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Enhancement of Stability and Antioxidant Activity of Mulberry Anthocyanins Through Succinic Acid Acylation

Running title: Acylation of Mulberry Anthocyanins

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SUMMARY

Research background. Anthocyanins possess valuable health-promoting activities with significant health benefits for humans. But, the instability of anthocyanins has been to limiting factor for its usage in functional foods and beverages.

Experimental approach. In this work, a new method to enhance the stability of anthocyanins from

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mulberry fruit through acylation by using succinic acid as a selected acyl donor was explored. The Box-Behnken design (BBD) of response surface methodology was applied to determine the optimized condition of the acylation processes.

Results and conclusions. The highest acylation conversion rate was 79.04 % under conditions of anthocyanins to succinic acid mass ratio of 1:8.96, acylation time duration of 3 h and acylation temperature of 50 °C. Structural analysis of acylated anthocyanins revealed that succinic acid introduces a C-O-C bond and hydroxyl group. The thermostability and light resistivity of mulberry anthocyanins scored significant improvement after acylation, and the antioxidant activities in terms of total reducing power and Fe^{2+}-chelating capacity of the acylated anthocyanins were also enhanced.

Novelty and scientific contribution. The increased stability and antioxidant abilities of anthocyanins as evidenced through succinic acid acylation, provides a novel method for stabilizing mulberry anthocyanins, facilitating its use in the food and nutraceutical industries.

Keywords: mulberry fruit; anthocyanins; acylation; stabilities; antioxidant activity

INTRODUCTION

Mulberry are rich dietary source of different nutrient composition, such as alkaloids, flavonoids and polyphenols (*1*). Anthocyanins, one of a flavonoid, possess valuable health-promoting activities with significant health benefits for humans (*2*), and has been ascribed with prevention of heart disease, antioxidant, antibacterial, anti-inflammatory and anticancer activities (*3-5*). Anthocyanins, with a C6-C3-C6 skeleton structure, generally occur in plants as glycosides and acyl-glycosides of anthocyanidins (aglycone), and differ from one another in the position of substitution of hydroxyl and methoxy groups in the β-ring (*6*). HPLC/ESI/MS analysis of mulberry fruits revealed the presence of four anthocyanins recognized as cyanidin 3-*O*-glucoside (C3G), pelargonidin 3-*O*-glucoside (P3G), cyanidin 3-*O*-rutinoside (C3R), and pelargonidin 3-*O*-rutinoside (P3R) (*7*). C3G and C3R are the major anthocyanins detected in mulberry fruit (*8*).

However, anthocyanins are extremely unstable and their composition is influenced by several factors during processing. Temperature plays an important role in affecting the stability of anthocyanins. It has been suggested that anthocyanins would exist in the chalcone structure. The anthocyanin's become slightly unstable and turns colorless when the temperature rises to 60 °C (*9*).

Another important factor that has been observed to have a strong influence on the stability of anthocyanin is pH value. Rein and Heinonen (*10*) demonstrated that there were four forms of anthocyanin aqueous solution interconversion and different pH values leading to the existence of different colors. Besides these, light (*11*), metallic ions (*12*), the content of sugar (*13*), and hydrogen peroxide (*14*) are also found to affect the stability of anthocyanins.

The relatively poor chemical stability of anthocyanins *in vitro* as well as in *vivo* (in plant cells or in the digestive tracts of animals) conditions has been a critical drawback and primary barrier to limit the wide and effective applications, raising up concerns on the importance of reducing the degradation of anthocyanins (*15,16*). To improve anthocyanins stability, several modification methods have been studied, including glycosyl acylation (*15*), glycosylation (*16*), microencapsulation (*17*), metal chelation (*18*), liposomes (*19*), and Fe_3O_4 magnetic nanoparticle (*20*). From the nutritional viewpoint, acylated anthocyanins have been reported to possess antioxidant activity (*21*). Studies have shown that the presence of anthocyanins recognized is different from plant to plant. To date, no information is available in the literature on the acylation of anthocyanins from mulberry fruit.

To predict the quality changes of anthocyanin during storage and processing, an acyl donor was selected for the acylation of mulberry anthocyanins in this study. A Box–Behnken design of response surface methodology was conducted for optimization of the acylation conditions. The thermostability and light resistivity of acylated anthocyanins were evaluated, and the antioxidant activity effects of acylated anthocyanins were demonstrated *in vitro*.

MATERIALS AND METHODS

Materials

Mulberry fruit collected from the plantation of the National Mulberry Orchard (Zhenjiang, China) were freeze-dried (EYELA FDU-2100, Tokyo Rikakikai Co., Ltd. Tokyo, Japan) and ground to powder.

The cyanidin 3-*O*-glucoside standard was purchased from Putian Genesis Biotechnology Co., Ltd (Beijing, China). Succinic acid, L-malic acid, oxalic acid, pyridine, formic acid, and acetonitrile were purchased from Sangon Biotech Co., Ltd (Shanghai, China). All other reagents used were of

analytical grade.

