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Direct analysis of anthocyanins-rich Mao fruit juice using sample dilution method based on chromophores/fluorophores of both cyanidin-3-glucoside and pelargonidin-3-glucoside

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Abstract

Anthocyanins are mostly classified as natural colorants in fruit, vegetables and other plant materials. The anthocyanin-rich foods or food products are of interest because of their health benefits. In Thailand, "Mao" (*Antidesma thwaitesianum* Muell. Arg.) the wild berry fruit with reddish violet pigment at ripening stage are well known as high antioxidants according to anthocyanins and polyphenolics content. Total anthocyanins in some Mao juice samples determined by pH-differential method (AOAC method 2005.02) were ranged between 35.1-578 mg/L Cya-3-glu equiv. and 22.6-357 mg/L PGD-3-glu equiv. depending on their chromogenic absorption. In this study, direct analysis of the anthocyanins in an appropriate diluted juice was developed attributing for the photoextinction of chromophores/fluorophores of the two standards by UV-Visible and fluorescence spectrophotometry. Concerning on their spectral characteristics of Cya-3-glu and PGD-3-glu, both of their calibration curves were constructed resulting in similar trends of the pigments determined, but gave too high contents according to its dilution factor, compared with those of the standard method. In fact, since their dilution factor and recovery study are certainly validated, the developed methods are undoubtedly sensitive, even if, due to negligibly biased spectral interferences. Advantage of the specific extinction of any major anthocyanins in the juice samples with at least 10^3-fold dilution factor is, therefore, unexpectedly reported.

Introduction

"Mao" (*Antidesma thwaitesianum* Muell. Arg.) is a wild tree found in the north-eastern area of Thailand and its compiled fruits are used for juice, wine and healthy food. Whole taste of the fruits are sour, much like cranberries, when unripe (green), acidic flavor and slightly sweet when fully ripe (orange-red turns to dark-purple). A recent study showed that the Mao juice was rich in the phenolics, flavonoids and ascorbic acid contents and correspondingly exhibited high antioxidant activity compared with other fruit juices (Sripakdee *et al*., 2015). Moreover, strong antioxidant properties of polyphenolics were found in its seed and marc extracts (Puangpronpitag *et al.*, 2008). This antioxidant activity is mostly related to its high anthocyanin content (Kukongviriyapan *et al*., 2015). It is reported that the Mao Luang fruits *(Antidesma bunius)* are rich in nutritional components such as carbohydrates, sugars, organic acids, proteins, minerals, vitamins, anthocyanins, flavonoids and phenolic acids (Butkhup and Samappito, 2008). Therefore, the fruit juice is another product that becomes more popular among Thai consumers as healthy nourishment (Nuengchamnong and Ingkaninan, 2010).

Anthocyanins are the natural colorants responsible for the red, blue, and purple colors of many fruits, vegetables and plants. They have several biological activities, including antioxidant, anti-inflammatory, anti-tumor, neuroprotective, anti-diabetic and cancer chemopreventive agents (Tarozzi *et al*., 2007; Sarić *et al*., 2009; Azevedo *et al*., 2010; Yao *et al*., 2010; Yang *et al*., 2010; Bishayee *et al*., 2011; Sun *et al*., 2012; Tremblay *et al.*, 2013; Rakić *et al*., 2014). Additionally, they are widely used as colorants in food industry (Arnnok *et al*., 2012). Anthocyanins are a subclass of flavonoids and represent one of the most widely distributed classes of flavonoids in various plants (Bondre *et al*., 2012). They are glycosides of anthocyanidins, which vary with different hydroxyl or methoxyl substitutions at the 3 and 5 positions on the A and C rings in their basic flavylium structure (Lee *et al*., 2008; Tang *et al*., 2014). The difference in chemical structure and color of anthocyanins depends on several factors including pH, temperature, light intensity, amount of pigments, metallic ions, ascorbic acid and sugars (glucose, galactose, rhamnose,

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xylose and arabinose). Moreover, their variations by acylation of the sugar groups with acids occur. In acidic conditions, there are four anthocyanin structures present in equilibrium: flavyliumcation, quinonoidal base, carbinol pseudobase and chalcone (Lee *et al*., 2008; Cevallos-Casalsand Cisneros-Zevallos, 2004). Anthocyanins can commonly be found in numerous fruits, especially various kinds of berries, their juices as well as wines (Jakobek *et al*., 2007; Roy *et al*., 2009).

