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Optimization of Aqueous Methanol Extraction Condition of Total Polyphenol from Spent *Lycium chinense* Miller to Develop Feed Additives for Pig

Shim, Kwan-Seob* · Na, Chong-Sam* · Oh, Sung-Jin** · Choi, Nag-Jin***

양돈용 사료 첨가제 개발을 위하여 구기자 부산물로부터 메탄올수용액을 이용한 총 폴리페놀 추출조건 최적화

심관섭 · 나종삼 · 오성진 · 최낙진

This study was conducted to develop a functional feed additive for pig with spent *Lycium chinense* Mill fruit. We investigated the optimum conditions for the extraction of polyphenol from spent *Lycium chinense* Mill using methanol. Methanol concentration as a solvent for extraction, extraction time and the volume of solvent per a gram of solid (ground spent Lyceum chinense Mill) were selected as parameters. Three levels of parameters were configured according to Box Behnken experiment design, a fractional factorial design, and total 15 trials were employed. Total polyphenol concentration from each trial was used as response from experiment system and effects of parameters on total polyphenol extraction efficiency were determined using response surface model. As a result, all terms in analysis of variance, regression ($p = 0.001$), linear ($p = 0.002$), square ($p = 0.017$) and interaction ($p = 0.047$) was significant and adjusted determination coefficient (R^2) was 94.7%. Total polyphenol extraction efficiency was elevated along increased methanol content and decreased solvent to solid ratio. However extraction time did not affect the efficiency. This study provides a primary information for the optimum extraction conditions to maximize total polyphenol recovery from spent Lycium chinens Mill fruit and this result could be applied to re-use of argo-industrial by-products and to develop of functional feed additives in organic farming.

Key words : *spent Lycium chinense Miller, aqueous methanol, total polyphenol, response surface methodology, fractional factorial design*

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* Department of Animal Biotechnology, Chonbuk National University
** Department of Animal Science, Chonbuk National University
*** Corresponding author, Department of Animal Science, Chonbuk National University(nagjin@jbnu.ac.kr)

Ⅰ. Introduction

An antibiotic has been frequently used for both disease control and health promotion because these features of antibiotic use are highly related to the improvement of animal production. However, residue of antibiotics in animal product has resulted in various health problems and in recent, the use of antibiotics is strictly restricted. Therefore, the development of new, natural origin antibiotic replacers is needed and many researches have been performed (Chang et al., 2010). For a possible antibiotics replacer, plant extracts have been used.

Fruits of *Lycium chinense* Miller are broadly distributed in subtropical regions of southeastern Asia and some European countries. It has gained attention because of its possible health benefit. Lycium chinese Miller fruit has been reported that it contains various phytochemicals such as betaine, cholin, physalien, rutin and zeazanthin and these compounds show antioxidant activity, antibacterial activity, anti-cancer effect, improvement of hepatic function and decreasing blood cholesterol in many of reports (Lonni et al., 2012; Zhang et al., 2010). Approximately, 566 ton of Lycium chinese Miller fruit is produced in 141 ha per a year in Korea and it is marketed in the form of manufactured products such as tea or beverage. Hence considerable amount of residue is produced as a byproduct and most of these residues are utilized as a fertilizer or dumped as a waste. However, there are remaining phytochemicals such as polyphenol and flavonoids that are soluble in organic solvent because most of *Lycium chinense* Miller food is produced by water extraction.

Phenolic compounds have been regarded as health beneficial plant extract because of their protective activities such as antioxidant, antibacterial activity and others (Rice-Evans et al., 1997). Phenolic compounds from plant or vegetables have been reported as valuable feed additives, which can improve the performance of animal or substitute the use of antibiotics in many studies (Jung et al., 2010; Oliveira et al., 2010). Phenolic compounds found in plant are generally water soluble and are composed of simple phenol, phenolic acid, phenyl propanoid and phenolic quinone(Kim et al., 2001). These phenolic compounds can also be extracted by organic solvents, particularly aqueous methanol (Mussatto et al., 2011). Different techniques have been applied to extract phenolic compounds from agro-industrial by-products and plants. Among these techniques, solid to liquid phase extraction is mainly employed because it showed high efficiency with low cost for processing. However, the extraction efficiency is influenced by several factors such as a type of solvent, concentration of solvent, reaction time and solvent to solid ratio (Chirinos et al., 2007). Therefore, the optimization of extraction conditions is very important in order to maximize the efficiency of extraction.

For the optimization of process, generally the conventional method involves investigating one parameter at a time, the while the others are held in constant. This technique is called as one factor at a time (OFAT) method. Some of disadvantages of OFAT are that it is time consuming and often missing the effect of interaction of parameters (Cohran et al., 2002). Response surface methodology (RSM) is applied to overcome the limitation of OFAT method (Cho et al., 2010; Kaushik et al., 2006; Soni et al., 2007). Fractional factorial design (FFD) is frequently employed to construct experimental design because the responses from FFD can be described by RSM and one of most representative FFD is Box Behnken design (Box et al., 1960) .

