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Chemical and biological variability of hot pepper fruits (*Capsicum annuum* var. *acuminatum* L.) in relation to maturity stage

Filomena Conforti *, Giancarlo A. Statti, Francesco Menichini

Department of Pharmaceutical Sciences, University of Calabria, I-87036 Rende (CS), Italy

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Abstract

The aim of the present work was to evaluate the chemical composition and the radical-scavenging and antioxidant activities of hot pepper fruits (*Capsicum annuum* L. var. *acuminatum*) at three maturity stages (small green, green and red). GC–MS analysis of *n*-hexane and chloroform fractions showed a different composition between the three stages of ripening. The first stage of maturation (small green) showed the highest radical-scavenging activity (IC_{50} of 129 μg/ml). Using the bovine brain peroxidation assay, the methanolic extract of green pepper showed significant antioxidant activity (IC_{50} of 522 μg/ml). Addition of methanolic extract of red and green pepper inhibited oxidation of linoleic acid. Methanolic extract of red pepper showed greater antioxidative potency than the others (IC_{50} of 3 μg/ml). The different composition of lipophilic compounds and the various amount of phenolics, showed in the three stage of ripening of *C. annuum* var. *acuminatum* fruits, modifies the antioxidant activity.

Keywords: *Capsicum annuum* L. var. *acuminatum*; Radical scavenger; Antioxidant activity; Biodiversity; Phenolic content

1. Introduction

Oxidative stress in cells can result from an increase in the levels of reactive oxygen species (ROS) (Halliwell & Gutteridge, 1989). ROS, such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH), are produced as a result of many biochemical reactions and are considered to be the prime causes of oxidative damage, including protein denaturation, mutagenesis, and lipid peroxidation in aerobic cells. Among these free radicals, the hydroxyl radical is one of the most aggressive found in living beings, reacting at a controlled diffusion rate with molecules such as DNA, lipids, proteins, and carbohydrates (Baskin & Salem, 2000). Many conditions that limit productivity, including ozone exposure, metal toxicity, exposure to radiation, wounding, chilling, drought, salinity, heat stress, pathogens, and senescence, result in the enhanced production of ROS (Nez, Romojaro, Mez, Lanos, & Sevilla, 2003). Oxidative damage, caused by the action of free radicals, may initiate and promote the progression of a number of chronic diseases, including cancer, cardiovascular diseases and inflammation.

Pepper is a vegetable of importance in human nutrition. Currently, one of the most interesting properties of natural products is their antioxidant content. In recent years, peppers have grown in popularity, and a wide number of varieties are now available in the grocery stores. This taxon includes both sweet cultivars eaten mainly as vegetables and hot ones, often used as a spice. Hot pepper, genus Capsicum, belongs to the Solanaceae family (Pignatti, 1982). Chemical composition of pepper fruit has been studied fairly well, mainly with respect to vitamin (C, E), β-carotene and carotenoid pigments content (Minguez-Mosquera & Hornero-Mendez, 1994; Palevitch & Craker, 1995).

Within hot varieties of pepper fruits, the capsaicinoids were also studied. Capsaicinoids are alkaloids that are important in the pharmaceutical industry for their neurological effectiveness. When used at low levels in the diet

* Corresponding author. Tel.: +39 0984 493063; fax: +39 0984 493298.
E-mail address: filomena.conforti@unical.it (F. Conforti).

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they significantly decrease serum, myocardial and aortic total cholesterol levels (Govindarajan & Sathyanarayana, 1991).

It is of great interest to know what is the contribution of an individual food product in the daily nutritional needs and how ripening and maturity affect nutritive composition and therefore biological properties (Gao, Ohlander, Jeppsson, Bjork, & Trajkovski, 2000). Peppers suffer a profound change during the course of ripening with the conversion of existing pigments. Thus, the green colour of the fruit is principally due to the presence of chlorophyll and to the carotenoids typical of the chloroplast, such as oxygenated carotenoids or xanthophylls, violaxanthin, neoxanthin, and lutein, as well as β-carotene (Mìnguez-Mosquera & Hornero-Méndez, 1994). Among the carotenoid pigments, capsanthin, capsorubin and capsanthin 5,6-epoxide are almost exclusive to the genus *Capsicum* and are responsible for the final red colour (Davis, Mathews, & Kirk, 1970). Red peppers contain the highest amount of provitamin A (β-carotene and β-cryptoxanthin) (Mìnguez-Mosquera & Hornero-Méndez, 1993). Regarding flavonoids, most of the studies on peppers have been concentrated only on flavonoid aglycones (quercetin and luteolin) obtained after hydrolysis (Howard, Talcott, Brenes, & Villalon, 2000; Lee, Howard, & Villalon, 1995). Materska et al. (2003) have recently identified two new flavonoids in hot pepper pericarp, and other flavonoids were found for the first time in pepper fruits. Howard et al. (2000) have studied the effects of pepper maturation on antioxidants content in different pepper types (*Capsicum annuum*, *C. frutescens*, and *C. chinense*). They found that the concentration of these antioxidant constituents increased as the peppers reach maturity.

