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A two-step factorial design for optimization of protein extraction from industrial rapeseed meal after ethanol-assisted reduction of antinutrients

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Abstract

Rapeseed meal is a by-product of oil production with relatively high antinutrient content which limits its application in both feed and food industries. A two-step procedure, including two-level full factorial and central composite designs, was used to assess the influence of five factors, namely pH (6 and 12), temperature (20 and 40°C), NaCl concentration (0 and 7.5%), rapeseed meal concentration (2.5 and 5%), and extraction longevity (15 and 60 min), on protein extraction from industrial rapeseed meal subjected to ethanol-assisted reduction of antinutrients. Data demonstrated that pH and its interaction with NaCl influenced protein extractability the most. While still significant, individual effects of NaCl, extraction longevity, temperature and its interaction with pH had a less significant effect on protein yield. Overall, higher protein yields were obtained in response to combining high pH values with low NaCl concentrations, and high pH values with high temperatures. The highest protein yield (59.56 ± 1.29%) was achieved after extraction of 5% ethanol-treated rapeseed meal at pH 12 with no NaCl addition, at 40°C and extraction longevity of 60 min. A second-order polynomial model for protein yield prediction was generated. The adequacy of the model was verified by coefficient of determination and residual analyses. A high correlation between experimentally obtained and predicted protein yields (R^2 = 0.95) was established. Since extraction conditions are strong determinants of protein characteristics, the predictive model generated in the present work is useful for the selection of factor combinations, which allows for the achievement of optimal protein yield of a product with desired techno-functional properties.

Introduction

Rapeseed meal is generated in significant quantities as a by-product of vegetable oil production. Recently, there is an increased demand for the oil, which is used either for food or technical purposes (Carré and Pouzet, 2014; Xu *et al*., 2016). Currently, the European Union is emerging as a global leader in biodiesel production where rapeseed oil is used as a feedstock (EUBIA, 2017).

Due to the high protein content (38%) and relatively balanced amino acid composition, rapeseed meal is used as an additive in the feed industry (Liu *et al*., 1994). However, rapeseed meal inclusion levels in diets are limited by fibre and antinutrient compound concentrations, which are presented in concentrations unfavourable for the animal growth. Alternatively, rapeseed meal can be used for generation of protein-rich ingredients for the food industry (Tan *et al*., 2011). This application is also restricted by the high content of antinutrients which may remain in the final products, thus worsening their quality and functional properties. Therefore, the reduction or removal of antinutrients is recommended to improve rapeseed meal applicability (Liu *et al*., 1994).

Various approaches for detoxification of rapeseed meal have been studied (Shahidi and Naczk, 1992; Barrett *et al*., 1998; Gu *et al*., 2011). Among them, pre-treatment with aqueous ethanol solution is one of the most commonly employed methods. Ethanol is allowed for use in the food industry (European Commission, 2012) and has a high potential to reduce certain antinutrient concentrations, including glucosinolates and phenols (Slawski *et al*., 2012;

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Adem *et al*., 2014). However, ethanol is a polar agent, which denatures proteins and alters their solubility (Von Der Haar *et al*., 2014). While the ethanol-treated rapeseed meal has been proven to be suitable as a feed additive in the fish diet (Slawski *et al*., 2012; Adem *et al*., 2014), the optimization of protein recuperation from this source has not been performed yet. Preliminary experiments in our laboratory indicated decreased protein extractability from ethanol-treated rapeseed meal as evaluated by Osborne fractionation. Therefore, factors influencing protein extraction from ethanol-treated rapeseed meal need to be studied in detail in order to optimize protein yield when this by-product is intended for further preparation of more protein-rich products with added value.

Response surface methodology (RSM) is a quick and efficient methodology that combines mathematical and statistical techniques, thus allowing for optimization of a specific response and generation of a model describing the process (Box *et al*., 1978; Baş and Boyacı, 2007; Morshedi and Akbarian, 2014). This approach has been proven to be successful when a relatively small number of experiments allows for the influence of multiple variables and their impact on the desired outcomes (Firatligil-Durmus and Evranuz, 2010; Nil Das Purkayastha and Mahanta, 2015; Mechmeche *et al*., 2017). The aim of the present work was therefore to evaluate the significance of pH, temperature, concentrations of NaCl and rapeseed meal, and extraction longevity on protein extraction from ethanol-treated industrial rapeseed meal, and to optimize the protein yield by using response surface methodology.

