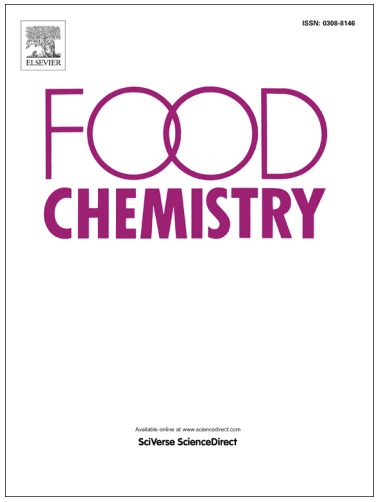

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Black Goji as a Potential Source of Natural Color in a Wide pH Range

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Abstract

Lycium ruthenicum Murr is a traditional Chinese herb widely distributed in Tibet. The fruit, known as black goji, is popular in traditional Chinese medicine. The objective of this study was to investigate its anthocyanin profile (by HPLC coupled to PDA and MS detectors) and the colorimetric and spectrophotometric properties. Black goji extracts contained abundant petunidin derivatives, with *cis* and *trans* isomers of petunidin-3-*p*-coumaroyl-rutinoside-5-glucoside. The colorimetric and spectrophotometric traits of black goji anthocyanins were significantly impacted by solid-phase-extraction, pH, and acylation. MCX cartridge removed considerable polyphenolics from fruit extracts, but attenuated the saturation of color expression. Petunidin-3-*trans*-*p*-coumaroyl-rutinoside-5-glucoside contributed most of the color expression of the black goji extract, and showed superior stability compared to other extracts over time. Acylation strengthened the petunidin derivatives color retention, and enhanced the color intensity and stability. Black goji anthocyanins produced various vivid hues over wide ranges of pH, making them promising candidates for natural colorants.

Keywords

Anthocyanins; Natural colorants; Petunidin; Black goji (*Lycium ruthenicum* Murr); Acylation

1. Introduction

Color plays a critical role in food quality and consumer perception. During the last few years, there have been increasing health concerns towards the use of synthetic dyes, which are suspected to cause behavior problems in children with attention deficit hyperactivity disorder (ADHD) (Sharma, McKone, & Markow, 2010). Started in 2010, the European Union (EU) demanded the warning labels for all the food products that contain synthetic food colorants (CSPI, 2010). One year later, the U.S. Food and Drug Administration (FDA) also conducted the review of scientific evidence on artificial colorants and possible association with ADHD in children (FDA, 2011). Although a causal relationship between color additive and ADHD has not been confirmed in this review, the condition of ADHD might be promoted by synthetic colorants. The increasing consumer demands for natural colorants and the current market trend of "clean label", therefore, have driven the food industry to focus on the replacement of artificial colorants with those natural alternatives.

Despite their various advantages over synthetic dyes, natural pigments have several weaknesses that limits their application in food. Generally speaking, naturally derived colorants are less stable compared to the artificial counterparts, and their stabilities are greatly influenced by food processing and storage conditions (Rodriguez-Amaya, 2018). Pigments sourced from vegetable extracts, for example red cabbage, red radish, and red beet, could also impart undesirable aroma and flavor, resulting in difficulties for their direct food application (Sigurdson, Tang, & Giusti, 2017). In addition, limited choices of naturally derived pigments are available to match the color traits of synthetic ones, especially for blue and green hues (Sigurdson et al., 2017a; Wrolstad & Culver, 2012). Therefore, it remains to be a huge challenge for food industry to explore, develop, and employ new sources of

naturally-existing colorants that possess extraordinary stability, bear strong tinctorial strength, and cover a wide range of vivid hues without unpleasant sensory attributes.

Lycium ruthenicum Murr. is a traditional Chinese herb widely distributed in Qinghai-Tibet plateau. The fruit, known as black goji, has a pleasant aroma and flavor, and is popular in traditional Chinese medicine for disease treatment, such as heart disease, abnormal menstruation and menopause (Jin et al., 2015; Zheng et al., 2011). Its health benefits have been associated with the antioxidant activities of the anthocyanins, which are responsible for the black-bluish color of the fruit (Potterat, 2010). Black goji fruit from Tibet contained 550-500mg anthocyanin per 100g FW, most of which (>80%) were acylated ones (Zheng et al., 2011). Five major anthocyanins were identified and petunidin derivative accounted for 95% of the anthocyanin content. Total polyphenol content (~1310 mg GA equivalents/100gFW) and strong antioxidant activity (~1060mg GA equivalents/100g FW) were also reported (Zheng et al., 2011). Several goji (*Lycium*) fruits were analyzed by HPLC mass spectrometry and their phenolic content identified and quantified, including pigments such as carotenoids and anthocyanins (Mocan et al., 2017, 2018; Rocchetti et al., 2018; Xin et al., 2017). With plentiful acylated anthocyanins and antioxidant capacity, black goji anthocyanins seem to be good candidates for natural colorants. Although it has been well studied in terms of anthocyanin content, antioxidant activity, and biosynthesis, the color properties of black goji pigments remain to be explored, especially the influence of purity, and acylation on the color expression.

Anthocyanins are an important group of water-soluble natural pigments found in fruits and vegetables. They render vivid red to blue color to plants, and have important application in coloring food products (Obón, Castellar, Alacid, & Fernández-López, 2009; Sigurdson, Tang, & Giusti, 2017). Anthocyanin colors are significantly affected by their chemical structures including chromophore methoxylation, hydroxylation, glycosylation and

acylation. Generally, more hydroxyl groups on B ring leads to the bluer shift in spectrum while more methoxyl groups causes the redder shift in spectrum (He & Giusti, 2010; Heredia, Francia-Aricha, Rivas-Gonzalo, Vicario, & Santos-Buelga, 1998). Acylation on anthocyanin structure was generally believed to enhance anthocyanin stability as the acyl group influences the pigment structure configuration and protects the chromophore from hydration, which explains the predominance of acylated anthocyanins in natural food colorants industry (M. Monica Giusti & Wrolstad, 2003; M. Giusti & Wrolstad, 1996). In addition, acylation substituent patterns, including various acyl moieties and attachment locations, could affect anthocyanin colorimetric and spectrophotometric properties (Ahmadiani, Robbins, Collins, & Giusti, 2016).

The objective of this study was to investigate the profile, color characteristics, and stability of the anthocyanins in black goji, as well as the influence of purity, pH and acylation on the colorimetric and spectrophotometric properties, providing a new potential natural color source for the food industry.

2. Materials and Methods

2.1 Materials & Reagent

Black goji pigments were extracted from dried black goji berries that were purchased from a grocery store (LianHua Supermarket) in Shanghai, China.

The chemicals and reagents (ACS or HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ USA), including acetone, chloroform, methanol, trifluoroacetic acid (TFA), ammonium hydroxide (NH_4OH), acetonitrile (HPLC and LC/MS grade), potassium hydroxide (KOH), citric acid, sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), potassium chloride, and sodium acetate. ACS grade ethyl acetate and formic acid

were obtained from Mallinckrodt Chemicals (Bedminster Township, NJ USA) and Honeywell (Morris Plains, NJ USA), respectively.

