# OPTIMIZATION (FACTORIAL EXPERIMENT WITH RSM) OF MICROWAVE-ASSISTED EXTRACTION OF POLYPHENOLS FROM SEA BUCKTHORN LEAVES

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*The aim of this study was to optimize the microwave-assisted extraction (MAE) of polyphenolic compounds from sea buckthorn leaves. The influence of three independent variables: extraction temperature (40-80°C), extraction time (300 - 600s) and stirring rate (600 – 1200 rpm) on the two measured responses (total polyphenolic content and antioxidant activity) was studied using a $2^3$ factorial experiment. The optimal microwave-assisted extraction conditions determined by statistical analysis for TPC and for antioxidant activity were 600 s extraction time, 80°C extraction temperature and 1200 rpm stirring rate, and respectively 300 s extraction time, 80°C extraction temperature are 1200 rpm stirring rate.*

**Keywords**: microwave-assisted extraction, sea buckthorn leaves, polyphenolic compounds

## 1. Introduction

Sea buckthorn (Hippophae rhamnoides L., family: Elaeagnaceae) is a thorny bush distributed throughout the temperate zone of Asia and Europe and all over the subtropical zones of especially at high altitude [1]. Sea buckthorn is generally known for the high nutritional value of its berries. The sea berries are an important source of vitamin C, E and A, yellow/orange pigments, polyphenolic compounds, amino acids and oil [2]. The recent study shown that sea buckthorn leaves have a high bioactive and antioxidant activity because their content in polyphenolic compounds include phenolic acids, flavonoids, and hydrolyzable tannins [3,4].

Polyphenolic compounds are secondary plant metabolites [5], in the last years being recognized for their nutritional value, anti-carcinogenic, anti-ulcer, antithrombotic, anti-inflammatory, anti-allergenic, immunemodulating, anti-aggregative, anti-microbial, vasodilatory and estrogenic effects [6].

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In the last decade, a lot of studies have been published on the extractions methods of polyphenolic compounds from plants. Traditional extraction methods have been studied, like maceration [7], conventional solvent extraction (Soxhlet extraction, reflux extraction [7-9] and un-conventional extraction methods, like ultrasound assisted extraction (UAE) [7, 9] microwave-assisted extraction (MAE) [10-12], ultrasound-microwave-assisted extraction (UMAE) [13], supercritical fluid extraction (SFE) [14], sub-critical water extraction (SCWE) [15]. The new extraction techniques, inclusively MAE, reduced the volume of organic solvents, reduced extraction time and increased extraction yield [12].

From our knowledge the MAE of polyphenolic compounds from sea buckthorn leaves was investigated for the first time by our research group [11].

The objective of this study was to optimize extraction of polyphenolic compounds with high antioxidant activity, from sea buckthorn leaves, as function of microwave extraction time, extraction temperature and stirring rate, using a $2^3$ factorial experiment with response methodology (RSM).

## 2. Experimental section

### 2.1 Plant material

Sea buckthorn (*Hippophae rhamnoides* L.) leaves used in this study were obtained from HOFIGAL EXPORT IMPORT SA. The leaves were harvested in June 2014. Before use, the leaves were dried in an oven at 60°C and the dried material was grinded in a fine powder, using a domestic grinder. The samples were stored in airtight bottles, in a dark place at 4°C.

### 2.2. Chemicals

All the chemicals were purchased from Sigma Aldrich and Chimreactiv, used as received, without further purification or distillation. The solutions were prepared using distillated water.

### 2.3. Extraction procedure

Extraction of total polyphenolic compounds from sea buckthorn leaves by microwave assisted extraction was performed using a microwave generator (Miniflow 200SS, 2450Hz, Sairem) with adjustable temperature and power settings. The stirring was provided by an external magnetic stirrer placed at the bottom of the microwave cavity (IKA – magnetic stirrer RH digital GmbH & Co KG, Staufen, Germany). All the extraction were carried out in a glass reactor with a volume of 30 mL, using a plant to solvent ratio of 1:20 and, as extraction solvent, ethanol at a 50 vol.%. The extraction were carried out varying the extraction temperature (40, 60 and 80°C), extraction time (300, 450 and 600 s) and the stirring rate (600, 900 and 1200 rpm). The extracts obtained were filtered over a Whatman No. 1 paper, and stored in airtight bottles, in a dark place at 4°C, until the next use. All the extractions were carried out in triplicate.