Mulberry anthocyanins extraction

Mulberry anthocyanins were extracted using acidified ethanol (ethanol and 1.0 N HCl, 85:15, V/V, pH=1), assisted by ultrasound (*22*). The partially purified extracts by active macroporous resin D-101 were evaporated to dryness at 50 °C using a rotating evaporator, and were re-dissolved in acidified ethanol. Individual anthocyanins were separated, dried by freeze-drier (EYELA FDU-2100), and quantified using high-performance liquid chromatography (HPLC) system (Agilent Technologies Inc., CA, USA). Anthocyanins structure was determined by Fourier Transform Infrared Transmission spectroscopy (FTIR, Varian Medical Systems, CA, USA) for scanning in the absorption spectrum range.

Preparation of glycosyl acylation anthocyanin

Acyl donors, including malic acid, succinic acid and oxalic acid were selected for anthocyanin acylation by the method of Xu *et al.* (*23*). The reaction system was mixed with 2 mL pyridine as catalyst and 5 mg dried mulberry anthocyanins in 40 mL of 50 % ethanol solvent, and incubated at 50 °C for 4 h. Acylated anthocyanins were then evaporated at 40 °C, using a rotating evaporator (EYELA N1001, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to remove the pyridine and ethanol, and dried using a vacuum freeze dryer.

Optimization of anthocyanin acylation conditions

The following three-step procedure was used to optimize the anthocyanin acylation indices: 1) Plackett-Burman (PB) design, to screen the 4 most influential factors that are known to affect anthocyanin acylation; 2) Single-factor, the acyl donors (malic acid, succinic acid and oxalic acid), the ratio of anthocyanin to donor (1:4, 1:8, 1:12, 1:16, and 1:20 mg/g), temperature (30, 40, 50, 60, and 70 ℃) and reaction time (3.0, 3.5, 4.0, 4.5, and 5.0 h) were screened to determine the level of each factor that significantly improves acylated anthocyanins production; and 3) Box-Behnken design, for

optimizing the conditions for anthocyanin acylation using response surface methodology. Three virtual variables were used to estimate standard error. Based on the results obtained using the PB design, the 3 most influential factors that cover a wide range were selected to estimate variation in anthocyanin acylation (*22*).

Structural characterization of anthocyanins

The absorption spectra were recorded using a UV spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan), scanning the spectrum range from 200 to 600 nm. The acylated anthocyanins were separated by their absorption peaks. Characterization of the structures of the acylated anthocyanins was achieved using a FTIR, by scanning the infrared absorption spectrum range from 4000 to 400 cm^{-1}.

Calculation of conversion yield

The acylated anthocyanin conversion yield was calculated as previously described as following (*24*).

$$C = \frac{A1}{A1+A0} \times 100 \qquad /1/$$

where *C*, is the acylated anthocyanin conversion yield (%); A_0, is the nonacylated anthocyanin peak area; and A_1, is the acylated anthocyanin peak area.

Stability tests of mulberry anthocyanins

Thermostability test

The thermostability of acylated anthocyanins was evaluated using 0.6 mg/mL of purified anthocyanins dissolved in 0.01 % HCl solution (pH=1). The effect of temperature on anthocyanins content was investigated in a water bath at 70, 80, and 90 °C for 10 h under the dark condition of each treatment. Anthocyanin content was determined using a UV-2450 UV/vis spectrophotometer, by measuring absorbance at 520 nm. Anthocyanin retention rate (*R*, %) was calculated as follows (*25*):

$$R = (A_t/A_0) \times 100 \qquad /2/$$

where A_0 represents absorbance at the start of heating (t = 0) and A_t, represents absorbance at time t.

Light resistivity test

To evaluate the light stability of the acylated anthocyanins, the anthocyanins solution as above (0.6 mL) was placed in open glass cuvettes under 450 W light at 20 °C. After exposure to the light for 2, 4, 6, 8, 10, and 12 day, the anthocyanins retention rate was determined as above.

Antioxidant activities

Antioxidant activity was represented by the DPPH· free radical scavenging capacity, total reducing power, and ferrous ion (Fe^{2+}) chelating capacity, assayed according to Wu *et al.* (*26*).

DPPH free radical scavenging capacity

Two mL of freshly prepared DPPH solution (1 mmol/L) was added into the anthocyanin extracts. The mixture was kept in the dark at 25 °C for 30 min and sample absorbance was measured at 517 nm with spectrophotometer (V-1800; Shimadzu Corp., Tokyo, Japan), using Trolox as the reference.

Ferrous ion (Fe^{2+}) chelating capacity

Two mM $FeCl_2$ solution (0.05 mL) and 5 mM ferrozine solution (0.1 mL) were added to 3 mL of anthocyanins extracts. The mixture was then shaken vigorously and incubated at room temperature for 10 min, and sample absorbance was measured at 562 nm with spectrophotometer (V-1800; Shimadzu Corp., Tokyo, Japan).

Total reducing power

Two mL of anthocyanins extracts was added to 2 μL of 1 % potassium ferricyanide. After mixed and incubated at 50 °C for 20 min, 2 mL of trichloroacetic acid was added. 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$. After incubating the mixture for 10 min,

sample absorbance was measured at 700 nm with spectrophotometer (V-1800; Shimadzu Corp., Tokyo, Japan).