2.2 Pigments Extraction

The extraction of black goji anthocyanins by acetone-chloroform partitioning method were based on Rodriguez-Saona & Wrolstad, (2005). Dried black goji berries (~50g) were mixed with liquid nitrogen, and powdered by using a food blender. The powders were then mixed with 100%(v/v) aqueous acetone with 0.01% HCl. The slurry was filtered through filter paper (Whatman No.4 filter paper, Whatman Incorporation. NJ, US) by water vacuum. After filtration, re-extraction was performed by adding 70%(v/v) aqueous acetone with 0.01% HCl until the slurry becoming faded. Two volumes of chloroform were then added into the aqueous acetone solution, and the whole filtrate was transferred into the separatory funnel. After the gentle mixing, samples were stored in 4°C refrigerator overnight. On the next day, after transferring the upper aqueous portion to flask, the acetone and chloroform were evaporated by rotary evaporator.

2.3 Pigments Purification

The C-18 cartridge purification method was adapted from Rodriguez-Saona & Wrolstad, 2005. Aqueous anthocyanin extracts obtained from the above procedure were pumped to pass through the Sep-Pak® C18 cartridge (Waters Corporation. Milford MA, USA) after activating the cartridge with methanol. Then the cartridge was washed with two column volumes of acidified water (0.01% v/v HCl), and anthocyanin was eluted into a boiling flask by adding 0.01% v/v HCl acidified methanol. After the removal of the methanol by rotary evaporator, the purified anthocyanin samples were re-dissolved by acidified distilled water (0.01% v/v HCl).

The remaining phenolics in the C-18 purified anthocyanin sample could be further removed by Oasis® MCX extraction cartridge (Waters Corporation. Milford MA, USA), a

novel solid-phase extraction method introduced by He & Giusti, 2011. The MCX cartridge was first activated by two columns of 0.1% TFA methanol and water, respectively. Then samples were loaded onto the activated MCX cartridge, and two washes of 0.1% TFA water followed by 0.1% TFA methanol were performed to remove the salts, sugars, and other phenolics. The anthocyanin samples were then recovered with 1% NH_4OH in methanol, and collected in a round flask containing formic acid that helps to neutralize the alkaline condition of eluent.

2.4 Pigments Saponification

Alkaline hydrolysis (saponification) was performed to cleave the ester bond between acyl group and anthocyanin glycoside (Giusti, Rodríguez-Saona, & Wrolstad, 1999). Purified anthocyanin samples were mixed with 10 ml 10% aqueous KOH in a capped test tube for 12 min. Then 2N HCl was added to the mixture to adjust the pH back to mildly acidic conditions. Samples were then purified by C-18 cartridge to remove acids as described above.

2.5 Pigments Identification and Isolation

Individual anthocyanin pigments in extracted and purified samples were then analyzed and identified by high performance liquid chromatography (HPLC) (Shimadzu, Columbia, MD) coupled to a SP-M20A Photodiode Array Detector (Shimadzu, Columbia, MD) and a LCMS-2010EV Liquid Chromatograph Mass Spectrometer. A reverse phase Symmetry C-18 (5µm, 4.6*150mm) column (Phenomenex, Torrance, CA US) was used. All of the extracts were filtered through a 0.22 um syringe filter (Phenomenex, Torrance, CA US) before injection into the HPLC. Samples were analyzed using a flow rate of 0.8 ml/min. The mobile phase consisted of solvent (A) 4.5% (v/v) formic acid and solvent (B) 100% acetonitrile. The linear gradient used in the analysis was from 8% B 0min-5min, 8%-15% B 5min-35min, 15%-35% B 35min-37min, 35%-8% B 37-40min, 8% B 40-45min.

Anthocyanin and all phenolics elutions were monitored at 500-530nm and 280-700nm, respectively. Total ion scans and selected ion monitoring (Mass/charge ratios of 271, 287, 303, 301, 317 and 331, corresponding to the most common anthocyanin aglycones) were conducted. The purity of the anthocyanins were calculated as dividing the total area under the curve of anthocyanins (monitored at 500-530nm) by that of phenolic compounds (monitored at 280-700nm).

The major pigment, petunidin-3-*trans-p*-cou-rut-5-glu, was isolated by Luna reverse-phase PFP column (5µm particle size and 100 Å pore size with 250*21.20 nm column size, Phenomenex, Torrance, CA US) and semi-prep reverse-phase HPLC (Shimadzu, Columbia, MD) composed of pumps (LC-6AD), autosampler (SIL-20A HT), column oven (CTO-20A), Photodiode Array Detector (SPD-M20A), and communication module (CBM-M20A). The flow rate was 10 ml/min, with mobile phase consisted of solvent (A) 4.5% (v/v) formic acid and solvent (B) 100% acetonitrile. The linear gradient used for pigment isolation was from 12% B 0min-2min, 12%-21% B 2min-25min, 21%-21% B 25min-30min, and 21%-30% B 30-50min. For non-acylated (saponified) petunidin derivative, the gradient was from 10% B 0min-1min, 10%-15% B 1min-31min. Each pigment was collected manually based on the real-time absorbance over 280-700nm. The collected pigments were purified by C-18 cartridge and their identification and purity were confirmed by Kinetex reverse-phase PFP column (2.6µm particle size and 100 Å pore size with 100*4.6nm column size, Phenomenex, Torrance, CA US) and the same pigment identification method as described above.

2.6 Pigments quantification

The monomeric anthocyanin content was determined by the pH differential method (Mónica Giusti & Wrolstad, 2005). Buffer solutions were prepared using 0.1M potassium chloride at pH 1.0 and 0.4M sodium acetate at pH 4.5. Absorbance of samples at pH 1.0 and 4.5 were measured at 700nm and its λmax (512nm) by using UV-vis Spectrophotometer

(Shimadzu corporation. Tokyo, Japan). Measurements were done in triplicates, and each black goji anthocyanin extracts or isolates was expressed as cyanindin-3-glucoside equivalence.

2.7 Buffer System and Sample Preparation

The buffer systems in this study were citric acid-Na_2HPO_4 buffer solutions for pH 3-7; Na_2HPO_4-NaH_2PO_4 buffer solution for pH8; Na_2CO_3-$NaHCO_3$ buffer solutions for pH9-10 (Dawson, Elliott, Elliott, & Jones, 1986). All the black goji extracts and isolates were diluted in these buffer solutions (pH3-10) at concentration of 25 μM. The pH condition after mixing was confirmed using pH meter (Mettler Toledo Inc, Columbus, OH US). The initial spectrophotometric and colorimetric measurement were performed after 1 hour equilibrium. Samples were stored at refrigerated condition in the dark for three weeks for stability tests. Analysis was done in triplicates.

2.8 Spectrophotometric Analysis

After 1 hour mixing of black goji samples with various buffer systems, 250 μL of each sample was aliquoted to poly-D-lysine coated polystyrene 96 well plates, and the spectrums were analyzed from 380 nm to 700 nm with 1 nm interval by using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale CA). The spectrophotometric analysis was also conducted on day 1 (24 hours after mixing), 2, 3, 7, 14, and 21.

2.9 Colorimetric Analysis

The spectral absorbance data for each sample from 380 nm to 700 nm as described above were converted into colorimeteric data using the ColorBySpectra software (according to CIE 1964 standard observer, D65 illuminant spectral distribution, and 10° viewer angle) (Farr, JE; Srivastava, A; Machiraju, R; Giusti, 2017). The samples were kept in refrigerated condition in the dark at 4°C for three weeks. The color changes were measured as ΔE. The coloration of all the samples were photographed at each time points as described above.

