



Scientific Research

Investigating the Water Holding Capacity and Fat Absorption Capacity of Hemp Protein Isolate (*Cannabis sativa* L.) Extracted by Alkaline Method

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ARTICLE INFO

ABSTRACT

Article History:

Received: 2024/11/28

Review: 2025/03/08

Accepted: 2025/11/10

Keywords:

Fat absorption capacity,
Water holding capacity,
Hemp protein isolate,
Alkaline extraction.

DOI: 10.48311/fsct.2026.83877.0

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Proteins play a crucial role both in human survival and food products. They have important roles in health and nutrition, and possess a wide range of functional properties. Hemp protein is considered a valuable plant protein source with excellent nutritional value and unique functional properties. It can be used in various food systems to create desirable textures. Maintaining freshness and enhancing oil absorption are key factors in the quality of many food products. The alkaline extraction method for hemp protein was evaluated using response surface methodology. In this study, the effects of various parameters such as alkaline pH (8.5, 10, 11.5), solid to liquid ratio (1:10, 1:15, 1:20), and centrifugation temperature (4, 4.5, 5 °C) were investigated on the water holding capacity and fat absorption capacity of isolated hemp protein. The highest value of water and fat absorption capacity were respectively 2.985 and 6.036 (g/g) with the desirability of 98%. The optimum point was obtained at alkaline pH of 11.5, isoelectric pH of 4, solid to liquid ratio of 1:12, and centrifugation temperature of 4 °C. The statistical analysis using t .test showed no significant difference between the experimental data and the predicted values ($p>0.05$), indicating the optimal functional properties of hemp protein for its potential application in the food industry.

1- Introduction

Plant-based diets are increasingly popular for several reasons, including environmental impacts, the high cost of animal protein production, and the growing trend of vegetarianism. Utilizing plant proteins is economically viable, sustainable, and environmentally friendly [1]. From both nutritional and functional perspectives, plant proteins are preferred over animal proteins to meet the diverse needs of consumers with varying ethnic, religious, dietary, and ethical preferences. Proteins extracted from oilseeds, cereals, legumes, and by-products are economically attractive, sustainable, and renewable alternatives to animal proteins [2]. These plant-derived proteins play specific functional roles in food formulations [3]. The desired functional properties vary depending on the application. For example, critical functional characteristics in producing plant-based meat with desirable texture include solubility, water-holding capacity (WHC), and fat absorption capacity (FAC). Key functional properties of proteins include protein-water interactions (WHC) and protein-fat interactions (FAC) [4]. Hemp, a member of the *Cannabaceae* family, has been used in food and pharmaceutical industries due to its nutritional value. Industrial hemp (*Cannabis sativa* L.) is an annual herbaceous plant rich in oil, protein, carbohydrates, insoluble fiber, vitamins, and essential minerals. Hemp proteins, in particular, have garnered significant scientific and industrial attention due to their excellent nutritional value and diverse techno-functional properties [5]. They are recognized as a good protein source with essential amino acids and high digestibility. Therefore, a deeper understanding of the functional properties of hemp protein is necessary to expand its

application as a valuable ingredient in the food industry [6]. Water-holding capacity (WHC) describes the ability of proteins to entrap and retain water molecules within their matrix against gravitational forces [7]. A protein's ability to interact with water facilitates solubility, which is crucial since other functional properties like emulsification and gelation often require the protein to be soluble in water [6, 8, 9]. WHC is particularly important in food products with low to moderate moisture content because water loss can lead to undesirable textures such as stickiness, dryness, or brittleness [10]. Retaining water within the protein matrix enhances mouth feel, preserves freshness, and minimizes undesirable moisture loss in food products [6, 7]. Despite these advantages, the WHC of hemp protein remains largely unknown and is highly dependent on the protein's structure. Fat absorption capacity (FAC), on the other hand, measures the ability of the protein's non-polar regions to bind oils [11]. The aim of this study was to optimize the water-holding capacity and fat absorption capacity of hemp protein isolates (HPI). Historically, hemp protein was considered a secondary by-product, and extraction methods rarely prioritized protein quality. Processing methods often degraded the protein. Hemp was mainly cultivated for fiber or oil, making its protein fraction a low-value by-product that had not been optimized. Consequently, industrial hemp's potential remains underdeveloped. This study sought to highlight the promising and emerging role of hemp protein as a valuable source for the food industry, focusing on optimizing its water and oil absorption functional properties.

2-Materials and Methods

2.1. Materials

Hemp seeds with hulls were obtained from the Agricultural Research Center of Isfahan, Iran, and stored in a refrigerator at 4°C until testing. The seeds were thoroughly cleaned to remove any impurities such as straw, debris, and stones. The dehulled seeds were then ground using a household mill and sieved through a 40-micron mesh sieve.

Subsequently, the hemp powder was defatted using hexane at a 1:4 (w/v) ratio and dried under a fume hood at room temperature with air circulation for 24 hours. The defatted hemp flour was mixed and sieved again to ensure homogeneity. All defatted and dried hemp flour was stored at 4°C until protein extraction [12].

2.2. Preparation (Extraction) of Hemp Protein Isolates

The defatted milled hemp powder was passed through laboratory sieves of varying mesh sizes to separate particles based on size. The selected material was used for protein extraction and isolation, and all solutions prepared during the experiments were made with deionized distilled water. The defatted hemp flour was mixed with distilled water at specific solid-to-solvent ratios (w/v) according to Table 1. The pH of the mixture was adjusted to the desired alkaline levels as shown in Table 1 by adding 1.0 M sodium hydroxide (NaOH), under continuous stirring for 30 minutes at room temperature. The mixture was then centrifuged at 9000 rpm for 30 minutes at temperatures specified in Table 1. The supernatant was collected, and its pH was adjusted to the isoelectric point using 1.0 M hydrochloric acid (HCl) according to Table 1, to precipitate the protein. This suspension was again centrifuged at 9000 rpm to facilitate

protein precipitation. The collected protein precipitate was re-suspended in deionized water, and the pH was adjusted to 6.8–7.0 using 0.1 M (NaOH). The resulting suspension was freeze-dried for 24 hours. The completely dried hemp protein isolates were stored in plastic bags and preserved at -40°C in a freezer for further studies [12, 13].

