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Optimization of extraction process of escin from dried seeds of *Aesculus hippocastanum L.* by Derringer's desirability function

Zorica Vujic[a*], Dragana Novovic[b], Ivana Arsic[c], Dusanka Antic[b]
[a] *University of Belgrade-Faculty of Pharmacy, Vojvode Stepe 450, 11 000 Belgrade, Republic of Serbia*
[b] *Alternativa medica, Manufecturer of phytopreparations, Zmaj Jovina 1, 15 300 Loznica, Republic of Serbia*
[c] *University of Niš-Faculty of Medicine, Department of Pharmacy, Bulevar dr Zorana Djindjica 81, 18 000 Nis, Republic of Serbia*
**Corresponding author E-mail address*: zvujic@pharmacy.bg.ac.rs *Tel.: +381-11-39-51-340; fax: +381-11-29-72-840.*

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1 SUMMARY

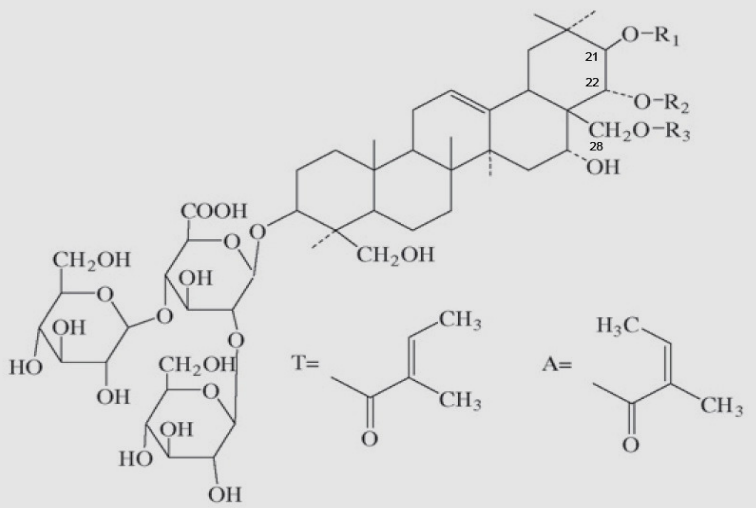
The optimization of the extraction procedure of escin from dried seeds of *Aesculus hippocastanum L.* was studied. Full factorial design with central point and Derringer's desirability function was employed to obtain the best possible combination of temperature (A: 25–45 ◦C), flow rate of organic solvent (B: 125-345 ml min^{-1}) and extraction time (C: 3–6 h) for maximum extraction yield of escin. The experimental data were fitted to a second-order polynomial equation using multiple regression analysis and also analyzed by appropriate statistical methods (ANOVA). In order to get the best chromatographic performance, the multicriteria methodology was employed by means of Derringer′s desirability function. The optimum extraction conditions were as follows: the temperature of 45°C, flow rate of 125 ml min^{-1} and extraction time 3h.

2 INTRODUCTION

Aesculus Hippocastanum L; Fam. Hippocastanacea is a large deciduous tree, commonly known as horse-chestnut or conker tree. *Aesculus Hippocastanum L.* is spread in the northern hemisphere, primarily in eastern Europe, eastern Asia and eastern north America. Semen Hippocastani consists of the dried ripe seeds of *Aesculus hippocastanum*, which present the plant material of medical interest.

The main active compound in horse chestnut is escin. Escin is a mixture of saponins with anti-inflammatory, vasoconstrictor and vasoprotective effects found in *Aesculus hippocastanum* (the horse chestnut).Horse chestnut seed extracts are indicated for treatment of complaints of chronic venous insufficiency such as feeling of pain, heaviness, and tension in the legs, nocturnal systems, swelling of the legs and pruritis (Wichtl, 2004). The principal ingredient of Horse Chestnut seed escin has an anti-excudative vascular tightening effect and reduction of vascular permeability which result in an antiedemic effect. The vein-toning properties of the Horse Chestnut seed extract also demonstrated improvement of venous return flow (Thomson Healthcare, 2007) Escin inhibits the activity of lysosomal hyaluronidase by preventing break-down of the main component hyaluronic acid, and thereby preserving the matrix that supports the capillary walls (Wichtl, 2004). According to German Commision E monographs average daily dose of horse chestnut seed preparations for oral use corresponds to 100 mg of escin,

two times per day (http://www.heilpflanzen-welt.de, 2012).
Aesculus hippocastanum contains an escin that is a natural mixture of triterpene saponins. The aglycons are derivatives of protoaescigenin, acylated by acetic acid at C-22 and by either angelic (A) or tiglic acids (T) at C-21 (Glensk *et al.*, 2011). The saponins in escin are made up of two series of isomers, β-escin and α-escin defined by the position of an acetyl group at C_{22} and hydroxyl group at C_{28}. β-escin (mainly made up of escin Ia and escin Ib) is the major active component in extracts of horse chestnut seed. α-escin, made up mainly of isoescin Ia and isoescin Ib, has less bioactivity (Wu *et al.*, 2012). β-escin and α-escin can be distinguished by melting point, specific rotation, hemolytic index and solubility in water (Glensk *et al.*, 2011).



