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Phenolic compounds, ascorbic acid, carotenoids and antioxidant properties of green, red and yellow bell peppers

Donglin Zhang and Yasunori Hamauzu*
*Science of Functional Foods, Graduate School of Agriculture, Shinshu University, 8304 Minami-minowa, Kami-ina, Nagano 399-4598 Japan. *email: hamauzu@gipmc.shinshu-u.ac.jp*

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Abstract
Different colored bell peppers (*Capsicum annuum* L.), green, red, and yellow were examined for their content of phenolic compounds, ascorbic acid and carotenoids as well as free radical scavenging activity using DPPH assay and antioxidant activity using ß-carotene/linoleic acid assay. Phenolic, ascorbic acid and carotenoid contents varied in different colored bell peppers. Green pepper had higher level of phenolics than red and yellow peppers. Red pepper had higher content of ascorbic acid than green and yellow peppers. Red and yellow peppers showed higher in carotenoid content than green peppers. In the methanol extracts red pepper showed a higher level of DPPH radical scavenging activity compared to green and yellow peppers, while green pepper showed the highest in the phenolic extracts. DPPH radical scavenging activity in the phenolic extracts accounted for 4.6-11.7% of that in the methanol extracts. The radical scavenging activity in the methanol extracts was mainly from ascorbic acid and phenolic compounds. In the antioxidant activity assay, green pepper showed the highest activity in the phenolic extracts among three coloured peppers. However, a pro-oxidant effect was observed with the methanol extracts.

Key words: Phenolic compounds, ascorbic acid, carotenoids, antioxidant activity, free radical scavenging activity.

Introduction
Fruits and vegetables are a good source of natural antioxidants, containing many different antioxidant components which provide protection against harmful free radicals and have been associated with lower incidence and mortality rates of cancer and heart diseases in addition to a number of other health benefits[1,2,3].

Peppers are one of popular vegetables because of the combination of colour, taste and nutritional value. Peppers contain a wide array of phytochemicals and are a good source of vitamin C and carotenoids that are important nutritional antioxidants found in the human diet. Vitamin C is hypothesized to prevent cancer by inhibiting the formation of N-nitroso compounds in the stomach, and by stimulation the immune system[4]. Vitamin C is also the most abundant water-soluble antioxidant in the human body[5]. Carotenoids are fat-soluble antioxidants found in many fruits and vegetables and are required for humanepithelial cellular differentiation[4]. In addition, peppers were reported to contain moderate to high levels of neutral phenolics or flavonoids, phytochemicals that are important antioxidant components of a plant-based diet, other than traditional nutrients, that may reduce the risk of degenerative diseases[6,7,8]. Flavonoids are widely distributed in the plants. Flavonoids are reported to possess many biochemical and pharmacological effects, including antioxidation, antiinflammation and antiallergy[9,10]. Flavonoid intake was reported to reduce cancer risk[11]. Flavonoids inhibited enzymes such as prostaglandin synthase, lipoxygense and cyclooxygenase, closely related to tumorigenesis[12,13].

As consumption continues to increase, peppers could provide important amounts of both nutritional and non-nutritional antioxidants to the human diet. Levels of the phytochemicals may be affected by maturity, genotype as well as storage and processing conditions so as to affect antioxidant properties that are important for dietary consideration as well as consumption. However, the understanding of phytochemicals and their antioxidant properties in bell peppers is limited. The objective of this study was to determine the levels of phenolic compounds, ascorbic acid and carotenoids, as well as free radical scavenging activity using DPPH assay and antioxidant activity using β-carotene/linoleic acid assay in different colored bell peppers.

Materials and Methods
***Plant materials**:* Green, red and yellow bell peppers (*Capsicum annuum* L.) at maturity stage of full fruit size were obtained from a local wholesale market in Minami-minowa, Nagano.

