TITLE: Optimization of ultrasoundassisted extraction of bioactive compounds from wild garlic (Allium ursinum L.)

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Optimization of ultrasound-assisted extraction of bioactive compounds from wild garlic (*Allium ursinum* L.)

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Abstract

Ultrasound-assisted extraction was used for extraction of bioactive compounds and for production of *Allium ursinum* liquid extract. The experiments were carried out according to tree level, four variables, face-centered cubic experimental design (FDC) combined with response surface methodology (RSM). Temperature (from 40 to 80 °C), ethanol concentration (from 30 to 70%), extraction time (from 40 to 80 min) and ultrasonic power (from 19.2 to 38.4 W/L) were investigated as independent variables in order to obtain the optimal conditions for extraction and to maximize the yield of total phenols (TP), flavonoids (TF) and antioxidant activity of obtained extracts. Experimental results were fitted to the second order polynomial model where multiple regression and analysis of variance were used to determine the fitness of the model and optimal condition for investigated responses. The predicted values of the TP (1.60 g GAE/100g DW), TF (0.35 g CE/100 g DW), antioxidant activity, IC_{50}(0.71mg/ml) and extraction yield, Y (38.1%) were determined at the optimal conditions for ultrasound assisted extraction as: 80°C temperature, 70% ethanol, 79.8 minutes and 20.06 W/L ultrasonic power. The predicted results matched well with the experimental results obtained using optimum extraction conditions which validated the RSM model with a good correlation.

Keywords: ultrasound-assisted extraction, *Allium ursinum*, total phenols, total flavonoids, antioxidant activity, response surface methodology

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1. Introduction

In the recent years there has been an increase of interest for the use of natural compounds in the prevention and treatment of various diseases such as cancer, arthritis, diabetes, hypertension, coronary diseases, etc. Beside application in the prevention and treatment of various diseases, the utilization of natural compounds from various sources has been increased in the food industry. This increase is in accordance with demand to replace all synthetic and toxic additives with natural and safe ones. As a source of health benefit compounds *Allium* species have been used in the traditional medicine for many centuries thanks to its wide-spread distribution and popularity as edible and medicinal plant. In the past few years interest for *Allium ursinum*, as wild aromatic plant, is significantly growing. The potential health benefits of *A. ursinum* have been attributed mainly to the sulfur containing compounds which are the most characteristic constituents in *Allium* plants [1]. *A. ursinum* belongs to methiin/alliin- type *Allium* species, which means it contains mainly a mixture of (+)-S-methyl-L-cysteine-sulfoxide (methiin) and (+)-*S*-allyl-L-cysteine-sulfoxide, alliin. According to Schmitt et al. [2] and Kubec et al. [3], by hydrolyzing these compounds, many volatile compounds are created. Compounds like thiosulphinates and (poly) sulfides (among them allicin, methyl-allyl- or dimethyl thiosulfinates) are responsible for *Allium* specific flavor and odor. Apart of sulfur-containing substances *A. ursinum* has been also reported as a good source of phenolic compounds [4]. The leaves containe free forms of galic, ferulic and vanillic acids, and bound forms of p-coumaric, ferulic and vanillic acids. In the bulbs free ferulic, p-hydroxybenzoic and vanillic acids, and bound forms of pcoumaric and ferulic acids were detected [5]. As far as qualitative profile is concerned, ramson is abundant predominantly in kaempferol derivatives (3-O-b-neohesperidoside- 7-O-[2-O-(trans-p-coumaroyl)]-b-D-glucopyranoside, 3-O-b-neohesperidoside-7-O-[2-O-(trans-p-feruloyl)]-b-D-glucopyranoside, 3-O-b-neohesperidoside-7-O-[2-O-(trans-p-coumaroyl)-b-D-glucopyranosyl]-b-D-glucopyranoside) [6–8]. Several biological activities of *A. ursinum* plants and extracts, such as antioxidant [9], cytostatic [10], antimicrobial [10] ,and antidiabetic [11] were reported. Broad spectrum of biological activities obtained from *A. ursinum* and its extracts, as well as the presence of chemical compounds with high terapeutical potential, makes this plant potential candidate for future development of various functional products and food supplements.

