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Determination of total phenolics and anthocyanin contents in the pericarp of hot chilli pepper (*Capsicum annuum* L.)

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Abstract: This study was aimed to determine the contents of both total phenolics and anthocyanin mainly in the pericarp part of hot chilli pepper, considerably attributing for an enzymatic browning effect. The extraction conditions for total phenolics and anthocyanin in the sample were separately investigated. Total phenolic compounds were extracted with a solvent ratio of methanol: 0.05% (v/v) aqueous HCl (90/10, v/v) prior to determination by Folin-Ciocalteu method. While anthocyanin was extracted with 2% (v/v) HCl in methanol and then cleaned up using C_{18} solid-phase extraction prior to analysis by HPLC-PDA. Both of the optimum extraction conditions and their determination procedures were also applied for other kinds of the chilli pepper samples. From the results, the contents of anthocyanin and total phenolics in these samples were ranged of 0.796–4.70 mg CGE kg^{-1} and 0.782–4.52 g GAE kg^{-1} fresh weight, respectively. Their contents of the dried ground samples were also determined and found to be in the range of 62.9–70.3 mg CGE kg^{-1} and 81.8–90.2 g GAE kg^{-1} dry weight, respectively. It is generally noted that the contents of total phenolics in the hot chilli peppers were relatively higher (about 10^3-folds) than those of anthocyanin.

Keywords: Anthocyanin, total phenolics, chilli pepper, optimization extraction, HPLC-PDA

Introduction

Enzymatic browning is a significant problem in a number of fruits and vegetables such as strawberry (Chisari *et al*., 2007), grape (Muñoz *et al*., 2004), potato (Lee and Park, 2007), and lettuce (Gawlik-Dziki *et al*., 2007). The discoloration in fruits and vegetables by enzymatic browning, resulting from conversion of phenolic compounds to *o*-quinones which subsequently polymerize to be a brown or dark pigment. The enzymes involved in these processes are polyphenol oxidase (PPO) and peroxidase (POD) (Jiang *et al*., 2004), since PPO and POD are the main enzymes involved in the phenolic oxidation of many fruits and vegetables. Phenolic compounds are a group of chemical substances in plants, which play an important role during enzymatic browning because they are substrates for the browning enzymes. The phenolics are normally complex organic substances, which contain more than one phenolic group. Polyphenolics can be divided into many different subcategories, such as flavonoids and non-flavonoid components. Anthocyanins are a subgroup of plant constituents known as flavonoids (Markakis, 1982). They occur in all higher plants, mostly flowers and fruits but also in leaves, stems and roots. Anthocyanins are plant pigments, which are responsible for red, blue and purple colors of many fruits, vegetables and flowers. These polyphenolic substances are glycosides of polyhydroxy- and polymethoxy-derivatives of 2-phenylbenzopyrylium or flavylium ion (Kong *et al*., 2003).

In nature, anthocyanidins are always found attached to one or more sugar and/or acylated sugars. Four substituted monosides are particularly common: glucose, galactose, rhamnose and arabinose. When anthocyanidins are coupled to sugars, anthocyanins are formed. Thus, an anthocyanin molecule normally consists of two parts: a light-absorbent (chromophore) termed an anthocyanidin, and a glycoside attachment. As sugars can be coupled at different places and many different sugars are present in plants, it is clear that a very large range of anthocyanins can be formed (Delgado-Vargas and Paredes, 2003). Nevertheless, the most common anthocyanin structure in plants is cyanidin-3-glucoside. Anthocyanins are becoming increasing important as antioxidant properties and health benefits, including anti-cancer, anti-inflammatory and vasoprotective effects, preventing coronary heart diseases and improving visual acuity (Giusti and Wrolstad, 2001; Stintzing *et al*., 2002; Chaovanalikit *et al*., 2003; Joniec *et al*., 2003; Kähkönen *et al*., 2003; Katsube *et al*., 2003; Kallithraka *et al*., 2005; Gómez-Plaza *et al*., 2006; Prata and Oliveira, 2007). They are important compounds not only because they have health related properties, but also because they are widely used as colorants in food industry (Giusti and Wrolstad, 2001; Muñoz *et al*., 2004; Gómez-Plaza *et al*., 2006; Prata and Oliveira, 2007). However, anthocyanins are

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unstable during processing and storage of fruits and vegetables. The browning of fruits and vegetables was generally thought to be a rapid degradation of anthocyanins caused by PPO and POD, producing the browning-colored by-products (Jiang *et al*., 2004; Jiménez-Atiénzar *et al*., 2005; Zhang *et al*., 2005).

Pepper fruits (*Capsicum annuum* L.) are popular vegetables because of the combination of color, test and nutrition. They are used as foods and spice. Moreover, the red pepper fruit has been used for many years as a source of pigments to add or change the color of foodstuffs. Fresh peppers are good source of vitamin C and E as well as provitamin A and carotenoid compounds with well known antioxidant properties (Markus *et al*., 1999; Krinsky, 2001; Perucka and Masterska, 2001; Navarro *et al*., 2006; Chatterjee *et al*., 2007; Conforti *et al*., 2007; Deepa *et al*., 2007; Serrano-Martínez *et al*., 2008). The carotenoid pigments in fresh peppers have been widely studied to improve color retention during processing and storage (Markus *et al*., 1999; Krinsky, 2001; Deepa *et al*., 2007). However, anthocyanin contents have never been studied in the relation with browning effect in the pepper fruits.

The present study was aimed to optimize for the extraction conditions for both anthocyanin and total phenolics mainly in the pericarp part of hot chilli peppers and to determine their contents by HPLC-PDA and Folin-Ciocalteu assay, respectively.

