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Optimization of the Isoelectric Precipitation Method To Obtain Protein Isolates from Amaranth (*Amaranthus cruentus*) Seeds

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This research was conducted to evaluate the effect of extraction pH (7.8−9.2) and precipitation pH (4.3−5.7) on four selected quality attributes of protein isolates from amaranth seeds (*Amaranthus cruentus*) such as protein content (PC), whiteness index (WI), enthalpy of transition (EN), and denaturation temperature (DT). Ten different treatments involving extraction and precipitation pH combinations were analyzed by a central composite design; the experimental data were fitted by a second-order model using a least-squares method for each one of the four dependent variables. Response surface methodology was used for the optimization process; in addition, a common optimum value for the four dependent variables was obtained utilizing the desirability method. A confirmatory test showed that the generated regression equations could adequately predict performance of this isoelectric precipitation method. The results indicate that extraction pH and precipitation pH showed an important effect on PC, WI, and EN. However, the different combinations did not significantly affect the DT. Values of 9.2 and 8.0 for extraction pH and 5.7 for precipitation pH produced the best overall result for all responses. Finally, the results have shown that it is possible to obtain protein isolates from *A. cruentus* seeds at optimized values of extraction pH and precipitation pH, which presented a high protein content and good physicochemical properties.

KEYWORDS: Isoelectric precipitation; protein isolates; *Amaranthus cruentus*; central composite design; response surface methodology; optimization process

INTRODUCTION

The ingestion of proteins is essential for maintaining health; humans, as animals do, use protein chiefly for its amino acid content and profile (*1*). Traditionally, egg, milk, and meat proteins have been considered as the best sources of high-quality proteins. However, proteins from animal sources are expensive and are not available in many countries for the majority of the inhabitants. Additionally, in recent years animal foods have been projected in negative terms because of an increased intolerance to milk and allergies to animal products in the world population; this has resulted in an interest in diets that do not contain animal protein. Thus, new alternative and cheap sources of good-quality proteins are necessary (*2*, *3*).

In this regard, in developing countries, cereals and legumes are the most important sources of dietary proteins (*1*). In this context, the crop-based plant foods help to meet the protein nutritional needs of various segments of the population, including infants and preschool children (*1*, *4*). Setting aside nutritional considerations, proteins are also used as food ingredients for their functional properties, which provide a certain specific function in the end product (*5*). Moreover, protein isolates have gained importance in the food industry because of their high protein contents, which can reach 90% as in legume protein isolates; this represents an alternative in the preparation of traditional foods and in the development of new foods as well. In addition, some antinutritional factors can be eliminated during the isolate production process (*6*).

The successful use of plant protein isolates depends on the versatility of their functional properties, which are influenced by intrinsic factors (composition and conformation of proteins), environmental factors (composition of the model system or food), and methods and conditions of isolation (*7*). In the latter point, protein isolates are obtained mainly following the traditional process known as isoelectric precipitation, which consists of a protein extraction from the defatted seed flour with dilute alkali (pH 8−11), followed by precipitation of the major protein fractions at the isoelectric point, which ranges from pH 4.5 to 5.0. It is important to emphasize that both alkaline extraction and acidic precipitation processes may induce physicochemical changes and affect functional properties in a positive

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or negative way. Furthermore, a decrease in the nutritional value of proteins can be observed (*6*, *8*, *9*).

