AN EXPERIMENTAL DESIGN FOR PHENOLIC COMPOUNDS EXTRACTION FROM FLAX SEEDS

Andreea LUPITU[1,2], Nicolae DINCA[1*]

[1]Faculty of Food Engineering, Tourism and Environmental Protection, "Aurel Vlaicu" University, Romania, 2 Elena Dragoi, Arad 310330, Romania
[2]Research Development Innovation in Natural and Technical Sciences Institute, "Aurel Vlaicu" University, Romania, 2 Elena Dragoi, Arad 310330, Romania
[*]Corresponding author email: nicolae.dinca@uav.ro

Abstract: *Response surface methodology (RSM) was used to determine the optimization function for the extraction of phenolic compounds from flax seeds. Our preliminary design includes tree independent variables at two levels (8 experiments): hydrolysis temperature (60 and 80°C), extraction time (3 and 4 h) and solvent composition (ethanol/water 60:40 and 80:20 v/v). The dependent variable used in order to evaluate the extraction process of the total phenolic content of extracts obtained from flax seeds was expressed in gallic acid equivalent (mg GAE/L). Using RSM, a polynomial equation was obtained by multiple regression analysis for predicting optimization of the extraction protocol. Maximum yield was obtained when hydrolysis temperature, extraction time and solvent composition were 80°C, 4 h and 60:40 (v/v), respectively. This relation can be useful in the development and optimization of industrial extraction processes.*

Keywords: response surface methodology, optimization, flax seeds, extraction of phenolics.

INTRODUCTION

Flax (*Linum usitatissimum* L.) has been used by humans for about 10,000 years. This was probably one of the first plants that were domesticated, about 6,000 years ago BC in Mesopotamia. The uses of flax seeds can be divided into three main groups: (1) the production of oils for edible purposes (as a natural product consumed as such, for cooking, as a baking ingredient, as an ingredient in margarine products), and industrial applications agent, paint, printer ink and paints), (2) fibre production for the textile industry, and (3) use of seeds as a raw material for obtaining value-added products (food supplements high in phenolic compounds and lignans), as an ingredient in the food industry (includes bakery products, pastries and cereals for breakfast), as a functional food (to provide protection against certain types of cancer, heart disease, hyperglycaemia, stroke, and thrombosis), as animal feed, in the form of a hull resulting from seed pressing (for buffaloes, cattle, horses, poultry, cats and dogs) (Jhala and Hall, 2010; Singh et al., 2011).

Flax seeds are the most significant source of lignans (the content in lignans is up to 800 times higher than in any other food source). Other sources of phenolic compounds are: grapefruits, cherries, kiwi, plum, mandarin, olives, orange, melon, grapes, banana, tomato, pineapple (Milder et al., 2005). Lignans are a class of secondary phenolic metabolites, which have a basic structure consisting of 2,3-dibenzylbutane (Cornwell et al., 2004; Meagher and Beecher, 2000; Milder et al., 2005; Smeds et al., 2007).

The chemical composition of flax seeds varies considerably between varieties and also depends on the environmental conditions in which the plant is grown. The main components present in flax seeds are: fatty acids (α-linolenic, linoleic, oleic, palmitic and stearic acid) (Hettiarachchy et al., 1990; Oomah and Mazza, 1993); water-soluble mucilage and insoluble fibres (Warrand et al., 2005) phenolic compounds (ferulic acid, synaptic acid, coumaric acid, hydroxy-benzoic acid and caffeic acid, gallic acid, secoisolariciresinol diglucoside, pinoresinol, matairesinol, lariciresinol (Oomah, 2003; Pag et al., 2014)).

Different extraction techniques are often used to extract phenolic compounds: conventional extraction, ultrasonic assisted extraction, microwave assisted extraction, supercritical fluid extraction, and enzyme assisted extraction (Akl et al., 2017; Kim and

Mazza, 2006; Renouard et al., 2010; Westcott and Muir, 1998).

Usually, phenolic compounds are extracted from air dried, defatted flaxseeds by different solvents: methanol, ethanol, acetone, ethyl acetate, distilled water, or a mixture of them (Anwar and Przybylski, 2012; (Oomah and Mazza, 2001; Zhang et al., 2007).

The amount of phenolic compounds extracted from flax seeds can be affected by solvent polarity. The mixture of water with ethanol is often recommended to prepare extracts because of their and their safety for human consumption and handling (Kim and Mazza, 2006; Zhang et al., 2007).

The purpose of this preliminary work was to study the effect of solvent composition, hydrolysis temperature and time over the phenolic compounds extraction from flax seeds using a two-level experimental model.