2.2. Determination of Water-Holding Capacity (WHC)

To measure the water-holding capacity (WHC), 1 ml of distilled water was added to 0.1 g of the protein isolate placed in pre-weighed centrifuge tubes. The resulting suspension was vortexed vigorously for 2 minutes and then left to stand at room temperature for 30 minutes. The mixture was then centrifuged at 3000 g for 20 minutes. The supernatant was carefully decanted, and the tubes were weighed again. The WHC was calculated using Equation (1) and expressed as the grams of water bound per gram of protein sample [14–16].

Equation (1):

$$\text{WHC} = (W_2 - W_1) / W_1$$

Where:

W_1 = Weight of the dry protein sample (g)

W_2 = Weight of the hydrated gel-like precipitate (g)

2.3. Determination of Fat Absorption Capacity (FAC)

Fat Absorption Capacity (FAC) is defined as the amount of oil absorbed per gram of protein tested [11]. To determine FAC, 1 ml of corn

oil was added to 0.1 g of protein isolate placed in pre-weighed centrifuge tubes. The suspensions were shaken for 2 minutes at 3000 rpm using a shaker and then allowed to stand at room temperature for 30 minutes. Afterwards, the samples were centrifuged at 3000 g for 20 minutes. The supernatant (oil) was removed, and the tubes were weighed again. FAC was calculated using Equation (2) and expressed as grams of oil bound per gram of protein [14–16].

Equation (2):

$$\text{FAC} = (W_2 - W_1) / W_1$$

Where:

W_1 = Initial weight of the sample (g)

W_2 = Final weight after decanting the supernatant (g)

3- Data Analysis

Statistical analysis was performed using the Central Composite Design (CCD) method in Design Expert software, version 11. The effects of the following independent

parameters were assessed on WHC and FAC of hemp protein isolate: Alkaline pH: (8.5, 10, 11.5), Solid-to-solvent ratio: (1:10, 1:15, 1:20), Isoelectric pH (4, 4.5, 5), Centrifugation temperature: (4°C, 14°C, 24°C). These variables were tested according to Table 2 in the study. The experimental design consisted of 39 runs, with 7 replicates at the central point and 2 replicates at axial points. The physical-chemical properties (WHC and FAC) of hemp protein were optimized based on maximizing these capacities. The model's predicted values were compared with the experimental results using the t-test at a 5% significance level. The percent error between predicted and actual experimental values was calculated using Equation (3):

Equation (3)

$$\text{Percent Error} = \frac{|\text{Actual Value} - \text{Predicted Value}|}{\text{Actual Value}} * 100$$

4- Results and Discussion

Table 1. Experimental design of independent and response variables

Treatment number	Solid to solvent ratio (g/ml)	Isoelectric pH	Alkaline pH	Centrifuge Temperature(°C)	Water holding capacity (g/g)	Fat absorption capacity (g/g)
1	15	4.5	10	14	2.3	5.3
2	10	4	8.5	24	1.6	5.5
3	10	4	11.5	24	2	5.3
4	15	4.5	10	14	2.3	5.3
5	10	5	11.5	4	2.5	5.5
6	15	4.5	8.5	14	2.6	5
7	20	4	11.5	24	1.4	5
8	20	4	8.5	4	2	5.2
9	15	4.5	10	14	2.3	5.3
10	20	4	8.5	24	1.8	5.5

11	15	4	10	14	2.2	5.4
12	15	5	10	14	2	5.9
13	15	4.5	10	14	2.3	5.4
14	20	4.5	10	14	2.3	4.9
15	20	5	8.5	24	3	5.7
16	15	4.5	10	4	2.1	5.4
17	20	5	11.5	24	2.6	6.3
18	15	4.5	11.5	14	3.4	4.9
19	10	5	11.5	24	1.5	6
20	15	4.5	11.5	14	3.4	4.9
21	10	4.5	10	14	1.8	5
22	15	4.5	10	14	2.3	5.3
23	15	4.5	10	4	2.2	5.3
24	15	4.5	10	24	1.5	5.8
25	10	4.5	10	14	1.8	5
26	10	5	8.5	4	0.7	5.4
27	10	5	8.5	24	1	5.1
28	20	5	11.5	4	3.3	5.3
29	15	5	10	14	2	5.9
30	15	4.5	8.5	14	2.6	5
31	15	4.5	10	14	2.3	5.4
32	15	4	10	14	2.2	5.4
33	15	4.5	10	14	2.2	5.3
34	20	4.5	10	14	2.3	4.9
35	10	4	8.5	4	2.1	5.6
36	20	4	11.5	4	3	4
37	20	5	8.5	4	2.3	5.4
38	10	4	11.5	4	4	4.8
39	15	4.5	10	24	1.5	5.8

Table 2. Independent variable ranges for hemp protein extraction

Factor	Name	Units	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Solid/Solvent	(g/ml)	10.00	20.00	-1 ↔ 10.00	+1 ↔ 20.00	15.00	3.63

B	Isoelectric pH		4.00	5.00	-1 ↔ 4.00	+1 ↔ 5.00	4.50	0.3627
C	Alkaline pH		8.50	11.50	-1 ↔ 8.50	+1 ↔ 11.50	10.00	1.09
D	Centrifuge temperature (°C)		4.00	24.00	-1 ↔ 4.00	+1 ↔ 24.00	14.00	7.25

Table 3. ANOVA of the quadratic model of the water holding capacity of hemp protein

