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ORIGINAL RESEARCH PAPER

OPTIMIZING CONDITIONS FOR MELANIN EXTRACTION FROM BLACK TEA LEAVES

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Melanin is a natural pigment with great development potential as a healthful food colorant. In this study, extraction conditions of natural melanin from black tea leaves were investigated. Temperature, duration and liquid-solid ratio were chosen to optimize extraction conditions using response surface methodology (RSM). The Box-Behnken experimental results showed the optimum extraction conditions as follows: a temperature of 97.92 °C, a duration of 44.93 min and a liquid-solid ratio of 106.76 ml/g. Under these conditions, the maximal melanin yield was 271.40 mg/100 g. The results of this study showed that black tea leaves could be used as a good source of the new-type natural melanin.

Keywords: black tea leaf, melanin, extraction condition, optimization

Introduction

Frequent discovery of the harmful effects of synthetic pigments on human health has led to public interest in natural pigments as alternative in food industry, resulting in an increasing number of studies on natural colorants. Melanin is an irregular, black or brown polymer produced from the oxidative polymerization of tyrosine or indolic compounds (Riley, 1997). Melanins, derived from such biological sources as Black-bone silky fowl (Chen *et al*., 2008), *Osmanthus fragrans* seeds (Wang *et al*., 2006), *Auricularia auricula* fruit-bodies (Zou *et al*., 2013), and *Hypoxylon archeri* (Wu *et al*., 2008) of microorganisms, show similar physical and chemical properties. These physico-chemical properties include strong light absorbance, unusual solubility and remarkable redox properties. As is shown in previous studies, melanin has a number of healthful functions, such as antioxidation (Chiarelli-Neto *et al*., 2011; Tu *et al*., 2009) and anti-HIV activity (Manning *et al*., 2003; Montefiori and Zhou, 1991). These functions promise natural melanin with great development potential as a healthful food colorant.

Black tea is a Chinese traditional and famous food. Black tea leaves are rich in melanin and are increasingly popular as a black food in China. There have been reports about the research on the extraction of melanin, and melanin is considered to be one of the most important functional components in these black foods (Cheng *et al*., 2009). However, there is little information available in literature about the investigation of melanin extraction from black tea leaves using an effective statistical technique.

This paper aims to develop economical and efficient extraction of melanin from black tea leaves. Response surface methodology (RSM) was employed to optimize extraction conditions (temperature, duration and liquid-solid ratio) in order to obtain the maximum melanin yield.

Materials and methods

Materials and reagents

Black tea leaves were purchased from a local market in Dalian City (Liaoning Province, China), pulverized and sifted through a 60-mesh sieve. The powder was stored in dark bags to be kept from moisture and light. Synthetic melanin was purchased from Sigma-Aldrich Chemicals Co. (St. Louis, USA).

Extraction of melanin

The extraction process of melanin was carried out according to the method of Zou *et al*. (2010) with proper modification. Two grams of black tea leaves powder was washed with running water at a ratio of 60 ml/g (water/raw materials) for 5 min, followed by centrifugation at 4000 rpm for 5 min. The precipitate was immersed into water and the initial pH was adjusted to 12with1 M NaOH. Then, the mixture was put into a conical flask for incubation extraction. After that, the sample was centrifuged at 4000 rpm for 5 min and the supernatant containing melanin was obtained and stored at 4 °C in the dark.

Experimental design

The Box-Behnken experimental design with three factors and three levels was employed to optimize the extraction conditions in order to obtain the highest melanin yield. Temperature (*A*), duration (*B*) and liquid-solid ratio (*C*) were chosen as independent variables in this design. *A* (60, 80 and 100°C), *B* (20, 40 and 60min) and *C* (80, 100 and 120 ml/g) were determined as critical levels with significant effect on melanin extraction. The complete design consisted of seventeen combinations including five replicates of the center point (Table 1).

Table 1. Independent variables and their coded and actual values used for optimization

Independent variable	Symbol	Code levels		
		-1	0	+1
Temperature (°C)	*A*	60	80	100
Duration (min)	*B*	20	40	60
Liquid-solid ratio (ml/g)	*C*	80	100	120

The experimental results were analyzed by quadratic stepwise regression to fit the second-order equation (1):

$$Y = \beta_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{3} B_{ij} X_i X_j \quad (1)$$

where Y stands for melanin yield, X_1, X_2, X_3 for independent variables, β_0 for the model intercept and B_i, B_{ii}, B_{ij} for regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively. The software Design-Expert 7.0.0 Trial (State-Ease Inc., Minneapolis, USA) was used to obtain the coefficients of the quadratic polynomial model.

