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1 **Subcritical water extraction of wild garlic (*Allium ursinum* L.) and process optimization by**
2 **response surface methodology**
3

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10

11 **Abstract**
12 Subcritical water extraction (SWE) was employed in order to obtain high valuable extracts from
13 wild garlic (*Allium ursinum*). The influence of temperature (120-200 °C), extraction time (10-30 min) and
14 added acidifier, HCl (0-1.5%) on extraction process was investigated. Analysis of variance was used to
15 determine the fitness of the model and optimal process parameters for SWE, in order to maximize
16 extraction yield, total phenolic compounds and total flavonoids content, and antioxidant activity. The
17 optimal conditions for SWE were determined at temperature of 180.92 °C, extraction time of 10 min, and
18 added acidifier at 1.09%. An insight into the development of Maillard reaction products during SWE was
19 provided through measurement of 5-hydroximethylfurfural(5-HMF) and furfural (F)in all obtained
20 extracts. No influence of 5-HMF and F on antioxidant activity was observed. Using HPLC-DAD,
21 kaempferol derivates were identified as the major phenolic compounds in extract obtained at optimal
22 condition.
23

24 **Keywords:** subcritical water extraction, *Allium ursinum*, total phenolic compounds, antioxidant activity,
25 response surface methodology, HMF
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27

28 **1. Introduction**

29 Extracts of herbs, vegetables, fruits and other plant materials are target of interest of
30 pharmaceutical and food industry as they contain a wide variety of compounds that may have beneficial
31 health effects. Application of such extracts thereby may improve the quality and nutritional value of
32 food, and may enable the creation of new functional products and dietetic supplements. Selection of
33 adequate extraction technology and setup of adequate extraction parameters are the key steps in the
34 isolation and recovery of valuable bioactive compounds and in the production of extracts with adequate
35 quality. Nowadays modern production implies implementation of new processes in accordance to the
36 requirements of "green chemistry". Therefore, beside production of quality final products, these
37 requirements are obligation of modern extracts production. Several extraction technologies are recognized
38 as "green extraction technologies" among subcritical water extraction (SWE) is one of the most
39 promising. According to Xu et al., this extraction technology is one of the best option for the isolation of
40 bioactive compounds from plants and food [1]. SWE exhibits a number of advantages over conventional
41 extraction technologies. Namely, SWE demonstrated the ability to selectively extract different classes of
42 compounds, with the more polar organics being extracted at lower temperatures, and less polar organics
43 being extracted at higher temperatures [2]. Extracts obtained by SWE contain no trace of toxic solvent
44 residues, because water is applied as extraction solvent, therefore no additional separation or purification
45 is needed. In such way obtained extracts can be used directly as semi-products or products for food and
46 pharmaceutical industry. In comparison to classical extraction technologies, extraction time in this
47 extraction technology is much shorter.

48 In SWE, at elevated process temperatures, the physiochemical properties of water are
49 considerably different from the properties of water at room temperature. Here, according to He et al., over
50 the range of temperatures the density of water decreases, dielectric constant decreases and ionization
51 constant increases [3]. As temperature increases, the physiochemical properties of water being resembled
52 to the properties of organic solvents, which increases a solubility of various organic molecules [4,5]. By
53 this, the main drawback of water (high polarity and low selectivity to the low polar and non-polar
54 constituents) is overcome. Beside, increased extraction of various organic compounds in SWE due to
55 application of high process temperatures, formation of various new compounds can be expected. Thus,
56 according to Zhang et al. [6] the heating in SWE could lead the appearance of non-enzymatic browning
57 reactions, for example the Maillard reaction, caramelisation and oxidation of phenolic compounds,
58 resulting in a typical dark brown color and formation of some antioxidant compounds. According to
59 Herrero et al. [7], this possibility of new antioxidant formation could further increase the interest of SWE
60 considering that this technique would be capable not only for recovering the naturally present
61 antioxidants, but also for allowing the generation of new antioxidant compounds during the SWE

62 process. However, during SWE process, also through Millard and caramelization reactions, formation of
63 5-hydroxymethylfurfural (5-HMF) could occur. As 5-HMF has been demonstrated to be cytotoxic in
64 higher concentration, it is of great interest to gain an insight on the formation of this compound during
65 SWE processes from natural matrices.

66 According to previous statements of advantages of SWE, it can be assumed that SWE can be
67 applied as efficient extraction technology for recovery valuable compounds from plants such as *Alium*
68 *ursinum*. *A. ursinum*, also known as wild garlic, possess wide range of biological activities such as
69 antioxidant [8,9], antiplatelet [10], cardio protective [11], cytostatic, antimicrobial [12], anti-inflammatory
70 [13] and antidiabetic [14]. The potential health benefits of *A. ursinum* have been attributed mainly to the
71 sulphur-containing compounds which are one of the most characteristic constituents of *Allium* plants. The
72 antioxidant activity of *Allium* species has been linked to the same compounds and their precursors, but it
73 is also related to other bioactive compounds such as phenolic compounds, flavonoid glycosides [15],
74 dietary fibres and microelements [13]. Moreover, in *A. ursinum* the presence of flavonols such as
75 kaempferol derivatives, which possess protecting properties against heart disease and cancer, and also
76 prevents oxidative damage to cells and DNA, was reported [16–18]. The short harvesting period *A.*
77 *ursinum* is the main obstacles in the wider use and application of this plant. Therefore, one of the best
78 ways to utilize *A. ursinum*, as well as its bioactive compounds, could be through application of
79 appropriate extraction technology and production of extracts with much higher concentration of bioactive
80 compounds compared to the raw material.

81 Therefore, in this study, possibility for application of SWE as environmentally friendly extraction
82 technology, for extraction of bioactive compounds, especially antioxidants, from *A. ursinum* was
83 investigated. Response surface methodology (RSM) and Box-Behnken (BBD) experimental design were
84 employed to investigate the effect of three process parameters (temperature, time and amount of added
85 acidifier) on the extraction of targeted compounds. Beside, effect of same parameters on formation of
86 Millard's reaction products was observed.
87
88 ## 2. Materials and methods
89
90 ### 2.1. Plant material
91 Dried *A. ursinum* was donated by a local tea factory (Fructus doo, Bačka Palanka, Serbia). Before
92 extraction material was ground in a blender, and the granulation of the obtained material was determined
93 (0.325 mm) using sieve sets (Erweka, Germany). The fraction of the same particle size was used in all
94 extraction runs. Moisture content (6.12%) was determined prior to extraction.