Applied extraction method can substantially affect the quality and concentration of targeted compounds in extract production. Extraction of herbs using ultrasound-assisted process was recommended by various authors as a one of the most efficient, inexpensive and simplest existing extraction systems which could be suitably operated for large-scale preparations [12], [13]. Ultrasonic waves after interaction with subjected plant material alter its physical and chemical properties. The use of ultrasound can enhance the extraction process by increasing the mass transfer between the solvent and plant material. The collapse of cavitation bubbles leads to better cell disruption through the formation of micro jets due to asymmetrical bubble collapse near a solid surface. Cavitation effect facilitates the release of extractable compounds and enhances the mass transport by disrupting the plant cell walls, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between the solid and liquid phase [14], [15]. Using ultrasound, extractions can be completed in short time with high reproducibility, reducing the consumption of solvent, simplifying manipulation and work-up, extraction at lower temperatures, giving higher purity of the final product, faster extraction rates and greater yields of product [16], [17].

In this study possibility to apply UAE for the production of quality *A. ursinum* extracts was investigated. Effect of different extraction parameters (solvent concentration, extraction temperature, extraction time and ultrasonic power) on properties of extracts was investigated and analyzed using response surface methodology (RSM). A three levels, four variables, face central composite design was employed to obtain the optimal conditions for the extraction of functional components from *A. ursinum*.

2. Materials and methods

2.1. Plant material

Dried *A. ursinum* was kindly donated by local tea factory, Fructus doo Bačka Palanka, Serbia. Before extraction material was grounded in the blender. The particle size of the grounded material (0.325 mm) was determined using sieve sets (Erweka, Germany).

2.2. Chemicals

1, 1-Diphenyl-2-picryl-hydrazyl-hydrate (DPPH), Folin-Ciocalteu reagent, (±)-catechin, were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Gallic acid was purchased from Sigma (Sigma, St. Luis, 91 MO, USA). All other chemicals and reagents were of analytical reagent grade.

2.3. Ultrasound-assisted extraction procedure

For ultrasound-assisted extraction sonication water bath (EUP540A, Euinstruments, France) with fixed frequency at 40 kHz was used. According to Rodriguez et al., due to sonication water bath use, ultrasound power was expressed as ultrasound power density W/L in order to obtain more prcise results [18]. In experimental runs, 5 g of grounded *A. ursinum* was mixed with 25 ml of selected solvent (according to Table 1) in 100 ml flasks. Flasks with condensers were always positioned in the same distance from the transducer without additional agitation. Ultrasonic power, temperature and extraction time were controlled from the panel of the instrument. After extraction, extracts were immediately filtered through the filter paper under vacuum. Extracts were collected into glass flasks and stored at 4 °C until further analysis in the shortest time.

2.4. Determination of total phenols content

The total phenolic content (TP) in obtained *A. ursinum* extracts was determined by the Folin–Ciocalteu procedure ([19], [20] using gallic acid as a standard. Absorbance was measured at 750 nm. Content of phenolic compounds was expressed as g of gallic acid equivalent (GAE) on dry weigh of *A. ursinum* (g GAE/100 g DW). All experiments were performed in three replicates.

2.5 Determination of total flavonoids content

The total flavonoids content (TF) was determined using aluminum chloride colorimetric assay [21]. Results were expressed as g of catechin equivalents (CE) on dry weight of *A. ursinum* (g CE/100 g DW). All experiments were performed in three replicates.

2.6. DPPH assay

DPPH radical scavenging assay, based on the reduction of the DPPH solution in the presence of a proton-donating substance, has been extensively employed to evaluate the free radical scavenging ability of varied samples [22]. The free radical scavenging activity of dry *A. ursinum* extracts were determined as described by Espin et al. [23]. Different amount of *A. ursinum* extract were mixed with methanol (95%) and 90 μM 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) in order to gain different final concentrations of the extract. After 60 min at room temperature, the absorbance was measured at 515 nm and expressed as radical scavenging capacity. Radical scavenging capacity (%RSC) was calculated by following equation:

$$\%RSC = 100 - (A_{sample} \times 100)/A_{blank} \qquad (1)$$

where: A_{sample} is the absorbance of sample solution and A_{blank} is the absorbance of control. This activity was also expressed as the inhibitory concentration at RSC value 50% (IC_{50}, the concentration of test solution required to obtain 50% of radical scavenging capacity).

