EVALUATION OF PARAMETERS FOR SUBCRITICAL WATER EXTRACTION OF ZINGIBER ZERUMBET USING FRACTIONAL FACTORIAL DESIGN

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Abstract

Zingiber zerumbet (Z. zerumbet) is recognized for decades for its usability as spice and condiment in food flavoring as well as having high medicinal properties. Up to date, there are limited literature on evaluation of the effects of multiple variables in details especially in pilot-scale subcritical water extraction (SWE) of Z. zerumbet. The aim for this study is to implement the fractional factorial design with five variables which are temperature (100-170°C), time (10-40 minutes), pressure (10-20 bar), particle size (0.89-3.56 mm) and solvent to solid ratio (20-40 ml/g) in SWE of Z. zerumbet. Analysis of variance for all responses stated that temperature, time, particle size and solvent to solid ratio are significant variables. Temperature is the most significant factor for zerumbone concentration and antioxidant activity with a p-value of <0.0001 and 0.0002, respectively. The solvent to solid ratio was the most significant factor for the yield of extraction with a p-value of 0.0002. Time and particle size were significant towards all responses, however pressure was not significant on zerumbone concentration and yield. Thus, the fractional factorial design could give a broad overview in selecting the significant variables for further optimization in SWE from the findings.

Keywords: Fractional factorial design, subcritical water extraction, Zingiber zerumbet, zerumbone concentration, antioxidant activity

Abstrak

Zingiber zerumbet (Z. zerumbet) dikenali berdekad lamanya, digunakan sebagai rempah dan bahan dalam perasa makanan serta mempunyai nilai perubatan yang tinggi. Sehingga kini, kajian yang terperinci berkenaan kesan pembolehubah untuk pengekstrakan air subgenting berskala pilot adalah terhad. Kajian ini dijalankan untuk melaksanakan rekabe untuk pembolehubah faktorial pembolehubah iaitu suhu (100-170°C), masa (10-40 minutes), tekanan (10-20 bar), saiz bahan (0.89-3.56 mm) dan nisbah pelarut kepada pepejal (20-40 ml/g) dalam pengekstrakan air subgenting bagi Z. zerumbet. Analisis varians menyatakan bahawa pembolehubah termasuk suhu, masa, saiz bahan dan nisbah larutan kepada pepejal adalah signifikan terhadap respons. Suhu adalah paling signifikan terhadap kepekatan zerumbone dan aktiviti antioksida dengan nilai-p <0.0001 dan 0.0002. Nisbah pelarut kepada pepejal paling signifikan untuk jumlah hasil ekstrak dengan...
1.0 INTRODUCTION

Zingiber zerumbet (Z. zerumbet) locally called “Lempoyang” is lies under the Zingiberaceae family and extensively utilized throughout Asia in food and beverages. Besides its application as a food flavor, Z. zerumbet is traditionally consumed as home remedies for treating stomach discomfort and fever [1, 2]. Z. zerumbet also possess high antioxidant activity which is beneficial for consumption [3, 4]. These important properties resulted from the bioactive compounds available in Z. zerumbet. Zerumbone is one of the major bioactive constituents in Z. zerumbet, which reported to possess the bioactivity beneficial for both in-vitro and in-vivo studies [5–8].

In order to obtain valuable properties of Z. zerumbet at its optimum condition, an extraction process is a critical aspect. Previously, several extraction processes of Z. zerumbet have been disclosed [9–11]. However, most of the methods are still implementing conventional extraction methods like Soxhlet extraction methods which involve organic solvent and hydrodistillation that take a longer time. Subcritical water extraction is an advanced extraction technique that uses water as extracting solvent at 100°C until 374°C with a pressure higher than atmospheric pressure in the range of 0.1 MPa to 22.1 Mpa to ensure the water at its liquid condition during the extraction process [12–14]. Subcritical water extraction also demonstrated shorter extraction time (5-60 minutes) than conventional methods which could take at least 8 hours using the Soxhlet extraction method [15, 16]. In comparison to the other non-conventional method, supercritical fluid extraction, it is also required shorter time of experiments, however, it needs selective solvent and operates at the supercritical condition, compared to subcritical water extraction, which only uses water in subcritical condition [17–19].