95

96 **2.2. Chemicals**

97 1, 1-Diphenyl-2-picryl-hydrazyl-hydrate (DPPH), Folin-Ciocalteu reagent, (±)-catechin and 2, 2′-
98 azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and standard substances
99 including gallic acid and kaempferol were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim,
100 Germany). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) (Sigma Aldrich GmbH,
101 Sternheim, Germany) was used as an antioxidant standard. Potassium persulfate (99%, p.a.) was obtained
102 from Acros Organics (Acros Organics, Geel, Belgium). All other chemicals and reagents were of
103 analytical and HPLC reagent grade.

104

105 **2.3. SWE procedure**

106 SWE was performed in a batch-type high-pressure extractor (Parr Instrument Company, Moline, USA)
107 with an internal volume of 450 mL and maximum operating pressure of 200 bar and temperature 350 °C,
108 connected with a temperature controller (4838, Parr Instrument Company, Miline, USA), previously
109 described elsewhere [19]. The extraction vessel jacket was heated electrically and stirring of the media
110 was performed by a magnetic stirrer (750 rpm). All extractions were maintained under isobaric condition
111 at 36 bar, using sample-to solvent ratio 1:10 (w/v). Temperature (120-200 °C), time (10-30 min) and
112 percent of acidifier, HCl (0-1.5%) were independent variables. During extraction period, temperature was
113 held constant depending on experimental run. Prior to each experiment, an extraction cell heat-up was
114 carried out for a given time, which changed according to extraction temperature. Time needed to reach
115 desired temperature of extraction (120, 160 and 200 °C) was approximately 20, 25, and 30 min,
116 respectively. Cooling phase in ice bath was the same for all experimental runs, approximately 11 min.
117 After cooling, extracts were immediately filtered thought filter paper Extracts were immediately filtered
118 through filter paper (4–12 μm pore size, Schleicher and Schuell, Germany) under vacuum (V-700,
119 Büchi, Switzerland) and stored at 4 °C until further analysis.

120

121 **2.4. Determination of total phenolic content**

122 In the obtained *A. ursinum* extracts content of total phenolic compounds (TP) was determined
123 using the Folin–Ciocalteu reagent [20]. Absorbance was measured at 750 nm using Janway 6300
124 spectrophotometar (Bibby Sciencific, France). Content of phenolic compounds was expressed as gallic
125 acid equivalents (GAE) on dry weigh of *A. ursinum* (g GAE/100 g DB). All experiments were performed
126 in three replicates.

127

128 **2.5. Determination of total flavonoids content**

129 In the obtained *A. ursinum* extracts content of total flavonoids (TF) was estimated using the
130 aluminum chloride colorimetric assay [21]. Results were expressed as catechin equivalents (CE) on dry
131 weight of *A. ursinum* (g CE/100 g DB). All experiments were performed in three replicates.

132

133 **2.6. Determination of phenolic compounds by HPLC**

134 For analysis dry extract of *A. ursinum* was dissolved in solvent mixture of methanol and 1%
135 formic acid in water (50:50, v/v) and ultrasonicated for 10 min. Solutions were filtered through 0.45 μm
136 regenerated cellulose membrane filters (Agilent, Paolo Alto, CA, USA) before injection into the HPLC
137 system.

138 HPLC analysis of phenolic compounds in extract obtained at optimal SWE conditions was
139 performed by a liquid chromatography (Agilent 1200 series, Paolo Alto, CA, USA) on an Agilent, Eclipse
140 XDB-C18, 1.8 μm, 4.6 × 50 mm column using a diode array detector (DAD), according to the method
141 of Mišan et al. (2011). The following solvent linear gradient program with solvent A (methanol) to
142 solvent B (1% formic acid in water) was used as follows: initial 85% B; 0–6.2 min, 85% B; 6.2–8 min,
143 85–75% B; 8–13 min, 75–61% B; 13–15 min, 61% B; 15–20 min, 61–40% B; 20–25 min, 40–0% B.A
144 flow rate of 1.000 mL min^{-1} was set. The run time and post-run time were 25 and 10 min, respectively.
145 The column was operated at 30 °C. Into the system, 5 μL of samples were injected, using an auto sampler.
146 The spectra were acquired in the range 190–400 nm and chromatograms plotted at 280, 330 and 350 nm.
147 Phenolic compounds in samples were identified by matching the retention time and their spectral
148 characteristics against those of standards. When standard was not available, the content of detected
149 compound is expressed as corresponding phenolic compound equivalent. For quantification, the external
150 standard method was used.

151

152 **2.7. DPPH test**

153 The free radical scavenging capacity of dry *A. ursinum* extracts were determined as described by
154 Espin et al. [23]. Different amount of dry *A. ursinum* extract were mixed with methanol (95%) and 90 μM
155 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) in order to gain different final concentrations of the extract. After
156 60 min at room temperature, the absorbance was measured at 515 nm (Janway 6300 spectrophotometar,
157 Bibby Sciencific, France) and expressed as radical scavenging capacity. Radical scavenging capacity
158 (%RSC) was calculated by following equation:

159 $$\%RSC = 100 - (A_{sample} \times 100)/A_{blank} \quad (1)$$

160 where: A_{sample} is the absorbance of the sample solution and A_{blank} is the absorbance of the control. This
161 capacity was also expressed as the inhibitory concentration at RSC value 50% (IC_{50}, the concentration of
162 test solution required to obtain 50% of radical scavenging capacity; mg/mL).
163

164 **2.8. ABTS test**

165 ABTS test was conducted to confirm antioxidant capacity of the obtained extracts. Experiments
166 were performed according to Miller et al. with some modifications [24–26]. ABTS and potassium
167 persulfate were dissolved in distilled water to a final concentration of 7 mM and 2.45 mM, respectively.
168 These two solutions were mixed and the mixture was left in the dark at room temperature for 12 h before
169 further use in order to produce $ABTS^+$. In this study the $ABTS^+$ solution was diluted with distilled water
170 to an absorbance of 0.700±0.02 at 732 nm. Samples or Trolox standards (final concentration 10–150 μM)
171 were added to diluted $ABTS^+$ solution and the absorbance was measured 3 min after mixing using a
172 spectrophotometer (Agilent Cary 60 UV-Vis, Aligent Technologies, USA). All measurements were done
173 in triplicate. Results were expressed as Trolox equivalents on dry weight of *A. ursinum* (mM TEX/100 g
174 DB).

