have studied the effect of some process variables on multiple emulsions containing whey protein concentrate or egg white powder. The manuscript is well written and I consider that the work was well conducted.

We would like to thank the Reviewer for the positive evaluation of our work and the valuable suggestions that helped in improving our manuscript.

I would recommend some changes before publishing this article:
Line 21: W1 and W1/O concentrations?

Factor ranges have been added in the abstract.

Lines 22-23: Which means "a better structuring ability"? A higher viscosity?

Yes, we refer to the higher apparent viscosity and consistency coefficient values obtained with EW with respect to WP. The sentence has been clarified.

Line 66: W1/O/W2 emulsion

The word “emulsion” has been added in the sentence as suggested.

Lines 66-68: Cite some products that these multiple emulsions can be applied and what is the desired rheological properties for these products.

The text has been implemented as suggested.

Lines 114-115: Were the emulsions destabilized when they were heated due to the decrease of the continuous phase viscosity?

The primary emulsions were not destabilized during heating because of the induced gelation of the protein contained in W_1. As reported by Perez-Moral et al. (2014), the increased viscoelasticity of the gelled droplets can possibly be more resistant to coalescence. Gelation procedure was performed according to Surh et al. (2007); the reference has been added.

Line 116: Which protein was added to the external water phase?

The external water phase contained the same protein used in the inner water phase. The sentence has been clarified.

Line 117: Why was NaCl used in the external phase in the same concentration than in the inner phase?
The same NaCl concentration was used in W_2 and W_1 in order to balance the osmotic pressure and avoid water migration phenomena. The information has been added in the text.

Line 143: Why was used a very high speed for centrifugation?
The high speed was necessary in order to induce the separation of W_2 and W_1/O. We chose the best *g* value in preliminary trials, following the indications given by Surh et al. (2007) and Perez-Moral et al. (2014).

Line 144: Add details about the spectrophotometer.
Details have been added.

Line 156: Why was this shear rate chosen?
The shear rate range was chosen based on preliminary trials, in order to have a good sensitivity of the rheometer and avoid emulsion system disruption. The information has been added in the text.

Line 163: Creaming stability (CS)
We used CS because the abbreviation was already explained in line 124 (now line 140).

Line 211 and Figure 1: Why did NaCl solutions also show the bands related to amide and other peptide linkages?
NaCl solution only shows the bands related to water, but, as reported in lines 226-227 (now lines 249-250), at 1,645 cm^{-1} the H_2O bending vibrational band overlaps with amide I band.

Lines 243-245: Can some differences occur due to the heat treatment that was applied in the multiple emulsions but not in the protein solutions?
The Reviewer is right, this is possible. The text has been modified accordingly. In any case, the specific examples commented in the following lines refer to emulsions in which the gelation of the inner water phase was not induced.

Line 259: 2,826 and 2,954?
Thank you for the observation. The text is right, whereas the bands indicated in Fig. 1 were wrong. Now we have corrected them.

Lines 259-260: Would not the change in the bands in relation to the pure oil indicate the interaction between oil and protein?
We agree with the Reviewer. Indeed, the explanation in the following lines refers to oil and protein interactions.

Line 262: Does EW not contain hydrophilic and hydrophobic regions?
The Reviewer is right: also EW contains hydrophilic and hydrophobic regions. However, the sentence refers to the emulsifying activity of WP. To the best of our knowledge, EW has not being reported in the literature as a good W/O emulsifier, maybe because it has a hydrophilicity higher than WP, as explained in the following lines.

Line 270 and Figure 3: What is PC1 and PC2?
The abbreviations have been explained.

Lines 283 and 290: W1 and W1/O concentrations?
There is not a specific concentration to indicate, because the models are valid in the whole range considered for the experimental factors.

Line 310: n = 1.11 indicates a shear thickening behavior

The Reviewer is right, but considering the error associated to the measurement we think that the behavior can be compared to that of a Newtonian fluid. Actually, the grand mean for WP samples was 1.04±0.05. This information has been added and the sentence has been modified accordingly.

Lines 312-313: It would be interesting to present the microscopy images of emulsions.
Unfortunately, for this work we had not the possibility to perform the microscopy analysis of all the samples.

Lines 340-342: Rheological behavior was already described earlier.
Thank you for the observation. The lines have been deleted.

Line 374: How can stability be greater than 100%?
The CS higher than 100 is only a predicted value, with no physical meaning. The sentence has been clarified.

Conclusions: More details about the main results should be added to the conclusions.
Some details have been added as suggested, but keeping in mind the guidelines of the Journal, which suggest "Do not repeat in detail data given in Results and Discussion section".

ANSWERS TO REVIEWER#4

Reviewer #4: General Comments.
The manuscript is well written, the design of experiments, methodology and analysis of results are appropriate, and the originality is acceptable. I think it should be accepted with minor corrections. See detailed comments.
The authors wish to thank the Reviewer for the positive evaluation of the work and the valuable suggestions given, which helped us to improve the manuscript.

Line 156. Why were these values of shear rate selected?
The shear rate range was chosen based on preliminary trials, in order to have a good sensitivity of the rheometer and avoid emulsion system disruption. The information has been added in the text.

Line 165. Explain briefly about the red oil layer.
Some details have been added as suggested.

Line 183. I guess the term beta11 is missing because it's a categoric factor. This should be mentioned.
The Reviewer is right and the explanation has been added in the text.

Line 335. Why "with W1 decreasing"? I understand it should say "increasing" or nothing.
We apologize for the mistake. The sentence has been corrected.

Line 374. I understand CS 105% is a predicted value with no physical meaning. It should be mentioned.
Thank you for the observation. The explanation has been added in the text.

Table 3. For values of coded coefficients use scientific notation with 3 significant digits.
Values have been modified as suggested.

Whey Protein Concentrate and Egg White Powder as Structuring Agents of Double Emulsions for Food Applications

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Abstract

This work aims at studying the effects of whey protein concentrate (WP) and egg white powder (EW) as structuring agents in double emulsions ($W_1/O/W_2$). A D-optimal design was developed considering the following factors: type (WP, EW) and concentration (0, 5, 10 g/100 mL) of protein used to gel the inner water phase (W_1), W_1 volume percentage (20, 30, and 40%) in primary emulsion (W_1/O), and W_1/O volume percentage (40, 50, 60%) in $W_1/O/W_2$. The 21 samples were investigated by FT-IR spectroscopy, which revealed different protein conformations depending on W_1 and W_1/O fractions, and a better interaction with oil of WP rather than EW. Highly significant ($p < 0.001$) multivariate models were computed for yield, rheological properties and creaming stability of $W_1/O/W_2$, being W_1 and W_1/O the most influent factors. Protein type significantly affected $W_1/O/W_2$ rheology, revealing a better structuring ability of EW with respect to WP, resulting in higher apparent viscosity and consistency coefficient values. A $W_1/O/W_2$ optimized for maximum values of apparent viscosity, yield, and creaming stability was developed, composed of 10 g/100 mL EW in W_1, 29% W_1, and 60% W_1/O, with an oil content of 42.6 mL/100 mL. The optimized emulsion gave results in good agreement with the predicted values, thus confirming the validity of the developed multivariate models for the design of double emulsions with desired features.

Keywords: D-optimal experimental design; FT-IR spectroscopy; reduced-fat food; rheology; stability; yield.

Introduction

An interesting approach for the development of reduced-fat foods is the application of water-in-oil-in-water double emulsions ($W_1/O/W_2$). They consist of small droplets of an inner water phase (W_1) entrapped in oil droplets (O) that are, in their turn, dispersed in another aqueous phase (W_2) (Muschiolik 2007). The concentration of W_1 droplets inside the primary emulsion (W_1/O) is affected by the ratio of W_1 to O, while the concentration of oil droplets in the final emulsion can be controlled by using different W_1/O to W_2 ratios. The main advantage of $W_1/O/W_2$ lies in the possibility to obtain a typical oil-in-water (O/W) emulsion structure, but with a reduced fat content. Actually, rheology of $W_1/O/W_2$ can be considerably different from that of an O/W emulsion due to the higher effective volume fraction of the particles, which is approximately represented by the sum of the volume fraction of O and W_1/O. As a result, highly viscous products can be made with lower fat content (McClements 2016). Another advantage of double emulsions is the possibility to encapsulate a bioactive component within one of the emulsion phases, in order to improve its release behaviour, oral administration and digestion (Artiga-Artigas et al. 2019; Lamba et al. 2015; Momeni et al. 2017).

One of the main issues connected to real food applications of $W_1/O/W_2$ is their high susceptibility to breakdown during storage or when exposed to the common stresses involved in food preparation (e.g., mechanical stresses, chilling, freezing, heating, etc.) (McClements 2016). In order to overcome this issue, gelation of W_1 may be a useful strategy (Oppermann et al. 2015; Perez-Moral et al. 2014), together with the use in W_1/O of a strong lipophilic emulsifier such as polyglycerol polyricinoleate (PGPR) (Artiga-Artigas et al. 2019; Muschiolik and Dickinson 2017). The incorporation in W_1 of a polymer able to form a network in the water phase droplets or a gel-like layer at the

inner water-oil interface (e.g., xanthan, alginate, gelatine, agarose, bovine serum albumin, sodium caseinate) has been proposed as a way of improving the long-term stability of $W_1/O/W_2$. For instance, Surh et al. (2007) studied the possibility of improving $W_1/O/W_2$ stability by thermal gelation of whey proteins contained within W_1. They demonstrated that gelling the internal aqueous phase of the primary emulsion does not facilitate the formation of the double emulsion, but it can improve the long-term stability of the system; however, the Authors suggest further investigations.

