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Optimization of enzyme-assisted extraction of anthocyanins from blackberry (*Rubus fruticosus* L.) juice using response surface methodology

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Preparations of pectic enzymes are used for more efficient extraction of desirable blackberry pigments, facilitating faster release. In this study, we validated the use of response surface methodology (RSM) for the optimization of enzymatic treatment for extraction of anthocyanins from blackberry juice. Tristimulus colorimetry was used to quantitatively and qualitatively evaluate the process. Our results showed that the optimal yield (639 g/L) of anthocyanins extracted from blackberries by this study's enzymatic mixture was obtained under the following conditions: enzyme loading 0.2% and 52°C for 1.1 h. The yield of anthocyanins showed high correlations with lightness (L*) (r = -0.833), chroma (C*) (r = 0.796) and hue angle (h) (r = 0.752), and was significantly affected by the extraction temperature (p = 0.0011).

Key words: Anthocyanins, blackberry, response surface methodology, optimization, pectic enzyme.

INTRODUCTION

Anthocyanins are found widely in higher plants in their roots, caudexes and leaves as well as in their flowers and fruits. They have been in high demand by the food industry as replacements for synthetic dyes due to legislative action against and consumer concerns about synthetic food additives (Francis, 1989; Fabre et al., 1993). Anthocyanins also possess pharmacological properties and are used by humans for therapeutic purposes. Increasing numbers of studies have shown that anthocyanins have beneficial effects in the context of myriad human diseases, including liver dysfunction, hypertension, vision disorders, microbial infections and diarrhea (Bors et al., 1998; Smith et al., 2000; Wang et al., 2000). Mechanical crushing of berries results in a highly viscous fruit puree from which it is difficult to directly extract juice by pressing. For this reason, the addition of pectinolytic enzyme preparations to the fruit pulp prior to pressing is a prerequisite to obtaining satisfactory juice yield and efficient use of the press in the industrial production of black currant juice and concentrates. Pectic enzymes that are used in the fruit juice industry and increasingly in wine manufacturing, originate largely from fungal sources, notably Aspergillus niger spp. (Grassin and Fauquembergue, 1996). These enzyme preparations are generally multicomponent since they contain various pectinolytic and other plant cell wall

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degrading enzymes. During the pressing of liquefied berry mash, the juice is separated from the skin and seeds. The resulting juice contains relatively high levels of phenolics with an intense dark-purple color from anthocyanins, but the press residue is still rich in anthocyanins and other phenolics (Landbo and Meyer, 2004).

Preparations of pectic enzymes are used for more efficient extraction of desirable fruit pigments and other phenol compounds bound in plant cells, accelerating their release. These preparations also shorten the time required for maceration, settling and filtration, resulting in quicker release of red grape pigments and aroma compounds (Meyer et al., 1998; Schieber et al., 2001; Capounova and Drdak, 2002; Muñoz et al., 2004).

Blackberries are a good source of anthocyanins, with a reported anthocyanin content ranging from 67 to 230 mg/100 g fresh weight (Sellappan et al., 2002; Benvenuti et al., 2004). Blackberry juice (like all fruit and vegetable juices) is considered an ingredient when used in foods as a colorant. Current studies on anthocyanins from blackberries are focused on their stability and antioxidant properties (Wang and Lin, 2000; Elisia et al., 2007; Wang and Xu, 2007). However, few details are available on extraction process parameters for anthocyanins from blackberries using pectic enzymes in the literature, suggesting that an optimization method is needed for the extraction process of anthoycanins from blackberries using pectic enzymes.

Response surface methodology (RSM) is an effective statistical technique for optimizing complex processes. It is widely used in optimizing process variables. The basic theoretical and fundamental aspects of RSM have been previously reviewed (Farooq et al., 1997; Chandrika and Fereidoon, 2005). Color is one of the most important attributes of natural colorants and can be mostly attributed to anthocyanin pigments. The application of colorimetric systems based on uniform (CIELUV and CIELAB) and non- (CIEXYZ) uniform color spaces is useful in the quantification and characterization of the color properties of pigments and foods. The correlation between some color parameters and pigment content in food has been evaluated in previous studies (Kammerer et al., 2004; Montes et al., 2005), but the relationship between the total anthocyanin content and the color parameters in blackberries have not been studied during the extraction process.

