Whey Protein-Pectin Conjugate by Wet-Dry Heating: Optimization using Response Surface Methodology with Box-Behnken Design

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Submitted: December 17, 2021; Revised: September 5, 2022, October 29, 2022; Accepted: October 31, 2022; Published: November 30, 2023

ABSTRACT

The recent progress in glycation of proteins utilizing saccharides through the Maillard reaction has garnered substantial attention, with a specific emphasis on Whey Protein Concentrate (WPC). Conjugation mode is frequently intricate and poses challenges when scaling up for large-scale production. Consequently, this investigation sought to optimize the conditions of the WPC-pectin conjugation process using Response Surface Methodology (RSM) in conjunction with Box-Behnken design (BBD). The experimentation was executed employing a cabinet dryer, incorporating both wet and dry heating procedures to yield a WPC-pectin conjugate exhibiting favorable functional properties. The independent variables investigated encompassed pectin concentration (ranging from 0 to 1%), pH (ranging from 6 to 8), and drying time (ranging from 2 to 6 hours), The measured responses encompassed the emulsion stability index (ESI), emulsifying activity index (EAI), and solubility. Analyzing the experimental data underwent scrutiny for model sufficiency through diagnostic plots, and a second-order polynomial equation was fitted through multi-response regression analysis, resulting in a high coefficient of determination (R^2) value. The most effective parameters were identified as a pectin concentration of 0.49%, pH 6.7, and a drying duration of 4.12 hours, yielding a peak ESI of 452.267 minutes, EAI measuring 49.95 m^2 g^{-1}, and solubility reaching 48.09%. Further experiments were conducted to validate these outcomes, and the presence of the Maillard reaction was confirmed using Fourier Transform Infrared Spectrum (FTIR). The et-dry method demonstrated efficacy in producing WPC-pectin conjugates with commendable functional properties.

Keywords: Box-Behnken design; emulsion properties; response surface methodology; wet-dry heating method; whey protein-pectin conjugate

INTRODUCTION

Glycating protein with reducing saccharides through Maillard reaction or conjugate protein-saccharide is a recent development to improve the functional properties of food protein (Liu & Zhong, 2013). Protein is widely used as an emulsifier, particularly in oil-in-water food emulsions (A'yun et al., 2020). Generally, protein conjugate with reducing sugars is carried out using wet and dry heating methods. The reaction process in both methods is time-consuming, leading to microbial spoilage (Liu & Zhong, 2013), as wet approach involves heating a mixed protein solution with saccharides, while the dry heating method is carried out by heating the protein dispersion with saccharides Previous study on conjugate protein-saccharide has been done on a small or laboratory scale, making it challenging to scale up for large production. The conjugate preparation begins with dissolving the material, mixing, freezing, and lyophilization, thereby impacting product price and

DOI: http://doi.org/10.22146/agritech.71301
ISSN 0216-0455 (Print), ISSN 2527-3825 (Online)

potentially undesirable reactions such as protein fibrils formation (A'yun et al., 2020).

Whey protein is a widely used source due to its essential amino acids and versatile functional properties (Liu & Zhong, 2013). There are two common commercial types of whey, namely Whey Protein Isolate (WPI) and Whey Protein Concentrate (WPC), which are more economical (A'yun et al., 2020). Moreover, the conjugate process of whey protein with saccharides is usually influenced by saccharides concentration, pH, and heating time. A previous investigation reported that the concentration of gum Arabic affected the conjugate process with whey protein, reaching saturation at higher concentrations (Chen et al., 2019). The initial pH also affects WPC-pectin conjugate, with more alkaline conditions leading to faster Maillard reaction and polymerization (A'yun et al., 2020). The duration of heating time significantly affects the formation of products from Maillard reaction. In a previous study, conjugate of WPI with gum Arabic was carried out for seven days and the results showed that the conjugate increased the emulsification properties followed by decreasing with the longer heating time (W. Chen, Lv, et al., 2019). Based on these investigations, it can be concluded that pH, heating time, and saccharide addition have a significant effect on WPC-pectin conjugate process. Therefore, these parameters were used for WPC-pectin conjugate, where pectin was selected as saccharide due to its good hydrocolloid properties, containing neutral sugars (rhamnose, galactose, and arabinose), with emulsifying properties related to various proteins and functional groups attached to galacturonic acid (Wefers et al., 2018).

The innovation of protein-saccharide conjugate aims to achieve a simple process on a large scale. In this study, WPC-pectin conjugate process used a cabinet dryer, involving wet and dry heating, respectively This dryer was selected because the temperature can cause a Maillard reaction, with a drying process initiating a reaction in dry heating (Candraningrum et al., 2022). Furthermore, the cabinet dryer was selected due to its simple structure, low-cost installation, and adaptability to almost all environmental conditions (Amanlou & Zomorodian, 2010).

The formation of the WPC-pectin conjugate involved a sequential process of wet heating followed by dry heating to identify the optimal procedural conditions and achieve optimal functional attributes, particularly in terms of emulsification. The determination of the optimal WPC-pectin conjugation process was facilitated through the utilization of Box-Behnken design (BBD) in conjunction with Response Surface Methodology (RSM). This modeling approach was instrumental in comprehensively assessing the effects of diverse factors that govern the responses (Song et al., 2021).

Building upon the aforementioned explanation, the primary objective of this study was to formulate and refine the conjugation process of WPC by employing a sequence of wet heating followed by dry heating, incorporating pectin, pH, and drying time as the influential factors. The investigation further delved into understanding the impact of these factors on the progression of the Maillard reaction, aiming to ascertain the optimal conditions for the WPC-pectin conjugation process and achieve the highest level of emulsifying properties.

