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Short communication: Effect of purple corn pigment on change of anthocyanin composition and unsaturated fatty acids during milk storage

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ABSTRACT

Unsaturated fatty acids (UFA) in milk give rise to radicals and lead to lipid oxidation during storage, reducing the commercial value of milk. The objective of this study was to observe the effect of anthocyanins from purple corn pigment on the oxidation of UFA in milk. Milk samples were randomly divided into 2 groups: (1) the control (without purple corn pigment) and (2) treatment (0.3% purple corn pigment), using a completely randomized design. The milk samples were placed into plastic tubes and stored at 4°C for a period of 0, 1, 3, and 7 d. Individual anthocyanin composition and UFA were detected by HPLC-mass spectrometry and gas chromatography-mass spectrometry, respectively. The results indicated that pelargonidin (0.258 vs. 0.054 μg/mL), cyanidin (5.550 vs. 1.808 μg/mL), petunidin (0.464 vs. 0.107 μg/mL), delphinidin (2.061 vs. 0.123 μg/mL), and total anthocyanin (8.332 vs. 2.091 μg/mL) significantly decreased in response to increasing storage day. Of interest, purple corn pigment had a significant effect on most of the UFA (C14:1n-5, C16:1n-7, C18:1n-9, C18:2n-6, C18:3n-3, C18:3n-6, C20:2n-6, C20:3n-3, and C20:4n-6), except for C17:1n-7 and C20:3n-6. Specifically, various stronger positive correlations were noted for anthocyanin composition and UFA (pelargonidin and petunidin with C14:1n-5, C17:1n-7, C18:2n-6, C20:2n-6, C20:3n-3, and C20:4n-6; and cyanidin and total anthocyanins with C14:1n-5, C16:1n-7, C17:1n-7). Collectively, the current study suggested that the addition of anthocyanins from purple corn pigment had the potential to maintain UFA concentrations in milk during the storage period.

Key words: purple corn pigment, anthocyanin, unsaturated fatty acid, milk oxidation

Short Communication

Milk is rich in protein, vitamins, minerals, and essential fatty acids, and is one of the most widely consumed resources from livestock that is used for human nutrition. However, milk is a complex biological system, in which UFA are prone to lipid oxidation (Singh and Gallier, 2017). Milk antioxidants have been shown to play a crucial role in preventing lipid peroxidation. The addition of antioxidants to milk to prevent milk oxidation has been reported in a previous study (Schiano et al., 2019).

Anthocyanins are important bioactive substances and antioxidants, which are good for human health, found in purple corn (*Zea mays* L.). In particular, anthocyanins, which are natural pigments, have strong antioxidant potential and the ability to scavenge oxygen free radicals (Tian et al., 2018). Lamothe et al. (2019) suggested that the combination of milk and polyphenol-rich beverages resulted in strengthened protection against the formation of lipid oxidation species.

Purple anthocyanin-rich milk is a good source of antioxidants. Specifically, anthocyanins have been widely used in food and beverages, including milk, in many countries. To our knowledge, studies on the effect of anthocyanin on UFA profiles in milk are scarce. We hypothesized that the addition of purple corn pigment to milk could maintain the level of UFA. Accordingly, the purpose of the current study was to observe the effect of anthocyanins from purple corn pigment on the degradation of anthocyanin composition, UFA profiles, and their correlation in milk during storage.

Purple corn pigment was purchased from Nanjing Herd Source Biotechnology Co., Ltd. (Nanjing, China). The 6 standard anthocyanin compositions were obtained from J&K Scientific (Beijing, China). The chromatographic grade of methanol and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). The individual UFA were purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China). The

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hydrochloric acid (HCl), chloroform, hexane, potassium hydroxide, and boron trifluoride were all of analytical grade.

Fresh milk was collected from Guizhou University Farm (Guiyang, China; July 6, 2019) and was immediately transported to the laboratory and filtered through 4 layers of cheesecloth. All of the procedures were approved by the Rules of the College of Animal Science, Guizhou University. All samples were divided into 2 groups by varying the different purple corn pigment levels using a completely randomized design, with 6 duplicates per treatment. The milk samples were prepared via the addition of 0 or 0.3% purple corn pigment and mixed until well combined, according to Serafini et al. (2009). As a result, there were 2 groups: (1) control, raw milk; and (2) treatment, the addition of 0.3% (wt/vol) purple corn pigment in raw milk, and the milk was not pasteurized. All samples were placed into 150-mL plastic tubes (Nanjing Metasequoia Technology Co., Ltd., Nanjing, China) and stored at 4°C for a period of 0, 1, 3, and 7 d.

