Optimization of ultrasonic-assisted extraction for monacolin K, antioxidant activity, pigment and citrinin of monascal waxy corn by response surface methodology

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Abstract

This study aims to investigate the ultrasonic extraction optimization of monascal waxy corn (MWC). An optimization procedure using a central composition design with two factors (ethanol concentration and temperature) consisting of 13 experimental runs with five replicates of the central points was applied and a second-order polynomial model was used to describe the effects of these parameters on extraction for monacolin K content, antioxidant activities, pigment intensity and citrinin content. The optimized conditions were 69.91% ethanol/49.95°C (monacolin K); 69.62% ethanol/49.15°C (DPPH); 70.67%ethanol/ 49.90°C (chelating ability on Fe^{2+}); 70.91% ethanol/50.80oC (Pigment); 85.00% ethanol/60oC (citrinin) with corresponding yields of 348.72 mg/kg dry weight; 2.65 mmol Trolox/ml; 96.76%; 4072.45 unit/g dry weight and 2.04 µg/kg dry weight, respectively. The maximal monacolin K content predicted by response surface analysis was 349.59 mg/kg dry weight. The experimental value was close to those predicted values, showing fitting of the model (R^2 = 0.924) applying RSM for optimizing the ethanol concentration and temperature on monacolin K content from MWC.

Keywords: Optimization, Ultrasonic, Monacolin K, Monascal waxy corn, Response surface methodology

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1. Introduction

Monascus purpureus is a species of mold that produces purplish-red pigment. It is also known as ang-kak rice mold, corn silage mold, maize silage mold, and rice kernel discoloration. This fungus is important because of its use (i.e. in the form of red yeast rice) in the production of certain fermented foods in China. However, discoveries of cholesterol-lowering statins produced by the mold have prompted research into its possible medical uses. It could produce a number of statins known as lovastatins and analogs which are called monacolins K, L, J, and also occur in their hydroxyl acid forms along with dehydroxymonacolin and compactin (mevastatin). The prescription drug lovastatin, identical to monacolin K, is the principal statin produced by Monascus purpureus. Only the open-ring Z (hydroxyl acid) form is pharmacologically active (Panda *et al*., 2010).

Commonly, *Monascus* pigment extraction by the conventional method is the most popular method as presented in a few types of research (Yang *et al*., 2005; Yang *et al*., 2006). However, a few drawbacks of shaking extraction were time consumption and needed many cycles of extraction to recover the whole yield of pigments and antioxidants (Yang *et al*., 2006). Ultrasonic method has ever been studied for monacolin K and *Monascus* pigment extraction to obtain the yield satisfaction. Yang *et al*. (2005) explained that ultrasonic method was used to enhance the extraction potential of monacolin K and Monascus extracellular pigment as an effect of cavitation. In addition, the efficacy of an extraction process is influenced independently and/or interactively by many factors such as solvent type, solvent concentration, extraction temperature, pH, storage time, solvent-to-solid ratio and particle size (Pinelo *et al*., 2005; Silva *et al*., 2007). Carvalho *et al*. (2007) have studied that difference of ethanol concentration affected the yield of Monascus pigment. Yim *et al*. (2012) further reported that extraction temperature affected the stability of antioxidant activity. Therefore, from above researches concerning the ultrasonic method, the drawbacks of conventional extraction are expected to rely on ultrasonic extraction. Nevertheless, ethanol concentration and temperature combined with an ultrasonic device have never been studied for the extraction for monacolin K, antioxidant activity, pigment and citrinin of monascal waxy corn (MWC).

Central composite design (CCD) and response surface methodology (RSM) techniques are significant tools to determine the optimal process conditions. These are powerful techniques for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. (Chang *et al*., 2002). Also, interactions between variables can be identified and quantified by such technique (Box and Wilson, 1951). Previous works on pigment and monacolin K fermentation were conducted using CCD and RSM (Chang *et al*., 2002; Silveira *et al*., 2008). However, the application of these techniques to

extraction optimization for ultrasonic methods of MWC is scant. Therefore, the objective of present study is to implement such these techniques on the optimization of ultrasonic extraction for monacolin K, antioxidant activities, pigment and citrinin of MWC.

2. Materials and methods

2.1 Raw materials

Waxy corn (*Zea mays* L. var. ceratina) was harvested between 67–70 days after planting in Sukhothai province, Thailand. It was peeled, cleaned and removed the seed from a pod by using a knife. The seeds were kept at -18°C until MWC fermentation.

2.2 Chemicals

Monacolin K, citrinin, monosodium glutamate (MSG), peptone, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*'-disulfonic acid monosodium salt hydrate (Ferrozine), 2,2-diphenyl-1-picrylhy-drazyl (DPPH), methanol, Hydrochloric acid (HCl) and 6-Hydroxy-2,5,7,8-tetramethylchro-man-2-carboxylic acid (Trolox) were obtained from Sigma–Aldrich (St. Louis, MO). Iron(II) sulfate 7-hydrate ($FeSO_4.7H_2O$) were obtained from BDH (Poole, UK). All chemicals and solvents were analytical reagent grade.