METHODS

Materials

WPC (Wheyco GmBH®) was donated from PT. Greenfields Indonesia Tbk. (Malang, Indonesia), containing 81.33% protein, 6.94% lactose, 6.50% fat, and 4.14% ash, according to specifications. Low Methoxyl Pectin (LMP) (CP Kelco, Grossenbrode, Germany) was extracted from citrus peel. Subsequently, commercial palm oil, acquired from a local supermarket in Yogyakarta, Indonesia, was employed to formulate the O/W emulsions.

The main equipment used included homogenizer (T50 Digital ULTRA-TURRAX®), cabinet dryer (Sinton Electric Co., Ltd., China), spectrophotometer UV-Visible (Genesys 10S UV-VIS), and Fourier Transform Infrared spectrophotometer (Nicolet iS5, Thermo Electron Corp., Madison, WI, USA).

Effect of Pectin Concentration on Protein-Pectin Conjugate Properties

WPC and LMP were individually dissolved in distilled water at a concentration of 25% (w/w) and LMP at 2% (w/w), and refrigerated overnight at (4 °C) before mixing. Subsequently, five additions of LMP were added to the solutions to obtain concentrations of 0%, 0.25%, 0.5%, 0.75%, and 1%. The pH of the solutions has been adjusted to 8 with 0.1 N NaOH, solutions underwent heating and drying in a cabinet dryer at 70 °C for 2 hours, followed by placing the conjugate in the freezer.

Effect of pH on Product Absorbance and Emulsion Properties

The stock solutions were mixed to obtain an LM Pectin concentration of 0.5%. The pH of the solutions has been adjusted to 6, 7, 8, and 9 with 0.1 N HCl and 0.1 N NaOH. Subsequently, solutions underwent heating and drying in a cabinet dryer at 70 °C for 2 hours and the conjugate was placed in the freezer.

Effect of Drying Time on Product Absorbance and Emulsion Properties

The stock solutions were mixed to obtain LMP concentration of 0.5% and the pH of the solutions was adjusted to 8 with 0.1 N NaOH. The solutions were heated and dried in a cabinet dryer for 2, 3, 4, 5, and 6 hours, and the conjugate was placed in the freezer.

Color Determination

Color determination was used to measure the brown hue and the development of intermediary products resulting from the Maillard reaction. A solution of WPC-pectin conjugate at a concentration of 2 mg mL^{-1} was prepared for the analysis, and a UV-VIS spectrophotometer (Setiowati et al., 2019 with modification) at 420 nm for the brown color and 294 nm for intermediate products, respectively.

Emulsifying Properties (Emulsion Stability Index and Emulsion Activity Index)

The turbidimetric method with modifications was employed to assess the Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI) of the samples (Wang et al., 2020). The specimen were dissolved in a phosphate buffer solution (10 mm, pH 7.0) to achieve a final conjugate concentration of 1%. Following emulsion formation, a blend of palm oil and conjugate solution (1:9, w/w) underwent homogenization at 10,000 rpm for a duration of 3 minutes. Subsequently, at 0 and 10 minutes post-homogenization, 50 µl of the emulsion was promptly extracted from the bottom of the beaker and diluted at a ratio of 1:200 in a 0.1% SDS solution. The absorbance of the thinned emulsion was subsequently gauged at 500 nm. In this investigation, the value for EAI and ESI were calculated using Equations 1 and 2, respectively.

$$EAI\ (\mathrm{m^2/g}) = \frac{(2\ \times 2.303 \times A_0 \times N)}{[\rho \times \vartheta \times (1-\emptyset)] \times 10000} \quad (1)$$

$$EAI\ (min) = \frac{\mathrm{A0}}{\mathrm{A0} - \mathrm{A10}} x10 \quad (2)$$

Where DF denotes the dilution factor (200), C represent the protein concentration (g ml^{-1}), φ stands for the optical path (1 cm), Θ is the oil volume fraction (0.25), as well as A_0 and A_{10} refer to the absorbances of the emulsion at 0 min and 10 min.

Solubility Measurement

WPC-pectin conjugate was dissolved in distilled water to obtain a protein concentration of 2 mg mL^{-1}. The conjugate solubility was measured by a modified Bradfrod method (Chen, Ma, et al., 2019) with Bovine Serum Albumin (BSA) (0-1.5mg mL^{-1}) as the standard. The solubility was defined as the percentage of the total protein concentration.

Optimization of WPC-Pectin Conjugate by RSM and Statistical Analysis

The optimization of the WPC-pectin conjugate proceeded through two distinct stages. Initially, a single-factor experiment was conducted to ascertain the range of factors—namely, pectin concentration, pH, and drying time—utilizing Emulsion Stability Index (ESI) and Emulsifying Activity Index (EAI) as the parameters. The designated ranges for each factor were 0–1% for pectin concentration, 6–9 for pH, and 2–6 hours for drying time. The statistical analyses, including Analysis of Variance (ANOVA) and regression analysis, were performed for this initial stage using SPSS (Version 22.0).

In the subsequent stage, Response Surface Methodology (RSM), specifically employing a three-level, three-factor Box-Behnken Design (BBD), was employed for the probationary design and optimization of the WPC-pectin conjugation process. This was executed using Design Expert® (Version 11, State-Ease Inc., Minneapolis, USA). In alignment with the findings from the single-factor experiments, the influence of the three primary variables on WPC-pectin conjugation was observed at three levels (-1, 0, +1). Table 1 illustrates the design matrix, both in coded and actual values, for each independent variable at the three levels (+1, 0, -1), along with the observed responses obtained from the WPC-pectin conjugate experiments conducted using the BBD.