Extraction: 10 g of fresh pepper tissues were sampled respectively, cut to small pieces and homogenized with 15 ml of 80% methanol. The homogenate was filtered with four layers of cheesecloth and the residue was added with 15 ml of 80% methanol for two successive extractions. The filtrates were combined and centrifuged at 4,000×g for 10 min. The supernatant was collected and 1 ml was taken as methanol extract for the Folin-Ciocalteu assay and the measurement of antioxidant properties. Methanol was removed from the supernatant by evaporation under vacuum at 35°C, pigments and fatty acids were eliminated by two successive extractions with petroleum ether (2:1, v:v). The aqueous phase was collected as a crude extract for purification of phenolics using Sep-Pak C18 column.

Analysis of phenolic compounds: The crude extract was acidified to pH 2.0 and loaded into a Sep-Pak C18 cartridge preconditioned with methanol and acidic water. After washing with distilled water, the cartridge was eluted with methanol. The methanol solution was concentrated under vacuum at 35ºC for determination of phenolics and antioxidant properties. Phenolics was determined by the Folin-Ciocalteu reagent as described by Singleton and Rossi[14] using gallic acid as a standard and expressed as mg gallic acid equivalent (GAE)/100 g fresh weight (FW). After filtered with

0.45μm Advantec filter, phenolic compounds were analyzed by HPLC according to the method of Lee et al.[7] with some modification. Samples were separated on a Luna 5 μC18 column (150×4.60 mm from Phenomenex) by HPLC (SHIMADZU LC-VP Liquid Chromatograph equipped with SPD-M10Avp Photodiode Array Detector). The mobile phase consisted of acidified distilled water (0.1% phosphoric acid) (solvent A) and acidified acetonitrile(0.1% phosphoric acid) (solvent B).
The flow rate was 1.0 ml/min. The best separation was obtained at 40ºC using the following gradient elution: 5% B at 0 min, 25% B at 15 min, 35% B at 25 min, 50% B at 30 min, 70% B at 40 min, and 70% B at 50 min. SHIMADZU SPD-M10Avp Photodiode Array Detector was used to record UV spectra of phenolic compounds. The spectra were compared with authentic luteolin. The contents of phenolic compounds were expressed in mg equivalent luteolin per 100 g fresh weight respectively.

Free radical scavenging activity: Free radical scavenging activity of the extracts was measured using the method of Brand-Williams et al.[15] with some modification. A 0.1mM solution of DPPH (1,1-diphenyl-2-picryl-hydrazyl) in methanol was prepared and 4 ml of this solution was added with 0.2 ml of the extract. The decrease in absorbance at 517 nm was measured at 60 min. A control was added with 0.2 ml of distilled water instead of the extract. Free radical scavenging activity was expressed as the percentage of DPPH decrease.

Antioxidant activity: Evaluation of antioxidant activity based on coupled oxidation of ß-carotene and linoleic acid was conducted as described by Taga et al.[16] with some modification. ß-carotene (6 mg) was dissolved in 20 ml of chloroform. A 3 ml of the solution was added to a conical flask with 40μl linoleic acid and 400 μl Tween 20. Chloroform was removed with a rotary evaporator under vacuum at 35ºC. Oxygenated distilled water (100 ml) was added to the ß-carotene emulsion and mixed well. 3 ml aliquot of the ß-carotene emulsion and 0.2 ml of the diluted extract were placed in a test tube and mixed well. The tubes were immediately placed in a water bath and incubated at 50ºC. Oxidation of ß-carotene emulsion was monitored by measuring absorbance at 470 nm. Sample absorbance was measured at 60 min after incubation. A control consisted of 0.2 ml distilled water, instead of the extract. The degradation rate of the extracts was calculated by first order kinetics: Sample degradation rate = ln (a/b) ×1/t, where: ln = natural log; a = initial absorbance at time 0; b = absorbance at 60 min; t = time (min). Antioxidant activity (AA) was expressed as % inhibition relative to the control using the equation:

$$AA(\%) = \frac{\text{Degradation of control} - \text{Degradation of sample}}{\text{Degradation of control}} \times 100$$

