Changes in Phytochemical and Antioxidant Activity of Selected Pepper Cultivars (*Capsicum* Species) As Influenced by Maturity

L. R. Howard,*,† S. T. Talcott,† C. H. Brenes,† and B. Villalon‡

Department of Food Science, University of Arkansas, Fayetteville, Arkansas 72704, and Texas Agricultural Experiment Station, Texas A&M Univeristy, Weslaco, Texas 78596

The effect of fruit maturation on changes in carotenoids, flavonoids, total soluble reducing equivalents, phenolic acids, ascorbic acid, and antioxidant activity (AOX) in different pepper types (*Capsicum annuum*, *Capsicum frutescens*, and *Capsicum chinese*) was determined. Generally, the concentration of these chemical constituents increased as the peppers reached maturity. Peppers contained high levels of L-ascorbic acid and carotenoids at maturity, contributing 124−338% of the RDA for vitamin C and 0.33−336 RE/100 g of provitamin A activity, respectively. Levels of phenolic acids, capxanthin, and zeaxanthin generally increased during maturation, whereas the level of lutein declined. Flavonoid concentrations varied greatly among the pepper types analyzed and were negatively correlated to AOX under the conditions of the β-carotene−linoleic assay. Model systems were used to aid in understanding the relationship between flavonoids and AOX. Significant increases in AOX were observed in pepper juice models in response to increasing dilution factors and the presence of EDTA, indicating a pro-oxidant effect due to metal ions in the system. In vitro models demonstrated that increasing levels of flavonoids in combination with constant levels of caffeic and ascorbic acid gave a resultant AOX that was either additive of the two compounds or competitive in their ability to scavenge peroxyl radicals. The model systems were in good agreement with the chemical composition of the pepper cultivars and reflected the interactions affecting AOX. More research is needed to understand the complex interactions that occur among various antioxidants present in pepper extracts.

Keywords: *Peppers; antioxidant activity; model system; carotenoids; flavonoids*

INTRODUCTION

Fresh peppers are an excellent source of vitamins A and C as well as neutral and acidic phenolic compounds, which are important antioxidants for a variety of plant defense responses. Levels of these compounds can vary by genotype and maturity and are influenced by growing conditions and losses after processing (Mejia et al., 1988; Howard et al., 1994; Lee et al., 1995; Daood et al., 1996; Simmone et al., 1997; Osuna-Garcia et al., 1998; Markus et al., 1999). Breeding for and retaining antioxidant compounds in fresh fruits and vegetables have important health-related implications. Epidemiological data have indicated possible roles of antioxidant compounds in the prevention of numerous chronic diseases including certain types of cancer, cardiovascular disease, stroke, atheroschlerosis, and cataracts (Block and Langseth, 1994; Steinmetz and Potter, 1996; Van-Poppel and Van Den Berg, 1997). Peppers are also good sources of the provitamin A carotenoids β-carotene, α-carotene, and β-cryptoxanthin, and numerous studies have focused on improving their retention during processing and storage (Minguez-Mosquera and Hornero-Mendez, 1994; Howard and Hernandez-Brenes, 1998; Markus et al., 1999). In addition to provitamin A carotenoids, peppers are also a good source of oxygenated carotenoids or xanthophylls, which can vary in composition and concentration due to differences in genetics and degree of ripening (Davies et al., 1970; Markus et al., 1999). These compounds, which do not possess provitamin A activity, have been shown to be effective free radical scavengers (Matsufuji et al., 1998) and may be important for the prevention of age-related macular degeneration and cataracts (Seddon et al., 1994). The role of ascorbic acid in the diet is also thought to be significant in preventing common degenerative conditions including cancer, heart disease, cataracts, and immune system functioning (Sauberlich, 1994). First isolated from paprika (Haworth and Szent-Gyorgyi, 1933), ascorbic acid is a required human nutrient that acts as an aqueous reducing agent in biological systems.

Peppers 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 (Hasler, 1998). Flavonoids are a large class of compounds, ubiquitous in plants, which exhibit antioxidant activity based on the number and location of hydroxyl groups present as well as the presence of a 2−3 double bond and 4-oxo function (Rice-Evans et al., 1996). Many epidemiological studies have indicated an inverse association between dietary intake of flavonoids and the risk of coronary heart disease (Hertog et al., 1993, 1995; Knekt et al., 1996), stroke (Keli et al., 1996), and lung cancer (Knekt et al., 1997; Garcia-Closas et al., 1998).

Many studies have demonstrated that peppers contain a wide array of phytochemicals, but many pepper species and cultivars have not been analyzed for these

* Author to whom correspondence should be addressed [telephone (501) 575-2978; fax (501) 575-2165; e-mail lukeh@comp.uark.edu].

† University of Arkansas.

‡ Texas A&M University.

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important compounds. The phytochemical changes that occur during maturation and the resultant effect on antioxidant activity are important dietary considerations that may affect the consumption of different pepper types. In this study we determined the effect of maturation on ascorbic acid, flavonoid, carotenoid, and total phenolic contents of peppers from three *Capsicum* species. Additionally, we determined how changes in chemical composition, in response to maturation, influenced antioxidant activity.