In this study, we investigated some of the potential main factors (enzyme loading, extraction temperature and time) that may be related to the extraction of anthocyanins from blackberries; the color properties of the anthocyanins extracts were established by tritimulus colorimetry. The aim of this study was to validate the use of RSM to optimize the process conditions for quantitative and qualitative (relative to color properties) extraction of anthocyanins from blackberries using a commercial pectic enzyme preparation.

MATERIALS AND METHODS

Blackberries were purchased from a local farm in Nanjing City and stored at -20°C until use. A commercial pectic enzyme preparation (Klerzyme-150) was purchased from Shanghai Chemical Reagent Co., Shanghai, China. This preparation is 1 to 10% liquid pectinase derived from *Aspergillus niger.*

Enzymatic treatment

Frozen blackberries were thawed at 25°C for 6 h, and then crushed for 30 s using a triturator (Model DS-1, Shanghai Specimen and Model Factory, Shanghai, China). Crushed berries were transferred to a 50 ml conical flask and pectic enzyme was added to the crushed berries with different enzyme loadings (0.0 to 0.5%, m/m). Mixtures were then put in a thermostatic water bath at specific temperatures (20 to 70°C) for varying periods of time (0 to 2.5 h). Afterwards, the mixtures were centrifuged at 4000 rpm for 15 min. The supernatant was collected and transferred into a 50 ml volumetric flask for the determination of anthocyanin yield. Twenty grams of each sample was used for each treatment condition.

Experimental design

First, the single factor experiment for extraction was performed analyzing the effect of three factors (enzyme loading, temperature and time) on the extraction of anthocyanins from blackberries. Then the optimization of extraction process parameters (temperature, enzyme loading and time) was performed using Box-Behnken design (Table 1) and a model of extraction efficiency incorporating the 3 process parameters was developed and validated. Finally, the composition of anthocyanins was detected in the blackberries by high performance liquid chromatography (HPLC).

RSM was used to determine the optimal conditions for anthocyanin extraction from blackberries using Klerzyme-150. Experimental design and statistical analyses were performed using Stat-Ease software (Design-Expert 6.0.10 Trial, Delaware, USA Echip, 1993). A three-level, three-factor Box-Behnken design was chosen to evaluate the combined effect of the three independent variables of enzyme loading, temperature and time, which were coded as A, B and C, respectively. The minimum and maximum values were set for extraction temperature at 40 and 60°C, extraction times of 0.5 and 1 h and enzyme loadings of 1 and 3% (m/m) (Table 1). The measured response values were anthocyanin pigment yield, L*, *C** and *h*. The complete design consisted of 17 combinations including five replicates of the center point (Table 3) (Myers and Montgomery, 2002). The response function (Y) was partitioned into linear, quadratic and interactive components:

$$Y = \beta_0 + \sum_{i=1}^{k} B_i X_1 + \sum_{i=1}^{k} B_{ii} X^2 + \sum_{i>j}^{k} B_{ij} X_i X_j ,$$

Where β_0 is defined as the constant, B_i the linear coefficient, B_{ii} the quadratic coefficient and B_{ij} the cross product coefficient. X_i and X_j were defined as the levels of the independent variables, while k equaled the number of tested factors (k = 3). Analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were

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

Independent variable	Units	Symbol	Code levels		
			-1	0	1
Enzyme loading	%	*A*	0.1	0.2	0.3
Temperature	°C	*B*	40	50	60
Time	h	*C*	0.5	1	1.5

Table 2. Effect of different conditions for extraction on anthocyanins yield.

Index	Effect of enzyme loading on blackberry anthocyanin yield (1 h, 40°C) [Enzyme loading (%, m/m)]					
	0	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
	376±18^{c}	413±11^{b}	452±12^{a}	461±11^{a}	462±15^{a}	428±10^{b}
	Effect of temperature on blackberry anthocyanin yield (2%, 1 h) [Temperature (°C)]					
	20	**30**	**40**	**50**	**60**	**70**
Anthocyanin yield (g/L)	442±16^{d}	496±11^{c}	535±17^{b}	568±12^{a}	541±15^{b}	542±18^{b}
	Effect of time on blackberry anthocyanin yield (2%, 50°C) [Time (h)]					
	0	**0.5**	**1**	**1.5**	**2**	**2.5**
	466±16^{c}	536±18^{b}	572±10^{a}	564±17^{a}	588±12^{a}	536±19^{b}

determined. The significances of all terms in the polynomial were statistically evaluated by computing the F-value at a probability (p) of 0.001, 0.01 or 0.05. The regression coefficients were then used to make statistical calculations to generate contour maps from the regression models.