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	16.04	13	1.23	1046.97	< 0.0001	significant
A-Solid/Solvent	1.25	1	1.25	1060.69	< 0.0001	
B-Isoelectric pH	0.0980	1	0.0980	83.16	< 0.0001	
C-Alkaline pH	2.74	1	2.74	2323.33	< 0.0001	
D-Centrifuge temperature	1.98	1	1.98	1683.95	< 0.0001	
AB	3.06	1	3.06	2598.69	< 0.0001	
AC	0.7225	1	0.7225	613.08	< 0.0001	
AD	0.1225	1	0.1225	103.95	< 0.0001	
BD	0.8100	1	0.8100	687.33	< 0.0001	
CD	1.96	1	1.96	1663.16	< 0.0001	
A ²	0.2442	1	0.2442	207.21	< 0.0001	
B ²	0.1455	1	0.1455	123.46	< 0.0001	
C ²	2.71	1	2.71	2303.13	< 0.0001	
D ²	1.00	1	1.00	850.91	< 0.0001	
Residual	0.0295	25	0.0012			
Lack of Fit	0.0159	11	0.0014	1.49	0.2381	not significant
Pure Error	0.0136	14	0.0010			
Cor Total	16.07	38				
Std. Dev.	0.0343					
Mean	2.22					
C.V. %	1.54					
R ²	0.9982					
Adjusted R ²	0.9972					
Predicted R ²	0.9954					

Adeq Precision 161.9018

Table 4. ANOVA of the quadratic model of the fat absorption capacity of hemp protein

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6.16	13	0.4740	214.71	< 0.0001	significant
A-Solid/Solvent	0.0500	1	0.0500	22.65	< 0.0001	
B-Isoelectric pH	1.15	1	1.15	521.80	< 0.0001	
C-Alkaline pH	0.0980	1	0.0980	44.39	< 0.0001	
D-Centrifuge temperature	0.8405	1	0.8405	380.70	< 0.0001	
AB	0.3025	1	0.3025	137.02	< 0.0001	
AC	0.0900	1	0.0900	40.77	< 0.0001	
AD	0.2500	1	0.2500	113.24	< 0.0001	
BC	1.10	1	1.10	499.38	< 0.0001	
CD	0.4900	1	0.4900	221.94	< 0.0001	
A ²	0.5129	1	0.5129	232.31	< 0.0001	
B ²	0.7428	1	0.7428	336.47	< 0.0001	
C ²	0.5129	1	0.5129	232.31	< 0.0001	
D ²	0.4800	1	0.4800	217.41	< 0.0001	
Residual	0.0552	25	0.0022			
Lack of Fit	0.0359	11	0.0033	2.37	0.0655	not significant
Pure Error	0.0193	14	0.0014			
Cor Total	6.22	38				
Std. Dev.	0.0470					
Mean	5.32					
C.V. %	0.8836					
R ²	0.9911					
Adjusted R ²	0.9865					
Predicted R ²	0.9781					
Adeq Precision	81.3445					

Based on the analysis of variance (ANOVA), the effects of the independent variables on the water-holding capacity (WHC) and fat absorption capacity (FAC) of hemp protein

are presented in Tables 3 and 4. The p-value for the quadratic model was less than 0.0001 with co-efficient of determination (R²) of 0.99, indicating the model was highly significant. Moreover, the p-value for the lack of fit was

greater than 0.05, suggesting that the model adequately fits the data without significant deviation. The adjusted R^2 (R^2_{adj}) and predicted R^2 (R^2_{pre}) were 0.9972 and 0.9954 for WHC, respectively, 0.9865 and 0.9781 for FAC, respectively. Values of R^2 closer to 1 indicate higher model accuracy. When the adjusted R^2 and predicted R^2 are close, the model demonstrates strong generalizability and predictive power for new data. The Adequate Precision values were 161.9018 for WHC and 81.3445 for FAC, both exceeding the desirable threshold, indicating high model efficiency in predicting responses. Additionally, the Coefficient of Variation (C.V.%) was low: 1.54% for WHC and 0.8836% for FAC, reflecting minimal data dispersion and high homogeneity.

4.1. Optimization of Water-Holding Capacity of Hemp Protein

According to Table 3 and Figures 1a to 1d, the interactive effects of independent variables had a significant influence on WHC ($p \leq 0.0001$). These interactions included Solid-to-solvent ratio \times Isoelectric pH, Solid-to-solvent ratio \times Alkaline pH, Solid-to-solvent ratio \times Centrifugation temperature, Isoelectric pH \times Centrifugation temperature and Alkaline pH \times Centrifugation temperature. The solid-to-solvent ratio was a critical factor, not only affecting the extent of protein extraction but also influencing the protein's molecular interactions and structure in combination with pH and temperature.

Figure 1a shows the interaction between solid-to-solvent ratio and alkaline pH. Higher alkaline pH levels unfold protein structures, exposing hydrophilic groups. At pH (11.5), the tertiary and quaternary protein structures

open up, presenting more polar groups to water. In combination with intermediate ratios like (1:15 or 1:17), appropriate viscosity is maintained, enabling water retention without excessive aggregation. Conversely, lower ratios at lower alkaline pH result in reduced efficiency due to unstable structures.

Figure 1b illustrates the interaction effect of solid-to-solvent ratio and isoelectric pH on the water holding capacity (WHC) of protein. The results indicate that WHC significantly decreases as the solid-to-solvent ratio decreases (i.e., the suspension becomes more dilute, for example, from 1:10 to 1:15) and as the pH moves away from the low isoelectric point (i.e., pH increases to values higher than 4.5). The reduced aggregation of proteins in more dilute systems leads to a decrease in the protein's ability to form cross-links, consequently limiting the internal space of the matrix and reducing WHC. In contrast, near the isoelectric point, the net charge on proteins approaches zero, minimizing electrostatic repulsion; this facilitates protein aggregation and reduces the available space for water retention. Consequently, the highest water holding capacity is achieved under conditions of a more dilute solid-to-solvent ratio combined with a pH that is substantially distant from the isoelectric point. These findings are fully consistent with well-established physicochemical principles of proteins, particularly the minimum solubility and water holding capacity observed near the isoelectric point.