175

176 **2.9. Millard reaction indicators: determination of hydroxymethylfurfural and furfural content**

177 The chromatographic separation and quantification of hydroxymethylfurfural (5-HMF) and
178 furfural (F) was performed using the HPLC method described by Ariffin et al. (2014). 5-HMF and
179 furfural content was measured by HPLC Agilent 1200 system equipped with a diode array detector
180 (DAD) (Agilent Technologies) with XDB Zorbax Eclipse C-18 column (50 mm × 4.6 mm i.d., 1.8 μm).
181 The mobile phase consisted of an isocratic mixture of methanol: water (0.1% formic acid), ratio 10:90
182 (v:v) at a constant flow of 0.75 mL/min. The injected volume was 2 μL and the temperature was set as 30
183 °C. Extracts were filtered through 0.45 μm pore size nylon filter (Rotilabo-Spritzenfilter 13 mm, Roth,
184 Karlsruhe, Germany) before injection into the HPLC system. The results were expressed as μg/mL.
185

186 **2.10. Browning index**

187 The color of the extracts was measured using a Minolta Chromameter (Model CR-400, Minolta Co.,
188 Osaka, Japan) calibrated on a white calibration plate with the D65 light standard. CIE $L^*a^*b^*$ coordinates
189 were measured, where L^* is the luminance component, while a^* and b^* are color coordinates related,
190 respectively, with the red/green and yellow/blue spectral ranges. A standard white calibration plate was

191 employed to calibrate the equipment. Results were expressed as browning index (BI) [28]. The browning
192 index (Eq. 2) was used to describe the colour changes at different extraction conditions. The browning
193 index (BI) was calculated according to the followed equation:

194 $$BI = \frac{(x - 0.31)}{0.172} \times 100 \qquad (2)$$

195 Where, x is calculated according to the following formula:

196 $$x = \frac{a_t + 1.75L_t}{5.645L_t + a_0 - 3.012b_t} \qquad (3)$$

197 Where a_0 is the initial colour measurement of calibration white plate and L_t, a_t, BT are the colour
198 measurements at the specified extraction conditions.

199

200 **2.10. Experimental design and statistical analysis**

201 The functional relationship between the SWE extraction parameters affecting the properties (TP,
202 TF and antioxidant activity) of *A. ursinum* extracts obtained by SWE was determined using Box-Behnken
203 (BBD) experimental design. The RSM was applied to evaluate the effects of extraction and to optimize
204 conditions for various responses. For the optimization of extraction parameters 15 experiments were
205 carried out in randomized run order. Three replicates at central point were carried out to establish the
206 experimental errors. Independent variables used in the experimental design were in the ranges:
207 temperature (120-200 °C), extraction time (10-30 min) and added acidifier (0-1.5%). Process parameters
208 and their experimental domain were chosen according to literature data [29].

209 Experimental data were fitted to the following second order polynomial model (Eq. 4):

210 $$Y = \beta_0 + \sum_{i-1}^{k} \beta_i X_i + \sum_{i-1}^{k} \beta_{ii} X_{ii}^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i \qquad (4)$$

211 where Y is the response variable, X_i and Xj are independent variables and β_i, β_{ii}, and β_{ij} are the regression
212 coefficients for intercept, linear, quadratic and interception terms, respectively. Regression coefficients
213 were obtained to describe relationship between the responses and the independent variables.

214 Experimental design and multiple regression analysis were analyzed using Design Expert
215 software v. 9 Trial (State-Ease, Minneapolis, Minnesota, USA) to determine the statistical significance of
216 the model. The significances of all terms in the polynomial equation were considered statistically
217 different when $p < 0.05$. The adequacy of the model was checked by accounting for the coefficients of
218 determination (R^2, $Adj\ R^2$). The relationship between the response and independent variables was
219 demonstrated using response surface 3D plots.

220

221 **3. Results and discussion**

222 The influence of three process parameters (temperature, addition of acid and extraction time) on 223 the efficiency of SWE process and the quality of obtained extracts was observed. The target compounds 224 of the extraction process were phenolic compounds (TP, TF), as well as the antioxidant capacity of 225 obtained extract. Antioxidant capacity was determined using DPPH and ABTS test. Table 1 presents the 226 experimental design and experimental values for each response under different setup of SWE parameters.

227 According to the obtained results presented in the Table 1, TP content varied from 0.97 to 4.00 g 228 GAE/100g DB. The highest TP content correspond the highest investigated SWE temperature, 200 °C, 229 the lowest investigated extraction time, 10 minutes, and the addition of 0.75% of HCl. The lowest TP 230 content was observed at the lowest investigated SWE temperature of 120 °C, while extraction time was 231 20 minutes, and in this case there was no acidifier added.

232 TF content ranged from 0.11 to 0.71 g CE/100g DB. The highest TF content was obtained at the same 233 operating conditions as TP (temperature of 200 °C, extraction time of 10 min, 0.75% of HCl). The lowest 234 TF content corresponds to the following setup of SWE process parameters: the lowest extraction 235 temperature (120 °C, as it was the case of lowest TP), extraction time of 10 minutes, and addition of 1.5% 236 acid.

237 Content of phenolic compounds of *A. ursinum* was investigated by various authors using different 238 methods of extraction and different extraction solvents [9,17,30–32]. In the study of Djurdjevic et al. [31] 239 phenolic compounds were extracted from *A. ursinum* using methanol and ethyl acetate as extraction 240 solvents, in two extraction techniques: maceration and ultrasound assisted extraction (UAE). Here UAE 241 was found to be much more efficient for extraction of TP from *A. ursinum* in comparison to classical 242 method of extraction-maceration. According to this study, TP in *A.ursinum* dry leaves was determined as 243 4.34 mg/g. In our previous study where UAE was applied for extraction of TP from *A. ursinum* highest 244 content of TP in *A.ursinum* dry leaves was measured as 1.4454 g GAE/100g DB [9]. Comparing those 245 results with the results obtained in this study it can be concluded that much higher TP content from 246 *A.ursinum* is obtained using SWE in comparison to UAE and maceration; extraction yield of TP was 247 several times higher in SWE than in the case of UAE. Similar was observed in the case of TF, where 248 SWE gave app. 2 times higher extraction yield in comparison to UAE.