Determination of melanin

The determination of melanin was performed according to the method of Santos and Stephanopoulos (2008). Melanin extract was first adjusted to pH 2.0 with 3 M HCl to precipitate melanin, followed by centrifugation at 10000 rpm for 20 min and the supernatant was discarded. The precipitated melanin was washed with chloroform, ethyl acetate and ethanol, and was then dissolved in 0.01 M NaOH. The optical densities of solution containing melanin at 400 nm were determined against a blank (0.01 M NaOH) using a UV-2802 diode array spectrophotometer (UNIC, Princeton, USA). The melanin content (mg/100 g) was calculated according to the standard curve prepared from synthetic melanin.

Statistical analysis

The experimental results obtained were expressed as means ± SD of triplicates. Statistical analysis was performed using Fisher's F-test. P<0.05 was regarded as significant and P<0.01 as very significant.

Results and discussion

Analysis of Box-Behnken experiment

The extraction conditions including temperature, duration and liquid-solid ratio as independent variables were optimized for the maximum melanin yield. The Box-Behnken design and the corresponding response values were shown in Table 2.

By using the Design Expert software, a second-order polynomial model describing the correlation between melanin yield and the three variables in this study was obtained in equation (2) below:

$$Y = -531.41 + 1.59A + 10.54B + 7.79C - 0.03A^2 - 0.13B^2 - 0.05C^2 + 0.03AB + 0.04AC - 0.01BC \quad (2)$$

The statistical significance of equation (2) was checked by F-test, and the results of analysis of variance (ANOVA) were shown in Table 3. The model F-value of 6.54 obtained by ANOVA indicated that the model was significant (P<0.05). For the model fitted, the coefficient of determination (R^2) was 0.9324, implying that the sample variation of 93.24% for the melanin yield was attributed to the independent variables, and only 6.76% of the total variation could not be explained by the model. These results suggested that the developed model could adequately represent the real relationship among the parameters chosen.

Table 2. Box-Behnken design and experiment data for melanin extraction

Run	*A*: Temperature (°C)	*B*: Duration (min)	*C*: liquid-solid ratio (ml/g)	Melanin yield(mg/100 g)	
				Observed value	Predicted value
1	0 (80)	-1 (20)	1 (120)	145.82±11.18	151.71
2	0	-1	-1 (80)	135.60±10.24	139.86
3	0	1 (60)	1	179.45±11.17	175.14
4	-1 (60)	1	0 (100)	143.57±10.28	138.10
5	0	0 (40)	0	218.55±14.70	234.90
6	-1	0	-1	165.22±12.47	176.51
7	0	0	0	266.54±18.32	234.90
8	1 (100)	1	0	226.56±10.19	242.08
9	0	0	0	200.52±12.21	234.90
10	1	0	1	269.41±19.98	261.09
11	0	0	0	253.65±17.19	234.97
12	1	0	-1	240.80±20.98	231.14
13	0	0	0	236.07±14.24	234.90
14	-1	-1	0	139.54±10.08	123.92
15	-1	0	1	138.66±9.87	148.26
16	0	1	-1	191.28±15.43	185.29
17	1	-1	0	182.50±12.91	187.40

Effects of temperature, duration and liquid-solid ratio on melanin extraction

As was shown in Table 3, temperature had very significant linear effect (P<0.01) on melanin extraction; duration had very significant quadratic effect (P<0.01). However, none of the independent variables (temperature, duration and liquid-solid ratio) interacted significantly (P>0.05).

Figure 1 showed the effect of temperature and duration on melanin extraction from black tea leaves at a constant liquid-solid ratio of 100 ml/g. At a fixed duration, the melanin yield increased rapidly when temperature was raised. This indicated that extraction temperature was the principal effect on the melanin yield, which agreed with results reported by Wang *et al.* (2008). The melanin yield increased when duration was extended from 20 to 45 min but slowly decreased when duration continued to be extended. This phenomenon could be explained in terms of colorant degradation. The melanin might degrade due to long time treatment under high temperature condition (Tiwari *et al.*, 2009). Therefore, duration should not exceed 45 min in the present work.