The effects of using double emulsions as fat replacers in food products have been studied so far for dairy products (Leong, Zhou, Kukan, Ashokkumar, & Martin, 2017; Lobato-Calleros, Rodriguez, Sandoval-Castilla, Vernon-Carter, & Alvarez-Ramirez, 2006; Lobato-Calleros et al., 2008; Lobato-Calleros, Recillas-Mota, Espinosa-Solares, Alvarez-Ramirez, & Vernon-Carter, 2009; Márquez & Wagner, 2010), meat products (Cofrades, Antoniou, Solas, Herrero, & Jiménez-Colmenero, 2013; Freire, Bou, Cofrades, Solas, & Jiménez-Colmenero, 2016), mayonnaise and dressings (Matsumoto & Kohda, 1980; Takahashi, Aizawa, Tamai, Yoshida, & Takahashi, 1986), but there are still numerous challenges to be addressed before a successful commercial use (McClements, 2016).

Since properties of $W_1/O/W_2$ are affected by several factors, Design of Experiments (DoE) techniques can be successfully applied to the systematic characterization of these systems, in order to acquire deeper knowledge about their structure and be able to design a $W_1/O/W_2$ emulsion with the desired features. The aim of this work was thus to study the effects of two different biopolymers (i.e., whey proteins and egg white), used as gelation agents, on yield, rheological properties, and creaming stability of $W_1/O/W_2$. A D-optimal design was developed considering as experimental factors the type and

concentration of biopolymer used to gel W_1, the W_1 volume percentage in the primary emulsion, and the W_1/O volume percentage in the final double emulsion. Whey proteins and egg albumen were selected as structuring agents because they are widely used natural food-grade ingredients, able to form thermo-irreversible gels upon heating. While few works deal with the use of whey proteins to gel the inner water phase of double emulsions (Balcaen et al. 2016; Opperman et al. 2015; Sağlam et al. 2011; Surh et al. 2007), to the best of our knowledge, no studies have been published so far about the use of egg white as a gelation agent for multiple emulsions.

Materials and Methods

Materials

Whey protein concentrate WPC80 (WP) and spray-dried egg white powder (EW) were kindly supplied by Milkiland EU (Ostrów Mazowiecka, Poland) and Lactosan-Sanovo Ingredients Group (Zeven/Aspe, Germany), respectively. According to label data, both WP and EW contained 80 g/100 g proteins in dry matter. Corn oil (Carrefour, Boulogne-Billancourt, France) was bought in a local supermarket. PGPR was kindly supplied by Lasenor (Barcelona, Spain; product commercial name: VEROL PR). NaCl, 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt hydrate (PTSA) and Oil red O dye were purchased from Sigma Aldrich (Saint Louis, MO, USA).

Experimental Design

Experiments were planned according to a D-optimal design in order to study simultaneously the main and interaction effects of four factors: type of protein powder (WP and EW); protein powder concentration in W_1 (0, 5, and 10 g/100 mL); W_1 volume

percentage (20, 30, and 40%) in the primary emulsion; W_1/O volume percentage (40, 50, and 60%) in the final $W_1/O/W_2$. Factor levels were chosen based on literature study and preliminary trials. A total of 21 experiments, comprising 3 replicates of the central point, were performed (Table 1). The order of experiments was fully randomized in order to avoid possible bias related to systematic effects and uncontrolled variations. Sample identification refers to the protein biopolymer code (WP or EW), followed by the actual level of the other three experimental factors considered (protein powder concentration, W_1 percentage, W_1/O percentage).

Preparation of Double Emulsions

Double emulsions were prepared according to the procedure suggested by Perez-Moral et al. (2014) slightly modified and adapted to the subsequent system characterizations. For rheological and Fourier-transform infrared (FT-IR) spectroscopy measurements, double emulsions (150 mL) were prepared as follows: WP or EW (5 or 10 g/100 mL) were dissolved in NaCl solution (0.4 g/100 mL) and stirred at room temperature (22 °C) for 1 h. These solutions were used as W_1 in W_1/O. When no proteins were required in W_1, NaCl solution (0.4 g/100 mL) without protein powder addition was used as W_1. W_1/O were prepared by adding the established amount of W_1 (20, 30 or 40%) to corn oil containing 4 g/100 mL PGPR. The phases were mixed using a heavy duty blender (Waring Laboratory, Torrington, CT, USA) at 18,000 rpm for 30 s and at 20,000 rpm for further 30 s. When W_1/O contained proteins, they were heated in a water bath at 80 °C for 20 min and cooled down in iced water for 15 min in order to induce gelation (Surh et al., 2007). External water phase (W_2) was prepared by dissolving in NaCl solution (0.4 g/100 mL) 5 g/100 mL of the same protein powder used in W_1, and stirring for 1 h

at room temperature. The same NaCl concentration was used in W_1 and W_2 in order to balance the osmotic potential and avoid water diffusion phenomena (Perez-Moral et al., 2014). $W_1/O/W_2$ were prepared by adding the given amount of W_1/O (40, 50 or 60%) to W_2 and mixing as previously described for W_1/O preparation.

When intended for yield determination, $W_1/O/W_2$ (150 mL) were prepared following the same procedure but using sodium phosphate buffer 5 mM with NaCl 100 mM (pH 7) and PTSA (0.2 g/100 mL) as W_1. The corresponding $W_1/O/W_2$ prepared without dye addition were used as blank.

For creaming stability (CS) evaluation, $W_1/O/W_2$ (50 mL) were prepared as previously described for rheological characterization, but colouring the oil phase with the Oil red O dye (0.0015 g/100 mL).

FT-IR Spectroscopy Analysis

FT-IR spectra of $W_1/O/W_2$, corn oil, protein solutions (EW and WP both at 5 and 10 g/100 mL in 0.4 g/100 mL NaCl solution) and NaCl solution (0.4 g/100 mL) were acquired by a Vertex 70 spectrometer (Bruker Optics, Milan, Italy) equipped with a Germanium multiple reflection ATR cell. Spectral data were collected in duplicate in the range 4,000-400 cm^{-1} at 20 °C, with a 4 cm^{-1} resolution and 32 scans for both background and samples. Opus software (v. 6, Bruker Optics, Ettlingen, Germany) was used for instrument control and spectral acquisition. Before elaboration, duplicated spectra were averaged.

Double Emulsion Yield

Yield, expressed as the percentage of emulsified W_1 droplets remaining inside the oil

droplets, was determined spectrophotometrically using PTSA as a tracer, following a method adapted from Perez-Moral et al. (2014). $W_1/O/W_2$ samples, after storage at 4 °C overnight, were centrifuged in a benchtop centrifuge (Centrikon T-42K, Neufahrn, Germany) at 44,800 x g (20,000 rpm) for 20 min at 10 °C. The concentration of dye appearing in W_2 was determined spectrophotometrically (V-650 spectrophotometer, Jasco Europe, Cremella, LC, Italy) at 374 nm by using a PTSA calibration curve. Yield was calculated trough the following equation adapted from Surh et al. (2007):

$$\text{Yield } (\%) = 1 - \left(\frac{C_f}{C_i - C_f} \cdot \frac{1 - \phi_{W_1O}}{\phi_{W_1} \cdot \phi_{W_1O}} \right) \quad (1)$$

where: C_i is the initial PTSA concentration in W_1 (0.2 g/100 mL); C_f is the final PTSA concentration in W_1 (g/100 mL); ϕ_{W_1O} is the volume percentage of W_1/O in $W_1/O/W_2$; ϕ_{W_1} is the volume percentage of W_1 in W_1/O.

Yield measurements were performed in quadruplicate.

Rheological Characterization of Double Emulsions

Flow curves of $W_1/O/W_2$ were measured in triplicate at 25 °C using a Physica MCR 300 rheometer (Anton Paar, Graz, Austria) equipped with coaxial cylinders (CC27), in a 150-500 s^{-1} range of shear rate. Preliminary trials were performed to choose a shear rate range able to guarantee a good sensitivity of the rheometer, while avoiding emulsion destabilization. Apparent viscosity was taken at 310 s^{-1}, while consistency coefficient (K) and flow behaviour index (n) were obtained by fitting the curves with the power law equation:

$$\tau = K \cdot \dot{\gamma}^n \quad (2)$$

where τ is the shear stress (mPa) and $\dot{\gamma}$ is the shear rate (s^{-1}) (Steffe 1996).