Evaluation of total anthocyanins in fruits and other plants are usually relied on spectrophotometric methods or, more accurately, HPLC analysis (Agati *et al*., 2013; Rakić *et al*., 2015). UV-Visible spectrophotometry is one of the most widespread of anthocyanin spectroscopic methods. The method was also applied for the structural change of anthocyanin under the influence of different physicochemical factors and the process of polymerization of anthocyanins (Drabentet *et al*., 1999). Since their color and stability depend on several factors, much attention has been focused on the development of sensitive and reliable analytical methods for nondestructive quantification of anthocyanins in fruit juices (Shah *et al.*, 2013). Fluorescence spectroscopy of anthocyanins has been less studied. In general, anthocyanins are weakly fluorescent in solution, probably due to quenching of the efficient excited state proton transfer to water. Lack of information of anthocyanins luminescence related to their pigments has been limited in the literature (Drabent *et al.*, 1999). Fluorescence properties of the chalkone isomer of malvidin 3,5-diglucoside in aqueous solution was studied (Figueiredo *et al.*, 1990). It was found that long-wavelength fluorescence (centered at 495 nm) observed in the fluorescence spectra of cis-chalcone is ascribed to emitting species formed during the excited state of the chalcone form (Figueiredo *et al*., 1990; Lima *et al.*,1994). The fluorescence of cyanidin and malvidin glycosides in aqueous environment was investigated and found that Cya-3-glc exhibits short-wavelength fluorescence at λ_{max}299 nm which was most effectively excited at 220 nm (Drabent *et al.*, 2007). Similar short-wavelength fluorescence was observed for Cya-3,5-diglc (λ_{max}308 nm) and Mv-3,5-diglc (λ_{max}293 nm) in a binary solvent system. Moreover, the fluorescence approach for measuring anthocyanins and derived pigments in red wine was also reported (Agati *et al.*, 2013).

The fluorescence is generally recognized as highly sensitive, more selective and nondestructive method as well. It is useful tool for monitoring of anthocyanins and/or polyphenolics (Agati *et al*., 2005; Agati *et al*., 2013; Rakić *et al.*, 2015). Therefore, the aim of this study was to determine total anthocyanins in the Mao juice from various production sources using sample dilution method in association with an external calibration curve under optimum conditions by UV-Visible and fluorescence spectrophotometry comparing with pH-differential method (AOAC method 2005.02).

Materials and Methods

Chemicals and instruments

Cyanidin-3-glucoside chloride (Cya-3-glu) was purchased from Sigma (Germany) and pelargonidin-3-glucoside chloride (PGD-3-glu) was purchased from Sigma (France). Potassium chloride, sodium hydrogen carbonate and sodium hydroxide were purchased from Carlo Erba (Italy). Sodium acetate anhydrous, di-potassium hydrogen phosphate anhydrous and potassium dihydrogen phosphate were purchased from QRëC® (New Zealand). All chemicals and solvents used such as methanol, acetic acid and hydrochloric acid were of analytical grade.

UV-Visible spectrophotometer (Agilent 8453, USA) and spectrofluorophotometer (RF-5301PC, Shimadzu, Japan) were used. pH meter (Proline B210, China) was used to measure pH solution of fruit juice. The absorption spectra were recorded using UV-visible spectrophotometer. Fluorescence measurements (excitation and emission spectra) were performed using a spectrofluorophotometer with slit widths of 5 and 10 nm for an excitation and an emission. A rectangular quartz cuvette with 1.0 cm optical path length was used for both spectrophotometry. All experiments were performed in triplicate at ambient temperature.