In recent years, there has been a resurgence of interest in grain amaranth (*Amaranthus* sp.) because it is one of the few species with high potential to become an important source of dietary protein. One of the attractive features of amaranth seeds is their protein content, which, at 14−18%, is higher than that of most commercial cereals (~10% protein), and different studies have indicated that amaranth protein is notably high in lysine and presents acceptable levels of the sulfur-containing amino acids (*1*, *3*, *9*−*11*). Yet, protein quality depends not only on the amino acid composition but also on bioavailability (digestibility). Protein digestibility, available lysine, net protein utilization, and protein efficiency ratio have been widely used as indicators of protein nutritional quality. In this context, the values for amaranth protein are definitively higher as compared to cereal grains and are close to those of casein (*1*, *3*). Therefore, amaranth proteins are a promising food ingredient, capable of complementing cereal or legume proteins (*1*, *3*, *9*, *10*). It is very important to point out that one health food niche for amaranth protein has been as a gluten- or prolamin-free protein. Celiac disease is characterized by sensitivity to the prolamin fraction of cereals, particularly hypersensitivity to the alcohol-soluble gliadins from wheat (*1*, *12*). Cardiovascular diseases have also been correlated to dietary habits. In regard to this, the 7S and 11S globulins, which are present mainly in legumes, have been related to nutraceutical effects; in particular, it has been suggested that the soybean globulins exert a cholesterol-lowering effect (*13*−*16*). Interestingly, the globulin proteins are one of the most important protein fractions in amaranth seeds, accounting together with albumins for ~67% of the total protein (*1*, *10*, *11*, *17*−*20*). On the other hand, *A. cruentus*, *A. caudatus*, and *A. hypochondriacus* all have the potential to become cereal-like grain crops (*17*). Although there are a few reports about protein isolates from amaranth seeds, it is important to emphasize that most of these research works have been done with *A. hypochondriacus*. Martínez and Añon (*9*) analyzed the effect of extraction and precipitation conditions on the electrophoretic and calorimetric behavior of amaranth protein isolates, finding that extraction pH values >9 and <5 resulted in a decrease of thermal stability and an increase in protein denaturation. Also, Fidantsi and Doxastakis (*8*) isolated amaranth proteins by isoelectric precipitation and dialysis and evaluated their emulsifying and foaming properties, suggesting that amaranth protein isolates can be used as effective stabilizing and foaming agents.

The objectives of the present work were to optimize the production process of protein isolates from *A. cruentus* by isoelectric precipitation using different extraction- and precipitation-pH conditions and to analyze isolates as to their protein content, whiteness index, enthalpy of transition, and denaturation temperature through estimated response surface methodology.

MATERIALS AND METHODS

Plant Materials. The seeds of *A. cruentus* utilized in the present research were grown at the Instituto de Ciencias Agrícolas de la U. de Guanajuato, Mexico.

Sample Preparation. *A. cruentus* seeds were ground on a mill (Tekmar A-10 S2) with a mesh screen. The seed flour (7.7% crude fat, dry weight basis) was defatted for 24 h using hexane at a flour/hexane ratio of 1:10 (w/v) under continuous stirring at 4 °C; the defatted meal (1.0% crude fat, dry weight basis) was then air-dried at room temperature and stored at 4 °C until use.

Influence of pH on Amaranth Meal Protein Solubility. Protein solubility was determined by preparing suspensions with water and defatted amaranth meal (10% w/v); the pH of the suspensions was adjusted to values in the range of 2−12 with 1 N HCl or 1 N NaOH; the suspensions were stirred for 1 h at 25 °C followed by centrifugation at 12000*g* for 15 min at 25 °C. Solubility was calculated as the percentage of crude protein (N × 5.85) present in the supernatant in regard to total protein in the meal (*21*). Protein nitrogen was determined by the Kjeldahl method (*22*, *23*).

Table 1. Dependent Variable Values at the 10 Combinations (Treatments) of Extraction and Precipitation pH

	pH					
treatment	extraction	precipitation	PC[a] (%)	WI[b]	EN[c] (J/g)	DT[d] (°C)
1	8.0	4.5	81.3	26.9	2.7	100.2
2	9.0	4.5	83.4	25.2	2.4	99.6
3	8.0	5.5	78.3	24.3	6.3	99.2
4	9.0	5.5	81.7	18.1	6.7	99.4
5	7.8	5.0	78.1	29.3	4.1	101.8
6	9.2	5.0	81.9	21.2	4.1	99.7
7	8.5	4.3	80.3	30.1	1.6	99.4
8	8.5	5.7	78.0	27.3	6.9	99.7
9	8.5	5.0	75.8	28.3	3.2	99.8
10	8.5	5.0	75.9	27.7	3.2	99.8

[a] Protein content (Y_1). [b] Whiteness index (Y_2). [c] Enthalpy of transition (Y_3). [d] Denaturation temperature (Y_4).

