DYNAMICS OF ANTIOXIDANT CHARACTER OF EXTRACTS FROM FLAX SEEDS UNDER VARIABLE CONDITIONS

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Abstract: *The antioxidant character of natural extracts is an important indicator of quality. In extracts from flax seeds, this character is due to a series of biologically active natural compounds. Our experimental design studies the dynamics of antioxidant character due to the variation of three independent variables at two and three levels: solvent composition (ethanol/water 60:40, 80:20, and 100:0 v/v) extraction time (2, 3, and 4 h), and hydrolysis temperature (60 and 80°C). The antioxidant character (dependent variable) of extracts was expressed in the percentage of DPPH inhibition. The polynomial equation obtained by multiple regression analysis indicates that the influence of the three factors on the studied intervals decreases in order: solvent ratio ethanol > extraction time > hydrolysis temperature. In the studied intervals of the variables, the antioxidant character varies between 40.8 - 75.9 % DPPH inhibition. Response surface graphics and calculated nomograms show how much temperature and time can be reduced in the process without the antioxidant character being substantially affected. The experimental design and data processing described herein may constitute a management model of an optimal industrial extraction process from flax seeds.*

Keywords: antioxidant character, flax seeds, multiple regression analysis, extraction optimization.

INTRODUCTION

Antioxidants may be defined as substances that inhibit oxidation. Antioxidants are produced naturally by the biological system and occur in many foods. In plants, via the shikimic acid pathway, phenolic and polyphenolic compounds are produced, which have antioxidant character.

In food products, antioxidants are deliberately added to delay lipid oxidation during processing and storage. They have unique properties of extending the shelf-life of food products without changing their sensory or nutritional qualities. (Shahidi and Ambigaipalan, 2015)

1, 1 - diphenyl - 2 - picrylhydrazyl (DPPH) free radical scavenging method evaluates the antioxidant character of a compound, an extract, or other biological sources. The DPPH method is rapid, simple, inexpensive, and widely used to measure the antioxidant character of foods and/or food products. (Kedare and Singh, 2011) This method has been utilized for investigating the antioxidant character of wheat grain, and bran, vegetables, herbs, edible seed oils, and flours in several different solvent systems including ethanol, aqueous acetone, methanol, aqueous alcohol, and benzene (Parry et al., 2005). It is a convenient method for the antioxidant assay of cysteine, glutathione, ascorbic acid, tocopherol, and polyhydroxy aromatic compounds (Nishizawa et al., 2005), for olive oil, fruits, juices, and wines (Sánchez-Moreno, 2002).

The determination of the antioxidant character of various types of samples using DPPH is comparable to other methods. (Kedare and Singh, 2011)

The purpose of our work was to study the effect of solvent composition, extraction time, and hydrolysis temperature over the dynamics of the antioxidant character of extracts obtained from flax seeds using a three-level experimental model.

MATERIALS AND METHODS

Extraction of phenolic compounds

Based on the previously reported extraction conditions (Chen et al., 2007; Lupitu and Dinca, 2019; Popova et al., 2009; Willfor et al., 2006), an design with 18 experiments was created to

find the level of antioxidant character depending on the extraction conditions. In order to obtain the crude extracts, 15 g portions of flax seed (Cosmin variety), milled, dried and defatted were extracted for 2, 3 and 4 hours at 60^0C, using three proportion of ethanol/water: 60:40, 80:20 and 100:0 *v/v* (Table 1). The obtained extracts were hydrolysed for 2 hours using hydrochloric acid at 60 and 80^0C, and then neutralized and filtered.

Reagents, solvents, and standards

Reagents and solvents used in the experiments were of adequate analytical grade and were obtained from Sigma Aldrich (Fluka, Switzerland), Merck (Darmstadt, Germany) and Chimreactiv (Romania).

Measurement of antioxidant character

The antioxidant character of the extracts was assessed using the DPPH method. Briefly, 3 mL of 0.2 mM DPPH in ethanol were mixed with 0.1 mL sample. This mixture was kept in the dark for 60 minutes. The absorbance was measured against a control sample, at 517 nm using a Specord 200 UV-VIS double-beam spectrophotometer (Analytik Jena Inc. Germany). The calculation of DPPH free radical inhibition, I (%), was performed according to the relation displayed in equation 1:

$$I(\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \qquad (1)$$

where: $Abs_{control}$ represents the absorbance of the control sample consisted of 0.2 mM DPPH in ethanol and Abs_{sample} represents the absorbance of 0.2 mM DPPH containing the investigated extract. All analyzes were performed in triplicate and the results were reported as mean ±SD. (Metzner Ungureanu et al., 2020)

Experimental design

An experimental design, with three variables X_1 (solvent composition), X_2 (extraction time) and X_3 (hydrolysis temperature), at two and three variation levels (Table 1), was used to study the effect in the extraction process. The corresponding encodings are symbolized x_1, x_2 and x_3 (Myers & Montgomery, 2002).