249 The antioxidant activity for obtained *A. ursinum* extracts, determined using DPPH test, was in the 250 range from 0.039 to 0.469 mg/mL, expressed as IC_{50}value. The highest antioxidant activity was 251 determined for extracts where the highest extraction yield of TP and TF was achieved. Antioxidant 252 activity of extracts obtained using SWE was much higher than activity of *A. ursinum* extracts obtained by 253 UAE in our previous study [9]. This fact also indicates that SWE is much more efficient method for 254 extraction of antioxidant compounds from *A. ursinum* than UAE. It is expected that correlation between

255 content of TP and TF exists, therefore, it is expected that same extraction parameters will exhibit similar
256 effects on these responses. Using the Pearson's correlation, moderate positive correlation was shown
257 between the antioxidant activity (ABTS and DPPH test) and TP (R^2=0.78 and R^2=0.75, respectively) and
258 between antioxidant activity (ABTS and DPPH test) and TF (R^2= 0.77 and R^2= 0.70, respectively).
259 According to Serpen et al.[33] the ABTS$\bullet^+$ is more sensitive to phenolic compounds than to Maillard
260 reaction products, MPR (HMF), therefore, both tests (DPPH and ABTS) were conducted. Kinalski and
261 Noreña [34] connected antioxidant activity of *A. urisinum* with the presence of thiosulfinates. As
262 moderate correlation of antioxidant activity and TF and TF was observed, it could be concluded that
263 others compounds as thiosulfinates could also be responsible and could contribute to the antioxidant
264 activity of the obtained extracts.
265

266 **3.1. Model fitting**

267 For analysis of SWE process parameters influence and their optimization on antioxidant activity
268 and extraction of TP and TF from *A. ursinum*, RSM and BBD experimental design were applied. The
269 statistical analysis indicated that the proposed model (Equation 4) was adequate according to the
270 significance of the F-test (p≤0.05) and not significant lack of fit (p>0.05). While descriptive statistics
271 parameters such as R^2 and adjusted R^2 and the coefficient of variation provided additional information
272 about model fitness. The regression coefficients of the intercept, linear, quadratic and interaction terms of
273 the model (Equation 4) were generated for all responses using statistical approach, the method of least
274 squares (MLS). The regression coefficients, the model for each response and the results of the analysis of
275 variance (ANOVA) are displayed in Table 2. The model equations provide good representation of
276 experimental values with satisfactory coefficients of determination (R^2=.92 to 0.99). To the model
277 signification contributed high value of adjusted R^2, which is in reasonable agreement with R^2, regression
278 for the model (p_m < 0.05), and non-significant lack of fit (p_{lf}> 0.05).
279 Influence of investigated SWE parameters on targeted responses is shown in following second
280 order polynomial equations:
281 $$EY = 50.1033 - 6.1412X_1 + 5.9725X_3 - 5.2979X_1^2 - 5.0454X_3^2 \quad (5)$$
282 $$TP = 3.6079 + 0.9061X_1 + 0.4971X_3 - 1.0237X_1^2 - 0.6032X_3^2 \quad (6)$$
283 $$TF = 0.5812 + 0.2190X_1 - 0.0649X_3 - 0.1329X_1^2 - 0.1516X_3^2 \quad (7)$$
284 $$DPPH = 0.0549 - 0.1802X_1 - 0.0306X_3 + 0.0339X_1X_3 + 0.1758X_1^2 \quad (8)$$
285 $$ABTS = 9.8388 + 4.4439X_1 - 3.7924X_1^2 \quad (9)$$
286 According to Petrović et al. [35] ratio of sum of squares and total sum of squares obtained by
287 ANOVA were used to calculate the corresponding contributions of investigated extraction parameters.
288

289 **3.2. Effects of extraction parameters**

290 The solubility of phenolic compounds is governed mostly by the chemical nature of the herbal
291 matrix and also of the polarity of used solvents. But, in general, the extraction temperature has a
292 significant effect on the recovery of phenolic compounds. Temperature is causing disruption of herbal
293 matrix increasing mass transfer through solid phase, enhancing the efficiency and rate of the extraction.
294 Therefore, analysis of temperature impact on extraction of target bioactive compounds and setup of
295 adequate extraction temperature for each plant material is necessary. As expected, in this study extraction
296 temperature showed to be the most affecting parameter. Analyzing contribution graph (Figure 1) and
297 contour lines (Figure 2) it could be seen that the temperature was the most dominant parameter during
298 SWE. The highest TP (4.00 g GAE/ 100g DB), TF (0.71 g CE/ 100 g DB), antioxidant activity, expressed
299 as IC_{50} (0.04 mg/mL) and antioxidant activity measured by ABTS test (13.805 mM TEX/ 100 g DB)
300 were observed at the highest investigated temperature.

301 The increase of temperature in SWE induced the increased extraction of TP and TF from *A.*
302 *ursinum*, and accordingly to that, antioxidant activity of obtained extracts also increased. Analyzing data
303 from Table 2, linear and quadratic terms of temperature have shown significant impact on all investigated
304 responses. Positive highly significant ($p < 0.01$) effect of linear term of temperature was indicated in the
305 case of TP, TF and antioxidant capacity expressed through ABTS test. Negative highly significant
306 influence of temperature was observed in the case of total extraction yield and antioxidant activity
307 expressed as IC_{50} which means better antioxidant activity. As it can be seen in Figure 2, increasing in
308 phenolic compounds content and antioxidant activity could be achieved by raising temperature. Higher
309 content of target compounds was observed when temperature was in range 160-200 °C. According to
310 Haghighi Asl and Khajenoori, and Xu et al., an increase in temperature favors the extraction of phenolic
311 compounds by enhancing the diffusion coefficient of solvent allowing a deep penetration of the solvent
312 into the matrix, solubility of solutes, diffusion rate of analytes, and reducing solvent viscosity and surface
313 tension [1,36]. Some authors concluded that increasing the extraction temperature beyond the certain
314 value may lead to oxidation and degradation of phenolic compounds [1,37]. On contrary, in our research
315 the highest values of TP, TF and antioxidant activity was observed at the highest temperature.