2.10 Statistical Analysis

The tables and corresponding statistical analysis were presented by using Prism software (GraphPad, La Jolla, CA US). One-way ANOVA (two-tailed, α=0.05) and post hoc Tukey's test (family-wise α=0.05) were conducted to evaluate the differences in L, C*, h* values among different black goji anthocyanin extracts/isolates at a certain pH condition.

3.Results and Discussion

The anthocyanin profiles, colorimetric & spectrophotometric properties, and stabilities were compared among various black goji anthocyanin extracts or isolates. In the following sections, "crude extract" refers to the black goji anthocyanin extracts without any solid-phase purification procedures; "C-18 purified extract" and "MCX purified extract" indicate the extracts purified by C-18 cartridge and C-18 & MCX cartridges, respectively; "*trans* isomer" represents the isolated petunidin-derivative: petunidin-3-*trans*-*p*-coumaroyl-rutinoside-5-glucoside; and "SPO" stands for the petunidin-3-rutinoside-5-glucoside, which was produced from the saponification of the *trans* isomer.

3.1 Identification of anthocyanin profiles in black goji

Four major anthocyanins accounting for 97% of the total peak area were separated based on the chromatograph prepared by RP-HPLC-PDA-MS (**Figure 1**). The relative peak area of the four pigments (peak 1 to peak 4) was approximately 18%, 4%, 7%, and 71%, respectively. Two aglycones, delphinidin (m/z 303) and petunidin (m/z 317) were found in black goji, between which petunidin was the most abundant (93%). According to the MS data, the majority of the black goji anthocyanins in crude extracts were acylated (80%), and they were identified to be petunidin-3-galactoside-5-glucoside (peak 1), delphinidin-3-*trans*-*p*-coumaroyl-rutinoside-5-glucoside (peak 2), petunidin-3-*cis*-*p*-coumaroyl-rutinoside-5-glucoside (peak 3), and petunidin-3-*trans*-*p*-coumaroyl-rutinoside-5-glucoside (peak 4), in

agreement with the identification of predominant pigments in a previous study (Zheng et al., 2011).

Petunidin-3-*trans*-*p*-coumaroyl-rutinoside-5-glucoside accounted for almost 71% of the total anthocyanins in black goji (peak 4 in **Figure 1**), which was barely reported in berry fruits (Wu & Prior, 2005). Taking into account the abundancy in black goji, it is reasonable to believe that this pigment would be representative in terms of overall black goji color expression and other properties including color stability (as described later). Besides, it is interesting to find both *cis* and *trans* petunidin isomers (relative peak area was 7% and 71%) with same structure block but different spatial configuration (peak 3 and peak 4 in **Figure 1**). Acyl groups in acylated anthocyanins are typically *trans* configured, and there were few *cis* form identified in nature (George et al., 2001; Hosokawa, 1995; Ichiyanagi et al., 2005). Thus, the co-existing of both plentiful *trans* and *cis* isomers from same source is limited. To this end, black goji may serve as a good subject to study the impact of acyl spatial configuration on anthocyanin color expression.

Acylation on the anthocyanin structure is generally believed to enhance anthocyanin stability since the acyl group could alter the pigment structure configuration and protect the chromophore from hydration, which explains the predominance of acylated anthocyanins in food natural colorants industry (M. Monica Giusti & Wrolstad, 2003; M. Giusti & Wrolstad, 1996). Unlike other acylated anthocyanins which are commonly found in large amounts in vegetables such as red cabbage (containing acylated cyanidin derivatives) and red radish (containing acylated pelargonidin derivatives), black goji as fruit are rich in acylated anthocyanin. Some scholars postulated that this high-level content of acylated pigments in black goji was due to the extreme altitude, ultraviolet rich environment, and harsh weather in Tibet. The abundant acylated anthocyanins would protect plants from the tough conditions

(Dixon & Paiva, 1995). Nevertheless, it is a promising advantage of black goji in that the vegetable extracts usually carry unique aroma and flavors while black goji extracts do not.

The major pigment, petunidin-3-*trans-p*-coumaroyl-rutinoside-5-glucoside, was isolated to investigate its contribution to the overall black goji color properties. In addition, saponification of this *trans* isomer yielded the petunidin-3-rutinoside-5-glucoside (labeled as "SPO" in **Figure 1**) as a non-acylated anthocyanin comparison.

3.2 Effect of purification on anthocyanin profiles in black goji

The behavior of pigments can be greatly affected by surrounding compounds, as other phenolic including phenol, phenolic acid, flavone, and tannin could significantly influence colorimetric properties as well as pigment stabilities through intermolecular co-pigmentation. (Fossen, Rayyan, Holmberg, Nimtz, & Andersen, 2007; Gómez-Míguez, González-Manzano, Teresa Escribano-BailóN, Heredia, & Santos-Buelga, 2006; Kunsági-Máté, Szabó, Nikfardjam, & Kollár, 2006; Pacheco-Palencia & Talcott, 2010). Anthocyanin-rich vegetables and fruits generally contain abundant polyphenols and lipids, which complicated the qualitative and quantitative analysis of anthocyanin (Rodriguez-Saona & Wrolstad, 2001). Thus, purity is a critical factor in pigment analysis. Unfortunately, the commonly used extraction methods are not specific for anthocyanin, and a solid-phase purification procedure would be necessary to exclude the extraneous interfering compounds. In this study, two resins were used to accomplish this goal: C-18 and MCX cartridges. The former one contains 18 carbon-chains that are bonded on a silicon base. Hydrophobic organic compounds such as anthocyanin and phenolic compounds would retain on the cartridge while acids and sugars would be washed out by acidified water. Phenolic other than anthocyanin then could be partially removed using ethyl acetate wash (Oszmianski & Lee, 1990; Rodriguez-Saona & Wrolstad, 2001). The MCX cartridge was implemented for anthocyanin purification by He &

Giusti, 2011. Basically, this isolation method used cation-exchange and reversed-phase mechanisms to isolate positive charged anthocyanin. It was found to exhibit higher anthocyanin selectivity than other solid phase extraction methods.

The crude extract, C-18 purified extract, and MCX purified extract were investigated regarding their anthocyanin and phenolic compound contents, which were monitored at 500-530 nm and 280-700 nm, respectively. As demonstrated in **Figure 1**, the C-18 cartridge purification reduced the complexity of the matrix as some of the phenolic compounds were removed from the crude extract. The purity of anthocyanins in C-18 cartridge purified sample was 50%, in contrast to 43% in crude extract. A following wash with the MCX cartridge significantly eliminated phenolic compounds (anthocyanin purity was 65%), demonstrating MCX as a powerful tool for anthocyanin purification. As expected, anthocyanin profiles and content before and after the above purifications steps were intact since the proportions of peaks remained unchanged.

3.3 Colorimetric properties of black goji anthocyanin extracts and isolates

Generally, all the black goji anthocyanin extracts (crude extract, C-18 purified extract, and MCX purified extract) and isolates (*trans* isomers and SPO) exhibited a similar "red-purple-blue" pattern of color expression as pH increased from acidic to alkaline (**Figure 2**). Red hue colors were found in acidic condition, and then they gradually became colorless in mildly acidic condition as the anthocyanin structure undergoes pH-dependent transformation from red flavylium cation form to colorless carbinol pseudobase. These extracts and isolates exhibited various vivid purple, blue, and greenlish-blue colors in neutral to alkaline pH environments, where anthocyanin is predominant in quinonoidal base form (Brouillard & Delaporte, 1977). At pH 10, all the extracts quickly faded in color, except the isolated *trans* isomers which still retained an attenuated tinctorial strength. Although the overall pattern

"red-purple-blue" was the same, the colorimetric properties of black goji anthocyanins were entirely influenced by purification procedures, and acylation, in that there were pronounced discrepancies among these extracts and isolates throughout the whole pH ranges tested (**Figure 2**).

