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Optimising functional properties and chemical composition of *Pinus halepensis* Mill. Seeds protein concentrates

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A B S T R A C T

Vegetable proteins are widely used in many food formulations due to their physico-chemical properties, low cost and availability. The main objective of this work is to study the chemical composition and properties of a protein concentrate of *Pinus halepensis*. Mill seeds (PHPC) and mainly to optimize the effect of pH, NaCl concentration and phosphate buffer (PB) molarity on functional properties (solubility, emulsifying activity index (EAI) and foaming capacity (FC)) of this concentrate by response surface methodology (RSM). The chemical composition was determined in terms of proteins, sugars, lipids, ash and moisture. The physico-chemical characteristics were studied by their water and oil holding capacity (OHC, WHC) and their surface hydrophobicity (SH). Finally, the functional properties of PHPC were studied in terms of solubility, EAI, FC, minimum gelling concentration (MGC) and finally heat coagulability (HC). A PHPC yield of 36.66 ± 0.7% was obtained. The WHC and OHC was 3.89 g water/g PHPC and 3.54 g oil/g PHPC respectively and a SH of 87.09 ± 0.78 was obtained. The optimization results showed that the optimal conditions for solubility, EAI and FC were: pH:10.88, NaCl:0 g/l, PB:0.078 M; pH:12, NaCl:0.55 g/l, PB:0.1M and pH:2, NaCl:0, PB:0 M respectively, having given a solubility of 87.13 ± 0.14%, an EAI of 36.82 ± 0.34 and a FC of 182.72. Then, the desirability of the three responses (solubility, EAI and FC) which was pH:12, NaCl: 0.55 g/l and PB of 0.1M was used to assess the stability of EAI and FC, to determine the MGC and HC. This study shows that Aleppo pine seeds are a good source of functional proteins, potentially applicable in the food industry and that pH, NaCl concentration and PB molarity have a major influence on functional properties.

1. Introduction

Vegetable proteins are a very good alternative to animal proteins whether for food or cosmetic application, because of their low cost, abundance and diversity of their sources (legumes, cereals and oilseeds), their adequate quality and nutritional value, their ease of digestion, their non-toxicity and finally for their functionality (S. Damodaran, 2000, p. 384; Rodrigues, Coelho, & Carvalho, 2012; Soria-Hernández, Serna-Saldívar, & Chuck-Hernández, 2015).

In America, 60% of the population relies heavily on the protein content of food when choosing their product, because among the three primary metabolites (carbohydrates, proteins and fats), proteins are the most beneficial for their health. Most adults perceive proteins as the most energy-efficient ingredient that is very healthy and improves muscle tone. They are macronutrients most considered in weight management diets (Cheatham, 2014).

In recent years, oilseed proteins have made a very significant contribution to protein intake in the diet. In 2004/2005, 380 million tonnes of oleaginous plants were produced and 207 million tonnes of protein meals were produced worldwide (Ash, Dohlman, & Davis, 2006).

The most commonly used proteins of oleaginous origin are that of soybean, peanut and rapeseed for their functionalities in food processing (additives and the protein film industry). With the awareness of their usefulness and therefore the increase in needs, new sources have been developed, such as cashew nut (Ogunwolu, Henshaw, Mock, Santros, & Awonorin, 2009), milk weed (Hojilla-Evangelista, Evangelista, & Victor Wu, 2009) and almost all oilseeds. *Pinus halepensis* Mill seeds, come from

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a very abundant tree belonging to the Pinaceae family which can found on all the Mediterranean relief especially in Algeria and Tunisia (Maestre, Cortina, Bautista, & Bellot, 2003). The bible's manual of medicinal plants reports that they have been used extensively in pastry making, especially in Tunisia, and are also used to cure diabetes and sexual weakness in the eastern region of the Mediterranean (Schiller, 2014). Kadri et al. (2015) studied the chemical composition of its seeds and found a protein percentage of 26.62 ± 0.129 which is a very high percentage especially compared to other species *Pinus pinea* L., *Pinus pinaster* and *Pinus canariensis*.

Functional proteins are those that when added to food, confer nutritional, sensory, physico-chemical and organoleptic properties (color, texture, flavor …). Functional properties could be classified according to their physico-chemical mechanisms as follows: hydration properties (water/oil retention and solubility) rheological properties (viscosity, elasticity, aggregation and gelation), and protein surface properties (emulsifying and foaming activities, surface hydrophobicity and whipping) (Moure, Sineiro, & Domínguez, 2001).

Food applications of proteins is limited by their low solubility (Moure, Sineiro, & Domínguez, 2001), it is known that the pH, presence or absence of salts and its concentration and thus the ionic strength of the medium, as well as electrostatic repulsions influence the functional properties of proteins (Soria-Hernández et al., 2015). For this purpose and taking into account that according to the databases consulted, no studies were carried out on the functional properties of Aleppo pine seed proteins, the physico-chemical characteristics of *P. halepensis* Mill. Seed concentrated proteins (PHPC) (approximate composition, water and oil holding capacity (WHC and OHC), surface hydrophobicity (SH)) were determined and the solubility, emulsifying activity index (EAI) and foaming capacity (FC) conditions were optimized using the Box Behnken Design (BBD) by the response surface methodology, to study the effect of the parameters considered (pH, NaCl concentration and phosphate buffer molarity) on each of the responses and the relationship between solubility and functional activities. Then the heat coagulability (HC) and the minimum gelling concentration (MGC) were determined under optimal conditions.

2. Material and methods

2.1. Plant material

The seeds of Aleppo pine (*Pinus halepensis* Mill.) were obtained from the Collo forest located in Skikda province of Algéria in May 2018. They were cleaned with bidistilled water; dried in an oven at 40 °C for 2 days and then finely crushed using an electric grinder (KIKA Labortech-nik M20, Germany) until it became a fine powder (<250 μm) which was delipidated by the Soxhlet method with petroleum ether.