Statistical analysis

All of the analyses in the present study were replicated thrice, and the results are expressed as mean values. Design-Expert v. 8.6 and GraphPad Prism v. 7 (*27*) were used for experimental design and data analysis.

RESULTS AND DISCUSSION

Spectral analysis of acylated and nonacylated anthocyanin

The acylated and nonacylated anthocyanins were detected by HPLC at 4.032 min and 15.465 min, respectively (Fig. 1). The conversion yield of acylated anthocyanin was calculated according to the peak area.

The values, 4.032 and 15.465 indicate the time of detection of acylated and nonacylated anthocyanins, respectively.

To examine changes in the molecular structure, UV-visible spectrophotometry was used to scan the visible range from 200 to 600 nm. Fig. 2a shows that there are absorption peaks at 321 nm and 295 nm for nonacylated anthocyanins and acylated anthocyanins, respectively, indicating that the structure of acylated anthocyanins is significantly different. The increase in the absorbance of acylated anthocyanins indicates that the acyl group in anthocyanins has been induced by succinic acid. It may be assumed that the acylation of anthocyanins was realized by introducing the acyl group whereas, the methylation of anthocyanins was realized by introducing the methyl group (*28*).

Infrared spectrometry was performed by scanning in absorption spectrum range from 4000 to 400 cm^{-1} (Fig. 2b). The absorption peaks of nonacylated and acylated anthocyanins differed significantly in location with fluctuations in three regions. The strongest absorption peak is located in the first region from 3271 to 3328 cm^{-1}. It corresponded to hydroxide radical stretching vibration peaks of aromatic ring and glycosyl in the structure. The absorption peaks at 1633 and 1438 cm^{-1}

corresponded to the aromatic and heterocyclic ring's skeletal vibration in the chromone of anthocyanins. The stretching vibration of acylated anthocyanins is enhanced relative to that of nonacylated anthocyanins. The third region is located from 1438 to 1043 cm^{-1}, which is the characteristic absorption peak region in acylated anthocyanins. The stretching vibration region of the C-O-C group is located at 1250 cm^{-1}, proving that acylation with succinic acid introduced the C-O-C and hydroxyl group.

In the case of methylation, the stretching vibration region of the C-H bond is located at 1395 cm^{-1}, proving that it introduced the C-H bond and methyl group (*28*). Giusti *et al*. (*29*) reported that anthocyanin stability was enhanced significantly through hydrophobic and "π−π" interactions. It could be concluded that based on the modification method, appropriate changes are induced on the relative chemical group leading to enhanced anthocyanin stability.

Optimization of anthocyanin acylation

Based on a previous study (*30*), certain extents of potential factors were performed to confirm the influencing internal tendency. The single-factor tests to assess anthocyanin conversion rate as depicted in Table 1 have shown that the acyl donor is succinic acid, anthocyanin to donor mass ratio is 1:8, acylation time is 3.5 h, and acylation temperature is 40 °C.

Response surface analysis was adapted to optimize the process conditions of anthocyanin acylation. The results of the regression analysis are shown in Table 2. Using multiple regression analysis, the results were fitted to a second-order polynomial equation. The regression equation between *Y* and A, B and C was established as follows:

$$Y=72.6-2.55A+2.05B+3.35C-5.60AB+8.90AC-15.90BC-24.85A^2-2.65B^2-9.55C^2 \quad /3/$$

To understand the significance of the linear relationship between the response and independent variables, ANOVA was done for the regression response surface model. Table 2 shows that the model accounted is for 94.56 % of the variability in the response, indicating that it is a good fit. The non-significance of the lack-of-fit parameter shows that the quadratic regression equation is a good estimator of the response. The "Prob>F" value was less than 0.001 (Table 2), indicating that the model

is significant. The model calibration coefficient value, R=0.9456, indicates that the model explains 94.56 % of the variability in the response. This suggests that the most critical independent factors were the mass ratio (a), acylation time (b), and acylation temperature (c), in that order.

To determine the optimal acylation conditions, graphs were plotted to compare the influential parameters (Fig. 3). The response surface graphs, each relating two out of the three factors, consist of ellipses with one center, regardless of their orientation; the edges of the surfaces formed vaults with peaks. The optimum acylation conditions using succinic acid were as follows: anthocyanins to succinic acid mass ratio 1:8.96, acylation time 3 h, acylation temperature 50 °C. Under these conditions, the predicted conversion yield was 79.04 %, and the validation value 80.7 %, indicating its high reliability.

Thermostability of acylated anthocyanins

Thermostability of acylated anthocyanins was evaluated following storage at different temperatures for 10 h in darkness. The absorbance of nonacylated anthocyanins from 460–560 nm at 70 (Fig. 4a), 80 (Fig. 4b), and 90 °C (Fig. 4c) showed the same trend of acylated anthocyanins lower than that of nonacylated anthocyanins. The retention rates of acylated anthocyanins were 98.41 %, 84.25 %, and 44.10 %, and those of nonacylated anthocyanins were 87.49 %, 72.15 %, and 27.44 %, respectively at 70, 80 and 90 °C (Fig. 4d). This indicates that the acylated anthocyanins were relatively more stable than nonacylated anthocyanins at high temperatures, and that mulberry fruit anthocyanins can be preserved well at these temperatures.