Ascorbic acid analysis: 10 g of fresh pepper tissues were sampled respectively, cut to small pieces and homogenized with 15 ml of 5% metaphosphoric acid. The homogenate was filtered with four layers of cheesecloth and the residue was added with 10 ml of 5% metaphosphoric acid for two successive extractions. The filtrate was combined and centrifuged at 4,000×g for 10 min. The supernatant was collected and made up to 50 ml and then filtered with 0.45mm Advantec filter for HPLC analysis. HPLC analysis was performed using SHIMADZU LC-10A Liquid Chromatograph with a Shodex Asahipak NH2P-50 4E column (250×4.60 mm from Showa Denko K.K. Japan) at 40ºC. The mobile phase consisted of acidified distilled water (0.1% phosphoric acid) (solvent A) and acetonitrile (solvent B), performed at the ratio 25:75. The flow rate was 1.0 ml/min. L-ascorbic acid was detected at 254 nm.

Carotenoid analysis: Carotenoids were analyzed according to the methods of Howard et al.[17] and Mizda et al.[18] with some modification. 15 g of fresh pepper tissues were sampled respectively, cut to small pieces and homogenized with 10 ml of -20ºC acetone. The homogenate was filtered with four layers of cheesecloth and the residue was added with -20ºC acetone for three successive extractions. The filtrate was combined and centrifuged at 4,000×g for 10 min. The supernatant was collected and then filtered with 0.45mm Advantec filter for HPLC analysis. The same HPLC system as used for analysis of phenolics was used for carotenoid analysis. The mobile phase consisted of acetonitrile-water (9:1, solvent A) and ethyl acetate (solvent B). The flow rate was 1.0 ml/min. The following gradient elution was used for separation: 0% B at 0 min, 50 % B at 20 min, and 50% B at 35 min. Samples were detected at 450 nm. Identification of carotenoids was tentatively achieved by their retention time and UV spectra recorded with SHIMADZU SPD-M10Avp photodiode array detector in comparison with authentic β-carotene and carotenoids identified in peppers according to Howard et al.[17] and Mizda *et al*.[18]. The content of carotenoids was expressed in mg equivalent β-carotene per 100 g fresh weight.

Results

Phenolic, ascorbic acid and carotenoid contents : Phenolics varied with different colored bell peppers. Bell peppers contained phenolics 9.2-15.4 mg GAE/100g FW (Table 1). In the phenolic extracts, green pepper had a significant higher level of phenolics than red and yellow peppers ($P<0.05$). The methanol extracts was also measured by the Folin-Ciocalteu method. Red pepper showed a higher level of phenolics in the methanol extracts than green and yellow peppers (Table 1). Furthermore, the methanol extracts were observed markedly higher in the measurements compared to the phenolic extracts. The phenolic extracts of different colored peppers were also separated and analyzed by HPLC. Two luteolin conjugates were major flavonoids in green peppers (Figure 1). Their spectra were very similar to luteolin ($R^2 = 0.989$ and 0.999 respectively) (Figure 2). The same conjugates were also found in red and yellow pepper, while some other phenolics occurred. However, flavonoid aglycone – luteolin was barely detected. The contents of major luteolin conjugates were shown in Table 2. The results show that green pepper contained greater levels of luteolin conjugates than red and yellow peppers ($P<0.05$). Luteolin conjugates in green peppers represented 62.1% of total phenolics detected, while luteolin conjugates represented 23.8% in red pepper and 17.6% in yellow pepper respectively. Ascorbic acid was extracted with 5% metaphosphoric acid. The level was found significantly different within green, red, and yellow peppers (Table 1). Red pepper had the highest level of ascorbic acid, while green pepper had the lowest. The ascorbic acid content in the methanol extracts was also measured by HPLC. The level in the methanol extract was found very high, just slightly lower than in the extraction using 5% metaphosphoric acid (Table 1). Peppers mainly contained violaxanthin, capsanthin, lutein, and β-carotene (Figure 3). The level of those compounds varied with different

Table 1. Phenolic, ascorbic acid and carotene contents in different colored peppers.