Preparation of Isoelectric Protein Isolates. The amaranth protein isolates were prepared using different pH values for extraction and precipitation of proteins as previously described (*21*, *24*) with some modifications. For protein extraction, amaranth meal was suspended in water (10% w/v) and the pH adjusted to the required value by adding 1 N NaOH; this value was in the range from 7.8 to 9.2 (**Table 1**). The suspensions were stirred for 2 h at 25 °C and then centrifuged during 60 min at 10000*g* at 25 °C. Supernatants were adjusted with 1 N HCl to different pH values (4.3−5.7) for protein precipitation (**Table 1**), and then the suspensions were left at 4 °C overnight to allow proteins to precipitate; after that, centrifugation at 10000*g* for 60 min at 4 °C followed. Protein precipitates were resuspended in water and freeze-dried in a Freezone 4.5 L Freeze-Dry System (Labconco), and then were stored at −20 °C until further study. Every treatment (pH combination) was carried out independently from other treatments.

Protein Determination. Crude protein of protein isolates was determined by the Kjeldahl procedure using a conversion factor of 5.85 (*22*, *23*).

Colorimetric Evaluations. The color of the resultant protein isolates was determined using a Minolta Chroma meter CR-200 (Minolta Co., Ramsey, NJ). The instrument was calibrated using a white standard color calibration plate. Measurements of L^* (lightness) and b^* (yellowness) values were taken. Whiteness index (WI) was calculated using the equation $WI = L^* - 3b^*$ as described (*25*).

Differential Scanning Calorimetry (DSC). DSC measurements were performed using a model 2010 DSC (TA Instruments). The equipment was calibrated at a heating rate of 10 °C/min by using indium as standard. Samples (2 mg) were suspended in 10 μL of water and then placed in preweighed aluminum sample pans. For all DSC runs, a sealed empty aluminum pan was used as inert reference. The scanning range was 30−180 °C at a rate of 10 °C/min. Thermal stability parameters such as the enthalpy of transition (EN) and denaturation peak temperature (DT) were calculated by a thermal universal analysis 1.7 software (*11*).

Statistical Analysis. A central composite design with two replicates at the center point was chosen to systematically study the relative contribution of the two independent variables, extraction pH and precipitation pH (X_1 and X_2, respectively), with five levels (values) each (**Table 1**). Extraction pH levels used were 7.8, 8.0, 8.5, 9.0, and 9.2. Precipitation pH values employed were 4.3, 4.5, 5.0, 5.5, and 5.7. Dependent variables included protein content ($PC = Y_1$), whiteness index ($WI = Y_2$), enthalpy of transition ($EN = Y_3$), and denaturation temperature ($DT = Y_4$). These dependent variables were expressed individually as a function of the aforementioned independent variables. All treatments were performed randomly. Experimental data were fitted

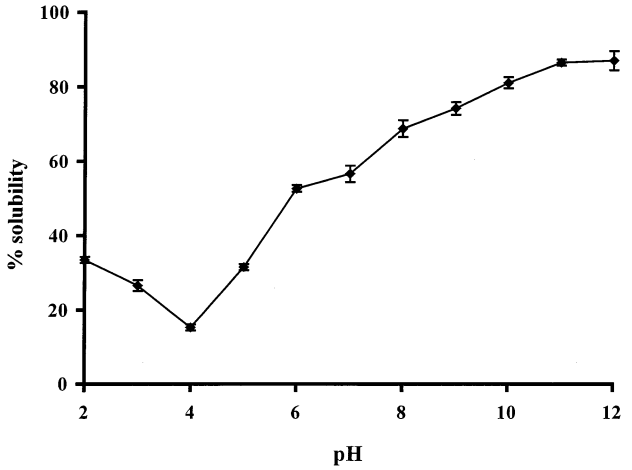

Figure 1. Protein solubility (percent) of amaranth meal proteins at different pH values. Mean values of three determinations and standard deviation bars are shown.