MATERIALS AND METHODS

Materials and Sample Preparation. Commercial pepper cultivars (*Capsicum* sp.) were grown at the Texas A&M Experimental Station at Weslaco, TX. All varieties received similar water and fertilizer treatments. Peppers were harvested at full fruit size at two maturity stages, immature (I), and mature (M), except for the habanero cultivars (cv.), which were harvested only at the mature stage. Pepper types analyzed included bell (*C. annuum*) cv. Yellow Bell 47 (I = green, M = yellow), cayenne (*C. annuum*) cv. Mesilla (I = green, M = red), cascabella (*C. annuum*) cv. Peto Cascabella (I = yellow, M = red), and long yellow (*C. annuum*) cv. Inferno (I = yellow, M = red). Tabasco peppers (*C. frutescens*, cv. Tabasco) (I = green, M = red) were obtained from the McIlhenny, Co. (Avery Island, LA), and habanero peppers [*C. chinense* cv. Francisca (M = orange) and Red Savina (M = red)] were obtained from GNS Spices Inc., (Walnut, CA). Peppers were shipped overnight to the University of Arkansas Food Science Department and, upon arrival, packed whole into metal cans under a blanket of nitrogen and frozen until analysis. For analysis, the frozen samples were homogenized prior to thawing in a kitchen-scale food processor. To simulate common consumption conditions, the seeds were retained in all pepper types except for Yellow Bell 47, which is typically consumed seedless.

Chemical Analyses. L-Ascorbic acid (LAA) was extracted and quantified by HPLC as described by Wimalasiri and Wills (1983). Carotenoids were extracted and separated using the method of Howard and Hernandez-Brenes (1998) with slight modifications. Saponification temperatures were reduced from reflux (~60 °C) to 40 °C for 30 min to maximize xanthophyll extraction. Samples were spiked with β-apo-8′-carotenal (Fluka Chemicals, St. Louis, MO) as an internal standard and used to confirm extraction efficiency. Individual carotenoids were quantified using external standards, which included capsorubin ($E^{1\%}_{1cm}$ = 2200 in benzene), capxanthin ($E^{1\%}_{1cm}$ = 2072 in benzene), lutein ($E^{1\%}_{1cm}$ = 2550 in 100% ethanol), zeaxanthin ($E^{1\%}_{1cm}$ = 2540 in 100% ethanol), β-cryptoxanthin ($E^{1\%}_{1cm}$ = 2287 in hexane), and α-carotene ($E^{1\%}_{1cm}$ = 2800 in hexane), each provided as a gift from Hoffman-La Roche Inc. (Basel, Switzerland). The β-carotene standard ($E^{1\%}_{1cm}$ = 2560 in hexane) was obtained from Sigma Chemical Co. (St. Louis, MO). Standard concentrations were determined spectrophotometrically, combined, and evaporated under nitrogen. The standards and pepper isolates were dissolved in 10 mL of acetone prior to HPLC analysis. A gradient mobile phase ran at 1.5 mL/min and consisted of acetonitrile, methanol containing 0.05 M ammonium acetate, dichloromethane, and triethylamine (75:20:5:0.05) in phase A with butylated hydroxytoluene (BHT) added at 0.1%. Phase B consisted of 100% methanol containing 0.1% BHT. A linear gradient ran from 25 to 75% phase A over 15 min and was then increased to 100% over 10 min for a total run time of 25 min with detection at 470 nm using a Water 996 photodiode array detector. The column was equilibrated to the original mobile phase concentration prior to the next sample injection.

Soluble components in peppers were extracted from 10 g of fruit by homogenizing in 50 mL of 50% methanol and then filtered through Miracloth (CalBiochem, La Jolla, CA). From this isolate, flavonoids were acid hydrolyzed according to the method of Lee et al. (1995) and aglycons separated by HPLC according to the method of Hertog et al. (1992). Quercetin and luteolin were quantified using external standards at 370 and 350 nm, respectively. Total soluble phenolics or total reducing compounds were also quantified from the isolate using the Folin−Ciocalteu assay (Swain and Hillis, 1959), with data expressed as chlorogenic acid equivalents. Individual phenolic acids were determined by filtering an aliquot of the isolate through a 0.45 μm filter and separated on a C_{18} Spherisorb ODS2 column (100 × 4.6 mm, 5 μm) connected to a Waters Nova-Pak C_{18} silica gel column (150 × 3.9 mm, 5 μm, Milford, MA). Mobile phase conditions were identical to those employed by Ramamurthy et al. (1992) with the following modification in the solvent program. Phase B ran from 0 to 30% over 20 min, from 30 to 50% over 10 min, from 50 to 70% over 20 min, and from 70 to 100% over 15 min for a total run time of 70 min at 0.8 mL/min. The column was washed with 100% of mobile phase B and equilibrated with 100% of mobile phase A prior to the next sample injection. Spectral analyses of detected compounds were compared to common cinnamic (caffeic, chlorogenic, and ferulic) and hydroxybenzoic acids (4-hydroxybenzoic, protocatechuic, and vanillic) for structural similarity. Moisture content was determined using AOAC Method 22.008 (AOAC, 1965).