Table 1 Independent variable values of the process, the corresponding levels, and their codification

Independent variable		Level		
	x_i	-1	0	1
Solvent composition (ethanol: water, *v/v*)	X_1	60:40	80:20	100:0
Extraction time (h)	X_2	2	3	4
Hydrolysis temperature (°C)	X_3	60	-	80

Data analysis

The multiple regression procedure and analysis of variance (ANOVA) from MS Excel 2019 software were used (Home Page of Excel 2019). The codified and experimental data (Table 2) were fitted to a polynomial model and regression coefficients were obtained. The generalized polynomial model used for establishing the importance and interaction of the studied factors was as follows:

$$Yi = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{123} x_1 x_2 x_3 \qquad (2)$$

where Yi is predicted response, β_0 is intercept, β_1, β_2, β_3, β_{11}, β_{22} *and* β_{33} is linear and quadratic effect terms, and β_{12}, β_{13}, β_{23} and β_{123} are interaction effects.

RESULTS AND DISCUSSIONS

Fitting the model

The multiple regression equation obtained with MS Excel 2019 is an empirical relationship between antioxidant character (% DPPH inhibition) and the three factors in coded units. The significance of each coefficient was appreciated using the *Student t* test and *p-value* calculated at 95% confidence interval. The corresponding variables will be more significant if the absolute *t* value is larger or the *p-value* is smaller (Home Page of NIST/SEMATECH, 2013). Consequently, the significance of the factors decreases in the order $x_1 > x_2 > x_3$ and the interaction between them in the order $x_1x_2 > x_1x_3 \approx x_1x_2x_3 > x_2x_3$ (Table 3). The minus sign of the coefficient indicates an inverse action of factor on the antioxidant character. The action of

the factor is stronger if the absolute value of its coefficient is greater. The high negative value of β_{11} shows that after the maximum, the decrease of the oxidizing character is abrupt with the increase of the ethanol concentration in the extraction solvent (Figures 1 and 2).

Table 2. The experimental design with three variables, the observed responses, and predicted values for antioxidant character, % inhibition of DPPH

	Variable levels			Experimental	Predicted
Treat	x_1	x_2	x_3	*Yi*	*Yi*
1	-1	-1	-1	36.20	32.43
2	0	-1	-1	29.18	36.80
3	1	-1	-1	-3.36	-6.73
4	-1	0	-1	50.56	50.36
5	0	0	-1	41.3	42.46
6	1	0	-1	-12.35	-13.34
7	-1	1	-1	62.42	68.29
8	0	1	-1	51.78	48.12
9	1	1	-1	-26.22	-19.95
10	-1	-1	1	50.3	52.50
11	0	-1	1	38.92	44.57
12	1	-1	1	-11.87	-11.26
13	-1	0	1	65.08	64.13
14	0	0	1	51.48	50.22
15	1	0	1	-13.81	-11.57
16	-1	1	1	72.95	75.75
17	0	1	1	59.44	55.88
18	1	1	1	-13.09	-11.88

Table 3 Significance of regression coefficient for antioxidant character (%DPPH inhibition)

Coefficients		*t Stat*	*p-value*
β_0	46.34	19.53	2.31E-07
β_1	-34.85	-26.81	2.58E-08
β_2	5.66	4.35	3.34E-03
β_3	3.88	3.66	8.09E-03
β_{12}	-9.12	-5.73	7.15E-04
β_{13}	-3.00	-2.31	5.44E-02
β_{23}	1.33	1.03	3.39E-01
β_{11}	-23.95	-10.64	1.42E-05
β_{22}	-1.49	-0.66	5.30E-01
β_{33}	0.000	-	-
β_{123}	3.15	1.98	-

The coefficients β_{23}, β_{22} and β_{33} are small so the corresponding terms can be neglected. The equation becomes:

$$Yi = 46.34-34.85x_1+5.66x_2+3.88x_3-9.12x_1x_2-3.00x_1x_3-23.95x_1^2+3.15x_1x_2x_3 \quad (3)$$

The verification of this relation was done by comparing the experimental values with the predicted value. The agreement of these values is illustrated by the high value of the correlation coefficient squared (0.9940). It also indicates that most of the variation of the response data is explained by the different input values.