2.2. Preparation of the protein concentrate

A mass of 10 g of delipidated powder was macerated under stirring for 20 min at room temperature. After filtration and centrifugation at 4 °C for 20 min at 6000 rpm, the supernatant was filtered again and its pH was adjusted to 6 (with 0.1 M HCl), $CaCl_2$ was added gradually until a concentration of 1 M, then centrifuged at 6000 rpm for 20 min. Finally, the recovered pellet was washed with distilled water and freeze-dried (Rotimi E Aluko, McIntosh, & Katepa-Mupondwa, 2005).

2.3. Chemical composition and yield

The extraction yield was expressed by the ratio of the amount of extract to the amount of defatted powder used. Ash, moisture and fat were determined according to AOAC (1998) methods, the protein content was determined by the Bradford (1976) method and the carbohydrates content was carried out by the Dubois, Gilles, Hamilton, Rebers, and Smith (1956) method using BSA and glucose for calibration curves respectively.

2.4. Water and oil holding capacity (WHC/OHC)

The Tan, Ying-Yuan, and Gan (2014) method was used to determine the capacity of the extract to retain water or oil. For this purpose, 100 mg of extract was suspended with the same amount of water or sunflower oil (1.5 ml), vortexed for 1 min, and then centrifuged at 3000 rpm for 20 min. The water and oil retention capacity was expressed in gram of water or oil retained per gram of extract.

2.5. Surface hydrophobicity (SH)

The bromophenol blue (BPB) binding method was used to study the surface hydrophobicity of the protein concentrate. A volume of 1 ml of protein concentrate suspension (5 mg/ml in 20 mM PB at pH 7) was added to 200 μl of BPB solution (1 mg/ml in distilled water). The mixture was vortexed for 10 min and directly centrifuged at 3000 rpm for 15 min. Finally, the supernatant absorbance was read at 595 nm. A control containing 1 ml of PB (20 mM, pH 7) and 200 μl of BPB solution was used (Mune & Sogi, 2016). The surface hydrophobicity is according to the following formula:

SH (%) = BPB bound (%) = (Absorbance control – Absorbance sample)/ Absorbance control X 100

2.6. Optimization of solubility conditions of PHPC and its functional properties

Before optimization of solubility conditions, three parameters (pH, NaCl concentration and PB molarity) were studied separately in the single-factor experiment, keeping the variables that were not studied constant each time in order to limite overall experimental work. After statistical analysis of the results of this preliminary study, three variables were selected as significant factors and three levels were used for each one. The study intervals were also determined for each parameter and then the response surface based on the Box Behnken Design was designed to obtain the conditions giving the best solubility, EAI and FC.

2.6.1. Protein solubility

A mass of 100 mg of PHPC was dispersed in 10 ml of different solutions prepared at the pH, NaCl concentration and molarity of PB determined according to the design of experiment. The dispersions were vortexed well for 15 min then centrifuged at 3000 g for 20 min. The protein content of the supernatant was determined by the Lowry method (Peterson, 1977) and the solubility was calculated as follows:

Solubility (%) = Protein content of supernatant x 100 /Total protein content

Total protein content represents 100% solubility and is determined in 3% NaOH (Chao, Jung, & Aluko, 2018).

2.6.2. Emulsifying properties

The emulsifying properties of the PHPC were determined using the method reported by Boye et al. (2010). A volume of 45 ml of protein solution (0.5% in different solution of pH, NaCl and PB) was added into 15 ml of sunflower oil. After homogenization of the emulsion with an ultra turrax (IKA T25, Staufen, Germany) for 1 min at 20,000 rpm, 50 μL of the prepared solution were diluted in 5 ml of sodium dodecyl sulfate (SDS) at 0.1%. Finally, the absorbance was recorded at 500 nm. The EAI was calculated using following equation:

$$EAI\ (m^2/g) = 2 \times 2303 \times A_0 \times DF\ /C \times \phi \times 10{,}000$$

where A_0 is the absorbance of the emulsion after emulsification, DF is the dilution factor, C is the weight of the protein per volume (g/mL), ϕ is

the volume fraction of the oil in the emulsion.

2.6.3. Foaming properties

Foaming capacity of PHPC was determined according to the method of Shahidi, Han, and Synowiecki (1995). A volume of 20 ml of protein concentrate solution at 0.1% (W/V) was homogenized using a Moulinex_R62 homogenizer to incorporate the air for 1 min at room temperature (25 ± 1 °C). The FC was expressed as percentage of volume increase after homogenization, which was calculated according to the following equation:

FC (%) = ((volume after whipping−volume before whipping) / volume before whipping) × 100

2.6.3.1. Experimental design. To optimize the factors affecting solubilization, foam capacity and emulsifying activity, the response surface methodology (RSM) with Box Behnken Design was studied using Minitab 17 (statistical analysis system Inc., SAS) software and the experimental values obtained for solubility, EAI and FC were compared to their values predicted based on the *t*-test ($p < 0.05$) (Table 8). In this study, fifteen tests were performed with the different values of pH (2, 7, 12), NaCl concentration (0, 0.275, 0.55 g/ml), and PB concentration (0, 0.05, 0.1 M) as shown in Table 2. The values were coded as follows: (+1) maximum value, (0) central value and (−1) minimum value. The experimental data were adjusted to a second order polynomial model and expressed by following equation:

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X_i^2 + \sum_{i>jk}^{k} B_{ij} X_i X_j$$

B_0 (constant coefficient); Bi, Bii, Bij (regression coefficients for intercepting, linear, quadratic and interaction terms, respectively); xi and xj (independent variables); k (number of optimized factors).

2.6.3.2. Validation of model. In order to draw conclusions from the validation of the model, the Minitab software provides the optimal conditions of the three factors (pH, NaCl, PB) from the three responses designed. The optimums responses obtained were used to test solubility, FC and EAI. Finally, the experimental optimums of each obtained response were verified by comparing them with the predicted values.