Creaming Stability of Double Emulsions

CS of $W_1/O/W_2$ was determined in triplicate according to a method adapted from Karaca et al. (2011). Immediately after preparation, samples stained with the Oil red O dye were poured into 10 mL graduated cylinders and stored at 4 °C. After 1 and 24 h, the height of the visible darker red oil layer creamed and separated from the bottom turbid layer was registered. CS was calculated through the following equation:

$$CS\,(\%) = 100 - \left(\frac{H_t}{H_e} \cdot 100 \right) \quad (3)$$

where H_t is the height of the red oil layer creamed after 1 or 24 h of storage, and H_e is the total height of the $W_1/O/W_2$.

Data Analysis

In order to obtain a high quality protein spectrum, reduced FT-IR spectra (considered ranges: 3,728-2,754 cm^{-1} and 2,272-1,000 cm^{-1}) were subtracted of the NaCl solution spectrum and pre-treated with smoothing (moving average, segment size = 5) and second derivative transformation (Savitzky-Golay algorithm, polynomial order = 2, smoothing points = 11) (Kong and Yu, 2007). Afterwards, the obtained spectra were analyzed by means of Principal Component Analysis (PCA) in order to investigate possible sample patterns and importance of spectral variables (The Unscrambler X software, v. 10.4.1, CAMO Process A/S, Oslo, Norway).

Data collected for DoE were elaborated by means of the Response Surface Methodology, postulating a quadratic model for each of the considered response variables:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \varepsilon \quad (4)$$

where y is the value of the considered response variable; x_1, x_2, x_3, and x_4 are, respectively, the type and the level of protein powder concentration, the volume percentage of W_1, and the volume percentage of W_1/O; β_0 is the model intercept; β_1, β_2, β_3, and β_4 are the linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are the interaction coefficients; β_{22}, β_{33}, and β_{44} are the quadratic coefficients (β_{11} is missing because of the categoric nature of the factor); ε is the random error.

Before model calculation, data were checked for normal distribution and skewness; apparent viscosity and K data were transformed in the inverse root square and log values, respectively. The significance of each coefficient was determined by one-way analysis of variance (ANOVA). When non-significant terms not counting to support hierarchy were present, a model reduction was considered. A multi-objective optimization of the double emulsion was also carried out, calculating an overall desirability function (Alamprese et al. 2007; Montgomery 2001). Data elaboration was performed by Design Expert 10.0.6 (Stat-Ease Inc., Minneapolis, MN, USA).

Results and Discussion

Double Emulsion Theoretical Composition

As expected, the lowest volume percentage of W_1 in W_1/O and the highest volume percentage of W_1/O in $W_1/O/W_2$ corresponded to the maximum theoretical oil content (48 mL/100 mL) in $W_1/O/W_2$ (Table 1). Increasing W_1 levels and decreasing W_1/O levels implied a progressive decrease in fat content, until reaching the minimum value (24 mL/100 mL) in the emulsions containing 40% of both W_1 and W_1/O. The lowest protein concentration (1.6 g/100 mL) was obviously obtained in samples with no protein

addition in W_1, for which the only protein contribution came from EW or WP dispersed in W_2.

FT-IR Spectroscopy Analysis

Fig. 1 shows FT-IR spectra of double emulsions, corn oil, dispersions of EW and WP (both at 5 and 10 g/100 mL) and NaCl solution (0.4 g/100 mL) after the spectral interval reduction and the baseline offset correction. In order to eliminate the less informative and noisiest regions, as well as the absorption band of CO_2, only the following spectral ranges were considered: 3,728-2,754 cm^{-1} and 2,272-1,000 cm^{-1}.

The broad band in the OH stretch region (3,000-3,700 cm^{-1}) is mostly related to water. In WP-based double emulsions (Fig. 1a), the height of this band is more variable than in EW-based samples, indicating a lower availability of water in some of the WP-based systems (Hesso et al. 2015). Moreover, in WP-based double emulsions a more pronounced shoulder around 3,270-3,280 cm^{-1} is evident, which can be related to an increase in hydrogen-bonded-associated chains of water with the emulsion proteins (Bock and Damodaran 2013). $W_1/O/W_2$ spectra are characterized also by two bands around 1,640 and 1,550 cm^{-1}, related to amide I and II, respectively, and due to the C=O and C-N stretching of the peptide linkages (Kong and Yu 2007; Mohammadian et al. 2018). The intensity of these absorption bands decreased in $W_1/O/W_2$ with respect to protein dispersions, due to a lower protein and water content of the multiphase systems (Hesso et al. 2015). Actually, at 1,645 cm^{-1} also the H_2O bending vibrational band shows a high absorbance, overlapping with amide I band (Kong and Yu 2007). Frequencies of the amide I band components are correlated to each secondary elements of proteins, while amide II band is much less sensitive to protein conformation changes.

In particular, the second derivative spectra between 1,600 and 1,700 cm^{-1} allow the identification of various secondary structures of the proteins, even if caution has to be exercised in the IR spectra interpretations (Kong and Yu 2007). After the subtraction of the NaCl solution spectrum and the second derivative (d2) transformation (Fig. 2), spectra of $W_1/O/W_2$ in the considered range (1,600-1,700 cm^{-1}) still showed a high numbers of extensively overlapped bands, thus deconvolution and peak quantification were not attempted, rather observing qualitative spectral changes.

WP and EW protein solutions showed major peaks around 1,631 and 1,637 cm^{-1}, respectively, which indicate proteins with a high content of β-sheet structure. A second high peak centered at 1,654 cm^{-1} was also visible in solutions of 5 g/100 mL WP and 10 g/100 mL EW, indicating a relevant content of α-helix structure. WP at 10 g/100 mL showed, instead, a relevant content of random coil structures (peak at 1,647 cm^{-1}, Fig. 2a). The different amount of water in the measured protein solutions accounts for the different protein conformations. In $W_1/O/W_2$ samples, shifts and different intensities of these peaks can be observed, indicating changes in protein conformation, probably due to heating eventually applied during emulsion preparation and the protein exposure to different amounts of water and oil. For instance, sample EW_00_20_60 (dotted line among double emulsion samples in Fig. 2b) has a low amount of proteins (1.6 g/100 mL), a similar amount of fat (48 mL/100 mL) and water (49.9 mL/100 mL) and it shows a low intensity of the amide I band, mostly constituted by random coil structures (d2 peak centered at 1,643 cm^{-1}). On the contrary, sample EW_00_40_60 (dashed line among the double emulsion sample in Fig. 2b), composed of the same amount of proteins (1.6 g/100 mL), but a higher amount of water (61.9 mL/100 mL) rather than fat (36 mL/100 mL), showed a bimodal distribution of d2 spectra, centered at 1,637 and

1,652 cm^{-1}, indicating similar content of β-sheet and α-helix structures.

The typical peaks of oil (2,800-3,030, 1,745, 1,000-1,490 cm^{-1}) resulted more evident in WP-based emulsions rather than in those containing EW (Fig. 1). This result indicates different interactions of the two types of proteins with oil. In particular, the appearance in WP-based $W_1/O/W_2$ spectra of the bands related to CH asymmetric and symmetric stretching of CH_2 (2,926 and 2,854 cm^{-1}, respectively) can indicate conformational changes of the oil alkyl chains in the oil/water interfaces, suggesting the involvement of CH_2 groups of oil with proteins (Guo et al. 2019). Actually, as reported in the literature, whey proteins naturally have hydrophobic and hydrophilic regions that make them surface-active, thus facilitating emulsion formation and stabilization (Cheong et al. 2015). Moreover, whey protein solutions have higher values of hydrophobicity than egg white protein solutions under the same pH conditions (Kuropatwa et al. 2009), thus supporting the hypothesis of a better interaction of WP with oil. As a further confirmation of the different interactions of WP and EW proteins with oil, a PCA applied to the reduced spectra after subtraction of the NaCl solution spectrum and the second derivative transformation showed that $W_1/O/W_2$ samples differentiated along the first principal component (PC1) depending on the type of protein used (Fig. 3a). Loading values revealed that the most influent variables in the differentiation of samples along PC1 corresponded to the absorption peaks typical of oils (2,800-3,030, 1,745 cm^{-1}; Fig. 3b).

Multivariate Models for Double Emulsion Characteristics

Double emulsion yield, rheological parameters and creaming stability are reported in Table 2, while the corresponding multivariate models are shown in Table 3. All the

calculated models resulted highly significant ($p < 0.001$). Adjusted R^2 values were higher than 0.89 for rheological variable models and around 0.72-0.75 for those of yield and stability. Predicted R^2 values resulted low for yield and CS, but the lack of fit was always not significant except for the model of n ($p = 0.01$), for which the response surface did not fit adequately the experimental domain. Adequate precision was always good, being much higher than 4. W_1 and W_1/O appeared to be the most influent factors. All the $W_1/O/W_2$ samples showed high yields (> 95%), meaning a very good retention of W_1 inside the oil droplets; no differences were observed between EW- or WP-based samples (Table 2). This result could be ascribed to the use of PGPR as lipophilic emulsifier, which ensure a high water encapsulation efficiency (Perez-Moral et al. 2014) due to a steric stabilization of the interfacial layer (Márquez al. 2010) and a smaller droplet size in W_1/O due to the high hydrophobicity (Artiga-Artigas et al. 2019). W_1 and W_1/O showed positive and highly significant ($p < 0.001$) coded coefficients for yield (Table 3), revealing a direct effect on W_1 retaining ability of $W_1/O/W_2$. The response surface (Fig. 4a) is characterized by a slight curvature, due to the significance ($p < 0.05$) of the interaction term between W_1 and W_1/O (β_{34}). This means that the effect of W_1/O on yield is more accentuated at low levels of W_1 (20%) rather than at high levels. Protein concentration and type did not significantly affect yield, contrarily to what reported by Opperman et al. (2015) and Perez-Moral et al. (2014) who found an increase in yield with W_1 gelation. The contrasting results may be due to the fact that the cited studies considered a constant $W_1/O/W_2$ formulation without investigating changes associated to the variation of oil and water volume fractions. In the present study, it is presumable that the effect on yield of oil and water fraction changes was more marked than the effect of W_1 gelation. Moreover, in this study, proteins were

added also to W_2 in order to further reduce water mobility in the system and this may have leveled the effect of W_1 gelation.