Materials and methods

Rapeseed meal was provided by a local company. It was produced after thermal treatment of rape seeds at 110 - 115°C followed by extraction with hexane at 60 - 65°C for approximately 1 h. Unified size particles (≤ 0.315 mm), obtained after grinding and sifting, were used for analyses. Ethanol treatment of the rapeseed meal was performed as described by Chabanon *et al*. (2007). Rapeseed meal was subjected to a 4-step treatment with 75% aqueous ethanol solution at a meal to solvent ratio of 25% (w/v) for 30 min at room temperature.

Experimental design

A two-step approach was used to assess the influence of factors on protein extraction from ethanol-treated rapeseed meal. In the first step, a two-level full factorial experimental design (Box *et al*., 1978) was used to evaluate the effect of five factors in the process of protein extraction. The factors and their low and high values are presented in Table 1. A total of $2^5 = 32$ experiments was performed in duplicate and randomly, according to the conditions indicated in Table 2 (runs 1 to 32).

In the second step, and after establishing the meaningless effect (not significant) of the rapeseed meal concentration, additional experiments were performed in order to couple a response surface methodology to the previously used factorial design. The effect of the significant four factors (x_1:pH, x_2:NaCl, x_3:Temperature, T and x_4: extraction longevity, EL) on protein yield was investigated by a Central Composite Design, CCD (Nemati *et al*., 2018), with the goal of obtaining a model for process optimization. The design consisted of three distinct sets of experimental runs (Table 2):

i. 16 experiments designed according to a 2^4 full factorial design, performed at the high and low limits of the four factors tested (runs 17 to 32 already performed);

ii. two experiments at the middle values (centre points) of the range of factors tested;

iii. eight experimental runs identical to the centre points except for one factor, which took on values symmetrically spaced at the lowest and at the highest values of the factors tested (axial points, called face cantered star points).

The three levels assigned for each factor were represented by the coded levels: -1 for the low value, 0 for the centre value and +1 for the high value. The CCD was composed of 26 experiments performed in duplicate and randomly, based on the conditions indicated in Table 2. The experiments were performed with a fixed value for rapeseed meal concentration of 5%.

Protein extraction and quantification

Proteins were extracted under constant agitation (1,000 rpm) and various combinations of factors as previously described. A water bath shaker (Gyrotory G76, New Brunswick Scientific Co. Inc., Enfield, CT, USA) was used to provide the desired temperature and agitation. pH was adjusted by using NaOH or HCl as needed. Extracted proteins were quantified by the Biuret method (AACC, 1983). Bovine serum albumin was used as a standard. Protein yield was calculated as a ratio of the amount of extracted protein, and protein content of the sample was evaluated by Kjeldahl method (AOAC, 1990). A coefficient of 6.25 was used to convert the amount of total nitrogen to protein.

Statistical analysis

The relationship between the response variable, the yield, and the four factors (independent variables) was described by a second order mathematical model using the following equation:

$$Yield = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j + \sum_{i=1}^{4} \beta_{ii} x_i^2$$

(Eq. 1)

where, Yield = predicted response, x_i = independent factors (i=1,…4), β_0 = intercept parameter of the model, β_i = parameters related to linear effects (i=1,…4), β_{ii} = model parameters related to quadratic effects (i=1,…4), and β_{ij} = parameters related to interactions between the variables β_{ij} (i=1,…3 and j=2,…4). The quality of the regression was assessed by residuals analysis. Independency, randomness, normality and homoscedasticity of the residuals were tested. The coefficient of determination (R^2) was also calculated.

Results from the two-level full factorial design and Central Composite Design were analysed by ANOVA procedures. The significance level assumed in all analyses was 0.05. Statistica© 6.0 (StatSoft, USA) was used for all regression procedures and statistical analysis.

Results and discussion

Evaluation of factor influence

Temperature, pH, NaCl concentration, rapeseed meal concentration, and extraction longevity are considered major factors that affect protein extractability (Ivanova *et al*., 2012; Cui *et al*., 2017). Due to lack of published data on protein extractability from rapeseed meal following ethanol treatment, the influence of the five previously mentioned factors and their interactions was studied in a first-step using a full factorial experimental design at two-levels. Low and high levels of the variables (Table 1) were chosen based on literature data. Since it is well established that high pH values favour protein extraction from oil-containing crops, experiments at acidic pH (pH < 6) were not considered (Pedroche *et al*., 2004; Ivanova *et al*., 2012; Lovatto *et al*., 2017). Although favouring protein solubilization, pH values higher than 12 were not included in the present work to avoid potential formation of lysinoalanine in final protein-rich products (Deng *et al*., 1990). Published reports regarding the effect of NaCl addition are controversial (Ivanova *et al*., 2013; Nil Das Purkayastha and Mahanta, 2015) which determined the choice of a wide concentration range from 0 to 7.5% NaCl. The temperature did not exceed 40°C to limit protein interaction with other substances.