Materials and Methods

Chemicals

All reagents used were at least analytical reagent (AR) grade including methanol, ethyl acetate, acetic acid, citric acid, hydrochloric acid and Folin-Ciocalteu's reagent were obtained from Carlo Erba (Italy). Methanol was of HPLC grade that obtained from Lab Scan (Thailand). Gallic acid was 99% purity obtained from Across (USA). Cyanin-3-glucoside which used as standard anthocyanin was HPLC grade and obtained from Fluka (USA). Polyvinylpyrrolidone was purchased from Fluka (USA). Sodium carbonate anhydrous was purchased from Ajax (Australia). Aqueous solutions were prepared with deionized water obtained from RiOsTM type 1 simplicity 185 (Millipore Waters, USA) throughout the experiments.

Instruments

The experiments were carried out on a Waters liquid chromatograph (Waters, USA). It consists of a Waters 600E Multisolvent Delivery System, a Waters In-Line Degasser AF, a Rheodyne injector with sample loop of 20 µL and a Waters 2996 Photodiode Array Detector (PDA). Empower software was used for data acquisition. A Vertisep AQS C_{18} column (250×4.6 mm i.d., 5 µm) and a guard column (10×4.6 mm i.d., 5 µm) were used. A spectrophotometric measurement was performed using an Agilent 8453 UV-visible spectrophotometer. The 1000 series centrifugal (Labquip, England), Model R-114 rotary evaporator (Buchi, Switzerland), VS-202D Shaker (Scientific Co. Ltd., Korea), HTS-1003 hotplate stirrer (LMS laboratory & material supplies, Japan) and Vortex (Model G-560 Vortex-2 Genie, Scientific Industries, USA) were used in the sample preparation step. The DSC-18 cartridge (20 mL/5 grams, 55 µm) was obtained from Supelco (USA). A Pipetman (Pipetman, France) micropipette and membrane filter, 0.45 µm (Whatman International Ltd., UK) were also used.

Hot chilli pepper samples

The type of samples to be studied were including pepper fruits, pepper fleshes, dried peppers, pepper with fish sauce and pepper with vinegar (Table 1). All samples used in this study were obtained from a local market in Khon Kaen, Thailand (Arnnok *et al*., 2010). The pepper fruits were washed several times with tap water and homogenized by using a homogenizer (Moulinex Optiblend 2000, France) for 2 min. In case of pericrap samples, the seeds and peduncle were removed and the pericarps were homogenized. For fish sauce with chilli and vinegar with chilli, fish sauce and vinegar were removed by washed several times with tap water. The pepper pieces were left drying at room temperature and were then subjected to homogenize in the same manner as mentioned above. Dry matter content of the homogenized sample was determined in an oven at 105°C for 2 h. The homogenized samples were kept storing in a plastic bag at 4°C for a week for further experiments.

Extraction optimization for total phenolics

The optimum conditions for extraction of total phenolics were studied. Actually, there were many extraction factors affecting the extraction of total phenolics in plant samples including extraction methods (magnetic bar stirring, batch shaking and vortex mixing), sample/solvent ratio, organic solvent/aqueous solution ratio, HCl concentration and extraction time. Thus, for most selective extraction method of the plant samples used, the following experimental parameters were subsequently investigated as a simple optimization method by comparing its response in terms of the contents of

total phenolics obtained.

Table 1. Sample description and dry matter content of the hot chilli pepper samples.

Sample code and type	Part used	Fruit wide (cm)	Fruit length (cm)	Fruit weight (g)	DM content (%)
RP1, Red pepper fruit	Pericarp	1.80	11.20	11.60	12.0
RP2, Red pepper fruit	Pericarp	1.00	6.90	3.10	21.0
RP3, Red pepper fruit	Pericarp	1.10	4.70	2.32	14.0
RP4, Red pepper fruit	Pericarp	2.08	13.40	22.00	10.0
RP5, Red pepper fruit	Pericarp	0.89	5.57	2.24	17.0
RF5, Red pepper fruit	Whole fruit	0.89	5.57	2.24	26.0
OP1, Orange pepper fruit	Pericarp	2.01	7.98	14.42	12.0
GP1, Green pepper fruit	Pericarp	1.80	13.60	18.78	9.0
GP2, Green pepper fruit	Pericarp	0.90	6.70	2.36	12.0
GP3, Green pepper fruit	Pericarp	1.10	4.70	1.99	13.0
GP4, Green pepper fruit	Pericarp	2.67	8.27	22.17	5.0
GP5, Green pepper fruit	Pericarp	1.65	9.71	11.44	9.0
GP6, Green pepper fruit	Pericarp	1.49	11.80	10.32	8.0
GP7, Green pepper fruit	Pericarp	0.83	4.76	1.49	15.0
GF7, Green pepper fruit	Whole fruit	0.84	4.76	1.49	16.0
GF8, Green pepper fruit	Whole fruit	0.65	3.72	0.92	15.0
FC, Fish sauce with red chilli	Pieces	-	-	-	6.0
VC, Vinegar with green chilli	Pieces	-	-	-	25.0
D1, Dried chilli pepper	Whole fruit	-	-	-	-
D2, Dried chilli pepper	Whole fruit	-	-	-	-

- : No data

The effect of sample/solvent ratio was studied by using methanol as the extraction solvent. Various amounts of the homogenized sample (0.3, 0.5, 1.0, 2.0 and 5.0 g) were shaken in 20 mL of the extraction solvent using a VS-202D shaker (Scientific Co. Ltd., Korea) for 30 min. The homogenate was filtered through a Whatman No. 42 filter paper and collected as the methanolic extract. Then, two grams of the homogenized sample were used to extract for 30 min in 20 mL of the extraction solvent containing various ratios of 0.1% (v/v) aqueous HCl in methanol. The organic solvent/aqueous ratios of 100, 99/1, 90/10, 50/50 and 10/90 were investigated. The concentration of HCl in the extraction solvent was also studied. The solvent was an aqueous HCl /methanol (10:90, v/v) with varying the acid concentrations in the range of 0.05 to 0.2% (v/v).