Viscosity results are very interesting for fat reduction strategies since they affect both air incorporation ability of the system (Márquez and Wagner 2010) and sensory perception of emulsion creaminess (van Aken et al. 2011). Apparent viscosity and K showed a comparable trend, with samples containing 60% W_1/O resulting in the highest values (Table 2). Emulsions containing EW were characterized by n ranging from 0.36 to 0.91, indicating a non-Newtonian pseudoplastic behavior of different intensity. On the contrary, WP-based samples showed n values in the range 0.94-1.11, with a grand mean and standard deviation value of 1.04±0.05, revealing a quite Newtonian behavior, as already reported in previous studies about single emulsions (Erçelebi and Ibanoğlu 2009). Shear thinning of EW-emulsions may be connected to the presence of aggregated droplets (Panagopoulou et al. 2017) that are deformed and disrupted as the shear rate increases (Demetriades et al. 1997). Thus, the differences observed between samples containing the two different protein powders could be linked to the higher ability of WP proteins in interacting with other components of the emulsion and creating a more homogenously dispersed phase, in agreement with the FT-IR results. Apparent viscosity was significantly influenced by all the experimental factors (Table 3), with a tendency to increase at higher concentrations of protein powder in W_1 ($p < 0.01$), higher level of W_1/O ($p < 0.001$), and lower percentages of W_1 ($p < 0.001$). The effect of protein concentration was in agreement with the findings of Oppermann et al. (2016) who demonstrated that gelation of W_1 in double emulsions allows to obtain viscosity values higher than those of simple O/W emulsions, despite the lower oil content of W_1/O/W_2. In addition, they found that all the fat-related mouth-feel and after-feel attributes

increase in gelled samples, balancing the effects of fat reduction. In the present work, also the protein powder type resulted significant ($p < 0.001$), demonstrating EW to be a better structuring agent with respect to WP (Fig. 4c-d). Actually, at equal levels of W_1 and W_1/O, changing the protein powder from WP to EW quite doubled the apparent viscosity of double emulsions (Table 2). Similarly, protein type and percentage of W_1/O had a strong positive effect ($p < 0.001$) on K, with EW-containing samples showing the highest values (Table 2). On the other hand, increasing W_1 led to a significant decrease ($p < 0.001$) of K due to the reduction of the oil content (Fig. 4b). Protein concentration in W_1 was involved in significant ($p < 0.05$) interactions with both W_1 (β_{23}) and W_1/O (β_{24}). In fact, the reduction of K values with W_1 increasing was more marked at high (10 g/100 mL) than at low (0-5 g/100 mL) protein concentrations. Similarly, at high and intermediate levels of protein powder concentration, the increase of K while increasing W_1/O was less marked than in absence of proteins. The reduction of K values with the increase in W_1 was higher at high levels of W_1/O, due to the significant and negative interaction β_{34} ($p < 0.05$; Fig. 4b). W_1 showed a direct effect ($p < 0.05$) on n, while W_1/O resulted in an opposite effect ($p < 0.01$): the less the W_1/O percentage, the higher the n values.

CS is of paramount importance for double emulsions intended as alternative fats in food formulation, since they have to be prepared beforehand and stored until use. All the samples showed no creaming phenomena 1 h after production (data not shown), while after 24 h samples with the lowest W_1/O level (40%) resulted in the lowest stability (< 80%), irrespective the protein type (Table 2). The only exception was sample WP_00_20_40, for which a CS of 88 ± 1% was registered. In particular, the highest water content (about 72 mL/100 mL) produced a stability lower than 50% in samples

EW_00_40_40, WP_05_40_40, and WP_00_40_40, suggesting the unsuitability of these emulsions as storable ingredients. CS was significantly ($p < 0.001$) improved as the percentage of W_1/O increased, while an inverse significant effect ($p < 0.01$) was found for W_1 volume fraction (Table 3). These findings are in agreement with previous studies demonstrating that 20-30% of W_1 in primary emulsions is the optimum phase volume ratio in terms of stability (Su et al. 2006). As reported above for yield, also for stability a significant effect of the W_1 gelling was not observed, confirming that the other factors had a stronger effect on the system.

Optimization of a Double Emulsion Intended as Shortening Replacer

The developed multivariate models can be used to design $W_1/O/W_2$ with peculiar characteristics. As an example, a $W_1/O/W_2$ intended for shortening replacement in reduced-fat baked goods was optimised by means of desirability function. The optimization criteria were chosen in order to obtain an emulsion with a good structure (maximum values of apparent viscosity and yield) and a high stability (maximum value of CS). The same level of importance was given to all the constraints and a linear desirability function was used (weight = 1). The calculated formulation had a very high desirability value (d = 0.990) and it was composed of 10 g/100 mL EW in W_1, 29% W_1 in W_1/O, and 60% W_1/O in $W_1/O/W_2$, thus reaching a content of oil and protein of 42.6 mL/100 mL and 3.5 g/100 mL, respectively. The optimized emulsion was produced and characterized in duplicate, giving results in good agreement with the predicted values (mean ± standard error): yield, 98.71±0.04% vs. 99.35± 0.30%; apparent viscosity, 70.4±4.7 vs. 63.1±0.1 mPa s; CS, 100±1% vs. 105±5% (the predicted value is higher than 100% just as a result of model extrapolation). The good results obtained confirmed

the validity of the developed multivariate models for the design of $W_1/O/W_2$ with given features.

Conclusions

WP and EW resulted to be good structuring biopolymers for double emulsions intended for food applications and possible encapsulation of bioactive compounds. In particular, despite a better oil interaction of WP assessed by FT-IR spectroscopy, the use of EW proved to be more effective in structuring $W_1/O/W_2$ emulsions, resulting in higher apparent viscosity and consistency coefficient values.

Highly significant multivariate models were computed for yield, rheological properties, and CS of $W_1/O/W_2$ emulsions as a function of protein type and concentration, W_1 and W_1/O volume percentages. W_1 and W_1/O were the most influent factors, allowing the tuning of the system characteristics. To the best of our knowledge, no papers in the literature deal with the study of $W_1/O/W_2$ properties in such a systematic and comprehensive approach, including all the factors considered in this research. Thus, the obtained multivariate models represent a useful starting point for the development of $W_1/O/W_2$ systems designed for targeted purposes, as demonstrated also by the good results obtained in the optimization example.

Declarations

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Availability of data and material: not applicable

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Authors' contributions. Maria E. Moriano: Methodology, Formal analysis, Investigation, Data curation, Writing-original draft preparation. **Cristina Alamprese:** Conceptualization, Methodology, Formal analysis, Writing-review and editing, Visualization, Supervision.

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Figure captions

Fig. 1 FT-IR spectra after interval reduction and baseline offset correction of: (a) corn oil (dotted grey line), dispersions of whey protein concentrate (WP) at 5 g/100 mL (black dashed line) and 10 g/100 mL (black line), NaCl solution at 0.4 g/100 mL (black dotted line), and WP-based double emulsions (grey lines); (b) corn oil (dotted grey line), dispersions of egg white powder (EW) at 5 g/100 mL (black dashed line) and 10 g/100 mL (black line), NaCl solution at 0.4 g/100 mL (black dotted line), and EW-based double emulsions (grey lines)

Fig. 2 FT-IR spectra in the 1,600-1,700 cm^{-1} range, after subtraction of the NaCl solution (0.4 g/100 mL) spectrum and second derivative transformation: (a) dispersions of whey protein concentrate (WP) at 5 g/100 mL (black dashed line) and 10 g/100 mL (black solid line), and WP-based double emulsions (grey lines); (b) dispersions of egg white powder (EW) at 5 g/100 mL (black dashed line) and 10 g/100 mL (black solid line), and EW-based double emulsions (grey lines, black dotted line, black dash-dot line). In Fig. 2b samples EW_00_20_60 (black dotted line) and EW_00_40_60 (black dash-dot line) are highlighted

Fig. 3 Results of Principal Component Analysis carried out on FT-IR reduced spectra of double emulsions after subtraction of the NaCl solution (0.4 g/100 mL) spectrum and second derivative transformation: (a) first (PC1) vs. second (PC2) principal component score plot of double emulsion samples containing whey proteins (squares) or egg white powder (circles); (b) PC1 (black line) and PC2 (grey line) loading plots