Due to the unknown molar absorptivity of the corresponding individual black goji anthocyanin, they were quantified as cyaniding-3-glucoside equivalent. Thus, a divergence in L* (lightness) and C* (chroma, saturation of the color) among extracts and isolates would be expected due to the different dilution factors for each pigment; but h* (hue angle) would still be comparable since h* was less variable in a small range of concentration. The colorimetric data are presented in **Table 1** and **Figure 2**.

The differences in L*, C* and h* among crude extract, C-18 purified extract, MCX purified extract, and *trans* isomer were small in acidic and mildly acid conditions from pH 3 to pH6. But this dissimilarity became very distinct between pH 7 and pH 9. As black goji passed through MCX cartridges, L* value increased and C* value decreased compared to that of crude extract, resulting in more pale and dull hues. However, there was no significant difference before and after C-18 cartridge purification in terms of the colorimetric property. It could be explained based on the chromatograph, as the polyphenolics and anthocyanin contents remained almost the same between the crude extract and C-18 purified sample, while the MCX cartridge considerably removed the interfering polyphenolics which were believed to impact anthocyanin color properties and stability by means of inter-molecular co-pigmentation (Gómez-Míguez et al., 2006; Malien-Aubert, Dangles, & Amiot, 2001). The removal of these polyphenolics would predictably alter the pigments color expression and other traits. On the other hand, the *trans* isomer displayed decreased L* and increased C* values compared to that of MCX purified extract, leading to a similar vivid and intensified purple or blue hues as crude extract. This similarity demonstrated the role of the *trans* isomer

as the major pigment in black goji and its leading contribution for black goji color expression. The *trans* isomer was even able to express color at pH 10 while other extracts faded away in color quickly after mixture. Nevertheless, the hue angle (h*) remained unchanged among all extracts and *trans* isomer.

A comparison between *trans* isomer and SPO demonstrated the importance of acylation in anthocyanin color expression (**Table 1** and **Figure 2**). The two isolates exhibited red hues in acidic and mildly acidic conditions, but acylation helped to strengthen and intensified the red color, as the C* values of *trans* isomer were larger than those of SPO. Compared to SPO, acylated petunidin suffered less color loss when pH arose from 3 to 6. The role of acylation affecting anthocyanins colorimetric properties are complicated and are suggested due to forming a folded structure called intramolecular co-pigmentation between acyl groups and chromophore (Galland, Mora, Abert-Vian, Rakotomanomana, & Dangles, 2007; M. Monica Giusti & Wrolstad, 2003; Malcıoğlu, Calzolari, Gebauer, Varsano, & Baroni, 2011; Malien-Aubert et al., 2001; Sigurdson, Tang, & Giusti, 2018; Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002). This co-pigmentation leads to steric hindrance and extension of the electron delocalization, protecting the chromophore from hydration (Goto & Kondo, 1991; Yoshida, Kondo, & Goto, 1992). The resulting increased pK_h (hydration constant) strengthens the color retention at higher pH. Therefore, SPO without acyl moiety protection would be more prone to hydration and structurally transformed into colorless carbinol pseudobase forms (George et al., 2001; M. Monica Giusti & Wrolstad, 2003). When pH increased to neutral and alkaline conditions, huge divergence in the h* value was found. The *trans* isomer showed purplish blue hues (267°-306°), whereas SPO expressed greenish blue hues (218°-274°). To this end, anthocyanin colorimetric properties were greatly influenced by the acylation on its structure.

3.4 Spectrophotometer properties of black goji extracts and isolates

Corresponding to the colorimetric data as discussed above, the spectrophotometric properties of black goji extracts were dependent on purity and pH (**Figure 3** and **Table 1**). At pH 3, the visible light absorption spectra of all the extracts and *trans* isomer displayed a unique single peak with the maximum absorbance found around 525 nm. Besides, the -3,5-glycosylation of black goji anthocyanin was well recognized by low ratio of $A_{440}/A_{vis\text{-}max}$ (Durst & Wrolstad, 2005). In mildly acidic condition from pH 4 to 6, the spectra flattened because of the anthocyanin structure hydration and the formation of colorless carbinol pseudobase. As pH further ascended to neutral and alkaline condition, bathochromic and hyperchromic shifts were observed, with the λ_{max} ranged from 558 nm at pH 7 to 578 nm at pH 9. While the MCX purified extract showed a similar absorption intensity at pH 3 as other extracts, a significant diminution in absorption was found from pH 7 to pH 9, corresponding to the relative pale and dull colors as described in previous section. All the extracts displayed a sharp peak in spectra when pH is greater and equal to 7, matching the vivid and highly intensified hues. Interestingly, a distinctive peak shoulder existed right to the λ_{max} at round 640 nm for all the extracts containing petunidin-3-*trans*-*p*-coumaroyl-rutinoside-5-glucoside, while it was absented in SPO.

There was an increasingly significant incongruity between *trans* isomer and SPO considering their visible light absorption spectra as pH arose from 3 to 9 (**Figure 3** and **Table 1**). The non-acylated petunidin derivative was documented by a single peak with λ_{max} being around 525 nm at pH 3, and a broad peak with $\lambda_{max} > 600$ nm above pH 8. The *trans* isomer, however, was characterized by its sharp peaks in the spectrum, which was linked to its vivid color expression. While the SPO exhibited greater λ_{max} than the *trans* isomer in alkaline conditions, it presented less λ_{max} throughout pH 3 to pH 6. Overall, the variations in λ_{max}

between the two isolates were small in acidic condition, but became considerable when pH was greater than 6.

3.5 Comparison of color stabilities among the black goji anthocyanin extracts and isolates

All the extracts and isolates were stored at refrigerated condition in dark for three weeks, and the color changes were expressed as ΔE (**Figure 4** and **Figure 5**). Black goji anthocyanin color stability was greatly influenced by its composition, acylation, and pH. Typically, black goji anthocyanins are stable at an acidic environment, and became vulnerable to degradation as pH increases. At pH 3 and 4, all of the extracts and isolates remained almost the same color in that ΔE's were < 5 throughout the testing time frame. At neutral and alkaline pH, the discrepancy enlarged. As expected, SPO degraded in the fastest pace among all the samples, while acylation significantly boosted stability from pH 7 to 9. Compared to other black goji extracts, the isolated *trans* isomer was extraordinary stable at alkaline condition as its purple-blue hue endured up to three weeks, illustrating the influential role of plant polyphenolics in anthocyanin color stability. The crude extract, C-18 purified extract, and MCX purified extract shared similar stability pattern, probably due to their similar anthocyanin profiles.