## 2.4. Sample analysis

***Determination of total phenolic content (TPC)***

The total phenolic content (TPC) in the extracted samples was determined by the modified Folin-Ciocalteu colorimetric method [16]. For the construction of the standard curve, gallic acid was used. All the samples extracted were tested in duplicate. Gallic acid was used as standard for the generation of a standard curve, with concentrations ranging from 1 to 4.5 mg/L. For the analysis, 0.5mL of diluted sample extract or gallic acid standard solution, 7.5 mL of distilled water, 0.5 mL of 1N Folin-Ciocalteu reagent and were added to the test tubes and stirred (at 300rpm) for 3 min. After stirring, 1.5 mL of sodium carbonate solution (200 g/L) was added and the mixture was kept in the dark for 1 hour. The absorbance was then measured at 765 nm using a spectrophotometer (Shimadzu UVmini 1240) and the results were expressed in milligram of gallic acid equivalents (GAE) per gram of dry plant. All analyses were carried out in duplicate.

***HPLC analysis***

Quantitative analysis of individual compounds was carried out using a system consisting of: UV-2075 Detector, PU-2080 plus Pump, LG-2080 Gradient Unit, DG-2080_4 Degasser (from Jasco Analytical Instruments). Separation was realised on Teknokroma Nucleosil 100 C18 column (250 x 0.4 mm, 10 μm). Analyses were performed at room temperature, with a flow rate of 0.5 mL/min using 2% v/v acetic acid (solvent A) and methanol (solvent B) under the following gradient program: 0-8 min 70% A, 8-19 min 60% A and 19-30 min 50% A. Identification of each compound was based on the retention time of polyphenols used as standards. The standard stock solutions were prepared using standard of gallic acid (1.3 mg), catechin (1.6 mg), caffeic acid (1.5 mg), p-cumaric acid (1.5 mg), ferulic acid ( 1.3 mg), rutin (1.6 mg), quercetin (1.5mg) and 16 mL ethanol 50%. The calibration curve was constructed using successively dilutions of the stock solutions.

Before HPLC analysis, an additional step of liquid-liquid extraction was required, because the extracted samples contained some non-polar compounds beside the polyphenolic compounds. A liquid-liquid extraction step using diethylether was employed in order to remove these non-polar compounds, which cause serious matrix interferences [17].

***Antioxidant activity***

The antioxidant activities of the samples extracted were determined theoretical based on the concentration of the compounds in the samples, determined by HPLC analysis. For the calculation of the antioxidant activities, the standard phenolic compounds antioxidant activities were used as a reference [18, 19].

The experimental values obtained by HPLC analysis, regarding the polyphenols concentration in the extracted samples are presented in another paper in pending publication.

## 5. Experimental design

To investigate and optimize the effect of three independent parameters for MAE, namely: extraction temperature ($x_1$, 40-80°C), extraction time ($x_3$, 300 – 600 s) and stirring rate ($x_2$, 600 – 1200 rpm) on the total polyphenolic content and antioxidant activity, a $2^3$ factorial experimental design with response surface methodology (RSM) was used. The statistical analysis have been performed using Statgraphics Centurion XVI Software.

## 3. Results and discussions

### 3.1. Fitting the model

The natural and coded values of the selected independent variables (temperature, stirring rate and extraction temperature) are presented in *Table 1.* TPC and antioxidant value for the 14 experiments, including six replicates as the center point, of the experimental design protocol are shown in *Table 2.*

*Table 1*

**Experimental ranges of the independent variables in the experimental design**

| Factor | Value / Level | | | | | |
|---|---|---|---|---|---|---|
| | Low | | Medium | | High | |
| | Natural (z) | Coded (x) | Natural (z) | Coded (x) | Natural (z) | Coded (x) |
| Temperature (°C) | 40 | -1 | 60 | 0 | 80 | +1 |
| Stirring rate (rpm) | 600 | -1 | 900 | 0 | 1200 | +1 |
| Extraction time (s) | 300 | -1 | 450 | 0 | 600 | +1 |