Sample preparation

In this work, a dilution method was used for sample preparation for direct analysis of total anthocyanins in Mao juices. The fruit juice samples from five different production sources: JS1: Chaiyaphum; JS2: Maoluang Saitong; JS3: Khok Srisuphan; JS4: Maoluang Phuphan; JS5: Chang Palangsong. There are three brands from Phuphan district (Maoluang Phuphan, Chang Palungsong and Maoluang Saitong) and one brand from Khok Srisuphan district, Sakon Nakhon province, and the other is from Chaiyaphum province, Thailand. These juices were diluted by an acidified methanol and an aqueous buffer solution (containing 12% methanol). The diluted sample solutions were used for evaluation of total anthocyanins by UV-Visible spectrophotometer and spectrofluorophotometer.

Effect of pH on color change of the Mao juice

It is widely known that stability of anthocyanins depends on pH of solution. Anthocyanin reversibly changes its color by varying of pH of the solution. In acidic media, anthocyanin exists as the flavylium ion only (AH^+). In base solutions, the quinonoidal base may be present as an anion A^- (Drabent *et al.*, 2007). The sample solution was diluted with buffer solutions of pH 1-11. The electrolyte solutions for pH 1-2 were prepared with 0.2MKCl and 0.2 M HCl. Acetate buffer solutions of pH 3-5 were prepared from 0.2 M CH_3COOH and 0.2 M CH_3COONa. Phosphate buffer solutions of pH 6-8 were consisted of 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 and carbonate buffer solutions of pH 9-11 were prepared from 0.05 M$NaHCO_3$ and 0.1 M NaOH. The certain pH value was measured with a pH-meter. Changes in colored sample solutions were measured spectrophotometrically. UV-Visible absorption spectrum was recorded at wavelength of maximum absorption for each sample. Fluorescence intensity of both Cya-3-glu and PGD-3-glu was measured at 306 nm with the excitation at 277 nm, while the fluorescence intensity of the sample solution was detected at 309 nm with the excitation at 280 nm.

Evaluation of total anthocyanins in Mao juice

pH-differential method

Total anthocyanins were determined using the pH-differential method (Lee *et al.,* 2005). This method is based on Lambert-Beer's law: $A = \varepsilon cl$. The juice samples were diluted in the solution pH 1.0 (0.025 M KCl) and the acetate (0.4 M) buffer solution pH 4.5. The certain pH values were adjusted to pH 1.0 and 4.5 with a drop wise of strong HCl or NaOH solution. The absorbance of each appropriate dilution of the fruit juices: Chaiyaphum 1:40 (v/v); Maoluang Phuphan 1:50 (v/v); Chang Palungsong 1:200 (v/v); Maoluang Saitong 1:20 (v/v) and Khok Srisuphan 1:20(v/v) was measured at their maximum wavelengths (λ_{max}) in the visible region and at 700 nm for haze background correction. The absorbance (A) of the diluted samples was calculated as followed:

$$A = (A_{\lambda max} - A_{700})\text{pH 1.0} - (A_{\lambda max} - A_{700})\text{pH4.5}$$

where, $A_{\lambda max}$ is the absorbance at the maximum wavelength in the visible region. The total anthocyanin expressed as Cya-3-gluand PGD-3-gluequivalents were calculated with the following formula:

$$Totalanthocyanins\ (mg/L) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

where, A is the absorbance obtained from pH-differential; MW is the molecular weight, 484.84 g/mol for Cya-3-glu and 464.84 g/mol for PGD-3-glu; DF is dilution factor; l is path length in cm; ε is molar extinction coefficient, 26,900 for Cya-3-glu and 31,620 for PGD-3-glu in $Lmol^{-1}cm^{-1}$; 10^3 is a factor for conversion from g to mg. The total anthocyanins are expressed as mg/100 mL of the juice sample.