Temperature and light degrade anthocyanin stability significantly and reduce biological activities (*31*). Thermal treatment is believed to break down anthocyanins or their conjugated sugars into small molecules like aldehydes, benzoic acid derivatives or synonymous anthocyanidins (*32,33*). In this study, the anthocyanin retention rate decreased sharply from 87.49 % at 70 °C to 27.44 % at 90 °C after 10 h. However, the retention rate of acylated anthocyanins decreased from 98.41 % at 70 °C to 44.10 % at 90 °C. These results are consistent with those of previous studies (*16,24,34*).

Light resistivity of acylated anthocyanins

To investigate the effect of acylation on the light resistivity of acylated anthocyanins, anthocyanin retention rate was measured after exposure to 400 W light for 2, 4, 6, 8, 10, and 12 d at 20 °C. The absorbance of anthocyanins (Fig. 5a) and acylated anthocyanins (Fig. 5b) from 460–560 nm were observed to significantly decrease in proportion to duration of exposure under 400 W light. The anthocyanin retention rate decreased steeply from 76.13 % to 65.97 % over 10 day (Fig. 5c). However, the acylated anthocyanin retention rate was 80.10 % for the 4 day period and 77.21 % for 10 day period, without significant difference. It indicates that the light resistivity of acylated anthocyanins is much better than that of methylated anthocyanins (*16*).

In terms of light resistivity, acylated anthocyanins synthesized by lipase-catalyzed transesterification were more stable than their non-acylated glucosides under illumination with white fluorescent light (*35*). Several studies have reported the potential ability of acyl groups to donate electrons to anthocyanins (*36,37*), enhancing the stability of acylated anthocyanins under light irradiation.

Antioxidant activities

The antioxidant properties that were assessed *in vitro* were DPPH radical scavenging, total reducing power and Fe^{2+}-chelating capacity (Fig. 6). Nonacylated and acylated anthocyanins exhibited these properties in a concentration-dependent manner. DPPH radical scavenging activity was similar in the two groups, without significant differences (Fig. 6a). However, total reducing power and Fe^{2+}-chelating capacity were detected as increasing rapidly with increasing concentration and being significantly higher in acylated anthocyanins. At 0.8 mg/mL Fe^{2+}, nonacylated and acylated anthocyanins reached levels of 62.2 % and 70.5 %, respectively (Fig. 6c).

Anthocyanin acylation using succinic acid as a donor significantly enhanced the retention rate of anthocyanins at a certain degree of temperature and light conditions, and improved their antioxidant activities in terms of total reducing power and Fe^{2+}-chelating capacity. The present result is in line with those of our previous reports (*16*). However, the exact mechanisms whereby acylation enhances

anthocyanin stability need to be explored further.

CONCLUSIONS

In this study, succinic acid was selected as an acyl donor for anthocyanin acylation, to improve anthocyanin stability. The optimized acylation conditions were as follows: anthocyanin to succinic acid mass ratio of 1:8.96, acylation time of 3 h, and acylation temperature of 50 °C. The acylation of anthocyanins by succinic acid may be attributed to the formation of a C-O-C bond and hydroxyl group. Our thermostability and light resistivity results show that acylation significantly enhances the preservation rate of anthocyanins, and improves their antioxidant activity. In the case of the light resistivity, the acylation process offered better results than the methylation for anthocyanins. Thus, acylation of anthocyanins may be a novel method for stabilizing anthocyanins in mulberry fruit, and to enable their use as a commercial food ingredient.

CONFLICT OF INTEREST

Authors state no conflict of interests with respect to the objective, interpretation and presentation of the results in this study.

AUTHORS' CONTRIBUTION

Bei Zhang, Xizhi Jiang, and Zhongzheng Gui conceived and designed the experiments. Bei Zhang, Xizhi Jiang, Gaiqun Huang, and Xiangdong Xin performed the experiments. Bei Zhang, Thomas Attaribo, Yueyue Zhang, and Ning Zhang analyzed the data. Zhongzheng Gui contributed to writing of the manuscript and approved the final manuscript.

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Table 1. Selection of potential explanatory factors for anthocyanin acylation

Acyl donors	Conversion rate/%	Mass ratio	Conversion rate/%
Succinic acid	71.53 ± 3.421*	1:4	60.00 ± 4.034
Malic acid	65.00 ± 2.066	1:8	71.57 ± 3.859*
Oxalic acid	23.50 ± 2.905	1:12	54.97 ± 4.565
		1:16	45.60 ± 3.804
		1:20	39.73 ± 3.400
Acylation temperature/°C	Conversion rate/%	Acylation time/h	Conversion rate/%
30	64.37 ± 3.800	3.0	64.47 ± 3.232
40	70.43 ± 2.359*	3.5	72.80 ± 2.551*
50	62.87 ± 2.631	4.0	62.70 ± 2.352
60	57.47 ± 2.386	4.5	59.73 ± 3.272
70	48.43 ± 3.009	5.0	52.57 ± 2.802