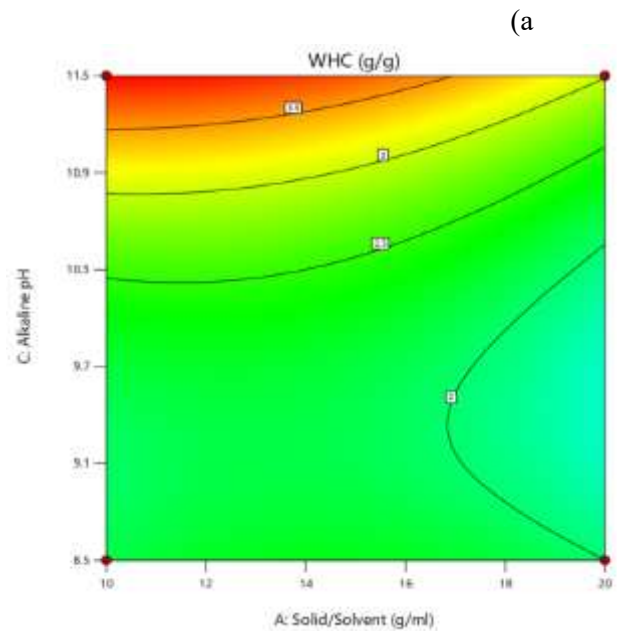
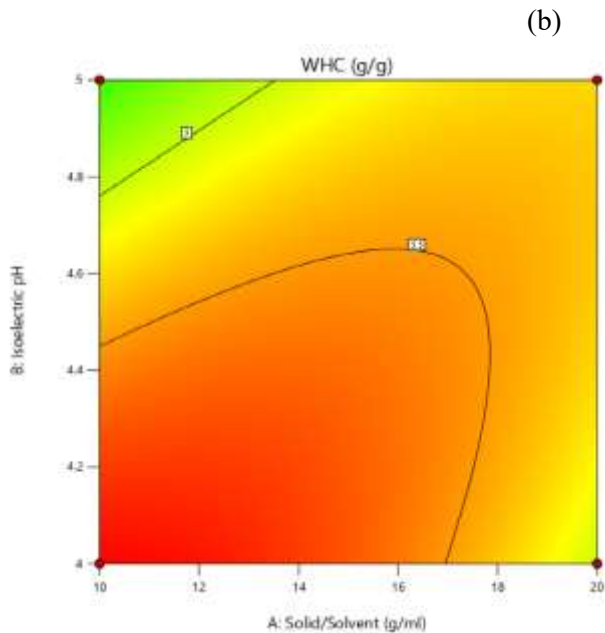
Figure 1c displays the interaction between solid-to-solvent ratio and centrifugation temperature. Lower temperatures, particularly 4°C, preserve protein structure and prevent the breakdown of the swollen matrix, improving WHC. At higher temperatures, increased

molecular motion releases water, reducing WHC, especially in concentrated samples where matrix space is limited.

Figure 1d illustrates the interaction effect of isoelectric pH and centrifugation temperature on the (WHC) of protein. The WHC of proteins increases at pH values further away from their isoelectric point. This is because the increased net charge on protein molecules results in stronger electrostatic repulsion between them. This repulsion prevents protein aggregation and creates a more open network structure capable of entrapping more water. In contrast, near the isoelectric pH, the net charge on the proteins is minimized, repulsion decreases, and the tendency to aggregate increases, which lowers WHC. This pattern is

fully consistent with established physicochemical principles of proteins, particularly the minimum solubility and WHC near the isoelectric point, as well as the influence of temperature on protein-water interactions.

Figure 1e shows the interaction between alkaline pH and centrifugation temperature. At high pH and temperatures, protein denaturation increases WHC by exposing hydrophilic groups. Combining these conditions with low temperature prevents secondary re-aggregation, allowing better water retention. However, at 24°C, even with high pH, part of this beneficial effect diminishes, leading to decreased WHC.