Determination of anthocyanin yield

The quantification of total juice anthocyanin content was determined by pH-differential methods as previously described by Giusti and Wrolstad (2001). Total anthocyanins were calculated as cyanidin-3-glucoside according to the following equation:

Total anthocyanins (mg/L) = A × MW × DF × 1000 / (ε × 1)

Where A = (A_{510} – A_{700}) pH 1.0 – (A_{510} – A_{700}) pH 4.5, molecular weight (MW) = 449.2 g/mol for caynidin-3-glucoside, DF = dilution factor, 1 = pathlength in cm, ε = 26,900 (molar extinction coefficient) in L/mol/cm for caynidin-3-glucoside and 1000 = conversion from g to mg. All analyses were done in triplicate (n = 3).

Other anathlytes

Brix was measured at 25°C using an Abbe refractometor (Atago, Tokyo, Japan). The turbidity of juice was measured by a STZ-A24 turbidimeter (Guangming Turbidimeter Plant, Wuxi, China) and expressed in nephelometric turbidity units (NTU). Proteins concentrations were determined by the micro-Kjeldahl nitrogen method × 6.25 (AOAC, 1990). Titratable acidity and total sugar were determined according to the standard method (AOAC, 1990).

Color coordinates

The weighted-ordinated method (constant intervals, Δλ = 2 nm) was applied to obtain tristimulus values, using as references the CIE Standard Illuminant D_{65}, the CIE 1964 Standard Observer, and water as the reference blank. Following the most recent recommendations made by the CIE, CIELAB System (the variables related with psychometric color attributes: L*, *C** and *h* for color specification was applied (Cevallos-Casals and Cisneros-Zwvallos, 2004).

Statistics

All trials were carried out in triplicate and all data were reported as means ± standard deviation (SD). Statistical significance was evaluated using Student's t-test and *P* values < 0.05, 0.01 or 0.001 were considered significant.

RESULTS AND DISCUSSION

Effect of enzyme loading, temperature and extraction time on anthocyanin yield

The effect of enzyme loading on extraction of anthocyanins was shown in Table 2. Anthocyanin yield increased when enzyme loading was increased from 0 to 0.2% (m/m), but did not significantly increase when the ratio was higher than 0.2%. These data suggested that the solvent-solid ratio of 0.2% was the optimal ratio for

Table 3. Observed and predicted values of L*, C*, *h* and anthocyanin yield obtained by the Box-Behnken experiment.

No.	Independent variables			Dependent variables			
	Enzyme loading (%, m/m)	Temperature (°C)	Time (h)	Anthocyanin yield (g/L)	L*	C*	*h*
1	0.1(-1)	40(-1)	1.0(0)	542±11	31.00±0.18	31.24±0.05	10.93±0.12
2	0.1(-1)	60(1)	1.0(0)	589±10	30.47±0.10	32.34±0.07	12.98±0.07
3	0.3(1)	40(-1)	1.0(0)	511±11	31.34±0.10	30.88±0.07	9.53±0.13
4	0.3(1)	60(1)	1.0(0)	579±10	30.72±0.09	31.02±0.03	12.02±0.03
5	0.2(0)	40(-1)	0.5(-1)	529±18	31.28±0.07	31.14±0.11	10.60±0.09
6	0.2(0)	60(1)	0.5(-1)	585±17	31.21±0.14	31.33±0.09	10.27±0.01
7	0.2(-1)	40(0)	1.5(1)	575±16	31.00±0.04	31.37±0.07	8.87±0.18
8	0.2(1)	60(1)	1.5(1)	589±12	30.54±0.09	32.37±0.05	12.72±0.07
9	0.1(-1)	50(0)	0.5(-1)	585±10	30.37±0.11	32.54±0.06	13.03±0.06
10	0.3(0)	50(0)	0.5(-1)	595±11	31.31±0.06	32.35±0.18	13.92±0.05
11	0.1(0)	50(0)	1.5(1)	589±14	30.20±0.04	32.37±0.07	13.99±0.09
12	0.3(1)	50(1)	1.5(1)	595±14	30.36±0.07	32.31±0.01	13.49±0.14
13	0.2(0)	50(0)	1(0)	619±18	29.32±0.09	32.35±0.09	13.92±0.07
14	0.2(0)	50(0)	1(0)	623±15	29.49±0.07	32.88±0.15	13.85±0.08
15	0.2(0)	50(0)	1(0)	624±10	29.31±0.06	32.28±0.03	13.24±0.11
16	0.2(0)	50(0)	1(0)	627±17	29.35±0.15	32.99±0.04	14.03±0.15
17	0.2(0)	50(0)	1(0)	631±14	29.31±0.07	32.35±0.02	13.30±0.05