Antioxidant activity (AOX) was determined on the same filtered isolate used for phenolic acid determination by HPLC using the coupled oxidation of β-carotene and linoleic acid assay described by Lee et al. (1995). Hydrogen peroxide (90 mM) was used as the oxidant source, and 50 μL of the isolate was tested for its ability to inhibit oxidation of the β-carotene−linoleate emulsion through scavenging of peroxyl radicals.

Model systems for in vitro analysis of antioxidant compounds and their interactions were prepared from authentic standards of caffeic acid, ascorbic acid, and quercetin obtained from Sigma Chemical Co. and luteolin obtained from Roth Chemical Co. (Karlsruhe, Germany). Additional studies to confirm AOX responses in peppers were performed on juice obtained from yellow bell peppers. The peppers (100 g) were thoroughly blended in a kitchen-scale food processor, and the slurry was filtered through Miracloth. The juice was immediately boiled for 20 min to inactivate enzymes and cooled in ice water. The juice was filtered through Whatman No. 4 filter paper to remove particulate matter and incrementally diluted with deionized water to obtain four dilutions (100, 75, 50, and 25% juice). AOX was then tested in triplicate on the four juice dilutions and on identical samples containing 500 mg/kg ETDA.

Statistical Analysis. Data represent the mean of three replicate samples for each pepper type and maturity stage. Model systems represent the mean of two repeated experiments with each model variant tested in duplicate. Multiple linear regression, analysis of variance, and Pearson correlations were conducted using JMP software (SAS Institute, 1996), and mean separation was performed using the LSD test ($P < 0.05$).

RESULTS AND DISCUSSION

Carotenoid Methodology. Saponification prior to HPLC analysis has been recommended to remove chlorophyll and to hydrolyze carotenol esters (Scott, 1992). The lack of saponification in peppers prior to analysis can result in underestimation of carotenoid values because these compounds are esterified to fatty acids in immature fruit (Minguez-Mosquera and Hornero-Mendez, 1994; Hart and Scott, 1995; Minguez-Mosquera and Perez-Galvez, 1998). Several investigators have noted greater carotenoid extraction at elevated saponification temperatures, but usually at the expense of xanthophyll recovery due to thermal degradation (Khachik et al., 1986; Scott, 1992). In our study, preliminary work determined that 40 °C was the optimal saponification temperature that maximized the retention of both xanthophyll and nonoxygenated carotenoids. Chromatograms of carotenoid standards and a