After optimization and validation of the experimental design, a compromise solution was obtained by using the desirability function. The desirability is an important function when multiple response optimization was carried out because it's not possible to optimize each one in separate way. For that, the overall solution must be included in optimal region leading to a certain degree of compliance with the proposed criteria for each variable of the system; namely, a compromise solution must be found. Desirability (d) always takes values between 0 and 1, where D = 0 for an undesirable response, and d = 1 represents a completely desirable value (Candioti, De Zan, Camara, & Goicoechea, 2014). The stability of the functional properties studied (FC and EAI) after 15, 30, 45 and 60 min and other functional properties (MGC and HC) were tested at the optimal conditions obtained by desirability.

Table 1
Solubility, emulsifying activity index and foaming capacity of PHPC on distilled water.

Functional properties	
Solubility	27.02 ± 0.52
EAI	20.89 ± 0.24
Foaming capacity	61.66 ± 0.66

Data are the mean ± SD of three analyses.

2.7. Stabilisation of foaming and emulsifying properties

To study the kinetic of the foaming and emulsifying activities depending on the time (15, 30, 45 and 60 min), the PHPC solution was prepared with the optimum of pH, NaCl and PB concentrations. The stability is expressed as a percentage of remaining of these two properties (Boye et al., 2010; Shahidi et al., 1995).

2.8. Minimum gelling concentration (MGC)

The method of O'Kane, Vereijken, Gruppen, and Van Boekel (2005) was used to determine the MGC with a slight modification. A volume of 5 ml of PHPC solution was prepared at the concentrations of 4–18% (w/v) and then heated in water bath at 95 °C for 10 min (in sealed tubes to avoid evaporation). After cooling, the tubes were placed at 4 °C for 12 h and then inverted. The MGC is the smallest concentration from which the contents of the inverted tube do not flow.

2.9. Heat coagulability (HC)

For Heat Coagulability (HC), the solubility method described above was used, the suspension of PHPC under optimal conditions was vortexed and the proteins of the supernatant were measured by the Lowry method. An aliquot of the supernatant was heated in a water bath at 100 °C for 30 min. After cooling and centrifugation at 3000 rpm for 15 min, a filtration was carried out on Whatman No. 2 filter paper, and concentration of proteins in the filtrate were again determined by the same method (Voutsinas, Nakai, & Harwalkar, 1983). The HC of the sample was calculated from the following equation:

% Heat Coagulability = Ps – Pf / Ps × 100

where:

Ps = % protein in supernatant
Pf = % protein in filtrate

3. Results and discussion

3.1. Proximate composition

As shown in Table 3, the extraction yield of PHPC was 36.66 ± 0.7%, of which approximately 69.33 ± 0.3% are proteins. Among the impurities, we found sugars representing 2 ± 0.2%, which can be justified by the presence of glycoproteins also reported by Kadri et al. (2015) and minerals (ash) found in the proportion of 4.9%. However, the lipids were found only in trace form, which confirms the good delipidation of the powder before extraction. The moisture test revealed a level of 2.4 ± 0.2% which is comparable to lyophilized extract dried by other methods such as Bambara concentrate in which the moisture content is of the order of 4% (Adeleke, Adiamo, & Fawale, 2018).

3.2. Water and oil holding capacities (WHC/OHC) and surface hydrophobicity

3.2.1. Water and oil holding capacities (WHC/OHC)

The Water and oil holding capacities of PHPC were evaluated and the results are represented in Table 4. The amount of water and oil that binds BPB depends on the polar and non-polar, ionized or deionized groups of proteins (Ghribi et al., 2015) and these properties mean that these proteins can be used as an additive to improve food quality (Tontul, Kasimoglu, Asik, Atbakan, & Topuz, 2018).

In our study, the WHC of PHPC was found at 3.89 g water/g PHPC (Table 4). This is in agreement with the WHC range of products with water retention capacity (1.49–4.71) (Kaur & Singh, 2007). This capacity can be explained by the large particle size of the extract as well as

Table 2
Box–Behnken design matrix and experimental and predicted data.

Run						pattern				
Run	pattern	Variables			Solubility	Pred Formula solubility	EAI	Pred Formula EAI	FC	Pred Formula FC
1	0−−	pH (X1)	C NaCl (X2)	PB M(X3)						
2	−0−	7	0	0	28	33.9310905	21.37	21.9767438	61.66	60.40375
3	+0−	2	0.275	0	44.11	49.4575682	24.177	24.515224	91.66	91.6725
4	0+−	12	0.275	0	26.14	24.5587616	18.731	19.3399277	50	62.485
5	−−0	7	0.55	0	21.4	13.3703086	23.84	22.1893426	16.66	2.08875
6	+−0	2	0	0.05	87.04	77.4362757	26.6	25.4061574	123.33	122.91375
7	000	12	0	0.05	81.6	77.4337243	27.71	26.7413426	106.66	102.03125
8	000	7	0.275	0.05	27.54	29.5491319	19.13	19.018366	41.66	41.66
9	000	7	0.275	0.05	29.12	29.5491319	18.84	19.018366	41.66	41.66
10	−+0	7	0.275	0.05	29.52	29.5491319	19.79	19.018366	41.66	41.66
11	++0	2	0.55	0.05	43.08	46.7025309	34.48	35.4261389	58.33	64.56875
12	0−+	12	0.55	0.05	57.4	67.5474691	24.14	25.3563611	93.33	93.74625
13	−0+	7	0	0.1	36.11	43.9489095	20.27	21.8257562	31.66	39.57125
14	+0+	2	0.275	0.1	21.18	24.406169	28.74	27.6611376	75	62.505
15	0++	12	0.275	0.1	73.85	70.1473624	24.91	24.1018413	100	99.9875

C NaCl: concentration of NaCl; PBM: Phosphate buffer molarity; EAI: emulsifying activity index; FC: foaming capacity. The codded values were (+): maximum value, (0): central value and (−): minimum value.

Table 3
Proximate chemical composition of PHPC.

	Rate (%)
yield	36.66 ± 0.7
Ash	4.9 ± 0.3
moisture	2.4 ± 0.2
proteins	69.33 ± 0.3
carbohydrates	2 ± 0.02
fats	–

Data are the mean ± SD of three analyses.