anthocyanin extraction for this case. Anthocyanin yield also increased with increasing temperatures from 20 to 50°C, but declined when the extraction temperature was above 50°C. These results indicated that 50°C was the optimal temperature for anthocyanin extraction from blackberries using Klerzyme-150 (Table 2). Table 2 showed the effect of different extraction times on anthocyanin yield: yield increased when the extraction time was extended from 0 to 1 h, but remained approximately the same when the time was extended from 1 to 2 h. Anthocyanin yield significantly declined when the extraction time was extended from 2 to 2.5 h, indicating that extraction times between 1 to 2 h were the optimal duration for anthocyanin extraction in this study.

Analysis of the Box-Behnken experiment

The results for each dependent variable and their coefficients of determination (R^2) were summarized in Tables 3 and 4. Statistical analyses indicated that the proposed model was adequate, possessing no significant lack of fit and with very satisfactory of the R^2 for all responses. The R^2 values for anthocyanin yield, L*, *C** and *h* were 0.955, 0.989, 0.883 and 0.986, respectively. Coefficient of variances (Table 4) for anthocyanin yield, L*, *C** and *h* were within the acceptable range. In general, a high coefficient of variance indicates that variation in the mean value is high and does not result in an adequate response model (Chandrika and Shahidi, 2005). The probability (*p*) values of all regression models were less than 0.05. The effects of enzyme loading, temperature and time on anthocyanin yield, L*, *C** and *h* were reported (Table 4) by the coefficient of the second-order polynomials. Response surface and contour plots were used to illustrate the effect of extraction temperature, extraction time and solid-liquid ratio on the responses. Response surfaces for anthocyanins yield were shown in Figures 1 to 3.

The effect of temperature and enzyme loading on anthocyanin yield was shown in Figure 1. When enzyme loading increased to approximately 0.2 %, temperature became a critical factor in improving anthocyanin yield. The fluctuation in temperature could lead to great differences in anthocyanin yield. It could be observed that the optimal temperature and enzyme loading for anthocyanins extraction were about 50°C and 0.2%. We consider that the dissolving of anthocyanins was inhibited when the temperature was lower than 50°C; and when the temperature was exorbitantly higher than 50°C, anthocyanins could be degraded and its structure could be rearranged (Chigurupati et al., 2002). When the optimal temperature was at about 50°C, the highest yield of anthocyanins could be achieved when the enzyme loading was about 0.2% and the yield slight decreased when the enzyme loading was higher than 0.2%. Considering the cost of enzyme loading, it was advisable that the enzyme loading should be set at 0.2%.

The effect of temperature and extraction time on anthocyanin yield was illustrated in Figure 2. When the

Table 4. Regression coefficients (R^2) and CV values for four dependent variables for anthocyanin extraction from blackberries.

Coefficient	Anthocyanin yield	L*	C*	*h*
β_0 (intercept)	636.20	23.96	32.57	13.67
Liner				
B_1	-3.12	0.21**	-0.24	-0.13
B_2	23.13**	-0.21**	0.15***	1.01**
B_3	6.75	-0.26***	-0.02	0.04
Quadratic				
B_{11}	-29.73**	0.54	-0.03	-0.27
B_{22}	51.23***	0.99***	-1.17	-2.04***
B_{33}	-15.47*	0.66***	-0.15	-1.02*
Cross product				
B_{12}	5.25	-0.02	-0.24	0.11
B_{13}	-1.00	-0.19*	0.03	-0.12
B_{23}	-10.50*	-0.10	-0.10	1.05**
R^2	0.955	0.989	0.883	0.936
CV	2.07	0.41	1.13	4.93
Probability (*p*)	0.0006***	<0.0001***	0.0145*	0.0021**