The objective of this study was to optimize the condition for the extraction of total phenolic compounds from spent Lycium chinese Miller fruit using solid to liquid extractions with aqueous methanol as solvent. Methanol concentrations, reaction time and solvent to solid ratios were optimized using RSM.

Ⅱ. Materials and Methods

1. Preparation of spent *Lycium chinense* Miller fruit

Dried *Lycium chinense* Miller fruit was purchased from domestic market in Jeonju, Korea. Spent material was obtained after hot water extraction. Dried fruit was added into ten volume of boiling water for 5 min and this extraction procedure was repeated for three times. Finally, the remaining fruit was achieved and dried at 60℃ for 48 hours. Then, the dried spent *Lycium chinense* Mill fruit was finely ground using cutter miller (Philips HR2860, Netherlands) and used for experiment.

2. Parameters for extraction condition and fractional factorial experiment design

In this study, methanol concentration (%) as a solvent, extraction time (min) and solvent to solid ratio (mL/g) were employed as parameters for the optimization of extraction condition and their three levels were assigned to 15 trials according to Box Behnken design. The configuration of parameters and their tested levels (coded and uncoded values) are shown in Table 1. The experiment was performed in triplicates.

Table 1. Box Behnken experimental design configuration and observed responses assigned to each trials

Trials	Variables						Responses
	Methanol		Reaction time		Solvent to solid ratio		Total polyphenol, g/L
	Coded	Uncoded, %	Coded	Uncoded, min	Coded	Uncoded, mL/g	Observed
1	-1	50	-1	30	0	20	0.397±0.006
2	1	100	-1	30	0	20	0.475±0.011
3	-1	50	1	90	0	20	0.406±0.003
4	1	100	1	90	0	20	0.488±0.011
5	-1	50	0	60	-1	10	0.644±0.019
6	1	100	0	60	-1	10	0.569±0.017
7	-1	50	0	60	1	30	0.319±0.001
8	1	100	0	60	1	30	0.444±0.013
9	-1	50	-1	30	-1	10	0.648±0.012
10	0	80	1	90	-1	10	0.616±0.011
11	0	80	-1	30	1	30	0.329±0.005
12	0	80	1	90	1	30	0.330±0.004
13	0	80	0	60	0	20	0.401±0.006
14	0	80	0	60	0	20	0.398±0.008
15	0	80	0	60	0	20	0.400±0.009

3. Extraction procedures

One gram of spent *Lycium chinense* Miller fruit was submerged into an erlenmeyer flask containing aqueous methanol and the extraction was performed at room temperature with shaking (150 rpm). After the extraction, the mixture was filtered through filter paper (Whatman No. 1) and the filtrate was used for total polyphenol analysis.

4. Determination of total polyphenol concentration

Total polyphenol concentration was determined spectrophotometrically using FollinCiocalteaus reagent (Juan et al., 2009). Briefly, 0.1 mL of extract solution was mixed with 1.0 mL of FollinCiocalteaus phenol reagent (Sigma, St. Louis, Mo, USA) and then the mixture was reacted for 3 min at room temperature. Then 0.3 mL of 1 N Na_2CO_3 was added and it was reacted for 90 min at room temperature. At the end of reaction, 2 mL of distilled water was added and then the optical density was determined at 720 nm using spectrophotometer (Option 2120UV, Korea). Gallic acid (Sigma, St. Louis, Mo, USA) was used as a standard compound. Total polyphenol concentration in sample was calculated by regression and it was described as gallic acid equivalent.

5. Statistical analysis

Construction of Box Behnken experiment design, analysis of variance and the calculation of response surface model were conducted using MINITAB® software (version 14, Minitab Inc., USA).

Ⅲ. Results and Discussion

The methanol concentration, extraction time and solvent to solid ratio are critical factors that affect the efficiency of phenolic compound recovery from plant materials or argo-industrial by-product and these factors can also affect the economical status of manufacturing process. In this study, these factors were investigated using a fractional factorial design that can evaluate main effects of parameters and their interactions, simultaneously. Total 15 trails with triplicated center points (13, 14 and 15 trials) were employed and the total polyphenol concentration as a response from each trial is shown in Table 1. The total polyphenol concentrations were varied from 0.330 g/L to 0.648 g/L. The difference between lowest and highest efficiency was approximately double. This large difference implied that the investigated parameters were strongly influencing the efficiency of system. Total polyphenol concentrations from each trial were calculated by RSM and following quadratic equation was achieved;

$$Y = 1.5125 - 0.0149MC(p = 0.015) + 3.0\times10\text{-}5ET(p = 0.991) - 0.0481SSR(p = 0.001)$$
$$+ 8.0\times10\text{-}5MC^2\ (p = 0.028) + 1.5\times10\text{-}5ET2(p = 0.769) + 5.7\times10\text{-}4SSR2\ (p = 0.009)$$
$$+ 1.0\times10\text{-}5MC\times ET(p = 0.696) + 0.0002MC\times SSR(p = 0.001) - 4.0E\times10\text{-}5ET\times SSR(p = 0.438)$$

Where Y is total polyphenol concentration. Terms of MC, ET and SSR are coded value of investigated parameters as methanol concentration (%), extraction time (min) and solvent to solid ratio (mL/g), respectively. Superscriptsin backet mean probabilities of terms.