Table 4
Water and oil holding capacities (WHC/OHC) and surface hydrophobicity of PHPC.

Parameters	
Water holding capacity (g of water/g of PHPC)	3.89 ± 0.06
Oil holding capacity (g of oil/g of PHPC)	3.54 ± 0.02
Surface hydrophobicity (%)	87.09 ± 0.78

Data are the mean ± SD of three analyses.

the capacity of the sugar and fibers found in the concentrate as impurities which are known for this capacity (Zhao et al., 2012). The capacity of this extract is greater than that of most protein extracts reported by the bibliography, for example, WHC chickpea protein concentrates were found at 3.65 for freeze-dried extract and in agreement with that found for rapeseed protein isolate (3.85 g water/g extract) (Yoshie-Stark, Wada, Schott, & Wäsche, 2006). Therefore, this extract can be used as an additive for viscous foods (Aletor, Oshodi, & Ipinmoroti, 2002).

The oil retention capacity was found in 3.54 g oil/g extract (Table 4), This is in the range of literature values (1.1–4.1) (Kaur et al., 2007). This good capacity can be explained by the hydrophobic properties of PHPC and the non-polarity of the side chains of its amino acids. This extract can therefore be used as an additive to confer an organoleptic quality to a fatty food such as dairy products (Ghribi et al., 2015).

3.2.2. Surface hydrophobicity (SH)

The SH informs us about the surface-active properties of the extract. Table 4 shows that SH of PHPC studied by binding to the BPB has been found in order of 87.09 ± 0.78%. It is higher than that found by Tontul et al. (2018) (60.98%), this means that our PHPC can have promising surface-active properties.

3.3. Optimization by RSM

3.3.1. Model analysis

Combination of the three studied factors (pH, concentration of NaCl and PB molarity) and the value of the corresponding response obtained in different experiments were presented in Table 2. It indicated that solubility was ranged from 21.18 to 87.04, the EAI from 18.84 to 34.48, while FC varied from 16.66 to 123.33. The values of the experimental results are consistent with the predicted values for the three responses.

3.3.2. Analysis of response surface

RSM based on BBD was applied to disclose optimal levels for the studied parameters (pH, NaCl concentration and PB molarity). Surface response models were the best method which illustrates the effects of independent variables and their interactions on the solubility of PHPC, their emulsifying activity and their foaming capacity. Experimental data were fitted to second order polynomial model.

Table 5
Analyze of variance (ANOVA) for the experimental results obtained by solubility.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	7	0,018057	0,002580	27,04	0,0002
Linear	3	0,002770	0,000923	9,68	0,0075
X1	1	0,000662	0,000662	6,93	0,0344
X2	1	0,001539	0,001539	16,13	0,0053
X3	1	0,000570	0,000570	5,98	0,0440
Square	3	0,009962	0,003321	34,81	0,0001
X1X1	1	0,005900	0,005900	61,84	0,0006
X2X2	1	0,001645	0,001645	17,24	0,0044
X3X3	1	0,002002	0,002002	20,99	0,0032
Interaction	1	0,005325	0,005325	55,82	0,0003
X1X3	1	0,000668	0,005325	55,82	0,0006
Error	7	0,000644	0,000095		
Lack of fit	5	0,000024	0,000129	10,83	0,0873
Pure error	2	0,018725	0,000012		
Total	14				
S	**0.0097674**				
R-sq	**0.9643**				
R-sq (adj)	**0.9287**				

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

3.4. Solubility

3.4.1. Analyze of the model of solubility

In Table 5, it has been shown that for solubility, all linear parameters have been significant; X1 and X3 ($p < 0.05$) and X2 ($p < 0.01$) therefore highly significant. Their quadratic parameters are also very highly significant ($p < 0.01$), as well as for the quadratic parameter X1, X3. However, all other parameters are not significant ($p > 0.05$) and the only significant interaction parameter is the X1X3 ($p < 0.01$). Taking into account only the significant parameters with $p < 0.05$, the predictive equation has been deduced.

$$\text{Solubility} = -0.18664 + 0.00909\ X1 - 0.01387\ X2 + 0.00844\ X3 + 0.03997 \times 1 \times 1 + 0.02111 \times 2 \times 2 - 0.02329 \times 3 \times 3 + 0.03649 \times 1 \times 3$$

Table 5 shows also, the variance analysis of the experimental results. The F value of the model was 27.04, this being said that the model is significant. The determination coefficient (R^2) was 0.9643 which means that only 3.57% of the variations could not be explained, and that 96.43% were attributed to the independent variables of solubility of PHPC.

However, the value of R^2 is not always synonymous with a good regression model, it must be comparable to the adjusted R^2, which is verified in our case as shown in Table 5 (adjusted $R^2 = 0.9287$). In addition, the value of lack of fit was 0.087 (whose value must be insignificant ($p > 0.05$) compared to the pure error). Finally, the low value of S which is the standard variation error (0.009) implies that the values obtained are close to the adjusted line. All these values and significance indicate that this model is well and truly validated and that it could work for the prediction of the solubility of PHPC.

3.4.2. Response surface of solubility

Fig. 1 (A, B, C) shows the three dimensional response surface profiles of multiple non-linear regressions of PHPC solubility.

The solubility depends mainly on pH because the linear and quadratic effect are significant and highly significant with ($p < 0.001$ and $p < 0.05$) respectively. As mentioned above, the three parameters studied (pH, NaCl and PB) are factors that significantly influence solubility and the interaction effect between pH and PB is also significant. A slight increasing of solubility was noted with increasing of pH value and decreasing then subsequently increasing of the NaCl concentration value. However, the solubility reaches its maximum when NaCl concentration decreases slightly and PB M increases significantly. On the other hand, it is result from the decreasing of pH value and very significant increasing of PBM, the increasing and then decreasing of solubility (Fig. 1 A, B, C). This is perfectly in agreement with the results of the preliminary study. The solubility of PHPC can be improved by varying the pH values; it increases as pH approaches the extreme pH values of 2 and 12. In general, as the pH increases, solubility decreases until it reaches the isoelectric domain, then increases. Because at pHs close to isoelectric pH, electrostatic repulsive forces favour the aggregation of proteins. The large volume of the aggregate and the bulk density therefore lead to the precipitation of these proteins and prevent their solubilization. However, at extreme pHs (far from the isoelectric domain) Electrostatic repellent forces help to separate positively charged proteins and increase interactions between them and the solvent (Mao & Hua, 2012). Protein solubility profile as a function of pH is used as an indicator of protein functionality, since functional properties are directly related to solubility (Ortiz & Wagner, 2002). Our results are similar to those found for the protein extracts from other plant matrices, as demonstrated by Hu et al. (2017) for walnut protein concentrate (Tontul et al., 2018), for chickpea protein isolate and Chao et al. (2018) for pea isolate.

