Optimization of Mixing Ratio to Improve Antioxidant Activity of Aged Garlic with Pine Needle Using Response Surface Methodology

Jae-Hee Park, Eunji Shin, and Eunju Park
Department of Food, Nutrition and Biotechnology, Kyungnam University, Gyeongnam 51767, Korea

ABSTRACT: Aged garlic has been reported to possess beneficial pharmacological activities, including anti-stress and anti-fatigue properties, and to exert protective effects on the cardiovascular system and liver. Pine needles are widely used in folk medicine and as food additives owing to their pharmacological properties such as anti-aging and anti-inflammatory effects. It has long been known that combining certain phytochemicals with other phenols or organic acids can produce synergistic effects. Therefore, the purpose of this study was to develop an optimal formula of aged garlic with added pine needle powder for improved antioxidant activity using the statistical technique of response surface methodology. The antioxidant activities of aged garlic mixed with pine needle powder were confirmed by measuring oxygen radical absorbance capacity and total polyphenol content. An optimized antioxidant formula was identified that contained 5.08 g aged garlic and 1.97 g pine needle powder. The antioxidant activities of the mixture prepared using this optimal formula were significantly higher than the predicted values according to an additive model. Hence, this study confirms that the addition of pine needle powder to aged garlic can improve its antioxidant activity. This study demonstrated an optimal mixing ratio to produce an aged garlic product with improved functionality through the addition of pine needle powder that could be successfully employed by the food industry to prepare functional foods.

Keywords: aged garlic, pine needle, response surface methodology, ORAC value, synergistic effect

INTRODUCTION

Currently, many garlic products are used in alternative medicine, including fresh garlic extract, garlic oil, aged garlic, and various organosulfur compounds derived from garlic (1). Aged garlic is produced by aging fresh garlic at a high temperature for at least 1 month. During the aging of garlic, non-enzymatic browning produces changes in physicochemical parameters including color, flavor, texture, and the contents of macro- and micronutrients (2). Aged garlic contains water-soluble allyl amino acid derivatives that account for most of its organosulfur content, stable lipid-soluble allyl sulfides, flavonoids, sapo-nins, and essential macro- and micronutrients (3). Further, pharmacological studies have found that aged garlic and its components possess antioxidative, antiaging, cardiovascular, and hepatoprotective effects (4).

Pine (Pinus densiflora) needles have been used in Asia to prepare folk medicines to treat conditions such as hypertension (5). Pine needle extracts were reported to show protective effects against oxidative DNA damage and apoptosis induced by hydroxyl radicals (6).

Studies involving the evaluation of synergistic effects of plant combinations are emerging and seem to highlight the potential for the mixtures to have enhanced functional properties when compared with the effects of each plant individually (7). Synergy assessment has become a key area in phytomedicine research in recent years to find a scientific rationale for the centuries-old, often observed therapeutic superiority of multidrug combinations over single constituents in traditional medicine (8).

Response surface methodology (RSM) is a statistical tool for performing, improving, and optimizing the independent factors that influence responses in a given set of experiments. RSM defines not only the effects of independent variables, but also their interaction effects (9). When multiple factors and interactions affect the desired responses for a given process, RSM is an effective technique for evaluating the process parameters with the fewest experiments (10,11).
The objective of this study was to investigate the optimal mixing ratio of aged garlic and pine needle powders for maximizing the functional properties of the resultant mixtures, including their total polyphenol content (TPC) and oxygen radical absorbance capacity (ORAC), using RSM.

MATERIALS AND METHODS

Samples and sample preparation
For the preparation of aged garlic, raw garlic cloves were put into an incubator within Chamaecyparis obtusa boxes and then fermented for more than 600 h in the temperature range of 25°C to 75°C. The aged garlic was dried to a moisture content of 40%, ground to pass through a 6-mm sieve, and then cooled to 50°C. It was then gelatinized at 80°C for 2 h, dried at 60°C, and ground again to pass through a 3-mm sieve. This procedure was repeated with grinding to pass through a 1-mm sieve. Finally, the mixture was dried to a final moisture content of approximately 3%. The pine needle powder was purchased at a local market (Garunara, Gyeonggi, Korea).

Measurement of TPC
TPC was determined according to the method of Park et al. (12). Briefly, each sample was mixed with 2 mL of 1 N Folin-Ciocalteu reagent and incubated at 25°C for 1 h. Then, 2 mL of 10% Na₂CO₃ was added, and the mixture was incubated at 25°C for 1 h. The absorbance at 690 nm was measured with a microplate reader (Tecan, Männedorf, Switzerland). TPC was expressed as gallic acid equivalents (GAE).