	Peppers*		
Components	Green	Red	Yellow
Phenolic content (mg/100g FW)			
Methanol extract	48.4±0.6c	64.5±0.7a	54.8±0.6b
Phenolic extract	15.4±0.2a	9.9±0.1b	9.2±0.1b
Ascorbic acid (mg/100g FW)			
5% metaphosphoric acid extracts	115.5±2.2c	191.2±2.3a	153.5±2.3b
Methanol extracts	109.4±2.0c	187.1±2.1a	151.5±2.2b
Carotenoids (mg/100g FW)			
Methanol extract	0.38±0.02b	0.44±0.02b	1.09±0.04a
Acetone extract	0.82±0.04c	1.48±0.08b	1.67±0.08a

*Data were the means of three duplicate experiments. Means in each row with similar letters not significantly different(P<0.05, Duncan's multiple range test).

Table 2 . Major luteolin conjugates in different colored peppers.

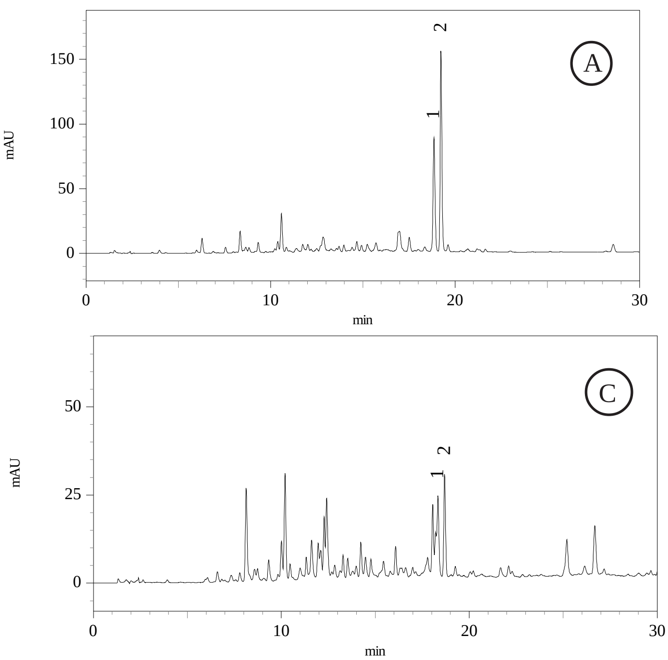
	Luteolin-conjugates (mg/100gFW)*	
Peppers	1	2
Green	1.36±0.05a	2.12±0.06a
Red	0.44±0.01b	0.65±0.02b
Yellow	0.34±0.01c	0.38±0.01c

*Data were the means of three duplicate experiments. Means in each column with similar letters not significantly different (P<0.05, Duncan's multiple range test).

Table 3. Free radical scavenging activities and antioxidant activities in different colored peppers*.

Peppers and compounds	Free radical scavenging activity (%)	Antioxidant activity (%)
Green		
Methanol extract	33.2±1.2	2.8±0.1
Phenolic extract	3.9±0.1	13.8±0.5
Red		
Methanol extract	52.3±1.9	-9.5±0.3
Phenolic extract	2.4±0.1	8.6±0.2
Yellow		
Methanol extract	45.5±1.5	-6.7±0.1
Phenolic extract	2.2±0.1	8.1±0.2
Ascorbic acid (2mg/100ml)	12.4±0.5	-11.4±0.4
β-carotene (1mg/100ml)	1.1±0.0	-
Luteolin (2.5mg/100ml)	31.2±1.1	55.2±1.8

*The extracts for free radical scavenging activity were tested at a level representing 5% of original fresh plant material, while antioxidant activity was tested at a level of 10%. Data are the means of three duplicate experiments.