Solubility also depends strongly on NaCl concentration but with a negative effect and very significant linear and quadratic effects ($p < 0.01$). The preliminary study showed that the salt concentration increases the solubility of the protein extract to the concentration of about 0.280 g/l and then decreases it to the minimum at 0.55 g/l, this result is very logical and can be explained by the phenomenon of salting in and salting out found by several authors such as Deng et al. (2011) for the protein isolate of *Ginkgo biloba* seeds. The optimum occurred without NaCl can be explained by the fact that at this pH and this molarity in phosphate buffer, the solubility reaches its maximum and the addition of NaCl does not affect it and this is confirmed by the very high significance of these two parameters interaction. These phenomena depend also on the conformational differences characteristic of proteins (Hu et al., 2017) and effect of NaCl on the ionic strength of the medium (Inyang & Iduh, 1996). Further, salts play an important role in a protein medium on the solubility of the protein, they reduce the charge of counter ions and therefore both those of attractions and electrostatic repulses (Bau, Mohtadi-Nia, Lorient, & Debry, 1985).

It was found that the molarity in PB has also a positive influence on solubility because its linear effect is significant ($p < 0.05$) and its quadratic effect is highly significant. This confirms the results of the preliminary study where we found that more the molarity of the phosphate buffer increases, more soluble the PHPC are at lower pH in the range studied. This may be due to the fact that the phosphate buffer on the stability of the protein (Pikal-Cleland, Rodríguez-Hornedo, Amidon, & Carpenter, 2000). Therefore the higher the molarity the more stable the protein is and therefore more soluble.

For this model, the optimal solubility conditions are: pH: 10.88; NaCl: 0; PB: 0.078. The protein extract was solubilized under these conditions and the actual solubility obtained was 87.13% ± 0.14 against a predicted value of 87.113% whose difference is not significant ($p < 0.05$). This solubility value is much better than the solubility obtained by dispersing these proteins in distilled water which was 27.02 ± 0.52 (Table 1).

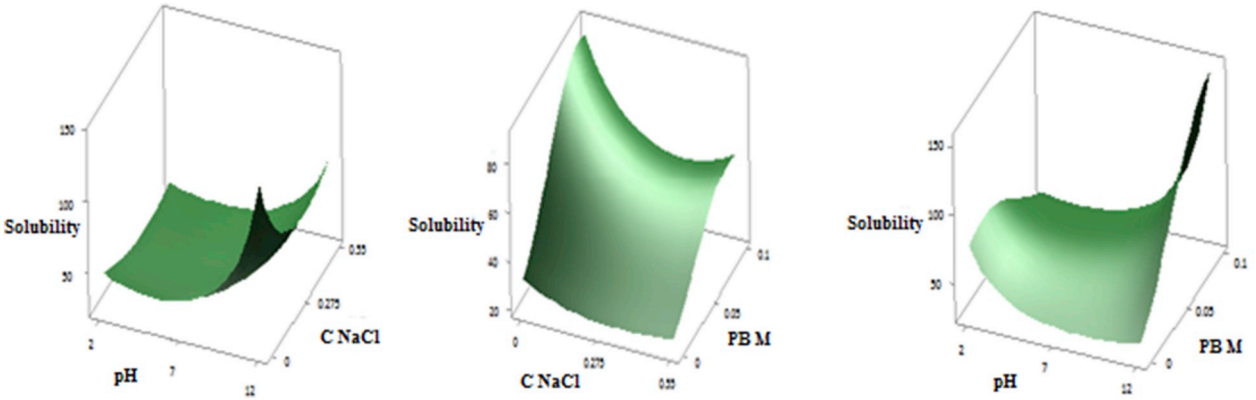

Fig. 1. Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on solubility of PHPC.

3.5. Emulsifying activity index

3.5.1. Analyze of the model of emulsifying activity index

For the emulsifying activity index, it has been shown that all linear parameters, their quadratic and their interactions were very highly significant ($p < 0.001$). The final predictive equation was obtained as follows:

$$(EAI\text{^}\lambda\text{-}1)/(\lambda \times g\text{^}(\lambda\text{-}1)) = 4.058 - 2.961\ X1 + 2.903\ X2 + 2.395\ X3 + 4.845 \times 1 \times 1 + 4.969 \times 2 \times 2 - 4.316 \times 1 \times 2 + 2.495 \times 2 \times 3$$

($\lambda = 3$; $g = 23{,}8189$ is the geometric mean of EAI).

Table 6 shows the variance analysis of the experimental results of EAI. The F value of the model was 53.18, this being said that the model is significant. The determination coefficient (R^2) was 0.9815 which means that only 1.85% of the variations could not be explained, and that 98.15% were attributed to the independent variables of emulsifying activity index of PHPC and the value of adjusted R^2 (0.9631) is quite close to R^2.

In addition, the value of lack of fit was 0.06 which is not significant. Finally, the standard variation error was 1,09919. All these values and significance indicate that this model is well and truly validated and that it could work for the prediction of the emulsifying activity of PHPC.