The aim of the present work was to determine the radical-scavenging and antioxidant activities of hot pepper (*C. annuum* L. var. *acuminatum*), widely consumed in diets of Mediterranean people, at different ripe stages. Immature green, green and red hot peppers were evaluated to estimate the activity against free radicals depending on the maturity stage. Total soluble phenolics were measured by the Folin-Ciocalteu assay. The antioxidant potential was determined by three complementary methods: DPPH radical-scavenging assay that evaluates the antioxidant capacity through hydrogen donating ability of antioxidants; bovine brain peroxidation assay that evaluates the attack of free radicals on membrane systems and the reduction of the extent of peroxidation when an antioxidant compound is incorporated in the lipid peroxidation assay reaction mixture; β-carotene bleaching test that evaluates the inhibition of the breakdown of lipid hydroperoxides.

2. Materials and methods

2.1. Chemicals

Methanol, ethanol, ethyl acetate, petroleum ether, diethyl ether, H_2SO_4, chloroform, HCl, KOH, butanol, silica gel 70–230 mesh, thin-layer chromatography plates (TLC) were obtained from VWR International s.r.l. (Milano, Itlay). Thiobarbituric acid (TBA), phosphate buffered saline (PBS), bovine brain extract, $FeCl_3$, ascorbic acid, butylated hydroxytoluene (BHT), propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, linoleic acid, Tween 20, Folin-Ciocalteu reagent, chlorogenic acid were obtained from Sigma–Aldrich S.p.a. (Milano, Italy).

2.2. Plant materials

The fruits of *C. annuum* L. var. *acuminatum* used in this study were harvested in Calabria (Italy) at the same time (September 2004) but at three successive stage of technological maturity on the basis of their size and colour as one ripening stage (not completely developed named small green) and two maturity stages (completely developed but not coloured named green, and at the stage of full ripeness named red). The not completely developed small green fruit showed a mean weight of 8.34 ± 0.88 g, while for green and red fruits this mean was 45.45 ± 2.09 g.

The samples were authenticated by Prof. Dimitar Uzunov, Natural History Museum of Calabria and Botanic Garden, University of Calabria, Italy.

2.3. Preparation of the extracts

Five hundred grams of each fresh sample had been washed and cut in small pieces and extracted with MeOH (5 l) through maceration (144 h × 3 times). The resultant extract was dried under reduced pressure to give 31.35 g of small green, 9.13 g of green and 41.51 g of red. In order to operate a separation of the chemical compounds, methanolic extracts were acidified with 2.5% aq H_2SO_4 (pH 1) and partitioned following the procedure proposed by De Vivar, Pérez, Vidales, Nieto, and Villaseñor (1996). The aqueous acid solution was extracted with *n*-hexane, basified with aq. NH_3 (to pH 10) and extracted with chloroform. The obtained fractions taken to be brought to dryness under reduced pressure to determine the weight and the yield % in comparison to the weight of the fresh fruits (Table 1).

2.4. GC–MS analysis

The *n*-hexane and chloroform fractions analysis was performed using a Hewlett–Packard gas-chromatograph, model 5890 equipped with a mass spectrometer, model 5972 series II, and controlled by HP software equipped with capillary column 30 m × 0.25 mm, static phase SE30, using programmed temperature from 60 °C to 280 °C (rate 16°/min); the detector and the injector were set to a temperature of 280 °C and 250 °C, respectively (split vent flow 1 ml min^{-1}). Compound identification was verified according to relative retention time and mass spectra with those of Wiley 138 library data of the GC–MS system (Hewlett–Packard Co.).

Table 1
Methanolic extracts, *n*-hexane and chloroform fractions yield of *Capsicum annuum* var. *acuminatum* at different ripening stages

Ripening stage	MeOH (g)	Yield %[a]	*n*-Hexane (g)	Yield %[a]	$CHCl_3$ (g)	Yield %[a]
Small green	31.35	6.27	0.19	0.04	1.55	0.31
Green	9.13	1.83	0.28	0.06	1.05	0.21
Red	41.51	8.30	0.41	0.08	0.9	0.18

[a] Percent in comparison to the weight of the fresh fruits (500 g).