2.3 2-step fermentation of MWC

Monascus purpureus TISTR 3090 was purchased from Thailand Institute of Scientific and Technological Research (TISTR). The strain was cultivated on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) at 25°C for 7 days before being used for the production of MWC. 100 g of waxy corn seeds and 12.08% MSG (equivalent to 1% N2 content) were transferred into a flask 500 ml. The mixture was sterilized by an autoclave at 121°C for 15 min and then left until cool down. The spore suspension of *M. purpureus* was obtained from actively growing slants in sterile water and diluted to a concentration of 106 spores/ml. 5 ml aliquot of the spore suspension was inoculated into sterilized waxy corn with and incubated for 12 days at 25°C (Kraboun *et al*., 2013). Then, MWC from the conventional method was repeatedly fermented with the second step fermentation as following. It was re-inoculated with 5 ml spore suspension of 10^6 spores/ ml of actively growing slants and continuously fermented with the same condition as the conventional method for 12 days.

2.4 Ultrasonic-assisted extraction (UAE)

UAE was performed in a sonication water bath (KH5200, Kunshan Ultrasonic Instrument Co., Ltd., Jiangsu, China). UAE was described by Deng *et al*. (2010) with some modifications. The working frequency and power were fixed at 40 kHz and 200 W. Water in the ultrasonic bath was circulated. The temperature and time of extraction were controlled from a panel. The ground powder of 1 g MWC was extracted in a 250 ml volumetric flask with the

volume of 10 ml of the ethanol concentration and temperature depended on the design. The flask was sealed by a plastic film to avoid loss of solvent. The extraction was controlled at 30 min. Then, the extract was obtained from filtration through Whatman no.4 filter paper. All of the measurements were carried out in triplicate. The extract was evaporated at 40°C to dryness. The dried extract was used for the analyses of monacolin K content, antioxidant activities pigment intensity and citrinin content.

2.5 Central composite design (CCD)

RSM was used to determine the optimum levels of ethanol concentration (%) and temperature (˚C) on five responses namely, monacolin K content, DPPH• scavenging and chelating abilities on Fe^{2+}, pigment intensity and citrinin content in MWC. The influence of ethanol concentration (X_1) and temperature (X_2) was evaluated using CCD (2^2) with four-starpoints (α =1.41) and five replicates at the centre points, resulting in a total number of 13 runs (Table 1 and 2). The experiments were performed in triplicate. A second order polynomial, equation (1), which included all interaction terms, was used to calculate the predicted response.

$$Y= \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where, Y represents response variable; β_0 is the interception coefficient; β_i is the coefficient of the linear effect; β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of interaction effect; Xi and Xj denote the coded levels of variable Xi and Xj investigated in the experiment.

The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test. All experimental designs and statistical data were analyzed by using software Design-Expert® 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA).

Table 1 Variables and their levels for the CCD

Symbols	Independent variables	Code levels				
		$-\alpha$ (-1.4142)	**-1**	**0**	**1**	**α (1.4142)**
X_1	Ethanol concentration (%)	54.82	60	72.5	85	90.18
X_2	Time (min)	35.86	40	50	60	64.14

Table 2 Experimental design and results of the CCD with observed experimental data

Run	Independent values		Dependent values (Responses)[a]				
	Ethanol concentration X_1 (%)	Temperature X_2 (%)	Y_1	Y_2	Y_3	Y_4	Y_5
1	54.82	50	250.21	1.53	61.34	2,600	4.22
2	60	40	250.97	1.5	63.35	2,400	5.45
3	60	60	267.87	1.57	74.44	3,100	8.77
4	72.5	35.86	265.56	1.6	72	2,967	7.45
5	72.5	64.14	258.66	1.55	63.43	2,844	5.77
6	85	40	249.67	1.47	56.34	2,231	3.54
7	85	60	220.56	0.14	50	1,650	2.33
8	90.18	50	100.33	0.04	49.35	978	1.08
9	72.5	50	374.00	2.60	95.44	4,050	11.80
10	72.5	50	354.12	2.66	95.89	4,010	12.88
11	72.5	50	303.00	2.55	96.19	4,022	11.78
12	72.5	50	345.00	2.78	97.00	4,064	11.99
13	72.5	50	355.00	2.31	95.22	4,013	12.45

Note: [a]Y_1 : Monacolin K content (mg/kg dry weight); Y_2: DPPH• scavenging ability (mmol Trolox/mL); Y_3: Chelating ability on Fe^{2+} (%); Y_4: Pigment intensity (unit/g dry weight); Y_5: Citrinin content (µg/kg dry weight)

2.6 Pigment intensity

The extract was immediately analyzed by spectrophotometer (Thermo spectrophotometer model Genesys 20) against methanol blank. The pigment concentration was measured at 500 nm (modified from Yongsmith *et al*., 2000). Pigment intensity was calculated from the following equation.

$$\text{Pigment intensity (Unit /g of substrate)} = \frac{\text{A 500 X dilution factor X Volume of methanol}}{\text{Weight of sample (g)}}$$

2.7 DPPH radical scavenging activity

The scavenging activity (H/e-transferring ability) against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was measured spectrophotometrically by following Velazquez *et al*. (2003). Each 1-20 mg/ml of an aliquot of 40 µl appropriately diluted extracts mixed with 200 µl of 0.02 mM DPPH solution and methanol 4 ml. Samples were kept for 15 min at room

temperature and the absorbance was measured at 517 nm. The absorbance of a blank sample containing the same amount of solvent was also measured. The extent of decolourisation is calculated as the percentage reduction of absorbance, and this is determined as a function of concentration and calculated relative to the 0.1–0.01 mM of equivalent Trolox concentration. The radical scavenging activity is expressed in mmol of equivalent Trolox per gram of sample (mmol Trolox /ml) with interpolation to 50% inhibition (IC_{50}).