316 Extraction time is depended on the temperature and the nature of the matrix and target
317 compounds. Prolonging extraction time can induce the increase of energy and operational costs of the
318 process. Also, long heating time during the extraction may cause the compound degradation. Therefore,
319 the second parameter of interest during SWE was extraction time. To determine the effect of extraction
320 time, SWE was performed for 10, 20 and 30 min and by varying temperatures and amount of acidifier in
321 the same time. According to analysis of variances (ANOVA), time had no significant impact on
322 investigated responses (Table 2). Insignificant impact of extraction time resulted that by prolonging

323 extraction time minor concentration increase of investigated responses was noticed. This suggests that in
324 the case of *A. ursinum,* SWE extraction time can be reduced, and this will have no significant impact on
325 extraction of bioactive compounds, but can effect positivelly on the reduction of the operation costs and
326 increase number of batch of SWE extractions.

327 Phenolic compounds release from the higher plant cell is interfered with the presence of cell-wall
328 and its characteristics, such as structural parameters, physical traits and chemical composition, as well as
329 phenolic chemical structure. Cell-wall cross-linked polysaccharides are the main barrier for the release of
330 intracellular substances [38]. Moreover, phenolic compounds may also be associated with other plant
331 components as are carbohydrates and proteins. Therefore, there is no universal extraction procedure
332 suitable for extraction of all plant phenolic compounds. In order to obtain the best phenolics recovery, it is
333 important to develop the adequate hydrolysis methods to degrade cell-wall and remain environment
334 friendly, low-cost and highly efficient. According to previous statements, one of the aims of the present
335 study was to increase the phenolic compounds release from the investigated material by the addition of
336 hydrochloric acid and to evaluate its effect on the extraction of bioactive compounds in the SWE of
337 *A.ursinum.*

338 According to the *p*-values of regression coefficients, linear terms of added acidifier had highly
339 significant positive influence ($p > 0.01$) on total extraction yield and significant positive influence on
340 extraction of TP, while influence on TF was moderate (p>0.1). Presumably, the hydrolysis reaction
341 affected flavonoid glycosides, which reflected on higher TF content. Addition of acidifier significantly
342 and positively impacted on antioxidant activity reducing the IC_{50} value, while there was no impact on
343 antioxidant activity measured by ABTS test. Decrease of IC_{50} with increase of added acidifier indicates
344 the increase of antioxidant activity. This is in accordance with increased TP and TF extraction caused by
345 increase addition of acidifier. Interaction between temperature and added acidifier had significant impact
346 only in the case of IC_{50} on DPPH radical, while in the case of other parameters this interaction had no
347 effect. Impact of acidifier is visualized in Figure 2. The positive impact of added acidifier is in accordance
348 with Gizer et al. study, where the impact of organic and mineral acid on anthocyanin extraction using
349 pressurized acidifier extraction was investigated. Gizir et al. noticed that extraction efficiency of target
350 compounds was significantly improved by adding acidifier, especially organic one [39]. According to
351 obtained results it has been also noticed that after a certain point of acidifier addition (app. lower and
352 higher than 0.75%) extraction of TP and TF from *A. ursinum* starts to decrease. This is especially
353 noticeable in the case of TF extraction. Solvent acidified with hydrochloric acid may hydrolyse acylated
354 flavonoides which leads to its lower concentration [40]. Therefore, to avoid or at least minimize the
355 breakdown of acylated flavonoides lower concentration of acid should be used.
356

357 **3.3. Indicators of Millard reaction**

358 There are several papers which indicate the accumulation of undesirable compounds generated
359 during non-enzymatic browning reactions that occur during SWE extraction [41]. Some of these
360 compounds are formed during the advanced stages of Maillard reaction, such as furfurals, which are
361 frequently measured to evaluate the intensity of the applied SWE extraction procedure, i.e. the intensity of
362 the heat treatment [42,43]. The formation of 5-HMF and furfural (F) is unwanted, because these
363 compounds are known to possess cytotoxic, mutagenic, carcinogenic and genotoxic effects [44,45],
364 therefore, their presence is undesired in the obtained SWE extracts. Besides measuring furfurals,
365 determination of browning index is frequently used for following the non-enzymatic browning reactions.

366 For obtaining an insight into development of Maillard reaction products (MRPs) during SWE
367 from *A. ursinum*, 5-HMF and F were measured in all investigated extracts (Table 5). 5-HMF content was
368 significantly different among the samples treated on various extraction conditions (from 10.63 to1135.5
369 μg/mL). According to obtained results the amount of HMF significantly differed between the extracts
370 obtained at 120 °C and 160 °C. After reaching its maximum, there was a remarkable decrease in 5-HMF
371 content for samples extracted at 200 °C. Longer time and added acidifier favors HMF production, but
372 opposit influence was observed at 200 °C. Lower 5-HMF content when temperature was higher than 210-
373 220 °C due to its degradation, polymerization and reaction with other compounds as concluded in study
374 [43,46]. In our study the same trend was observed in F formation. The highest F content was observed for
375 the extracts obtained at 160 °C when acidifier was added (around 800 μg/mL) while drastic decrease in F
376 content was detected for samples treated at 120 °C and 200 °C. Herrero et al. [7] studied the contribution
377 of HMF present in the olive leave extracts, obtained using SWE at different temperatures, to antioxidant
378 activity and found that HMF did not contribute to the overall antioxidant capacity of the SWE olive leave
379 extracts. Same observation was noticed in our research, as there was no correlation between HMF and F
380 formation and antioxidant activity measured by applied antioxidant tests. Similarly to polyphenols content
381 and antioxidant activity, HMF and F were also fitted to a second-order polynomial model, however,
382 applied model was unable to adequately describe these responses ($p > 0.05$).

383 In addition, browning index is frequently used parameter for following the non-enzymatic
384 browning reactions. From the obtained results, the browning intensity of all investigated extracts
385 significantly increased with temperature rise (Table 5). These results suggest that the development of
386 brown colour is associated with all parameters (temperature, time and acidifier). The remarkable increase
387 in BI was measured at 160 °C, while above 160 °C there was no significant increase in BI.