*Table 2*

**$2^3$ full factorial design with their observed responses**

| | Levels | | | Observed responses | |
|---|---|---|---|---|---|
| Run | $x_1$ (Temperature) | $x_2$ (Stirring rate) | $x_3$ (Extraction time) | TPC (mg GAE/g of plant) | Antioxidant activity (μmol Trolox/g of plant) |
| 1 | -1 | -1 | -1 | 87.37 | 53.244 |
| 2 | +1 | -1 | -1 | 128.83 | 63.926 |
| 3 | -1 | +1 | -1 | 93.66 | 60.095 |
| 4 | +1 | +1 | -1 | 145.56 | **83.021** |

| | | | | | |
|---|---|---|---|---|---|
| 5 | -1 | -1 | +1 | 102.87 | 55.795 |
| 6 | +1 | -1 | +1 | 145.08 | 67.377 |
| 7 | -1 | +1 | +1 | 110.44 | 63.894 |
| 8 | +1 | +1 | +1 | **157.63** | 81.901 |
| 9 | 0 | 0 | 0 | 119.61 | 66.900 |
| 10 | 0 | 0 | 0 | 120.11 | 67.010 |
| 11 | 0 | 0 | 0 | 119.58 | 66.534 |
| 12 | 0 | 0 | 0 | 118.64 | 67.040 |
| 13 | 0 | 0 | 0 | 118.08 | 66.720 |
| 14 | 0 | 0 | 0 | 117.71 | 66.880 |

The TPC values range from 87.37 to 157.63 mg GAE/ g of plant, the best results were obtained using an extraction temperature of 80°C, extraction time 600 s and stirring rate 1200 rpm. The antioxidant activity expressed as μmol Trolox/g of plant obtained under MAE was in the range from 53.24 to 83.021, and the maximum value was obtained using the fallowing parameters: extraction temperature of 80°C, extraction time of 300 s and a stirring rate of 1200 rpm.

Linear models with two factor interactions were generated to describe the empirical relationship between the two responses and the three tested variables.

For TPC:

$$Y_{TPC} = 120.3700 + 22.8454x_1 + 5.3936x_2 + 7.5749x_3 + 1.9274x_1x_2 - \\ - 0.4949x_1x_3 - 0.3599x_2x_3 \quad (1)$$

and for antioxidant activity:

$$Y_{Trolox} = 0.066450 + 0.007899\,x_1 + 0.006071\,x_2 + 0.001085\,x_3 + \\ + 0.002333\,x_1x_2 + 0.000502\,x_1x_3 - 0.000415\,x_2x_3 \quad (2)$$

ANOVA gave a coefficient of determination ($R^2$) of 99.41% for TPC and 99.29% for antioxidant activity, which indicate a close agreement between experiment and predicted values.

### 3.2. Optimization of extraction conditions

The Pareto chart (Fig.1 and Fig.2) presents the importance and the statistical significance of the effects (linear and interactions between variables) of all variables on the response obtained. Fig.1 shows that TPC is influenced significantly by 3 main effects: temperature was the most affecting factor, followed by time and stirring rate. Also, the interaction between temperature and stirring rate is important for efficient extraction. In the case of antioxidant activity (Fig.2) there are four main effects: temperature is the most significant effect followed closely by the stirring rate; the interaction between temperature and

stirring rate is important for antioxidant activity of the extracted samples. The interaction between stirring rate and time has an antagonist effect on the antioxidant activity. Our result, regarding the increase of the TPC with the temperature is in concordance with other studied published about MAE of polyphenolic compounds from plants [20, 21].