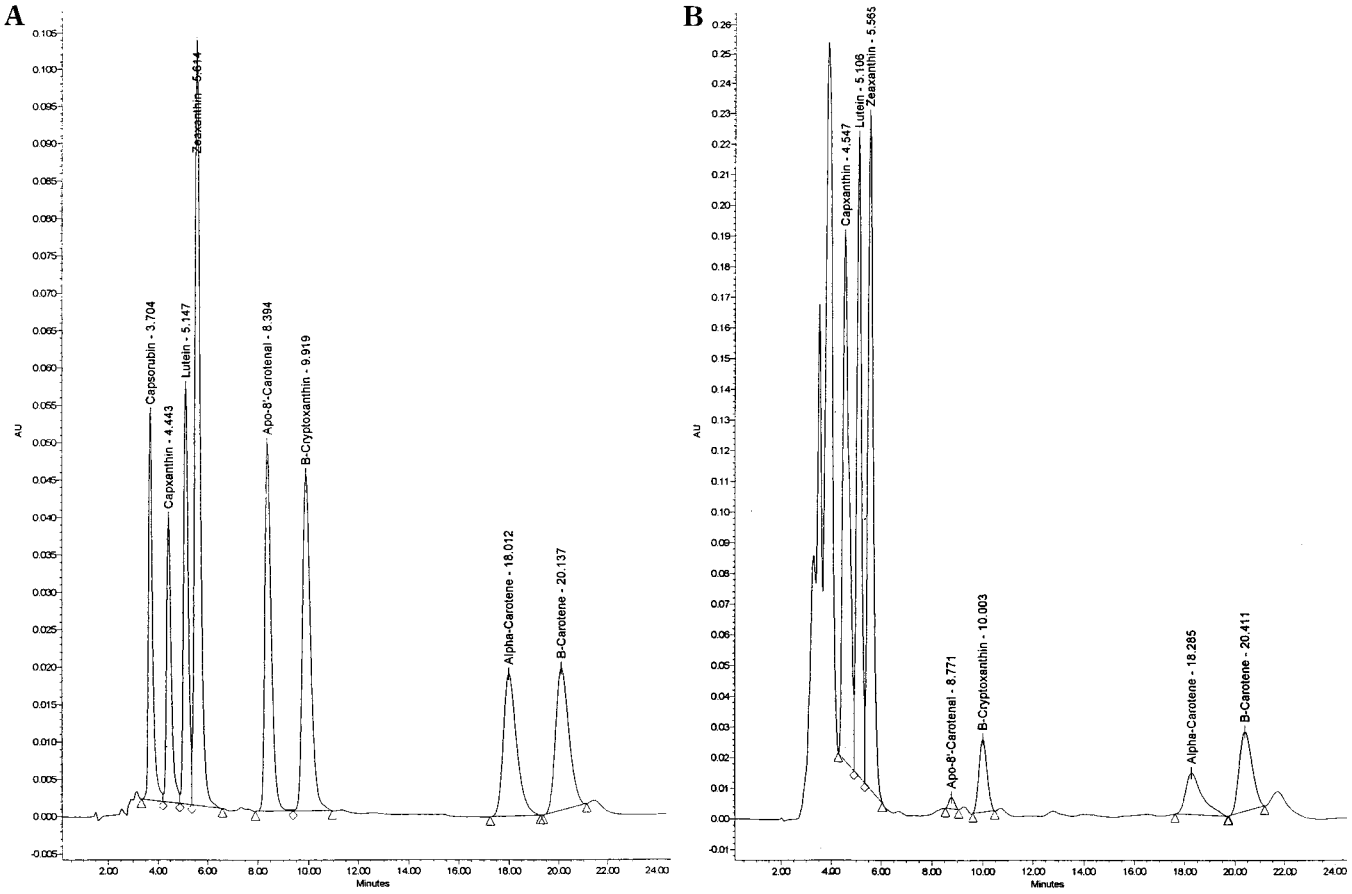

Figure 1. HPLC chromatograms of (A) carotenoid standards and (B) a typical pepper profile (B).

Table 1. Carotenoid, Provitamin A, and Retinol Equivalents of Fresh Pepper Fruit As Affected by Maturity[a]

species	type	cultivar	β-cryptoxanthin (μg/100 g)		α-carotene (μg/100 g)		β-carotene (μg/100 g)		retinol equiv (RE/100 g)		provitamin A[b] (% RDA)	
			I[Bc]	M[A]	I[B]	M[A]	I[B]	M[A]	I[B]	M[A]	I[B]	M[A]
C. annuum	bell	Yellow Bell	4b[d]	195a	NDb	2127a	186b	386a	31.3b	257a	3.13b	25.8a
C. annuum	cascabella	PETO Cascabella	3b	131a	NDb	710a	23b	403a	4.08b	137a	0.48b	13.7a
C. annuum	long yellow	Inferno	3b	245a	10b	332a	18b	337a	4.08b	104a	0.48b	10.4a
C. annuum	cayenne	Mesilla	9b	973a	ND	ND	247b	800a	41.9b	214a	4.19b	21.4a
C. frutescens	tabasco	Tabasco	NDb[e]	414a	136b	1252a	107b	1187a	29.1b	336a	2.92b	33.7a
C. chinense	habanero	Francisca	NA[f]	ND	NA	ND	NA	2	NA	0.33	NA	0.03
C. chinense	habanero	Red Savina	NA	353	NA	222	NA	861	NA	191	NA	19.1

species	type	cultivar	capsanthin (μg/100 g)		lutein (μg/100 g)		zeaxanthin (μg/100 g)	
			I[B]	M[A]	I[A]	M[B]	I[B]	M[A]
C. annuum	bell	Yellow Bell	ND	ND	765b	955a	35b	483a
C. annuum	cascabella	PETO Cascabella	NDb	5076a	61a	NDa	26b	293a
C. annuum	long yellow	Inferno	NDb	7443a	129a	NDb	18b	459a
C. annuum	cayenne	Mesilla	NDb	20861a	748a	NDb	NDb	956a
C. frutescens	tabasco	Tabasco	NDb	14434a	361a	NDb	73b	1958a
C. chinense	habanero	Francisca	NA	984	NA	ND	NA	15
C. chinense	habanero	Red Savina	NA	6754	NA	ND	NA	470

[a] See Materials and Methods for color designation of immature and mature fruit of each pepper cultivar. [b] Based upon RDA for males = 1000 mg/RE. [c] Similar upper case letters indicate that overall maturity effect is not significantly different (LSD test, $P < 0.05$). [d] Similar lower case letters indicate maturity effect for each pepper cultivar is not significantly different (LSD test, $P < 0.05$). [e] ND, none detected. [f] NA, sample not available.

typical pepper extract are shown in parts A and B of Figure 1, respectively.