2.5. Determination of total phenolics content

Total phenolics content of the MeOH extracts was determined by the Folin-Ciocalteu assay and chlorogenic acid was used as standard (Singleton & Rossi, 1965). Fifty milligrams of each extract was weighed into 50 ml plastic extraction tubes and vortexed with 25 ml extraction solvent (40 ml acetone:40 ml methanol:20 ml water:0.1 ml acetic acid). Then, the sample with the extraction solvent was heated at 60 °C (water bath) for 1 h, allowed to cool to room temperature, and homogenized for 30 s with a sonicator at setting 6. Two hundred microlitres (μl) (three replicates) were introduced into screw cap test tubes; 1.0 ml of Folin-Ciocalteu's reagent and 1.0 ml of sodium carbonate (7.5%) were added. The tubes were vortexed and allowed to stand for 2 h. The absorption at 726 nm was measured (Perkin–Elmer Lambda 40 UV/Vis spectrophotometer) and the total phenolic content was expressed as chlorogenic acid equivalents in mg per g dry material.

2.6. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Free radical-scavenging activity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay which was adapted from Wang et al. (1998). Briefly in an ethanol solution of 2,2-diphenyl-1-picrylhydrazyl radical (final concentration was 1.0×10^{-4} M) test samples were added at different concentrations. The absorbance of the resulting solutions was measured in 1 cm cuvettes using a Perkin–Elmer Lambda 40 UV/Vis spectrophotometer at 517 nm against blank, which was without DPPH. All tests were run in triplicate and averaged. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical-scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated in the equation:

$$\%\ \text{DPPH radical} - \text{scavenging} = \text{sample absorbance/control absorbance} \times 100$$

The DPPH solution without sample solution was used as control. Ascorbic acid was used as the positive control.

2.7. Bovine brain peroxidation assay

The in vitro antioxidant activity test was carried out using the TBA test (Fernandez, Perez-Alvarez, & Fernandez-Lopez, 1997). The TBA test detects aldehydic products such as malondialdehyde (MDA) resulting from lipid oxidation. MDA reacts with TBA to yield a coloured product, which in an acid environment absorbs light at 532 nm and is readily extractable into organic solvents. The intensity of colour is a measure of MDA concentration.

Methanol extracts of all samples were tested for their antioxidant activity against liposomes which were prepared from bovine brain extract in phosphate buffered saline (5 mg/ml). Peroxidation was started by adding $FeCl_3$ (1 mM) and ascorbic acid (1 mM) followed by incubation at 37 °C for 20 min. TBA, 25% HCl and BHT were added to the tubes and heated at 85 °C for 1 h to develop the colour. BHT in ethanol was added to prevent lipid peroxidation during the TBA test itself. The red chromogen, expression of the MDA:TBA adduct formation, was extracted with *n*-butanol; after brief centrifugation to favour organic phase separation, the upper *n*-butanol layer was removed and read spectrophotometrically at 532 nm against an appropriate blank using a Perkin–Elmer Lambda 40 UV/Vis spectrophotometer. Propyl gallate (Jacobi, Hinrichsen, Web, & Witte, 1999) was used as a positive control.

2.8. β-Carotene bleaching test

Antioxidant activity was determined using β-carotene bleaching test (Amin, Zamaliah, & Chin, 2004). Briefly 1 ml of β-carotene solution (0.2 mg/ml in chloroform) was added to 0.02 ml of linoleic acid and 0.2 ml of 100% Tween 20. The mixture was then evaporated at 40 °C for 10 min by means a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 100 ml of distilled water. The water was added slowly to the mixture and agitated vigorously to form an emulsion.

Five millilitres of the emulsion were transferred into different test tubes containing Two hundred microlitres of samples in 70% ethanol at different concentrations (2.6, 1.3, 0.65, 0.26, 0.13, 0.026, 0.013 and 0.0065). 0.2 ml of 70% ethanol in 5 ml of the above emulsion was used as control. Standard (propyl gallate) at the same concentration as samples was used for comparison. The tubes were then gently shaken and placed at 45 °C in a water bath for 60 min. The absorbance of the samples, standard and control was measured at 470 nm using a Perkin–Elmer Lambda 40 UV/Vis spectrophotometer against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at initial time ($t = 0$) and successively at 30 and 60 min. All samples were assayed in triplicate and averaged.

Table 2
Chemical composition of *n*-hexane extracts from *Capsicum annuum* at different stage of ripening: (A) red; (B) green; (C) small green