Table 1. Factors and level values used in a 25 full factorial experimental design.

Factor	Low value (-1)	High value (+1)
pH	6	12
Concentration of NaCl, %	0	7.5
Temperature (T), °C	20	40
Extraction longevity (EL), min	15	60
Concentration of rapeseed meal (RMC), %	2.5	5.0

Pareto chart of standardized effects (Figure 1) demonstrated that pH and its interaction with NaCl addition influenced protein extractability the most. While still significant, individual effects of NaCl, extraction longevity, temperature and temperature's interaction with pH affected protein yield by a much smaller extent. Rapeseed meal concentration in the range from 2.5% to 5.0% was not found significant. Increasing pH (Figure 2A), extraction longevity (Figure 2C), and temperature (Figure 2D) positively influenced the protein yield, while increasing NaCl concentrations decreased protein yield as evidenced by plots of the marginal means (Figure 2B). Our results agreed with Ivanova *et al*. (2013) who observed a decrease in solubility of sunflower protein isolates in response to augmentation of NaCl concentrations up to 0.25 M in a pH range from 2 to 8.5. Nil Das Purkayastha and Mahanta (2015) established an increase in protein recuperation from rapeseed meal following addition of NaCl but only to a low extent (7.65%). The evaluation of combined effects pH-NaCl and pH-T demonstrated the positive influence of the high pH level, chosen in the present work, on protein yield in both cases. In the limits studied, higher protein yields were obtained for high pH values and low NaCl concentrations (Figure 3A), and for high pH values and high temperatures (Figure 3B). Gerzhova *et al*. (2016) also noticed the inversed influence of pH and NaCl additions on protein extractability. According to them, it is most probably due to differences in the extractability of cruciferin and napin fractions (major proteins in canola/rapeseed) in the presence of NaCl, as evidenced by SDS-PAGE analyses.

As indicated in Table 2, the highest protein yield (59.56 ± 1.29%) was obtained following extraction of

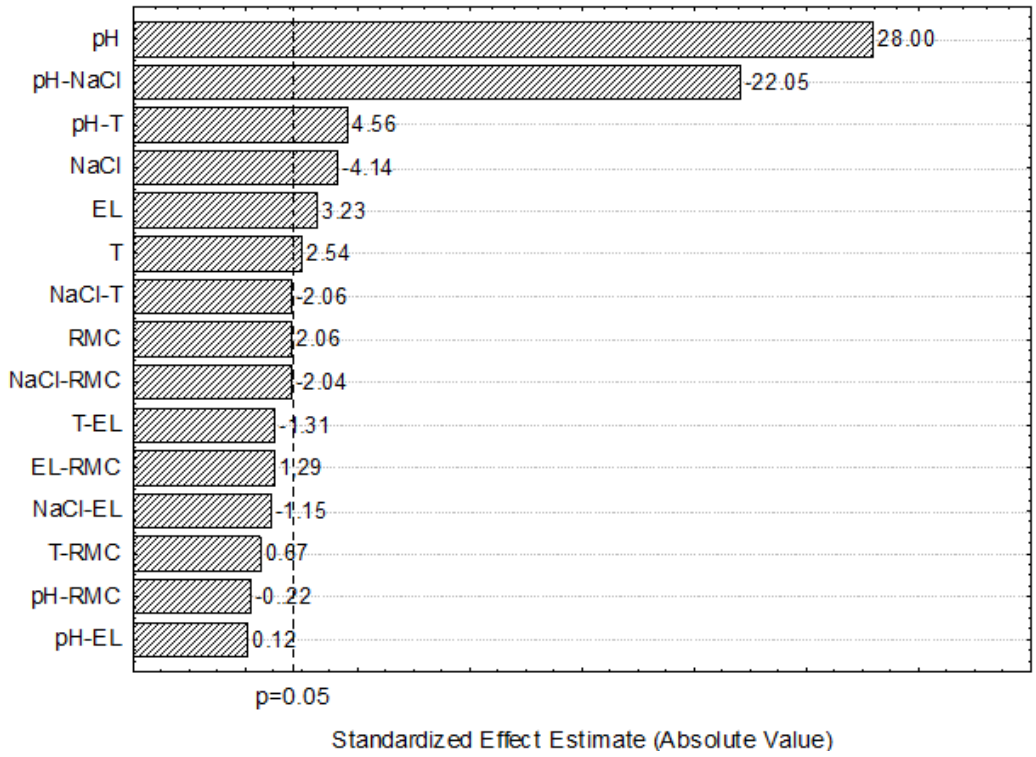

Figure 1. Pareto chart of standardized effect estimate of main variables and interactions obtained from a 2^5 full factorial design. Vertical dotted line indicates the statistical significance of the effects (p = 0.05).