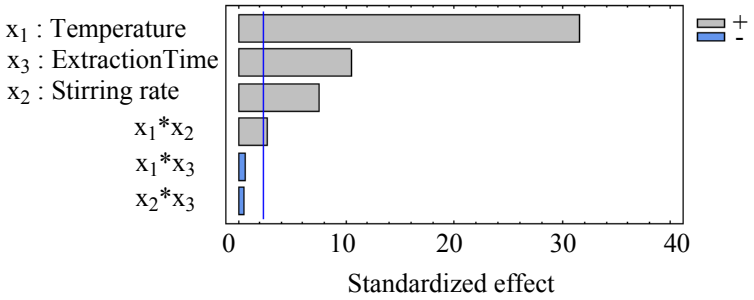


Fig. 1. Pareto Chart for Total polyphenolic content

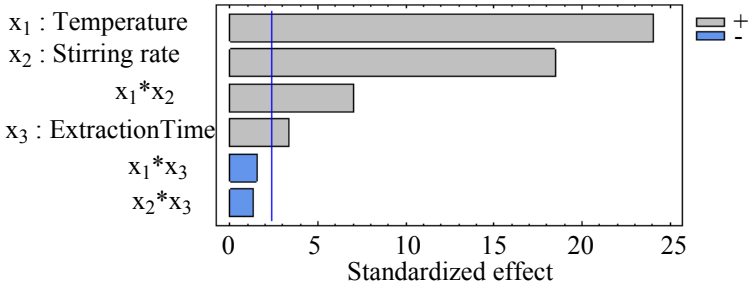


Fig. 2. Pareto Chart for Antioxidant activity

By analyzing the surfaces plots obtained for TPC content and antioxidant activity as a function of temperature and stirring rate (Fig.3 and Fig.4), we can notice the followings: a) in case of TPC, the optimum settings for the independent parameters that result in the highest values of the TPC can be found around the higher level value of both factors in the experimental domain. In fact, the optimum identified in the frame of the statistical software, starting at best predicted point, is the value of 157.80 mg GAE/g of plant for TPC, at a temperature of 80 °C, 1200 rpm and 601 s extraction time. These results are very close to the results obtained under the experimental conditions from run 8: 80°C, stirring rate 1200 rpm and extraction time 600 s, with a TPC = 157.63 mg GAE/g of plant (see *Table 2*). b) In the case of the antioxidant activity, the optimum settings for the two independent parameters that result in the highest values of the antioxidant activity (83.13 µmol TROLOX/g of plant) can be found around the higher level value of both factors (temperature and stirring rate) in the

experimental domain, but in this case, the extraction time indicated by the statistical software is 300s. Again, this is in perfect agreement with run 4 (see table 2). The software estimated a 0.995 value of desirability for TPC and 0.997 for antioxidant activity.

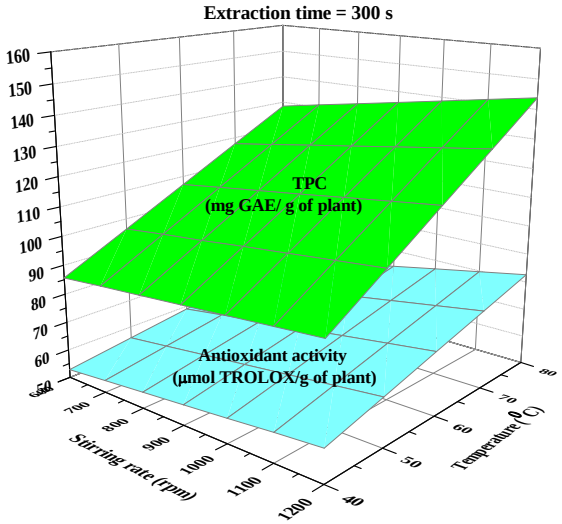


Fig.3. Response surface plots for TPC and antioxidant activity depending on the temperature and the stirring rate for 300 s extraction time

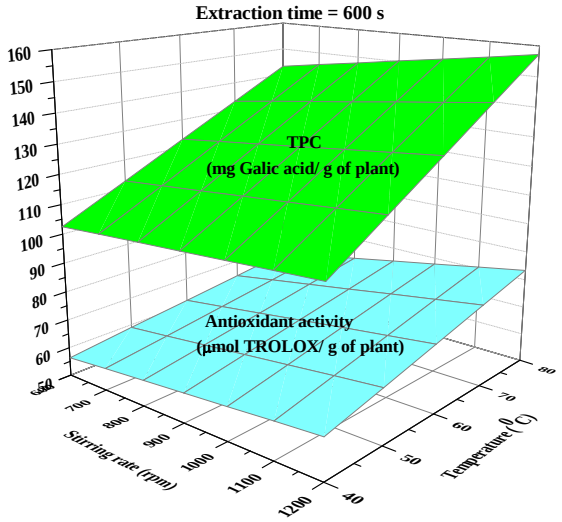


Fig.4. Response surface plots for TPC and antioxidant activity depending on the temperature and the stirring rate for 600 s extraction time.