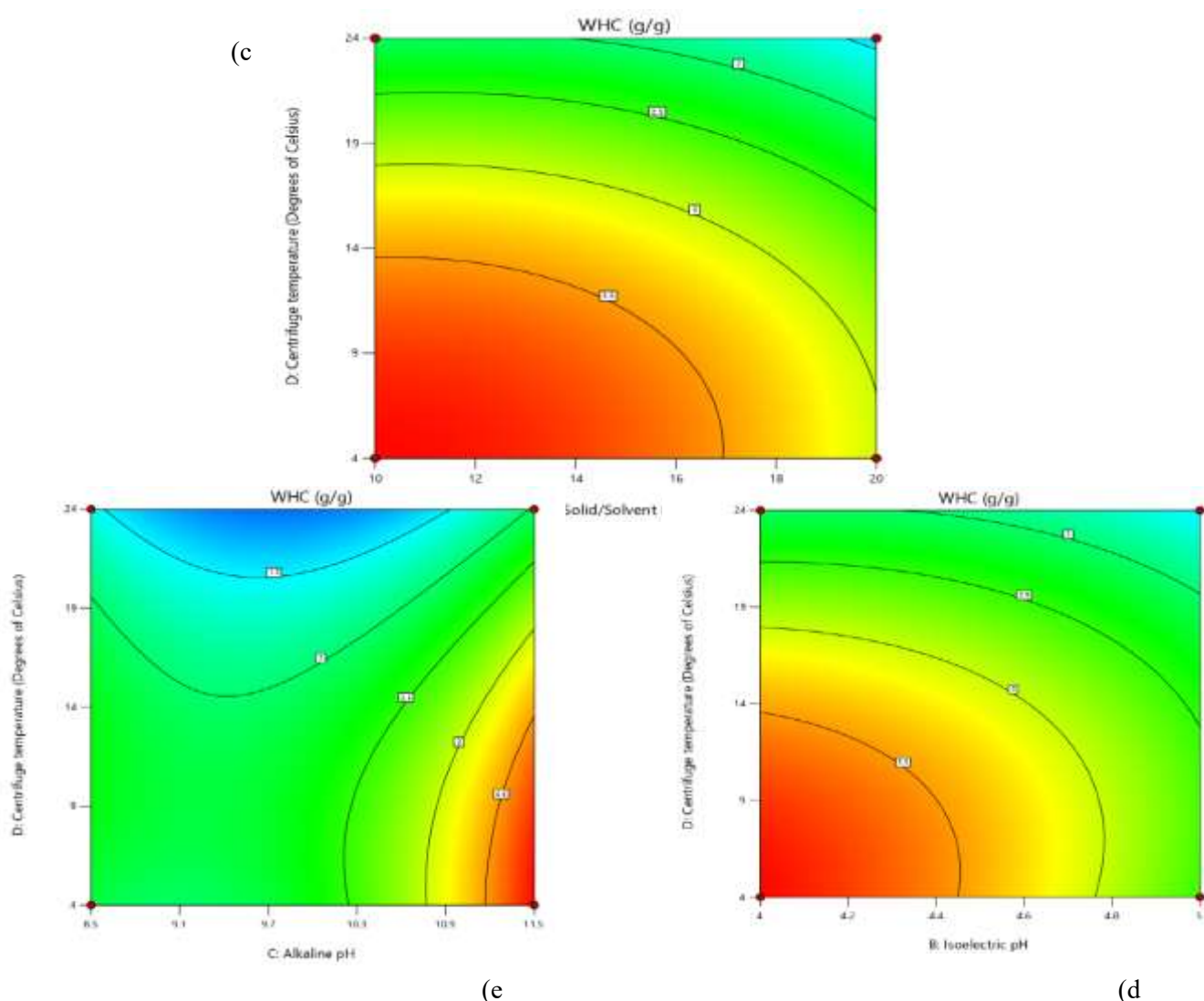


Fig 1. Two-dimensional contour diagrams a) The interaction effect of solid to solvent ratio and alkaline pH, b) The interaction effect of solid to solvent ratio and isoelectric pH, c) The interaction effect of solid to solvent ratio and centrifuge temperature, d) The interaction effect of isoelectric pH and centrifuge temperature, e) The interaction effect of alkaline pH and centrifuge temperature on water holding capacity of hemp protein

Based on the previous findings, the water-holding capacity (WHC) of hemp protein was modeled using the following Equation (4):

$$Y = 2.27529 + 0.25 A - 0.07 B + 0.37 C - 0.315 D + 0.4375 AB - 0.2125 AC + 0.0875 AD + 0.225 BD - 0.35 CD - 0.219202 A^2 - 0.169202 B^2 + 0.730798 C^2 - 0.444202 D^2$$

Where Y = Water-holding capacity of hemp protein (g/g), A = Solid-to-solvent ratio (g/mL), B = Isoelectric pH, C = Alkaline pH, D = Centrifugation temperature (°C).

4.2. Optimization of Fat Absorption Capacity (FAC) of Hemp Protein

According to Table 4 and Figures 2a to 2d, the interactive effects of the independent variables had a significant influence on the fat absorption capacity (FAC) of hemp protein ($p \leq 0.0001$). The significant interactions included Solid-to-solvent ratio \times Isoelectric pH, Solid-to-solvent ratio \times Alkaline pH, Solid-to-solvent ratio \times Centrifugation temperature, Isoelectric pH \times Alkaline pH and Alkaline pH \times Centrifugation temperature. In more concentrated ratios (1:10), the higher density of protein in solution led to protein aggregation, reducing hydrophobic interactions and thereby limiting FAC.

Figure 2a the interaction effect between the solid-to-solvent ratio and isoelectric pH is observed. Fat absorption capacity is higher at the higher isoelectric pH. At lower solid-to-solvent ratios (more diluted systems), conditions for effective protein precipitation at the isoelectric pH are improved. In this case, the protein structure becomes more swollen and extended, exposing more hydrophobic groups to contact with the oil, which consequently increases the fat absorption capacity. In contrast, at higher (more concentrated) ratios (e.g., 1:10), the high protein density leads to the formation of compact protein aggregates, which may reduce surface contact with the oil phase and limit the fat absorption capacity (FAC). When the solid-to-solvent ratio is high at the isoelectric pH, severe protein aggregation causes a large portion of the hydrophobic groups to become inaccessible to the oil. Whereas at lower ratios, the surface contact area increases, resulting in greater oil-binding ability.

Figure 2b the interaction effect between the solid-to-solvent ratio and alkaline pH is evident. High alkaline pH opens up the protein structure, exposing hydrophobic groups to the oil. A high alkaline pH (11.5) causes protein denaturation and unfolding of the structure. This unfolding is much more effective at intermediate ratios (1:15–1:17), as the appropriate medium density allows for optimal contact between the protein's non-polar groups and the oil. At ratios lower than 1:15 (more diluted systems), although denaturation occurs more effectively, the relative decrease in protein concentration may reduce effective contact with the oil.