Compound [a]	R_t [b]	A[c]	B[c]	C[c]
2-Heptanal (*E*)	7.53	–	–	0.496 ± 0.031
2-Decenal (*E*)	12.3	–	–	0.205 ± 0.021
2,4-Decadienal (*E*,*E*)	12.7	–	–	0.659 ± 0.045
Decadienal	12.9	–	–	0.863 ± 0.067
2-Undecenal	13.4	–	–	0.080 ± 0.009
Tetradecane	13.8	–	0.071 ± 0.009	–
Nonanoic acid, 9-oxo-, methyl ester	14.2	–	–	0.167 ± 0.018
Hexadecane, 2,6,10,14-tetramethyl-	14.4	–	–	0.043 ± 0.007
Pentadecane	14.8	–	0.108 ± 0.011	0.067 ± 0.008
Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl	15.0	–	0.117 ± 0.014	0.033 ± 0.003
Heptacosane	15.3	0.219 ± 0.041	–	–
Farnesol	15.5	0.248 ± 0.045	–	–
1-Hexadecene	15.7	–	0.071 ± 0.011	–
Tetradecanal	16.4	0.231 ± 0.042	–	0.046 ± 0.004
Heptadecane	16.6	0.549 ± 0.058	0.154 ± 0.012	0.176 ± 0.231
Myristic acid, methyl ester	16.8	0.609 ± 0.061	0.259 ± 0.031	0.060 ± 0.008
9-Octadecene (*E*)-	17.0	0.460 ± 0.062	–	–
1-Pentadecene	17.0	–	0.304 ± 0.041	–
Undecane	17.1	–	0.128 ± 0.015	–
Hexadecane	17.2	–	0.088 ± 0.011	0.113 ± 0.011
Oleic acid	17.3	–	0.095 ± 0.012	–
Octadecane	17.5	–	0.168 ± 0.014	0.110 ± 0.013
Oleic acid, methyl ester	17.6	–	0.306 ± 0.032	–
Pentadecanoic acid, methyl ester	17.7	0.324 ± 0.033	0.362 ± 0.037	0.057 ± 0.009
Pentadecanoic acid	17.7	0.638 ± 0.068	1.06 ± 0.241	–
Neophytadiene	17.8	–	–	0.050 ± 0.021
2-Pentadecanone, 6,10,14-trimethyl-	17.9	–	–	0.880 ± 0.067
2-Decene,7-methyl- (*Z*)	18.0	1.01 ± 0.271	–	–
Pentadecanoic acid, 14-methyl-, methyl ester	18.2	0.315 ± 0.029	0.743 ± 0.069	0.104 ± 0.014
Palmitoleic acid, methyl ester	18.3	1.03 ± 0.221	1.702 ± 0.231	
Palmitic acid, methyl ester	18.5	15.1 ± 0.976	8.64 ± 0.721	8.07 ± 0.701
Palmitic acid	18.9	–	1.71 ± 0.231	–
Palmitic acid, 14-methyl-, methyl ester	18.9	0.168 ± 0.025	1.37 ± 0.219	0.093 ± 0.022
Palmitic acid, ethyl ester	19.0	0.354 ± 0.031	–	–
Cycloeicosane	19.1	–	1.06 ± 0.191	–
Margaric acid, methyl ester	19.2	0.657 ± 0.066	–	–
5-Octadecene (*E*)	19.7	1.13 ± 0.231	–	–
8,11-octadecadienoic acid, methyl ester	19.8	13.5 ± 0.956	13.8 ± 0.978	3.64 ± 0.589
Linolenic acid, methyl ester	19.8	13.0 ± 0.945	4.99 ± 0.651	–
Phytol	19.9	4.61 ± 0.598	8.87 ± 0.739	1.61 ± 0.231
Stearic acid, methyl ester	20.0	2.53 ± 0.331	1.07 ± 0.191	1.37 ± 0.211
Hexadecanamide	20.4	–	–	0.370 ± 0.031
Eicosane	20.5	–	–	0.246 ± 0.027
Octadecanal	20.7	–	–	0.376 ± 0.032
Nonadecanoic acid, methyl ester	20.7	–	1.27 ± 0.231	–
1-Octadecene	21.1	0.668 ± 0.051	0.960 ± 0.062	0.159 ± 0.018
Linoleic acid	21.2	–	0.841 ± 0.059	–
Docosane	21.2	–	–	0.379 ± 0.036
Arachidic acid, methyl ester	21.3	0.230 ± 0.021	0.981 ± 0.067	0.444 ± 0.041
9-Octadecenamide (Z)-	21.7	–	1.43 ± 0.231	2.25 ± 0.331
1-Heneicosyl formate	21.7	0.256 ± 0.021	–	–
Octadecanamide	21.8	–	–	0.479 ± 0.038
Heneicosanoic acid, methyl ester	22.0	–	0.597 ± 0.047	–
Cyclodocosane, ethyl-	22.0	–	–	0.568 ± 0.043
4-Hexenoic acid, 3-methyl-2,6-dioxo-	22.2	–	–	0.139 ± 0.012
Cyclotetracosane	22.4	–	–	0.274 ± 0.022
Behenic acid, methyl ester	22.4	–	0.469 ± 0.037	0.701 ± 0.062
Pelargonic acid vanillylamide	22.5	–	0.453 ± 0.034	–
Palmitic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester	22.6	–	–	1.88 ± 0.231
Octadecane	23.2	–	–	0.123 ± 0.013
Tricosanoic acid, methyl ester	23.4	–	0.109 ± 0.011	0.144 ± 0.014
Linoleic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	24.0	–	2.042 ± 0.321	0.905 ± 0.081
2-Monolinolenin	24.1	–	0.698 ± 0.058	–

Table 2 (*continued*)