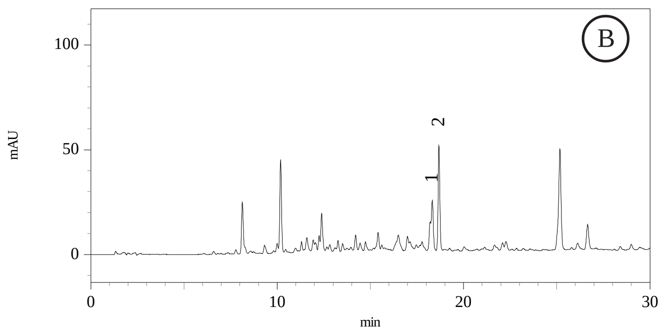


Figure 1. HPLC chromatogram of phenolic compounds from green (A), red (B) and yellow (C) peppers at 280 nm. Peak 1 and 2: luteolin conjugates.

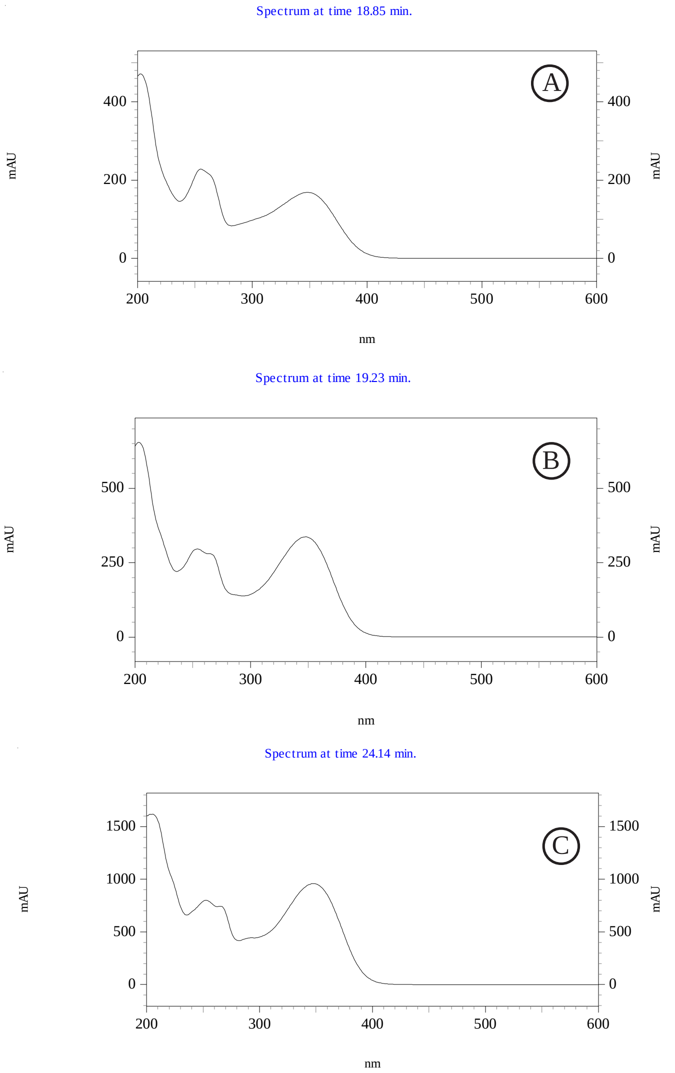


Figure 2. Spectra of luteolin conjugates from peppers and luteolin standard recorded by diode array detector. A: peak 1, B: peak 2, and C: luteolin.