Carotenoids. The concentrations of β-cryptoxanthin, α-carotene, β-carotene, capsanthin, and zeaxanthin increased extensively in all pepper types during maturation, whereas the concentration of lutein declined to nondetectable levels during maturation in all pepper types except for Yellow Bell 47, in which lutein increased as the pepper changed from green to yellow (Table 1). These results were consistent with other studies quantifying pepper carotenoids as a function of maturity (Davies et al., 1970; Minguez-Mosquera and Hornero-Mendez, 1994; Markus et al., 1999), and our data provide supplemental information on the carotenoids specific to the genotypes analyzed in this study. The provitamin A carotenoids (α-carotene, β-carotene, and β-cryptoxanthin) contributed 0.5−4.2% RDA for vitamin A at the immature stage, increasing from 10.4

Table 2. Flavonoid and Total Soluble Phenolic Contents (Milligrams per Kilogram) of Fresh Pepper Fruit As Affected by Maturity[a]

									total soluble phenolics			
			quercetin		luteolin		total flavonoids		Folin−Ciocalteu		HPLC	
species	type	cultivar	I[Ab]	M[A]	I[A]	M[A]	I[A]	M[A]	I[B]	M[A]	I[B]	M[A]
C. annuum	bell	Yellow Bell	22.39a[c]	12.64b	9.32a	10.51a	31.71a	23.15b	3085a	2846b	178.4a	178.7a
C. annuum	cascabella	PETO Cascabella	42.42a	23.96b	15.72a	5.96b	58.14a	29.92b	3685b	5788a	198.2b	283.3a
C. annuum	long yellow	Inferno	68.27a	64.47a	17.22a	16.83a	85.49a	81.30a	2565b	3156a	148.7b	347.7a
C. annuum	cayenne	Mesilla	11.01b	24.78a	6.16b	17.32a	17.17b	42.10a	3548b	5707a	133.4b	290.7a
C. frutescens	tabasco	Tabasco	2.22a	0.88b	43.65a	35.67a	45.87a	36.55a	5244a	5136a	287.7b	351.2a
C. chinense	habanero	Francisca	NA[d]	4.63	NA	0.87	NA	5.50	NA	4316	NA	253.38
C. chinense	habanero	Red Savina	NA	1.36	NA	0.39	NA	1.75	NA	4042	NA	208.51

[a] See Materials and Methods for color designation of immature and mature fruit of each pepper cultivar. [b] Similar upper case letters indicate that overall maturity effect is not significantly different (LSD test, $P < 0.05$). [c] Similar lower case letters indicate maturity effect for each pepper cultivar is not significantly different (LSD test, $P < 0.05$). [d] NA, sample not available.

to 33.7% of the RDA at the mature stage. Francisca and Red Savina peppers contributed 0.03 and 19.1% of the RDA, respectively, in their mature forms. These values were similar to RDA values reported for numerous pepper types and cultivars (Simmone et al., 1997; Howard et al., 1994; Mejia et al., 1988). The oxygenated carotenoids capxanthin and zeaxanthin increased considerably upon maturation as a result of color change. Specifically, the pigment capsanthin was not detected in the immature fruit and has been associated only with pepper types containing the genetic capacity to synthesize red pigments upon maturation (Davies et al., 1970; Matus et al., 1991; Minguez-Mosquera and Hornero-Mendez, 1994). Capsanthin levels in mature Tabasco and cayenne peppers were much higher than values reported for paprika, which is known to be a good source of the compound (Biacs and Daood, 1994). The oxygenated carotenoids may be important compounds for human health and nutrition due to their radical scavenging capacity (Matsufuji et al., 1998) and thus may afford protection against age-related macular degeneration and cataracts (Seddon et al., 1994).

Flavonoids. Flavonoid aglycons were quantified in pepper isolates after acid hydrolysis with standard recoveries of 78 and 99% for quercetin and luteolin, respectively. Flavonoid concentration varied greatly among peppers analyzed in this study with levels in Yellow Bell 47 and Peto Cascabella decreasing with maturity, whereas levels in Mesilla increased 2.5-fold. Remaining cultivars were unaffected by maturity (Table 2). A decline in flavonoid concentration was also observed for *C. frutescens* cultivars during maturation (Sukrasno and Yeoman, 1993). Flavonoid losses during maturation may reflect metabolic conversion to secondary phenolic compounds (Barz and Hoesel, 1977) or degradation via enzyme action (Jimenez and Garcia-Carmona, 1999; Miller and Schreier, 1985a,b). The cultivar Inferno had the highest concentrations of quercetin and luteolin of the peppers analyzed in this study. As a whole, mature *C. annuum* and *C. frutescens* cultivars were appreciably higher in total flavonoids than *C. chinense* cultivars at the mature stage. The extremely low flavonoid concentration in these pungent peppers may indicate diversion of phenolic precursors from flavonoids to capsaicinoids. Of particular interest was the luteolin content in the cultivar Tabasco, which contained twice the levels of any other pepper tested. Luteolin has been shown to be an effective free radical scavenger (Rice-Evans, 1996), and artichoke extracts high in luteolin were shown to prevent oxidation of low-density lipoprotein (Brown and Rice-Evans, 1998).