Compound [a]	R_t [b]	A[c]	B[c]	C[c]
Stearic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	24.2	–	–	0.394 ± 0.031
Lignoceric acid, methyl ester	24.22	–	0.437 ± 0.036	0.397 ± 0.032
Cholest-5-en-3-ol (3β)	30.0	–	0.244 ± 0.023	0.817 ± 0.054
Vitamin E	30.1	4.87 ± 0.678	0.292 ± 0.027	0.328 ± 0.029
Ergost-5-en-3-ol, (3β,24*R*)-	32.3	0.944 ± 0.072	2.908 ± 0.398	8.22 ± 0. 651
Stigmast-5,22-dien-3-ol (3β,22*E*)	33.2	–	0.714 ± 0.061	1.28 ± 0.231
Stigmast-5-en-3-ol, (3β,24*S*)	34.7	1.79 ± 0.231	4.040 ± 0.619	12.8 ± 0. 978
β-Amyrin	35.9	–	0.645 ± 0.059	1.9 ± 0.231
Viminalol	37.3	–	1.113 ± 0.189	–
Stigmast-4-en-3-one	39.1	–	–	0.321 ± 0.029

[a] Compounds listed in order of elution from SE30 MS column.
[b] Retention time (as min).
[c] Relative area percentage (peak area relative to total peak area %).

The antioxidant activity (AA) was measured in terms of successful bleaching of β-carotene by using the following equation.

$$AA = \left(1 - \frac{A_0 - A_t}{A_0^o - A_t^o}\right) \times 100$$

where A_0 and A_0^o are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, while A_t and A_t^o are the absorbance values measure in the samples/standard and control, respectively, at $t = 30$ min and $t = 60$ min.

2.9. Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by using Student's *t*-test. Differences were considered significant at $P \leqslant 0.05$. The inhibitory concentration 50% (IC_{50}) was calculated by nonlinear regression analysis using the Prism Graphpad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA (www.graphpad.com). The dose–response curve was obtained by plotting the percentage of inhibition versus the concentrations.

3. Results

GC–MS analysis of the *n*-hexane and chloroform fractions of *C. annuum* var. *acuminatum* showed a different composition between the three stage of ripening. The *n*-hexane fraction red fruits showed a major content in vitamin E (4.87%) compared to green and small green fruits (0.29% and 0.33%, respectively). Green fruits possess a high content of phytol, an acyclic diterpene alcohol (8.87%) compared to red and small green fruits (4.61% and 1.61%, respectively). Finally the *n*-hexane fractions were rich in sterols, particularly cholest-5-en-3-ol, ergost-5-en-3-ol, stigmast-5,22-dien-3-ol and stigmast-5-en-3-ol (Table 2). Small green fruits showed the major content of all sterols. The amount of cholest-5-en-3-ol decreased from 0.817% in small green to 0.244% in green while it is absent in red fruits. Ergost-5-en-3-ol varied widely in the different stage of ripening, 8.22%, 2.91% and 0.94%, in small green, green and red fruits, respectively. The same results was obtained for stigmast-5-en-3-ol which is present at 12.8%, 4.04% and 1.79% in small green, green and red fruits, respectively, and stigmast-5,22-dien-3-ol which is present at 1.28% and 0.714% in small green and green fruits, respectively.

Table 3
Determination of capsaicin and dihydrocapsaicin in *Capsicum annuum* var. *acuminatum* at different stage of ripening: (A) red; (B) green; (C) small green

Compound	R_t [a]	A[b]	B[b]	C
Capsaicin	23.1	40.80 ± 1.56	1.46 ± 0.231	–
Dihydrocapsaicin	23.3	35.0 ± 1.32	1.24 ± 0.126	–

[a] Retention time (as min).
[b] Relative area percentage (peak area relative to total peak area %).

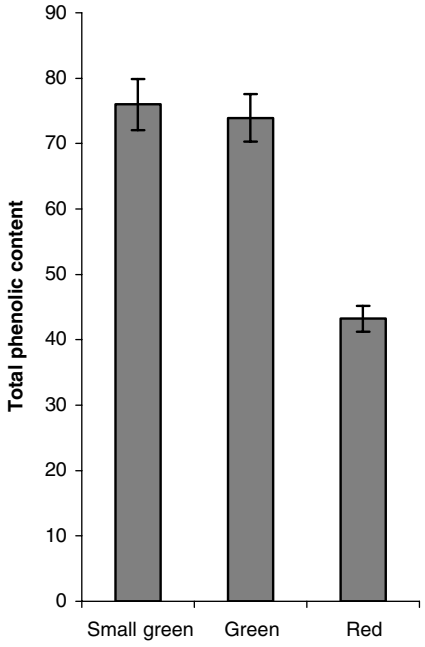

Fig. 1. Total phenolic content of MeOH extracts using Folin-Ciocalteau method. Values expressed as chlorogenic acid/g of extract ± SEM ($n = 3$).