Determination of total anthocyanins by UV-Visible spectrophotometry

Total anthocyanins are calculated as Cya-3-glu equivalent or PGD-3-glu equivalent by means of their calibration curve obtained. Standard solutions of Cya-3-glu (5.0-30 mg/L) in an acidified methanol (1%HCl in methanol) and PGD-3-glu (5.0-25 mg/L) diluted with 1%HCl in deionized water were used for each calibration curve. The absorbance of each sample dilution including Chaiyaphum 1:20 (v/v); Maoluang Phuphan 1:100 (v/v); Chang Palangsong 1:200 (v/v); Maoluang Saitong 1:20 (v/v) and Khok Srisuphan 1:40 (v/v) was measured at 528 nm and 497 nm for Cya-3-glu and PGD-3-glu, respectively. Total anthocyanins were expressed mg/100 mL Cya-3-glu and PGD-3-glu equivalent.

Determination of total anthocyanins by spectrofluorophotometry

Cya-3-glu and PGD-3-glu were chosen as the reference compounds. They were diluted in 12% methanol solution pH 2.0 (Agati *et al.*, 2013). This pH was chosen according to preliminary study on the effect of pH on fluorescence intensity of the anthocyanins. Excitation spectra were recorded from 220 nm to 300 nm for the emission at 306 nm, whereas emission spectra were measured between 310 nm and 450 nm with the excitation at 277 nm. For each sample, excitation spectra were also recorded from 220 nm to 300 nm for the emission at 309 nm, whereas emission spectra were measured between 310 nm and 450 nm with the excitation at 280 nm. Both the excitation and the emission slit width of monochromator were adjusted to 5 and 10 nm, respectively.

Cya-3-glu (1.0-5.0 mg/L) and PGD-3-glu (0.5-2.0 mg/L) were diluted in buffer solution pH 2.0 for plotting of their calibration curves. All samples were diluted with the same solvent. For both standard and sample solutions, the fluorescence spectra were recorded with the excitation/emission of 277/306 nm for Cya-3-glu and the excitation/emission of 282/311 nm for PGD-3-glu by spectrofluorophotometer. All experiments were performed in triplicate at ambient temperature.

Method validation

The external calibration method of both UV-Visible spectrophotometry and spectrofluorophotometry was validated in terms of linearity, accuracy and precision (IUPAC, 2002).

Results and Discussion

Effect of pH on color change of anthocyanins

The changes in color of anthocyanins of the Mao juices were varied upon pH range of the buffer solutions used (data not shown). Generally, difference in absorbance of anthocyanins at an appropriate maximum wavelength is related to its content between pH 1.0 and pH 4.5 as the pH-differential method (AOAC, 2005.02). These plant pigments have red-orange at lower range of pH values (pH 1-5). Under acidic pH, the anthocyanin exists primarily in the form of flavylium cation in red (Amelia *et al*., 2013). By stepwise pH increase until 8.0, the color gradually changes toward more bluish tones due to a rapid loss of proton to form quinoidal base. Moreover, the increasing of pH (pH > 9) causes the hydration of the flavylium cation to form a carbinal (pseudobase) or chalcone, which are given yellowish shade or faintly colored form (Fossen *et al*., 1998; Darias-Martín *et al*., 2002; Tirupula *et al.*, 2009). Furthermore, the absorbance readings for all samples exhibited high absorption in the pH range of 1-2 (Figure 1A) in accordance with fluorescence data of the samples, which showed high fluorescence intensity in the same pH range (Figure 1C). From Figure 1B, these results showed no significant difference in the fluorescence intensity of anthocyanins (Cya-3-glu and PGD-3-glu), also giving high intensity in the pH range of 1-2. According to the results, the solution pH 2 was chosen as a suitable solvent for the determination of total anthocyanins.