2.8 Chelating ability on Fe^{2+}

Chelating ability on ferrous ions was evaluated spectrophotometrically by a slightly modified method of Kuo *et al.* (2009). Three hundred µl of 2 mM $FeSO_4.H_2O$ were mixed with 1–20 mg/ml of each aliquot of 500 µl test samples before addition of 600 µl of 5 mM ferrozine. After the incubation at room temperature for 10 min, 5 ml of ethanol was added and the absorbance was measured at 562 nm against methanol as a blank. The chelating ability was calculated as follows.

$$\text{Chelating ability on } Fe^{2+} (\%) = \frac{\Delta A562 \text{ of control} - \Delta A562 \text{ of sample}}{\Delta A562 \text{ of control}} \text{ x}100$$

2.9 Monacolin K analysis

The sonicated pigment extract was filtrated through a 0.2 µm membrane and the extract was 201 analyzed by HPLC. The HPLC system consisted of Shimadzu LC-10AT VP Liquid 202 Chromatograph, FCV-10AL VP pump, an LDC Analytical Spectro Monitor 3100 detector set at 238 nm and an LDC Analytical CI-4100 integrator. A chromatography column Ascentis C18, 5µm, 250 × 4.6 mm was connected to a 20 µl loop injector. An isocratic mobile phase of acetonitrile: water in the ratio of 65:35 (by vol.) was used. The flow rate and temperature were 1.0 ml/min and 28°C, respectively (Friedrich *et al.*, 1995). Monacolin K dissolved in 70% ethanol was used as a standard.

2.10 Citrinin analysis

Citrinin analysis was described by Lim *et al.* (2010). The sonicated pigment extract was centrifuged at 1,600 g for 10 min followed by filtration through a 0.45 µm PTEE filter unit (National Scientific, Rockwood, TN). The citrinin was determined by HPLC using a chromatography column Ascentis C18 column (4.6 x 250 mm). The mobile phase consisted of methanol/acetonitrile/ phosphoric acid (0.1%) (3:3:4, v:v:v) and the analysis was performed with a fluorescence detector set at excitation and emission wavelengths of 330 and 500 nm, respectively. The flow rate was 0.6 ml/min and the sample was spiked to confirm the presence of citrinin.

3. Results and discussion

3.1 Fitting the models

Monacolin K content, DPPH• scavenging ability, Chelating ability on Fe^{2+}, pigment intensity and citrinin content of the MWC pigment extract after sonication with different ethanol concentrations and temperatures are shown in Table 2. Both F-test and *p*-value statistical parameter are used to confirm the significance of factors studied. The obtained results demonstrated that the extraction was more significantly affected by ethanol concentration ($p<0.05$) (Table 3). The larger the regression coefficient in a model with significant p-value indicates a more significant effect on the respective response variables (Yang *et al*., 2009). The ANOVA of the regression model demonstrated that each model is highly significant due to the evident from the calculated F values and a very low probability value which values of "Prob>F" less than or equivalent to 0.05 indicate model terms are significant while values greater than 0.1000 indicate the model terms are not significant (Montgomery, 1991). The results indicated that ethanol concentration had statistically significant on all responses ($p<0.05$). Higher percentages of ethanol concentration increased in the extraction for monacolin K, pigment intensity, both antioxidant activities and citrinin content. However, the ethanol concentration reached 90.18 % affected those decreased responses. The effect of temperature was not statistically significant ($p>0.05$) of all responses except DPPH• scavenging ability ($p<0.05$), which showing that 35.86oC was enough for the extraction. This was agreement with Carvalho *et al*. (2007), which reported that 2, 22, 32, 39 and 58ºC did not significantly affect the extraction of Monascus pigment. Moreover, the set of extraction temperatures in this experiment was not too high to affect the antioxidant activity (Yang *et al*., 2006; Kraboun *et al*., 2013). Dufosse *et al*. (2005) suggested that Monascus complex compounds containing pigment and monacolin K would be destroyed when the extraction temperature was suboptimal. The interactions between factors were statistically significant at a 95% confidence level except for monacolin K. The fit of all models was checked by the coefficient of determination R^2, R^2 values of monacolin K, pigment, DPPH free radical scavenging ability, Chelating ability on Fe^{2+} and citrinin were calculated to be 0.924, 0.994, 0.962, 0.985 and 0.973, respectively. These values indicated that not less than 92.40 % of the variability in the response could be explained by the models.