Table 4
IC_{50} values of radical-scavenging and antioxidant activities of methanolic extract of hot pepper at three ripening stages (*Capsicum annuum* L. var. *acuminatum*) ($n = 3$)

Extract	DPPH	IC_{50} (μg/ml)		
		Bovine brain peroxidation	β-Carotene bleaching test	
			30 min of incubation	60 min of incubation
Red	419 ± 4.23	>1000	4 ± 0.391	3 ± 0.173
Green	389 ± 3.27	522 ± 4.83	24 ± 0.872	19 ± 0.782
Small green	129 ± 1.18	>1000	>100	>100
Propyl gallate[a]	–	7 ± 0.53	1 ± 0.098	1 ± 0.086
Ascorbic acid[a]	2 ± 0.231	–	–	–

[a] Propyl gallate and ascorbic acid were used as positive control.

About chloroform fractions, red fruits showed the major content of capsaicin (40.8%) and dihydrocapsaicin (5.0). Green fruits also showed trace of capsaicin while it was absent in small green fruits (Table 3).

Total soluble phenolic constituents of the MeOH extracts of *C. annuum* var. *acuminatum*, measured by Folin-Ciocalteu method, were similar in the first and second stage of ripening (76.0 mg/g in small green fruits, 73.8 mg/g in green fruits) while varied widely in the last stage of maturity (43.2 mg/g in red fruits) (Fig. 1).

The scavenging effects of methanolic extract of three maturity stages of hot pepper on DPPH were examined at different concentrations (25, 50, 100, 250, 500 and 1000 μg/ml). As shown in Table 4 and Fig. 2, the first stage of maturation (small green) showed the highest activity (IC_{50} of 129 μg/ml) while the other two stages, green and red, showed similar activity (IC_{50} of 389 and 419 μg/ml, respectively).

Using the bovine brain peroxidation assay, the methanolic extract of green pepper showed the highest antioxidant activity (IC_{50} of 522 μg/ml) (Table 4 and Fig. 3). Methanolic extract of red pepper and small green pepper did not show significant activity ($IC_{50} > 1$ mg/ml).

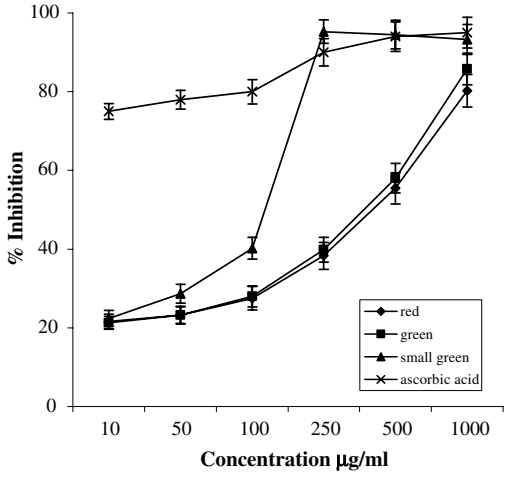

Fig. 2. Radical-scavenging activity of methanolic extract of three ripening stages of hot pepper (*Capsicum annuum* L. var. *acuminatum*) using DPPH assay. All samples were assayed in triplicate and averaged.

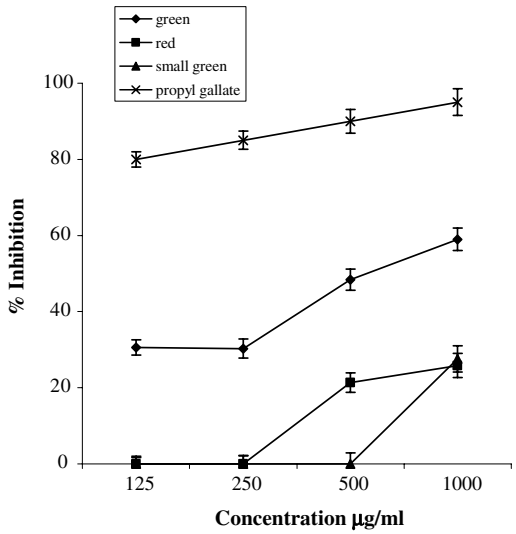

Fig. 3. Antioxidant activity of methanolic extract of three ripening stages of hot pepper (*Capsicum annuum* L. var. *acuminatum*) using TBA test. All samples were assayed in triplicate and averaged.