*Significant at 0.05; **significant at 0.01; ***significant at 0.001

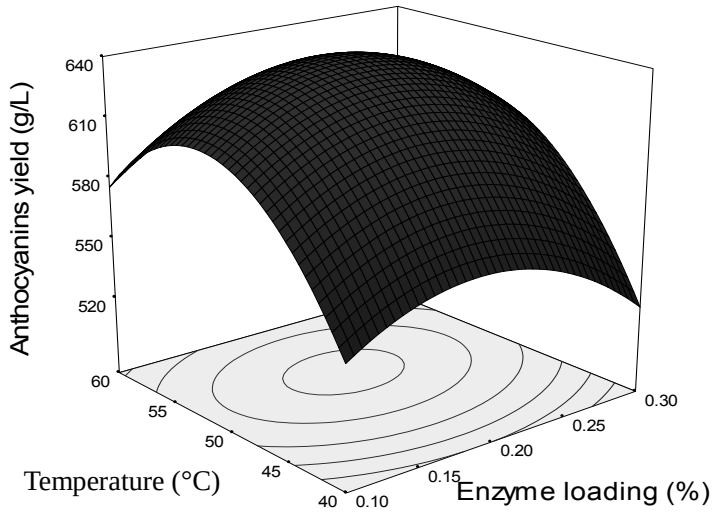

Figure 1. The effect of enzyme loading and temperature for enzyme extraction on anthocyanin yield.

extraction time reached approximately 1 h, temperature became the critical factor for improving anthocyanin yield. Fluctuation in temperature could lead to large differences in anthocyanin yield. It can be seen from Figure 2 that the optimal temperature and time for anthocyanin extraction was approximately 50°C and 1 h, respectively. If the extraction time was shorter than 1 h, the dissolution of anthocyanins did not reach equilibrium with the anthocyanins remaining in the blackberry. When the time was longer than 1 h, the anthocyanin yield slightly decreased. This may be due to the long time of exposure for dissolved anthocyanins to oxygen, light and microorganisms

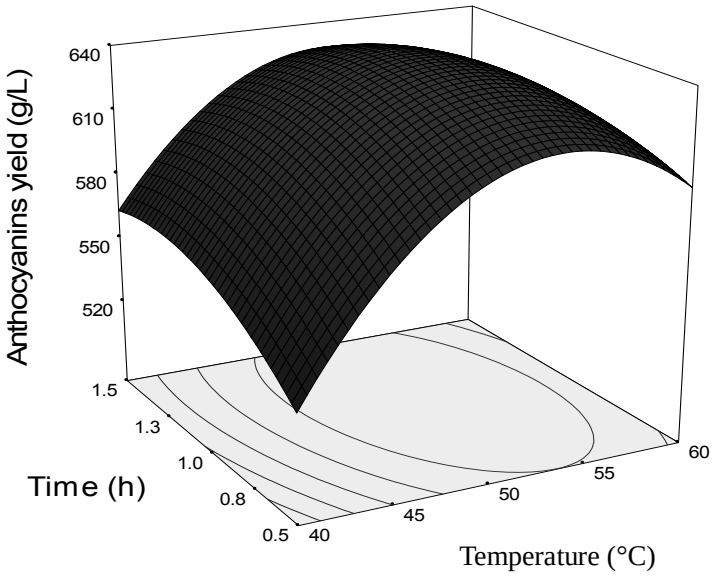

Figure 2. The effect of temperature and extraction time on anthocyanin yield.