The Box-Behnken Design (BBD) was employed for process optimization with a minimized number of runs, totaling 17 runs and encompassing five replicated center points. This approach aimed to explore the interactions among the three factors and their associated responses by fitting the data using the quadratic Equation 3.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (3)$$

In this context Y represents for the response, b_0 signifies the cut off, and b_1 to b_{23} represent the regression coefficient derived from the observations of individual response observations. Meanwhile, X_1 to X_3 symbolize the coded levels corresponding to the factors (where X_1 refers to pectin concentration, X_2 stands for pH, and X_3 stands for drying time).

Fourier Transform Infrared Spectrum

Fourier-Transform Infrared spectroscopy (FTIR) was conducted using the potassium bromide (KBr) pellet

Table 1. BBD with experiment values for ESI, EAI, and solubility

Run	X_1 (%)	X_2	X_3 (hours)	Y_1 (ESI (min))	Y_2 (EAI (m^2/g)	Y_3 Solubility (%)
1	0.50 (0)	8 (+1)	5 (+1)	352.92	39.01	36.80
2	0.75 (+1)	7 (0)	3 (-1)	372.84	40.95	37.55
3	0.50 (0)	7 (0)	4 (0)	452.67	52.69	49.99
4	0.25 (-1)	6 (-1)	4 (0)	412.62	44.28	42.82
5	0.25 (-1)	7 (0)	5 (+1)	403.25	43.82	41.96
6	0.25 (-1)	7 (0)	3 (-1)	369.52	40.24	37.30
7	0.50 (0)	8 (+1)	3 (-1)	347.71	41.05	36.55
8	0.50 (0)	7 (0)	4 (0)	446.68	50.02	48.93
9	0.50 (0)	6 (-1)	5 (+1)	407.35	43.60	42.64
10	0.50 (0)	7 (0)	4 (0)	447.34	51.00	49.11
11	0.25 (-1)	8 (+1)	4 (0)	381.32	41.96	38.61
12	0.50 (0)	7 (0)	4 (0)	442.34	49.99	48.19
13	0.75 (+1)	6 (-1)	4 (0)	418.51	44.92	42.99
14	0.50 (0)	6 (-1)	3 (-1)	395.14	42.64	39.79
15	0.75 (+1)	7 (0)	5 (+1)	389.33	41.78	41.60
16	0.75 (+1)	8 (+1)	4 (0)	343.03	38.71	35.43
17	0.50 (0)	7 (0)	4 (0)	443.24	49.52	48.07

method with a Nicolet iS5 Fourier-Transform Infrared spectrophotometer (Thermo Electron Corp., Madison, WI, USA). Samples weighing 2 mg were combined with 0.5 g KBr, forming KBr pellets for subsequent measurements. The FTIR analysis was performed in transmission mode within the frequency range of 4000-400 cm^{-1}, with a resolution of 4 cm^{-1}.

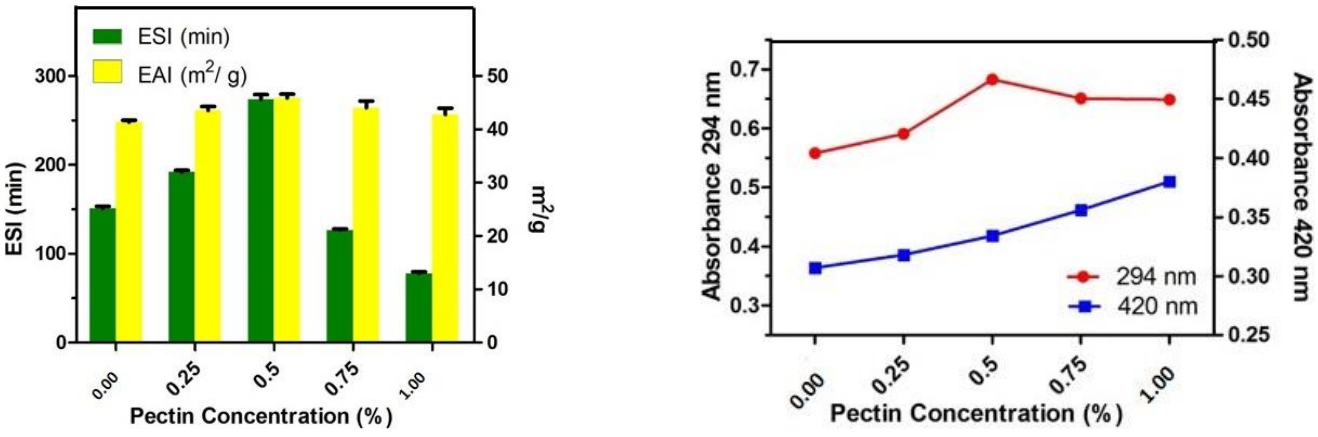
RESULTS AND DISCUSSION

Single Factor Experiment

The experiment aimed to track the progression of the Maillard reaction, which encompassed early, intermediate, and final stages. Intermediate stage products exhibited a yellow hue, while the final stage products displayed a brown coloration (Nooshkam & Varidi, 2020). The formation of this brown color was quantified via absorbance measurements of the aqueous sample at 420 nm. Additionally, absorbance at 294 nm was utilized to quantify the intermediate products in an aqueous sample. Given the observed inferior functional properties at the final stage (Shang et al., 2020), the products resulting from the Maillard reaction were correlated with the assessment of emulsifying properties.