The quercetin and luteolin content of peppers in this study compared favorably with values reported for vegetables. Peterson and Dwyer (1998) proposed a botanical classification scale for flavonoid concentration that rates foods as low (0.1−39.9 mg/kg), moderate (40−99.9 mg/kg), and high (>100 mg/kg). From this scale, both immature and mature peppers of Inferno and immature fruit from Peto Cascabella contained moderate levels of the flavonol quercetin, whereas the immature Tabasco contained moderate levels of the flavone luteolin. All other cultivars and maturity levels were classified as low for both quercetin and luteolin, but *Capsicums* appear to be unique in that they contain both of these flavonoids in appreciable concentrations.

Total Phenolics. Total soluble phenolics determined by the Folin−Ciocalteu assay and individual phenolic acids determined by HPLC were quantified from identical pepper isolates (Table 2). Due to the diverse array of individual phenolics present (40 compounds) from both the flesh and seeds, positive identification could not be conclusively made for each peak in the chromatograph. A significant number of the compounds exhibited absorption spectra similar to those of cinnamic acid derivatives ($\lambda_m = 320-330$ nm) and hydroxy-substituted benzoic acids ($\lambda_m = 255-295$ nm), in both monomeric and polymeric forms. Numerous compounds were also detected that exhibited absorption spectra similar to those of flavonoid glycosides ($\lambda_m = 350-370$ nm); however, these compounds were excluded from quantification in an effort to estimate only the phenolic acids present. For simplicity, and lack of positive identification, total peak areas were summed for each sample (HPLC phenolics) and quantified as chlorogenic acid equivalents. Total reducing equivalents or total soluble phenolics measured by the Folin−Ciocalteu assay correlated well with the HPLC phenolics quantified in this manner ($r = 0.97$), although amounts measured were substantially different.

The total soluble phenolics present in Yellow Bell 47 were relatively low compared to those in the other pepper types, and it was the only cultivar in which soluble phenolics decreased with maturity. The low level of total soluble phenolics in this cultivar was attributed to seed removal prior to extraction. Seeds from various plant sources have been shown to be rich in phenolics, which contribute significantly to AOX (Velioglu et al., 1998). Total phenolics in all other pepper types, which included seeds, generally increased with maturation regardless of the analytical method employed. Greater levels of total phenolics were found in peppers using the Folin−Ciocalteu assay as compared to HPLC quantification and likely reflected the additional detection of

Table 3. L-Ascorbic Acid Content of Fresh Pepper Fruit As Affected by Maturity[a]

species	type	cultivar	mg/100 g		% RDA[b]	
			I[Bc]	M[A]	I[B]	M[A]
C. annuum	bell	Yellow Bell 47	114.0a[d]	135.2a	190.1a	225.4a
C. annuum	cascabella	PETO Cascabella	171.7a	202.4a	286.2a	337.5a
C. annuum	long yellow	Inferno	91.69b	137.5a	152.8b	229.2a
C. annuum	cayenne	Mesilla	63.24b	102.4a	105.4b	170.7a
C. frutescens	tabasco	Tabasco	15.06b	74.55a	25.11b	124.3a
C. chinense	habanero	Francisca	NA[e]	122.02	NA	203.4
C. chinense	habanero	Red Savina	NA	115.16	NA	191.9

[a] See Materials and Methods for color designation of immature and mature fruit of each pepper cultivar. [b] Based upon highest RDA for males and females = 60 mg/100 g. [c] Similar upper case letters indicate that overall maturity effect is not significantly different (LSD test, $P < 0.05$). [d] Similar lower case letters indicate maturity effect for each pepper cultivar is not significantly different (LSD test, $P < 0.05$). [e] NA, sample not available.

capsaicinoids (Bajaj and Kaur, 1979), minor phenolics, reducing sugars, ascorbic acid, and flavonoids.

L-Ascorbic Acid. LAA content in the pepper cultivars either increased or remained constant as fruit matured (Table 3), confirming previous studies that reported increases during pepper ripening (Rahman et al., 1978; Howard et al., 1994; Osuna-Garcia et al., 1998). Light intensity has been shown to increase concentrations of ascorbic acid and glucose, the precursor to ascorbic acid (Mozafar, 1994), whereas reducing sugars have been shown to increase in peppers during ripening (Osuna-Garcia et al., 1998). All cultivars analyzed in this study were considered to be excellent sources of LAA and exceeded RDA values for vitamin C. Peto Cascabella had the highest level of LAA, contributing 286 and 337% of the RDA at the immature and mature stages of maturation, respectively, whereas Yellow Bell 47, Inferno, and Francisca all exceeded 200% of the RDA in mature fruit. The immature Tabasco was the only cultivar analyzed that fell below the RDA for vitamin C, but it is usually consumed in the mature form. Our values for mature peppers are consistent with other reports for peppers ranging from 25 to 461% of the RDA for vitamin C (Simmone et al., 1997; Osuna-Garcia et al., 1998; Howard et al., 1994; Lee et al., 1995).