Three extraction methods of total phenolics were used including magnetic bar stirring, batch shaking and vortex mixing. Two grams of the homogenized sample were extracted in 20 mL of 0.05% (v/v) aqueous HCl/methanol (10:90, v/v) for 30 min. The homogenate was filtered through a Whatman No. 42 filter paper. The magnetic bar stirring method was used. Then, the extraction time was also carried out using 2 g sample in 20 mL of 0.05% (v/v) aqueous HCl : methanol (10:90, v/v) by varying the extraction time for 15, 30, 45, 60, 90 and 120 min. The investigated parameters with their range for the extraction of total phenolics from the chili pepper sample are summarized in Table 2.

Table 2. The investigated parameters with their range for the extraction of total phenolics from chilli pepper sample

Parameter	Variation range
Sample/solvent ratio, gm L^{-1}	1/4, 1/10, 1/20, 1/40, 1/60
Methanol/dilute HCl ratio, %	100, 99/1, 90/10, 50/50, 10/90
HCl concentration, %	0.01, 0.05, 0.10, 0.15, 0.20
Extraction time, min	15, 30, 60, 90, 120
Extraction method	shaking, magnetic stirring, vortex mixing

Determination of total phenolics by Folin-Ciocalteu method

Stock standard solution (1,000 mg L^{-1}) of gallic acid was prepared by accurately dissolving of 1.0 g with methanol and then made up volume to 10 mL. The stock solution was transferred to a dark vial and kept cool at 4°C prior to use. Working standard solutions were prepared by appropriate dilution of the stock solution.

The contents of total phenolics of the hot chilli pepper samples were determined by Folin-Ciocalteu method using gallic acid as a standard compound. The sample extract (0.2 mL) was mixed with 2.6 mL of deionized water, 2 mL of 7% (w/v) Na_2CO_3, and 0.2 mL of the Folin-Ciocalteu reagent. After incubation at room temperature for 90 min, the absorbance of the reaction mixture was measured at 745 nm against the blank sample contained the same mixture solution without the sample extract. Using a five-point calibration curve (20 - 100 mg L^{-1}), the total phenolics were determined by a comparison of the values obtained with the calibration curve of gallic acid. The results were expressed as gallic acid equivalents (GAE) in grams per kg of sample.

Optimization for the extraction of anthocyanin

In order to evaluate the optimum conditions for anthocynin extraction from hot chilli pepper, a representative sample (RP2) was chosen to optimize the extraction conditions. Various extraction parameters affecting the yield of anthocyanin as the response factor expressed as their relative peak area obtained from HPLC separation were investigated including amount of sample, sample/solvent ratio, HCl concentration in methanol and extraction time.

Since no report was found about the anthocyanin content in hot chilli pepper grown in our local cultivating areas, and the preliminary study was done giving quite small amounts of the anthocyanin in the fruit samples, it was thus necessary to evaluate the optimal sample size which was used in the anthocyanin extraction in order to get the amount of

anthocyanin in the linear range of calibration curve and avoid the use of large amount of sample. Thus, various amounts of the homogenized sample (2.0, 5.0 and 10 g) were extracted by using magnetic bar stirring with 1% (v/v) HCl in methanol as an extraction solvent for 15 min. The volumes (mL) of the solvent used were at ten times of the sample weight (g). Next, different ratios of the sample/solvent were tried out. Ten grams of the homogenized sample were extracted with various volumes of the extraction solvent (30, 50, 70 and 100 mL). As mentioned above, the sample was extracted using 1% (v/v) HCl in methanol as the extraction solvent. It is evident that an acidified methanol has mostly been used to extract anthocyanin from the hot chilli pepper. So, an addition of the acid in the extraction solvent may increase the stability of anthocyanin (Escribano-Bailón and Santos-Buelga, 2003). It is also known that the use of acid is necessary to obtain the flavylium cation form, which is red and stable in an acid medium, and the maximum anthocyanin stability is obtained at pH 1.8 (Markakis, 1982). But at low pH levels or high concentrations of HCl can make any partial hydrolysis and anthocyanin decomposition. In addition, the effect of the extraction time (15-120 min) was also studied by using 10 g sample with 50 mL of 2% (v/v) HCl in methanol as the extraction solvent. The investigated parameters with their range for the extraction of anthocyanin from chilli pepper sample by using magnetic stirring method are summarized in Table 3.

Table 3. The investigated parameters with their range for the extraction of anthocyanin from chilli pepper sample by using magnetic stirring method

Parameter	Variation range
Amount of sample, g	2, 5, 10
Sample/solvent ratio, gmL^{-1}	1/3, 1/5, 1/7, 1/10
HCl concentration, %	0.1, 0.5, 1.0, 1.5, 2.0, 3.0
Extraction time, min	15, 30, 60, 90, 120

Since solid-phase extraction (SPE) mostly used for clean up the sample was necessary to remove matrices from the analyte prior to analysis, C_{18} cartridge was selected to equilibrate with 10 mL methanol, followed by 10 mL of 0.01% (v/v) HCl in deionized water. Five mL of the extract solution was diluted with 5 mL of deionized water and introduced into the conditioned C_{18} cartridge. The water soluble compounds and other phenolics were removed by washing the cartridge with 10 mL of 0.01% (v/v) HCl followed by 20 mL of ethyl acetate. The anthocyanin was then eluted with 10 mL of methanol containing 0.01% (v/v) HCl.

The extraction efficiency of anthocyanin from the samples was also investigated. The method was applied for the determination of anthocyanin in a red chilli fruit (RP2) and a green chilli fruit (GP2). The chilli fleshes were subjected to extract and clean up under the optimized conditions as mentioned above. The recovery was studied by spiking 10 µg and 20 µg of the anthocyanin standard solution into the samples before extraction, clean up and analysis by HPLC.