Extraction process includes several parameters that need to be considered, and it could be temperature, time, pressure and others depending on the designated research. The screening process is the first step in determining the significant factors towards optimization [20]. There is a screening processes design to be chosen, such as fractional factorial, D-optimal, Plackett-Burman, and Taguchi OA. Wong et al., 2014 successfully screened the significant variables in the extraction of palm kernel by implementing fractional factorial design for the further optimization process [21]. By utilizing screening process design, the number of the experiment will reduce, thus minimizing the usage of sample and solution related to the research [22, 23].

The present study explained the interaction between parameters including temperature, time, pressure, particle size, and solvent to solid ratio by employing fractional factorial design to analyze the most significant parameters in the pilot-scale subcritical water extraction of Z. zerumbet. This comprehensive research will give a short review of the interaction of multiple variables towards responses.

2.0 METHODOLOGY

2.1 Chemicals and reagents

Methanol HPLC grade and acetonitrile HPLC grade were purchased from Friendemann Schmidt Pty Ltd, Washington, US. 2,2-Diphenyl-1-picryl hydrazil (DPPH) was purchased from Sigma-Aldrich, US, and ethanol from J-Kollin, UK.

2.2 Preparation of Zingiber zerumbet

Z. zerumbet rhizomes were procured from a farm at Kuala Krau, Pahang, Malaysia. The samples were washed to remove soils and sliced to 1 mm thickness using the industrial slicer. Then, the sliced Z. zerumbet was dried in the oven at 50°C for three days. The final moisture of dried Z. zerumbet was measured using OHAUS Moisture Analyzer (MB25), USA to make sure it is dry enough to prevent the growth of microorganisms and it lengthens the shelf life for storage purposes [24]. Before the extraction process, dried Z. zerumbet was ground to obtain a smaller size, and sieved. The sieved Z. zerumbet was categorized by mean particle size, which is calculated based on the sizes of sieve that dried Z. zerumbet entrapped (4.75 mm, 2.36 mm, 1.18 mm, 600 µm). For a mean particle size of 0.89 mm, the dried Z. zerumbet was entrapped between sieve sizes of 600 µm and 1.18 mm.
2.3 Subcritical water extraction of *Zingiber zerumbet*

Subcritical water extraction of *Zingiber zerumbet* utilized in this research comprises of one extraction and one collection vessels. Both vessels equipped with pressure gauge and thermocouple for measuring pressure and temperature, respectively. The schematic diagram of 5 L subcritical water extraction is as illustrated in Figure 1.

![Schematic diagram of 5 L subcritical water extraction](image)

The first step in this process was to insert the dried *Z. zerumbet* into a sample holder placed in the extraction vessel and filled with distilled water. The volume of distilled water was fixed at 2.5 L; meanwhile, the weight of dried *Z. zerumbet* was varied based on the solvent to solid ratio for each run, as stated in Table 2. A fitted cover was fixed on top of the extraction vessel to prevent any pressure loss during the extraction process. Oxygenated oxygen available in the solution was purged with nitrogen gas for one minute. Then, the required pressure was maintained until the experiment completed. Finally, the extracts were streamed into the cooling vessels to be collected for the analysis.

2.4 Fractional factorial design

The fractional factorial or two-level half factorial design (2<sup>5-1</sup>) was employed using Design-Expert software (Version 7.1.6, Stat-Ease, Inc., MN). There are five variables namely temperature, time, pressure, particle size and solvent to solid ratio on pilot scale subcritical water extraction of *Z. zerumbet* [10, 25, 26]. Three responses were evaluated from the experiments, which are yield, the concentration of zerumbone and antioxidant activity of the extracts. In order to perform these experiments, nineteen runs were required to identify all possible combinations of variables. The independent variables used in the fractional factorial design and the respective levels are shown in Table 1.