388

389 **3.4. Optimization of SWE process**

390 SWE process optimization is very important in order to increase extraction of biological active 391 compounds and keep extraction parameters in optimal condition due to profitability of the process. 392 Multiple response optimizations were performed in order to simultaneously satisfy optimal level of 393 independent variables with desirable response of goals. Only compounds of great interest were involved 394 in optimization process. Optimized extraction condition for maximized EY (50.85%), TP (4.11g 395 GAE/100g DB), TF (0.66 g CE/100g DB), ABTS (1223 mM TEX/100g DB), and minimized IC_{50} value 396 (0.013 mg/mL), i.e. maximized antioxidant activity, were determined. For the desirable values of 397 responses optimal condition were temperature 179 °C, extraction time 10 min and 1.09% of acid 398 modifier, HCl. Determination of optimal conditions and predicted values was based on desirability 399 function, D=0.93. New extraction conditions were submitted to the same experimental procedures applied 400 as those from the commencement of this study. There was no significant difference between the estimated 401 and observed values, indicating a good fit between the models to the experimental data.

402

403 **3.5. HPLC of phenolic compounds in extract obtained under optimal SWE conditions**

404 Chromatogram of the separated phenolic compounds in extract obtained at SWE optimum conditions is 405 shown at Figure 4 and the spectra maximum and concentration of identified compounds are presented in 406 Table 4.The well-known fact is that all flavonoids have an absorption maximum between 240− 290 nm 407 and also an another absorbance maximum between 300–550 nm [47]. Based on obtained spectra shown 408 in Table 4, available standards and literature data, the profile of phenolic compounds in the sample was 409 determined. In analyzed sample, only the peak of catehnin was confirmed when it has been matched with 410 the spectra of standard (peak 6). Due to the lack of standards, other compounds present in extract were 411 identified according to their UV spectra in comparison to the literature data. Oszmiański et al. found that 412 *A. ursinum* is rich in kaempferol derivatives, namely kaempferol-hexosyl-acetyl-deoxyhexose-hexoside 413 derivatives and a numerous kaempferol-hexose derivatives. Identification of separated compounds was 414 performed using LC MS, and along with that data authors presented spectra maximums obtained using 415 DAD [17]. Those spectra data were used for the identification of separated compounds in our sample, and 416 thus our results are expressed as kaempferol equivalents. According to the same authors when acidified 417 methanol is used as a extraction solvent, broad spectrum of kaempferol derivates could be isolated. In our 418 research acidified subcritical water was used which at these set up conditions has dielectric constant close 419 to methanol [48] and therefore, we may also conclude that flavonoids could be present in our sample. The 420 most abundant compounds in extract were 9, 21 and 22 (1.85-88.19 µg kaempferol equivalent/mL 421 extract). According to Santos-Buelga et al. [47] the locations of absorption maximum are affected by the

422 number of hydroxyl groups in rings A and B as well as by the glycosylation of the flavonoid and acyl
423 substituents in glycosyls which cause an additional maximum in the UV spectrum of flavonoid
424 glycosides. The content of compound 2 is expressed as gallic acid equivalent, since its maximum was at
425 274 nm. The UV spectra of compounds 8, 9, 11, 13, 17, and 19 had a maximum at 348 nm, while the UV
426 spectra of compounds 27, 28, 29, 30 and 31 showed maximum at 318 nm. All other peaks had a
427 maximum between 316 and 318 nm (Table 4). Vlase et al. used 70% of ethanol as a solvent for the
428 extraction of phenolic compounds from *A. ursinum* and using UV detector, *p*-cumaric and ferulic acids
429 were identified [49]. However, these compounds were not present in our sample.

430

431 **4. Conclusion**

432 The application of SWE is an economical alternative to the conventional extraction methods, due
433 to facts that for efficient extraction shorter extraction time need to be applied, that this technology used
434 widely available water as extraction solvent, and due to the possibility to directly and without further
435 process of separation or purification use obtained extracts as semi-products or products for food or
436 pharmaceutical industry. Therefore, in this study SWE was employed for recovery of antioxidant
437 compounds and preparation of functional extracts from *A. ursinum*. Effect of extraction parameters
438 (temperature, extraction time, added acidifier) on recovery of bioactive compounds of interest was
439 evaluated. According to the statistical and graphical analysis, the most dominant effect in this extraction
440 process was the effect of extraction temperature. Temperature had notable influence on each targeted
441 response. However, this study also proved that acid hydrolysis can enhance the extraction concentration
442 of bioactive compounds, especially phenolics present in *A. ursinum*. Contrary, extraction time has shown
443 no significant influence during extraction and the shortest investigated time for all investigated responses
444 was determined as optimal time. Short extraction time goes in favour of process cost and production of
445 investigated extracts. Optimal condition of SWE, needed for preparation of *A.ursinum* extracts of most
446 desirable characteristics, were determined as temperature of 179 °C, extraction time 10 min and 1.09 % of
447 acid modifier, HCl. In extract obtained under optimal extraction conditions the major compounds,
448 identified by HPLC-DAD technique, were kamepferol derivates.

449 Millard product indicators as are BI, HMF and F were also investigated, but they were not taken
450 into account when optimization was conducted. In obtained extracts concentration of one of them, 5-
451 HMF, ranged between 10.63 to 11135.50 µg/mL, and it was significantly different among the samples
452 treated on various extraction conditions. Also, remarkable decrease, probably due to its degradation has
453 been noticed for this indicator on the highest investigated temperature of 200 ºC.