Determination of anthocyanin by reversed phase HPLC-PDA

The separation condition of anthocyanin by HPLC was also optimized. Several mobile phases have been described in the literatures for the analysis of anthocynin using the reversed phase column (Da Costa *et al*., 1998; Stintzing *et al*., 2002; Longo *et al*., 2007; Zhang *et al*., 2007). In this study, the organic solvent selected for the preliminary experiments was methanol due to the solubility of anthocyanin. The mobile phases containing various percents of methanol in aqueous acetic acid were then investigated.

In order to evaluate the potential of the present method for quantitative analysis, linearity, LOD, LOQ, precision and recovery were investigated. According to this method, the calibration curve for the determination of anthocyanin was constructed under optimum conditions using cyanin-3-glucoside as a standard compound. The anthocyanin content was determined by comparison of the peak area obtained with the calibration curve of cyanin-3-glucoside. The results were expressed as µg of cyanin-3-glucoside equivalents (CGE) per kg sample. Both LOD and LOQ of the present method were deduced based on the concentration of the analyte which produced a signal to noise ratio of three times and ten times, respectively. The repeatability and reproducibility were investigated in terms of relative standard deviation (RSD) of both retention time and peak area. The repeatability was deduced from ten replicates within one day (intra-day precision, n = 10) and reproducibility was calculated from the experiment in five consecutive days (inter-day precision, n = 5×3).

Results and Discussion

Extraction optimization and determination of total phenolics

To optimize the extraction conditions for total phenolic compounds, the extraction parameters including sample/solvent ratio, methanol/water ratio, HCl concentration, extraction method and extraction time were investigated. The yield of total phenolics as a function of the solvent/sample ratio was evaluated. As methanol is the most frequently used in the phenolics extraction, it was chosen as a solvent for

the following step. The homogenized sample such as 2 g was shaken in 20 mL of methanol for 30 min. The extraction yield of the phenolic compounds was not significantly different when the solvent/sample ratio increased from 4 to 40 and the solvent/sample ratio of about 60 gave the highest extraction yield (data not shown). However, the solvent/sample ratio of 10 was chosen to reduce amounts of organic waste.

The use of water in combination with organic solvents contributes to the creation of a moderate polar medium that ensures the extraction of phenolic compounds (Lapornic *et al*. 2005; Liyana-Pathirana and Shahidi, 2006). However, the use of water as only the solvent yields an extraction with a high content of impurities such as organic acids, sugars and soluble proteins, which can interfere in the phenolic identification and quantification. The effect of the solvent/water ratio on the extraction yield of phenolic compounds was thus investigated. Then the extraction in solvents contained various percents of methanol in 0.01% (v/v) aqueous HCl.

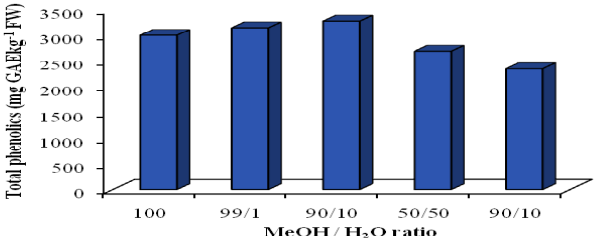

Figure 1. Effect of methanol/water ratio on the extraction yield of total phenolics

As the results shown in Figure 1, the proportion of methanol in the extraction medium had a significant effect on the extraction yield of the phenolic compounds. The highest values were obtained when the methanol/water ratio was 90/10 (v/v). It was suggested that the water proportions that equal or are higher than 50% do not ensure the recovery of the phenolic compounds from hot chilli pepper. Moreover, a minimum of 70% methanol has been reported needed to be inactivating PPO, which is widely distributed in plants, and to allow high recovery of phenolic compounds (Robards *et al*., 1999). Due to the results obtained, it was decided to continue working with 0.01% (v/v) HCl in 90% methanol for the evaluation of other parameters.

The effect of acidified solvents on the extraction of the chilli pepper phenolics was evaluated using aqueous HCl in 90% methanol as the solvent. A small quantity of water (10%, v/v) was added to the methanol in order to create a moderate polar medium to facilitate the extraction of phenolic glycosides which are more water soluble. The suitably acidified conditions for the extraction of phenolic compounds were 0.05% (v/v) HCl (Figure 2). At HCl concentrations higher than 0.05%, the extraction yields of total phenolics decreased probably due to acid hydrolysis and decomposition (Revilla and Martín-Oetega, 1998). Thus, 0.05% (v/v) aqueous HCl in 90% methanol was chosen for further experiments.

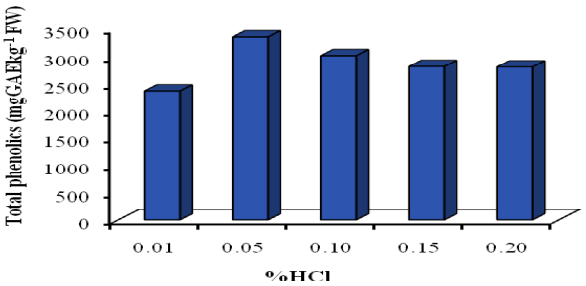

Figure 2. Effect of HCl concentration on the extraction yield of total phenolics

According to extraction method, three simple extracting methods were compared for the extraction of total phenolics from hot chilli pepper. There are common shaking, magnetic bar stirring and vortex mixing (data not shown). From the results, high extraction efficiency was obtained using magnetic bar stirring method. Therefore, the magnetic stirring was used to extract the total phenolics in this study. In addition, the effect of extraction time was also studied with the extraction solvent containing methanol: 0.05% (v/v) aqueous HCl (90/10, v/v) and magnetic bar stirring as the extraction method. No significant differences were found when the extraction time increased from 15 to 120 min (data not shown). Therefore, the extraction time of 15 min was chosen for routine analysis in order to reduce analysis time. Therefore, the optimum conditions for total phenolics extraction in the chilli pepper samples were summarized as follow: 2 g sample size, sample/solvent ratio of 1/10, methanol: 0.05% (v/v) aqueous HCl (90:10, v/v) as an extraction solvent, 15 min extraction time, and using magnetic bar stirring as the extraction method.