Antioxidant Activity. The AOX of the different pepper cultivars was determined on the basis of the peroxyl radical quenching properties of compounds soluble in methanol/water (50:50). Compounds not readily soluble in this solvent, specifically carotenoids, were logically excluded in their contribution to AOX. Utilizing a universal isolate from the peppers, instead of a purified isolate, allowed for an overall estimation of soluble compounds influencing AOX. All of the pepper types generally exhibited AOX (Table 4), as reported previously by Lee et al. (1995) for various *C. annuum* cultivars. However, a relatively low AOX was found in immature Yellow Bell 47, which may be partially attributable to seed removal prior to extraction. Chemical contributors to AOX in peppers are numerous and may include ascorbic acid, flavonoids, capsaicinoids, and a wide variety of phenolic acids. An indirect comparison of AOX using the β-carotene−linoleate assay can be made to other established methods for AOX (Cao and Prior, 1999) by comparing activities for standards. Because the β-carotene bleaching assay has essentially an upper limit at 100% inhibition, the degree of inhibition is highly dependent on the nature of a particular antioxidant. For example, 1 mM standards of caffeic and ascorbic acid resulted in 18.7 and 3.1% inhibition of β-carotene bleaching under the conditions of the assay, respectively. Additional standard values for percent inhibition of β-carotene bleaching included BHT (0.04 mM = 62%), Trolox (0.04 mM = 86%), and α-tocopherol (0.02 mM = 64%). However, these antioxidant compounds seem to inhibit β-carotene bleaching according to a a first-order reaction rate, so direct comparison with other methods was not considered to be practical.

Table 4. AOX of Fresh Pepper Fruit As Affected by Maturity[a]

species	type	cultivar	% inhibition of β-carotene bleaching[b]	
			I[Ac]	M[A]
C. annuum	bell	Yellow Bell	42.75b[d]	66.98a
C. annuum	cascabella	PETO Cascabella	87.18b	92.89a
C. annuum	long yellow	Inferno	78.89b	84.05a
C. annuum	cayenne	Mesilla	80.36a	68.92b
C. frutescens	tabasco	Tabasco	86.34b	91.85a
C. chinense	habanero	Francisca	NA[e]	68.03
C. chinense	habanero	Red Savina	NA	94.10

[a] See Materials and Methods for color designation of immature and mature fruit of each pepper cultivar. [b] Peppers were diluted 6-fold as a result of extraction. Percent inhibition of β-carotene bleaching for common standards included 0.04 mM BHT = 62%, 0.04 mM Trolox = 86%, and 0.02 mM α-tocopherol = 64%. [c] Similar upper case letters indicate that overall maturity effect is not significantly different (LSD test, $P < 0.05$). [d] Similar lower case letters indicate maturity effect for each pepper cultivar is not significantly different (LSD test, $P < 0.05$). [e] NA, sample not available.

AOX of the peppers significantly increased with maturation except for Mesilla, for which an appreciable decline was observed. As a group, the only significant correlation to AOX was found with total soluble phenolics ($r = 0.41$). However, this comparison was not considered to be accurate due to compositional diversity present in the different pepper types, so compositional data from each cultivar were correlated to AOX (Table 5). By separating the individual pepper types, a better explanation of the complex interactions among the independent variables was obtained. Data from these individual regression models indicated an unexpected inverse relationship between flavonoid concentration and AOX as the peppers matured. The only exception was Tabasco, which exhibited a positive relationship to AOX with increasing luteolin and ascorbic acid concentrations. When HPLC phenolics were factored into models, a positive correlation to AOX always existed. Contrary to these results, Lee et al. (1995) observed a linear relationship between flavonoid concentration and AOX in peppers. In their study, polar, interfering compounds were removed prior to analysis by use of reverse phase C_{18} cartridges. In our study, the presence of these polar analytes, such as ascorbic acid and metal ions, and their interaction with less polar compounds may have influenced AOX. Similarly, Gazzani et al. (1998) observed a pro-oxidant response in unfraction-

Table 5. Multiple Linear Regression Models for Prediction of AOX of Pepper Extracts

species	type	cultivar	regression model[a]	independent variable (s)	R^2
C. annuum	bell	Yellow Bell 47	$Y = 97.428 - 2.430X$	X = quercetin	0.96
C. annuum	cascabella	PETO Cascabella	$Y = 74.835 + 0.063X$	X = HPLC phenolics	0.86
			$Y = 99.120 - 0.274X$	X = quercetin	0.84
			$Y = 95.239 - 0.480X$	X = luteolin	0.72
C. annuum	long yellow	Inferno	$Y = 90.072 - 0.210X_1 + 0.022X_2$	X_1 = quercetin X_2 = HPLC phenolics	0.97
C. annuum	cayenne	Mesilla	$Y = 86.695 - 1.027X$	X = luteolin	0.83
			$Y = 89.140 - 0.810X$	X = quercetin	0.80
C. frutescens	tabasco	Tabasco	$Y = 76.157 + 0.193X_1 + 0.118X_2$	X_1 = luteolin X_2 = ascorbic acid	0.98
C. chinense	habanero[b]	Francisca + Red Savina	$Y = 99.733 - 6.232X$	X = quercetin	0.76

[a] Regression models were generated using the STEPWISE procedure of SAS and the variables ascorbic acid, quercetin, luteolin, and the sum of phenolics by HPLC. [b] Habanero cultivars were combined for linear regression analysis.