MATERIALS AND METHODS

Extraction of phenolic compounds

Based on the previously reported extraction conditions (Chen et al., 2007; Popova et al., 2009; Willfor et al., 2006), an experimental design was created to find the optimal conditions for phenolic compounds extraction.

In order to obtain the crude extracts, 15 g of flax seed (Cosmin variety), milled, dried and defatted were extracted for 3 and 4 hours at 60^0C using the proportion of solvent for each sample according to the extraction protocol. The obtained extracts were hydrolysed for 2 hours using hydrochloric acid at 60-80^0C, 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 total phenols

Total phenolic content of the extracts obtained was determined using Folin-Ciocalteu method slightly modified (Pag et al., 2014). Briefly, the obtained extracts were diluted using distilled water (1:25). 0.5 mL Folin-Ciocalteu reagent, 2 mL Na_2CO_3 (20%) and 5 mL distilled water were added to 1 mL sample. The mixture was kept in the dark for 90 minutes. The absorbance was measured against a blank prepared in the same conditions, at 765 nm, using a UV-VIS double beam spectrophotometer (Specord 200, Analytik Jena Inc., Jena, Germany). Gallic acid was used as reference. A calibration curve for gallic acid was obtained (20, 40, 100, 160, 200 mg/L), then the regression equation and the correlation coefficient were calculated and the results were expressed in mg GAE/L. All experiments were performed in triplicates.

Experimental design

An experimental design, with three variables X_1 (solvent composition), X_2 (extraction time) and X_3 (hydrolysis temperature), at two variation levels (Table 1), was used to study the effect in the extraction process. Experiments were randomized in order to maximise the effects of unexplained variability in the observed responses due to extraneous factors (Myers & Montgomery, 2002).

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

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

Data analysis

The multiple regression procedure and analysis of variance (ANOVA) from MS Excel 2016 software were used (Home Page of Excel 2016). 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:

$$Yi=\beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_12x_1x_2 +\beta_{13}x_1x_3+\beta_{23}x_2x_3 \quad (1)$$

where Yi is predicted response, β_0 is offset term, β_1, β_2 *and* β_3 is linear effect terms, and β_{12}, β_{13} and β_{23} are interaction effects.

RESULTS AND DISCUSSIONS

Fitting the model

The multiple regression equation obtained with MS Excel 2016 is an empirical relationship between total phenols yield 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 $x_1>x_3>x_2$ and the interaction between them in the order $x_1x_3> x_1x_2>x_2x_3$ (Table 3). The minus sign of the coefficient indicates an inverse action of factor on the phenol's extraction yield. The action of the factor is stronger if the absolute value of its coefficient is greater.

Table 2. The experimental design with three variables, the observed responses, and predicted values for protein yield

	Variable levels			Experimental	Predicted
Treat	x_1	x_2	x_3	Yi	Yi
1	-1	-1	-1	878.9	887.8
2	1	-1	-1	216.8	223.9
3	-1	1	-1	921.4	912.5
4	1	1	-1	206.3	199.2
5	-1	-1	1	1069.1	1079.8
6	1	-1	1	240.8	253.3
7	-1	1	1	1115.2	1104.5
8	1	1	1	241.1	228.6

Table 3 Significance of regression coefficient for predicted total phenols content yield

Coefficients		*t Stat*	*P-value*
β_0	611.202	679.136	0.00094
β_1	-384.961	-427.749	0.00149
β_3	55.350	61.502	0.01035
β_{13}	-40.659	-45.179	0.01409
β_{12}	-12.366	-13.740	0.04625
β_2	9.808	10.898	0.05825
β_{23}	1.781	1.9787	0.29790

The interaction of x_2 with x_3 is weak so the term $1.781x_2x_3$ and $9.808x_2$ can be neglected. The equation becomes:

$$Yi = 611.20 - 384.96x_1 + 55.350x_3 - 12.366x_1x_2 - 40.659x_1x_3 \quad (2)$$

The verification of this relation was initially done by comparing the experimental values with the predicted value. The agreement of these values is also illustrated graphically (Fig. 1) and by the high value of the correlation coefficient squared (0.9993). It also indicates that most of the variation of the response data is explained by the different input values.