Table 3 Regression coefficients for ultrasonic extraction for monacolin K and citrinin contents, pigment intentsity, DPPH free radical scavenging ability and chelating ability on Fe^{2+}

Factor	Regression coefficient	Std. err	F-value	P-value
Monacolin K content				
Mean	346.20	11.91	17.37	< 0.00*
% ethanol (A)	-32.57	9.42	11.95	0.01*
Temperature (B)	-2.75	9.42	0.08	0.77
A^2	-78.32	10.1	66.24	< 0.00*
B^2	-34.90	10.1	14.75	< 0.00*
AB	-11.50	13.32	0.74	0.51
Pigment intensity				
Mean	4010	43.96	250.1	< 0.00*
% ethanol (A)	-489.10	34.76	197.98	< 0.00*
Temperature (B)	-6.86	34.76	0.03	0.84
A^2	-1111	37.27	888.3	< 0.00*
B^2	-552.75	37.27	219.88	< 0.00*
AB	-320.25	49.15	42.44	< 0.00*
DPPH free radical scavenging ability				
Mean	2.66	0.08	59.27	< 0.00*
% ethanol (A)	-0.44	0.06	48.54	< 0.00*
Temperature (B)	-0.16	0.06	6.75	0.07
A^2	-0.94	0.06	187.6	< 0.00*
B^2	-0.54	0.06	63.06	< 0.00*
AB	-0.35	0.09	14.95	< 0.00*
Chelating ability on Fe^{2+}				
Mean	95.89	1.33	94.65	< 0.00*
% ethanol (A)	-6.05	1.05	32.89	< 0.00*
Temperature (B)	-0.92	1.05	0.76	0.41
A^2	-20.39	1.13	325.04	< 0.00*
B^2	-14.21	1.13	157.8	< 0.00*
AB	-4.35	1.49	8.53	0.02*
Citrinin content				
Mean	12.18	0.40	51.57	< 0.00*
% ethanol (A)	-1.60	0.32	25.39	< 0.00*
Temperature (B)	-0.033	0.32	0.011	0.91
A^2	-4.67	0.34	188.14	< 0.00*
B^2	-2.69	0.34	62.36	< 0.00*
AB	-1.13	0.45	6.37	0.03

Note: * Significant factors $p \leq 0.05$

3.2 RSM of monacolin K content

Ethanol concentration has an effect on monacolin K content with a good regression coefficient (R^2 = 0.924) (Figure 1A). The relationship between monacolin K content and the extraction parameters is shown in Eq. (2) as follows:

$$\text{Monacolin K} = 346.20 - 32.57\ X_1 - 2.75\ X_2 - 78.32\ X_1^2 - 34.90\ X_2^2 - 11.50 X_1 X_2 \quad (2)$$

Ethanol concentration had significant effect (p<0.05) except temperature but also interaction effect between ethanol concentration and temperature affected significantly on monacolin K (p<0.05) (Table 3). Figure 1A illustrates response surface plot of ethanol concentration and temperature on monacolin K content. The optimal ethanol concentration for the extraction was in the range of 66.25–78.75 %. Out of this range, monacolin K contents were decreased. At 72.50% ethanol concentration, the temperature was increased from 45 to 55°C, higher monacolin K content was obtained before showing signs of decreasing. Therefore, high monacolin K content could be obtained through extraction at moderate temperature (40–55°C) with an ethanol concentration of 66.25–78.75%. The result of monacolin K content in this optimized extraction condition was much higher than those reported by Manzoni *et al*. (1999), Su *et al*. (2003) and Yim *et al*. (2012). Carvalho *et al*. (2007) reported that higher extract content was obtained as appropriate polarity, density and dielectric between solvents and solutes (Deng *et al*., 2010). The higher extraction temperature could improve the mass transfer resulting in the increased solubility of monacolin K and the decreased viscosity of the solvent (Deng *et al*., 2010). Moreover, ultrasonic wave increases more effective extraction for monacolin K since cavitation phenomenon generates the number of acoustic cavitation bubbles created by ultrasound (Yang *et al*., 2005). This indicates that UAE effectiveness is enough for destroying cell wall of the fungus so that leaked monacolin K is high from the cell, which resulting high monacolin K content as well.

The maximal monacolin K content predicted by response surface analysis (RSA) was 349.59 mg/kg dry weight with such the optimum ethanol concentration of 69.90% and an extraction temperature of 49.95°C.