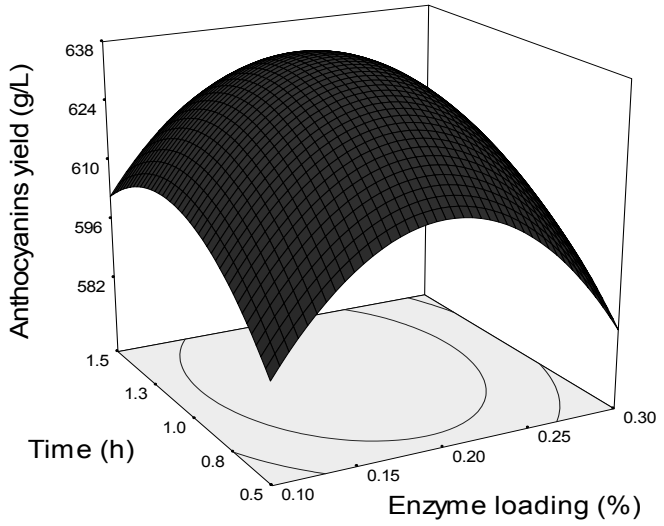

Figure 3. The effect of enzyme loading and extraction time on anthocyanin yield.

in the environment leading to increased chances of oxidation or degradation of dissolved anthocyanins (Chigurupati et al., 2002).

The effect of enzyme loading and time on anthocyanin yield was shown in Figure 3. Both enzyme loading and time had a significant, quadratic effect ($p < 0.05$) on anthocyanin yield. Yield increased when the enzyme loading was increased from 0.1 to 0.2%, but did not continue increasing when the enzyme loading was higher than 0.2%. The response surface shows the optimal conditions for the extraction process relative to the anthocyanin yield. It was observed that the optimal conditions for anthocyanin yield were slightly different. There were multiple combinations of variables that could give maximum levels of anthocyanin yield. Since the optimal response for each dependent variable did not fall in exactly the same region, all the response surfaces were superimposed. During anthocyanin extraction, the

Table 5. Design and results of confirmatory trials.

Trial	Enzyme loading (%, m/m)	Temperature (°C)	Time (h)	Anthocyanin yield (g/L)	
				Observed value	Predicted value
Optimal condition	0.2	52	1.1	620±10	639
Random condition 1	0.2	40	1.5	550±15	564
Random condition 2	0.2	60	1	590±12	608

parameters of enzyme loading, temperature and time are important. Therefore, the best combination of process variables for the response functions was found. The process variables resulting in the best combination of response functions were enzyme loading of 0.2% at a temperature of 52°C and a time of 1.1 h. The response functions were calculated from the final polynomial, resulting in a response of 639 g/L for anthocyanin yield, 29.32 for L*, 32.54 for *C** and 13.80 for *h*.

Correlations between anthocyanin yield and color parameters

Correlations between the yield of anthocyanins and color parameters were also explored in this study. A negative correlation (r = -0.833) was found between yield and L*, indicating that higher L* values correlated with lower yield of anthocyanins. Positive correlations were identified between the anthocyanin yield and chroma (*C**) (r = 0.796). This positive correlation indicated that high *C** values correlated with high anthocyanin yield. These results are in agreement with Montes et al. (2005) who evaluated the correlations between anthocyanin yield and *C** and L* in Jaboticaba fruit. Similarly, the correlation between yield and *h* was positive (r = 0.752).

Verification of the model

Within the scopes of the variables investigated in the Box-Behnken design, additional experiments were performed under different conditions for anthocyanins extraction to assess the validity of the model (Equation 1). The design and results of the confirmatory trials were shown in Table 5. It was demonstrated that there was a high degree of fit between the values observed in the experiment and the values predicted by Equation 1.

Conclusion

The use of pectolytic enzymes can give high yields of anthocyanins from blackberries. Testing different process conditions (enzyme loading, temperature and time) for the extraction of anthocyanins revealed that the extraction temperature significantly affected the yield of anthocyanins from blackberries processed with Klerzyme-150. The relationship of yield to extraction conditions can be modeled by second-order polynomials. The optimization of the extraction process using Klerzyme-150 was performed by RSM, and the optimal conditions for anthocyanin yield from blackberries were found to be an enzyme loading of 0.2% (m/m) at a temperature of 50C for a time of 1.1 h under these study conditions. The correlations between anthocyanin yield and the color parameters (L*) were relatively high (r = –0.833). The results of this study show the utility of RSM as a process optimization approach for extraction. Future studies are necessary to make broader conclusions about the optimization of different process parameters on anthocyanin extraction by testing and controlling for blackberries of different origins, and for different pectolytic enzyme mixtures with well-controlled purity, activity and composition.

Conflict of interest

Authors declare that they have no conflicts of interest.