These figures denoted the effects of the stirring rate and temperature on the TPC and antioxidant activity at a fixed extraction time of 300 s respectively 600 s. For both cases we can observe an increase of the TPC and antioxidant activity with the temperature and stirring rate. Also, the figures showed that the increase of the antioxidant activity is lower compared with the TPC at high temperature and high stirring rate. The high TPC at long extraction times can be the result of extraction of some nonphenolic compounds like amino acids, proteins, nucleotide bases, unsaturated fatty acids, carbohydrates, etc., which are also reactive towards the Folin-Ciocalteu reagent [22].

## 4. Conclusions

In the present work the microwave-assisted extraction of polyphenolic compounds from sea buckthorn dry leaves was optimized. The polyphenolic content of the extracted samples was determined using Folin-Ciocalteu method. The antioxidant activity was estimated, using the polyphenolic concentration determined by HPLC analyses for each sample. Depending on the independent parameters like temperature, stirring rate and time, the conditions for microwave-assisted extraction were optimized using statistical analysis. The results obtained confirmed the experimental trends observed:

- For TPC, the highest values were obtained at 80°C, stirring rate 1200 rpm and extraction time 600 s. The most important parameters are extraction temperature and extraction time.

- For the antioxidant activity, the highest values were obtained at 80°C, 1200 rpm and extraction time 300 s. In this case, the most important parameters are the extraction temperature and the stirring rate.

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## R E F E R E N C E S

[1]. *T. A. Wani, S.M. Wani, M. Ahmad, M. Ahmad, A. Gani, F.A. Masoodi*, „Bioactive profile, health benefits and safety evaluation of sea buckthorn (*Hippophae Rhamnoides L.*): A review", Cogent Food Agric., 2016, 2: 1128519 **http://dx.doi.org/10.1080/23311932.2015.1128519]** .

[2]. *T. Beveridge, T.S. C. Li, B. D. Oomah, A. Smith*, „Sea buckthorn products: manufacture and composition", J. Agric. Food Chem., Vol. 47, 1999, pp. 3480−3488.

[3]. *T. Fatima, V. Kesari, I. Watt, D. Wishart, J. F. Todd, W. R. Schroede, G. Paliyath, P.Krishna,* „Metabolite profiling and expression analysis of flavonoid, vitamin C and tocopherol biosynthesis genes in the antioxidant-rich sea buckthorn (*Hippophae Rhamnoides L.*)", Phytochemistry, Vol. 118, 2015, pp. 181–191.

[4]. *T. Michel, E. Destandau, G. L. Floch, M. E. Lucchesi, C. Elfakir*, „Antimicrobial, antioxidant and phytochemical investigations of sea buckthorn (*Hippophae Rhamnoides L.*) leaf, stem, root and seed", Food Chem., Vol. 131, 2012, pp. 754–760.

[5]. *M. Leopoldini, N. Russo, M. Toscano*, „The Molecular Basis Of Working Mechanism Of Natural Polyphenolic Antioxidants", Food Chem., Vol. 125, 2011, 288–306.

[6]. *J. Valls, S. Millán, M. P. Martí, E. Borràs, L. Arola*, „Advanced separation methods of food anthocyanins, isoflavones and flavanols", J. Chromatog. A, vol. 1216, 2009, pp. 7143–7172.

[7]. *C. C. Crişan, I. Călinescu, C. Zălaru, Z. Moldovan,* „Techniques for extracting polyphenols from Coreopsis Tinctoria nutt.fruits", U.P.B. Sci. Bull., Series B, Vol. 75, Iss. 4, 2013, pp.169-178.