3.5.2. Response surface of emulsifying activity index

Fig. 2 (A, B, C) shows the three dimensional response surface profiles of multiple non-linear regressions of emulsifying activity index of PHPC. The parameters pH, NaCl and PB have significant effects ($p < 0.05$) on emulsifying activity, and the interaction effects between pH and PB, pH and NaCl as well as PB and NaCl are also significant ($p < 0.05$). For this activity, the EAI is maximal when NaCl concentration increases significantly and pH decreases and then increases. While the EAI increases slightly despite the significant increasing of PBM and NaCl concentration. Finally, the EAI increases very significantly when pH decreases significantly and PBM increases significantly (Fig. 2 A, B, C). Such as solubility, the emulsifying activity is also pH dependent because the linear and quadratic effects are very highly significant ($p < 0.001$) and it affects it in the same way as solubility (high activities at extreme pH and lower activities at neutral pH levels), as demonstrated by several authors such as Hu et al. (2017), Inyang, et al. (1996) and Tontul et al. (2018). The pH has also greatly influenced the emulsifying activity, this activity depends on the hydrophilic-lipophilic balance (Wu, Wang, Ma, & Ren, 2009) which in turn depends on the pH. At the oil-water interface, the lipophilic protein molecules are directed towards the lipid phase (oil) and the hydrophilic molecules towards the aqueous phase. The surface tension is thus reduced. At pH levels close to pHi (where protein solubility is reduced), protein adsorption is controlled by diffusion. Which is not the case at extreme pH levels (better protein solubility), the activation energy barrier does not allow protein migration to give way to diffusion, so protein solubility allows for improved interactions between the oil phase and the aqueous phase (Mao et al., 2012).

Although, most authors reported a more pronounced effect at basic pHs than at Acid pHs, contrary to the results obtained, whose optimum has been found at acid pH which can be explained by the interaction effect between pH and NaCl which is very highly significant ($p < 0.05$).

The same observation for the salt concentration, which also affects emulsifying activity with a very high significance ($p < 0.001$) and with a positive effect, the higher the salt concentration the higher the emulsifying activity increases. Our results are in agreement with those reported by other authors such as Deng et al. (2011), Hu et al. (2017) and (Inyang et al., 1996). The effect of salt on emulsifying activity could be due to its ability to form charged layers around the oil drips, which would promote repulsion between the droplets dispersed in the emulsion (Hu et al., 2017). The phosphate buffer affects positively the emulsifying activity with a very high significance.

The optimal emulsifying activity conditions for this model are: pH: 2; C NaCl: 0.55 g/l; PB: 0.1 M the EAI of the protein extract has been investigated under these conditions and the actual EAI obtained was 36.82 ± 0.34 against a predicted value of 36.65 whose difference is not significant. These optimal conditions have significantly improved the emulsifying activity of PHPC compared to its activity in distilled water (20.89 ± 0.24) (Table 1).

Table 6
Analyze of variance (ANOVA) for the experimental results obtained by Emulsifying activity.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	7	449.795	64.2564	53.18	0.0001
Linear	3	183.440	61.1468	50.61	0.0004
X1	1	70.119	70.1186	58.03	0.0001
X2	1	67.419	67.4188	55.80	0.0003
X3	1	45.903	45.9031	37.99	0.0005
Square	2	166.959	83.4796	69.09	0.0001
X1X1	1	87.187	87.1870	72.16	0.0002
X2X2	1	91.694	91.6936	75.89	0.0005
Interaction	2	99.395	49.6976	41.13	0.0001
X1X2	1	74.496	74.4961	61.66	0.0002
X2X3	1	24.899	24.8990	20.61	0.0033
Error	7	8.457	1.2082		
Lack of fit	5	8.251	1.2082	16.01	0.0600
Pure error	2	0.206	0.1031		
Total	14	458.252			
S	**1.09919**				
R-sq	**0.9815**				
R-sq (adj)	**0.9631**				

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

3.6. Foaming capacity

3.6.1. Analyze of the model of foaming capacity

The results of the analysis of variance of the pH effect, NaCl and PB on foaming capacity are represented in Table 7.

The results show that for linear parameters, only X2 is significant ($p < 0.01$). For quadratic parameters, X1X1 is very highly significant and X3X3 is significant, and finally the significant interaction parameters are X1X3 and X2X3. The equation of prediction was as follows:

$$FC = 3.7295 + 0.0008\ X1 - 0.2806\ X2 + 0.0518\ X3 + 0.8413 \times 1 \times 1 + 0.0492 \times 2 \times 2 - 0.2327 \times 3 \times 3 + 0.1538 \times 1 \times 2 + 0.2234 \times 1 \times 3 + 0.3137 \times 2 \times 3$$

The model of FC as shown in Table 7 is significant at F value of 19.763. The R-sq is at 0.9726 which means that only 2.74% of the variation could not be explained by the model and then 97.26% were attributed to the independent variables used. This value of R-sq is very comparable to the value of R-sq adjusted (0.9234). Finally, the value of S is also very low (0.1555). However, for this response (FC) the value of lack of fit was significant ($0.012 < 0.05$) but this does not prevent the validation of the design given the validity of the other R^2 and adjusted R^2 parameters as well as S. Moreover, several authors have demonstrated that the significance of the value of lack of fit does not necessarily invalidate the design because it can be due to the value of the pure error which can be very small or zero due to the accuracy of the repeat measurements (Ahmad, Yusup, Bokhari, & Kamil, 2014; Bashir, Aziz, Yusoff, & Adlan, 2010; Marković et al., 2018). According to the obtained results, we can say that this model can be used for prediction of the effect of pH, NaCl and PB molarity on FC.