It is noteworthy to find that the stabilities of black goji extracts and isolates were superior at pH 8 than that in any other pH environment, which might be explained by their pH-dependent transformation in structure configuration. As pH arises from acidic to mildly acidic or neutral conditions, the red flavylium cation form either undergoes hydration and turns into colorless carbinol pseudobase, or bears deprotonation and exists in blue-purple quinoidal base form. As the pH further increases, the quinoidal base form could be ionized and becomes one or two negatively charged forms (Brouillard & Delaporte, 1977; He &

Giusti, 2010). A previous study has reported that the pK_{a2} and pK_{a3} (dissociation constants for the structure transformation from quinoidal base to one negatively charged form and from one to two charged form, respectively) of petunidin aglycones were pH 6.99 and 8.27 (León-Carmona, Galano, & Alvarez-Idaboy, 2016). Although these two numbers would not be the same as the dissociation constants of petunidin-derivative pigments in black goji, it is still rational to postulate that a larger proportion of two negatively charged quinoidal base forms exists at pH 8, and therefore the isolated pigments were more resistance to degradation in this condition. Nevertheless, the isolated *trans* isomers showed excellent stability at alkaline condition, demonstrating its potential capability as various stable blue-hues natural pigments in food application.

4. Conclusion

Black goji extracts contained abundant petunidin derivatives, with petunidin-3-*p*-coumaroyl-rutinoside-5-glucoside being the main pigment (~80% of the total pigment). The colorimetric and spectrophotometric traits of black goji anthocyanin were greatly influenced by purification procedures, pH, and acylation. MCX cartridge removed considerable polyphenolics from fruit extracts, and attenuated the saturation of color expression. The predominate petunidin-3-*trans*-*p*-coumaroyl-rutinoside-5-glucoside contributed most of the black goji anthocyanin color properties, and showed a better color stability compared to other extracts over time. Acylation not only strengthened the color retention in mildly acidic condition, but also enhance the tinctorial strength and stability of pigments. Nevertheless, these dissimilarities shed light on the chemical attributes that could be used to manipulate the color expression in natural pigment application.

This study demonstrated that black goji is a promising source for natural colorants, producing various vivid hues over a wide range of pH. The major pigment, petunidin-3-*trans*-

p-coumaroyl-rutinoside-5-glucoside, with strong stability and attracting vibrant hues, would broaden the choices of natural pigments in food industry, catering to the current trends of shifting from artificial colorant to natural alternatives.

5. Conflicts of interests

The authors were not aware of any conflicts of interests.

Figure and Table Captions:

Figure 1: Black goji anthocyanin extracts and isolates chromatograms at 520 nm and 280-700nm, and their identifications.

Figure 2: Color expression of black goji anthocyanin extracts and isolates at pH 3-10 (1 hour after mixing with buffers); a) overall colorimetric properties at various pH conditions; b) colorimetric data of MCX purified extract, *trans* isomers, and SPO as expressed on color space (a*, b*).

Figure 3: Spectral characteristics of black goji anthocyanin extracts and isolates at pH 3-10. Pictures were taken 1 hr after pH adjustment with the buffers.

Figure 4: Color changes of black goji extracts and isolates that were stored under refrigerated condition in dark within three weeks testing period. Pictures were taken 1 hr after pH adjustment with the buffers.

Figure 5: Color changes (described as ΔE) of black goji extracts and isolates at pH 3,4,7,8,9, and 10. Samples were stored under refrigerated condition in dark within three weeks testing period

Table 1: Colorimetric (CIE-L*, a*, b*) and spectrophotometric (λ_{max}) data of black goji anthocyanin extracts and isolates. Different superscripts indicate a significant difference ($p<0.05$) among extracts and isolates at certain pH condition.

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Black Goji as a Potential Source of Natural Color in a Wide pH Range

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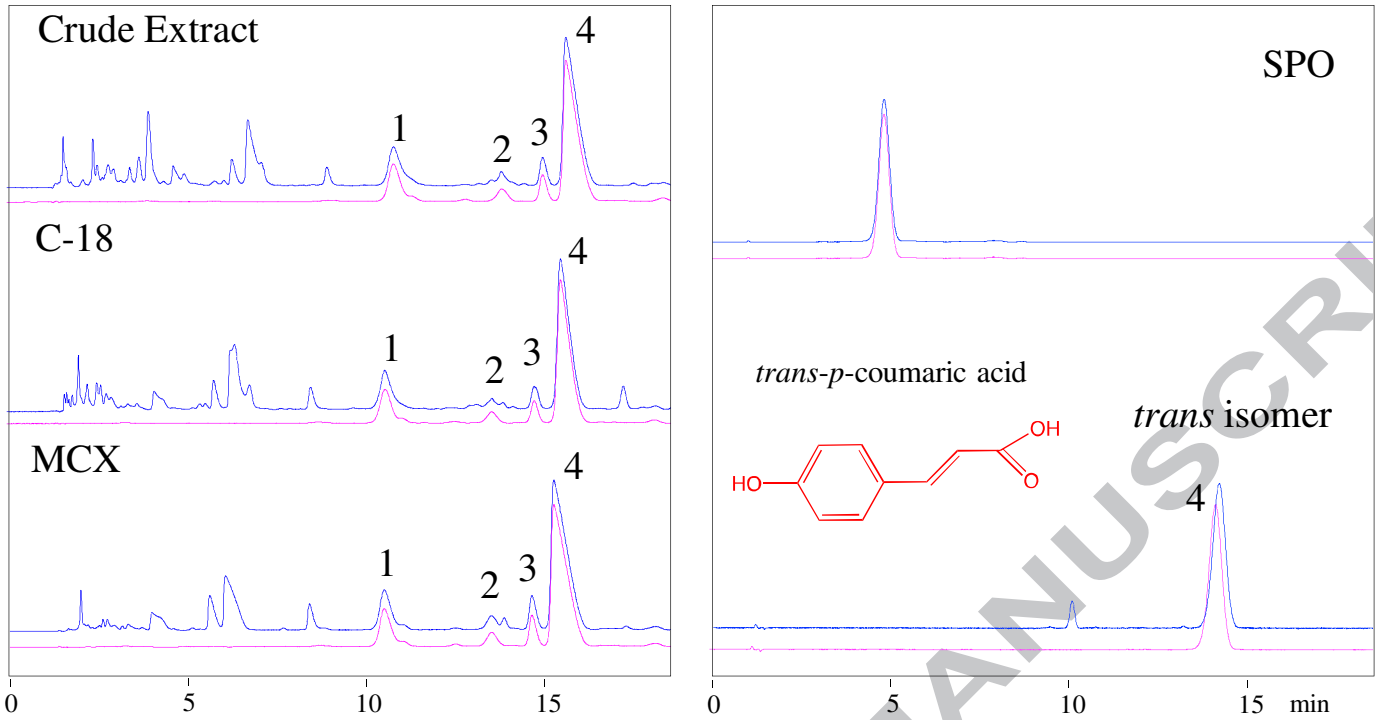