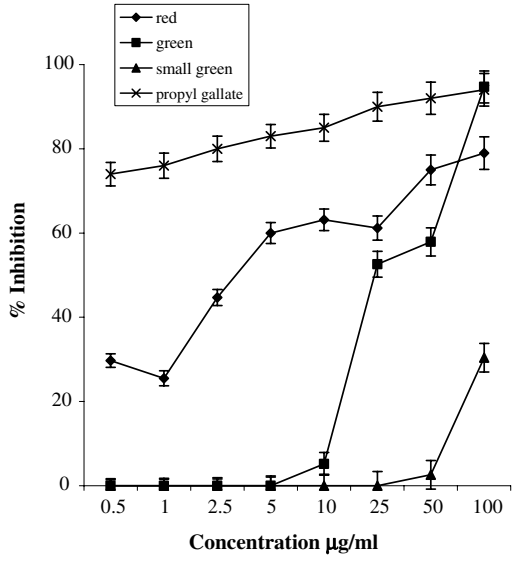

Fig. 4. Antioxidant activity of methanolic extract of three ripening stages of hot pepper (*Capsicum annuum* L. var. *acuminatum*) using β-carotene–linoleate system after 30 min of incubation. All samples were assayed in triplicate and averaged.

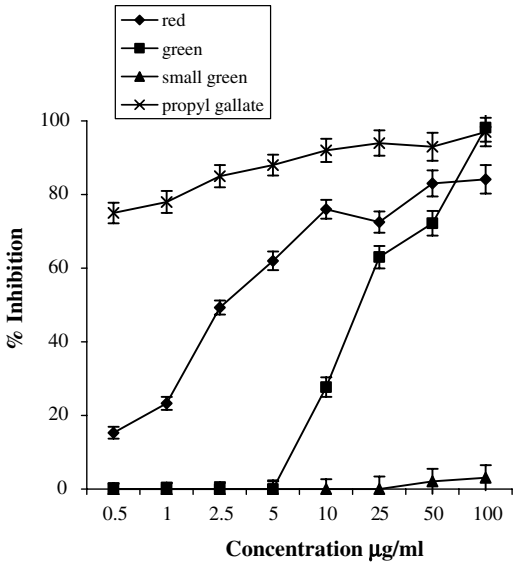

Fig. 5. Antioxidant activity of methanolic extract of three ripening stages of hot pepper (*Capsicum annuum* L. var. *acuminatum*) using β-carotene–linoleate system after 60 min of incubation. All samples were assayed in triplicate and averaged.

Antioxidant activity, which reflected the ability of the samples to inhibit the bleaching of β-carotene, was measured and compared with that of the control which contained no antioxidant component. It was demonstrated that addition of methanolic extract of red and green pepper inhibit oxidation of linoleic acid. As shown in Fig. 4 and Fig. 5, methanolic extract of red pepper showed greater antioxidative potency than the other extracts, and the IC_{50} of 4 µg/ml after 30 min of incubation and 3 µg/ml after 60 min of incubation, indicated that their activity were not correlated with time of heating.

4. Discussion

The phytochemical changes that occur during maturation and the resultant effect on antioxidant activity are important dietary considerations that may effect the consumption of different pepper types. In this study, we determined the effect of maturation on the concentration of specific constituents and on total phenolic content of hot pepper fruits from *C. annuum* var. *acuminatum*. Additionally, we determined how changes in chemical composition, in response to maturation, influenced antioxidant activity.

Hot spice red pepper is heavily consumed throughout the world and valued for its colourants, flavors, and pungency principles. The pigment of hot pepper consists of red and yellow carotenoids. The colouring capacity and colour stability of paprika products determined by the content of mono- and diesters (Biacs, Czinkotai, & Hoschke, 1992; Daood, Vinkler, Ma'rkus, Hebshi, & Biacs, 1996). Also hot pepper distributes considerable amounts of fat-soluble antioxidants such as tocopherols (mainly vitamin E) (Biacs et al., 1992). Another quality attribute in red pepper is the hot flavour caused by capsaicinoids, the pungency principles. These alkaloids have been intensively investigated for their physiological and pharmaceutical importance (Govindarajan & Sathyanarayana, 1991; Surh & Lee, 1996).

Red fruits showed the higher content in vitamin E compared to green and small green fruits which exhibited similar low content. Because of climatic ripening of hot pepper fruit, vitamin E tended to increase markedly. One of the most important ripening processes in climacteric fruits and vegetables is the induction of light-independent fat-soluble antioxidants biosynthesis which often occurs simultaneously with the destruction of the chloroplast system (chlorophyll destruction) (Daood et al., 1996; Hornero-Mendez, Gomez-Ladron, & Minguez-Mosquera, 2000). Different behaviour was observed for sterols content. The content of sterols, cholest-5-en-3-ol, ergost-5-en-3-ol, stigmast-5,22-dien-3-ol and stigmast-5-en-3-ol, decreases to increase of stage of ripening of the fruits.