Total anthocyanins in the Mao juices

In this work, the total anthocyanins of Mao juices were comparatively determined by two aspects of spectrophotometric method including absorption and fluorescence. The pH-differential method (AOAC 2005.02) and external calibration curve both were carried out by UV-Visible spectrophotometry. The other method was conducted by spectrofluorophotometry also expressed as external calibration curve. The absorption spectra of Cya-3-glu and PGD-3-glu are presented in Figure 2(A-B). At the pH 1.0, the absorption maximum of Cya-3-glu in an acidified methanol was shown at 528 nm, while that of PGD-3-glu in an acidified solution appeared at 496 nm. Both two maximum peaks of

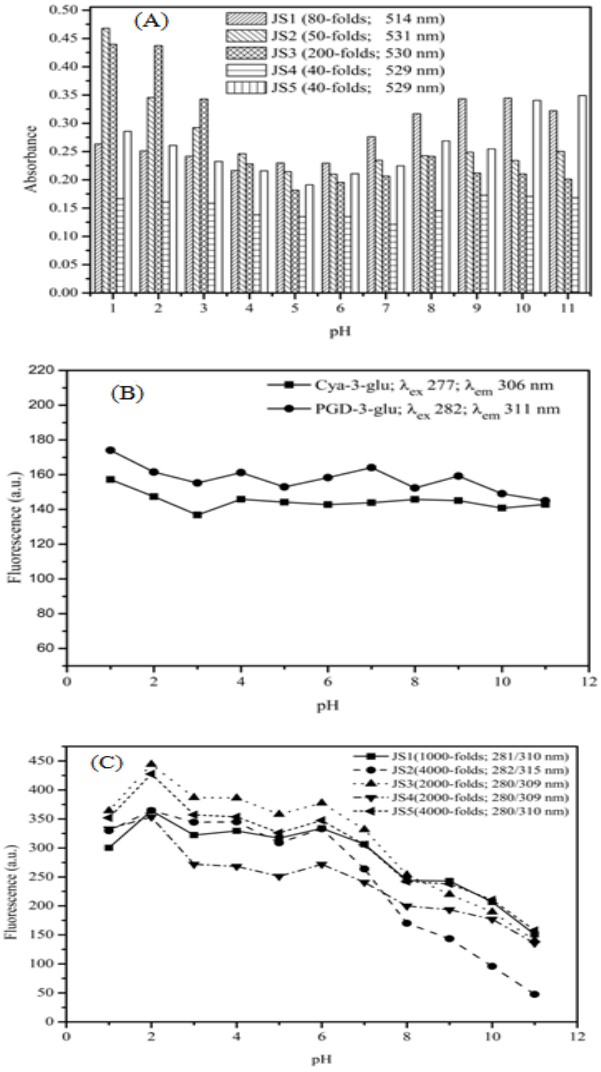

Figure 1. Effect of pH on (A) absorbance of anthocyanins in Mao juice solutions, (B) fluorescence intensity of Cya-3-Glu and PGD-3-Glu solutions, and (C) fluorescence intensity of different dilution folds of Mao juice samples at $\lambda_{ex}/\lambda_{em}$: 280-282/309-315 nm

Cya-3-glu and PGD-3-glu correspond to previous report (Santiago *et al.*, 2014), at which of this pH the flavylium cation is essentially the only existing in the solution (Brouillard, 1982; Mazza and Brouillard, 1987).

The total anthocyanins of five samples of Mao juices obtained from three methods are summarized in Table 1. For pH-differential method, the Mao juice of Chang Palangsong showed the highest amounts of anthocyanins (578 mg/L Cya-3-glu equiv. and 357 mg/L PGD-3-glu equiv.), followed by Maoluang Phuphan (269 mg/L & 165 mg/L), Khok Srisuphan (73.5 mg/L & 41.9 mg/L), Maoluang Saitong (50.5 mg/L & 29.3 mg/L) and Chaiyaphum (35.1 mg/L & 22.6 mg/L). It is clearly shown that the Mao juices obtained from different production sources have different anthocyanins contents, probably due to