454

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600 **Figures**

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602 **Figure 1** Contribution of extraction set-up parameters on investigated responses

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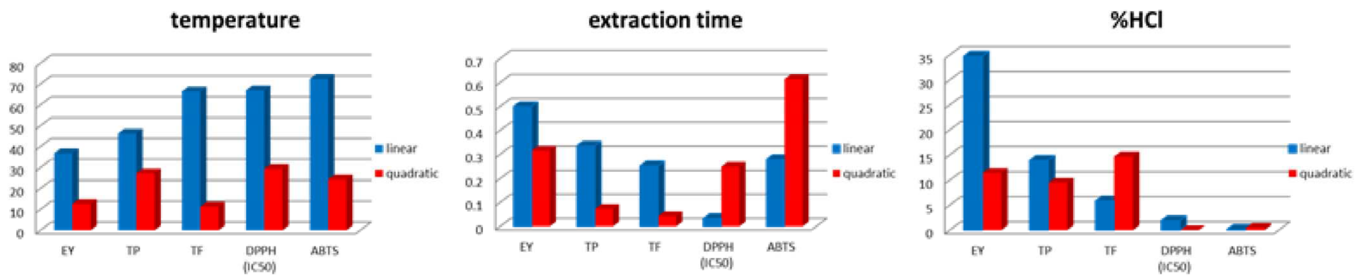

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605 **Figure 2** Contour lines of significant parameters, temperature and added acidifier, showing their

606 combined effects on EY, TP, TF, DPPH and ABTS

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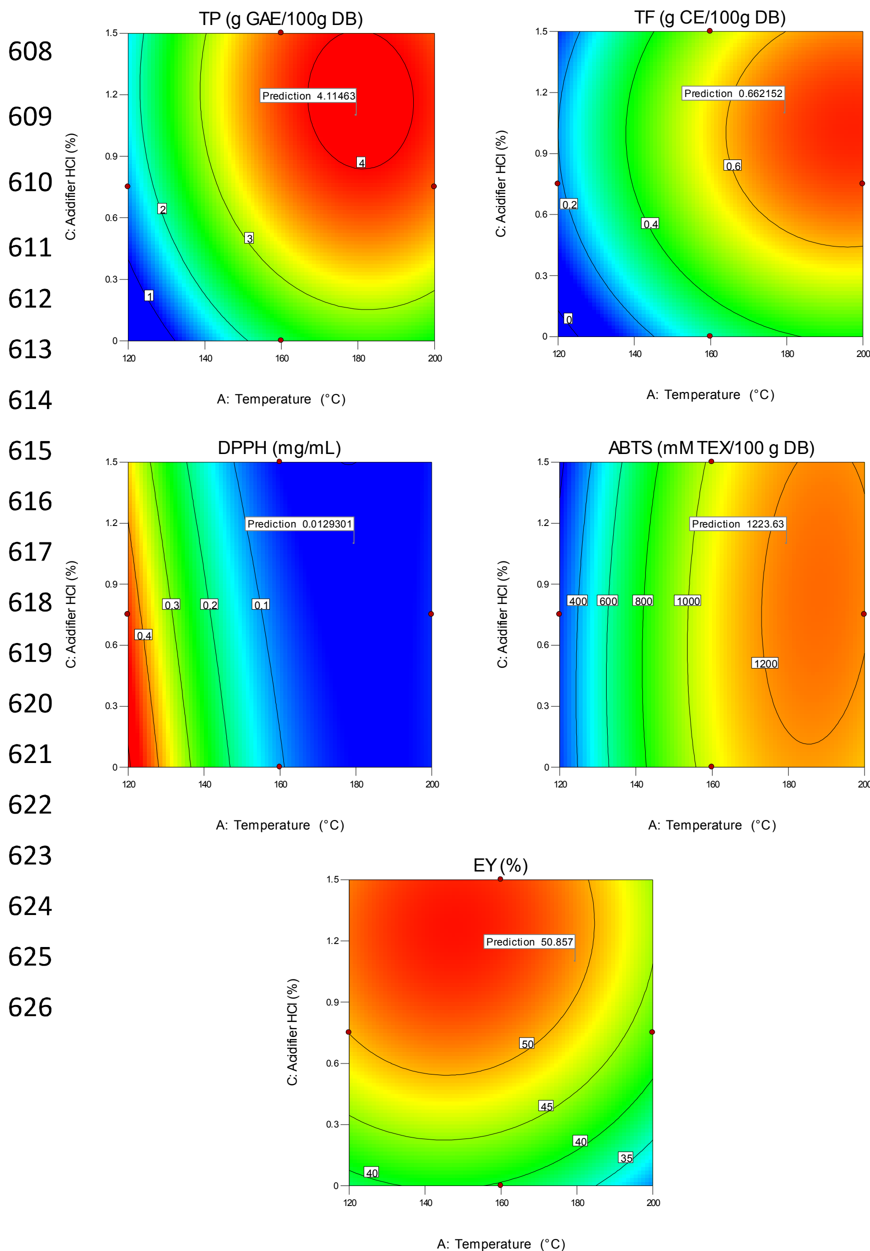

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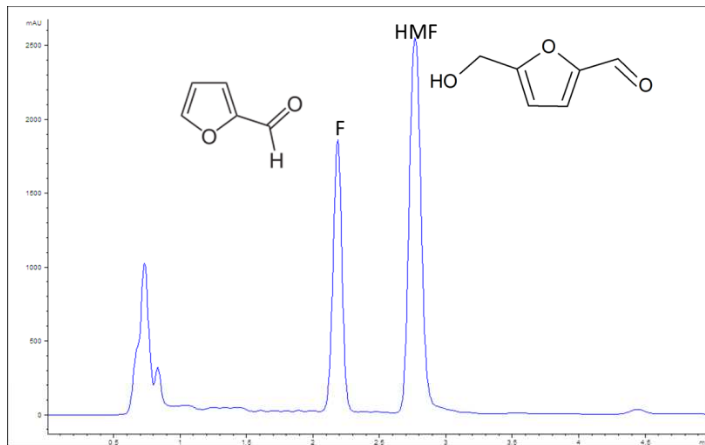
627 **Figure 3** Chromatogram and chemical structure of 5-HMF and F at 280 nm detected in investigated
628 extract

629 

630 **Tables**

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632 **Table 1** Box- Behnken design of the three-levels and three-variables with observed responses under
633 different experimental conditions