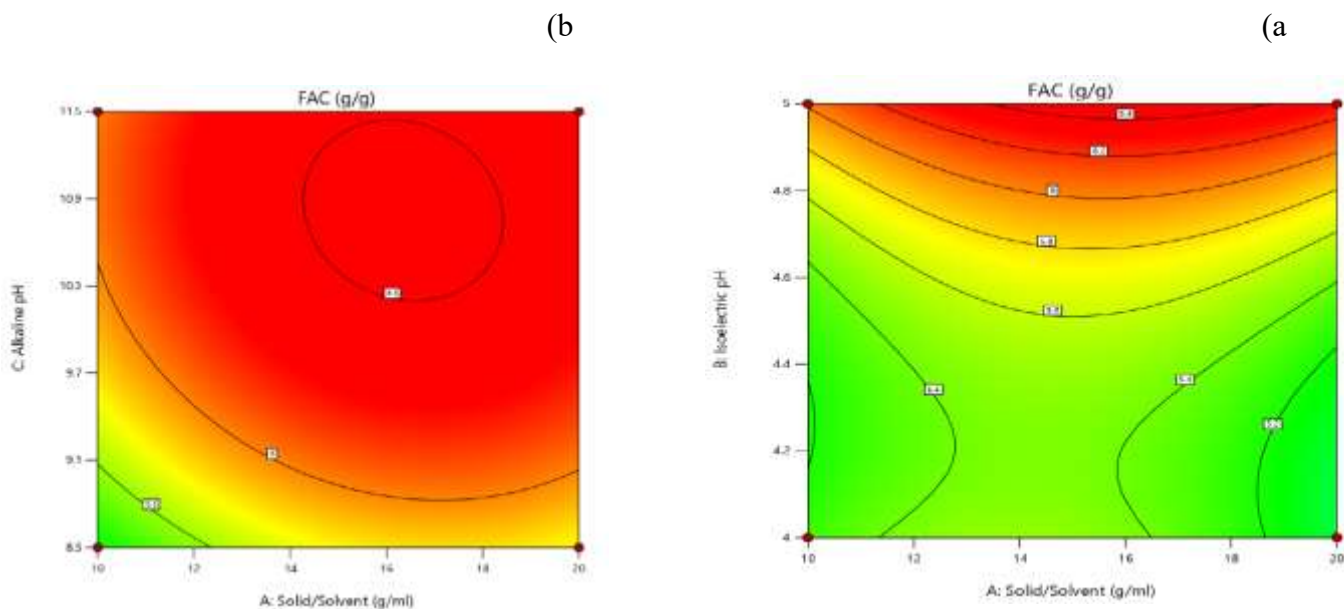
Figure 2c the interaction effect between the solid-to-solvent ratio and centrifuge temperature is illustrated. As observed, higher temperatures lead to the unfolding of the protein structure and an increase in oil absorption. At low temperature (4°C) and dilute ratios, protein structures are better preserved, preventing excessive degradation, which is effective in maintaining hydrophobic characteristics. However, at lower solid-to-solvent ratios and higher temperatures (14–24°C), partial denaturation and increased molecular mobility result in enhanced fat absorption capacity (FAC). This phenomenon is particularly amplified at high pH.

Figure 2d the interaction effect between alkaline pH and centrifuge temperature is demonstrated, revealing a synergistic influence of high pH and high temperature that significantly increases fat absorption capacity. The effect of high alkaline pH (11.5) causes the protein structure to denature and unfold, exposing more hydrophobic (non-polar) groups to contact with the oil. These groups play a key role in fat absorption. At low centrifuge temperature (4°C), molecular

movement slows down, reducing protein re-aggregation. As a result, the denatured unfolded structure remains open, keeping hydrophobic groups accessible. These conditions lead to a substantial increase in fat absorption capacity (FAC).

Figure 2e the interaction effect between isoelectric pH and alkaline pH on fat absorption capacity. The results indicate that at a lower isoelectric pH (4), extensive protein aggregation causes many functional groups to become trapped within the internal structure, reducing contact with the lipid phase and thereby decreasing fat absorption capacity. In contrast, at a higher isoelectric pH (5), protein

precipitation is milder, allowing the protein structure to remain more open. When this condition is combined with a high alkaline pH (11.5), greater denaturation occurs, intensifying the exposure of hydrophobic groups and resulting in increased fat absorption. The synergistic effect of the two pH values is most pronounced when both the alkaline pH and the isoelectric pH are at their highest levels; under these conditions, the protein exhibits its maximum potential for interaction with the lipid phase. This demonstrates that simultaneous control of these two variables can play a decisive role in enhancing fat absorption capacity (FAC).