by a second-order model using a least-squares method for each one of the four dependent variables. Statistical analysis was performed using Statgraphics software (*26*). The objective in the optimization process was to find a common optimum value for the four dependent variables; thus, we used the desirability method (*27*). The four fitted models can be evaluated at any point $X = (X_1, X_2)$ of the experimental zone, and as a result four values were predicted for each model, namely, $\hat{Y}_1(X)$, $\hat{Y}_2(X)$, $\hat{Y}_3(X)$, and $\hat{Y}_4(X)$. Then, each $\hat{Y}_i(X)$ is transformed into a value $d_i(X)$, which falls in the range [0,1] and measures the desirability degree of the response in reference to the optimum value intended to be reached. In our case, we wanted all dependent variables to be as high as possible. Thus, the transformation is

$$d_i(X) = \begin{cases} 0 & \text{if } \hat{Y}_i(X) \le Y_{i*} \\ \dfrac{\hat{Y}_i(X) - Y_{i*}}{Y_i^* - Y_{i*}} & \text{if } Y_{i*} \le \hat{Y}_i(X) \le Y_i^* \\ 1 & \text{if } \hat{Y}_i(X) \ge Y_i^* \end{cases} \quad (1)$$

The value Y_{i*} corresponds to the highest value of all dependent variables; therefore, it is the minimum acceptable. Once the four individual desirabilities were calculated, the next step was to obtain the global desirability for the four dependent variables, using the mathematical function of transformation

$$D = (d_1 d_2 d_3 d_4)^{1/4} \quad (2)$$

where the ideal optimum value is $D = 1$; an acceptable value for D can be between 0.6 and 0.8 ($0.6 < D < 0.8$). This acceptable value was found by utilizing the Gauss system (*28*).

RESULTS AND DISCUSSION

Influence of pH on Amaranth Meal Protein Solubility. The solubility profile of amaranth meal proteins at various pH values (**Figure 1**) is similar to those of other vegetable proteins such as soybean, rice bran, and pea proteins (*16*, *29*−*31*). Those profiles are characterized by a solubility minimum around protein isoelectric points (pH ∼4.0), because protein−protein interactions increase as the net electrostatic charges of the molecules are at a minimum and less water interacts with the protein molecules (*32*). In addition, only 35% of amaranth meal proteins are soluble at pH values <4.0, whereas a nearly linear increase in solubility was observed above that pH. In general, the highest solubility values were obtained at alkaline pH values. Above pH 10.0, protein solubility continued to increase, although at a slower rate. It is important to emphasize that these results are very similar to those reported by Marcone and Kakuda (*25*) for amaranth globulin isolates; they found that the maximum solubility occurred at extremely high alkaline (8−9) and low acidic pH values (3−4), whereas the minimum solubility occurred at pH values near the isoelectric point of the amaranth globulins, that is, 5−6.

Table 2. Significant Regression Coefficients of Models and Models from Analysis of Variance for Isoelectric Precipitation

independent variable	PC[c] (%)	WI[d]	EN[c] (J/g)	DT[e] (°C)
constant	75.88	28.01	3.21	99.82
linear				
X_1[a]	1.37	−2.42	0.01*	−0.42*
X_2[b]	−0.99	−1.72	1.94	−0.10*
second order				
X_1^2	2.45	−2.21	0.54	0.34*
X_2^2	2.03	−0.50*	0.59	0.21*
X_1X_2	0.34*	−1.13*	0.17*	−0.27*
R^2 (%)	91	73	99	64

[a] Extraction pH. [b] Precipitation pH. [c] Significant models for p value <0.01. [d] Significant model for p value <0.05 without coefficient (∗). [e] Nonsignificant model. Significant regression coefficient for p value <0.05 except for (∗).