ORAC assay
According to the method of Kim et al. (13), ORAC assays were performed using a GENios multi-functional plate reader (Infinite® F500; Tecan) with fluorescent filters (excitation wavelength 485 nm and emission wavelength 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) (20 mM) as a peroxyl radical generator in peroxyl radical-scavenging capacity (ORAC ROO ·) assays. Trolox (1 μM) was used as a control and prepared fresh daily. The analyzer was programmed to record the fluorescence signal every 2 min after AAPH was added. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated based on the difference in the area under the fluorescence decay curve between the blank and each sample. ORAC ROO · values were expressed as μmol of Trolox equivalents (TE). One ORAC unit was equivalent to the net antioxidant effect provided by 1 μM Trolox.

Experimental design and statistical analysis
The tested mixing ratios of aged garlic and pine needle powders are given in Table 1. Aged garlic powder (X₁) and pine needle powder (X₂) were chosen as independent variables. The range and center point values of these two independent variables, as presented in Table 1, are based on the results of preliminary experiments. The TPC and ORAC values were selected as responses for the combination of the independent variables given in Table 2. Two experiments for each ratio were carried out, and the mean values are reported as the observed responses. The mixtures of aged garlic and pine needle powders (total weight 5 g) were extracted with ethanol (100 mL) for 72 h, and the extracts were filtered through Whatman No. 1 filter paper (Whatman, Maidstone, UK). The extraction solvents were removed with an evaporator (Eyela N-1000, Tokyo Rikakikai Co., Tokyo, Japan), and the dry residue was re-dissolved in dimethyl sulfoxide to a concentration of 50 mg/mL and kept at 20°C until use.

The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

The variables were coded according to the equation:
\[ x = \frac{(X_i - X_0)}{\Delta X} \]  
(Eq. 1)

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 \( \Delta X \) is the increment of \( X_i \) corresponding to a variation of 1 unit of \( x \).

The mathematical model corresponding to the compo-

| Table 1. Response of dependent variables to mixing ratio for independent variables |
|-----------------------------------------------|
| **Treatment no.** | **Actual independent variables** | **Dependent variables** |
|                  | Pine needle | Aged-garlic | ORAC (μM TE) | TPC (mg GAE/100 g) |
| 1                 | 0.3         | 3.0         | 1.73          | 1,869.2           |
| 2                 | 1.7         | 3.0         | 2.14          | 1,450.8           |
| 3                 | 1.0         | 5.4         | 1.71          | 1,394.1           |
| 4                 | 1.0         | 4.0         | 1.80          | 1,572.8           |
| 5                 | 1.0         | 4.0         | 1.83          | 1,616.9           |
| 6                 | 2.0         | 4.0         | 2.21          | 1,847.6           |
| 7                 | 0.0         | 4.0         | 1.49          | 1,507.5           |
| 8                 | 1.0         | 4.0         | 1.83          | 1,620.0           |
| 9                 | 1.0         | 2.6         | 2.01          | 1,720.3           |
| 10                | 1.0         | 4.0         | 1.86          | 1,644.0           |
| 11                | 0.3         | 5.0         | 1.47          | 1,874.9           |
| 12                | 1.0         | 4.0         | 1.73          | 1,385.0           |
| 13                | 1.7         | 5.0         | 1.97          | 1,722.4           |

ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents; TPC, total polyphenol content; GAE, gallic acid equivalents.
RESULTS AND DISCUSSION

In this study, the ORAC and TPC values of aged garlic powder combined with pine needle powder at various ratios were modeled using RSM. The ORAC assay, which closely reflects the antioxidant capacity of a test substance in biological systems (12), is a method for assessing total antioxidant activity. Polyphenols and other antioxidant compounds may contribute to the health-promoting effects of fruits, vegetables, whole grains, and nuts. Revealing an association between the amount of polyphenols and their potential health benefits requires an accurate assessment of the content of these compounds in plant foods. Significant positive correlations have been reported between ORAC and TPC, indicating that a high antioxidant activity could be attributed to a high content of total polyphenols (14-17). For this reason, two dependent variables, namely ORAC and TPC, were chosen in this experimental design. In a study by Quispe-Fuentes et al. (18), only the ORAC value was chosen as a dependent variable for the optimization of antioxidant activity from Maqui berries.

The experimental data for all responses using different mixing ratios are presented in Table 1. Aged garlic in the range of 2.6~5.4 g was mixed with pine needle powder in the range of 0~2.0 g. Values of the dependent variables were within the range of 1.47~2.21 µM TE for the ORAC value and the range of 1,385.0~1,874.9 mg GAE/100 g for the TPC. The experimental data were used to calculate the coefficients of the second-order polynomial equation. The regression coefficient and significance of the coefficients of the models are presented in Table 2. The coefficient of determination ($R^2$) indicates the degree to which a model fits the experimental data (19), and the calculated $R^2$ values are also presented in Table 2. A linear relationship with a good regression coefficient between ORAC and the mixing ratio was found; this relationship is shown in Eq. 3.

$$\text{ORAC}=1.81+0.24103 X_1 -0.10678 X_2 \quad (\text{Eq. 3})$$

The correlation coefficient of the regression equation was 0.98, indicating a good fit between the experimental data and the regression model for the ORAC values, with a non-significant lack of fit (Table 2). Hence, it can be concluded that the proposed model approximates the response surfaces and is suitable for predicting any values of the parameters within the experimental domain. The addition of increasing concentrations of pine needle powder showed a significant positive linear relationship with ORAC values. The addition of aged garlic had a linear negative influence, and higher contents of aged garlic resulted in lower ORAC values. The quadratic and interaction effects had no significant influence. The response surfaces of the ORAC values are shown in Fig. 1.