3.3 RSM of DPPH• scavenging ability and Chelating ability on Fe^{2+}

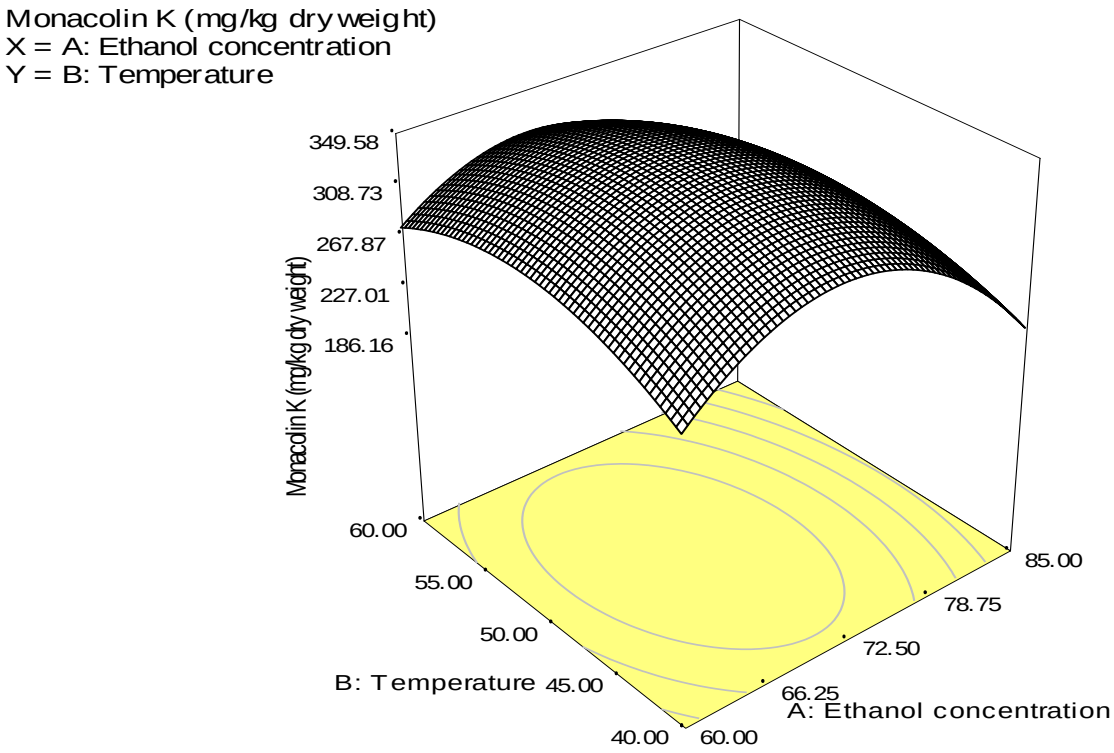
The relationships between DPPH• scavenging ability and extraction parameter (R^2 = 0.962) and between Chelating ability on Fe^{2+} and extraction parameter (R^2 = 0.985) are good regression coefficients, respectively (Figures 1B and 1C), and Eq. (3) and (4) show the relationships as follows:

$$\text{DPPH• scavenging ability} = 2.66 - 0.44\ X_1 - 0.16X_2 - 0.94\ X_1^2 - 0.54\ X_2^2 - 0.35\ X_1X_2 \quad (3)$$

$$\text{Chelating ability on } Fe^{2+} = 95.89 - 6.05X_1 - 0.92X_2 - 20.39\ X_1^2 - 14.21\ X_2^2 - 4.35X_1X_2 \quad (4)$$

Ethanol concentration showed significant effect except for temperature on DPPH• scavenging ability and chelating ability on Fe^{2+}, whereas significant interaction effects of both responses were observed between extraction time and temperature (p<0.05) (Table 3). Both responses had a similar pattern of response surface plot. Figures 1B and 1C show the response surface plots of the effect of ethanol concentration and temperature on DPPH• scavenging ability and Chelating ability on Fe^{2+}, respectively, indicating that both responses increased with the increase of ethanol concentration from 60 to 72.50%, but they decreased with ethanol concentration beyond 72.50% and temperature beyond 50°C. Likewise, it was noted that increasing extraction temperature from 40°C to 50°C affected higher both antioxidant activities of the extracts. From 50°C to 60°C, these responses fell slightly as increased extraction time. The increase in the working temperature favors extraction, enhancing both the solubility of solute and the diffusion coefficient, but beyond the antioxidants in MWC could be denatured (Yim *et al*., 2012). Compound stability may be affected due to chemical and enzymatic degradation or losses by thermal decomposition. This may be the main mechanism which causes less stability in DPPH• scavenging ability and Chelating ability on Fe^{2+} as reported by Kiassos *et al.* (2009). At this point, if ethanol concentration was suboptimal concentration, it would cause their activities reduced. In addition, the ultrasonic wave can efficiently destroy the cell wall of fungus so that important substances, i.e. monacolin K, α-aminobutyric acid (GABA) and phenolic compounds, can be better released from the cell, which affecting higher antioxidant activities. Yang *et al*. (2005) reported that cavitation mechanism obtained from ultrasonic wave has been used as a stimulated tool for an enhancement of monacolin K content. However, total phenolic and GABA contents were not determined in our study.

The optimal DPPH• scavenging ability predicted by RSA was 2.64 mmol Trolox/ml and with the ethanol concentration of 69.62% and an extraction temperature of 49.15°C; and 96.39% of chelating ability on Fe^{2+} was obtained by the ethanol concentration of 70.67% and extraction temperature of 49.90°C.