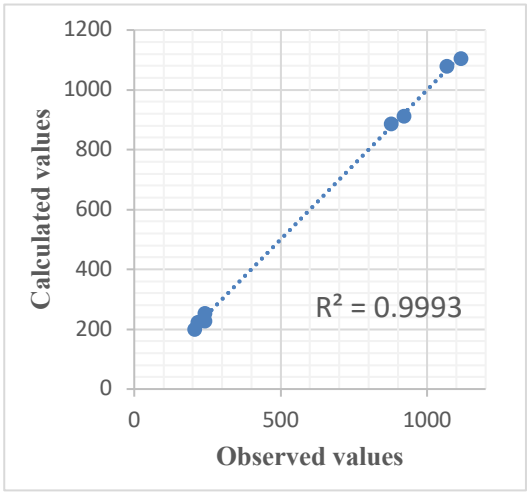

Fig. 1 Comparison between predicted and observed extraction yield.

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 total phenols content yield.

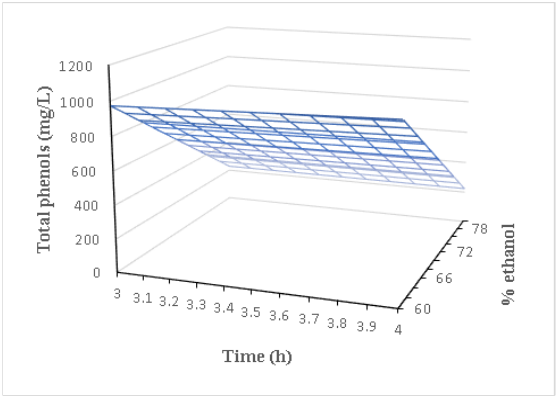

Fig. 2 Response surface plot showing the effect of extraction time and ethanol concentration at a constant hydrolysis temperature course of 70°C on total phenolic content.

The graphs in Fig. 2 and 4 show a very weak influence of the extraction time between 3 and 4 h. The largest slope of the surface is due to the percentage of ethanol in the extraction solvent (Figs. 2 and 3). This is the most influential factor of the process in the studied intervals.

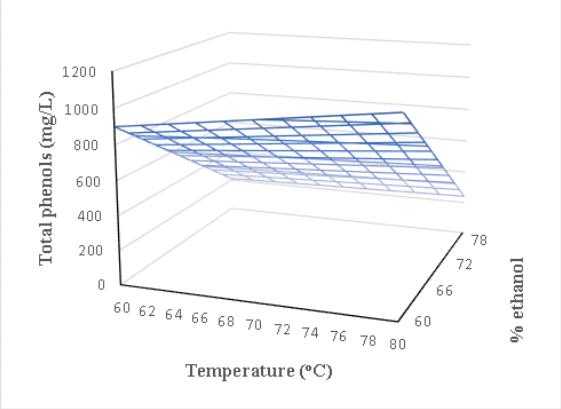

Fig.3 Response surface plot showing the effect of hydrolysis temperature and ethanol concentration at a constant extraction time course of 3.5 h on total phenolic extract.

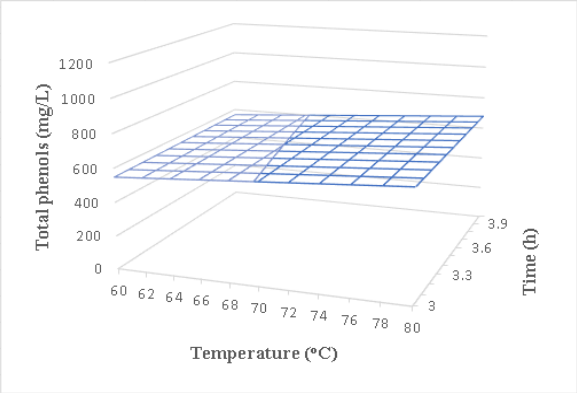

Fig.4 Response surface plot showing the effect of hydrolysis temperature and extraction time at a constant solvent composition course of 70% ethanol on total phenolic yield.

The coordinates of the highest points on the surfaces correspond to the values of the factors that ensure the maximum extraction yield (total phenols, 1114 ± 4 mg GAE/L): solvent composition (X_1, 60% ethanol), extraction time (X_2, 4 h) and hydrolysis temperature (X_3, 80°C) in the studied intervals. It is necessary in the following studies to investigate the time interval of the extraction before the low influence level between 3 and 4 h. It could thus be established how much time can be reduced without the extraction yield being substantially affected. Also, a new experimental design on three levels would highlight the curvature of the response surfaces allowing a more precise assembly of the physical-chemical optimization equations with those of economic optimization.

CONCLUSIONS

The response surface methodology was successfully employed to optimize the extraction of phenolic compounds from flax seeds. The influence of the three factors studied decreases in order: solvent ratio ethanol > hydrolysis temperature > extraction time. The resulted polynomial model gave a satisfactory description of the experimental data and allows the physico-chemical optimization of conditions for maximum extraction of total phenols. This relation can be useful in the development of industrial extraction processes.

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