Total soluble phenolics were measured by the Folin-Ciocalteu assay. Generally, the concentration of these chemical constituents increased as the pepper reached maturity regardless of the analytical method employed, as previously reported (Howard et al., 2000). In disagreement with Howard et al. (2000) phenolic content of the MeOH extracts of *C. annuum* var. *acuminatum* decreases with the increase of maturity of the fruits. These changes in chemical composition, in response to maturation, influenced antioxidant activity. The antioxidant potential of the total extracts was determined by three complementary methods. These assays differ from each other in terms of substrates, probes, reaction conditions, and quantification methods. The activity of a food extract, which may contain different chemical compounds, is reflected in the contest of specific reaction conditions such as pressure, temperature, reaction media, coreactants, and reference point. The antioxidant activity measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay. Therefore, it is appropriate to use different assays to evaluate the inhibition of different mechanisms of oxidation.

DPPH radical-scavenging activity of all samples was measured. The preparations were able to reduce the stable free radical DPPH to the yellow-coloured 2,2-diphenyl-1-picrylhydrazyl. The first stage of maturation (small green fruits) showed the highest activity on DPPH radical while the other two stages, green and red fruits, showed similar activity. The activity of small green fruits could be due to the higher level of phenols and to the major content of sterols as previously reported (Yoshida & Niki, 2003).

The high level of phenolic content and of a acyclic diterpene alcohol, phytol, in green pepper fruits is at the base of inhibition of lipid peroxidation, showed only for this stage of ripening. Several studies have reported on the relationships between phenolic content and antioxidant activity. Some authors found a correlation between the phenolic content and the antioxidant activity, while others found no such relationship. Velioglu, Mazza, Gao, and Oomah (1998) reported a strong relationship between total phenolic

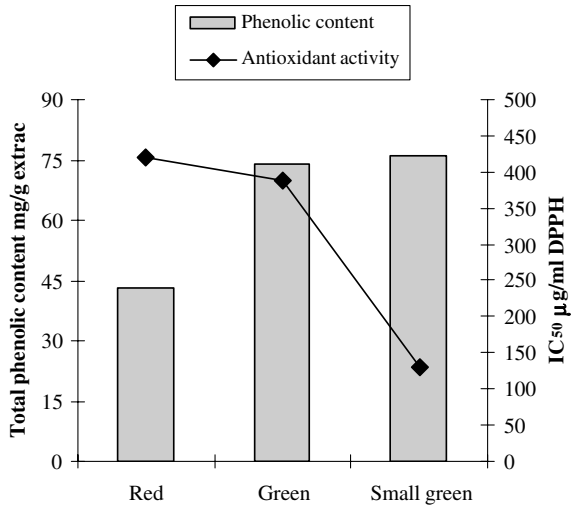

Fig. 6. Comparison of radical-scavenging activity and total phenolic content.

content and antioxidant activity in selected fruits, vegetables and grain products. No correlation between antioxidant activity and phenolic content was found in the study by Kähkönen et al. (1999) on some plant extracts containing phenolic compounds. In this study, this correlation was found for small green and red extracts in the DPPH test while no correlation was found in β-carotene bleaching test and bovine brain peroxidation assay. As shown in Fig. 6 the radical-scavenging activity increased with increasing of phenolics content, for small green and red extracts. Nevertheless, if we compare total phenolics content and radical-scavenging activity of small green and green extracts, no correlation was found. This fact may be explained in numerous way, in fact, the total phenolics content does not incorporate all the antioxidants. In addition, the synergism between the antioxidants in the mixture makes the antioxidant activity not only dependant on the concentration, but also on the structure and the interaction between the antioxidants.

The extracts exhibited a significant antioxidant capacity also in the β-carotene–linoleic acid test system. Methanolic extract of red pepper fruits showed greater antioxidative potency than other. The activity on bleaching of β-carotene of red fruits could be due to the presence of capsaicin and dihydrocapsaicin, which are absent in the other stage of ripening, and to the major content of vitamin E.

5. Conclusions

The present work showed for the first time the free radical-scavenging and the antioxidant activities of methanolic extract of hot pepper fruit (*C. annuum* L. var. *acuminatum*) at three ripening stages (small green, green and red). The free radical-scavenging activity was assessed by DPPH method while antioxidant activities were assessed by bovine brain peroxidation test and β-carotene bleaching test, which allow, respectively, the primary and the secondary step of oxidation to be followed (Gordon, 1990). A dose–response relationship was observed for all samples. The different composition of lipophilic compounds and the various amount of phenols showed in the three stage of ripening of *C. annuum* fruits modifies the antioxidant activity.

The results of this study showed that red pepper fruits has potent antioxidant property with β-carotene bleaching test. Methanolic extract of green pepper showed significant activity in the lipid peroxidation with protection of MDA formation. While methanolic extract of small green showed the most activity as radical scavenger.

These results showed different behaviour of the samples as radical scavenger, oxidation inhibitor linoleic acid and as oxidation inhibitor of membrane lipids. This discrepancy may be due to the different mechanisms involved in the different stages of oxidation.

Pepper is a vegetable of importance in human nutrition. It is very interesting to know what the contribution of an individual food product is to daily nutritional needs and how maturity affect nutritive composition and so biological properties.

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