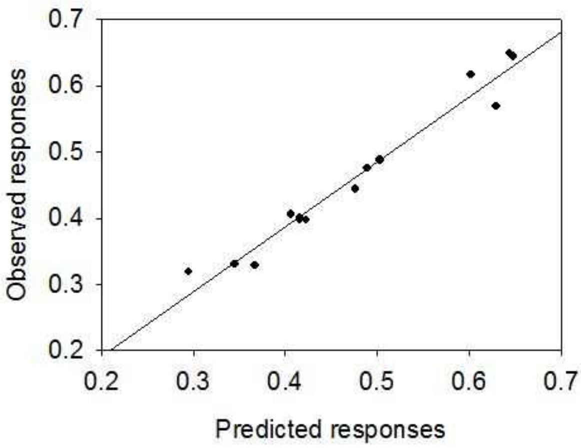

Figure 1. Predicted and observed responses from this experiment.

The predicted and observed responses are plotted in Figure 1 and adjusted determination coefficient (R^2) was 94.7%. This result means that the model from experiment system was a close agreement between observed results and predicted responses by model. Statistical analysis of the responses from experiment system revealed significance in regression model ($p = 0.001$), linear effect ($p = 0.002$), square effect ($p = 0.017$) and interaction ($p = 0.047$). The interaction among parameters is described using contour plots in Figure 2. Total polyphenol extraction efficiency was increased when the methanol concentration was increased but there was no effect of extraction time (Figure 2A). Improved extraction efficiency was achieved when solvent to solid ratio was decreased (Figure 2B). In the interaction between extraction time and solid to solvent ratio, only solvent to solid ration showed linear effects (Figure 2C).

Different organic solvents have been applied for the extraction of phytochemicals from plant materials and different solvents show different efficiency relevant to chemical nature of target compounds (Kaushik et al., 2006). For example, high yield of flavonoid extraction can be achieved by using ethyl alcohol and terpenes are well extracted by ethyl acetate or aliphatic ketones. Phenolic compounds is known to be recovered efficiently by aqueous methanol, ethyl alcohol and acetone and aqueous methanol is regarded as an economical solvent (Mussatto et al., 2011). Therefore, the selection of appropriate solvent system or extraction conditions was very important.

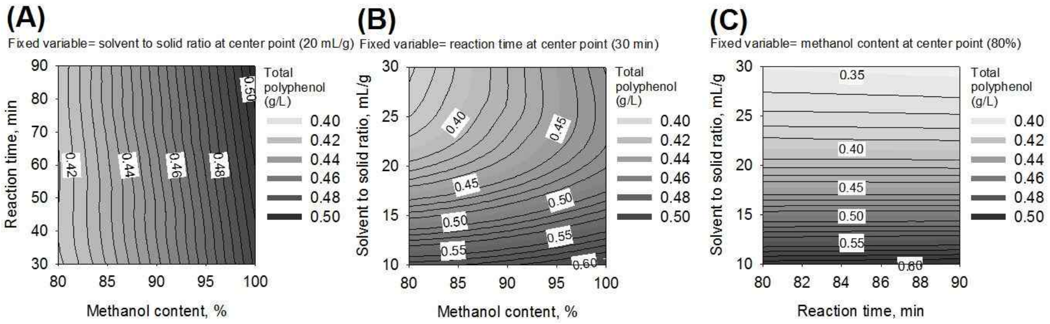

Figure 2. Contour plots for the analysis of interaction between variables. (A) Interaction between methanol content and reaction time with fixed solvent to solid ration to center point. (B) Interaction between methanol content and solvent to solid ratio with fixed reaction time to center point. (C) Interaction between reaction time and solvent to solid ratio with fixed methanol content to center point.

Ⅳ. Conclusion

In this study, aqueous methanol was used for extraction of polyphenol from spent Lyceum chinense Mill fruit and the effects of extraction conditions including methanol concentration, extraction time and solvent to solid ratio were investigated using a statistical method. Finally increased methanol concentration up to 100% and deceased solvent to solid ratio down to 10 mL/g showed increased yield of extraction. While, there was no effect of reaction time. The present result in this study could provide primary information for the utilization of argo-industrial by-products as a beneficial and valuable feed additive for organic farming.

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