2.7. Experimental design and statistical analysis

In this study a three levels, four-variable central composite face-centered design (FCD) was employed to investigate and validate the extraction parameters affecting the extraction of *A. ursinum* total phenols, total flavonoids and antioxidant activity. For the optimization of extraction parameters design consisted of 29 experiments, including five replicates in a central point. The parameters employed in the experimental design were in the ranges: temperature (40-

80 °C), ethanol concentration (30- 70%), ultrasonic power (19.2- 38.4 W/L) and extraction time (40- 80 min). Variables were coded according following equation [24]:

$$i \quad 0 \qquad (2)$$

where, X is the coded value, x_i is the corresponding actual value, x_0 is the actual value in the center of the domain, and Δx is the increment of x_i corresponding to a variation of 1 unit of X. The coded and original values of independent variables used in this experiment are listed in the Table 1.

Experimental data were fitted to the following second order polynomial model (Eq. 3). Regression coefficients were obtained to describe relationship between the responses and the independent variables.

$$(3)$$

where Y is the response variable, X_i and X_j are independent variables and β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linear, quadratic and interception terms respectively.

Experimental data were analyzed using Design Expert software v. 9 Trial (State-Ease, Minneapolis, Minnesota, USA). Analysis of variance (ANOVA) and response surface analysis were used to determine the statistical significance of the model. The significances of all terms in the polynomial were considered statistically different when $p< 0.05$. The adequacy of the model was checked by accounting for the coefficient of determination (R^2). The relationship between the response and independent variables was demonstrated using a response surface plot.

3. Results and discussion

3.1. Determination of process parameters

For quality utilization of material of interest, analysis of process parameters influence and optimization of process need to be provided. UAE is affected by several parameters, such as ultrasonic power, solvent concentration, and extraction temperature [25]. Dominant factor which

affect all extraction process, regardless on applied extraction techniques, is extraction solvent. Extraction solvent should be selected based on the compounds targeted by the process, based on its selectivity, safety, cost and availability. Extraction solvents as ethanol and ethanol/water mixtures are widely recognized by a number of investigations as extraction solvent adequate for extraction of antioxidant compounds, especially phenolic ones [26].

Application of longer extraction time than needed can induce the increase of energy and operational costs of the process. Therefore, the extraction time should be considered carefully because the excess of the time during UAE, with others process parameters of extraction can influence significantly on process and quality of extracts [15]. Use of UAE is advisable for thermolabile compounds [27]. Extraction at higher temperatures for a longer extraction time can cause the degradation of certain extracted compounds. Therefore, for quality utilization of *A. ursinum* influence of four process parameters was investigated. Those four independent variables (temperature, solvent concentration, time of extraction and ultrasonic power) were involved in the optimized experiment according to face centered design. Table 2 presents the experimental design and experimental values for each response under different UAE parameters.

The regression coefficients of the intercept, linear, quadratic and interaction terms of the model (Eq. 2) were generated for all responses using statistical approach called the method of least squares (MLS). MLS represents a multiple regression technique used to fit a mathematical model to a set of experimental data generating the lowest residual possible [28]. The regression coefficients, the model for each response and the results of the analysis of variance (ANOVA) are displayed in Table 3. According to the values coefficients of multiple determination (R^2) for TP, TF, IC_{50} and EY (extraction yield), varying from 0.68 to 0.99. Although R^2 in designed experiment was quite low for TP (0.71) and IC_{50} (0.68) mathematical models were statistically acceptable due to high significant regression for the model (p_m<0.05), moderate significant (p_m<0.1) and non-significant lack of fit (p_{lf}>0.05).

3.2. Effects of extraction parameters on extraction yield

The yields of extractions are presented in Table 2. The regression analysis of the data showed positive highly significant effect of linear term of temperature on total extraction yield of *A. ursinum* UAE. Effects of temperature and ultrasonic power interaction, as well as quadratic term of ethanol concentration, were significant and negative. Quadratic term of ultrasonic power affect moderate significantly on total extraction yield of investigated process. Other teams were insignificant. Therefore, the final predictive equation for describing the efficiency of extraction of total extractable compounds of *A. ursinum* using significant terms is as follows

$$Y = 36.6612 + 1.4423X_1 - 2.0503X_1X_4 - 1.6418X_2^2 - 1.0705X_4^2 \quad (4)$$

The best way to express the effects of any independent variables on the extraction yield of targeted compounds is to generate surface response plots of the model which were done by varying two variables in the experimental range under investigation and holding the other two variables at its fixed level [29]. Figure 1 shows the surface plots which are presenting the influence of investigated UAE parameters on the extraction yield. Presented plots enable us to have better visualization of influence and interaction between each two parameters in investigated design.