Different studies have shown that an increase of the extraction pH can induce conformational changes in the proteins, because of a decrease in the number of electrostatic interactions, which exposes more hydrophobic groups (*8*, *9*, *32*). Besides this, it is well-known that stronger alkaline treatments (pH >10.0) can negatively affect essential amino acids such as lysine, generating lysinoalanine, and ultimately resulting in a loss of protein digestibility and biological value (*33*). On this basis, we decided to employ pH values in the range of 8.0−9.0 for protein extraction and in the range of 4.0−6.0 for protein isoelectric precipitation, where protein solubilities show values of 70−80 and 15−50%, respectively, to optimize the production process of protein isolates from *A. cruentus* seeds. As a result, we expected to obtain protein isolates with a high protein content and acceptable physicochemical properties.

Effect of Extraction and Precipitation pH Combinations on the Protein Content and Physicochemical Properties of Protein Isolates. The protein contents and physicochemical properties of the protein isolates obtained by isoelectric precipitation of *A. cruentus* seed proteins are presented in **Table 1**. These isolates resulted from 10 different treatments involving extraction and precipitation pH combinations.

Protein Content (PC). Experimental PC values ranged from 75.8% at intermediate levels of extraction and precipitation pH values (8.5 and 5.0, respectively) to 83.4% at a combination involving a higher extraction pH (9.0) and a lower precipitation pH (4.5) (**Table 1**). This increase in PC is a desirable event from an efficiency process viewpoint, and it is most probably due to an actual decrease of the other seed components (e.g., starch, nondigestible fiber, and antinutritional components). In this context, the removal of undesirable components is essential to improve the nutritional quality of protein isolates and to effectively exploit their full potential as functional ingredients. (*6*, *34*). Moreover, our results are similar and even higher than those of other isoelectric protein isolates of amaranth, rice bran, and mung bean; the PC values of these protein isolates were 67.0, 74.5, and 81.0%, respectively (*8*, *29*, *35*). The analysis of variance showed that the model for PC was significant for a p value <0.01 for both the linear and quadratic terms. This model accounts for 91% of total variation (**Table 2**). It is worth mentioning that protein yield (i.e., the percentage of the amaranth meal total protein actually recovered in the isolate)

ranged from 53.4% (pH combination 8.5−5.0) to 61.0% (pH combination 9.0−4.5).

Whiteness Index (WI). Color in protein isolates is a property that derives from small nonprotein phenolic compounds; the most common of these are syringic, coumaric, caffeic, and sinapic acids and vanillin. A number of these compounds are colorless or slightly colored, but oxidation by phenoloxidases or by other means converts them to highly colored polymers (*36*, *37*). These compounds bind proteins in aqueous media through a variety of complex-forming mechanisms including hydrogen bonding, covalent bonding, hydrophobic interactions, and ionic bonding; this makes them difficult to remove. Also, the protein−polyphenol reaction is affected by pH, oxygen level, time, and temperature (*36*−*38*). The presence of phenolic compounds has been associated with a decrease in the biological value of proteins, due to a decrease in the protein digestibility (*1*, *36*, *38*).

The WI is the attribute by which an object is judged to approach the preferred white; this index departs from white toward yellow (*36*). The highest value for WI of the amaranth protein isolates was 30.1 for samples obtained with extraction and precipitation pH values of 8.5 and 4.3, respectively, whereas the lowest value was 18.1 and was obtained at high extraction and precipitation pH values (9.0 and 5.5, respectively) (**Table 1**). Low WI values are probably due to trace amounts of phenolic compounds and their oxidation products, present in the amaranth seed coat, coprecipitating with the proteins during preparation of the amaranth isolates, leading to the perceived difference in color; as mentioned above, the elevated pH is probably accelerating the protein−polyphenol reaction (*25*, *37*). Also, Xu and Diosady (*38*) evaluated the removal of phenolic compounds in canola protein isolates; these authors found that a decrease of 72.5% in phenolic compounds produces an increase of 24.8% in the whiteness of these isolates with respect to the control.

Commercial soybean protein isolates are some of the most widely used food ingredients. Waggle et al. (*36*) studied 15 different commercial soybean protein isolates and found that they showed WI values in the range from 16.3 to 46.6. In this context, amaranth protein isolates obtained in the present work show WI values falling within this range.