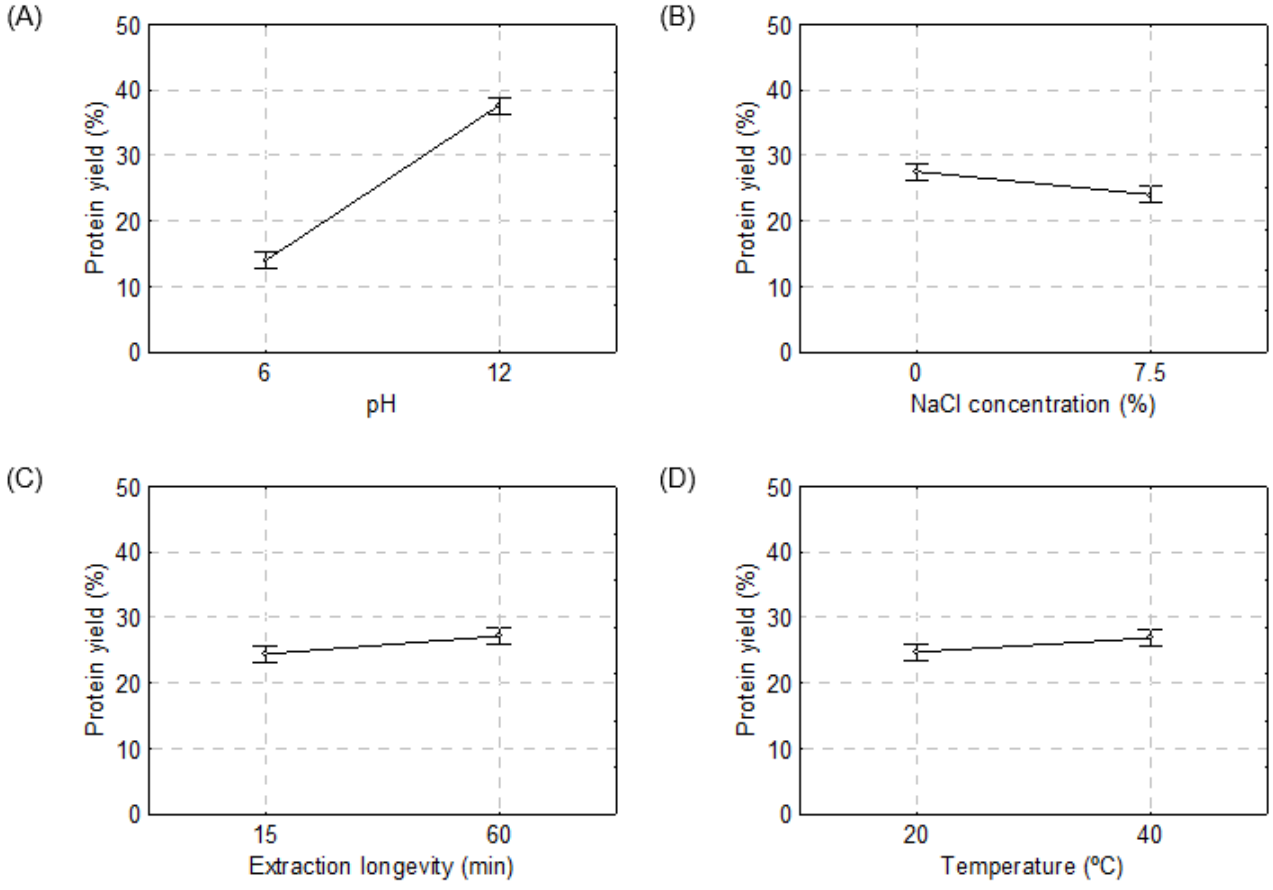

Figure 2. Plots of marginal means of protein yield as influenced by pH (A), NaCl concentration (B), extraction longevity (C), and temperature (D).