The sample of anthocyanin was extracted using a 15-mL extraction solution of 1.5 *M* HCl dissolved in 95% methanol solution (15:85, vol/vol), using an ultrasonic water bath at 4°C for 30 min. The supernatant was collected after centrifugation at 10,000 × *g* at 4°C for 5 min (Hunan Kaida Scientific Instrument Co., Ltd., Changsha, China). Next, 7 mL of HCl was added, and the mixture was incubated at 90°C for 40 min, then diluted to 60 mL. In this study, 1 g and 5 mL of purple corn pigment and the milk sample were extracted, respectively. Each sample was passed through a 0.22-μm filter membrane and analyzed by HPLC (Agilent Technologies, Santa Clara, CA) and tandem MS (SCIEX-6500Qtrap; AB Allen-Bradley, Milwaukee, WI). The HPLC conditions were as follows: anthocyanins were separated on a Poroshell 120 SB-C18 reversed-phase column (2.1 × 150, 2.7 μm), with a column temperature of 35°C; mobile phase: A = acetonitrile and B = 0.1% formic acid/1% phosphoric acid in deionized water, in A:B (3:7); elution gradient under isocratic and gradient elution; and injection volume was 2 μL. The MS conditions were as follows: positive ion ionization mode, multiple-reaction monitoring scan mode, a curtain gas pressure of 15 psi, ion spray voltage of +4,500 V, nebulization gas pressure of 65 psi, aux gas pressure of 70 psi, and nebulization temperature of 350°C. Six anthocyanins were detected: pelargonidin, peonidin, cyanidin, malvidin, petunidin, and delphinidin, and the sum of the values was defined as total anthocyanins.

Milk fatty acids were detected using the following procedures. A 0.5-mL milk sample was added to a 10-mL centrifuge tube, then 5 mL of methanol was transferred into the milk sample, and the mixture was shaken vigorously. The mixture was kept for 2 h at room temperature, with frequent vortexing via a rotary evaporator (Shanghai NAI Precision Instrument Co., Ltd., Shanghai, China) and then centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatant was transferred to a 10-mL tube and was mixed with 5 mL chloroform, kept for 2 h at room temperature, and then centrifuged (10,000 × *g* at 4°C for 10 min), and the chloroform phases were combined. Each sample was dried using a dry nitrogen blower machine (Shanghai NAI Precision Instrument Co., Ltd., Shanghai, China), filtered, and transferred into a 10-mL vial, with 1 mL of hexane to dissolve the residue. Next, 1 mL of 5% potassium hydroxide methanol solution was added, mixed uniformly for methylation, and incubated in a 60°C water bath for 30 min. Then, 3 mL of 14% boron trifluoride-methanol solution was added and heated at 60°C for 30 min to methyl-esterify the saponified fatty acids. After cooling to room temperature, 2 mL of hexane was added, and the mixture was vortexed. The individual UFA were detected using GC (7890A, Agilent Technologies Inc.) and MS. The GC conditions were as follows: UFA were separated using an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) and flame ionization detector, with methyl heptadecanoate as the internal standard; the injection temperature was 280°C; split ratio was 20:1; temperature program was an initial temperature of 60°C for 1 min, a 6°C/min rise to 260°C and held for 5 min; the carrier gas was helium, and the flow velocity was 1.0 mL/min. The MS conditions were as follows: the ionization temperature was 230°C; MS Quard temperature was 150°C; electron ionization, the energy of ionization was 70 eV; the scanning quality range was 35–800 μ; and the injection volume was 1 μL.

All calculations were analyzed using the SAS System Version 9.1.3 (SAS Institute Inc., Cary, NC) according to the following model: $Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the observation, μ is the overall mean, A_i is the effect of anthocyanins, B_j is the effect of the storage time, $(AB)_{ij}$ is the effect of the interaction of anthocyanins and time, and ε_{ijk} is the random error with mean 0 and variance σ^2. The means were calculated via the LSMEANS statement, and the options after the slash specify the calculation of standard errors and tests of differences between least squares means using Tukey's test. Pearson correlation coefficient (r) was used to analyze the relationship between individual anthocyanin and UFA in milk (Kaps and Lamberson, 2004). A *P*-value of <0.05 was considered statistically significant.