Fig. 4 Response surfaces of double emulsion characteristics: (a) yield (constant factors: egg white protein; 5 g/100 mL protein in inner water phase); (b) consistency coefficient K (constant factors: egg white powder; 5 g/100 mL protein powder in inner water phase); (c) apparent viscosity (constant factors: whey protein concentrate; 5 g/100 mL protein powder in inner water phase); (d) apparent viscosity (constant factors: egg white powder; 5 g/100 mL protein powder in inner water phase). W_1 (%), inner water phase volume percentage; W_1/O (%), primary emulsion volume percentage

Figure captions (color version for online only)

Fig. 1 FT-IR spectra after interval reduction and baseline offset correction of: (a) corn oil (yellow line), dispersions of whey protein concentrate (WP) at 5 g/100 mL (black dashed line) and 10 g/100 mL (black line), NaCl solution at 0.4 g/100 mL (black dotted line), and WP-based double emulsions (blue lines); (b) corn oil (yellow line), dispersions of egg white powder (EW) at 5 g/100 mL (black dashed line) and 10 g/100 mL (black line), NaCl solution at 0.4 g/100 mL (black dotted line), and EW-based double emulsions (red lines)

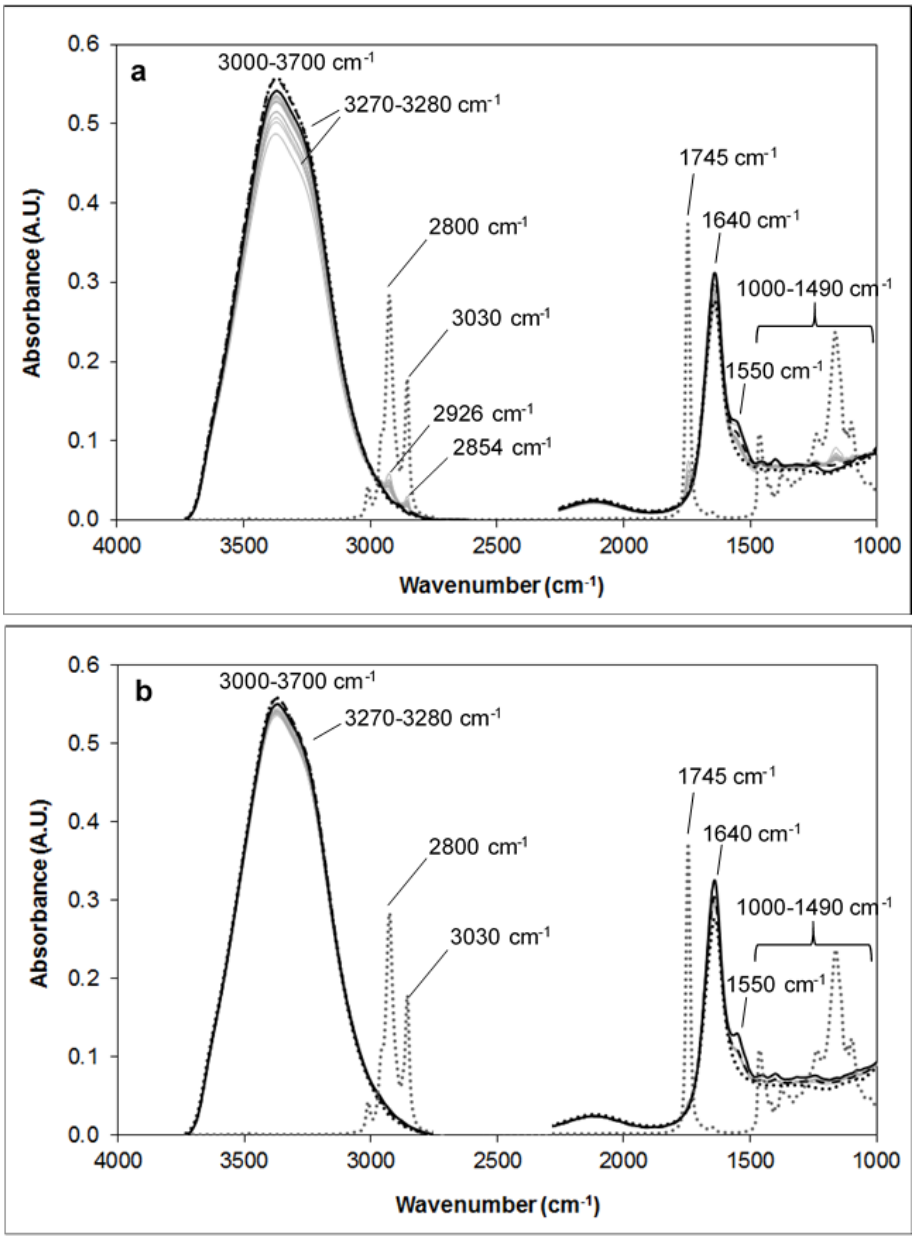
Fig. 2 FT-IR spectra in the 1,600-1,700 cm^{-1} range, after subtraction of the NaCl solution (0.4 g/100 mL) spectrum and second derivative transformation: (a) dispersions of whey protein concentrate (WP) at 5 g/100 mL (black dashed line) and 10 g/100 mL (black line), and WP-based double emulsions (blue lines); (b) dispersions of egg white powder (EW) at 5 g/100 mL (black dashed line) and 10 g/100 mL (black line), and EW-based double emulsions (red and green lines). In Fig. 2b samples EW_00_20_60 (green

dotted line) and EW_00_40_60 (green dashed line) are highlighted

Fig. 3 Results of Principal Component Analysis carried out on FT-IR reduced spectra of double emulsions after subtraction of the NaCl solution (0.4 g/100 mL) spectrum and second derivative transformation: (a) first (PC1) vs. second (PC2) principal component score plot of double emulsion samples containing whey proteins (blue squares) or egg white powder (red circles); (b) PC1 (black line) and PC2 (grey line) loading plots

Fig. 4 Response surfaces of double emulsion characteristics: (a) yield (constant factors: egg white protein; 5 g/100 mL protein in inner water phase); (b) consistency coefficient K (constant factors: egg white powder; 5 g/100 mL protein powder in inner water phase); (c) apparent viscosity (constant factors: whey protein concentrate; 5 g/100 mL protein powder in inner water phase); (d) apparent viscosity (constant factors: egg white powder; 5 g/100 mL protein powder in inner water phase). W_1 (%), inner water phase volume percentage; W_1/O (%), primary emulsion volume percentage