**Table 1 Factors and levels of independent variables in a fractional factorial design**

| Independent variables | Notation | Levels |
|-----------------------|----------|--------|
| Temperature (ºC)      | A        | 100, 135, 170 |
| Time (min)            | B        | 10.0, 25.0, 40.0 |
| Pressure (bar)        | C        | 10.0, 15.0, 20.0 |
| Mean particle size (mm) | D      | 0.89, 1.77, 3.56 |
| Solvent to solid ratio (ml/g) | E | 20.0, 30.0, 40.0 |

2.5 High-performance Liquid Chromatography for Zerumbone Concentration

The concentration of zerumbone was analyzed using High-Performance Liquid Chromatography, HPLC (Waters 600-MS, USA) with Photodiode Array Detector (PDA) (Waters, USA) and Lichrocart 250-4, 6 Purospher Star RP-8E (5 Mym) column (Merck, Germany). The mobile phases employed in this research are 100% methanol (solvent A) and 100% acetonitrile (solvent B) according to the modified procedure of [10]. The separation was carried out in isocratic elution with 35% (solvent A) and 65% (solvent B), monitored by PDA detector at a flow rate of 1 ml/min and wavelength of 254nm.
2.6 Antioxidant Activity of Zingiber zerumbet Extract

In order to identify the antioxidant activity of Z. zerumbet extracts, DPPH (1,1-diphenyl-2-picrylhydrazyl hydrate, Sigma-Aldrich, Germany) was used to analyze the radical scavenging activity against the stable. 0.012 g of DPPH was dissolved in 300 ml of 80% ethanol, and the solution was then kept in an amber glass bottle with a screw cap to prepare an ethanolic solution of DPPH. The absorbance of ethanolic DPPH as a control solution was taken at 517 nm using a spectrophotometer (UV-1800, Shimadzu, Japan) equipped with a quartz cell (optical path length, 1 cm) was utilized to measure the absorbance.

The liquid Z. zerumbet extracts were prepared by diluting in ethanol at a ratio of 1:4. These solutions were shaken vigorously for 1 minute using a vortex (Heidolph, Germany) and incubated in a dark environment at room temperature. After 1 hour, the absorbance of these solutions was measured at the same wavelength of the control solution. The percentage of antioxidant activity was calculated using Equation 1:

\[ \text{Antioxidant activity (\%) } = \frac{A - B \times 100}{A} \]  
(Equation 1)

Where A is the absorbance of the DPPH control solution, and B is the absorbance of the DPPH control solution mixture with the Zingiber Zerumbet extract.

2.7 Yield of Zingiber zerumbet Extract

The yield of Zingiber Zerumbet extracts was obtained by a freeze-drying process to eliminate the solvent. The extracts were put in a beaker and stored in a freezer at -80°C. Then, the extracts were freeze-dried for three days to obtain the dried powder. The dried powder collected was placed in a freezer at the temperature of -20°C. The percentage of yield calculated based on weight to weight basis (w/w) as stated in Equation 2:

\[ \text{Yield (\%) } = \frac{W_t (g) - W_i (g) \times 100}{W_i (g)} \]  
(Equation 2)

Where \( W_t \) is a weight of beaker with dried Zingiber zerumbet after the freeze-dried process completed, \( W_i \) is the weight of empty beaker (g) and \( W_t \) is the weight of dried and ground Zingiber zerumbet.

3.0 RESULTS AND DISCUSSION

3.1 Influence of Extraction Variables on Zerumbone Concentration

The research outcomes are stated in Table 2, and the results were analyzed for each response studied. The most striking result to emerge from the data is that temperature was the most significant factor in zerumbone concentration as the p-value was <0.0001 with percentage contribution of 43.55% as recorded in Table 3. To compare the results on zerumbone concentration, only pressure was found to be insignificant as the p-value was 0.2646, as stated in Table 4. For a variable or model to be significant, the p-value must less than 0.05.

The final equation in coded factors for zerumbone concentration is stated in Equation 3.

Concentration = +8.05 +2.04 *A +1.08 *B -0.89 *D -1.04 *E +0.39 *A*B +0.18 *A*C -0.51 *A*D -0.58 *A*E +0.034 *B*C -0.96 * B * D -0.32 * B * E +0.30 *C*D -0.61 * D * E  
(Equation 3)