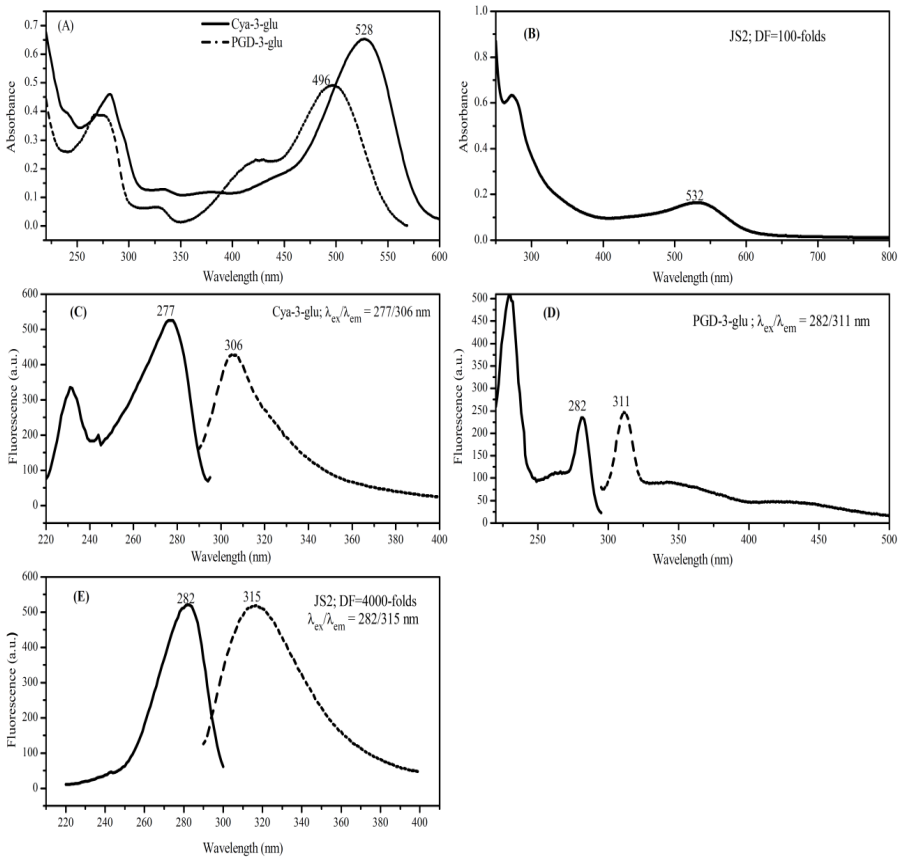

Figure 2. Absorption and fluorescence spectral characteristics of two standard anthocyanins and Mao juice sample (JS2): (A) Absorption spectra of Cya-3-glu (λ_{max} 528 nm) and PGD-3-glu (λ_{max} 496 nm), (B) Absorption spectrum (λ_{max} 531 nm) of JS2, (C) and (D) Fluorescence excitation and emission spectra of PGD-3-glu ($\lambda_{ex}/\lambda_{em}$: 282/311 nm) and Cya-3-glu ($\lambda_{ex}/\lambda_{em}$: 277/306 nm), respectively, and (E) Fluorescence excitation and emission spectra of JS2 ($\lambda_{ex}/\lambda_{em}$: 282/315 nm)

their maturity of the berries. Since these Mao juices are commercially available, it is rather difficult to trace back through their production process. Further study of its maturity would be clarified in detail. However, as compared with other fruit juices using pH-differential method, the content of anthocyanins of Chang Palangsong sample was higher than that of strawberry (13.6 mg/L), raspberry (336.7 mg/L), sour cherry (369.4 mg/L) and sweet cherry (256.6 mg/L) (Lee *et al*., 2005; Jakobek *et al.*, 2007).

The results obtained from the external calibration curve by UV-Visible spectrophotometry (Table 1) also gave similar trends, but higher (about a few up to 10 folds) than those determined by pH-differential method. Since the pH-differential method was specified only the chromophores of monomeric anthocyanins (as flavylium cation) absorbed at pH 1.0, while those of the external calibration curve method under the optimized conditions were generally included both monomeric and polymeric anthocyanins (Humadi and Istudor, 2009).