Fig. 1



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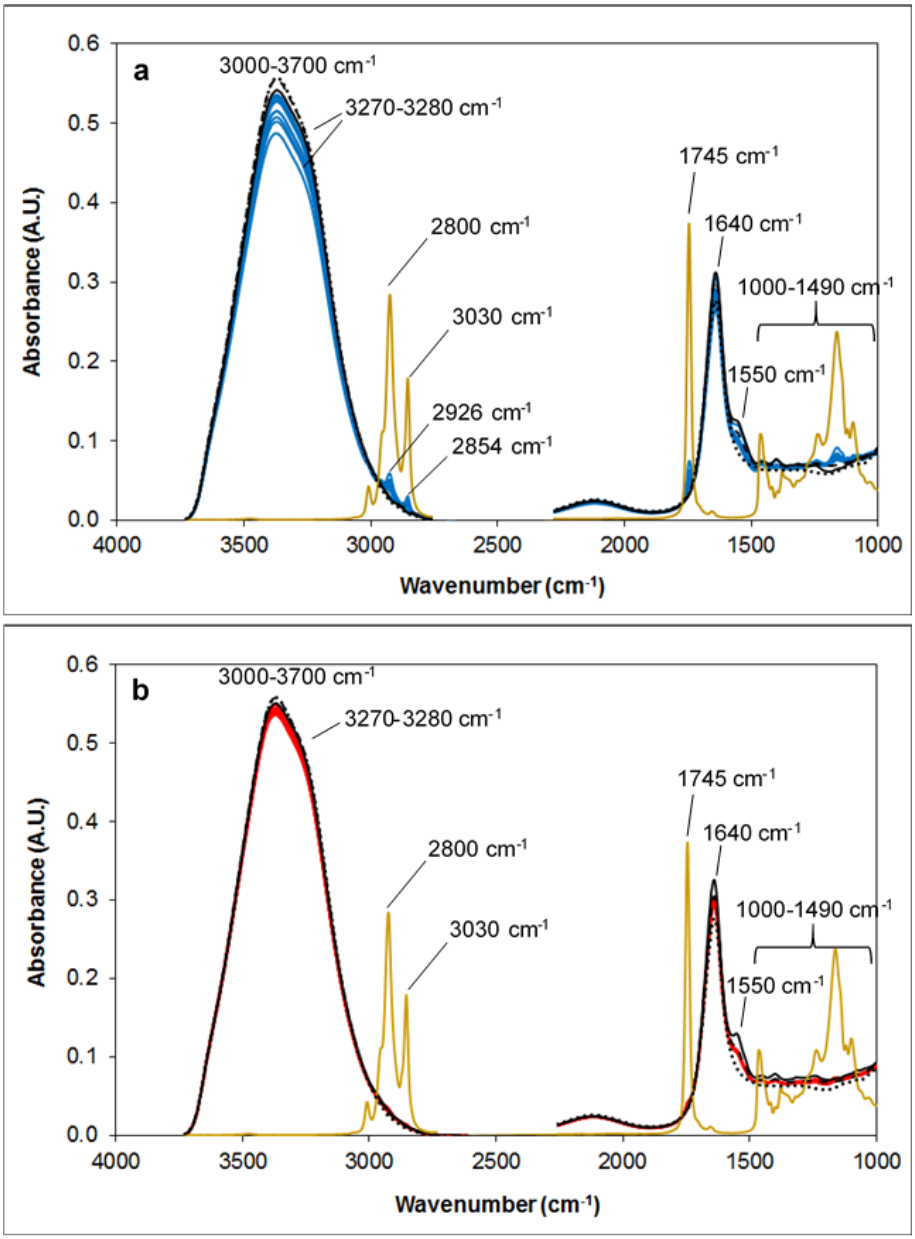
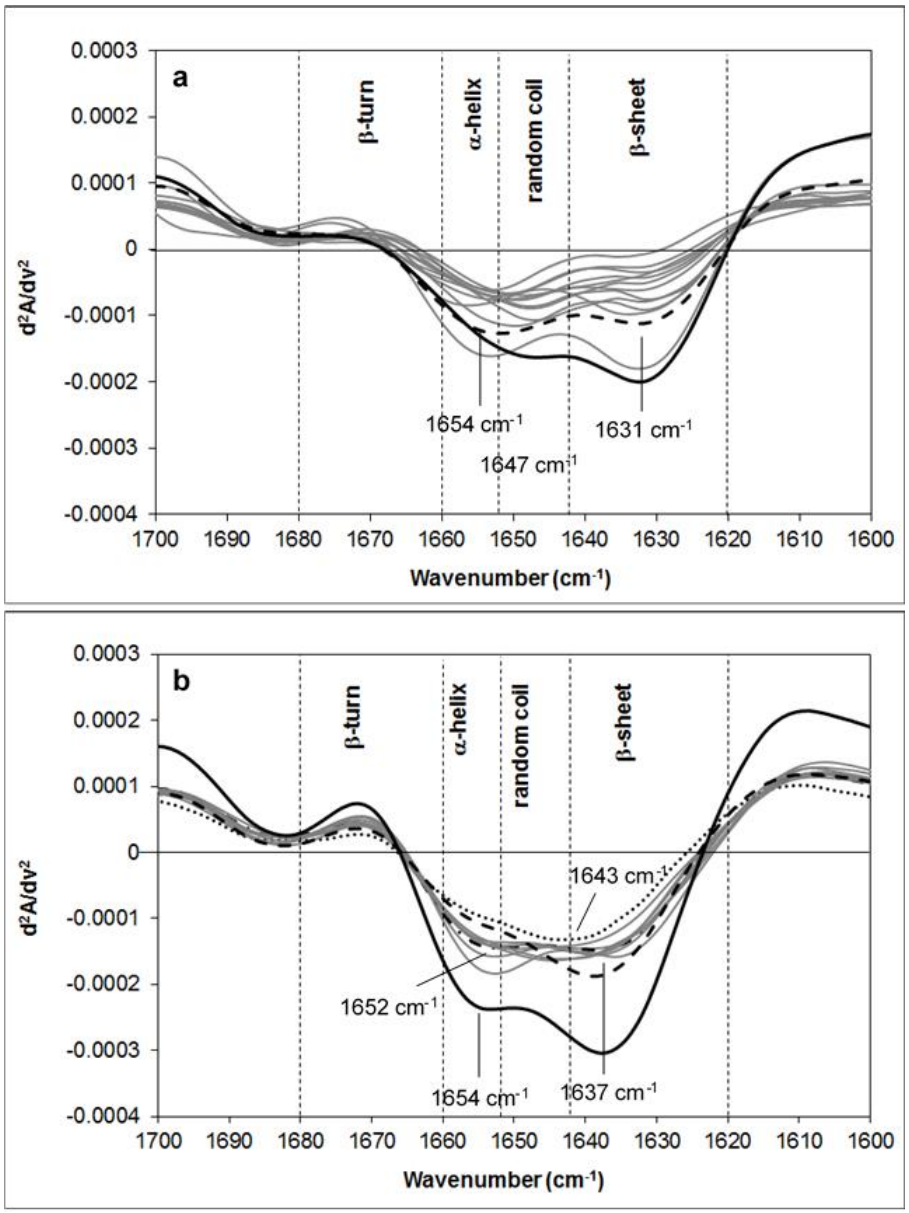


Fig. 2



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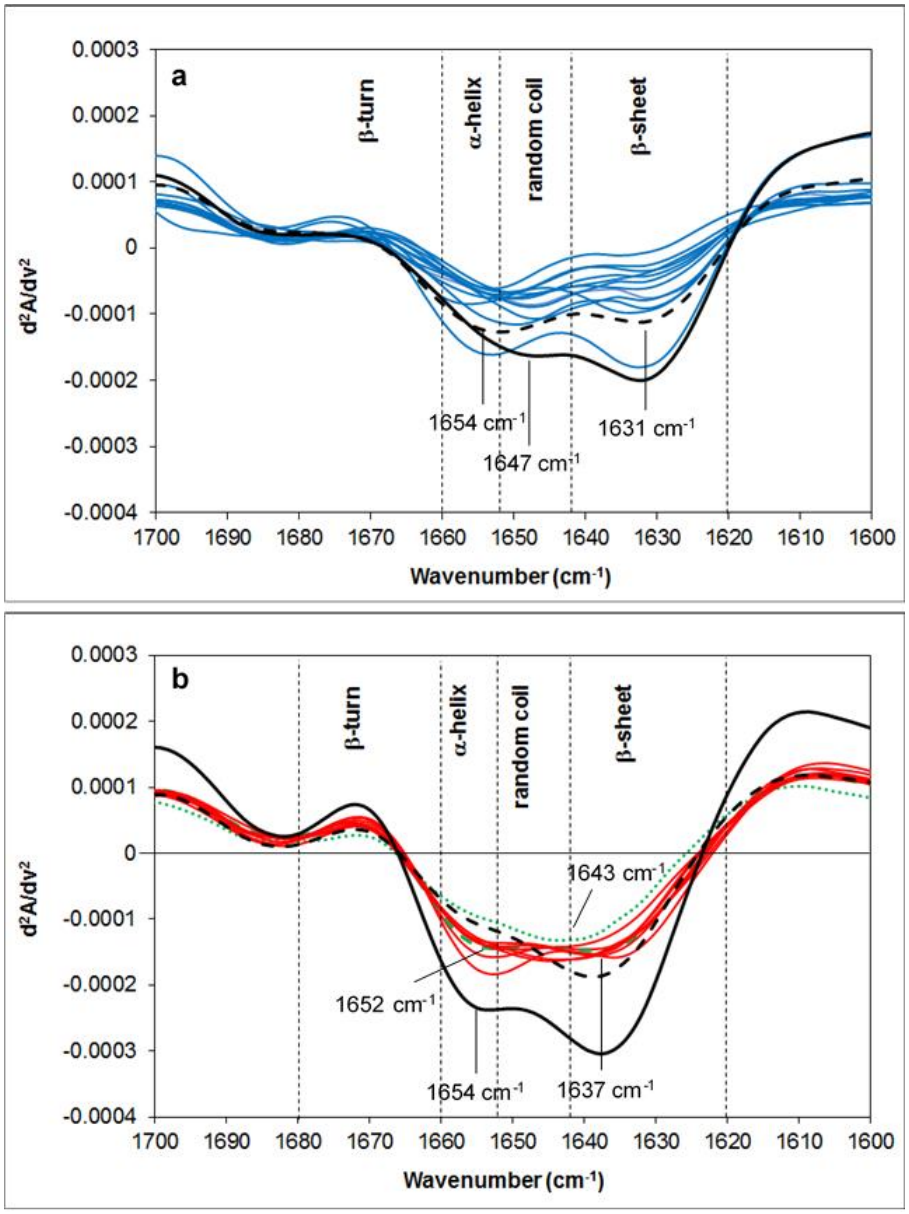
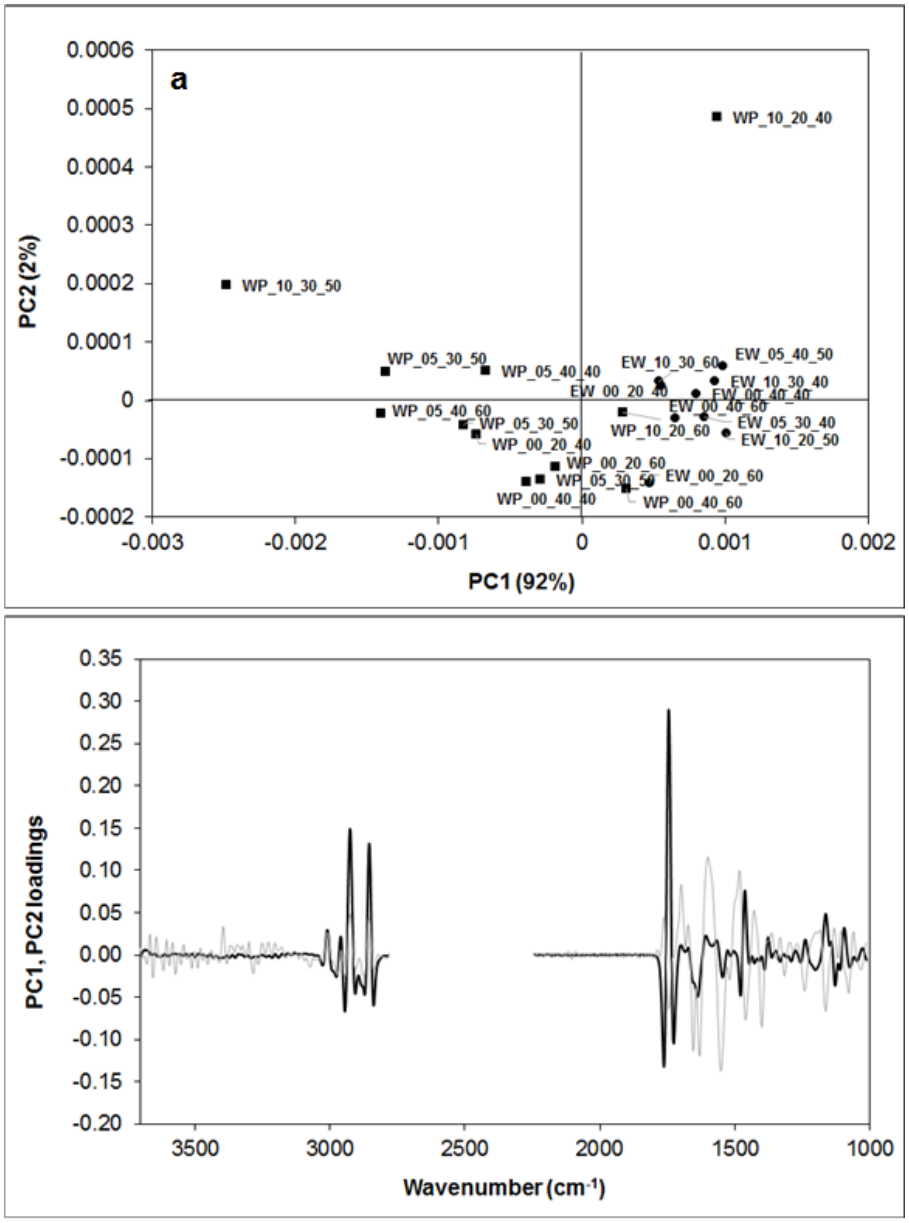