The conversion ratio of anthocyanin to acyl donor mass ratio, acylation temperature and acylation time were presented under succinic acid as the donor

Table 2. ANOVA results for the regression response surface model

Source	Sum of Squares	df	Mean Square	F Value	P Value Prob>F
Model	4814.92	9	534.99	13.53	0.0012**
A	52.02	1	52.02	1.32	0.2891
B	33.62	1	33.62	0.85	0.3872
C	89.78	1	89.78	2.27	0.1756
AB	125.44	1	125.44	3.17	0.1181
AC	316.84	1	316.84	8.01	0.0254*
BC	1011.24	1	1011.24	25.58	0.0015**
A^2	2600.09	1	2600.09	65.76	0.0001**
B^2	29.57	1	29.57	0.75	0.4158
C^2	384.01	1	384.01	9.71	0.0169*
Residual	276.78	7	39.54		
Lack of Fit	188.22	3	62.74	2.83	0.1701
Pure Error	88.56	4	22.14		
Cor Total	5091.70	16			
R^2	0.9456				
Adj-R^2	0.8758				

*P<0.05; **P<0.01

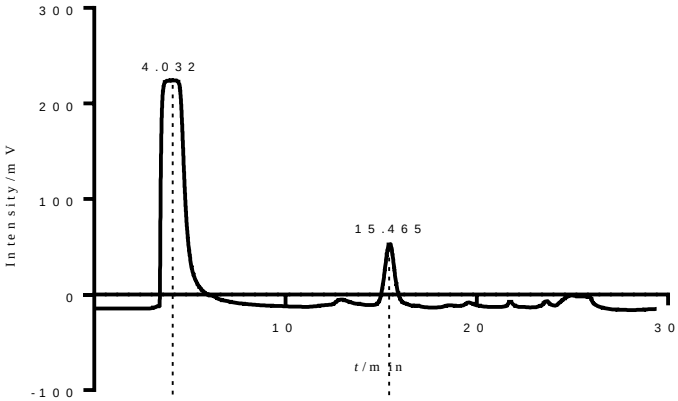


Fig. 1. HPLC analysis of acylated and nonacylated anthocyanins

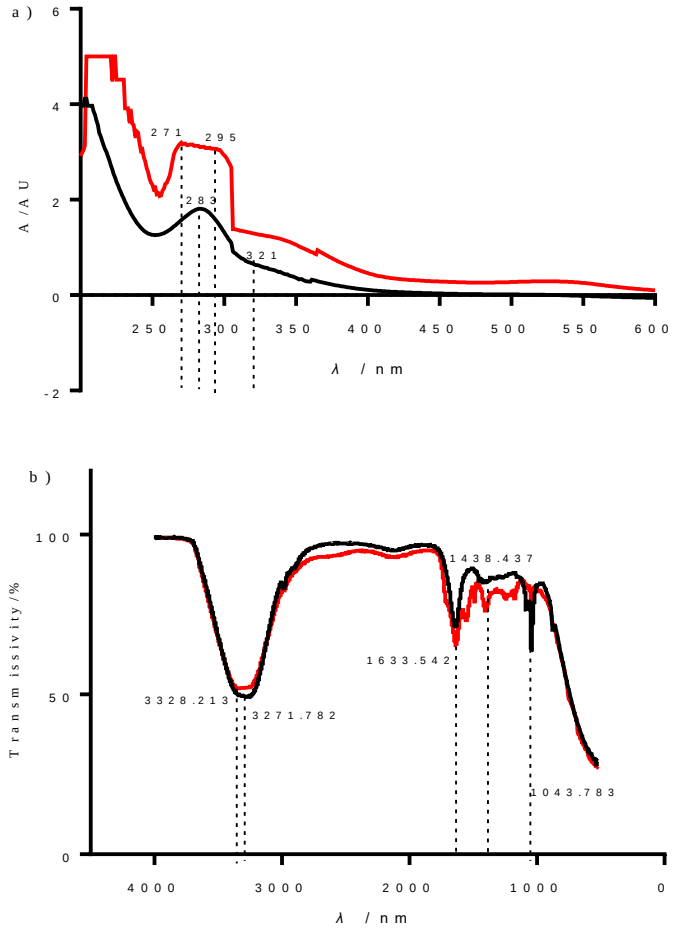


Fig. 2. UV/Vis scanning and FTIR characterization of acylated and nonacylated anthocyanins. a) UV-visible spectrophotometry scan of the visible range from 200 to 600 nm, b) Infrared spectrometry absorption spectrum range (4000 to 400 cm^{-1})