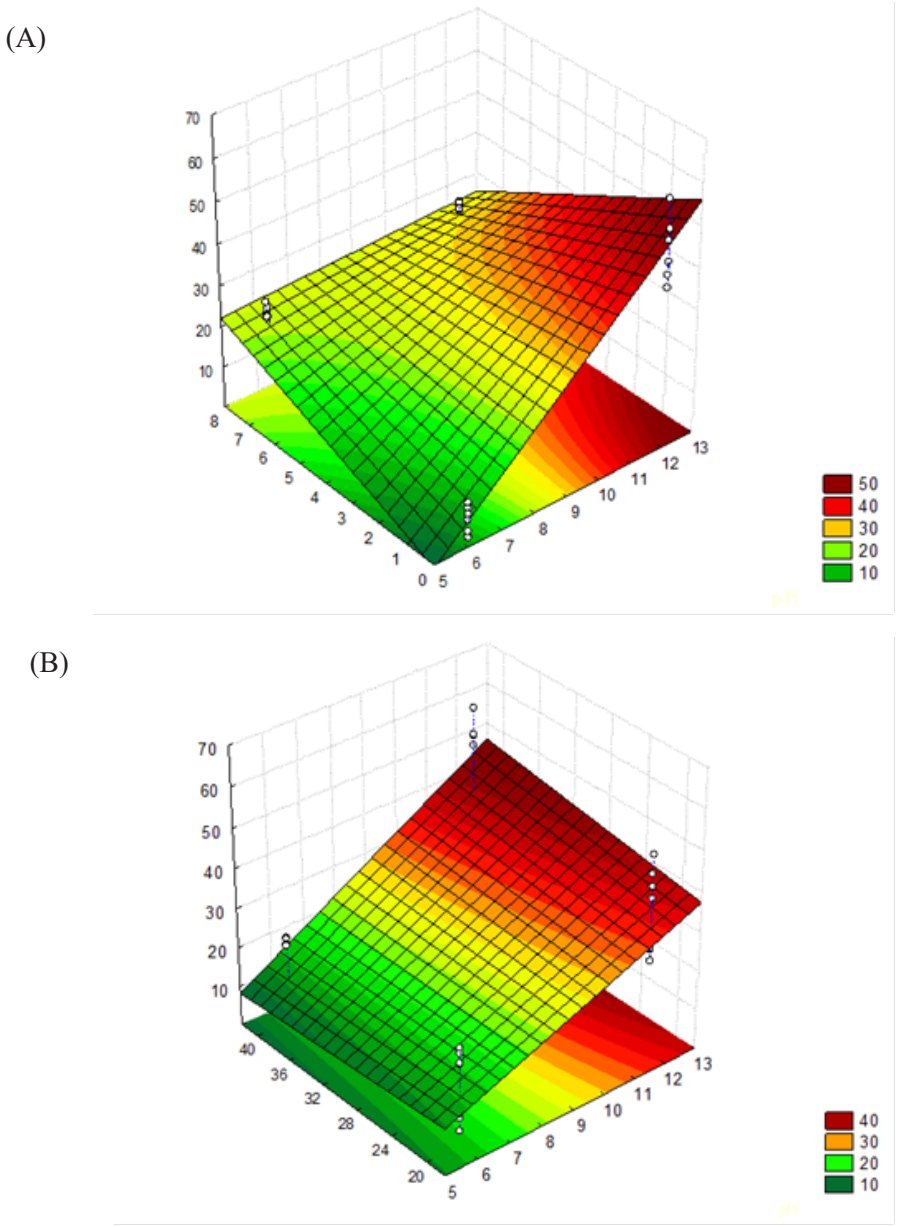

Figure 3. Combined effect pH-NaCl (A) and pH-T (B) on protein yield.

5% ethanol-treated rapeseed meal at pH 12, no NaCl addition, at 40°C and extraction longevity of 60 min. Our result is close to that reported by Pedroche *et al*. (2004) and Gerzhova *et al*. (2016) who achieved 63% and 58% protein yield respectively while using non-treated canola meal. These extraction conditions could be used for further preparation of either protein isolate or concentrate depending on purity, functional properties and the targeted application (Ivanova *et al*., 2017; 2018; Kalaydzhiev *et al*., 2019).

Model for protein yield prediction

Since the rapeseed meal concentration did not significantly affect the protein yield, its value was set at 5.0% when performing further experiments according to a central composite design. After running 26 trials, the second-order polynomial model obtained for protein yield prediction was as follows:

$$Protein\ yield = 32.072 - 6.598\ pH + 0.626 pH^2 + 9.837\ NaCl - 0.194 NaCl^2 - 1.457 T + 0.020 T^2 + 0.274\ EL - 0.002 EL^2 - 0.811\ pH\ NaCl + 0.059\ pH\ T + 0.007\ pH\ EL - 0.036\ NaCl\ T - 0.014\ NaCl\ EL - 0.001\ T\ EL \quad \text{(Eq. 2)}$$

The coefficient of determination (R^2) was 0.95, demonstrating that 95% of all variability could be explained by the model. In addition, the adequacy of model fit was assessed by analysis of the residuals. They were randomly distributed showing homoscedasticity. Normality of residual distribution was verified as well (results not shown).