Fig. 3



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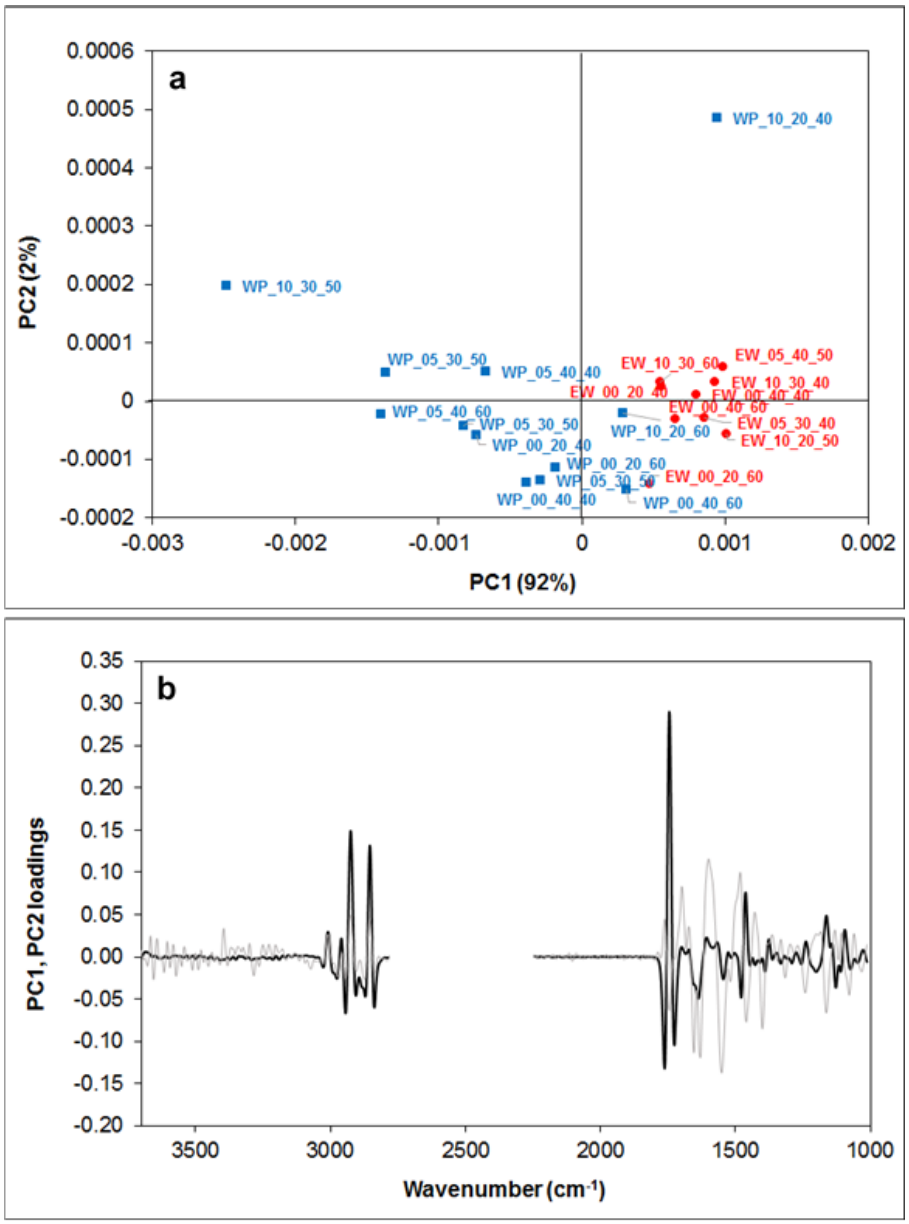
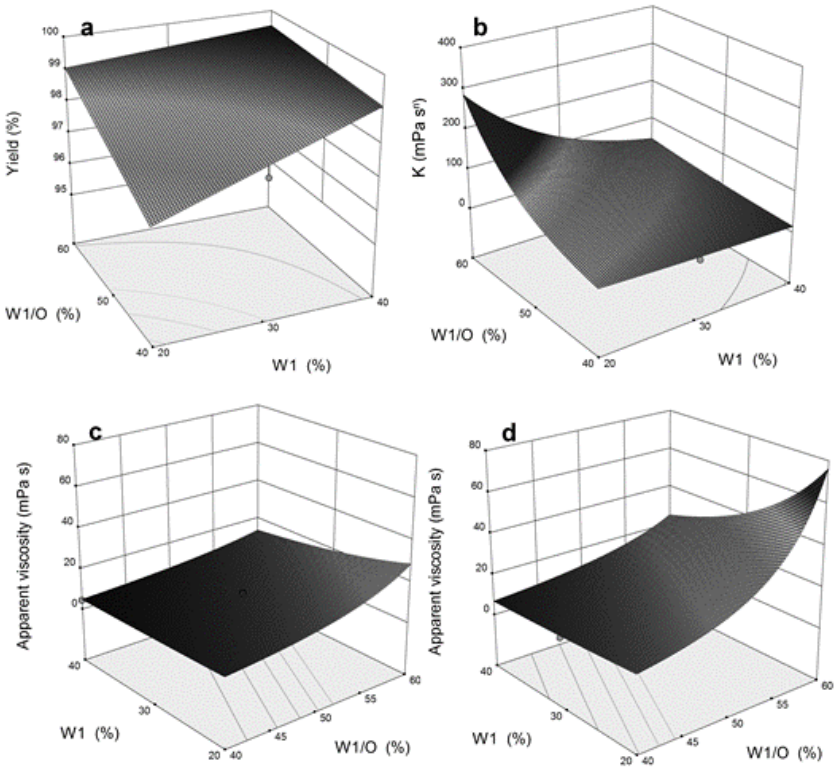


Fig. 4.