Saponins	R1	R2	R3
Escin Ia	T	-COCH3	-H
Escin Ib	A	-COCH3	-H
Isoescin Ia	T	-H	-COCH3
Isoescin Ia	A	-H	-COCH3

Figure 1: Chemical structures of saponins

During the last decades, many analytical methods including spectrophotometry (DAB, 1991; Acar and Paksoy, 1993), HPTLC methods (Constantini, 1999; Apers *et al.*, 2006), liquid chromatography-mass spectrometry (Griffini *et al.*, 1997), multivariate calibration methods such as partial least squares (PLS) regression (Donmez *et al.*, 2006) have been proposed for the determination of escin or its isomers. The saponins escin Ia, escin Ib, isoescin Ia and isoescin Ib have been analyzed using the method based on High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD) and positive ion electrospray-time of flight mass spectrometry (Chen *et al.*, 2007) or Liquid Chromatography-Tandem Mass Spectrometry LC-MS/MS (Liu *et al.*, 2010). In the German Pharmacopeia (DAB) the content of escin of *Aesculus hippocastanum* and its extract are determined by means of a colorimetric method. This method provides determination of only the total saponins content and is non-specific towards individual escin and has been used in the present study for optimization of method extraction of horse chestnut seed. The classical method of studying one variable at a time may be effective in some processes, but fails to consider the combined effects of several factors involved. Therefore, it is necessary to use an optimization method that can be used to determine all the factors as well as the possible interactions among these independent variables (Li *et al.*, 2011). Optimization through factorial design and Derringer's desirability function particularly fulfills this requirement. In order to evaluate the effect of the most important factor, a 2^3 full factorial design (FFD) with three replicates at the zero level was chosen. Derringer′s desirability function as multicriteria approach which provides an establishing a set of controlled experimental factors and getting the optimum response was applied (Vujic *et al.*, 2012).

3 MATERIALS AND METHODS

3.1 Materials and reagents : The seeds were collected in gardens at Vrnjacka Banja in Serbia. All the reagents such as methanol, ethanol, chloroform and acetic acid of analytical grade and were obtained from local chemical suppliers. Standard escin was obtained from Santa Cruze Biotechnology California.

3.2 Sample preparation : The horse chestnut seeds were dried at room temperature and ground into powder with a cyclone mill and passed through a number 1400 sieve. The hippocastani seed powder was treated with 67 % *v*/*v* of ethanol. The process of extraction was carried out in a laboratory by the extractor Dig Maz 5, Samtech, Austria. The ratio of drug to the extract was 2:5. Photometric determination of triterpene glycosides (calculated as total escin amounts) is based on the color reaction of the products from the reaction with mixture of acetic acid, sulfuric acid and conc.ferry chloride.

3.3 Experimental design and statistical analysis: This study was aimed to optimize the extraction conditions of escin from dried horse chestnut seeds investigating the effects of extractions parameters such as the extraction temperature, flow rate and the extraction time. The content of the escin was analyzed using pharmacopeia method (DAB, 1991).

A three-variable, two-level full factorial design (FFD) was applied in order to evaluate the influence of critical factors on process of extraction and in order to obtain maximum extraction yield in examined experimental domain. On the basis of preliminary experiments three factors were chosen as independent variables: extraction temperature (°C, A), flow rate of solvent (ml min^{-1}, B) and extraction time (h, C). Factors and their "low" (−1), "high" (+1) and "zero" (0) values are presented in Table 1.

Table 1: Independent variables and limit levels.

Factors		**Factor levels**		
		(−1)	**(0)**	**(+1)**
A	Extraction temperature (°C)	25	35	45
B	Flow rate (ml min^{-1})	125	235	345
C	Extraction time (h)	3	4.5	6

Regression analysis was performed for the experiment data and was fitted in a second-order interaction model with the following form:

$$y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{123}ABC \quad (1)$$

Where b_0 is the intercept, b_i (b_1, b_2, b_3), b_{ij} (b_{12}, b_{13}, b_{23}) and b_{ijk} represent the regression coefficient and *A*, *B*, *C* represent independent variables. Appropriate calculations were done with the Design-Expert 7.0 software (Stat-Ease Inc. Minneapolis, MN, USA).