Effect of Pectin Concentration on Maillard Reaction and the Emulsion Properties

The increase in pectin concentration from 0% to 1% significantly promoted WPC-pectin conjugate, causing a gradual decrease in ESI and EAI, as shown in Figure 1B. The highest values of ESI and EAI were reached when conjugate was carried out with 0.5% pectin. As presented in 1A, the values of ESI decreased sharply and EAI slightly declined with 0.75-1.00% pectin. This result aligned with a previous study (Dong et al., 2020) that showed a slight decline in EAI and a sharp reduction in ESI due to an increase in the forming of advanced Maillard products at higher pectin concentrations. This phenomenon was also linked to the formation of polymerization products from pectin, resulting in reduced molecular mobility and a subsequently slower absorption of the conjugate at the oil/water interface (Dong et al., 2020). ESI decreased due to the saturation of glycating reaction as the concentration of whey protein reduced with 0.75%-1.00% pectin. When the reaction was saturated, the free amino acids were not available to assess with the carbonyl group of pectin. However, increasing carbonyl groups was



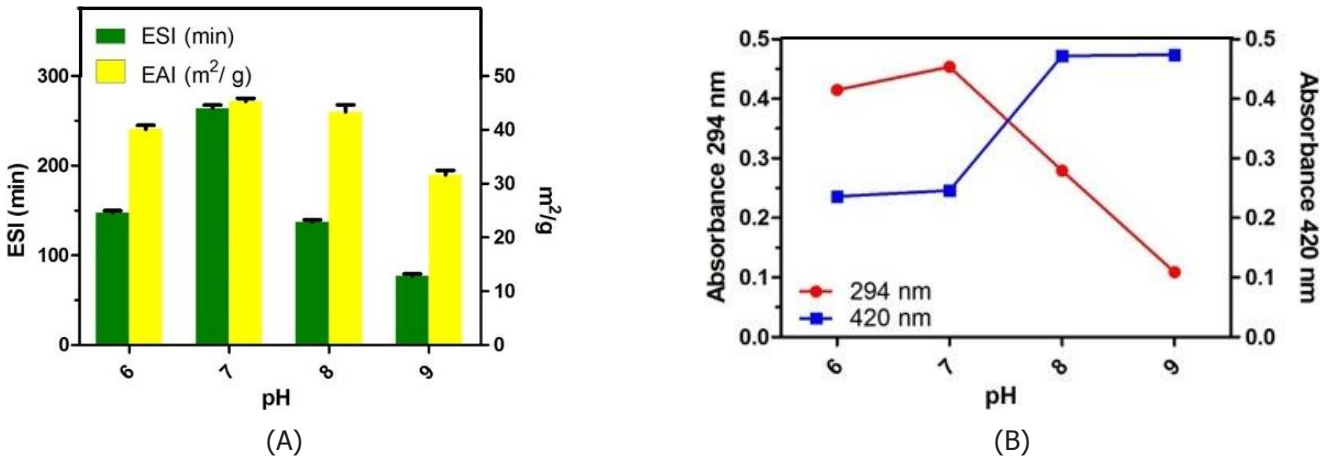
(A) (B)

Figure 1. The effect of pectin addition on emulsifying properties (A) and the absorbance of WPC-pectin conjugate at 294 nm and 420 nm (B) at drying temperature 70 °C for 2 hours, pH 8, and various pectin concentrations (p<0.05)

more accessible to the free amino acid groups and the glycosylation saturation increased with higher polysaccharides concentration (Chen, Ma, et al., 2019). The addition of 0% pectin caused conjugate or the formation of Maillard reaction due to the presence of lactose, a simple sugar in the whey protein, enabling the reaction of whey amino acids with the lactose carbonyl group (A'yun et al., 2020). At the same pH and drying time, the absorbance at 420 nm increased at pectin addition from 0% to 1% (Figure 1B). The absorbance at 294 nm increased at pectin addition from 0% to 0.5% and decreased by further addition. The increasing browning intensity aligned with the previous study, where more polysaccharides in conjugate resulted in a higher initial rate of brown color formation during incubation (Setiowati et al., 2016). This indicated that the development of Maillard reaction rate at 0%-0.5% pectin addition was lower than 0.75% and 1.00% pectin addition. This caused the alteration of the Amadori compound to melanoidin to be faster at 0.75%-1% compared to the 0-0.5% addition of pectin. When the Amadori compound changed to melanoidin, the absorbance at 294 nm decreased due to the reduction in the content of the compound. Therefore, 0.25%-0.75% was selected as the suitable pectin concentration range for further optimization.

Effect of pH on Maillard Reaction and the Emulsion Properties

The initial pH for the conjugate process affected the development of Maillard reaction, which influenced



(A) (B)

Figure 2. Absorbance of WPC-pectin conjugate at 294 nm and 420 nm (A) and the emulsifying properties (B) at 0.5% pectin, drying temperature of 70 °C for 2 hours, and various initial pH (p < 0.05)