Finally, the model for WI was significant with a p value <0.05 without the quadratic terms of the precipitation pH and of the interaction between extraction and precipitation pH values. This model accounted for 73% of the total variation; the coefficient of determination value resulted from the fact that there are no significant quadratic terms in the WI model (**Table 2**).

Enthalpy of Transition (EN) and Denaturation Temperature (DT). DSC is a valuable tool for assessing the potential of protein isolates as functional ingredients in different food systems, where heat processing is required. Because the functional properties of proteins are greatly influenced by their conformation and DSC is a technique highly sensitive to conformational changes, we applied it to our protein isolates (*39*, *40*). By analyzing DSC thermograms, two dependent variables (EN and DT) are determined. EN is calculated from the area above the transition peak and correlates with the extent of ordered structure in a protein; DT is a measure of the thermal stability of a given protein (*9*, *39*). These parameters have been well documented in amaranth proteins; the thermodynamic properties of amaranth globulins have been reported by Gorinstein et al. (*39*), who obtained values of 4.25 and 2.08 J/g for native and denatured amaranth globulins, respectively. Chen and Paredes-López (*11*) reported a DT of 99 °C for the 11S globulin from amaranth seeds; similarly, Martínez and Añon (*9*) and Gorinstein et al. (*39*) found DT values of 64 and 94−101 °C for the albumin and globulin fractions, respectively, whereas peaks at 70 and 96 °C were reported for the glutelins of amaranth. Furthermore, EN values were higher for the 11S globulin in comparison with the values observed for albumins and glutelins; the lower value of EN for glutelins could be attributed to a partial denaturation induced by the strong extraction conditions (*9*, *39*). On the other hand, Martínez and Añon (*9*) evaluated the thermal stability of amaranth protein isolates; these authors observed a decrease in the enthalpy of transition of the isolates obtained by precipitation at low pH. A possible explanation is that the increase of positive charges at lower pH could induce unfolding of protein molecules. Also, Kiosseoglou et al. (*40*) found that isoelectric precipitation at pH 4.5 in lupin protein isolates caused structural rearrangements which encompass a wide molecular weight distribution, judging from the extensive endothermic event. In our study, EN values ranged from 6.85 to 1.55 J/g (**Table 1**); a combination of relatively high extraction and precipitation pH values (8.5 and 5.7, respectively) produced an isolate showing the highest EN value, whereas lower precipitation pH values produced a decrease in EN. This behavior may be attributed to a low bonding energy conformation, a high proportion of hydrophobic interactions, or aggregation, all due to partial protein denaturation by effect of the isolate preparation method (*9*, *39*). Analysis of variance showed a strong dependence ($p < 0.01$) of EN on the precipitation pH linear term and on the extraction pH and precipitation pH quadratic terms (**Table 2**). In contrast, the different combinations of extraction and precipitation pH values in the production of amaranth protein isolates had no effect on the DT, as shown in **Table 1**. As a result, the model for DT was not significant and none of the factors had an effect (**Table 2**). Therefore, an adequate value for this model is $\hat{Y}_4(X) \cong 100$. Comparison of the thermal stability values of our isolates with those reported for amaranth protein fractions (*9*, *11*, *39*) supports the presence of albumins, globulins (mainly 11S globulin), and glutelins in the amaranth isoelectric protein isolates.

Optimization. As mentioned above, the objective in the optimization process was to find the pH combination that resulted in the optimum values for the four dependent variables. To predict the dependent variables at the optimum value, we considered the complete models in the desirability function (*27*). The nonsignificant terms had a slight effect on the accuracy of the results in the optimization process. The common optimum values for the four dependent variables were obtained at a desirability value of 0.63, as a result of pH combination 7.8−4.4 (**Table 3A**). Obviously, the EN response was not completely satisfactory; this result could be explained by the opposite signs in the regression coefficients between WI and EN (**Table 2**). Therefore, to obtain a better value in this response, the level curves of the desirability function were created (**Figure 2**). From this figure, different scenarios were created to improve the EN response without negatively affecting the other three dependent variables. Different values for extraction pH and precipitation pH were chosen at a desirability value of 0.8 (**Table 3B**), where better values for EN were observed without affecting the remaining dependent variables. In accordance with these results, we decided to employ case 1 (i.e., pH combination 9.2−5.7; **Table 3B**) and case 4 (i.e., pH combination 8.0−5.7; **Table 3B**) to produce amaranth protein isolates by isoelectric precipitation. Additionally, the efficiency of these results was evaluated through five experimental confirmatory tests at the selected pH values (**Table 3C**).