Run order	Independent variables			Investigated responses				
	Temperature [°C]	Extraction time [min]	Acidifier HCl [%]	EY [%]	TP [g GAE/100g DB]	TF [g CE/100g DB]	DPPH·, IC_{50} [mg/mL)	ABTS•$^{+}$[mM TEX/100 g DB]
1	160	10	1.50	53.26	3.924	0.560	0.052	1031.458
2	120	20	0.00	38.44	0.971	0.106	0.469	391.162
3	120	10	0.75	49.36	1.349	0.171	0.455	245.765
4	160	20	0.75	48.64	3.680	0.580	0.050	936.546
5	160	20	0.75	52.56	3.684	0.610	0.051	1058.255
6	200	10	0.75	41.16	4.002	0.707	0.039	1364.475
7	200	30	0.75	37.00	3.325	0.662	0.065	1048.623
8	160	20	0.75	49.11	3.460	0.553	0.063	1016.741
9	200	20	0.00	26.20	2.476	0.482	0.071	943.665
10	200	20	1.50	41.88	2.847	0.498	0.052	910.760

11	160	30	0.00	40.62	2.430	0.358	0.073	925.295
12	160	10	0.00	42.16	1.999	0.231	0.098	908.867
13	120	20	1.50	52.52	1.630	0.101	0.315	201.659
14	160	30	1.50	47.54	3.453	0.537	0.046	1180.121
15	120	30	0.75	55.05	1.449	0.220	0.429	196.073

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636 **Table 2** Estimated coefficients of the fitted second-order polynomial model for EY, TP, TF, IC50 and
637 ABTS analysis of variance ANOVA of the investigated experimental design

Term	Regression coefficient				
	EY	TP	TF	DPPH test	ABTS test
Intercept					
β_0	50.1033	3.608	0.5812	0.0549	9.8388
Linear					
β_1	-6.1412*	0.9062*	0.2190*	-0.1803*	4.4439*
β_2	-0.7162	-0.0772	0.0135	-0.004	-0.2755
1β3	5.9725*	0.4971**	-0.0649***	-0.0307**	0.2131
Interaction					
β_{12}	-2.4625	-0.1942	-0.0236	0.0129	-0.7317
β_{13}	0.4	-0.0719	0.0051	0.0339**	0.4305
β_{23}	-1.045	-0.2257	-0.0375	0.0047	0.3635
Quadratic					
β_{11}	-5.2979**	-1.0237*	-0.1330**	0.1759*	-3.7924**
β_{22}	0.8371	-0.053	-0.0082	0.0162	0.6021
β_{33}	-5.0454**	-0.6033**	-0.1516**	-0.0039	-0.5186
$R^{2\ a}$	0.9560	0.9367	0.9356	0.9918	0.9231
R2 Adj	0.8967	0.8228	0.8196	0.9769	0.7846

CV [c]	6.08	15.97	20.79	16.37	24.29
p_m-value [d]	0.0068	0.0160	0.0167	0.0001	0.0251
p_{lf}-value [e]	0.3434	0.0521	0.0617	0.0510	0.0752

638 *p<0.01

639 **0.01≤ p <0.05

640 ***0.05≤ p <0.1

641 [a] coefficient of multiple determination

642 [b] coefficient of variance [%]

643 [c] probability of F value for the model

644 [d] probability of F value for the lack of fit

645

646 **Table 3** Millard reaction indicators (BI, HMF and F) under different experimental conditions

Run order	Temperature [°C]	Extraction time [min]	Acidifier HCl [%]	BI	HMF [μg/mL]	F [μg/mL]
1	160	10	1.50	136.27[a]	515.65[b]	920.85[b]
2	120	20	0.00	35.66[a]	12.25	5.56
3	120	10	0.75	45.71	16.63	28.62
4	160	20	0.75	133.12[a]	1057.90[a]	764.54[a]
5	160	20	0.75	127.07[a]	1045.80[a]	795.47[a]
6	200	10	0.75	130.36[a]	70.07	135.94[a]
7	200	30	0.75	153.82	568.69[a]	668.50[b]

8	160	20	0.75	128.34^{a}	1035.10^{a}	832.63^{a}
9	200	20	0.00	146.16	608.04^{b}	140.58^{a}
10	200	20	1.50	153.58	10.63	10.46
11	160	30	0.00	139.32^{a}	477.08^{b}	47.27^{b}
12	160	10	0.00	145.71^{b}	122.69^{b}	7.10^{a}
13	120	20	1.50	97.85^{b}	1116.73^{a}	94.38^{a}
14	160	30	1.50	141.89^{b}	397.65	700.41^{a}
15	120	30	0.75	85.10^{a}	1135.50^{a}	59.66

Values are means of three determinations±standard deviation.
Values in the same column with the a in superscript are not statistically different ($p<0.05$).
Values in the same column with the a in superscript are not statistically different ($p <0.1$)

Table 4 Characterization of *A. ursinum* phenolic compounds using HPLC-DAD in extract obtained at optimal conditions

Peak	retention time	λ	Area	Concentration [µg/ml]
1	0.74	230, 260	430.7	32.97
2	0.91	234, 256, 274	360.2	27.57
3	1.26	296	117.3	8.99
4	1.59	232, 270, 362	53.04	4.07
5	2.32	234, 298	44.83	3.44
6	4.28	234, 280	94.44	7.24
7	5.44	234, 316	86.34	6.62
8	6.55	240, 266, 348	153.09	11.73
9	7.42	232, 264, 348	1152.4	88.19
10	8.41	232, 310	27.69	2.13
11	8.96	232, 266, 348	56.02	4.30
12	9.38	246, 323	24	1.85

13	9.88	232, 266, 348	34.97	2.69
14	10.17	232, 266, 346	113.68	8.71
15	10.56	232, 266, 346	238.38	18.25
16	10.87	232, 266, 346	277.22	21.22
17	12.41	232, 266, 348	103.39	7.92
18	14.61	230, 266, 312, 354sh	118.75	9.10
19	15.20	232, 266, 348	215.19	16.48
20	16.10	232, 266, 316	388.86	29.77
21	16.29	234, 266, 316	1017.4	77.86
22	16.95	234, 266, 316	1145.91	87.69
23	17.45	232, 266, 316	267.07	20.45
24	17.68	234, 266, 316	221.23	16.94
25	17.96	230, 266, 316, 348sh	211.02	16.16
26	18.01	230, 266, 316	304.52	23.31
27	18.38	230, 266, 318	122.54	9.39
28	18.50	230, 266, 318	127.98	9.81
29	19.01	230, 266, 318	126.93	9.72
30	19.38	230, 266, 318, 345sh	126.93	9.72
31	19.85	230, 266, 318	60.24	4.62

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654 **Figures**

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