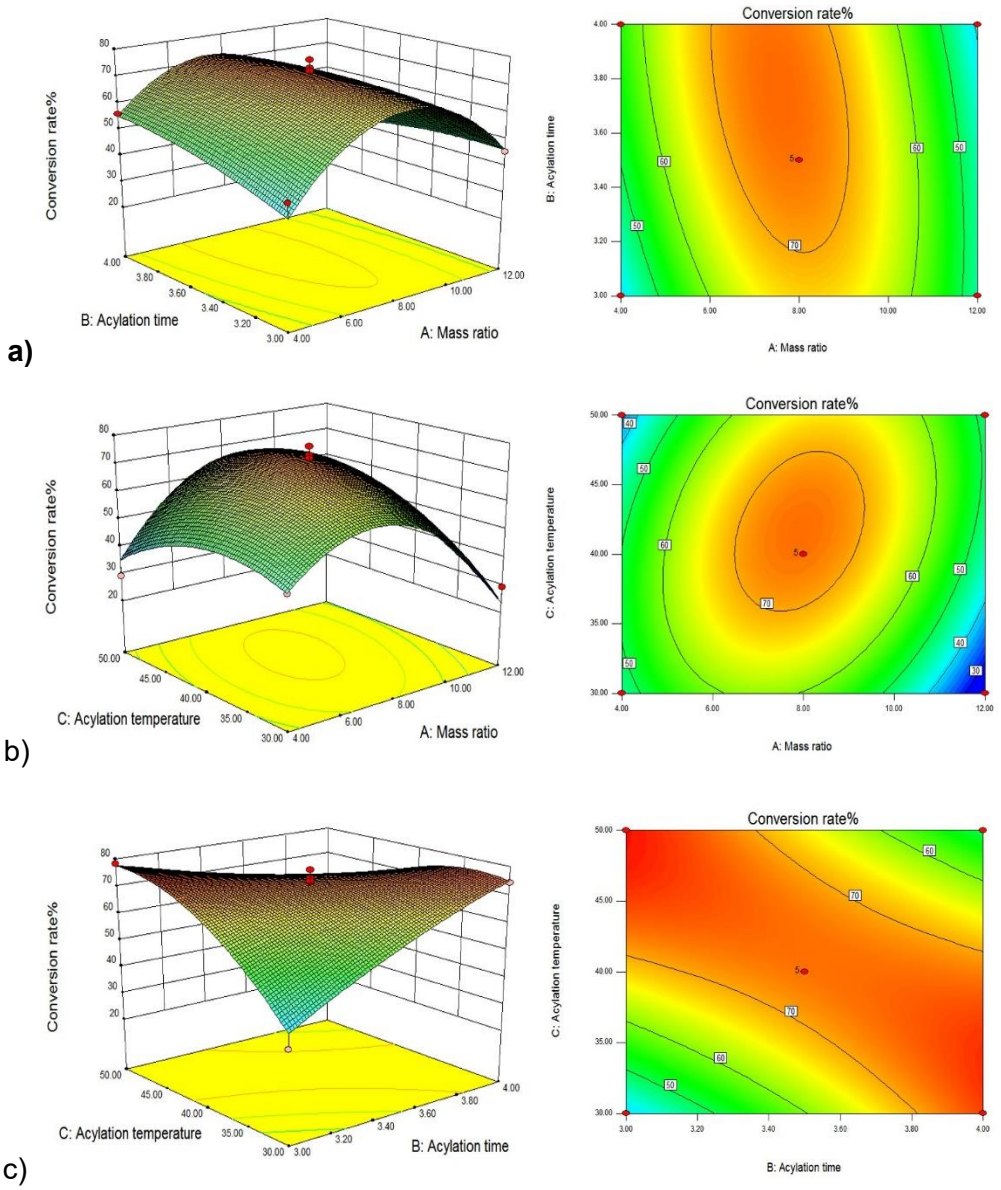
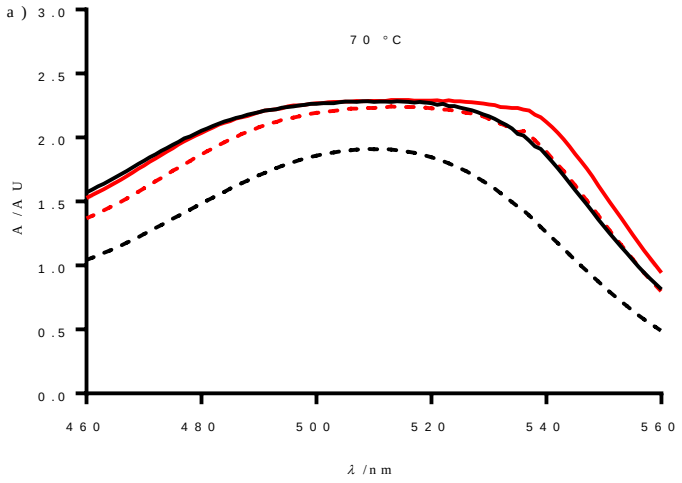
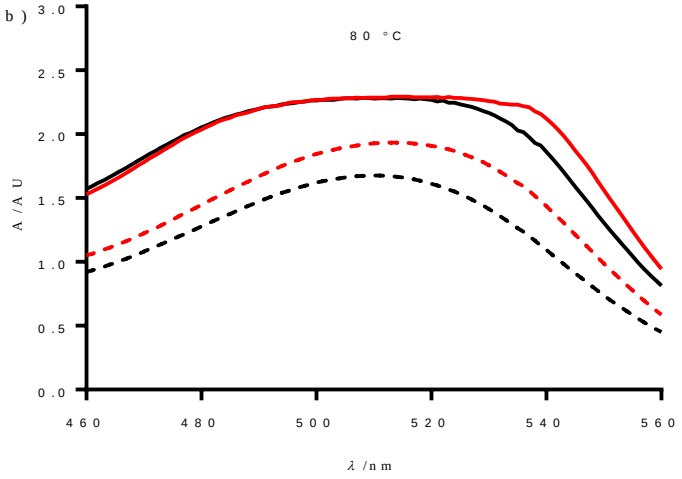