4 RESULTS AND DISCUSSION

The investigation was carried out in two steps. The objective of the first step was to perform a screening of the factors that could potentially influence to process of extraction (to reduce yield of extraction) and the second was employing the multicriteria methodology in order to get the best operating conditions. Since the number of influencing factors is three and since it does not require an excessive number of experimental runs, full factorial design (FFD) was chosen. A total of 11 experiments were designed. Each experiment were performed in triplicate and average extraction yield (Ey) was chosen as the response, Y. Experimental layout designed by Design-Expert and corresponding experimental values of responses is presented in Table 2.

Table 2: Factorial design matrix and results of experiments.

Factor variables			
A (temperature,°C)	**B (flow rate, ml min^{-1})**	**C (extraction time, h)**	**Extraction yield (Ey) of escin (%)**
45 (1)	125(−1)	3(−1)	50
25(−1)	125(−1)	6(1)	40
25(−1)	125(−1)	3(−1)	28.33
45(1)	125(−1)	6(1)	29.17
35(0)	235(0)	4.5(0)	29.17
45(1)	345(1)	6(1)	27.5
45(1)	345(1)	3(−1)	44.17
35(0)	235(0)	4.5(0)	29.17
25(−1)	345(1)	6(1)	36.67
25(−1)	345(1)	3(−1)	26.67
35(0)	235(0)	4.5(0)	28.33

The repetition of the central experimental point provided a precise estimation of the experimental errors and the measure of the adequacy of the models (lack of fit). The results were analyzed by ANOVA method and the results are presented in Table 3.

Table 3: Statistical parameters of models obtained by ANOVA.

	SS_{models}	$SS_{models/df}$	F	p	R^2	R^2_{adj}
Model	540.21	77.17	328.11	0.003	0.9991	0.9961
A: Temperature	45.94	45.94	195.31	0.0051		
B: Flow rate	19.50	19.50	82.91	0.0118		
C: Extraction time	31.32	31.32	133.18	0.0074		
AB	0.79	0.79	3.35	0.2088		
AC	437.64	437.64	1860.7	0.0005		

BC	0.78	0.78	3.30	0.2111		
ABC	4.25	4.25	18.06	0.0511		

The lack of fit test was determined by performing Fischer-F test. The high value of F with a very low probability (only model terms with corresponding *p*-value lesser than 0.05 are significant at 95% confidence level) implies that there was no evidence of the models lack-of-fit and the models could be accepted as an adequate representation of the data. In addition, the values of R^2 and R^2 adjusted taking into account the degrees of freedom indicated that the regression model fits the data well. The mathematical relationship between the three independent variables and response can be presented by the following second-order polynomial equation (Eq.2).

Final Equation in Terms of Coded Factors:

$$Ey = 35.31+2.4A-1.56B-1.98-0.31AB-7.40AC+0.31BC+0.73ABC \quad (2)$$

The data collected from the performed FFD design led to the following conclusions: it was noticed that interaction of temperature and extraction time (AC) has the largest influence on extraction yield. This influence has a minus sign, which means that the higher value of interaction will reduce yield. E.g. extraction at an elevated temperature for a long time can lead to degradation of active substances. Considering the influences of factors separately it could be seen that increasing of temperature and decreasing of extraction time will improve extraction procedure (Fig.2). Since the selected response was not affected in the same manner an additional optimization procedure was needed.

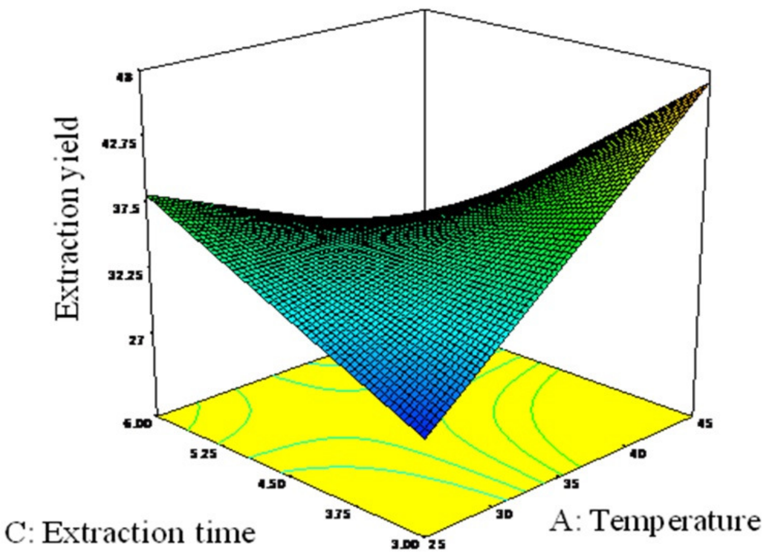


Figure 2: Response surface (3-D) showing the effect of the extraction time and extraction temperature on the response (Extraction yield); Flow rate =235 ml min^{-1}