The correlation coefficient (0.21) of the TPC model was lower than that of the ORAC model, and the lack-of-fit value was significant (Table 2). Therefore, this model could not be used for the prediction of TPC. A quadratic relationship with a poor regression coefficient ($R^2=0.21$) was found between the TPC values and the mixing ratio with a significant lack of fit. Moreover, the concentrations of aged garlic and pine needle powders showed no significant effect on TPC. The response surfaces of the TPC are shown in Fig. 1.

Response optimization using RSM helped to determine
Fig. 1. Response surface plots for the oxygen radical absorbance capacity (ORAC) value (A) and total polyphenol contents (TPC) (B) in aged-garlic powder (AB) added with pine needle powder (PN).

In conclusion, RSM proved to be a useful tool for establishing the optimum mixing ratio for producing a high ORAC value. The optimal mixing ratio for producing a high ORAC value was an aged garlic powder content of 5.08 g and a pine needle powder content of 1.97 g. Using this mixing ratio, ORAC and TPC values of 2 μM TE and 1,798.7 mg GAE/100 g were predicted, respectively. The predictive capacities of the developed models were tested and validated using this optimal mixing ratio and actual ORAC and TPC values of 6.4 μM TE and 2017.8 mg GAE/100 g were obtained, respectively. The actual ORAC and TPC values of the mixture prepared using the optimal formula of aged garlic with pine needle powder were significantly higher than the predicted values (P<0.05).

ACKNOWLEDGEMENTS

Following are results of a study on the “Leaders in INDUSTRY—University Cooperation +” Project, supported by the Ministry of Education and National Research Foundation of Korea.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

1. Capasso A. 2013. Antioxidant action and therapeutic efficacy of *Allium sativum* L., *Molecules* 18: 690-700.
2. Nencini C, Menchiari A, Franchi GG, Micheli L. 2011. *In vitro* antioxidant activity of aged extracts of some Italian *Allium* species. *Plant Foods Hum Nutr* 66: 11-16.
3. Amagase H. 1998. Intake of garlic and its components. Abstract No 4 presented at Nutritional and Health Benefits of Garlic as a Supplement Conference, Newport Beach, CA, USA.
4. Morihara N, Sumioka I, Ide N, Moriguchi T, Uda N, Kyo E. 2006. Aged garlic extract maintains cardiovascular homeostasis in mice and rats. *J Nutr* 136: 777S-781S.
5. Kim KY, Chung HJ. 2000. Flavor compounds of pine sprout tea and pine needle tea. *J Agric Food Chem* 48: 1269-1272.
6. Jeong JB, Seo EW, Jeong HJ. 2009. Effect of extracts from pine needle against oxidative DNA damage and apoptosis induced by hydroxyl radical via antioxidant activity. *Food Chem Toxicol* 47: 2135-2141.

The authors declare no conflict of interest.
15. Wang Y, Zhu J, Meng X, Liu S, Mu J, Ning C. 2016. Comparison of polyphenol, anthocyanin and antioxidant capacity in four varieties of *Lonicera caerulea* berry extracts. *Food Chem* 197: 522-529.

16. Hwang SJ, Yoon WB, Lee OH, Cha SJ, Kim JD. 2014. Radical-scavenging-linked antioxidant activities of extracts from black chokeberry and blueberry cultivated in Korea. *Food Chem* 146: 71-77.

17. Zheng W, Wang SY. 2001. Antioxidant activity and phenolic compounds in selected herbs. *J Agric Food Chem* 49: 5165-5170.

18. Quispe-Fuentes I, Vega-Gálvez A, Campos-Requena VH. 2017. Antioxidant compound extraction from maqui (*Aristolochia chilensis* [Mol] Stuntz) berries: optimization by response surface methodology. *Antioxidants* 6: 10.

19. Patel TM, Bhatt NM. 2016. RSM and MLR model for equivalent stress prediction of Eicher 11.10 chassis frame: a comparative study. In Proceedings of First International Conference on Information and Communication Technology for Intelligent Systems. Satapathy SC, Das S, eds. Springer, Dordrecht, Netherlands. Vol 2, p 390.