3.6.2. Response surface of foaming capacity

Fig. 2 (A, B, C) shows the three dimensional response surface profiles of multiple non-linear regressions of Foaming capacity of PHPC. The NaCl parameters have a significant effect on foaming activity, and the interaction effects between pH and PB and PB and NaCl are also

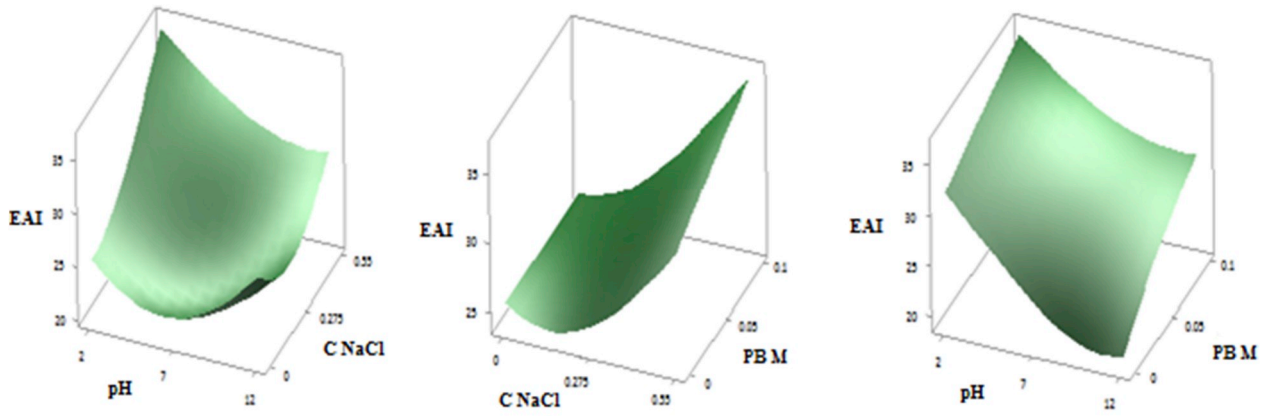

Fig. 2. Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on emulsifying activity (EAI) of PHPC.

Table 7
Analyze of variance (ANOVA) for the experimental results obtained by foaming capacity of PHPC.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	9	4.31315	047924	19.76	0.0023
Linear	3	0.65130	0.21710	8.95	0.0192
X1	1	0.00001	0.00001	0.00	0.9895
X2	1	0.62985	0.62985	25.97	0.0048
X3	1	0.02144	0.02144	0.88	0.3904
Square	3	2.97391	0.99130	40.87	0.0015
X1X1	1	2.97391	2.61342	107.74	0.0004
X2X2	1	0.00892	0.00892	0.37	0.5715
X3X3	1	0.19986	0.19986	8.24	0.0358
Interaction	3	0.68794	0.22931	9.45	0.0177
X1X2	1	0.09463	0.09463	3.90	0.1055
X1X3	1	0.19970	0.19970	8.23	0.0353
X2X3	1	0.39361	0.39361	16.23	0.0105
Error	5	0.12129	0.02426		
Lack of fit	3	0.00000	0.04043		0.0120
Pure error	2	4.43444	0.00000		
Total	14				
S	**0.1555747**				
R-sq	**0.9726**				
R-sq (adj)	**0.9234**				

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

Table 8
Regression coefficient, standard error, and *t*-test results of response surface for solubility, EAI and FC.

	Regression coefficients	Standard error	t- value	P- value
Solubility				
Constant	−0,18664	0,00564	−33,10	0,0002
X1	0,00909	0,00345	2,63	0,0344
X2	−0,01387	0,00345	−4,02	0,0053
X3	0,00844	0,00345	2,44	0,0440
X1X1	0,03997	0,00508	7,86	0,0001
X2X2	0,02111	0,00508	4,15	0,0044
X3X3	−0,02329	0,00508	−4,58	0,0032
X1X3	0,03649	0,00488	7,47	0,0003
EAI				
Constant	4058	0,528	7,68	0,0001
X1	−2961	0,389	−7,62	0,0001
X2	2903	0,389	7,47	0,0003
X3	2395	0,389	6,16	0,0005
X1X1	4845	0,570	8,49	0,0002
X2X2	4969	0,570	8,71	0,0005
X1X2	−4316	0,550	−7,85	0,0002
X2X3	2495	0,550	4,54	0,0033
FC				
constant	3,7295	0,0899	41,48	0,0023
X1	0,0008	0,0551	0,01	0,9895
X2	−0,2806	0,0551	−5,10	0,0048
X3	0,0518	0,0551	0,94	0,3904
X1X1	0,8413	0,0811	10,38	0,0004
X2X2	−0,0492	0,0811	0,61	0,5715
X3X3	−0,2327	0,0811	−2,87	0,0358
X1X2	0,1538	0,0779	1,98	0,1055
X1X3	0,2234	0,0779	2,87	0,0353
X2X3	0,3137	0,0779	4,03	0,0105

EAI: emulsifying activity index; FC: foaming capacity.

significant. The foaming capacity depends also on the pH with a very highly significant quadratic effect. On the concentration of NaCl with a very highly significant linear effect, and on the phosphate buffer with a significant quadratic effect, the interaction effects are significant for the pH-PBM and NaCl-PBM parameters. The value of FC decreases after significant decreasing of pH and slightly increasing of NaCl concentration. When NaCl concentration decreases very significantly and PBM increases, the FC increases slightly then quickly decreases. At last, the FC is maximal with significant decreasing of pH value and decreases with increasing of pH at the same time that PBM (Fig. 3 A, B, C). The effect of pH and NaCl on FC has also been reported by several authors (Hu et al., 2017; Inyang et al., 1996). Foaming capacity is highly related to solubility and Kinsella (1979) reported that only soluble proteins contribute to the formation of foams. This explains the better capacity at extreme pH (pH = 2) but ionic forces could depress the foams by reducing the coulombic forces of the polypeptides of protein molecules (Altschul & Wilcks, 1985).

The optimal FC conditions for this model are: pH: 2; NaCl: 0 g/l; PBM: 0 M the FC of the protein extract has been investigated under these conditions and the actual FC obtained was 183.55 ± 2.03 against a predicted value of 182.72 with no significant differences ($p < 0.05$). The foaming capacity obtained under these optimal conditions is much higher than that obtained in distilled water (61.66 ± 0.66) (Table 1).