Estimated values by the model (Eq. 2) are included in Table 2 for the conditions tested. The plot

Table 2. Design matrix of conditions used, measured and predicted responses of protein yield.

Run	Uncoded and coded (in parentheses) values of independent variables					Response	
	pH	NaCl	T	EL	RMC	Observed protein yield, %	Predicted protein yield, %
Factorial points							
1	12 (+1)	7.5 (+1)	40 (+1)	60 (+1)	2.5 (-1)	28.36 ± 0.72	-
2	12 (+1)	7.5 (+1)	40 (+1)	15 (-1)	2.5 (-1)	27.99 ± 0.09	-
3	12 (+1)	7.5 (+1)	20 (-1)	60 (+1)	2.5 (-1)	26.06 ± 0.33	-
4	12 (+1)	7.5 (+1)	20 (-1)	15 (-1)	2.5 (-1)	23.29 ± 0.33	-
5	12 (+1)	0 (-1)	40 (+1)	60 (+1)	2.5 (-1)	50.04 ± 0.63	-
6	12 (+1)	0 (-1)	40 (+1)	15 (-1)	2.5 (-1)	52.81 ± 1.28	-
7	12 (+1)	0 (-1)	20 (-1)	60 (+1)	2.5 (-1)	44.87 ± 0.86	-
8	12 (+1)	0 (-1)	20 (-1)	15 (-1)	2.5 (-1)	41.84 ± 1.57	-
9	6 (-1)	7.5 (+1)	40 (+1)	60 (+1)	2.5 (-1)	21.46 ± 0.63	-
10	6 (-1)	7.5 (+1)	40 (+1)	15 (-1)	2.5 (-1)	19.89 ± 0.57	-
11	6 (-1)	7.5 (+1)	20 (-1)	60 (+1)	2.5 (-1)	24.49 ± 0.48	-
12	6 (-1)	7.5 (+1)	20 (-1)	15 (-1)	2.5 (-1)	20.88 ± 0.59	-
13	6 (-1)	0 (-1)	40 (+1)	60 (+1)	2.5 (-1)	2.81 ± 0.41	-
14	6 (-1)	0 (-1)	40 (+1)	15 (-1)	2.5 (-1)	2.49 ± 0.68	-
15	6 (-1)	0 (-1)	20 (-1)	60 (+1)	2.5 (-1)	8.03 ± 0.24	-
16	6 (-1)	0 (-1)	20 (-1)	15 (-1)	2.5 (-1)	3.75 ± 0.54	-
17	12 (+1)	7.5 (+1)	40 (+1)	60 (+1)	5.0 (+1)	28.05 ± 0.16	29.84
18	12 (+1)	7.5 (+1)	40 (+1)	15 (-1)	5.0 (+1)	26.38 ± 0.46	27.99
19	12 (+1)	7.5 (+1)	20 (-1)	60 (+1)	5.0 (+1)	26.04 ± 0.16	26.11
20	12 (+1)	7.5 (+1)	20 (-1)	15 (-1)	5.0 (+1)	26.48 ± 0.27	22.04
21	12 (+1)	0 (-1)	40 (+1)	60 (+1)	5.0 (+1)	59.56 ± 1.29	56.51
22	12 (+1)	0 (-1)	40 (+1)	15 (-1)	5.0 (+1)	52.40 ± 0.79	52.71
23	12 (+1)	0 (-1)	20 (-1)	60 (+1)	5.0 (+1)	49.89 ± 0.6	49.28
24	12 (+1)	0 (-1)	20 (-1)	15 (-1)	5.0 (+1)	38.94 ± 0.68	43.26
25	6 (-1)	7.5 (+1)	40 (+1)	60 (+1)	5.0 (+1)	21.96 ± 0.2	21.01
26	6 (-1)	7.5 (+1)	40 (+1)	15 (-1)	5.0 (+1)	20.00 ± 0.52	19.36
27	6 (-1)	7.5 (+1)	20 (-1)	60 (+1)	5.0 (+1)	23.11 ± 0.34	25.01
28	6 (-1)	7.5 (+1)	20 (-1)	15 (-1)	5.0 (+1)	20.50 ± 0.23	21.15
29	6 (-1)	0 (-1)	40 (+1)	60 (+1)	5.0 (+1)	9.40 ± 0.21	10.27
30	6 (-1)	0 (-1)	40 (+1)	15 (-1)	5.0 (+1)	6.63 ± 0.16	6.67
31	6 (-1)	0 (-1)	20 (-1)	60 (+1)	5.0 (+1)	10.81 ± 0.34	10.78
32	6 (-1)	0 (-1)	20 (-1)	15 (-1)	5.0 (+1)	6.81 ± 0.16	4.96
Axial points							
33	6 (-1)	3.8 (0)	30 (0)	37.5 (0)	5.0*	33.27 ± 0.18	39.46
34	12 (+1)	3.8 (0)	30 (0)	37.5 (0)	5.0*	23.81 ± 0.4	17.68
35	9 (0)	7.5 (+1)	30 (0)	37.5 (0)	5.0*	23.83 ± 0.38	18.30
36	9 (0)	0 (-1)	30 (0)	37.5 (0)	5.0*	16.57 ± 0.74	22.15
37	9 (0)	3.8 (0)	40 (+1)	37.5 (0)	5.0*	25.01 ± 0.05	26.17
38	9 (0)	3.8 (0)	20 (-1)	37.5 (0)	5.0*	24.87 ± 0.31	23.76
39	9 (0)	3.8 (0)	30 (0)	60 (+1)	5.0*	22.23 ± 0.41	23.48
40	9 (0)	3.8 (0)	30 (0)	15 (-1)	5.0*	21.17 ± 0.23	19.98
Centre points							
41	9 (0)	3.8 (0)	30 (0)	37.5 (0)	5.0*	22.94 ± 0.2	22.94
42	9 (0)	3.8 (0)	30 (0)	37.5 (0)	5.0*	23.09 ± 0.33	22.94