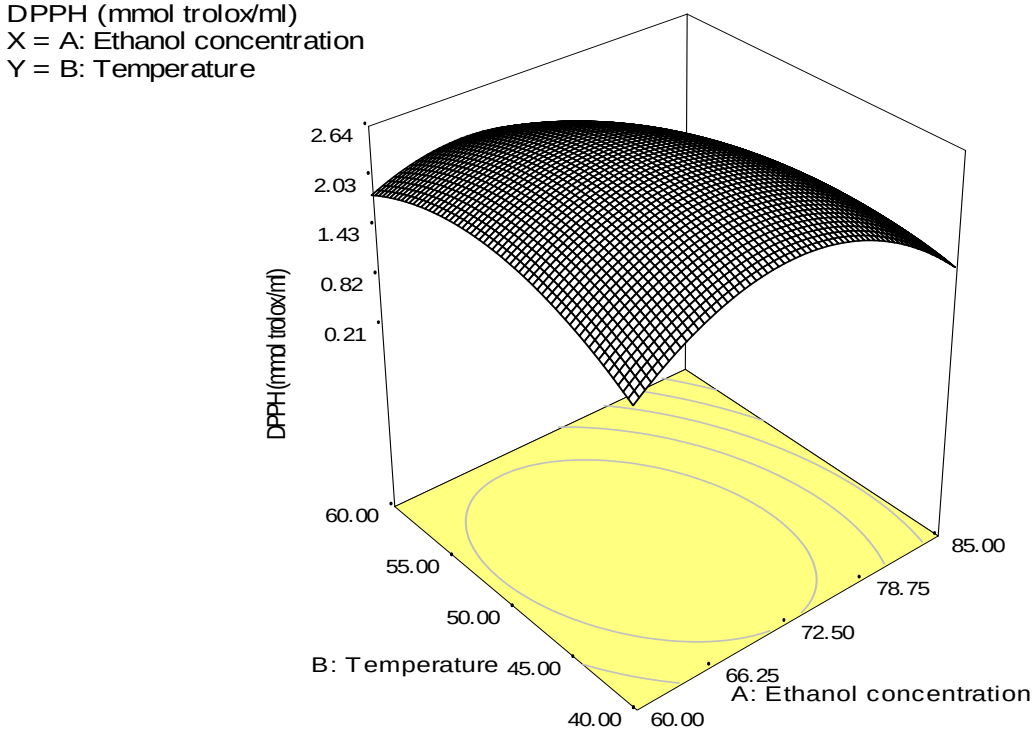


Figure 1 Response surface for extraction of parameters (A, monacolin K; B, DPPH•scavenging ability; C, chelating ability on Fe^{2}; D, pigment; E, citrinin) as a function of ethanol concentration (%) and temperature (^{o}C).