REFERENCES

AOAC (1990). Official methods of analysis, 15th ed. Washington, DC: Association of Official Analytical Chemists.

Benvenuti S, Pellati F, Melegari M, Bertelli D (2004). Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of Rubus, Ribes, and Aronia. J. Food Sci. 69(3):164-169.

Bors W, Heller W, Michel C (1998). Flavonoids in Health and Disease. Marcel Dekker, New York, pp. 111-136.

Capounova D, Drdak M (2002). Comparison of some commercial pectic enzyme preparations applicable in wine technology. Czech. J. Food Sci. 20:131-134.

Cevallos-Casals BA, Cisneros-Zevallos L (2004). Stability of anthocyanin-based aqueous extracts of Andean purple corn and redfleshed sweet potato compared to synthetic and natural colorants. Food Chem. 86:69-77.

Chandrika LP, Fereidoon S (2005). Optimization of extraction of phenolic compounds from wheat using response surface methodology. Food Chem. 93:47-56.

Chigurupati N, Saiki L, Gayser C, Dash AK (2002). Evaluation of red cabbage dye as a potential natural color for pharmaceutical use. Int. J. Pharm. 241:293-299.

Elisia I, Hu C, Popovich DG, Kitts DD (2007). Antioxidant assessment of an anthocyanins-enriched blackberry extract. Food Chem. 101:1052-1058.

Fabre CE, Santerre AL, Loret MO, Baberian R, Pareilleux A, Goma G,

Blanc PJ (1993). Production and food applications of the red pigments of Monascus rubber. J. Food Sci. 58:1099-1111.
Farooq AM, Imran T, Khaled AS (2002). Response surface methodology: A neural network approach. Eur. J. Oper. Res. 101:65-73.
Francis F (1989). Food colourants: Anthocyanins. Crit. Rev. Food Sci. Nutr. 28:273-314.
Giusti MM, Worsltad RE (2001). Characterization and measurement of anthocyanins by UV–visible spectroscopy. Curr. Protoc. Food Analyt. Chem. F: F1:F1.2.
Grassin C, Fauquembergue P (1996). Fruit juices. Industrial Enzymology, 2nd edition, MacMaillan Press, London UK. pp. 225-264.
Kammerer D, Carle R, Schieber A (2004). Quantification of anthocyanins in black carrot extracts (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) and evaluation of their properties. Eur. Food Res. Technol. 219:479-486.
Landbo AK, Meyer AS (2004). Effect of different enzymatic maceration treatments on enhancement of anthocyanins and other phenolic in black currant juice. Innov. Food Sci. Emerg. Technol. 5:503-513.
Meyer AS, Jepsen SM, Sorensen SS (1998). Enzymatic release of antioxidants for human low-density lipoprotein from grape pomace. J. Agric. Food Chem. 46:2439-2446.
Montes C, Vicario IM, Raymundo M, Fett R, Heredia FJ (2005). Application of tristimulus colorimetry to optimize the extraction of anthocyanins from *Jaboticaba* (*Myricia jaboticaba* Berg.). Food Res. Int. 38:983–988.
Myers RH, Montgomery DC (2002). Response surface methodology: Process and product optimization using designed experiments. 3rd Edition. Wiley, New York. pp. 301-310.
Schieber A, Stintzing FC, Carle R (2001). By-products of plant food processing as a source of functional compounds-recent developments. Trends Food Sci. Technol. 13:401-413.
Sellappan S, Akoh CC, Krewer G (2002). Phenolic compounds and antioxidant capacity of Georgia-grow blueberries and blackberries. J. Agric. Food Chem. 50(8):2432-2438.
Smith M, Marley K, Seigler D, Singletary K, Meline B (2000) Bioactive properties of wild blueberry fruits. J. Food Sci. 65:352–356.
Wang C, Wang J, Lin W, Chu C, Chou F, Tseng T (2000). Protective effect of Hibiscus anthocyanins against tert-butyl hydroperoxide-induced hepatic toxicity in rats. Food Chem. Toxicol. 38(5):411–416.
Wang SY, Lin HS (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. J. Agric. Food Chem. 48:140–146.
Wang WD, Xu SY (2007). Degradation kinetics of anthocyanins in blackberry juice and concentration. J. Food Eng. 82:271-275.