Fig. 3. Response surface and contour plots for anthocyanins acylation. a) Interaction of volume ratio to acylation time; b) Interaction of acylation time to temperature; and c) Interaction of mass ratio to acylation temperature





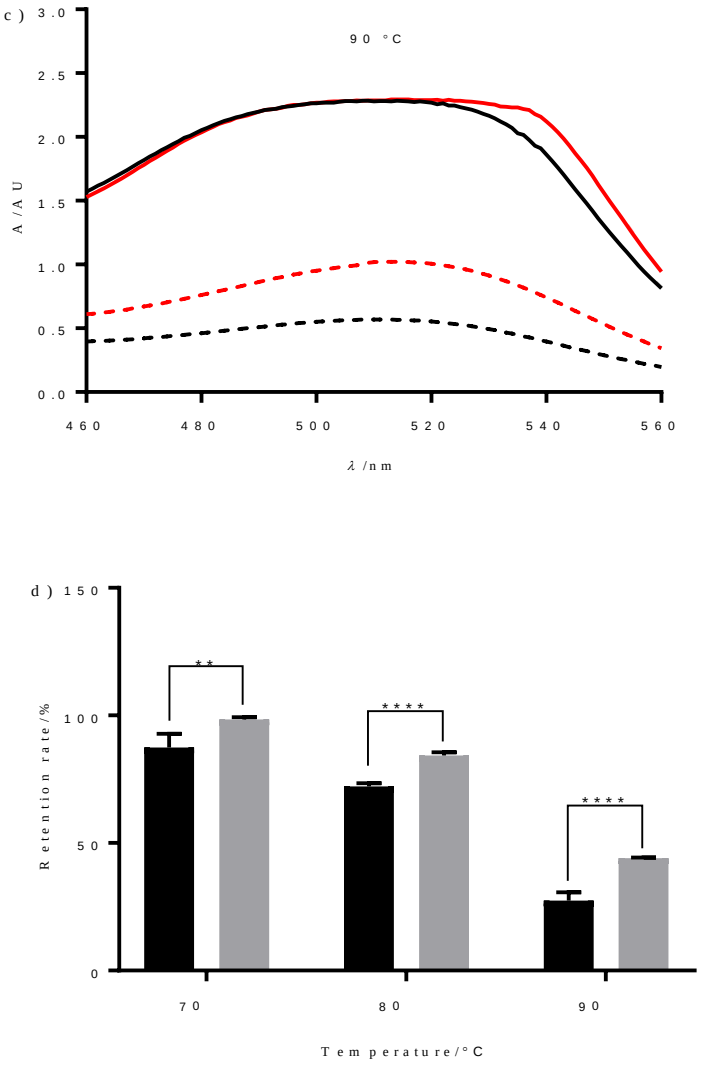


Fig. 4. Thermostability of acylated and nonacylated anthocyanins. a) Anthocyanins at absorbance from 460 – 560 nm under 70 °C, for 10 h in darkness; b) Anthocyanins at absorbance from 460–560 nm under 80 °C, for 10 h in darkness; c) Anthocyanins at absorbance from 460–560 nm under 90 °C, for 10 h in darkness; d) Retention rate of anthocyanins at different temperatures.