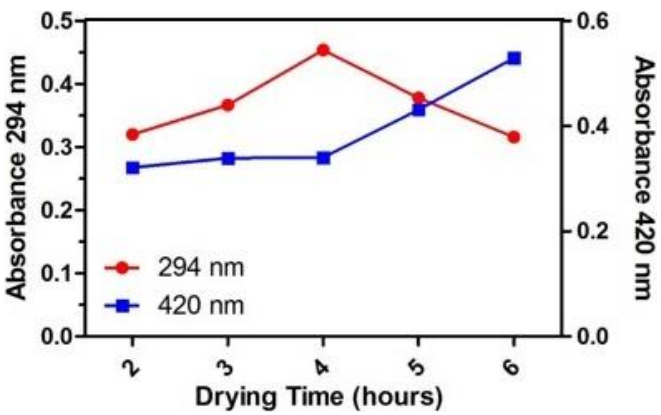
emulsifying properties of the conjugate. Under the alkaline condition, Schiff-base formed quickly and promoted the further Maillard reaction (Shang et al., 2020). More alkaline initial pH of conjugate also caused an increase in Maillard reaction rate (A'yun et al., 2020). However, further increasing the preconditioning pH to 10 led to the transformation of some intermediate products into brown polymers. The insoluble complexes of brown polymers were formed due to the advanced stages of Maillard reaction (A'yun et al., 2020; Shang et al., 2020). As shown in Figure 1A, both ESI and EAI of WPC-pectin conjugate were improved up to pH 7 due to the development of the glycation reaction and decreased with a further increase in pH. This might be due to higher pH causing acceleration of Maillard reaction and the insoluble complex was formed by the rapidly advanced stage (A'yun et al., 2020). Therefore, at the same drying time and pectin content, Maillard reaction rate at pH 8 and 9 was higher than pH 6 and 7, resulting in faster transformation of the Amadori compound, as shown in Figure 2B. This transformation caused a decrease in the absorbance at 294 nm due to the reduction in Amadori compound content after being converted into advanced-stage products. The absorbance at 420 nm also significantly increased at pH 8 and 9, indicating a rise in advanced products. This caused ESI and EAI as emulsifying properties of WPC-pectin conjugate to decrease due to the high content of advanced products.

At pH 6 and 7, the early-stage products effectively adsorbed at the oil and water interface, leading to increased emulsification properties (Wang et al., 2020). Therefore, the initial pH in the range of 6-8 was selected for further optimization.

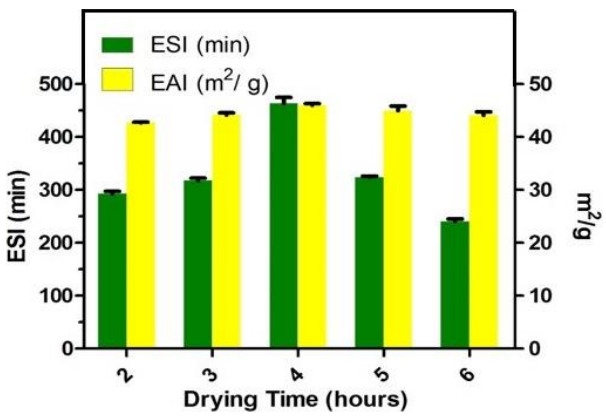
Effect of Drying Time on Maillard Reaction and the Emulsion Properties

The optimal duration of drying plays a crucial role in the wet-dry heating process of WPC-pectin conjugate. As depicted in Figure 3A, there is a discernible surge in absorbance at 294 nm after 4 hours of drying, followed by a gradual decline with extended drying up to 6 hours. A distinctive trend is evident in the development of brown color during the Maillard reaction, as illustrated in Figure 3A. This deviation is attributed to the likelihood that most intermediate products undergo polymerization, resulting in the formation of brown pigments, thereby yielding only a minimal quantity of intermediate substances (Pirestani et al., 2017). As presented in Figure 3B, ESI and EAI gradually increased with a rise in drying time from 2 hours to 4 hours. However, a significant decrease was observed with a further increase in drying time due to the advanced Maillard reaction stage occurring in 5 to 6 hours, as shown in the browning intensity (420 nm) in Figure 3A.

The progression into the Maillard reaction stages, particularly with an extended drying time, leads to polymerization. The resultant products diminish the molecular mobility and reduce the conjugate's capacity to adsorb at the oil and water interface. This effect is manifested by an escalation in insolubility and surface hydrophilicity of the conjugate with increasing drying time (Chen, Lv, et al., 2019; Dong et al., 2020). The elevation in IAE and ISE values can be attributed to the proteins' capability to expose hydrophobic groups and lysyl residues, subsequently reacting with the reducing-end carbonyl groups (Dong et al., 2020). Therefore, 3-5 hours were selected for further optimization using the BBD.



(A)



(B)

Figure 3. Absorbance of WPC-pectin conjugate at 294 nm and 420 nm (A) and the emulsifying properties (B) at 0.5% pectin, pH 7, and drying temperature of 70 °C for various times (p<0.05)

Optimization of WPC-Pectin Conjugate Process with RSM

Data Analysis and Formulation Optimization

RSM represents an empirical modeling technique utilized for the estimation of actual outcomes. Employing a three-factor, three-level Box-Behnken Design (BBD), this study engaged in experimental design and process optimization. The configuration of the BBD was specifically adopted to derive a suitable model for optimizing the WPC-pectin conjugation process. The experimental design aimed to delineate the influences of three key variables—namely, pectin concentration, pH, and drying time—on the emulsifying properties (ESI and EAI) and solubility of the WPC-pectin conjugate, serving as the responses. The most fitting model terms discovered for all three factors were quadratic polynomial models, signifying a direct impact of independent variables on the dependent responses. A comparative assessment of all observed responses was conducted to identify the optimal experimental parameter. ANOVA results displayed highly significant p-values for the model (p<0.0001). and individual factors, signifying substantial influence on each response. Notably, the lack of fit in this model was deemed non-significant, indicated by a relatively high p-value (p>0.05 suggesting the credibility and accuracy of the employed quadratic model. The final regression equations generated by the applied quadratic model for the individual responsesI (*Y1*), EAI (*Y2*), and solubility (*Y3*) produced by RSM using DesignExpert® software were shown in Equation 4 to 6.