It has been reported that the phenolic compounds of plant materials were subjected to be a substrate of the enzymes (Richard-Forget and Gauillard, 1997; Zhang *et al*., 2005; Chisari *et al*., 2007; Duan *et al*., 2007; Mohapatra *et al*., 2008). Thus, it is an important to consider the relation between the total phenolics and the enzyme activities of the hot chilli peppers studied (Arnnok *et al*., 2010). The total phenolic contents of the methanolic extracts were determined using Folin-Ciocalteu method. A five-point calibration curve of gallic acid in the range of 20 - 100 mg L^{-1} ($y = 0.0052x - 0.0002$ with r^2 of 0.9993) was constructed.

Extraction optimization and determination of anthocyanin

HPLC-PDA was used for the quantification of anthocyanin in the hot chilli pepper samples. Under the optimal isocratic conditions (methanol / 2% (v/v) acetic acid in deionized water: 65:35, v/v) as the mobile phase and 0.7 mL min^{-1} flow-rate), anthocyanin was separated within 5 min with its retention time of 4.2 min as shown in Figure 3(a). In the present study, the PDA detection allows the identification of the compound using the absorption spectrum and peak purity (Figure 3(b)). Using PDA detector, with the maximum wavelength at 520 nm, an identification of anthocyanin was achieved by comparing retention time and absorption spectrum of the standard compound and the sample extract. As cyanidin-3-glucoside is the most common anthocyanin in plants, it was chosen as the anthocyanin standard. Quantification was done based on the basis of calibration curve of the anthocyanin standard. The linear range for cyanin-3-glucoside was 5 to 45 mg L^{-1} and the linear regression equation was as follows: $y = 0.1359x - 0.3691$, with a correlation coefficient (r^2) greater than 0.9995. LOD and LOQ were found to be 0.7 and 5.0 mg L^{-1}, respectively. The repeatability and reproducibility of the retention times for anthocyanin were in good precision which their RSD were lower than 0.4% while the RSD of the peak area were lower than 7.0%.

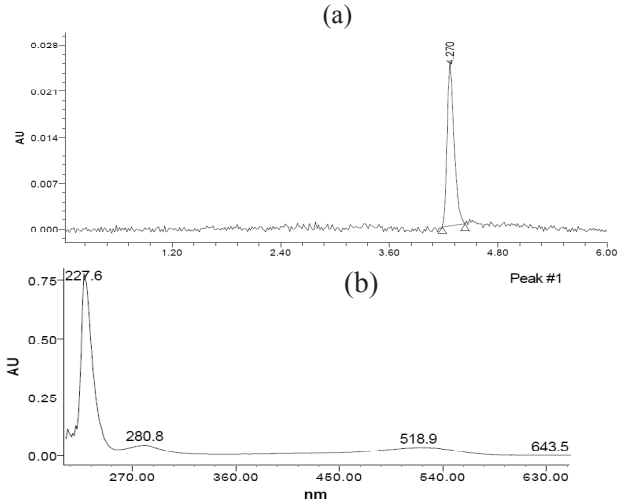

Figure 3. Chromatogram of cyanidin-3-glucoside standard, 10 mg L^{-1} (a) and its UV-Visible spectrum (b)

As expected, an increase in the amounts of sample resulted in an increase in the peak area of the anthocyanin. Ten grams of the sample used gave the highest yield of anthocyanin and its content was in the range of the calibration curve. Nevertheless, the sample size used was less than 10 g gave the peak area of anthocyanin under limit of the linear range of the calibration curve. Thus, the sample size of 10 g was chosen for the following extraction steps. The extraction yield of anthocyanin was increased with an increasing of the volumes of the solvent. However, the yield was not significantly different when the sample/solvent ratio was less than the ratio of 1:5 by volume (data not shown). Therefore, the sample/solvent ratio of 1:5 was chosen. From the results (Figure 4), the yield of anthocyanin was increased with an increasing of HCl concentrations until 2% (v/v) HCl which the yield stayed nearly constant. Thus, 2% (v/v) HCl in methanol was chosen for the evaluation of other parameters.

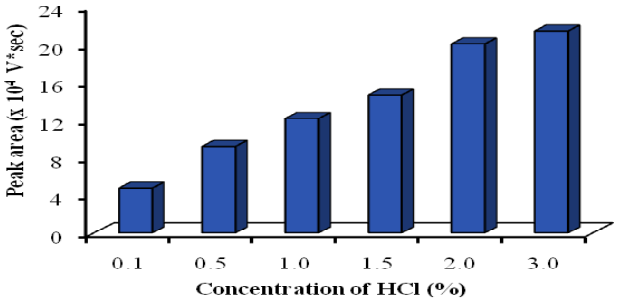

Figure 4. Effect of HCl concentration on the extraction yield of anthocyanin

Anthocyanin content was found the highest in terms of the peak area when the extraction time was 15 min and it decreased at higher extraction times, probably due to the decomposition of anthocyanin. The anthocyanin was successfully eluted with 10 mL of methanol containing 0.01% (v/v) HCl. The obtained sample from this process gave a clear chromatogram when injected into HPLC. It was an indication of the efficiency of SPE for clean up of anthocyanin. Additionally, the percentage recoveries of anthocyanin for two types of the samples used were found in the range of 75.8 - 95.2% (data not shown). The optimum conditions for anthocyanin extraction in hot chilli pepper samples were summarized as follow: 10 g sample size, sample/solvent ratio of 1/5, 2% (v/v) HCl in methanol as the extraction solvent, 15 min extraction time, and also using magnetic bar stirring as the extraction method.