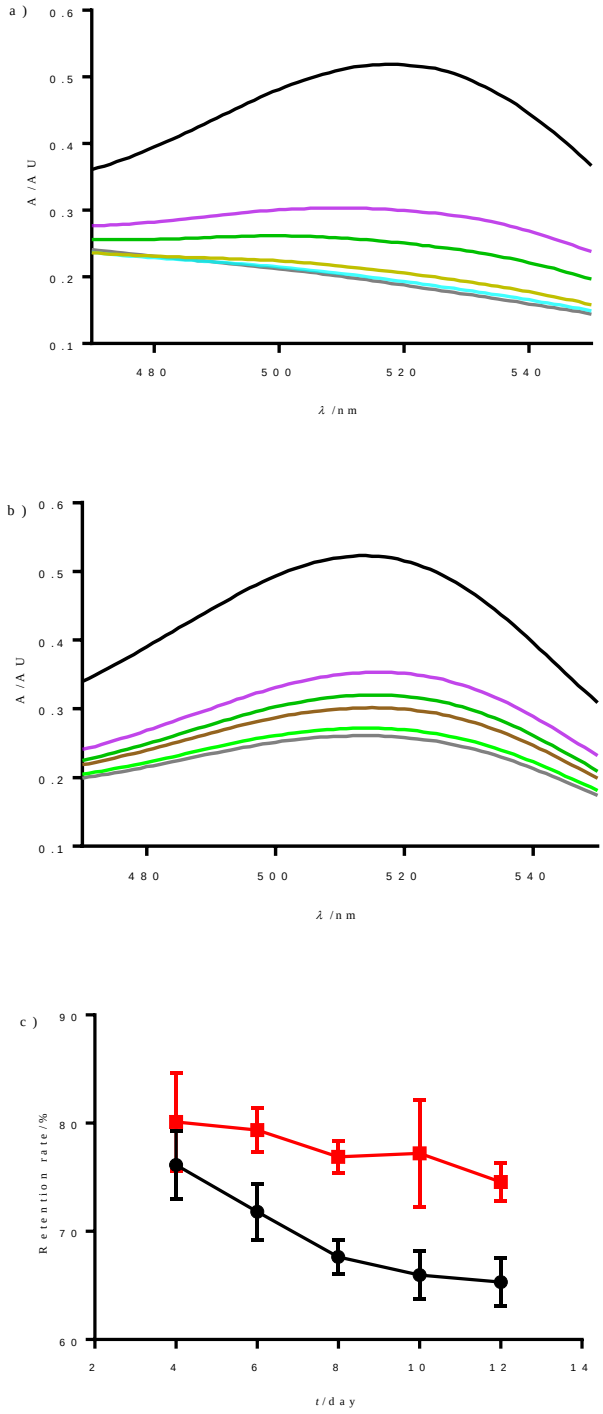
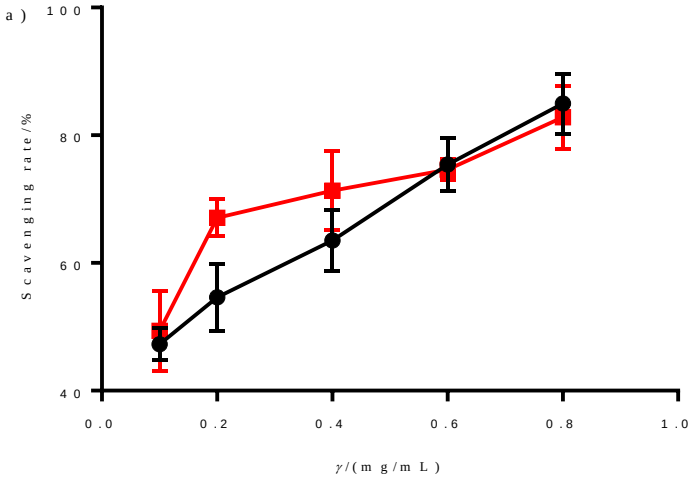
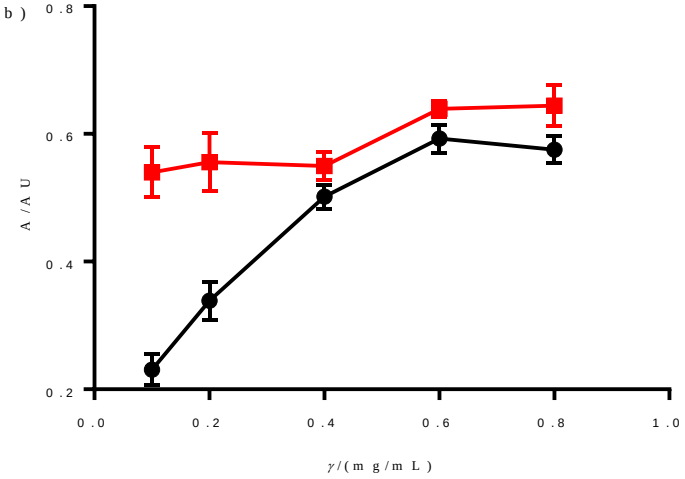


Fig. 5. Light resistivity on acylated and nonacylated anthocyanins. a) Nonacylated anthocyanins absorbance at 460–560 nm when exposed to 400 W light; b) Acylated anthocyanins absorbance at 460–560 nm when exposed to 400 W light; c) Retention rate of anthocyanins exposed to 400 W light for different durations





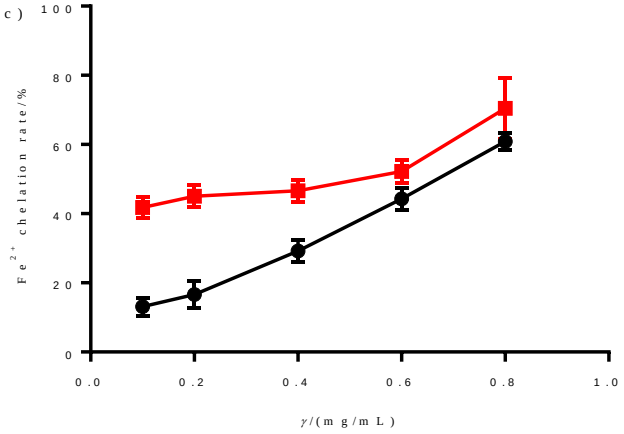


Fig. 6. Antioxidant abilities of acylated and nonacylated anthocyanins. a) DPPH free radical scavenging; b) Total reducing power; and c) Fe^{2+}-chelating capacity