$$Y_1 = 446.45 - 5.37 \times X_1 - 26.08 \times X_2 + 8.46 \times X_3 - 11.05 \times X_1X_2 - 4.31 \times X_1X_3 - 1{,}76 \times X_2X_3 - 24.81 \times X_1^2 - 32.77 \times X_2^2 - 37.91 \times X_3^2 \quad (4)$$

$$Y2 = 50.64 - 0.49 \times X_1 - 1.84 \times X_2 + 0.42 \times X_3 - 0.98 \times X_1X_2 - 0.69 \times X_1X_3 - 0.75 \times X_2X_3 - 4.03 \times X_1^2 - - 4.15 \times X_2^2 - 4.92 \times X_3^2 \quad (5)$$

$$Y3 = 48.86 - 0.38 \times X_1 - 2.60 \times X_2 + 1.47 \times X_3 - 0.83 \times X_1X_2 - 0.15 \times X_1X_3 - 0.64 \times X_2X_3 - 4.12 \times X_1^2 - 4.77 \times X_2^2 - 5.14 \times X_3^2 \quad (6)$$

Effect of Independent Factors on Emulsion Stability Index

ESI of all the runs ranged from 343.03 to 452.67 min. Analysis of the regression equation indicated that the addition of pectin (X_1), pH (X_2), and drying time (X_3) had a notable impact on ESI (Y_1). The three-dimensional response surface plot offered comprehensive insights into the influence of diverse independent factors on Emulsion Stability Index (ESI), as illustrated in Figures 4 A-C. The plot displayed an initial rise in Emulsion Stability Index (ESI) when adjusting the pectin concentration (X_1) from level -1 to 0, succeeded by a notable decline with a subsequent increase from 0 to +1. This pattern could be attributed to the initial increase in pectin concentration, which caused a higher rate of brown color formation as a sign of advanced Maillard reaction products (Setiowati et al., 2016). Based on previous study, the higher concentration of polysaccharides in the WPC-pectin conjugate provided more carbonyl groups to access the amino acid groups. However, the addition of a polysaccharide in a high concentration caused saturation of the glycation reaction due to insufficient amino groups to react with the carbonyl group.

The three-dimensional response surface plot also depicted a noteworthy improvement in Emulsion Stability Index (ESI) with an increase in pH (X_2), succeeded by a decrease with further elevation. This phenomenon had a correlation with the progression of the Maillard reaction, which inhibited advanced reaction products (ARPs) at a lower pH below a value of 8. By the wet heating method, the ARPs, such as Nε-(carboxymethyl) lysine (CML), pyrraline, pentosidine, and imidazolones, were formed rapidly when pH was uncontrolled. These compounds reduced protein functionality and caused decreasing emulsion stability. Meanwhile, the values of ESI increased with increasing drying time, followed by a decrease with a further rising in the drying time due to higher browning formation (Setiowati et al., 2017). The three-dimensional plot revealed that the interaction between factors A and B, B and C, as well as A and C collectively influenced ESI.

Effect of Independent Factors on Emulsion Activity Index

As presented in Figure 4 D-F, EAI values were between 38.70 to 52.69 $m^2\ g^{-1}$. The three-dimensional response surface plot revealed a notable improvement in EAI values with an increase in pectin concentration, followed by a decrease with further elevation, similar to the observed pattern in ESI. Furthermore, the high polysaccharide concentration caused saturation of Maillard reaction due to the group of amino acids being unavailable to access the carbonyl group.

The increase of saccharides in the conjugate led to the formation of intermediate products of Maillard reaction, yielding melanoidin, a compound that was difficult to dissolve. This compound also caused a decrease in the performance of the conjugate as an emulsifier. Based on a previous study, the conjugate of