The optimal conditions were determined by maximizing desirability using the Minitab prediction profiler. In order to verify the predictive capacity of the model, the results of the maximized conditions were used for a solubility test of the PHPC, their EAI and their FC. The optimal conditions obtained were: pH 12, NaCl concentration 0.55 g/l. molarity in PB 0.1 M. The experimental values for solubility, EAI and FC were 78.07 ± 0.98, 30 ± 0.52, 110 ± 2 respectively with composite desirability value of 0.77, intermediate values of desirability between (0–1) indicate more or less desirable response (Candioti et al., 2014). These experimental results were in agreement with the predicted values corresponding to 77.4, 30.05 and 111 respectively (no significant difference).

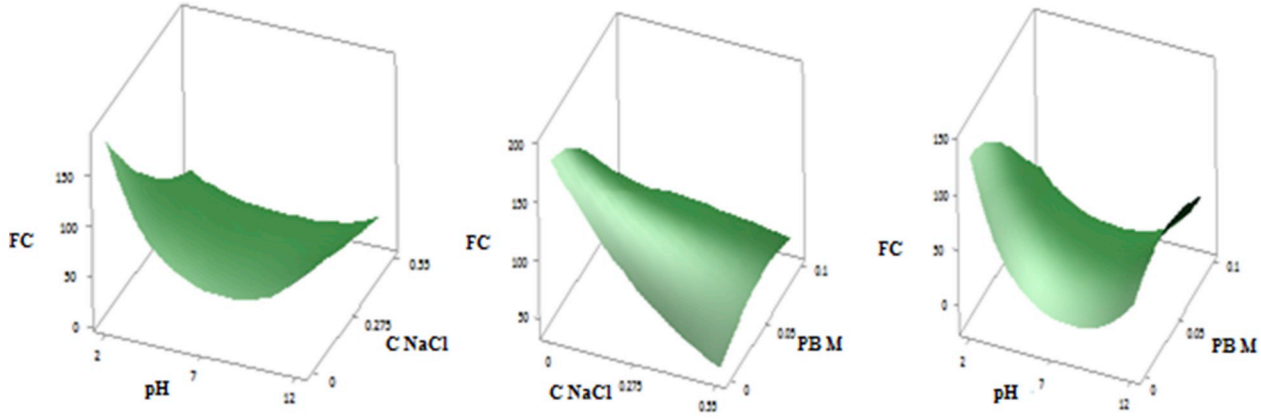

Fig. 3. Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on foaming capacity (FC) of PHPC.

3.7. Stabilisation of foaming and emulsifying properties

The optimal desirability conditions of the three responses (solubility, EAI and FC) were used to study the stability of the emulsion and that of the foam formed. The results of stability percentages are represented in Table 9.

A very good stability was exhibited for both the emulsion and the foam compared to that of sesame (Inyang et al., 1996). The stability of these two activities depends primarily on the solubility of the extract but also on the ionic strength of the medium.

3.8. Minimum gelling concentration (MGC)

The temperature of 95 °C was used for the study of the gelation of *Pinus halepensis* Mill. proteins because for the formation of a protein gel, a denaturation of the proteins is necessary (Srinivasan Damodaran, 1988). According to Arntfield and Murray (1981), the denaturation temperatures of oat, fababean, field pea and soybean were 112, 88, 86 and 93 °C respectively.

The MGC of PHPC under optimal conditions of functional properties was around 6% of PHPC, it's showing the very good gelling capacity of these proteins under these conditions. The gelation phenomenon is a phenomenon much more associated with temperature. Heat treatment allows the denaturation of proteins by cleavage of the structure of the disulfide bonds and thus the deflagration of proteins or an activation of the sulfide groups buried inside the molecule. these sulfide groups can give intermolecular disulfide bonds (exchange of the disulfide season) which causes a deployment of the protein molecules followed by an aggregation and association step thus forming the gel (Bau et al., 1985), the optical and rheological properties of thermally irreversible gels are therefore obtained (Ziegler & Foegeding, 1990). The parameters that can improve gelling are the increase in time, temperature, pH and protein concentration (Coffmann & Garciaj, 1977). Sun and Arntfield (2010) reported also that the salt concentration improves significantly the gelling properties of proteins. In our study, the high pH and high salt concentration may have influenced this capacity. In addition to the solubility of its proteins which is 77.39%, the minimum concentration obtained is more interesting than that reported by most other protein extracts. O'Kane et al. (2005), Coffmann and Garciaj (1977) and (A. M. Altschul, 1958) obtained MGC of 16% for pea protein, 10% for mung bean protein and 8% for soy protein respectively.

3.9. Heat coagulability (HC)

A relatively high HC was demonstrated by PHPC which was 24% compared to concentrate of *Brassica juncea* mustard seeds and *Sinapis alba* (Rotimi E. Aluko, McIntosh, & Katepa-Mupondwa, 2005) and canola isolate seeds (Rotimi E Aluko & McIntosh, 2001) as well as soybean isolate and pea isolate (Voutsinas et al., 1983) which have not shown any coabulability to heat. Our result is comparable to the HC of sunflower isolate (22.5%). Voutsinas et al. (1983); Rotimi E Aluko et al. (2001) and Rotimi E. Aluko et al. (2005) explain that HC depends mainly on the solubility as well as the surface hydrophobicity of the protein extract, which justifies the heat coagulation of our PHPC under the conditions used which solubilize them at 77.39%.

Table 9
Percentages of foam and emulsion stability of PHPC.

	15 min	30 min	45 min	60min
Emulsion Stability index (%)	75 ± 2.3	68.5 ± 1.5	53 ± 2	51 ± 0.8
Foam stability (%)	90 ± 4.6	87 ± 2.2	83 ± 1.9	73 ± 4.3

Data are the mean ± SD of three analyses.

4. Conclusion

In conclusion, this study confirmed that the functional properties are strongly related to the pH of the medium, its NaCl concentration and its PB molarity. In addition, the results show that Aleppo pine seeds are a good source of protein whose functional activities have been improved by the three parameters influencing them (pH, NaCl concentration and PB molarity). These proteins can therefore be used as food ingredients and the variation in pH, NaCl concentration and PB molarity can be employed as an effective processing method to improve the use of proteins as functional ingredients in food product formulations.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2019.105416.

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