In the current study, Pel, Cya, Pet, Del, and TA decreased significantly ($P < 0.001$; Table 1) in response

Table 1. Effect of purple corn pigment on change of anthocyanin composition in milk during storage (μg/mL)[1]

	Storage period, d											
	0		1		3		7			*P*-value[3]		
Item[2]	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	SEM	P	T	P × T
Pel	0.115	0.143	0.040	0.078	0.016	0.051	0.013	0.041	0.0123	***	***	NS
Peo	ND	ND	ND	ND	ND	ND	ND	ND	—	—	—	—
Cya	0.276	5.274	0.147	2.865	0.112	1.835	0.095	1.713	0.3070	***	***	***
Mal	ND	ND	ND	ND	ND	ND	ND	ND	—	—	—	—
Pet	0.214	0.250	0.091	0.123	0.045	0.092	0.023	0.084	0.0219	***	***	NS
Del	0.207	1.854	ND	0.566	ND	0.237	ND	0.123	0.1934	***	***	—
TA	0.811	7.521	0.279	3.632	0.173	2.214	0.131	1.960	0.4905	***	***	***

[1]Values represent the means of 6 replicates (n = 6). ND = not detected or detected at <0.01 μg/mL.

[2]Pel = pelargonidin; Peo = peonidin; Cya = cyanidin; Mal = malvidin; Pet = petunidin; Del = delphinidin; TA = total anthocyanins.

[3]P = effect of purple corn pigment; T = effect of the storage period; P × T = effect of purple corn pigment and storage period interactions.

***$P < 0.001$.

Table 2. Effect of purple corn pigment on change of UFA in milk during storage (mg/kg)[1]

	Storage period, d											
	0		1		3		7			*P*-value[3]		
Item[2]	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	SEM	P	T	P × T
C14:1n-5	26.27	26.89	23.98	26.08	23.48	26.31	23.94	25.88	0.3248	***	**	NS
C16:1n-7	13.36	13.89	12.86	13.42	13.00	13.80	12.97	13.81	0.1353	**	NS	NS
C17:1n-7	5.70	5.98	5.00	5.81	5.10	5.54	4.77	5.71	0.1201	NS	NS	NS
C18:1n-9	148.58	147.88	144.65	147.57	141.77	146.72	142.04	153.16	1.1146	**	NS	*
C18:2n-6	172.22	173.92	138.46	165.45	137.54	171.98	134.58	169.38	3.4466	***	***	***
C18:3n-3	3.37	3.22	2.32	3.13	2.22	3.32	2.45	3.31	0.1153	***	**	**
C18:3n-6	735.21	731.84	639.38	721.98	620.92	729.92	626.10	739.81	10.2385	***	***	***
C20:2n-6	8.83	8.90	6.06	7.16	5.51	6.40	5.08	6.61	0.3950	***	***	NS
C20:3n-3	11.24	11.18	9.20	11.07	8.67	10.32	8.41	10.48	0.3051	***	**	NS
C20:3n-6	4.28	4.37	4.49	4.26	4.32	4.44	4.25	4.44	0.0646	NS	NS	NS
C20:4n-6	5.13	5.10	4.47	4.95	4.51	4.68	4.03	4.79	0.1037	**	***	*
MUFA	193.91	194.64	186.50	192.87	183.35	192.37	183.72	198.56	1.2903	***	*	**
PUFA	940.28	938.53	804.39	918.01	783.68	931.06	784.91	938.83	14.2272	***	***	***

[1]Values represent the means of 6 replicates (n = 6).

[2]MUFA = all MUFA from C14:1n-5 to C18:1n-9 with single double bond; PUFA = all PUFA from C18:2n-6 to C20:4n-6 with 2 or more double bonds.

[3]P = effect of purple corn pigment; T = effect of the storage period; P × T = effect of purple corn pigment and storage period interactions.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

to increasing storage time, perhaps because anthocyanin was unstable to various factors of pH, addition amount, and dilution rate (Tian et al., 2019b). The intermolecular co-pigmentation of anthocyanins had a sterically compact structure, increasing the protective intramolecular mechanism to become more stable (Eiro and Heinonen, 2002). These results agreed with the observation of Campos et al. (2017) in which the level of anthocyanins in milk decreased by approximately 40% at 28 d of storage.