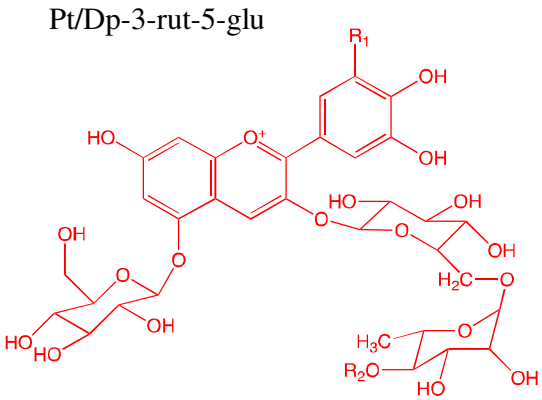

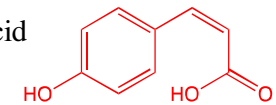

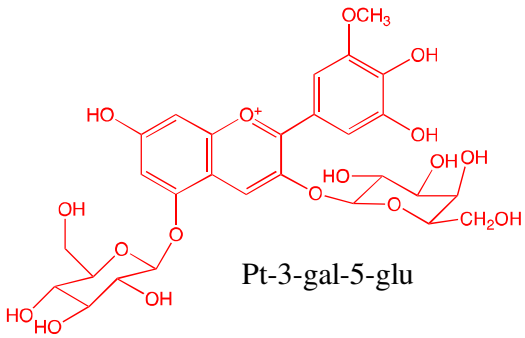

Peak	M⁺	Anthocyanin	R1	R2
1	641(317)	Petunidin-3-O-galactoside-5-O-glucoside	OCH_3	H
2	919(303)	Delphinidin-3-O-rutinoside-(*trans-p*-coumaroyl)-5-O-glucose	OH	*trans-p*-coumaric acid
3	933(317)	Petunidin-3-O-rutinoside-(*cis-p*-coumaroyl)-5-O-glucose	OCH_3	*cis-p*-coumaric acid
4	933(317)	Petunidin-3-O-rutinoside-(*trans-p*-coumaroyl)-5-O-glucose	OCH_3	*trans-p*-coumaric acid
SPO	787(317)	Petunidin-3-O-rutinoside-5-O-glucose	OCH_3	H

Figure 1: Black goji anthocyanin extracts and isolates chromatograms at 520 nm and 280-700nm, and their identifications.

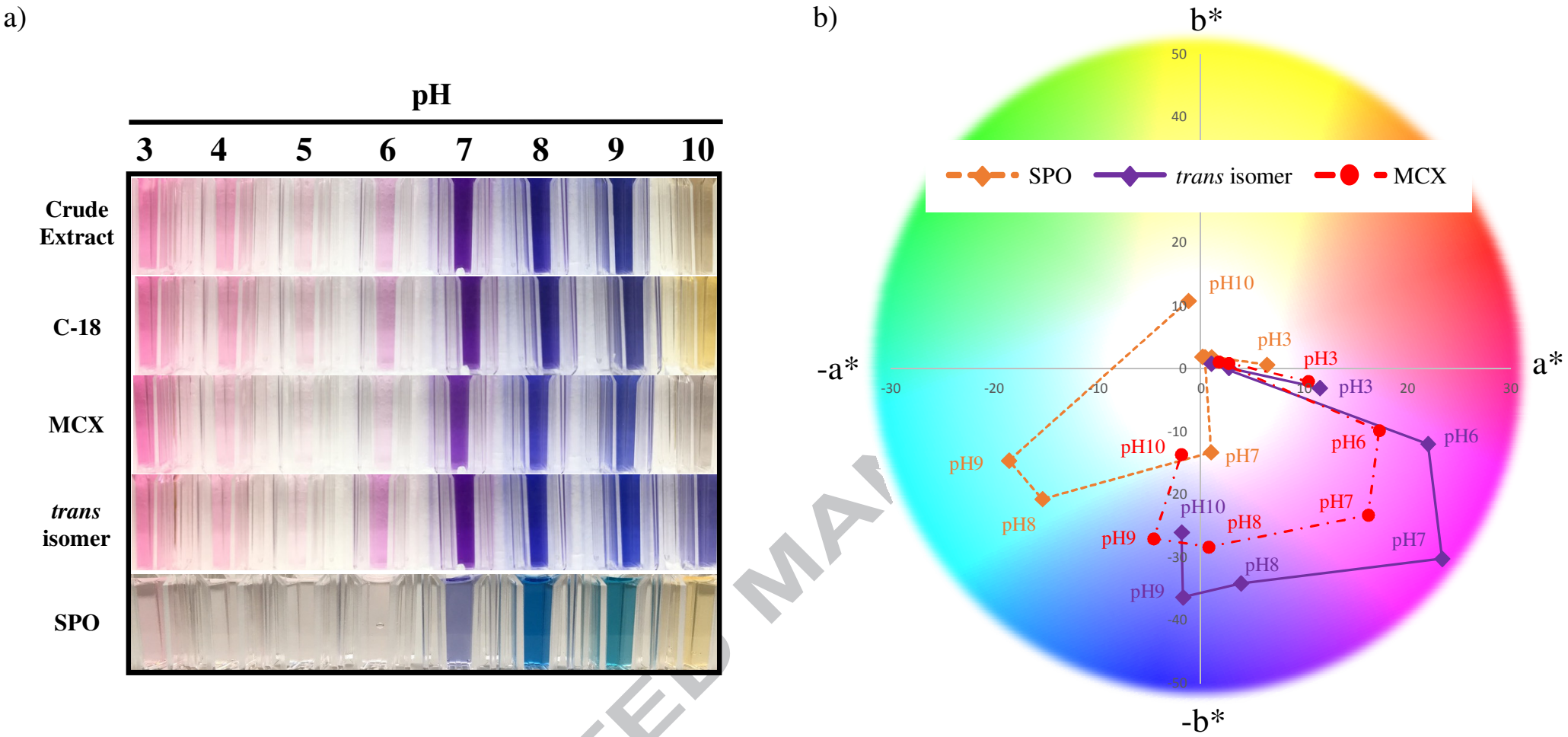

Figure 2: Color expression of black goji anthocyanin extracts and isolates at pH 3-10 (1 hour after mixing with buffers); a) overall colorimetric properties at various pH conditions; b) colorimetric data of MCX purified extract, *trans* isomers, and SPO as expressed on color space (a*, b*).