Table 3. Analysis of variance (ANOVA) of the response surface regression model

Source	Sum of squares	Degree of freedom	Mean squares	*F*-value	*P*-value
Model	32102.06	9	3566.90	6.54	0.0112
A	14019.75	1	14019.75	25.34	0.0015
B	2370.16	1	2370.16	4.28	0.0772
C	1.44	1	1.44	0.01	0.9607
A^2	454.32	1	454.32	0.82	0.3949
B^2	11227.08	1	11227.08	20.30	0.0028
C^2	1728.71	1	1728.71	3.13	0.1204
AB	410.06	1	410.06	0.74	0.4178
AC	846.81	1	846.81	1.53	0.2559
BC	121.00	1	121.00	0.22	0.6542
Residual	3872.11	7	553.16		
Pure Error	2779.70	4	694.92		
Corrected Total	35974.17	16		$R^2 = 0.9324$	

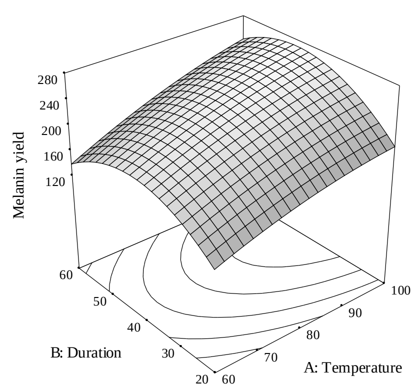

Figure 1. Response surface plot showing the effect of temperature and duration on melanin extraction from black tea leaves. The liquid-solid ratio was constant at 100 ml/g

Figure 2 showed the effect of temperature and liquid-solid ratio on melanin extraction from black tea leaves at a constant duration of 40 min. When the liquid-solid ratio was set, the melanin yield increased rapidly when temperature was raised, which also implied that the melanin yield was significantly influenced by temperature. At a fixed temperature, the variety of melanin yield was slight when the liquid-solid ratio increased, especially when the temperature exceeded 95°C. In addition, increasing extraction temperature might result in more solvent volatilization, more energy cost and more impurities extraction (Zhang and Liu, 2008). Therefore, the optimum extraction temperature should be about 95°C in the present study.

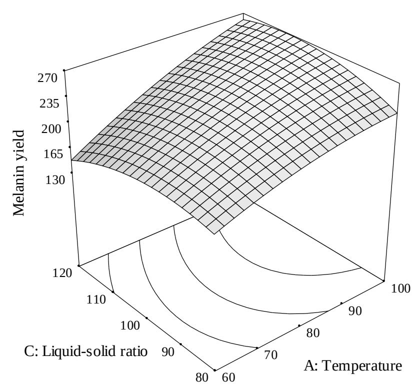

Figure 2. Response surface plot showing the effect of temperature and liquid-solid ratio on melanin extraction from black tea leaves. The duration was constant at 40 min

Figure 3 showed the effect of duration and liquid-solid ratio on melanin extraction from black tea leaves at a constant temperature of 80°C. The increase of both liquid-solid ratio and duration accelerated the extraction of melanin. However, above the optimal liquid-solid ratio (about 105 ml/g) and duration (about 45 min), the increase in liquid-solid ratio and duration would not further increase the melanin yield. This result was similar to those previously reported by Rodrigues *et al*. (2008). These findings make the whole process of melanin extraction economically more feasible and efficient in the potential application in food industry.

Optimization of extraction conditions and verification of model

According to the RSM test results, the optimal extraction conditions to obtain the highest melanin yield were determined as follows: a temperature of 97.92°C, a duration of 44.93 min and a liquid-solid ratio of 106.76 ml/g. The melanin yield was 271.40±13.24 mg/100 g, and this value was not significantly different (P>0.05) from the predicted value of 272.26 mg/100 g. These data proved that the model designed in this study was valid.

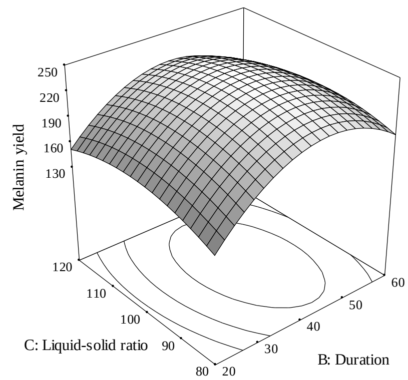

Figure 3. Response surface plot showing the effect of duration and liquid-solid ratio on melanin extraction from black tea leaves. The temperature was constant at 80°C

Conclusions

In this study, temperature, duration and liquid-solid ratio were chosen to optimize melanin extractions by a three variable, three level Box-Behnken experiment design. The combination of temperature (97.92°C), duration (44.93 min) and liquid-solid ratio (106.76 ml/g) was determined to obtain the highest melanin yield (271.40 mg/100 g). The experimental results showed that black tea leaves could be used as a good source of the new-type natural melanin.

Acknowledgments

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