One study did show that the stages of unsaturated lipid peroxidation in milk were initiation, propagation, and termination; PUFA molecules could transform into hydroperoxides, conjugated dienes, and peroxy radicals (Lindmark-Månsson and Åkesson, 2000). In the present trial, except for C17:1n-7 and C20:3n-6, purple corn pigment significantly ($P < 0.05$; Table 2) affected the remaining of UFA (C14:1n-5, C16:1n-7, C18:1n-9), C18:2n-6, C18:3n-3, C18:3n-6, C20:2n-6, C20:3n-3, and C20:4n-6), indicating that anthocyanins might inhibit unsaturated lipid peroxidation, maintaining the levels of UFA in milk. This was in accordance with the results of an earlier study, in which anthocyanin-rich grape seed could inhibit UFA oxidation in sheep milk (Correddu et al., 2015). Anthocyanins could act as hydrogen atom donors to the peroxy radical, thereby inhibiting the oxidation of UFA by chain radical termination (Narayan et al., 1999). In addition, phenolic compounds and other natural antioxidants showed synergistic action against oxidation (Milde et al., 2007). Interestingly, the milk of Saanen dairy goats that received anthocyanin-rich feed was found to contain dietary anthocyanins, thus increasing the amount of superoxide dismutase (Tian et al., 2019b). In addition, anthocyanins might bind to fat for easy absorption, which could protect compounds' recovery and stability during duodenal digestion (Zhang et al., 2014). Predictably, for consumers receiving anthocyanin-rich milk, anthocyanins and their metabolic products in the body of humans could effectively prevent the macromolecules from oxidation damage, thus improving an organism's immune function and enhancing the antioxidant effect. As a consequence, the supplement of anthocyanin-rich roughage in dairy ruminant feedstuff or the addition of anthocyanins in milk not only maintained UFA levels, but also was found to be essential to prevent anthocyanin stability in the body.

Polyphenolic compounds could act as hydrogen donors in the reaction because the phenol groups form radical intermediates with delocalized electrons (Nimse and Pal, 2015). One study did report that free radicals, such as superoxide radicals, hydroxyl radicals, and peroxide radicals, could be controlled via the milk antioxidant system (Tsopmo et al., 2011). Considering anthocyanins could enhance the level of milk antioxidants (Tian et al., 2019a), the addition of anthocyanins to milk might prevent free radicals from damaging milk quality. Gad and Sayd (2015) reported that adding polyphenolic compounds to dairy products might be beneficial to extend their shelf life by inhibiting the

Table 3. Pearson correlation coefficient (r) between anthocyanin composition and UFA in milk[1]

Item[2]	C14:1n-5	C16:1n-7	C17:1n-7	C18:1n-9	C18:2n-6	C18:3n-3	C18:3n-6	C20:2n-6	C20:3n-3	C20:3n-6	C20:4n-6
Pel											
r	0.8063	0.5634	0.8059	0.4715	0.7444	0.6643	0.6977	0.9788	0.8551	0.1067	0.8654
P	*	NS	*	NS	*	NS	NS	***	**	NS	**
Peo											
r	—	—	—	—	—	—	—	—	—	—	—
P	—	—	—	—	—	—	—	—	—	—	—
Cya											
r	0.7378	0.7594	0.7714	0.4377	0.6521	0.5555	0.6143	0.6040	0.6593	0.0661	0.6026
P	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS
Mal											
r	—	—	—	—	—	—	—	—	—	—	—
P	—	—	—	—	—	—	—	—	—	—	—
Pet											
r	0.7647	0.5282	0.7751	0.4734	0.7177	0.6291	0.6687	0.9840	0.8279	0.0369	0.8674
P	*	NS	*	NS	*	NS	NS	***	*	NS	**
Del											
r	0.8739	0.4105	0.8585	0.3206	0.3967	0.4892	0.2138	0.5940	0.5050	0.0803	0.5184
P	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
TA											
r	0.7357	0.7286	0.7678	0.4077	0.6442	0.5412	0.5982	0.6626	0.6678	0.0361	0.6287
P	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS

[1]Values represent the means of 6 replicates (n = 6).

[2]Pel = pelargonidin; Peo = peonidin; Cya = cyanidin; Mal = malvidin; Pet = petunidin; Del = delphinidin; TA = total anthocyanins.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

rate of hydrolysis and lipid oxidation. In addition, the use of phenolic compounds as functional ingredients to enhance the oxidative stability of dairy products has been advocated (O'Connell and Fox, 2001). Hence, various anthocyanin compositions and UFA were positively ($P < 0.05$; Table 3) correlated in the current study. Results of this study were consistent with those of Correddu et al. (2016), who suggested that the inclusion of anthocyanin-rich grape seed could be useful to increase the concentration of UFA in milk of Sarda dairy sheep.

The current study indicated that the addition of anthocyanins from purple corn pigment had the potential to maintain UFA content in milk during the storage, which seemed to be an effective way to retard lipid oxidation. Our observations provide an insight into the beneficial effects of anthocyanin on milk UFA profiles.

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