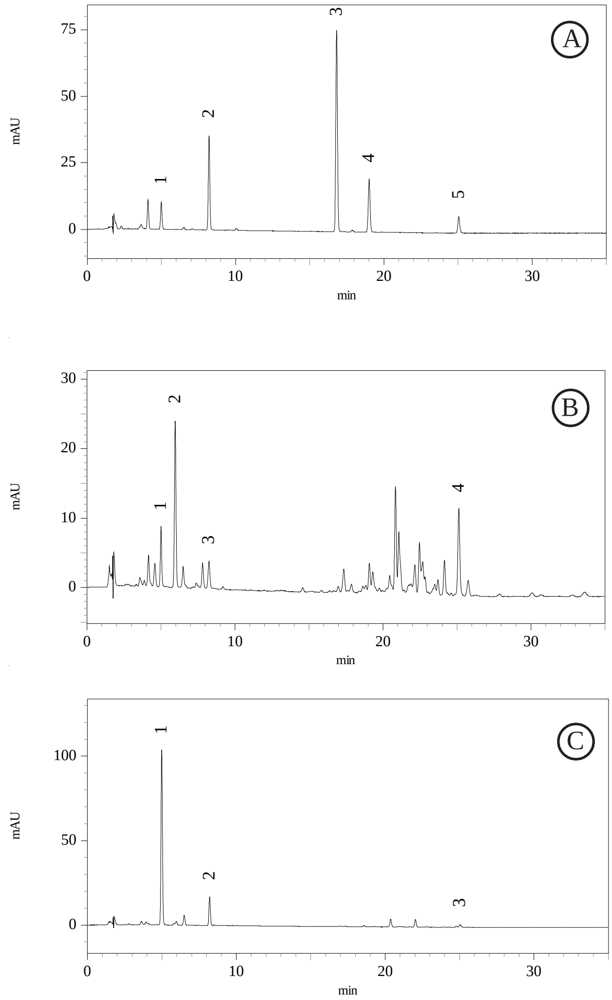


Figure 3. HPLC chromatogram of carotenoids from green (A), red (B) and yellow (C) peppers at 450 nm. In A, peak 1: violaxanthin, peak 2: lutein, peak 3 and peak 4: chlorophyll a and b, peak 5: β-carotene; in B, peak 1: violaxanthin, peak 2: capsanthin, peak 3: lutein, peak 4: β-carotene; in C, peak 1: violaxanthin, peak 2: lutein, peak 3: β-carotene.

colored peppers. The carotenoid contents were shown in Table 1. Yellow pepper had the highest level of carotenoids, while green pepper had the lowest. The methanol extracts were also analyzed by HPLC for carotenoid content. The methanol extracts were seen lower in the carotenoid contents than the extracts by acetone. In the methanol extracts, yellow pepper had the highest level of carotenoids, while green pepper was the lowest.

Free radical scavenging activity and antioxidant activity Free radical scavenging activity in different colored bell peppers is shown in Table 3. Free radical scavenging activity varied in different colored bell peppers. In the phenolic extracts, green pepper showed the highest radical scavenging activity, while yellow pepper had the lowest. However, in the methanol extracts, red pepper was the highest, while green pepper was the lowest. In all the colored peppers, the methanol extracts also showed much higher in radical scavenging activity than phenolic extracts. At a level representing 5% of original fresh plant material, DPPH radical scavenging activity in phenolic extracts accounted for 4.6-11.7% of the methanol extracts. Antioxidant activity was measured using the coupled oxidation of β-carotene/linoleic acid method. The results showed that antioxidant activity varied in different colored bell peppers (Table 3). In the phenolic extracts, green pepper showed the highest antioxidant activity, while yellow pepper had the lowest. However, a pro-oxidant effect was observed with the methanol extracts. Green pepper methanol extract showed a low level of antioxidant activity than the phenolic extracts. Red and yellow pepper methanol extracts showed promotion of bleaching of the β-carotene emulsion.

Discussion

Vinson et al.[19] found bell peppers contained 46.4 mg catechin equivalent/100 g fresh weight. Lee et al.[7] reported that fresh peppers contained total soluble phenolics from 178.0 to 384.9 mg chlorogenic acid equivalent/100 g fresh weight. The different values in the literature may be due to different cultivars, different extraction methods and the ways to express the results. In our study, the methanol extracts of all three colored peppers were found to exhibit much higher in phenolic levels than Sep-Pak C18 column-purified extracts. The phenolic content in Sep-Pak C18 column-purified extracts accounted for 15-32% of the methanol extracts.