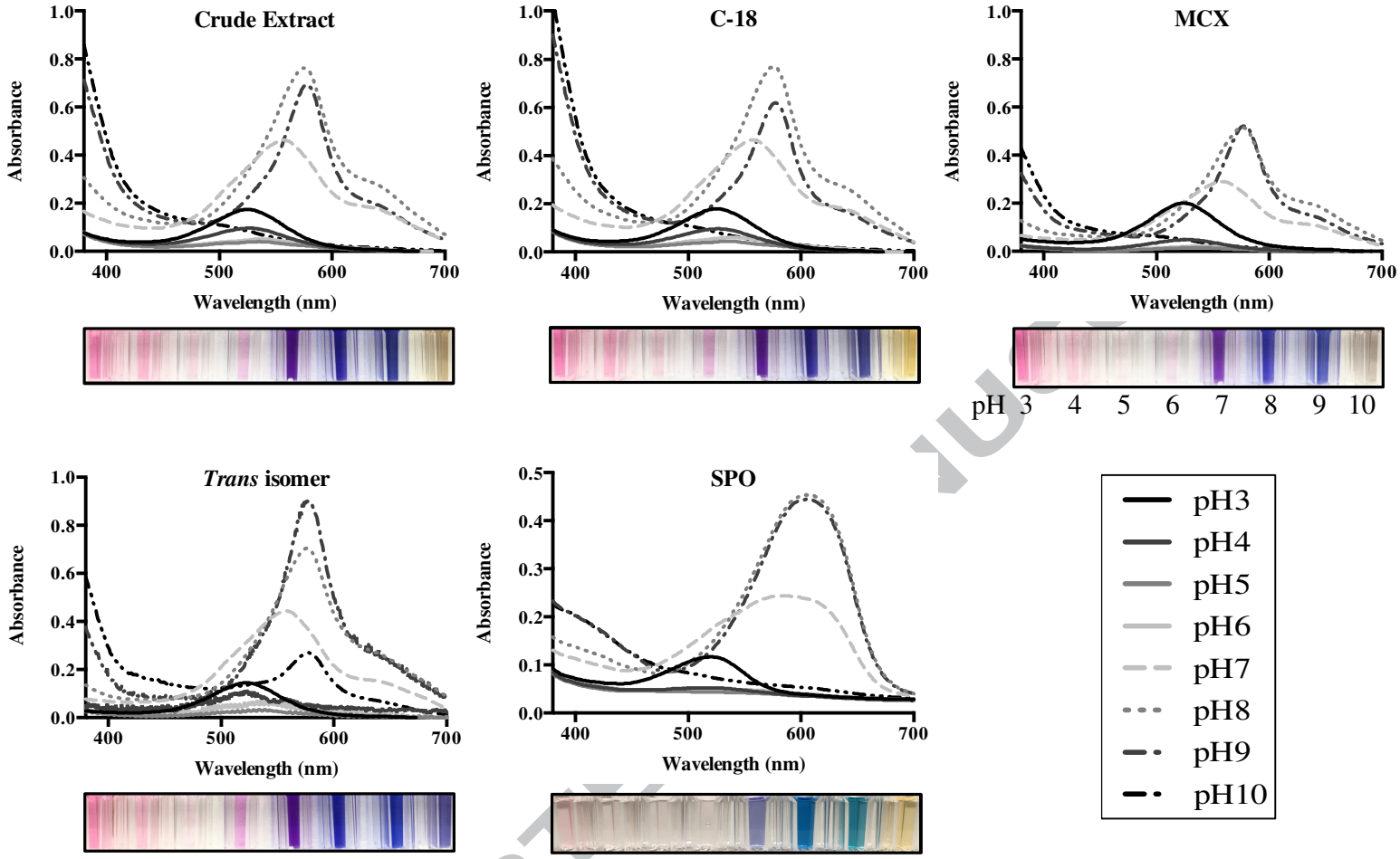

Figure 3: Spectral characteristics of black goji anthocyanin extracts and isolates at pH 3-10. Pictures taken 1 hr after pH adjustment with the buffers.

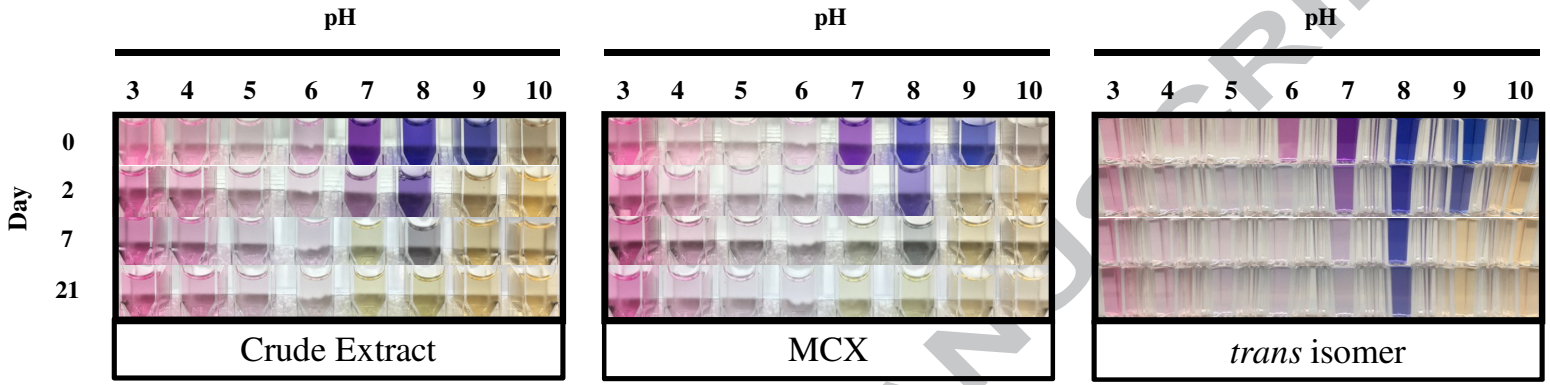

Figure 4: Color changes of black goji extracts and isolates that were stored under refrigerated condition in dark within three weeks testing period.

Pictures taken 1 hr after pH adjustment with the buffers.

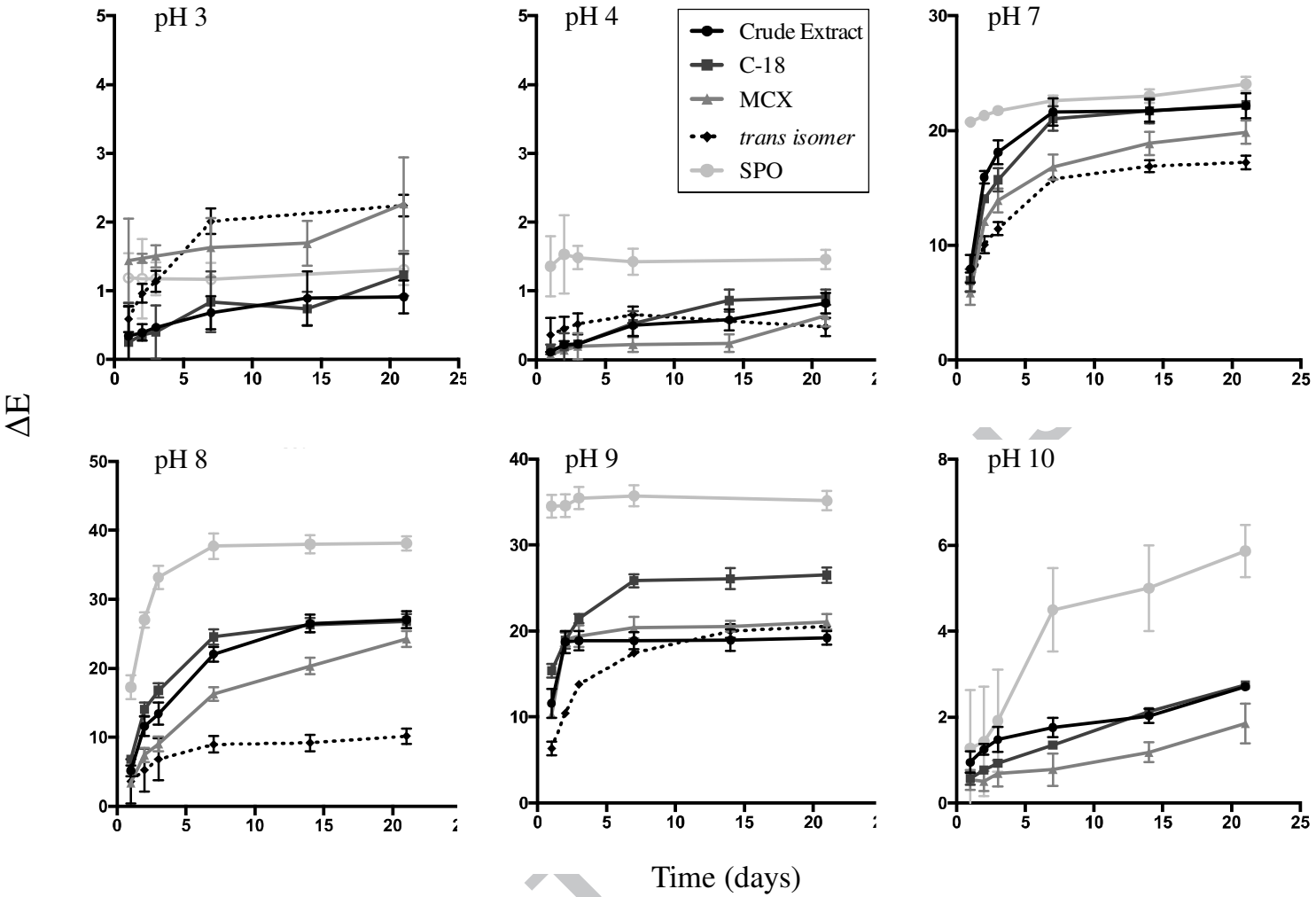

Figure 5: Color changes (described as ΔE) of black goji extracts and isolates at pH 3,4,7,8,9, and 10. Samples were stored under refrigerated condition in dark within three weeks testing period