Visual analysis of surface plots is in accordance with multiple regression analysis. Positive temperature influence on total yield can be observed in plots A, B, C (Figure 1). The negative effect of quadratic terms of ethanol concentration and ultrasonic power can be observed from plots D, E, F. The ethanol concentration indicates a saddle point and the highest yield is observed at approximately 50%. Negative impact of quadratic term of ethanol concentration can be explained by the fact that additional of water to ethanol improves extraction rate. But, according to Spigno et al. the high water content can brought an increased concomitant extraction of other compounds, and this can lower phenols concentrations in the extracts [30].

The negative effect of interaction between temperature and ultrasonic power may suggest using lower ultrasonic power in combination with high temperature what will prevent the degradation of bioactive compounds from *A. ursinum*. Inclined surfaces to the side to the higher investigated temperatures are in accordance with statement that the higher system energies could

increase the solubility of target compounds, and consequently improve their liberation from the sample matrix by destroying the integrity of connective and structural tissues [31].

3.3. Effects of extraction parameters on total phenols content

Figure 2 shows the surface plots which are presenting the influence of investigated UAE parameters on the extraction of TP from *A. ursinum*. Presented plots enable us to have better visualization of influence and interaction between each two parameters in investigated design. The efficiency of TP extraction, in design varied from 0.92 to 1.44 g GAE/100 g DW. The lowest TP was obtained using 50% ethanol as extraction solvent, at the highest temperature and for the lowest extraction time, while ultrasonic power was 28.8 W/L. Using same extraction solvent (50% ethanol) at same extraction temperature (80 °C) and ultrasonic power (28.8 W/L) the highest yield of TP was achieved. But, in this case extraction time was much higher (80 min). In accordance with *p*-values the regression coefficients terms with significant effect are presented in Eq. 5. Various process factor affecting the efficiency of TP extraction from *A. ursinum*. Among them, the most dominant is the effect of temperature.

$$Y = 1.2643 + 0.0314X_3 + 0.0569X_1 X_2 + 0.1476X_1 X_3 - 0.1072X_1 X_4 - 0.528X_1^2 \quad (5)$$

In equation describing extraction of TP from *A. ursinum* it can be seen that the linear term of time have a moderate positive impact on extraction of TP, meaning that with the increase of the extraction time extraction of TP from investigated material will be slightly increased. Interaction between time and ethanol concentration is characterized with moderate significance to, therefore it will have the same moderate effect on the increase of TP extraction. According to the same equation interactions between temperature and extraction time and temperature and ultrasonic power had highly significant influence on TP extraction. The effect of temperature and time interaction was positive, what means that increase of parameters which are elements of these interactions will increase the extraction of TP from *A. ursinum*. Unlike this interaction, interaction of temperature with ultrasonic power will affect significantly, but negatively, meaning that in interaction these two parameters could decrease extraction of TP from *A. ursinum.* Significant but negative effect of quadratic term of temperature, causing decrease of TP

extraction, can be explained by possible degradation, at higher temperatures, of certain thermo sensitive phenolic constituents presented in investigated *A. ursinum*.

From a visual analysis of surface plots (A, B and C) the influence of temperature is also noticeable. Interaction between temperature and time leads to the higher TP content. Influence of interaction of these two factors could be explained with multiple effect of temperature on mass-transfer process such as improved diffusion, degradation of the plant matrix and improvement of solvent penetration during the time [32]. Thus, as it has obvious from the equation above, if the thermo sensitive compounds are present in the material of investigation the effect of temperature could be revers. The influence of extraction time best represents plot B with a positive slope to greater time extraction. Significant negative influence between temperature and ultrasonic power in observed in Figure 2. Plot C.

3.4. Effects of extraction parameters on total flavonoids content

Compared with the results obtained using conventional maceration, results obtained by UAE provide higher values of TF. The same observation was made by Gîtin et al. [4], but different extraction parameters were set.