Interestingly for fluorophore attribution of the diluted sample, Cya-3-glu and PGD-3-glu were chosen as the reference compounds. The fluorescence characteristics of both Cya-3-glu and PGD-3-glu, and the diluted juice solution at pH 2.0 were investigated in details. It was found that the excitation and emission maximum wavelengths of both Cya-3-glu and PGD-3-glu were at 277 nm and 306 nm, respectively, while those of excitation and emission of the juice samples were found a few longer in the ranged of 280-283 nm and 310-312 nm, respectively, indicating almost similar fluorophores of anthocyanins (Figure 2C-E). Thus, it was implied that the excitation wavelength of 277 nm and emission wavelength of 306 nm were used for determination of total anthocyanin in Mao juices. However, the total anthocyanins of the Mao juices were found to be very high amounts, more than 100-folds compared with those of the two absorption methods mentioned above (Table 1). Question was raised up for any specific fluorophores that attributing for the spectral bias of the method, although this method was validated by the recovery study of the matrix effects and linearity of both calibration curves used. In fact, sensitivity of the fluorophores in a diluted solution is much more prominent (about

Table 1. The contents (mg/L) of total anthocyanins in Mao juice samples determined by pH-differential method and both calibration curves using UV-Visible spectrophotometry and spectrofluorophotometry

Juice sample	pH-differential method		Calibration curve using UV-Visible spectrophotometry		Calibration curve using spectrofluorophotometry	
	Cya-3-glu	PGD-3-glu	Cya-3-glu	PGD-3-glu	Cya-3-glu	PGD-3-glu
JS1	35.1 ± 7.9	22.6 ± 5.9	281.4 ± 11	237.4 ± 9.9	1,523 ± 42.2	5,487.7 ± 458
JS2	50.5 ± 5.7	29.3 ± 2.9	206.4 ± 4.6	150.1 ± 2.1	3,555 ± 90.4	7,425.1 ± 66.9
JS3	73.2 ± 4.2	41.9 ± 4.5	332.7 ± 6.9	241.3 ± 3.7	8,087 ± 283	27,175 ± 149
JS4	269 ± 41	165 ± 30	726.9 ± 1.8	580.7 ± 3.8	6,770 ± 123	19,365 ± 842
JS5	578 ± 55	357 ± 43	1,950 ± 12	1,146 ± 4.6	4,210 ± 67.3	13,340 ± 737

JS1: Chaiyaphum; JS2: Maoluang Saitong; JS3: Khok Srisuphan; JS4: Maoluang Phuphan; JS5: Chang Palangsong

Table 2. Linear equations for two calibration curves of the standard anthocyanins used and the curves of dilution factor of the Mao juice samples

Anthocyanins / Juice solution	Concentration range (mg/L)/ Dilution factor (fold)	UV-Visible spectrophotometry Linear equation	Correlation coefficients (R^2)	Concentration range (mg/L)/ Dilution factor (fold)	Spectrofluorophotometry Linear equation	Correlation coefficients (R^2)
Calibration curve						
Cya-3-glu	5.0-30.0	y = 0.04824x + 0.0013	0.998	1.0-5.0	y = 142.66x + 163.89	0.997
PGD-3-glu	5.0-25.0	y = 0.0454x + 0.0169	0.994	0.5-2.0	y = 142.96x + 121.41	0.998
Curve of dilution factor						
JS1	200-1600	y = 0.013x + 0.007	0.998	1000-64000	y = 0.2059x + 135.10	0.999
JS2	200-1600	y = 0.0261x + 0.0054	0.997	1000-64000	y = 0.7201x + 161.61	0.997
JS3	200-1600	y = 0.0266x + 0.0137	0.993	1000-64000	y = 0.4135x + 149.60	0.993
JS4	200-1600	y = 0.0297x + 0.0017	0.999	1000-64000	y = 0.3452x + 144.83	0.994
JS5	200-1600	y = 0.0144x + 0.00057	0.997	1000-64000	y = 0.7679x + 162.60	0.994