The contents of anthocyanin and total phenolics in the hot chilli pepper samples

The contents of anthocyanin found in the hot chilli samples were expressed as mg CGE kg^{-1} of fresh weight (Table 4) and dry weight (Table 5). The anthocyanin contents in hot chilli fruits (RP1 – GF8) were ranged of 0.796 – 4.70 mg CGE kg^{-1} fresh weight. The highest anthocyanin contents in the chilli fruits based on fresh weight was found in the sample GF8, while the lowest was found in the sample RP1. On the other hand, the anthocyanin contents in the sample FC and VC were found to be 1.84 and 0.803

mg CGE kg^{-1} fresh weight, respectively. When the results were expressed as dry basis (Figure 5), it was suggested that the anthocyanin contents in the grinded chilli samples (D1 and D2) were higher than that in the chilli fruits. It has been reported that the browning index of the grinded sample increased with an increasing of storage time from 1 to 10 days (Göğűş and Eren, 1998). From the results obtained, it could be possibly implied that the dark color of the grinded chilli pepper was resulted from the formation of the anthocyanin. In case of flower, the accumulation of anthocyanin in blotches of the petals caused the color of the blotches darker than that of the non-blotches (Zhang *et al*., 2007)

Table 4. Anthocyanin and total phenolics contents in the hot chilli pepper samples based on fresh basis (n = 3)

Sample code	Anthocyanin content (mg kg^{-1}FW)	Total phenolics content (mg kg^{-1}FW)
RP1	0.795 ± 0.008	1993.4 ± 35.7
RP2	1.82 ± 0.019	3304.5 ± 181
RP3	2.78 ± 0.132	2728.7 ± 269
RP4	0.842 ± 0.014	1581.7 ± 38.6
RP5	1.35 ± 0.074	4516.2 ± 135
RF5	1.80 ± 0.096	4297.1 ± 185
OP1	0.968 ± 0.015	2227.7 ± 13.6
GP1	0.894 ± 0.01	897.49 ± 41.2
GP2	1.73 ± 0.102	1590.0 ± 33.1
GP3	1.86 ± 0.029	2370.6 ± 66.0
GP4	0.892 ± 0.022	1244.2 ± 49.8
GP5	1.01 ± 0.024	1255.9 ± 63.8
GP6	1.28 ± 0.012	782.07 ± 19.7
GP7	2.28 ± 0.178	2898.6 ± 172
GF8	4.70 ± 0.458	3094.3 ± 129
FC	1.84 ± 0.022	678.85 ± 27.5
VC	0.803 ± 0.013	132.20 ± 4.10

Total phenolics of the methanolic extracts of the samples were determined using Folin-Ciocalteu method. The results were expressed as mg GAE kg-1 sample. The total phenolics contents were calculated based on fresh weight (Table 4). The total phenolics contents in the chilli fruits (RP1-GF8) were found to be 782.07 – 4516.2 mg GAE kg-1 fresh weight. The highest total phenolics content based on both fresh weight and dry weight (Table 5) was found in the sample RP5 and the lowest was found in sample VC. In addition, it can be suggested that the total phenolics contents in the grinded chilli peppers were lower than that always of the chilli fruits which was calculated based on dry weight (Figure 6). The phenolic compounds are susceptible towards temperature, oxygen and UV-light. Temperature may cause the loss of total phenolics due to decomposition. Light may also has a similar effect. Oxygen may destroy phenolics, as do other oxidizing reagents (Escribano-Bailón and Santos-Buelga, 2003).

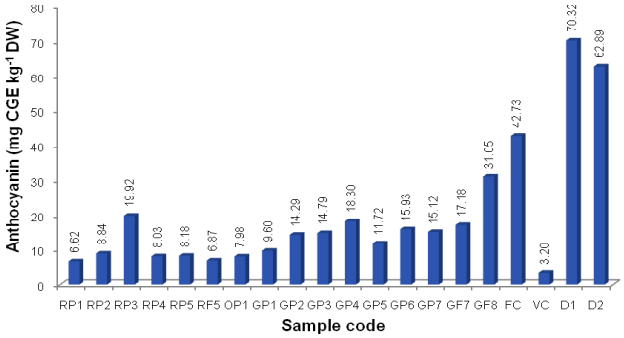

Figure 5. Anthocyanin contents in the hot chilli pepper samples based on dry weight

Table 5. Anthocyanin and total phenolics contents in the hot chilli pepper samples based on dry basis (n = 3)

Sample code	Anthocyanin content (mg kg^{-1}DW)	Total phenolics content (mg kg^{-1}DW)
RP1	6.62 ± 0.06	16,596 ± 296.8
RP2	8.84 ± 0.11	16,021 ± 877.1
RP3	19.9 ± 0.83	19,642 ± 1938
RP4	8.03 ± 0.05	15,195 ± 371.0
RP5	8.18 ± 0.48	26,671 ± 798.4
RF5	6.87 ± 0.33	16,493 ± 710.7
OP1	7.98 ± 0.12	18,176 ± 110.8
GP1	9.60 ± 0.10	9,628.7 ± 441.8
GP2	14.3 ±0.85	13,125 ± 273.3
GP3	14.8 ± 0.26	18,766 ± 522.4
GP4	18.3 ± 0.49	25,486 ± 1020
GP5	11.7 ± 0.32	14,500 ± 736.6
GP6	15.9 ± 0.18	9,782.8 ± 246.4
GP7	15.1 ± 0.87	19,558 ± 1159
GF7	17.2 ± 2.11	17,484 ± 1105
GF8	31.0 ± 3.02	20,452 ± 851.2
FC	42.7 ± 0.22	2,723.4 ± 110.3
VC	3.20 ± 0.03	2,199.6 ± 69.00
D1	70.3 ± 4.39	9,016.0 ± 510.3
D2	62.9 ± 2.77	8,1839 ± 694.1

Therefore, total phenolic compounds in the grinded chilli peppers could be lost during processing and storage which exposed to high temperature, UV-light and oxygen. The relationship between anthocyanin and total phenolic contents in these samples is illustrated in Figure 7. Since anthocyanin is a subgroup of total phenolic compounds in plants, a similar tendency of the anthocyanin content and total phenolics content was observed. In addition, the total phenolics contents were found to be about 10^{-3} folds of the anthocyanin contents in the chilli pepper samples.