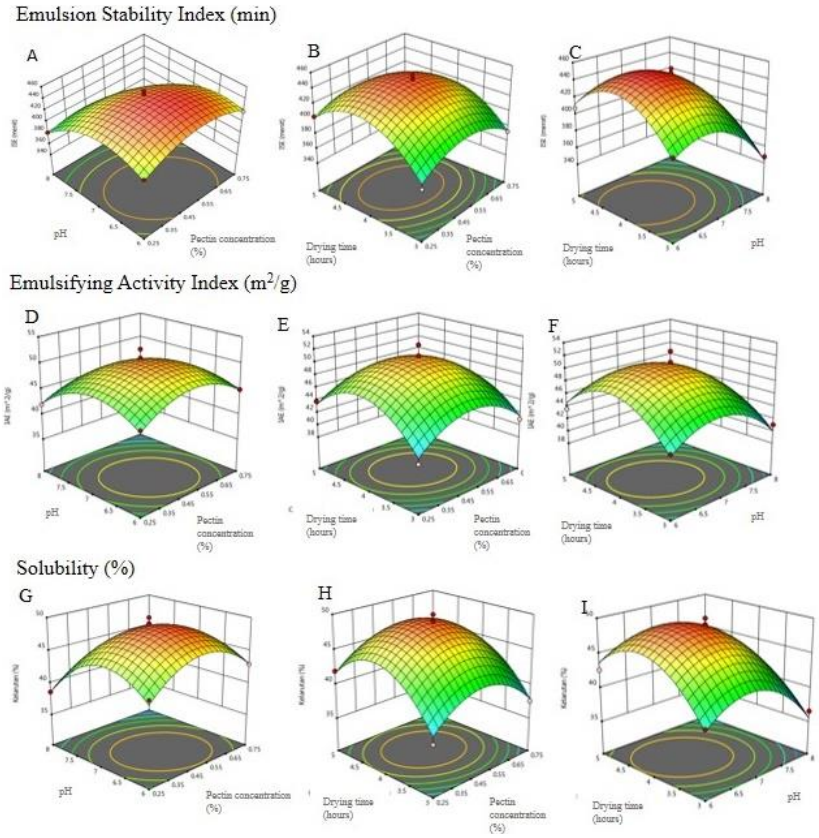


Figure 4. a three-dimensional response surface plot for the optimization of the formulated solutions, illustrating the influence of various formulation variables (independent factors); (A-C) depicting the impact of X_1, X_2, and X_3 as well as the interaction terms on ESI, (D-F) illustrating the effect of *X1*, *X2*, and *X3* along with the interaction terms on EAI, and (G-I) showcasing the effect of X_1, X_2, and X_3 and the interaction terms on solubility.

WPI with LMP in different ratios showed that increasing LMP content caused a rise in the rate of brown color formation with a further increase in LMP. The longer incubation time of the WPI-LMP also led to an increase in browning colors (Setiowati et al., 2016). However, this conjugate of WPC-pectin had the highest value of IAE in a particular pectin concentration and drying time due to several amino acids reacting with the carbonyl group up to a certain concentration of polysaccharides. When there were high concentrations of polysaccharides, Maillard reaction was saturated. In another investigation (Sedaghat Doost et al., 2019), wet heating at pH 7 and 9 with a duration of up to 20 hours showed that WPC-pectin conjugate with initial pH of 7 and reaction time of 10-15 hours had better emulsification properties than pH 9. In the cabinet dryer, the water content of the solution decreased, as caused by the conjugate changes from wet to dry heating. Moreover, in wet heating process, the conjugate occurred faster due to the higher formation of the final products. The highest EAI value was noted when the interaction terms X_1X_2, X_1X_3, and X_2X_3 were set at their central point values.

Effect of Independent Factors on Solubility

The results showed that the solubility of WPC-pectin conjugate in all prepared formulations ranged from 35.43-49.99%. As shown in Figure 4 G-I, the 3D response surface plot provided detailed information on the effect of various independent factors on solubility. The graph showed an increase in solubility with increasing pectin concentration, pH, and drying time, followed by a significant decrease with a further rise in each factor. This response occurred due to protein denaturation and polymerization by increasing drying time. The polymerization products also had poor solubility and the conjugate protein with a higher molecular weight of the saccharide caused a decrease in the solubility of the conjugate (Guo et al., 2019).

A previous study established that a lower molecular weight saccharide accelerated the development of Maillard reaction (Xue et al., 2013). In WPC-pectin conjugate, the amino acids reacted with lactose first, and the other amino acids reacted with pectin, while pH affected WPC-pectin conjugate solubility. WPC-pectin

conjugate with ribose and glucose was also found to be at pH 7 and 9, resulting in the best solubility with initial pH 7 (Sedaghat Doost et al., 2019).

Optimization and Point Prediction

After conducting a statistical analysis and assessing the influence of individual factors on the responses, the optimization of the formulated conjugate was performed. This optimization yielded precise parameter conditions to attain the specified objectives for the selected responses. In the quest for optimal conditions, all responses were set to maximize, and numerical optimization using Box-Behnken Design (BBD) suggested the most suitable values for the variables: 4.9% for pectin concentration, pH 6.7, and a drying time of 4.12 hours. The anticipated responses for the optimized formulation were 451.89 minutes, 50.84 m^2 g^{-1}, and 49.33% for Emulsion Stability Index (ESI), Emulsifying Activity Index (EAI), and solubility, respectively. Upon verification, the ESI, EAI, and solubility of the WPC-pectin conjugate were