JS1: Chaiyaphum; JS2: Maoluang Saitong; JS3: Khok Srisuphan; JS4: Maoluang Phuphan; JS5: Chang Palangsong

Table 3. The recovery results of Mao juice samples obtained from direct analysis of their dilute solution by UV-Visible spectrophotometry and spectrofluorophotometry

Juice sample	UV-Visible spectrophotometry Recovery (%)				Spectrofluorophotometry Recovery (%)			
	Dilution fold/ Spiked level (mg/L)	Cya-3-glu	Dilution fold/ Spiked level (mg/L)	PGD-3-glu	Dilution fold/ Spiked level (mg/L)	Cya-3-glu	Dilution fold/ Spiked level (mg/L)	PGD-3-glu
JS1	40/5	54.40	40/2.5	79.80	1000/1.0	60.50	5000/0.5	25.10
	40/10	91.16	40/5	85.71	1000/2.5	66.22	5000/1.0	27.22
	40/30	93.40	40/25	88.00	1000/5.0	80.10	5000/2.0	49.30
JS2	100/5	66.97	100/2.5	92.28	4000/1.0	37.83	20,000/0.5	24.72
	100/10	94.40	100/5	94.25	4000/2.5	78.51	20,000/1.0	36.00
	100/30	96.61	100/25	101.5	4000/5.0	80.47	20,000/2.0	50.00
JS3	40/5	51.33	40/2.5	71.68	2000/1.0	66.28	10,000/0.5	28.34
	40/10	74.44	40/5	83.59	2000/2.5	68.50	10,000/1.0	35.32
	40/30	94.71	40/25	89.65	2000/5.0	74.48	10,000/2.0	49.41
JS4	20/5	60.19	20/2.5	65.80	2000/1.0	68.61	5000/0.5	24.71
	20/10	64.59	20/5	91.42	2000/2.5	73.02	5000/1.0	34.17
	20/30	96.42	20/25	91.44	2000/5.0	78.79	5000/2.0	50.01
JS5	40/5	60.23	40/2.5	88.43	4000/1.0	48.35	20,000/0.5	27.64
	40/10	90.75	40/5	91.14	4000/2.5	59.86	20,000/1.0	31.27
	40/30	94.71	40/25	97.86	4000/5.0	65.57	20,000/2.0	48.23

JS1: Chaiyaphum; JS2: Maoluang Saitong; JS3: Khok Srisuphan; JS4: Maoluang Phuphan; JS5: Chang Palangsong

10^3 folds) than that of the chromophores of the compounds. The results are, therefore, agreed with these chromophores/fluorophores concerns.

The linearity was evaluated by creating external calibration curves for each reference compound and the dilution factor of the samples. Both the reference compounds and the sample solutions showed high correlation coefficients (R^2) better than 0.995 (Table

2). These results indicated that the external standard calibration could be applied for quantification of total anthocyanins.

Accuracy of the method was evaluated by recovery study of total anthocyanins by standard addition method at three spiking levels (low, medium and high of their calibration curve) of each standard anthocyanin as shown in Table 3. From the results, the recoveries were obtained within the range of 51-96% (as Cya-3-glu equiv.) and 72-101% (as PGD-3-glu equiv.) for the UV-Visible spectrophotometry and the range of 38-81% (as Cya-3-glu equiv.) and 25-50 (as PGD-3-glu equiv.) for spectrofluorophotometry.

Conclusion

A sample dilution method for determination of total anthocyanins from Mao juice was successfully developed and validated using both UV-Visible spectrophotometry and spectrofluorophotometry. The method was applied to the Mao juices from different sources. These results were compared with those of the pH-differential method as standard method. Interestingly, both external calibration curves of the two standard anthocyanins using spectrofluorophotometry for determining total anthocyanins in the Mao juice samples gave abruptly very high content ranges, negligibly spectral bias of the method. It is evident that this direct analysis associated with diluted sample solution using such a facile spectrophotometry can be considered for total anthocyanins determination in fruit juices.

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