The coefficient of determination for this method (R^2) was very high, 0.99, which implies an adequate correlation between the model and the experimental results for the chosen parameters. Efficiency of TF extraction from *A. ursinum* oscillated from 0.09 to 0.40 g CE/100 g DW, as it is presented in Table 2. The lowest TF was obtained using 30% ethanol as extraction solvent at the temperature of 60 °C and ultrasonic power of 38.4 W/L, while extraction time was set for 60 minutes.

Based on the *p*-value from the Table 3. effects of investigated process parameters on extraction of TF from *A. ursinum* can be presented by the following equation:

$$Y = 0.13 + 0.1398X_2 - 0.0074X_3 + 0.1031X_2^2 \quad (6)$$

The ANOVA results (Table 3) are showing that solvent concentration has dominant effect on the extraction of TF from *A. ursinum*. Its linear term impacted highly significant and positive on the TF yield. The same case was with quadratic terms of ethanol concentration. This imply that increase of ethanol concentration leads to increase of TF extraction from *A. ursinum in* investigated range. Results indicate that the solvent employed is important when tailoring an ultrasonic extraction. There is noted sharper flavonoids content rise started from approximately 50% ethanol concentration (A, D, E). High increase of flavonoid extraction with increase of ethanol concentration in ethanol/water mixture might be due to the solvent which provides the most suitable polarity for extraction of *A. ursinum* flavonoids [33]. It has been reported that the polarity of solvent used in extraction directly affects not only the quantity of TF, but also the composition of polyphenolic and flavonoids compounds [34]. According to the Table 3 time has a moderate significant and negative influence ($p \leq 0.0\ 5$) which can be noted also by observing plots (Figure 4). Although the other process variables were insignificant on TF some conclusion may be done. From the plot analysis, it was found that we can obtain similar yields of using shorter time of extraction. Plots C, E, F indicate that prolonged sonication did not result in further improvements of extraction efficiency, therefore medium strength should be enough to obtained desirable yield. This implicate that we can use mild conditions (time, ultrasonic power and temperature) to gain similar yield, but saving time and energy.

3.5. Effects of extraction parameters on antioxidant activity of obtained extracts

During cavitation, hydroxyl radicals can be produced and these sonochemically generated radicals can react with easily oxidable food compounds. Depending on the process and the matrix, the chemical effects of acoustic cavitation may be either beneficial or detrimental [35]. The experimental data (Table 2) showing the antioxidant activity of extracts obtained by UAE under investigated setup of extraction parameters. Antioxidant activity of *A. ursinum* extracts was in the range from 0.71 to 1.12 mg/ml. Practically, a lower IC_{50} value corresponds to stronger antioxidant activity of tested samples [36]. Thus, the best antioxidant results were obtained in run 11 (0.71 mg/ml) in extract obtained using 30% ethanol at temperature of 60 °C, ultrasonic power of 28.8 W/L during 40 minutes of extraction.

The results of regression analysis indicated that the main extraction parameters affecting the antioxidant activity of analyzing extracts are linear terms of temperature and ethanol concentration, and an interaction term between temperature and ultrasonic power. The final equation describing the effects of process parameters (within investigated range) on antioxidant activity of obtained *A. ursinum* extracts is as following:

$$Y = 0.9100 - 0.0839X_1 + 0.0567X_2 + 0.1547X_1X_4 \quad (7)$$

Effect of ultrasonic power is highly significant in interaction with temperature, which is visible from plot C (Fig. 4). Higher ultrasonic power could have damaged more cell walls, releasing more antioxidants, including phenolic compounds to the solvents. But, in the same time it could lead to the degradation of certain sensitive constituents present in *A. ursinum*. An increase in temperature increases target compound solubility, solvent diffusion rate and mass transfer [37], but, as like ultrasonic power, higher temperatures could lead to the degradation of thermo sensitive compounds present in *A. ursinum*. In this case, as ultrasonic power temperature interaction effect highly significant and positive on response characterizing antioxidant activity (IC_{50}) it can be concluded that their increase will lead to increase of IC_{50} therefore it will decrease antioxidant activity of extracts. Couse of this decrease could be temperature and ultrasonic power caused degradation of *A. ursinum* sensitive compounds with antioxidant properties. According to negative temperature slope on the plots A, B, C (Figure 4) it can be concluded that with increasing temperature, as IC_{50} is decreasing, antioxidant activity is rising. This is in accordance with equation 7 and results present in Table 3, which are showing highly significant effect of linear term of temperature indicating that increase of temperature will lead to decrease of IC_{50}, thus increase of obtained extract antioxidant activity. Therefore, if the target of investigated process is the production of *A. ursinum* extracts with highest antioxidant properties higher temperatures at lower ultrasonic power should be applied.