[8]. *F. Damsa, A. Woinaroschy, C. L. Badarau*, „Anthocyanins extraction from purple potato (Gălăneşti blue-violet variety)", U.P.B. Sci. Bull., Series B, Vol. 76, Iss. 3, 2014, pp. 123-128

[9]. *M.F. Barrera Vázquez, L.R. Comini, R.E. Martini, S.C. Núñez Montoya, S. Bottini, J.L. Cabrera*, „Comparisons between conventional, ultrasound-assisted and microwave-assisted methods for extraction of anthraquinones from Heterophyllaea Pustulata Hook F. (*Rubiaceae*)", Ultrason. Sonochem. Vol. 21, 2014, pp. 478–484.

[10]. *A. Alupului, I.Calinescu, V.Lavric*, „Microwave extraction of active principles from medicinal plants", U.P.B. Sci. Bull., Series B, Vol. 74, Iss. 2, 2012, pp. 129-142.

[11]. *I. Asofiei, I. Calinescu, A. Trifan, I. G. David, A. I. Gavrila*., „Microwave Assisted Batch Extraction of Polyphenols from Sea Buckthorn Leaves", Chem. Eng. Comm., 1563-5201, (online)]

[12]. *D.J.Bhuyan, Q.V. Vuong, A.C. Chalmers, I. A. van Altena, M.C. Bowyer, C. J. Scarlett*, „Microwave-assisted extraction of *Eucalyptus robusta* leaf for the optimal yield of total phenolic compounds", Ind. Crops Prod., Vol. 69, 2015, pp. 290–299.

[13]. *Z. Lianfu, L. Zelong,* „Optimization and comparison of ultrasound/microwave assisted extraction (UMAE) and ultrasonic assisted extraction (UAE) of lycopene from tomatoes", Ultrason. Sonochem., Vol. 15, 2008, pp. 731–737.

[14]. *L. D. Kagliwal, A.S. Pol, S. C. Patil, R. S. Singhal¸ V. B. Patravale,* „Antioxidant-rich extract from dehydrated seabuckthorn berries by supercritical carbon dioxide extraction", Food Bioprocess Tech.,Vol.5, 2012, pp. 2768-2776.

[15]. *P. Budrat, A. Shotipruk*, „Enhanced recovery of phenolic compounds from bitter melon (*Momordica charantia*) by subcritical water extraction", Sep. Pur. Tech., Vol. 66, 2009, 125-129.

[16]. International Standard ISO 14502-1 .

[17]. *M. Bittová, E. Krejzová, V. Roblová, P. Kubáň, V.Kubáň*, „Monitoring of HPLC profiles of selected polyphenolic compounds in sea buckthorn (*Hippophaë rhamnoides L.*) plant parts during annual growth cycle and estimation of their antioxidant potential", Cent. Eur. J. Chem.,Vol.12, 2014, pp.1152-1161.

[18]. *J. Tabart, C. Kevers, J. Pincemail, J.-O. Defraigne, J. Dommes*, „Comparative antioxidant capacities of phenolic compounds measured by various tests", Food Chem., Vol. 113, 2009, 1226–1233.

[19]. *M.-E. Cuvelier, H. Richard, C. Berset*, „Comparison of the Antioxidative Activity of Some Acid-phenols: Structure-Activity Relationship", Bioscience, Biotech. Biochem, **Vol.56**, Iss.2, 1992, pp. 324-325.

[20]. *P. Putnik, D. B. Kovacevic, M. Penic, M. Feges*, *V.Dragovic-Uzelac*, „Microwave-assisted extraction (MAE) of Dalmatian sage leaves for the optimal yield of polyphenols: HPLC-DAD identification and quantification", Food Anal. Methods, DOI 10.1007/s12161-016-0428-3.

[21]. *A. Taamalli, D. Arraez-Roman, E. Ibanez, M. Zarrouk, A. Segura-Carretero, A. Fernandez-Gutierrez*, „Optimization of Microwave-Assisted Extraction for the Characterization of Olive Leaf Phenolic Compounds by Using HPLCESI-TOF-MS/IT-$MS^2$", J. Agr. Food. Chem., **Vol. 60**, 2012, pp.791−798.

[22]. *J.D. Everette, Q.M. Bryant, A.M. Green, Y.A. Abbey, G.W. Wangila, R.B. Walker*, „A thorough study of reactivity of various compound classes towards the Folin-Ciocalteu reagent", J. Agric. Food Chem., Vol.58, Iss. 14, 2010, pp. 8139–8144.