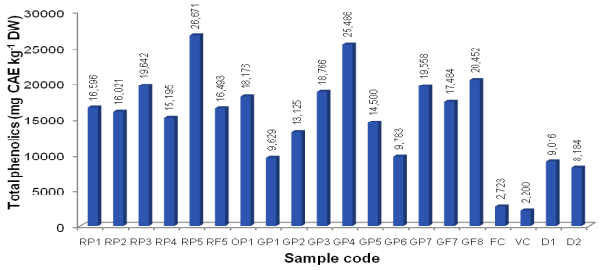

Figure 6. Total phenolics contents in the hot chilli pepper samples based on dry weight

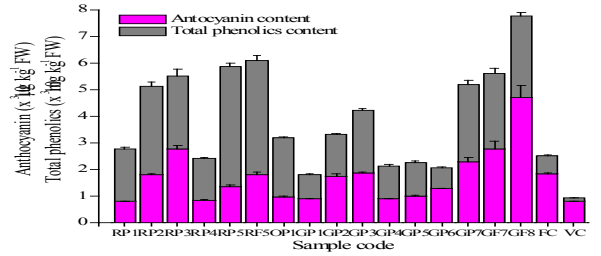

Figure 7. Ratio of anthocyanin and total phenolics found in the hot chilli pepper samples

Conclusion

The chromatographic method for the analysis of anthocyanin and sample preparation method for extraction and clean up of anthocyanin from hot chilli pepper sample were reported. The optimum conditions for the extraction of total phenolics in the chilli pepper were investigated. The total phenolics of the methanolic extract were estimated by the Folin-Ciocalteu method. Both contents of anthocyanin and total phenolics in these chilli samples were ranged of 0.796–4.70 mg CGE kg^{-1} and 0.782–4.52 g GAE kg^{-1} fresh weight, respectively, and the contents of the grinded samples were also determined and found to be 62.9–70.3 mg CGE kg^{-1} and 81.8–90.2 g GAE kg^{-1} dry weight, respectively. The contents of both anthocyanin and total phenolics of the samples were made in comparison. The total phenolics contents in the grinded chilli peppers were less than that of the chilli fruits based on dry weight. The correlation between anthocyanin content and total phenolics content in the chilli pepper samples were also discussed attributing to their browning effect.

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References

Arnnok, P., Ruangviriyachai, C., Mahachai, R., Techwongstien, S. and Chanthai, S. 2010. Optimization and determination of polyphenol oxidase and peroxidase activities in hot pepper (*Capsicum annuum* L.) pericarb. International Food Research Journal 17(2): 385-392.

Chaovanalikit, A., Dougherty, M.P., Camire, M.E., Darokar, and Briggs, J. 2003. Ascorbic acid fortification reduces anthocyanins in extruded blueberry-corn cereals. Journal of Food Science 68: 2136-2140.

Chatterjee, S., Niaz, Z., Gautan, S., Adhikari, S., Pasad, S.V. and Sharma, A. 2007. Antioxidant activity of some phenolic constituents from green pepper (*Piper nigum* L.) and fresh nutmeg mace (*Myristica fragrans*). Food Chemistry 102: 515-523.

Chisari, M., Barbagallo, R.N., and Spagna, G. 2007. Characterization of polyphenol oxidase and peroxidase and influence on browning of cold stored strawberry. Journal of Agricultural and Food Chemistry 55: 3469-3479.

Conforti, F., Statti, G.A. and Menichini, F. 2007. Chemical and biological variability of hot pepper fruits (*Capsicum annuum var. acuminatum* L.) in relation to maturity stage. Food Chemistry 102: 1094-1104.

Da Costa, C.T., Nelson, B.C., Margolis, S.A. and Horton, D. 1998. Separation of blackcurrent anthocyanins by capillary zone electrophoresis. Journal of Chromatography A 799: 321-327.

Deepa, N, Kaur C., George, B., Singh, B. and Kapoor, H.C. 2007. Antioxidant constituents in some sweet pepper (*Capsicum annuum* L.). LWT-Food Science and Technology 40: 212-219.

Delgado-Vargas, F. and Paredes, O (Eds) 2003. Natural Colorants for Food and Nutraceutical Uses. Washington, D.C.: CRC Press.

Doğan, S. and Doğan, M. 2004. Determination of kinetic properties of polyphenol oxidase from Thymus (*Thymus logicaulis* subsp. *chaubardii* var. *chaubardii*). Food Chemistry 88: 69-77.

Duan, X., Su, X., You, Y., Qu, H., Li, Y. and Jiang, Y. 2007. Effect of nitric oxide on pericarp browning of harvested longan fruit in relation to phenolic metabolism. Food Chemistry 104: 571-576.

Escribano-Bailón, M.T. and Santos-Buelga, C. 2003. Method in Polyphenol Analysis. United Kingdom: The Royal Society of Chemistry.

Gawlik-Dziki, U., Złotek, U. and Świeca, M. 2007. Characterization of polyphenol oxidase from butter lettuce (*Luctuca sativa* var. *capitata L.*). Food Chemistry 107: 129-135.

Giusti, M.M., and Wrolstad, R.E. 2001. Current Protocols in Food Analytical Chemistry. New York: John Wiley & Sons.

Göğűş, F., and Eren, S. 1998. Effect of temperature and pH on nonenzymic in minced dried pepper during storage. Turkish Journal of Engineering Environmental Science 22: 33-38.