3.6. Optimization of the extraction process

The aim of this study was to maximize extraction yield of compounds within extraction parameters. Based on the experimental results and statistical analysis, numerical optimizations

have been conducted in order to establish the optimum level of independent variables with desirable response of goals. In the current study, for all responses only one optimal condition was obtained: 80 °C temperature, 70% ethanol, 79.8 minutes and ultrasonic power of 20.06 W/L. Determination of optimal conditions and predicted values was based on desirability function, D=0.878. In order to verify predictive mathematical model of the investigated process experimental confirmation of obtained was performed on estimated optimal conditions. Predicted and the observed values are presented in Table 4. The predicted results matched well with the experimental results obtained at optimal extraction conditions which were validated by the RSM model with as good correlation.

4. Conclusions

Optimization of UAE of *A. ursinum* was performed by varying different solid-liquid extraction process parameters and using RSM as mathematical tool for extraction process optimization. The second-order polynomial model provided adequate mathematical description of UAE of targeted responses: TP, TF, extraction yield and antioxidant activity. Therefore, optimization of extraction conditions in order to provide maximum yields for each observed response (TP, TF, antioxidant activity and extraction yield) was successfully performed. Generally, obtained results and statistical analysis qualify UAE as appropriate extraction technique for extraction of antioxidant compounds, such are TP and TF, from medicinal plant such is *A. ursinum*. The most dominant effect on the extraction process of *A. ursinum* was the effect of extraction temperature followed by concentration of ethanol in extraction solvent. Statistical and graphical analysis showed that the temperature has notable influence on each targeted response, except on the TF extraction. Temperature had dominant effect, sole or in interaction, on extraction of TP from *A. ursinum*. Temperature ultrasonic power interaction leads to decrease of TP extraction, probably due to degradation of certain sensitive constituents of *A. ursinum* as may be gallic and vanillic acid. In the cases of temperature interaction with time the efficiency of TP extraction increases, what leads to the conclusion that for efficient extraction of TP from *A. ursinum* higher temperatures and lower ultrasonic power should be applied. Ethanol concentration played most important role in extraction of flavonoid compounds from *A. ursinum,* probably due to the adequate polarity of extracting bioactive compounds. In the case of flavonoids extraction from *A. ursinum* ultrasonic power and temperature had no influence.

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Figure Caption

Figure 1 Response surface plots showing the combined effects of the extraction parameters on extraction yield

Figure 1 Response surface plots showing the combined effects of the extraction parameters on extraction yield of total flavonoids

Figure 2 Response surface plots showing the combined effects of the extraction parameters on extraction yield of antioxidant activity

Figure 3 Response surface plots showing the combined effects of the extraction parameters on extraction yield of antioxidant activity

Tables

Table 1 Experimental domain of face-centered design (FCD)

Independent variable	Factor levels		
	-1	0	1
Temperature, X_1 [°C]	40	60	80
Ethanol concentration, X_2 [%]	30	50	70
Extraction time, X_3 [min]	40	60	80
Ultrasonic power, X_4 [W/L]	19.2	28.8	38.4

Table 2 Face-centered central composite cube design of the three-levels and four-variables and observed responses under different experimental conditions