NaCl: NaCl concentration, %
T: Temperature, °C
EL: Extraction longevity, min
RMC: Rapeseed meal concentration, %

of experimental protein yield versus estimated values is shown in Figure 4, revealing prediction ability. However, the predictive model expressed by Eq. 2 did not have a maximum (or minimum) value within the limits imposed for the studied factors. Results from CCD analyses reinforced that pH was the main factor influencing the protein yield, followed by the combined effect of pH with NaCl. The increase in pH increased the protein yield, which indicated the highly alkaline area as the most appropriate for maximal solubilization and extraction of proteins from the ethanol-treated rapeseed meal. This finding is in accordance with the common perception that alkaline conditions are the most suitable for extraction of proteins from oilseed meals (Tan *et al*., 2011). By studying protein extractability from *Brassica carinata* at pH 10, 11 and 12, Pedroche *et al*. (2004) established achievement of highest nitrogen yield at pH 12. However, the protein isolates obtained from oilseed meals under highly alkaline conditions are characterized with dark brown colour due to interactions of the proteins with polyphenols which make them inappropriate as additives in the food and feed industries (Von Der Haar *et al*., 2014). The predictive model, generated in the present work, allows for the selection of a combination of factors which determine the achievement of not only an optimal protein yield but also a product with desired characteristics. Thus, the detrimental effect of high pH values on protein quality could be avoided by conducting the extraction at low pH but with increased concentrations of NaCl, while still achieving relatively high protein yield (Figure 5A).

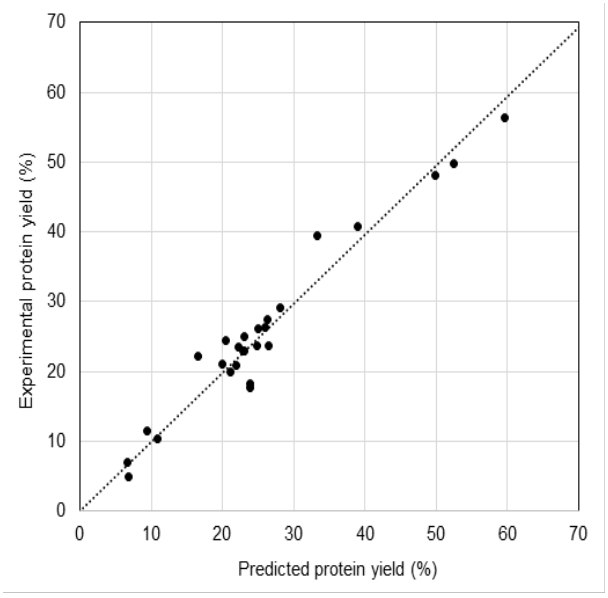

Figure 4. Experimental protein yield versus predicted protein yield by the model (Eq. 2).