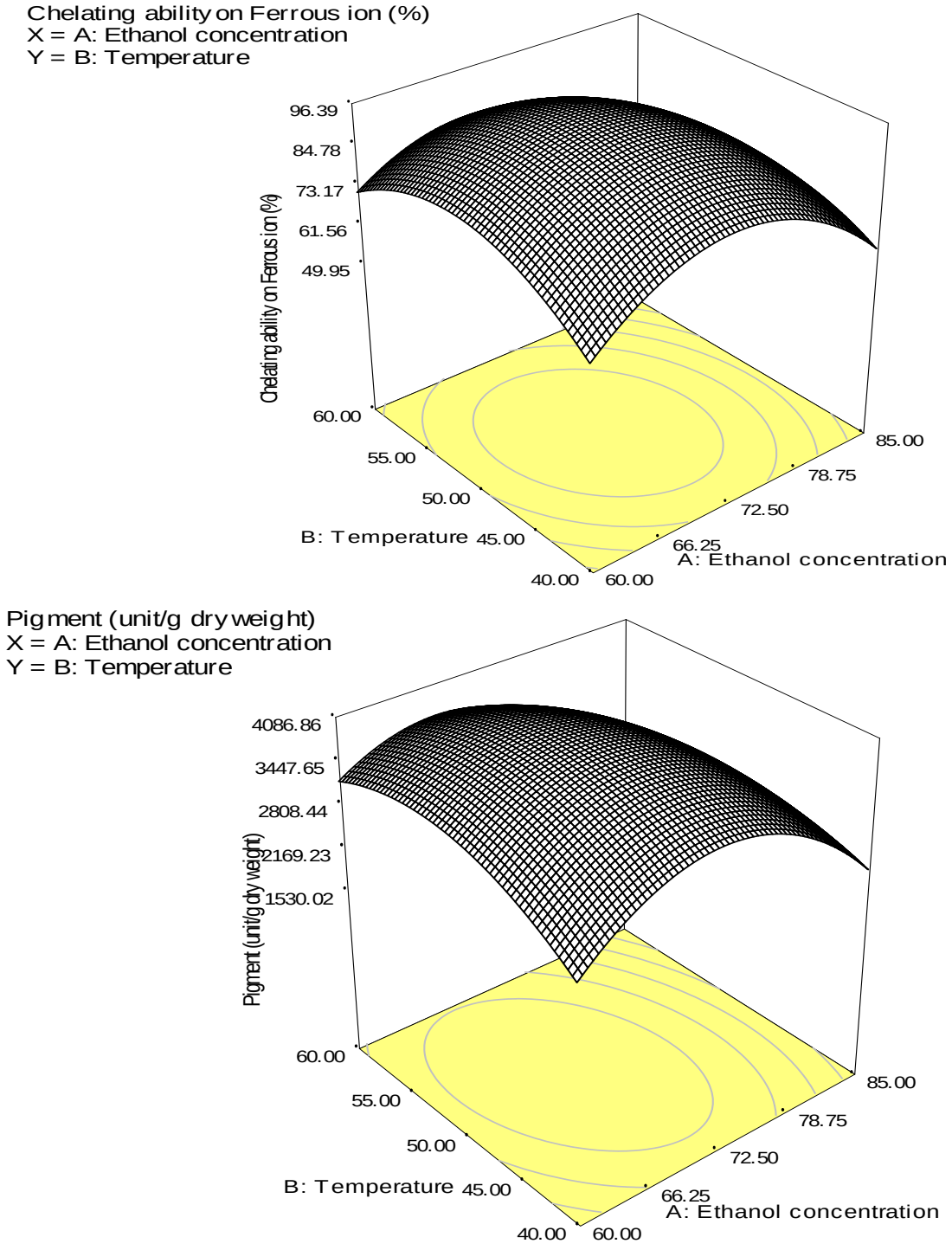


Figure 1 (Cont.) Response surface for extraction of parameters (A, monacolin K; B, DPPH•scavenging ability; C, chelating ability on Fe^{2}; D, pigment; E, citrinin) as a function of ethanol concentration (%) and temperature (^{o}C).

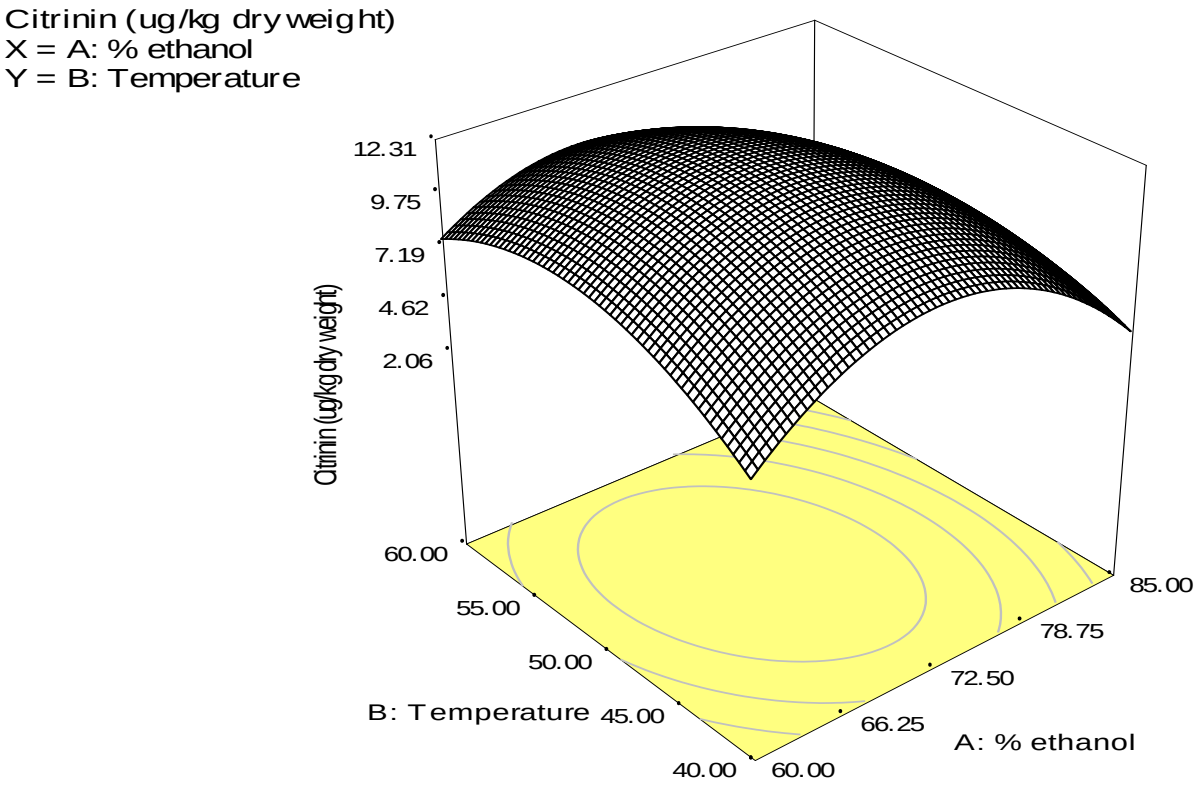


Figure 1 (Cont.) Response surface for extraction of parameters (A, monacolin K; B, DPPH° scavenging ability; C, chelating ability on Fe^{2}; D, pigment; E, citrinin) as a function of ethanol concentration (%) and temperature (°C).