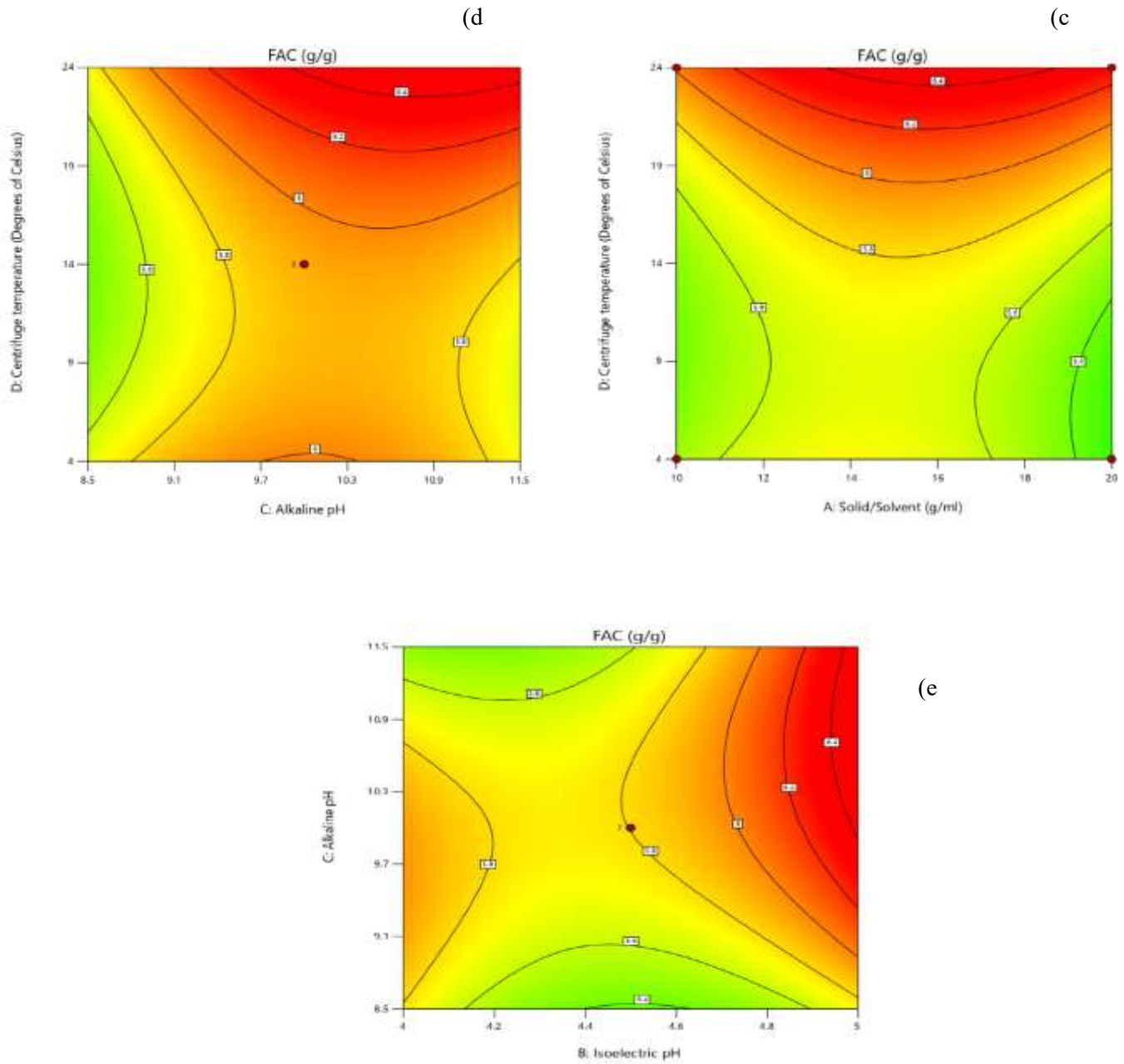


Figure 2. Two-dimensional contour diagrams a) Interaction of solid to solvent ratio and isoelectric pH, b) Interaction of solid to solvent ratio and alkaline pH, c) Interaction of solid to solvent ratio and centrifuge temperature, d) Interaction of alkaline pH and centrifuge temperature, e) Interaction of alkaline pH and isoelectric pH on fat absorption capacity of hemp protein

Based on the previous findings, the fat absorption capacity (FAC) of hemp protein was modeled using the following Equation (5):

$$Y = 5.29011 - 0.05A + 0.24B - 0.07C + 0.205D + 0.1375AB - 0.075AC + 0.125AD + 0.2625BC + 0.175CD - 0.317681A^2 + 0.382319B^2 - 0.317681C^2 + 0.307319D^2$$

Where Y = Fat absorption capacity of hemp protein (g oil/g protein), A = Solid-to-solvent ratio (g/mL), B = Isoelectric pH, C = Alkaline pH, D = Centrifugation temperature (°C).

This equation quantitatively describes how the interaction of these variables influences the oil-binding capacity of hemp protein.

Table 2. Comparison of experimental and predicted data

Parameters	Predicted data	The average of the experimental data	p- value	Δ
Water-holding-capacity	2,985	2.866±0.04	0,98 ^{ns}	%4
Fat absorption capacity	6,036	5,966±0.04	0,92 ^{ns}	%1

Mean ±SD (n=3), ns: not significant

4.3. Evaluation of Water-Holding Capacity (WHC) of Hemp Protein

The results of this study demonstrated that the independent variables had a positive effect on the water-holding capacity (WHC) of hemp protein. As the independent variables particularly the solid-to-solvent ratio and isoelectric pH increased, the WHC of hemp protein improved. Additionally, the interaction effects between solid-to-solvent ratio × alkaline pH, Solid-to-solvent ratio × centrifugation temperature, and isoelectric pH × centrifugation temperature were all significant in influencing WHC. A study on the water-holding capacity of flaxseed proteins [18, 19] showed that protein hydration under alkaline pH conditions can enhance the protein chain length and hydration volume, ultimately reducing WHC. In another study on pea protein, it was found that a greater number of hydrophilic groups exposed at alkaline pH led to easier hydration but actually reduced WHC compared to hemp

proteins [20]. This difference is attributed to the spherical protein matrix of hemp, which is stabilized by disulfide bonds, allowing more polar groups to be retained within the structure, thereby increasing the WHC. Regarding the interaction between solid-to-solvent ratio and alkaline pH, higher alkaline pH caused further protein unfolding, exposing hydrophilic groups to water and increasing WHC. A similar pattern was observed with isoelectric pH at lower isoelectric pH values, intense protein aggregation limited the exposure of hydrophilic groups to water. Therefore, WHC was higher at isoelectric pH values closer to the upper limit, as illustrated in Figure 1a. The interaction between temperature and alkaline pH (Figure 1d) showed that higher pH combined with higher temperatures facilitated protein denaturation and unfolding, exposing hydrophilic groups and consequently increasing WHC [14]. When analyzing the interaction of solid-to-solvent ratio with isoelectric pH, it was observed that more diluted ratios (e.g., 1:20) combined with higher isoelectric pH (5) reduced protein aggregation and increased the exposure of polar groups to water leading to

higher WHC. Simultaneously, the combined effect of solid-to-solvent ratio and alkaline pH indicated that at higher alkaline pH (11.5) and intermediate to lower solid-to-solvent ratios (1:15–1:20), protein denaturation enhanced structure opening and accessibility to hydrophilic groups. Furthermore, centrifugation at lower temperatures (4°C) in combination with a diluted solid-to-solvent ratio prevented protein re-aggregation, forming a swollen and stable protein matrix suitable for water retention. These findings align with the results reported by Nasrollahzadeh et al. (2022) and Struik et al. (2000), confirming the effectiveness of these conditions for optimizing WHC in hemp proteins [14, 20].