Table 2 Fractional factorial design for five factors in the experiment

| Run  | Independent variables | Responses |
|------|-----------------------|-----------|
| A    | B         | C    | D    | E    | Y1  | Y2  | Y3  |
| 1    | 100        | 40   | 10.0 | 3.56 | 40.0 | 5.78 | 26.7 | 9.70 |
| 2a   | 135        | 25.0 | 15.0 | 1.77 | 30.0 | 7.52 | 37.4 | 12.6 |
| 3a   | 135        | 25.0 | 15.0 | 1.77 | 30.0 | 8.15 | 33.4 | 12.0 |
| 4    | 100        | 40.0 | 10.0 | 0.89 | 20.0 | 9.43 | 34.2 | 14.0 |
| 5    | 100        | 40.0 | 20.0 | 3.56 | 20.0 | 7.77 | 45.8 | 13.5 |
| 6    | 100        | 10.0 | 10.0 | 0.89 | 40.0 | 4.78 | 29.5 | 9.90 |
| 7    | 100        | 10.0 | 10.0 | 3.56 | 20.0 | 4.39 | 27.3 | 12.5 |
| 8    | 170        | 40.0 | 10.0 | 0.89 | 40.0 | 11.9 | 44.6 | 14.0 |
| 9    | 170        | 40.0 | 20.0 | 3.56 | 40.0 | 6.50 | 41.9 | 12.3 |
| 10   | 170        | 40.0 | 10.0 | 3.56 | 20.0 | 9.17 | 45.8 | 12.9 |
| 11   | 170        | 10.0 | 10.0 | 3.56 | 40.0 | 7.63 | 31.6 | 11.8 |
| 12   | 170        | 10.0 | 20.0 | 3.56 | 20.0 | 11.6 | 43.1 | 13.4 |
| 13   | 100        | 40.0 | 20.0 | 0.89 | 40.0 | 7.11 | 39.0 | 12.3 |
| 14   | 100        | 10.0 | 20.0 | 3.56 | 40.0 | 4.73 | 25.7 | 8.40 |
| 15a  | 135        | 25.0 | 15.0 | 1.77 | 30.0 | 8.56 | 36.2 | 12.4 |
| 16   | 170        | 40.0 | 10.0 | 0.89 | 40.0 | 8.04 | 35.0 | 12.8 |
| 17   | 170        | 10.0 | 20.0 | 0.89 | 20.0 | 4.48 | 30.4 | 11.2 |
| 18   | 170        | 40.0 | 20.0 | 0.89 | 20.0 | 15.8 | 56.3 | 16.0 |
| 19   | 170        | 10.0 | 10.0 | 0.89 | 20.0 | 10.6 | 47.3 | 14.0 |

A – Temperature (°C); B – Time (min); C – Pressure (bar); D – Mean particle size (mm); E – Liquid to solid ratio (ml/g); Y1 – Zerumbone concentration (mg/g); Y2 – Antioxidant activity (%); Y3 – Yield (%)

3.2 Influence of Extraction Variables on Antioxidant Activity

For antioxidant activity, all variables were found to be significant as the temperature was found to be the major influencing factor of 38.36%, followed by time (21.01%), solvent to solid ratio (15.87%), pressure (4.63%) and mean particle size (3.57%). The final equation in coded factor is stated in Equation 4.

Antioxidant activity = +37.35 +5.44 *A +4.03 *B +1.89 *C -1.65 *D -3.50 *E -1.01 *A*C -0.81 *A*D - 1.41 *A*E +2.08 *B*C -0.23 *B*E +1.26 *C*D -0.72 *C*E -0.99 * D* E  
(Equation 4)
One surprising variable that was found to be the most significant associated with the yield of subcritical water extraction of Z. zerumbet was solvent to solid ratio with a percentage contribution of 30.29%, which was slightly higher compared to temperature (28.10%). It was compared with temperature because the temperature was found to be the most significant factor for zerumbone concentration and antioxidant activity in this research. The pressure was found to be insignificant since it is contributed only 0.14% towards the yield of extraction. The final equation for the yield of Z. zerumbet extract is stated in Equation 5.

\[ \text{Yield} = +12.37 +0.98 \times A +0.67 \times B -0.59 \times D -1.02 \times E -0.27 \times A \times B +0.16 \times A \times C -0.19 \times A \times D +0.34 \times A \times E +0.37 \times B \times C -0.38 \times B \times D +0.019 \times C \times D -0.24 \times D \times E \] (Equation 5)

3.4 Analysis of the Effects of Variables on Zerumbone Concentration, Antioxidant Activity and Yield of Extraction

The temperature was found to be the most significant factor for both zerumbone concentration and antioxidant activity. It could be seen from the large gradient of slope on the perturbation graph for the effect of temperature in Figure 3a, 3b and 3c for zerumbone concentration, antioxidant activity, and yield respectively. These results are in line with previous studies on subcritical water extraction that mentioned the temperature was one of the most significant factors [28, 29]. As water is used as a solvent in subcritical water extraction, temperature plays an important role. During the extraction process, once the temperature is increased, the viscosity and surface tension decrease, thus increasing diffusion characteristic of water [30, 31].