3.4 RSM of pigment intensity

The relationship between pigment intensity and extraction parameter has a good regression coefficient (R^2 = 0.994) (Figure 1D) and Eq. (5) shows the relationship:

$$\text{Pigment} = 4{,}010.00 - 489.10\ X_1 - 6.86X_2 - 1{,}111.00\ X_1^2 - 552.75\ X_2^2 - 320.25\ X_1X_2 \quad (5)$$

Ethanol concentration had significant (p<0.05) but temperature did not affect on pigment intensity whereas significant interaction effect (p<0.05) was found between ethanol concentration and extraction temperature. Figure 1D shows response surface plot of pigment intensity which indicating that it was drastically increased with temperature 40°C–50°C temperature and 60.00 to 72.50% of ethanol concentration. At 72.50% of ethanol concentration and 50°C of extraction temperature, the highest pigment intensity was observed. From this standpoint, higher ethanol concentration and temperature supported more effective extraction. The results were disagreement with Carvalho *et al.* (2007), indicated maximum Monascus pigment obtained when using 60% ethanol as a solvent. However, differences in the polarity of

the extracting solvents could result in a wide variation in the pigment extraction (Carvalho *et al*., 2007).

The optimal extraction conditions were predicted to be ethanol concentration of 70.58% and temperature of 51.91°C. The optimal pigment extraction predicted by RSA was 4067.93 unit/g dry weight.

3.5 RSM of citrinin content

The coefficient of determination (R2) was calculated to be 0.973 for citrinin extraction (Figure 1E) and Eq. (6) showed the relationship between citrinin content and extraction parameters of ethanol concentration and temperature.

$$\text{Citrinincontent} = 12.18 - 1.60X_1 - 0.033X_2 - 4.67X_1^2 - 2.69X_2^2 - 1.13X_1X_2 \quad (6)$$

The significant effect was ethanol concentration but temperature was significant, and a significant interaction between ethanol concentration and the temperature was found (Table 3). Figure 1E shows response surface plot of citrinin content which gradually increased when extracted between 66.25% and 72.50% of ethanol concentration, and beyond 72.50% of ethanol concentration, their contents were descended. Extraction temperature between 40°C and 50°C led to increased citrinin content and a further increase of extraction temperature, it was slightly decreased since the applied temperatures were suboptimal. Therefore, to obtain low citrinin content, higher ethanol concentration and extraction temperature were suggested. The increased level of ethanol concentration and extraction temperature could enhance both the solubility of solute and the diffusion coefficient, but beyond a certain extend significant substances could be denatured (Yim *et al*., 2012).

The minimal citrinin content predicted by response surface analysis (RSA) was 2.06 µg/kg dry weight with the optimum ethanol concentration of 85.00% and an extraction temperature of 60°C.

3.6 Verification of predictive model

Yim *et al*. (2012) suggested that verification step was done to ensure that the predicted results were not biased towards the practical value with the objective of each response to obtain maximum yield using deduced optimal condition. Table 4 indicates five individual verification experiments for Y1 : Monacolin K content (mg/kg dry weight); Y2: DPPH• scavenging ability (mmol Trolox/ml); Y3: Chelating ability on Fe^{2+} (%); Y4: Pigment intensity (unit/g dry weight) and Y5: Citrinin content (µg/kg dry weight) were carried out under respective optimal extraction conditions within the experimental range. The experimental values of monacolin K content, DPPH• scavenging ability, Chelating on Fe^{2+}, pigment intensity and citrinin content were 348.72 mg/kg dry weight, 2.65 mmol Trolox/ml, 96.76%, 4,072.45 unit/g

dry weight and 2.04 µg/kg dry weight, respectively, which were very close to the value predicted by the regression models with the CV between 0.06 and 0.97%.

Table 4 Experimental data of the verification of predicted extraction parameters

Dependent variables[a]	Ethanol concentration (%)	Temperature (°C)	Predic value	Experimental value[b]	% Different (CV)
Y_1	69.91	49.95	349.59	348.72±5.23	0.25
Y_2	69.62	49.15	2.64	2.65±0.03	0.37
Y_3	70.67	49.90	96.39	96.76±7.76	0.38
Y_4	70.91	50.80	4,074.93	4,072.45±10.64	0.06
Y_5	85.00	60.00	2.06	2.04±0.18	0.97

Note: [a] Y_1 : Monacolin K content (mg/kg dry weight); Y_2: DPPH• scavenging ability (mmol Trolox/mL); Y_3 : Chelating ability on Fe^{2+} (%); Y_4: Pigment intensity (unit/g dry weight); Y_5: Citrinin content (µg/kg dry weight)

[b] Results were expressed as mean±standard deviation (n = 3).

4. Conclusion

Optimization of ultrasonic extraction conditions to MWC with a maximum of monacolin K content, antioxidant activity and pigment intensity, but minimal citrinin content was carried out and adequately performed by using RSM. The experimental values of the optimized extraction parameters were well related to the predicted values. This study indicates an insight on the extraction condition (ethanol concentration and temperature) optimized for the maximum levels of monacolin K, antioxidant activity and pigment intensity but low citrinin content of MWC. The highest antioxidant activities were obtained under these respective optimized ethanol concentration and temperature of 69.62% and 49.15°C (DPPH); 70.67% and 49.90°C (Chelating ability on Fe^{2+}), respectively. Besides, the maximal monacolin K content and the minimal citrinin content were based on optimal ethanol concentration and temperature of 69.91% and 49.95°C and 85% and 60°C, respectively.

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