Although found to be not significant, the combined effect pH-T (Figure 5B) and pH-extraction longevity (Figure 5C) showed an increase in the protein yield with the increase of the variables as evidenced by the estimated contour plots.

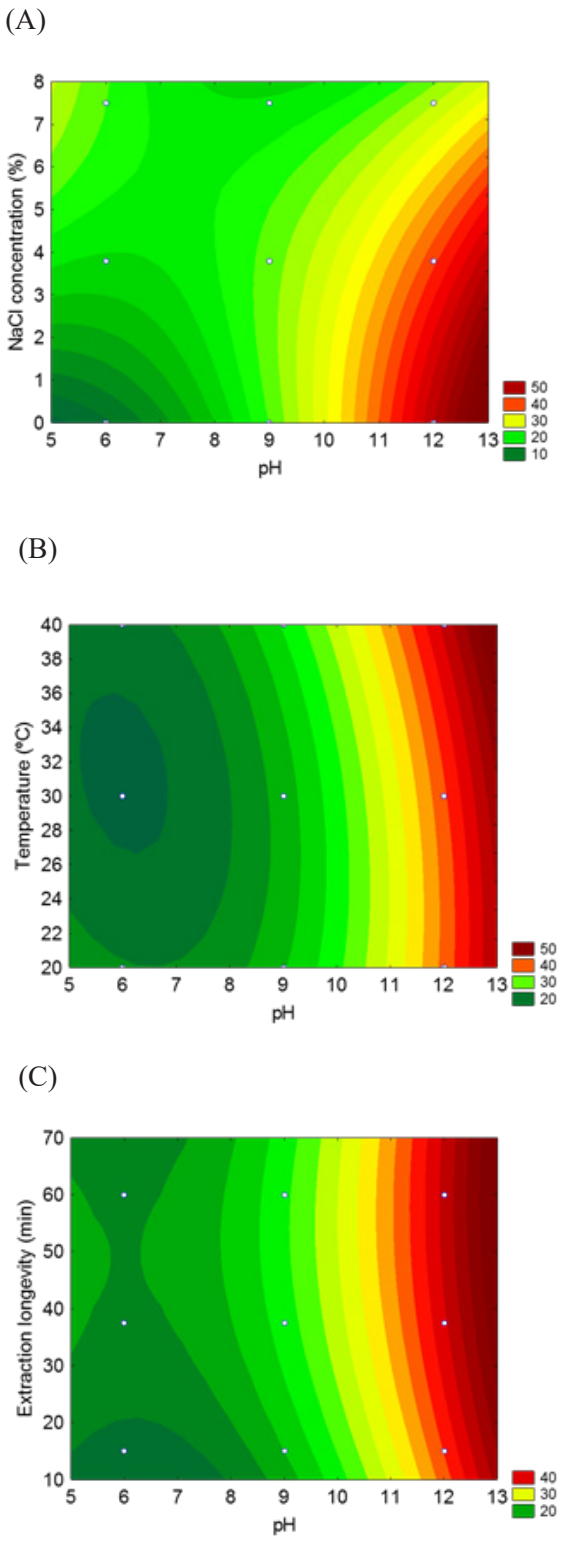

Figure 5. Estimated contour plot for the combined effect pH-NaCl (A), pH-T (B), and pH-extraction longevity (C) on protein yield.

Conclusion

A Central Composite Design, preceded by a full factorial experimental analysis at two levels, was successfully applied to model and optimize the protein yield response to four variables, namely pH, temperature, concentration of NaCl, and extraction longevity, when ethanol-treated industrial rapeseed meal was used as a raw material. The present work demonstrated prevailing influence of pH and its interaction with NaCl addition on protein yield. Higher protein yields were obtained by combining high pH values and low NaCl concentrations, and high pH values and high temperatures. Although leading to reduced protein yields, lower pH values and enhanced NaCl concentrations could be used to avoid the denaturing effect of highly alkaline conditions. For all variables studied, the highest protein yield (59.56 ± 1.29%) was obtained following extraction with 5% ethanol-treated rapeseed meal at pH 12, no NaCl addition, at 40°C and extraction longevity of 60 min. Since the extracting conditions are strongly related to protein fractional profile and characteristics, the mathematical model generated in the present work would facilitate the choice of factor combinations which lead to preparation of products with desired techno-functional properties while achieving optimal protein yield.

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