4.4. Evaluation of Fat Absorption Capacity (FAC) of Hemp Protein

The findings of this study revealed that the interactive effects of the independent variables led to an increasing trend in the fat absorption capacity (FAC) of hemp protein. Specifically, the interactions between Solid-to-solvent ratio \times Isoelectric pH, Solid-to-solvent ratio \times Alkaline pH, Solid-to-solvent ratio \times Centrifugation temperature, Alkaline pH \times Isoelectric pH, Alkaline pH \times Centrifugation temperature had a significant impact on FAC of the hemp protein isolate. The results indicated that FAC is highly dependent on pH changes, which affect the hydrophobic-hydrophilic balance of proteins. According to Malomo et al. (2014), protein interactions at pH 7.0 expose more binding sites for volatile compounds compared to pH 9.0, where the protein structure is more open but with fewer volatile-binding sites. This explains why the FAC of hemp protein isolates was relatively higher compared to their WHC under certain

pH conditions [21]. Due to the high protein content in hemp protein isolates, protein molecules, rather than insoluble polysaccharides, predominantly contribute to hydrophobic interactions with the lipid phase, thereby enhancing FAC [24]. Furthermore, the impact of extraction conditions on FAC aligns with the findings of Jiang et al. (2010) and Dapčević Hadnađev et al. (2019), who studied how extraction methods influence the FAC of hemp protein isolates. They reported that alkaline-extracted protein isolates exhibited higher FAC, likely due to partial denaturation of the protein during alkaline extraction, which increases surface hydrophobicity and thus improves FAC [25, 26]. Regarding the interaction between solid-to-solvent ratio and alkaline pH, higher alkaline pH levels resulted in greater protein unfolding, exposing hydrophobic groups and enhancing oil absorption. A similar pattern was observed with isoelectric pH, where lower isoelectric pH caused intense protein aggregation, reducing exposure of hydrophobic groups, while higher isoelectric pH improved FAC (as shown in Figure 2a). The interaction between temperature and alkaline pH (Figure 2d) also indicated that combining higher pH and higher temperatures increased the likelihood of protein denaturation and structural opening, leading to greater exposure of hydrophobic groups and, consequently, enhanced oil absorption. Therefore, the interaction of the solid-to-solvent ratio with pH and temperature variables played a determinant role in modulating the FAC of hemp protein isolates. Selecting a solid-to-solvent ratio of 1:15–1:17, combined with high alkaline pH and low centrifugation temperatures, provided optimal conditions for exposing non-polar groups and maximizing oil absorption [23, 24].

4.5. Conclusion

In this study, the water-holding capacity (WHC) and fat absorption capacity (FAC) of hemp protein isolate were evaluated and optimized using the Response Surface Methodology (RSM), aiming to maximize both capacities. The findings demonstrated that the alkaline extraction method significantly affected the functional properties of WHC and FAC in hemp protein isolate. The optimized conditions achieved WHC 2.985 g water/g protein, FA 6.036 g oil/g protein under the following parameters Solid-to-solvent ratio: 1:17, Isoelectric pH: 5, Alkaline pH: 11.5 and Centrifugation temperature 19°C. There was no significant difference ($p > 0.05$) between the predicted data from the software and the experimental results, confirming the model's validity. Hemp protein extracted via the alkaline method generally exhibits relatively high WHC due to its amphipathic structure and hydrophilic groups, as well as high FAC due to the presence of hydrophobic groups within its structure. Thus, optimizing these functional properties can enhance the potential of hemp protein isolate as a functional food ingredient suitable for various applications in the food industry.

Data Availability

The data used to support the finding of this study are available from the corresponding author upon request.

Conflict of Interest

The authors have no conflicts of interest to report.

Funding Statement

The researchers did not receive any specific grant from funding agencies the public, commercial or not-for-profit sectors.

Author contributions

Kimia Sahba contributed to the analysis, funding, resources, methodology, preparation of the main draft, writing– review & editing, and Nafiseh Zamindar contributed to conceptualization, project management, validation, software, writing, and review and editing.; All authors read and approved the submitted version.

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بررسی ظرفیت نگهداری آب و ظرفیت جذب روغن ایزوله پروتئین شاهدانه (*Cannabis sativa* L.) استخراج شده به روش قلیایی

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اسلامی؛ اصفهان؛ ایران

اطلاعات مقاله	چکیده
تاریخ های مقاله :	پروتئین ها هم در حفظ زندگی انسان و هم در محصولات غذایی نقش اساسی دارند و علاوه بر نقش مهمی که در سلامت و تغذیه دارند، دارای خواص عملکردی فراوانی هستند. پروتئین شاهدانه به عنوان منبع پروتئین گیاهی با ارزش غذایی عالی و خواص تکنولوژیکی- عملکردی متنوع شناخته می شود. حفظ تازگی و ظرفیت جذب روغن از عوامل کلیدی در کیفیت بسیاری از غذاها است. در این پژوهش تاثیر روش استخراج قلیایی بر پروتئین شاهدانه با استفاده از روش سطح پاسخ ارزیابی شد. اثر پارامترهای مستقل pH قلیایی (۱۱/۵، ۱۰، ۸/۵)، نسبت جامد به حلال (۱:۲۰، ۱:۱۵، ۱:۱۰)، pH ایزوالکتریک (۴، ۴/۵، ۵) و دمای سانتریفیوژ کردن (۴، ۱۴، ۲۴ °C) بر پارامترهای ظرفیت نگهداری آب و ظرفیت جذب روغن پروتئین شاهدانه بررسی شد. بیشترین جذب آب و روغن در شرایط pH قلیایی (۱۱/۵)، نسبت جامد به حلال (۱:۱۲)، pH ایزوالکتریک (۴) و دمای سانتریفیوژ (۴ °C) به ترتیب با مقدار ۲/۹۸۵ و ۶/۰۳۶ (گرم/گرم) با مطلوبیت ۹۸٪ ملاحظه شد. تفاوت معنی داری بین داده های تجربی و پیش بینی شده با استفاده از آزمون student. مشاهده نشد ($p > 0.05$) که نشانه کاربردی بودن این شرایط بهینه برای صنایع غذایی است.
تاریخ دریافت: ۱۴۰۳/۰۹/۰۸	
تاریخ داوری: ۱۴۰۳/۱۲/۱۸	
تاریخ پذیرش: ۱۴۰۴/۰۸/۱۹	
کلمات کلیدی:	
ظرفیت جذب روغن، ظرفیت نگهداری آب، ایزوله پروتئین شاهدانه، استخراج قلیایی.	
DOI: 10.48311/fsct.2026.83877.0	
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