Turning now to the experimental evidence for the concentration of zerumbone resulting from the subcritical water extraction of Zingiber zerumbet in Table 2. Run 18 (170°C) shows the maximum concentration of zerumbone at 15.76 mg/g compared to Run 7 (100°C), which was the lowest of 4.39 mg/g. However, further temperature increment will cause caramelization, Maillard reaction and hydrolysis to happen [32]. The interaction between temperature and time in the 3D surface graph also illustrated in Figure 4a, 4b and 4c for each response. Each graph gives the same illustration pattern in which as the temperature and time increased, the values of each response also increased.

Solvent to solid ratio was found to be the most significant factor for the yield of the extraction process, and the large slope gradient illustrated it for the solvent to solid ratio in perturbation graph in Figure 3c. From the finding, 20 ml/g of solid to solvent ratio produced the highest yield as in Run 18 (16%) compared to 40 ml/g, which only yielded 8.4% extracts as in Run 14. These results are consistent to the previous study on subcritical water extraction of Zingiber zerumbet in laboratory scale, which found the highest yield at 20 ml/g solvent to solid ratio compared to 10 ml/g [25]. These findings are significant, as it is in line with most of the paper that the sufficient solid-to-solvent ratio improved the contact between the sample and the solution, thereby improving the extraction process [33-35]. In addition, the maximum yield obtained in this subcritical water extraction is higher than the yield of Zingiber zerumbet extracts obtained by Soxhlet extraction from the previous study [3].

### Table 3 Percentage contribution of variables on responses

| Independent variables | Percentage contribution, 100% |
|-----------------------|-------------------------------|
|                      | Y<sub>1</sub> | Y<sub>2</sub> | Y<sub>3</sub> |
| A                    | 43.55          | 38.36          | 28.10          |
| B                    | 12.12          | 21.01          | 13.05          |
| C                    | 0.220          | 4.630          | 0.140          |
| D                    | 8.400          | 3.570          | 10.37          |
| E                    | 11.38          | 15.87          | 30.29          |
| AB                   | 1.580          | 0.006          | 2.110          |
| AC                   | 0.350          | 1.320          | 0.710          |
| AD                   | 2.770          | 0.860          | 1.100          |
| AE                   | 3.580          | 2.570          | 3.450          |
| BC                   | 0.012          | 5.620          | 3.970          |
| BD                   | 9.650          | 0.003          | 4.240          |
| BE                   | 1.050          | 0.066          | 0.001          |
| CD                   | 0.960          | 2.060          | 0.010          |
| CE                   | 3.850          | 0.670          | 0.010          |
| DE                   | 0.001          | 1.280          | 1.730          |

A = Temperature (°C); B = Time (min); C = Pressure (bar); D = Mean particle size (mm); E = Liquid to solid ratio (ml/g); Y<sub>1</sub> = Zerumbone concentration (mg/g); Y<sub>2</sub> = Antioxidant activity (%); Y<sub>3</sub> = Yield (%)

### Table 4 p-value of respective variables and responses

| Response | p-value for response | p-value for response | p-value for response |
|----------|----------------------|----------------------|----------------------|
|          | Y<sub>1</sub> | Y<sub>2</sub> | Y<sub>3</sub> |
| Model    | 0.0008 | 0.0026 | 0.0014 |
| A        | <0.0001 | 0.0002 | 0.0002 |
| B        | 0.0007 | 0.0009 | 0.0011 |
| C        | 0.2646 | 0.0214 | 0.4330 |
| D        | 0.0014 | 0.0338 | 0.0016 |
| E        | 0.0008 | 0.0017 | 0.0002 |
| R<sup>2</sup> | 0.9947 | 0.9788 | 0.9927 |

A = Temperature (°C); B = Time (min); C = Pressure (bar); D = Mean particle size (mm); E = Liquid to solid ratio (ml/g); Y<sub>1</sub> = Zerumbone concentration (mg/g); Y<sub>2</sub> = Antioxidant activity (%); Y<sub>3</sub> = Yield (%)

### 3.3 Influence of Extraction Variables on Yield

The pressure was found to be the maximum yield obtained in this study for subcritical water extraction that depended on subcritical water extraction that was in line with previous studies on subcritical water extraction that mentioned the temperature was one of the most significant factors [28, 29]. As water is used as a solvent in subcritical water extraction, temperature plays an important role. During the extraction process, once the temperature is increased, the viscosity and surface tension decrease, thus increasing diffusion characteristic of water [30, 31].