Table 3. Protein Content and Physicochemical Properties of Amaranth Protein Isolates at Different Desirability Values from the Optimization Process and Confirmatory Test in the Laboratory

		pH					
		extraction	precipitation	PC (%)	WI	EN (J/g)	DT (°C)
A	desirability = 0.63[a]	7.8	4.4	83.4	26.5	3.1	101.8
B	desirability = 0.80[a]						
	case 1	9.2	5.7	86.0	16.7	8.7	99.6
	case 2	8.5	5.7	81.3	24.5	7.2	99.1
	case 3	8.2	5.7	78.5	26.4	7.8	99.8
	case 4	8.0	5.7	79.0	29.4	7.4	99.6
C	confirmatory test[b]						
	case 1	9.2	5.7	89.2 ± 0.8	19.2 ± 0.3	5.3 ± 0.5	99.6 ± 0.2
	case 4	8.0	5.7	83.3 ± 0.3	26.1 ± 0.1	7.6 ± 1.4	99.1 ± 0.5

[a] Obtained from mathematical models. [b] Experimental test carried out in the laboratory.

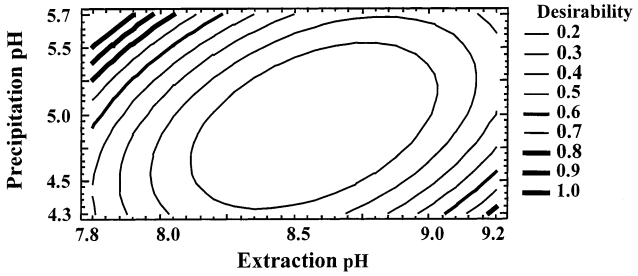

Figure 2. Different desirability scenarios for protein content, whiteness index, enthalpy of transition, and denaturation temperature of amaranth protein isolates.

Confirmatory Tests. The experimental confirmatory tests allowed us to contrast the values estimated by the model and oriented by the desirability function with those obtained once again in the laboratory. Thus, in **Table 3C** it can be observed that, at high values of extraction and precipitation pH values (9.2 and 5.7, respectively), a very good result for PC and a relatively high value of EN were obtained, although the WI value was relatively low. The difference between the EN value expected from the desirability function (8.7 J/g) and that obtained in the confirmatory test (5.3 J/g) may be attributed to the presence of a higher proportion of glutelins in the isolate. These proteins are extracted at higher pH, and this experimental condition causes partial protein denaturation leading to lower EN values, as previously noted by Martínez and Añon (*9*). Cases 1 and 4 in **Table 3B** produced adequate results for the four dependent variables, and these were confirmed by the additional laboratory tests (**Table 3C**). Finally, we could state that extraction pH values of 9.2 or 8.0 and a precipitation pH of 5.7 produced the best overall result (common optimum) for all dependent variables in the isoelectric precipitation process for amaranth protein isolates.

Conclusions. Our results indicate that extraction and precipitation pH values showed an important effect on PC, WI, and EN. However, the different combinations of extraction and precipitation pH values did not significantly affect DT. The best processing conditions from the optimization method for the four dependent variables were obtained when the extraction pH was 9.2 or 8.0 and the precipitation pH employed was 5.7. The PC, WI, EN, and DT values of the amaranth protein isolates produced under optimal conditions in the confirmatory tests were located within the ranges of the expected values. The results showed that it is possible to obtain functional protein isolates, with high protein content and good physicochemical properties, from *A. cruentus* seeds, through an isoelectric precipitation method. Currently, a more detailed physicochemical, functional, and nutritional characterization of these amaranth protein isolates is being carried out.

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