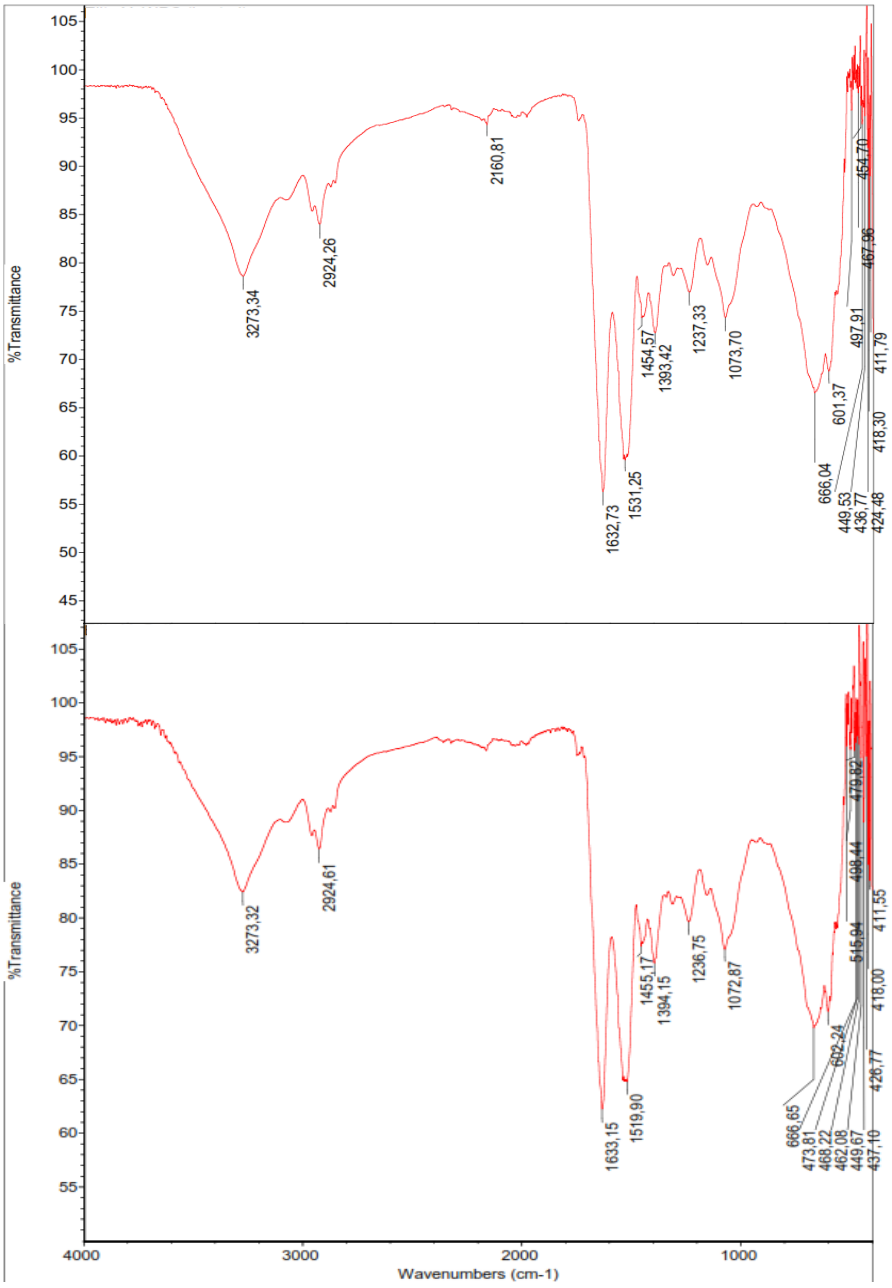


Figure 5. FTIR spectra of WPC (A) and the obtained WPC-pectin conjugate from the optimum process by BBD (B). Condition of conjugate was at 4.9% pectin, pH 6.7, and 4.12 hours for drying time

determined to be 452.267 minutes, 49.95 m^2 g^{-1}, and 48.09%, respectively. These results closely aligned with the predicted values, thereby confirming the efficacy of the WPC-pectin conjugation process.

Fourier Transform Infrared Spectrum

Protein-carbohydrate interaction was investigated by FTIR to ensure the formation of Maillard reaction products in WPC-pectin conjugate process (Q. Liu et al., 2021). FTIR indicated alterations in the structure of proteins through the assessment of hydrogen bonding forces. The proteins exhibited distinctive structural features in their peptide bonds, encompass C=O, C=N, and N-H bonds, giving rise to three characteristic absorption bands. These bands include the amide I group in the 1700-1600 cm^{-1} range, corresponding to N-H bending; the protein structure group in the 1500-1550 cm^{-1} range; and the amide III group in the 1300-1200 cm^{-1} range, corresponding to N-H bending and C-N stretching vibrations (Wang et al., 2020). As shown in Figure 4, the peaks were detected at 1632.72 cm^{-1}, 1531.23 cm^{-1}, and 1237.33 cm^{-1} in the spectrum of WPC, which belongs to amide I (C=O stretching), amide II (N-H bending), and amide III (C-N stretching and N-H deformation). After glycation at 4.90% addition of pectin, 4.12 hours drying, and preconditioning pH at pH 6.7, the main absorption bands of WPC at 1632.72 cm^{-1} and 1531.23 cm^{-1} shifted to 1633.15 cm^{-1} and 1519.90 cm^{-1}, respectively. According to Chen, Ma, et al., (2019), the covalent binding between protein and polysaccharides occurred through Maillard reaction mechanism, leading to the loss of –NH2 groups (Qu et al., 2018). The peak intensities exhibited a slow increase in the glycated WPC, indicating changes in the degree of grafting through Maillard reaction (Q. Liu et al., 2021). Moreover, after glycation of WPC, the absorption bands at 1072.87 cm^{-1} with high intensities indicated that a strong stretching vibration of newly C-N covalent bond formation occurred in conjugate (Qu et al., 2018). FTIR result also provided important evidence for the covalent conjugate of WPC.

CONCLUSION

In conclusion, this study showed that the design and optimization of WPC-pectin conjugate process using a single-factor and BBD-RSM obtain good properties of the conjugate. A cabinet dryer was applied for the conjugate process to ensure simplicity. The ANOVA resullts for each response (ESI, EAI, and solubility) exhibited a high coefficient of determination value (R^2), indicating a well-fitted secondary-order polynomial regression model with the experimental data. There was also an interaction between pectin concentration, pH, and drying time as factors. Based on the results, the optimal WPC-pectin conjugate process was a pectin concentration of 4.9%, pH 6.7, and a drying time of 4.12 hours. Under these optimized conditions, ESI, EAI, and solubility values were verified near the predicted values. Therefore, this optimal condition of WPC-pectin conjugate was valid to obtain WPC-pectin conjugate with good properties.

ACKNOWLEDGMENT

The authors are grateful to the Faculty of Agricultural Technology for providing good laboratory facilities and services.

CONFLICT OF INTEREST

The authors assert that there are no conflict of interest.

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