Turning now to the experimental evidence for the concentration of zerumbone resulting from the subcritical water extraction of Zingiber zerumbet in Table 2. Run 18 (170°C) shows the maximum concentration of zerumbone at 15.76 mg/g compared to Run 7 (100°C), which was the lowest of 4.39 mg/g. However, further temperature increment will cause caramelization, Maillard reaction and hydrolysis to happen [32]. The interaction between temperature and time in the 3D surface graph also illustrated in Figure 4a, 4b and 4c for each response. Each graph gives the same illustration pattern in which as the temperature and time increased, the values of each response also increased.

Solvent to solid ratio was found to be the most significant factor for the yield of the extraction process, and the large slope gradient illustrated it for the solvent to solid ratio in perturbation graph in Figure 3c. From the finding, 20 ml/g of solid to solvent ratio produced the highest yield as in Run 18 (16%) compared to 40 ml/g, which only yielded 8.4% extracts as in Run 14. These results are consistent to the previous study on subcritical water extraction of Zingiber zerumbet in laboratory scale, which found the highest yield at 20 ml/g solvent to solid ratio compared to 10 ml/g [25]. These findings are significant, as it is in line with most of the paper that the sufficient solid-to-solvent ratio improved the contact between the sample and the solution, thereby improving the extraction process [33-35]. In addition, the maximum yield obtained in this subcritical water extraction is higher than the yield of Zingiber zerumbet extracts obtained by Soxhlet extraction from the previous study [3].
Time was found to be significant after temperature and solid to solvent ratio for all responses [36]. Contact time of solvent and sample during the experiment is very important. Shorter extraction time will lead to an ineffective extraction; meanwhile, longer extraction time will cause degradation of the bioactive compound to be extracted [37]. It was proved that for 10 minutes of extraction, the results of zerumbone concentration in Run 7, together with both antioxidant activity and yield in Run 14, give the lowest values in results that are believed to be inadequate time to extracts all valuable properties in the sample. In contrast, for 40 minutes of extraction, highest zerumbone concentration was recorded at Run 18. This finding proves that by utilizing the subcritical water extraction, the extraction time was shorter than conventional methods to extract the valuable properties of the sample, especially in the extraction of *Z. zerumbet* [38, 39].

In this research, particle size was found to be significant in all responses studied. In most research, the smallest particle size affected the result the most [12, 40]. It was verified in this research as the smallest mean particle size 0.89 mm at Run 18 resulted in the highest value of zerumbone concentration since smaller particle size will increase the surface area of the sample, thus increased the research efficiency [41].

The pressure was insignificant for both zerumbone concentration and yield of extraction, however
significant for antioxidant activity. In most studies, pressure changes designed was identified to be not significant toward subcritical water extraction, however, sufficient pressure for subcritical water to happen is important in order to extracts the targeted bioactive compound in the sample [26]. Only the slight changes in pressure did not significantly affect the responses studied.

4.0 CONCLUSION

The present research aimed to screen the significant factors using the fractional factorial design of subcritical water extraction of Zingiber zerumbet. The findings clearly showed that temperature was the most significant factor for zerumbone concentration and antioxidant activity with 43.55% and 38.36% contribution respectively, meanwhile solvent to solid ratio plays the highest contribution with 30.29% in yield of Z. zerumbet extract. The contribution of each variable could also be observed from the perturbation graph, as if the contribution of factor is significant, the graph will illustrate the higher slope gradient. Time and particle size were also found to be significant toward responses. However, the pressure was found to be insignificant toward zerumbone concentration and yield of extraction, but significant toward antioxidant activity. In this research, the fractional factorial design successfully analyzed temperature, solvent to solid ratio, time and particle size as affecting variables for subcritical water extraction of Z. zerumbet and can be carried forward for the optimization process. The valuable findings of this research give a broad overview of the effects of each variable toward responses, thus highlighting the significant variables in subcritical water extraction for the further optimization procedure.

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