Run order	Independent variables				Investigated responses			
	Temperature [°C]	Ethanol concentration [%]	Extraction time [min]	Ultrasonic power [W/L]	EY [%]	TP [g GAE/100g DW]	TF [g CE/100g DW]	IC_{50} (mg/ml)
1	0	0	0	0	37.67	1.2711	0.1195	0.8644
2	0	0	1	-1	33.93	1.2508	0.1082	0.9882
3	1	0	1	0	41.42	1.4417	0.1329	0.7649
4	-1	0	1	0	34.95	1.1453	0.1219	1.0301
5	0	1	0	-1	34.73	1.2767	0.3527	0.8653
6	1	-1	0	0	35.13	1.1621	0.0942	0.8268
7	0	0	0	0	35.62	1.2879	0.1331	0.9000
8	0	-1	0	1	33.34	1.1576	0.0901	0.8841
9	0	0	0	0	36.50	1.2576	0.1381	0.8333
10	1	1	0	0	35.74	1.3306	0.3613	0.8723
11	0	-1	-1	0	34.27	1.2441	0.0931	0.7121
12	1	0	0	1	33.71	1.0992	0.1447	1.1166
13	0	0	0	0	36.88	1.2890	0.1288	0.9107
14	-1	-1	0	0	36.18	1.2261	0.0910	1.0352
15	0	-1	1	0	36.77	1.2419	0.0966	0.8282
16	0	1	0	1	34.33	1.2115	0.3481	0.9953
17	0	0	-1	1	34.64	1.3070	0.1393	0.9266
18	-1	0	0	-1	34.15	1.0756	0.1326	1.1256
19	0	-1	0	-1	34.88	1.1958	0.0920	0.8399
20	1	0	0	-1	39.50	1.3261	0.1502	0.7330
21	0	0	1	1	36.64	1.1992	0.1103	0.8115
22	1	0	-1	0	38.85	0.9240	0.1425	0.7710
23	0	1	-1	0	34.23	1.2879	0.4006	1.0773

24	0	1	1	0	34.65	1.2958	0.3771	1.0000
25	0	0	-1	-1	34.41	1.2160	0.1294	0.9525
26	-1	0	0	1	36.56	1.2778	0.1044	0.8903
27	-1	1	0	0	32.00	1.1666	0.3950	0.9974
28	-1	0	-1	0	33.19	1.2183	0.1312	1.0135
29	0	0	0	0	36.63	1.2160	0.1309	0.8239
standard deviation					1.98	0.09	0.10	0.11

Table 3 Estimated coefficients of the fitted second-order polynomial model for TP, TF, IC_{50} and Y and analysis of variance ANOVA of the investigated syste

Term	Regression coefficient			
	EY	TP	TF	IC_{50}
Intercept				
β_0	36.6612	1.2643	0.1301	0.9100
Linear				
β_1	1.4423*	0.0145	0.0042	-0.0840*
β_2	-0.4076	0.0284	0.1398*	0.0568**
β_3	0.7304	0.0314***	-0.0074***	-0.0025
β_4	-0.1976	-0.0074	-0.0023	0.0100
Interaction				
β_{12}	1.1955	0.0569***	-0.0092	0.0208
β_{13}	0.2019	0.1476*	-0.0001	-0.0057
β_{14}	-2.0503**	-0.1072*	0.0057	0.1547*
β_{23}	-0.5194	0.0025	-0.0068	-0.0483
β_{24}	0.2851	-0.0067	-0.0007	0.0215
β_{34}	0.6211	-0.0356	-0.0019	-0.0377
Quadratic				
β_{11}	0.2511	-0.0528**	0.0044	0.0448
β_{22}	-1.6418**	-0.0024	0.1031*	0.0144
β_{33}	-0.1791	0.0055	0.0000	0.0072
β_{44}	-1.0705***	0.0278	0.0004	0.0388
R^2 [a]	0.7398	0.7880	0.9911	0.6864
CV [b]	4.0895	5.0200	8.2188	8.5588
p_m-value [c]	0.0300	0.0098	< 0.0001	0.0056
p_{lf}-value [d]	0.0659	0.0570	0.0566	0.0677

*p<0.01
**0.01 ≤ p <0.05
***0.05 ≤ p <0.1

[a] coefficient of multiple determination
[b] coefficient of variance [%]
[c] probability of F value for the model
[d] probability of F value for the lack of fi

Table 4 Estimated predicted and observed values and confidence

Response	Predicted Mean	95% PI low	Observed value	95% PI high
TP [g GAE/100 DW]	1.60	1.37	1.61	1.83
TF [g CE/100 g DW]	0.35	0.30	0.41	0.41
IC_{50} [mg/ml]	0.71	0.44	0.73	0.98
EY [%]	38.07	32.68	36.70	43.47