Gómez-Plaza, E., Miñano, A. and Lóprz-Roca. 2006.

Comparison of chromatic properties, stability and antioxidant capacity of anthocyanin-based aqueous extracts from grape pomace obtained from different vinification methods. Food Chemistry 97: 87-94.

Jiang, Y., Duan, X., Joyce, D., Zhang, Z. and Li, J. 2004. Advances in understanding of enzymatic browning in harvest litchi fruit. Food Chemistry 88: 443-446.

Jiménez-Atiénzar, M. Escribano, J., Cabanes, J., Gandía-Herrero, F. and García-Carmona, F. 2005. Oxidation of the flavonoid eriodictyol by tyrosinase. Physiology and Biochemistry 43: 866-873.

Joniec, C.M., Mes, P. and Myers, J.R. 2003. Characterization and inheritance of the anthocyanin fruit (Aft) Tomato. Journal of Heredity 94: 449-456.

Kähkönen, M.P., Heinämäki, J., Olliainen, V. and Heinoneh, M. 2003. Berry anthocyanins: isolation, identification and antioxidant activities. Journal of the Science of Food and Agriculture 83: 1403-1411.

Kallithraka, S., Mohdaly, A.A.A., Makris, D.P. and Kefalas, P. 2005. Determination of major anthocyanin pigments in Hellenic native grape varieties (*Vitis vinifera* sp.): associated with antiradical activity. Journal of Food Composition and Analysis 58: 375-386.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K. and Kobori, M. 2003. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. Journal of Agricultural and Food Chemistry 51: 68-75.

Kong, J-M., Chia, L-S., Goh, N-G., Chai, T-F. And Brouillard, R. 2003. Analysis and biological of anthocyanins. Phytochemistry 64: 923-933.

Krinsky, N.I. 2001. Carotenoids as antioxidation. Nutrition 17: 815-817.

Lapornic, B., Prošek, M. and Wonder, A.G. 2005. Comparison of extract prepared from plant by-products using different solvents and extraction time. Journal of Food Engineering 71: 214-222.

Lee, M-K. and Park, I. 2007. Studies on inhibition of enzymatic browning in some foods by Du-Zhong (*Eucommia uimoides* Oliver) leaf extract. Food Chemistry 1114: 154-163.

Liyana-Pathirana, C., and Shahidi F. 2006. Optimization of extraction of phenolics from wheat using response surface methodology. Food Chemistry 93: 45-56.

Longo, L., Scardino, A. and Vasapollo, G. 2007. Identification and quanitification of anthocyanins in the berries of *Pistacia lentiscus* L., *Phillyrea latifolia* L. and *Rubia peregrine* L. Innovative Food Science and Emerging Technologies 8: 360-364.

Markakis, P. 1982. Anthocyanins as Food Colors. New York: Academic Press.

Markus, F., Daood, H.G., Kapitany, J. and Biass, P.A. 1999. Change in the carotenoid and antioxidant content of spice red pepper (paprika) as a function of ripening and some technological factors. Journal of Agricultural and Food Chemistry 47: 100-107.

Mohapatra, D., Frias, J.M., Oloveira, Z.M., Bira, Z.M. and Kerry, J. 2008. Development and validation of a model to predict enzymatic activity during storage of cultivated mushrooms (*Agaricus bisporus* spp.). Journal of Food Engineering 86: 39-48.

Muñoz, O., Sepúlveda, M. and Schwaerz, M. 2004. Effects of enzymatic treatment on anthocyanin pigments from grapes skin from Chilean wine. Food Chemistry 87: 487- 490.

Navarro, J.M., Flores, P., Garrido, C. and Martinez, V. 2006. Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. Food Chemistry 96: 66-73.

Perucka, I. and Masterska, M. 2001. Phenylalanine ammonia-lyase and antioxidant activities of lipophilic fraction of pepper fruits *Capsicum annuum* L. Innovative Food Science and Emerging Technologies 2: 189-192.

Prata, R.B.A.E. and Oliveira L.S. 2007. Fresh coffee husks as potential sources of anthocyanins. LWT-Food Science and Technology 40: 1555-1560.

Revilla, E., Ryan, J.M. and Martín-Oetega, G. 1998. Comparison of several procedures used for the extraction of anthocyanins from red grapes. Journal of Agricultural and Food Chemistry 46: 4592-4597.

Richard-Forget, F.C. and Gauillard, F.A. 1997. Oxidation of chlorogenic acid catechins and 4-methylcatechol in model solution by combination of pear (*Pyrus communis* Cv. Williams) polyphenol oxidase and peroxidase: a possible involvement of peroxide in enzymatic browning. Journal of Agricultural and Food Chemistry 45: 2475-2476.

Robards, K., Prenzler, P.D., Ticker, G., Swatsitang, P. and Glover, W. 1999. Phenolic compounds and their role in oxidative process in fruits. Food Chemistry 66: 401-436.

Serrano-Martínez, A., Fortea, F.M., Del Amor, F.M. and Núñez-Delicado, E. 2008. Kinetic characterization and thermal inactivation study of partially purified red pepper (*Capsicum annuum* L.) peroxidase. Food Chemistry 107: 193-199.

Stintzing, F.C., Stintzing, A.S., Carle, B.F and Wrolstad, R.E. 2002. Color and antioxidant properties of cyanidin-based anthocyanin pigments. Journal of Agricultural and Food Chemistry 50: 6172-6181.

Zhang, J., Wang, L., Shu, Q., Liu, Z., Li, C., Zhang, J., Wei, X. and Tian, D. 2007. Comparison of anthocyanins in non-blothes and blotches of the petals of Xibei tree peony. Scientia Horticulturae 114: 104-111.

Zhang, Z., Pangi, X., Xuewu, D., Zuoliang, J. and Jiang, Y. 2005. Role of peroxidase in anthocyanin degradation in litchi fruit pericarp. Food Chemistry 90: 47-52.