Table 1: Colorimetric (CIE-L*, C*, h*) and spectrophotometric ($\lambda_{vis\text{-}max}$) data of black goji anthocyanin extracts and isolates. Different superscripts indicate a significant difference ($p<0.05$) among extracts and isolates at certain pH condition.

	pH3	pH4	pH5	pH6	pH7	pH8	pH9	pH10
				L* (lightness)				
Crude	90.7(2.1) ^{a}	93.1(2.1) ^{a}	93.8(1.4) ^{a}	82.2(2.1) ^{b}	70.4(1.4) ^{c}	68.0(1.1) ^{d}	68.9(2.3) ^{c}	76.6(1.3) ^{c}
C-18	90.4(3.1) ^{a}	93.0(1.1) ^{a}	93.6(1.1) ^{a}	81.9(1.1) ^{b}	70.2(0.1) ^{c}	68.1(1.2) ^{d}	69.7(1.4) ^{c}	77.1(1.1) ^{c}
MCX	93.2(1.1) ^{a}	95.4(1.2) ^{a}	96.0(1.1) ^{a}	95.8(1.1) ^{a}	88.7(2.2) ^{a}	85.9(1.2) ^{a}	87.2(1.1) ^{a}	94.9(1.2) ^{a}
trans	93.7(2.3) ^{a}	95.0(1.2) ^{a}	95.8(1.2) ^{a}	95.2(1.3) ^{a}	85.1(2.1) ^{b}	82.3(1.1) ^{b}	82.2(1.1) ^{b}	91.2(1.1) ^{a}
SPO	93.9(0.3) ^{a}	96.0(0.1) ^{a}	96.3(0.1) ^{a}	95.7(0.4) ^{a}	83.0(1.7) ^{b}	78.3(2.0) ^{c}	79.9(1.9) ^{b}	93.6(0.5) ^{a}
				C* (Chroma)				
Crude	11.1(1.1) ^{a}	4.9(0.5) ^{b}	3.5(1.1) ^{a}	33.5(1.5) ^{b}	41.4(1.9) ^{a}	38.7(1.9) ^{a}	32.4(1.3) ^{a}	20.8(1.7) ^{b}
C-18	11.4(0.9) ^{a}	5.0(0.5) ^{b}	3.7(1.1) ^{a}	39.7(1.7) ^{a}	40.7(1.4) ^{a}	36.4(1.1) ^{a}	24.8(1.9) ^{b}	27.7(1.1) ^{a}
MCX	10.6(2.1) ^{a}	8.2(0.3) ^{a}	2.0(1.3) ^{ab}	19.9(1.3) ^{d}	28.4(1.2) ^{b}	28.5(1.6) ^{b}	27.5(2.3) ^{b}	13.8(0.8) ^{c}
trans	11.9(1.1) ^{a}	2.7(0.1) ^{c}	1.4(0.9) ^{ab}	27.8(1.1) ^{c}	38.2(1.1) ^{a}	34.4(1.1) ^{a}	36.4(1.2) ^{a}	26.1(0.1) ^{a}
SPO	6.4 (0.5) ^{b}	2.1(0.2) ^{c}	0.9 (0.1) ^{b}	0.8(0.2) ^{e}	13.4(1.8) ^{c}	25.8(2.0) ^{b}	23.6(2.2) ^{b}	10.8(1.3) ^{c}
				h° (Hue angle)				
Crude	350.0(2.9) ^{a}	16.5(2.4) ^{c}	30.0(1.8) ^{c}	330.8(1.8) ^{b}	307.5(2.9) ^{a}	276.8(1.8) ^{a}	261.0(1.2) ^{b}	279.6(2.9) ^{a}
C-18	350.2(2.4) ^{a}	16.5(1.8) ^{c}	30.1(2.4) ^{c}	327.7(1.7) ^{a}	306.6(1.7) ^{a}	275.6(1.4) ^{a}	259.3(1.8) ^{b}	282.6(1.4) ^{a}
MCX	348.9(3.2) ^{a}	16.5(1.1) ^{c}	30.1(2.7) ^{c}	330.3(1.6) ^{b}	304.8(1.3) ^{a}	271.5(1.3) ^{b}	260.4(2.1) ^{b}	262.2(1.8) ^{c}
trans	343.5(2.5) ^{b}	123.8(1.3) ^{a}	39.3(1.3) ^{c}	330.6(1.7) ^{b}	306.0(1.1) ^{a}	275.8(1.2) ^{a}	267.0(1.2) ^{a}	266.8(2.1) ^{b}
SPO	5.3(1.8) ^{c}	60.2(2.0) ^{b}	85.2(3.5) ^{a}	76.9(0.3) ^{c}	274.2(1.2) ^{b}	233.5(1.0) ^{c}	218.3(0.4) ^{c}	96.3(0.8) ^{e}
				$\lambda_{vis\text{-}max}$				
Crude	525(0) ^{b}	527(0.7) ^{b}	536(0.7) ^{a}	537(0.7) ^{a}	557(0) ^{b}	575(0) ^{d}	578(0) ^{c}	NA
C-18	525(0.7) ^{b}	527(0) ^{b}	536(0.7) ^{a}	539(1.4) ^{a}	558(0.7) ^{b}	578(0) ^{c}	578(0) ^{c}	NA
MCX	525(1.4) ^{b}	527(0) ^{b}	536(0) ^{a}	539(0) ^{a}	558(0) ^{b}	578(0) ^{c}	578(0) ^{c}	NA
trans	523(0) ^{b}	529(0) ^{b}	535(1.2) ^{a}	540(0.7) ^{a}	558(0) ^{b}	578(0) ^{c}	578(1.2) ^{c}	578(0) ^{b}
SPO	519(1.2) ^{c}	514(0.6) ^{c}	NA	511(1.9) ^{c}	582(2.1) ^{a}	604(0.6) ^{b}	604(1.2) ^{b}	NA

Black Goji as a Potential Source of Natural Color in a Wide pH Range

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Highlights

- Black goji was rich in pt-3-p-coum-rut-5-glu (~85% of total pigment).
- color and spectra were greatly influenced by purity, pH and acylation.
- Acylation increased color retention, tinctorial strength and pigment stability.
- Pure pt-3-p-coum-rut-5-glu showed improved color stability over time.
- Black goji produced vivid hues over a wide pH range and could serve as colorant.