In order to get the best chromatographic performance, the multicriteria methodology was employed by means of Derringer's desirability function. It is based on constructing desirable ranges for each response (individual desirable function, d_i) and establishing an overall desirability function (the Derringer desirability function). The Derringer's desirability function is defined as the geometric mean of individual desirability functions and can be expressed by Equation (3):

$$D = (d^{p1}_1 \times d^{p2}_2 \times .... d^{pn}_n)^{1/n} \quad (3)$$

Where n is the number of responses, and p^n is the weight of the responses. Weight of the response is the relative importance of each individual functions d_i and may range from 0.1 to 10. With a weight of 1, d_i varies in a linear way. In this study, weights equal to 1 was selected. Individual desirability functions range from 0 (undesired response) to 1 (a fully desired response). A value of D close to 1 means that the combination of different criteria is globally optimal. If any of the responses or factors falls outside their desirability range, the overall function becomes zero. There are several ways for calculating the desirability function depending on the goal desired. The goal of multicriteria optimization is shown in Table 4.

Table 4: Criteria for multivariate optimization of the response

	Goal	Lower limit	Upper limit	Weight	Importance
Temperature	In range	25	45	1	
Flow rate	In range	125	345	1	
Extraction time	In range	3	6	1	
Extraction yield	maximize	26.67	50	1	5

Desirability function calculations were performed using Design-Expert® 7.0. Obtained results are graphically presented in Fig. 3.

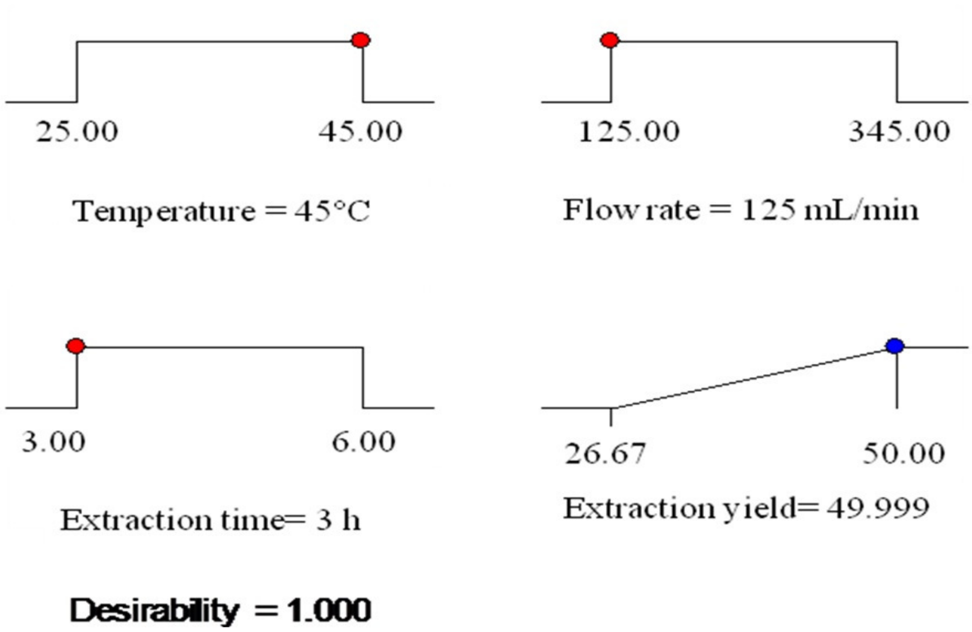


Figure 3. Graphical representation of the constraints accepted for the determination of optimal conditions

The coordinates related to the maximum of extraction yield are selected as the best operating conditions. The maximum extraction yield is achieved at the temperature of 45°C, flow rate of 125 ml min^{-1} and extraction time 3h.

5 CONCLUSION

The extraction conditions have significant effects on the extraction yield of escine.Using the FFD was effective for estimating the effect of three independent variables (extraction temperature, flow rate of organic solvent and extraction time). The optimum set of the independent variables was obtained by Derringer′s desirability function in order to obtain the desired extraction yield. The optimal experimental extraction yield of 49.999±0.67 % was obtained at the temperature of 45°C, flow rate of 125 ml min^{-1} and extraction time 3h. The main advantage of FFD and Derringer′s desirability function is that the number of experimental trials can be reduced. These results demonstrated that optimization method was efficient, less laborious and time-consuming compared to traditional methods which is based on "trial-and-error" postulate.

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