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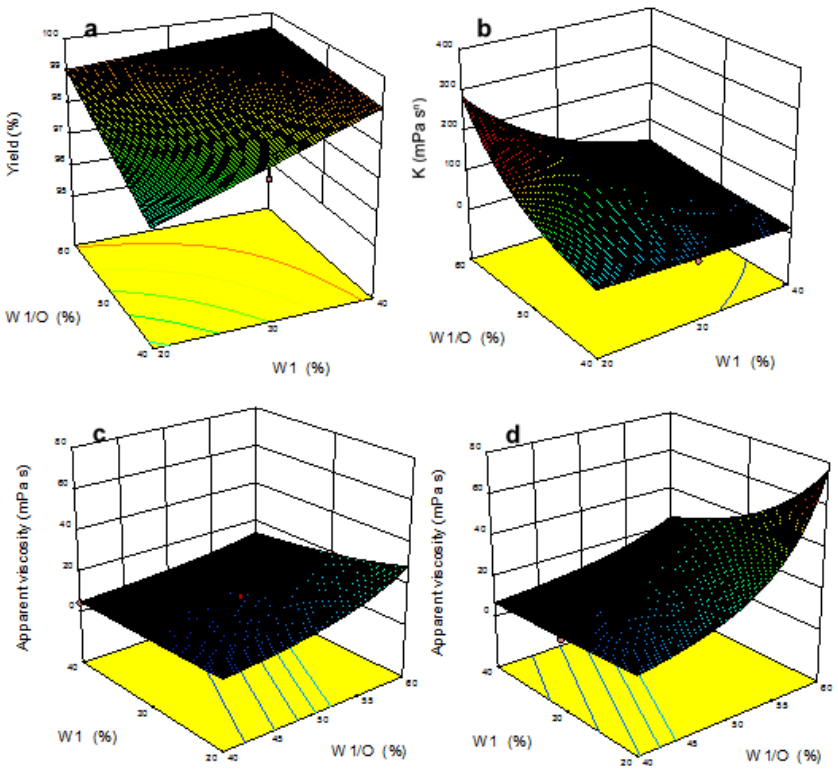

Table 1. D-optimal experimental design matrix developed to investigate the effects on the properties of double emulsions of four experimental factors: protein powder type (whey protein concentrate, WP; egg white powder, EW) and concentration in the internal water phase (W_1), volume percentage of W_1 in the primary emulsion (W_1/O), and volume percentage of W_1/O in the final double emulsion ($W_1/O/W_2$). Sample identification, factor levels, and theoretical composition of experimental emulsions in run order.

Sample	Experimental factors				Theoretical composition		
	Protein powder type	Protein powder concentration in W_1 (g/100 mL)	W_1 in W_1/O (%)	W_1/O in $W_1/O/W_2$ (%)	Water (mL/100 mL)	Proteins (g/100 mL)	Oil (mL/100 mL)
EW_00_40_40	EW	0	40	40	72.9	2.3	24
EW_10_30_40	EW	10	30	40	67.7	3.3	28
WP_05_40_40	WP	5	40	40	72.1	3.0	24
WP_10_20_60	WP	10	20	60	48.7	2.5	48
WP_00_20_60	WP	0	20	60	49.9	1.6	48
WP_00_40_60	WP	0	40	60	61.9	1.6	36
EW_05_40_50	EW	5	40	50	66.4	2.7	30
WP_05_30_50	WP	5	30	50	61.6	2.5	35
WP_05_30_50	WP	5	30	50	61.6	2.5	35
WP_10_20_40	WP	10	20	40	64.1	3.0	32
EW_00_20_40	EW	0	20	40	64.9	2.3	32
WP_10_30_50	WP	10	30	50	60.9	3.1	35
EW_10_30_60	EW	10	30	60	54.1	3.0	42
EW_00_40_60	EW	0	40	60	61.9	1.6	36
EW_10_20_50	EW	10	20	50	56.4	2.7	40
WP_05_30_50	WP	5	30	50	61.6	2.5	35
WP_05_40_60	WP	5	40	60	60.7	2.5	36
EW_05_30_40	EW	5	30	40	68.3	2.8	28
WP_00_20_40	WP	0	20	40	64.9	2.3	32
WP_00_40_40	WP	0	40	40	72.9	2.3	24
EW_00_20_60	EW	0	20	60	49.9	1.6	48

Table 2. Characteristics of double emulsions (mean ± standard deviation values[a]) formulated according to the D-Optimal experimental design described in Table 1.

Sample	Yield (%)	Apparent viscosity (mPa s)	K (mPa s^n)	n	CS (%)
EW_00_40_40	99.23 ± 0.03	6.8 ± 0.1	16.4 ± 1.1	0.847 ± 0.008	50 ± 1
EW_10_30_40	98.84 ± 0.06	13.7 ± 0.4	43.8 ± 5.1	0.809 ± 0.016	73 ± 1
WP_05_40_40	98.39 ± 0.13	4.3 ± 0.1	2.4 ± 0.1	1.105 ± 0.003	40 ± 1
WP_10_20_60	98.53 ± 0.05	32.7 ± 0.3	47.2 ± 0.4	0.935 ± 0.001	80 ± 1
WP_00_20_60	99.31 ± 0.06	28.7 ± 0.5	39.7 ± 1.8	0.943 ± 0.005	100 ± 1
WP_00_40_60	99.45 ± 0.06	13.0 ± 0.3	13.1 ± 0.5	0.999 ± 0.002	80 ± 1
EW_05_40_50	98.69 ± 0.05	9.9 ± 0.1	17.5 ± 0.3	0.900 ± 0.001	80 ± 1
WP_05_30_50	98.88 ± 0.05	9.6 ± 0.3	7.5 ± 0.2	1.045 ± 0.001	100 ± 1
WP_05_30_50	99.16 ± 0.18	11.1 ± 0.2	9.2 ± 0.2	1.032 ± 0.001	89 ± 1
WP_10_20_40	96.60 ± 0.11	9.1 ± 0.1	7.0 ± 0.2	1.046 ± 0.001	75 ± 1
EW_00_20_40	97.23 ± 0.04	8.6 ± 0.3	19.6 ± 2.7	0.857 ± 0.018	60 ± 1
WP_10_30_50	98.74 ± 0.10	11.0 ± 0.4	8.3 ± 0.4	1.050 ± 0.002	89± 1
EW_10_30_60	99.30 ± 0.02	63.1 ± 3.7	3089 ± 440	0.357 ± 0.061	100 ± 1
EW_00_40_60	99.45 ± 0.02	18.1 ± 0.3	85.4 ± 0.8	0.731 ± 0.001	90 ± 1
EW_10_20_50	95.47 ± 0.08	22.2 ± 0.9	142 ± 13	0.677 ± 0.008	90 ± 1
WP_05_30_50	98.79 ± 0.03	11.4 ± 1.2	8.6 ± 0.6	1.039 ± 0.005	90 ± 1
WP_05_40_60	99.21 ± 0.02	9.7 ± 0.2	7.4 ± 0.2	1.049 ± 0.001	100 ± 1
EW_05_30_40	97.62 ± 0.02	7.5 ± 0.4	13.0 ± 2.5	0.905 ± 0.025	63 ± 4
WP_00_20_40	95.83 ± 0.23	5.2 ± 0.3	3.3 ± 0.2	1.082 ± 0.002	88 ± 1
WP_00_40_40	99.01 ± 0.06	3.8 ± 0.1	2.5 ± 0.1	1.103 ± 0.001	47 ± 1
EW_00_20_60	98.61 ± 0.05	43.1 ± 0.2	545 ± 2	0.559 ± 0.001	100 ± 1

[a] Number of replicates (n): yield, n=4; apparent viscosity, K, n, and CS, n=3.
K, coefficient of consistency; n, flow behavior index; CS, creaming stability.

Table 3. Multivariate model coefficient values (in terms of coded factors) and results of one-way analysis of variance for the D-optimal design developed to study double emulsions structured by whey protein concentrate and egg white powder.

Response variable	β_0	β_1	β_2	β_3	β_4	β_{23}	β_{24}	β_{34}	R^2	Adj. R^2	Pred. R^2	Adequate precision	LOF
Yield (%)	9.86E+01	6.94E-02^{ns}	7.87E-02^{ns}	6.79E-01***	6.51E-01***			-4.42E-01*	0.791***	0.723	0.561	9.873	0.114^{ns}
1/√Apparent viscosity (1/√mPa s)	2.89E-01	-3.43E-02***	-2.62E-02**	4.09E-02***	-9.04E-02***				0.934***	0.920	0.882	24.97	0.201^{ns}
Log K [Log(mPa s^n)]	1.29E+01	3.92E-01***	5.14E-04^{ns}	-2.86E-01***	3.60E-01***	-1.32E-01*	-1.08E-01*	-1.11E-01*	0.967***	0.946	0.871	23.55	0.127^{ns}
n	9.11E-01	-1.24E-01***	-7.20E-003^{ns}	3.36E-02*	-5.92E-02**				0.915***	0.891	0.836	18.24	0.010**
CS (%)	8.31E+01	-2.04E+00^{ns}	4.92E+00^{ns}	-9.29E+00**	18.1E+00***				0.806***	0.751	0.652	12.46	0.298^{ns}

β_0, model intercept; β_1, β_2, β_3, β_4, linear coefficients for protein powder type, protein powder concentration, volume percentage of inner water phase, and volume percentage of primary emulsion, respectively; β_{23}, β_{24}, β_{34}, interaction coefficients. R^2, coefficient of determination; Adj. R^2, adjusted R^2; Pred. R^2, predicted R^2; LOF, lack of fit (p-value). Significance levels: $^{n.s.}$ not significant; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$

Graphical abstract