Analysis of response surfaces

The relationship between independent and dependent variables is illustrated in three-dimensional representation of the response surfaces generated by the models for the antioxidant character. As well as multiple regression coefficients, the graphs show a weaker influence of extraction time and hydrolysis temperature between 2-4 h and respectively 60-80°C. The largest slope of the surface is due to the percentage of ethanol in the extraction solvent (Figs. 1 and 2). This is the most influential factor of the process in the studied intervals.

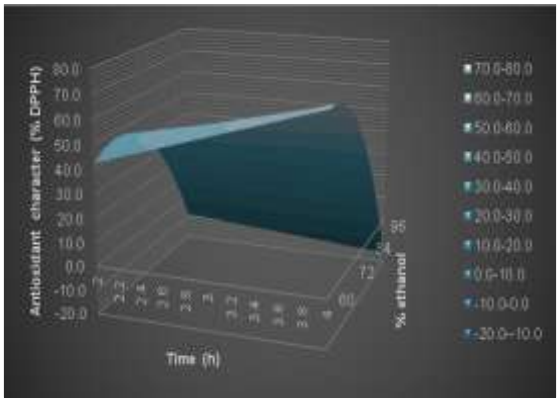

Fig. 1 Response surface plot showing the effect of extraction time and ethanol concentration at a constant hydrolysis temperature course of 70°C, on antioxidant character of extract from flax seeds.

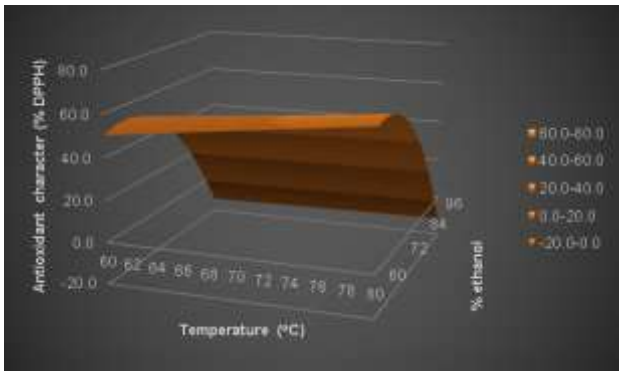

Fig.2 Response surface plot showing the effect of hydrolysis temperature and ethanol concentration at a constant extraction time course of 3 h, on antioxidant character of extract from flax seeds.

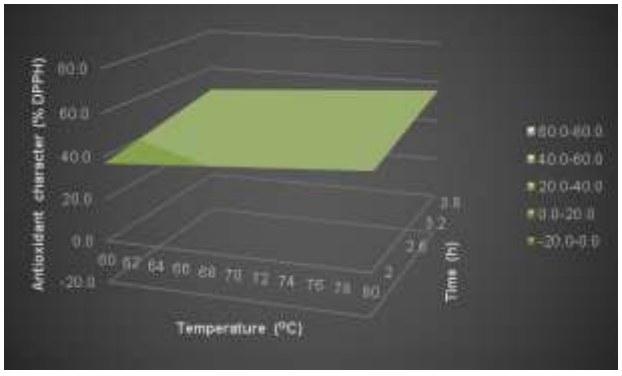

Fig.3 Response surface plot showing the effect of hydrolysis temperature and extraction time at a constant solvent composition course of 80% ethanol, on antioxidant character of extract from flax seeds.

The coordinates of the highest points on the surfaces correspond to the values of the factors that ensure the maximum antioxidant character of extract. The surface in the Fig.3 indicates that the maximum is reached at a marginal point: extraction time (X_2, 4 h) and hydrolysis temperature (X_3, 80°C). The curvature of the response surface on the axis of the extraction solvent shows that the maximum is obtained within the range, at a composition over 60% ethanol (X_1). This maximum point can be calculated by equalling with zero the partial derivative in relation to x_1 of the function (2) for x_2=1 and x_3=1. Equation (3) becomes:

$$Yi_{(x1)} = 55.88\text{-}43.82\ x_1\text{-}23.95x_1^2 \quad (4)$$

$$Y'i_{(x1)} = -43.82\text{-}47.90x_1 = 0 \quad (5)$$

$$x_1 = -0.9\ 149$$

$$X_1 = 61.7\ \%\ \text{ethanol}$$

$$Yi_{max} = 75.9\pm4.5\ \%\ \text{DPPH inhibition}$$

Using this calculation technique can determine the coordinates for any maximum point placed on the surface ridge (Figs. 1 and 2). For example, the maximum level of antioxidant character and the optimal alcohol concentration can be calculated for the situation in which, to save energy and time, is working on X_2=2h and X_3=60°C. In this case, the encoding is x_2=-1 and x_3=-1. Equation (3) becomes:

$$Yi_{(x1)} = 36.80\text{-}19.58\ x_1\text{-}23.95x_1^2 \quad (6)$$

$$Y'i_{(x1)} = -19.58\text{-}47.90x_1 = 0 \quad (7)$$

$$x_1 = -0.4088$$

$$X_1 = 71.8\ \%\ \text{ethanol}$$

$$Yi_{max} = 40.8\pm4.5\ \%\ \text{DPPH inhibition}$$

Such a reduction in energy consumption substantially reduces the antioxidant character of the extract (from 75.9 to 40.8 % DPPH) and requires solvent with a higher concentration of ethanol (71.8 % instead of 61.7%). It is a convenient situation because more concentrated alcohol is recirculated with less energy and time. For optimal management of the process in any intermediate variant of the above extremes, nomograms can be used (Figs. 4 and 5). These can be calculated and plotted for the entire code palette using the regression function (2) and its partial derivatives. MS Excel 2019 software is enough to achieve this. E.g., for a moderate reduction of energy consumption (X_2=3.1 h and X_3=73.9 °C), the graphical method indicates an optimal value X_1=64.6 % ethanol (Fig.4) which determines an antioxidant character of the

extract Yi_{max} = 62.8±4.5 % DPPH inhibition (Fig.5).

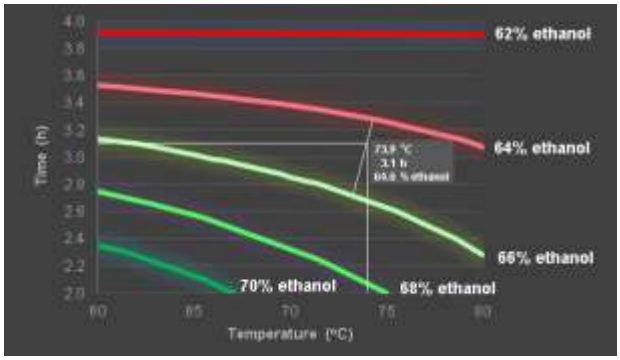

Fig.4 The nomogram with level curves of the optimal ethanol concentration (which ensures a maximum antioxidant character for the extract) according to temperature and time.

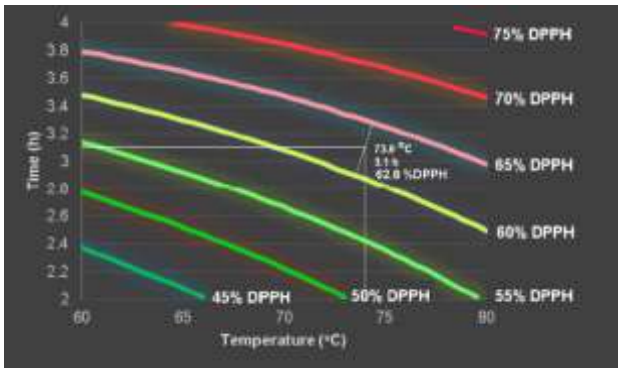

Fig.5 The nomogram with level curves of the antioxidant character according to temperature and time, using an optimal ethanol concentration.

It could thus be established how much temperature and time can be reduced without the antioxidant character being substantially affected.

CONCLUSIONS

The multiple regressions and the response surface methodology were successfully employed to control the antioxidant character of alcoholic extract from flax seeds. The influence of the three factors on the studied intervals decreases in order: solvent ratio ethanol > extraction time > hydrolysis temperature. The experimental design and data processing described herein may constitute a management model of an optimal industrial extraction processes from flax seeds.

ACKNOWLEDGEMENTS

Part of this work was supported by a grant of the Romanian Ministry of Education and Research, CNCS - UEFISCDI, project number PN-III-P1-1.1-PD-2019-0349, within PNCDI III.

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