This is likely due to a high level of ascorbic acid remaining in the methanol extracts (Table 1) as well as some other reducing components. These compounds capable of being oxidized by the Folin-Ciocalteu reagents of total phenolic assay yield the reduced (coloured) forms of the reagents and appear as a phenolic[20]. Reverse phase Sep-Pak C18 column can absorb flavonoid glycosides as well as other phenolic compounds efficiently[7]. Our results indicate that after removal of interfering components from the extract with Sep-Pak C18 column, the phenolic content of all three peppers tested in our experiment were much lower than the methanol extracts and the results of other research[7,19]. Thus, the effect of ascorbic acid on determination of phenolic content should be considered. The results also show that green pepper was 35.7% and 40.3% higher in phenolic content than red and yellow peppers respectively ($P<0.05$), which are consistent with the results by HPLC which is that green peppers contained higher amount of luteolin conjugates than the red and yellow peppers. The results suggest that the level of phenolic compounds varies within different colored peppers or cultivars. It has been reported that phenolic compounds, ascorbic acid and carotenoids can contribute to antioxidant activity[3,4,5]. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation caused by free radicals. In our study, radical scavenging activity in the phenolic extracts was related to the level of phenolic compounds. The green pepper contained a greater level of phenolics and showed higher scavenging activity. DPPH radical scavenging activity in the methanol extracts was found higher than that of the phenolic extracts. This is likely due to a high level of ascorbic acid remaining in the methanol extracts (Table 1). Ascorbic acid is a potent reducing agent and possesses strong radical scavenging ability[21,22]. Our result also showed that ascorbic acid was strong as a radical scavenger (Table 3). However, ascorbic acid in the methanol extracts was not quite stable. The level was reduced by storage temperature and duration after extraction. In addition, carotenoids were soluble in the methanol, representing 29.7-65.3% of the carotenoids tested in the extract with acetone (Table 1). Though carotenoids such as β-carotene are fat soluble and show a weaker radical scavenger in the test using DPPH method (Table 3), they would still make contribution to radical scavenging activity. Capsaicins may occur in the methanol extracts, which could also contribute to radical scavenging activity since capsaicins can directly scavenge various radicals[23,24]. Therefore, we suggest that the radical scavenging activity in the bell peppers was from different antioxidants, mainly from ascorbic acid and phenolic compounds. In the β-carotene/linoleic acid assay, antioxidant activity of the phenolic extracts varied in different colored peppers. These results suggest that antioxidant activity of the phenolic extracts was related to the level of phenolic compounds, which are consistent with the results of Lee et al.[7]. However, a pro-oxidant effect was observed with all the methanol extracts. This is also likely due to ascorbic acid remaining in the methanol extracts. Ascorbic acid was found to promote bleaching of the β-carotene emulsion[25,26]. Our result also showed that ascorbic acid had pro-oxidant effect (Table 3). Therefore, we suggest that the antioxidant activity in the methanol extracts should be the reflection of antioxidant effect of phenolic compounds and pro-oxidant effect of ascorbic acid. The results obtained HPLC suggest that luteolin conjugates were main phenolic compounds in fresh bell peppers, flavonoid aglycone – luteolin rarely occurred. Luteolin has been shown to be an effective free radical scavenger[27] and to prevent oxidation of low-density lipoprotein[28]. Lu and Foo[22] reported that flavonoid glycoside conjugates were better than epicatechin, phloridzin, chlorogenic acid, ascorbic acid and vitamin E as DPPH free radical scavengers, and flavonoid aglycone such as quercetin had higher radical scavenging activity than its glycosides. Though radical scavenging activity in the phenolic extracts only accounted for 4.6-11.7% of the methanol extracts, flavonoid glycoside conjugates would be degraded to aglycones by human intestinal flora[29] and could provide higher radical scavenging activity and antioxidant activity. Furthermore, some amounts of other phenolic compounds were also seen in the peppers, which could also make contribution to antioxidant properties.

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