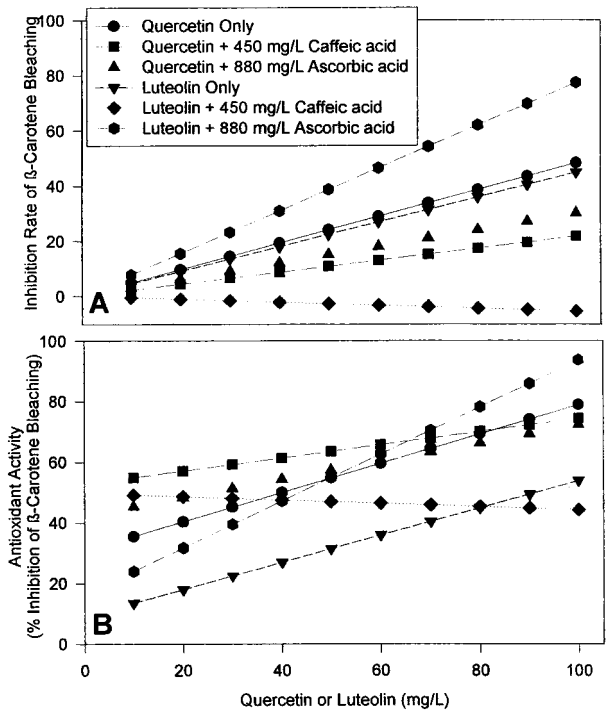

Figure 2. Model systems tested for antioxidant activity (AOX) using a β-carotene−lineoleate emulsion containing increasing concentrations of the antioxidant compounds quercetin and luteolin at a constant level of caffeic and ascorbic acid: (A) rate of inhibition of β-carotene bleaching (slope of the experimental curve) as affected by the antioxidant compounds; (B) linear regression analysis of the model systems tested for AOX. AOX values of 450 mg/L caffeic acid and 880 mg/L ascorbic acid solutions were 47, and 15%, respectively.

ated bell pepper juice using a β-carotene−linoleate assay. Therefore, we hypothesized that different antioxidants present in aqueous pepper extracts may interact in the sample matrix at various concentrations and influence the overall AOX.

In Vitro Models. Investigations with model systems were conducted using a constant level of caffeic acid (450 mg/L) and ascorbic acid (880 mg/L) in combination with increasing levels of quercetin and luteolin (10−100 mg/L). This model attempted to simulate chemical concentrations similar to those found in peppers, which would aid in determining if interactions existed. Figure 2A graphically demonstrates the resultant rates of inhibition of β-carotene bleaching (slope of the experimental curve) in relation to each model combination. It is important to note that, individually, caffeic and ascorbic acid maximally inhibited the oxidation of β-carotene at approximately 200 and 300 mg/kg, respectively. Adding higher concentrations of these compounds did not result in increased AOX; therefore, concentrations used in the model systems were purposefully higher than these thresholds, simulating in vivo conditions. A steady increase in AOX was observed as the concentrations of both quercetin and luteolin were individually increased, following a first-order inhibition rate over the range of concentrations tested. However, when caffeic and ascorbic acid were combined with quercetin and luteolin, the resultant AOX did not always exhibit an additive or synergistic effect equal to the compounds tested individually, indicating interaction among compounds in the model.

Linear regression analyses for the model systems are included in Figure 2B. Experimentally, no additive or synergistic effects were observed when caffeic acid was combined with any concentration of luteolin used in the model. With this combination, the resultant AOX was never >50%, a value that could be accounted for by the AOX of caffeic acid alone (47%). This may indicate that caffeic acid and luteolin are either competitive in their ability to scavenge peroxyl radicals or that caffeic acid interferes with the electron donating capabilities of luteolin. However, when either caffeic or ascorbic acid was combined with quercetin at concentrations up to 50 mg/L, an additive effect between the individual compounds was observed. At quercetin levels >50 mg/L, this effect was not observed and the individual effect of quercetin could account for the resultant AOX. The initial additive effect may indicate a separate mechanism by which radicals were quenched in the system, but at higher quercetin concentrations it appeared that competition for the remaining radicals existed. Conversely, the effect of adding ascorbic acid to an increasing level of luteolin resulted in an additive response between the compounds, resulting in a continuous increase in AOX up to the highest luteolin level studied. It was unclear if the two compounds were simply targeting the peroxyl radical by separate modes of action or if ascorbic acid reduced luteolin, indicating possible synergistic responses between the compounds. The polarity of each molecule and the mode of electron transfer in solution may be additional factors responsible for increased AOX in the β-carotene−linoleate emulsion.

In Vivo Models. It has previously been reported that both pepper juice and solvent extracts could exhibit prooxidant properties in the β-carotene−linoleic acid assay (Gazzani et al., 1998; Lee et al., 1995). The observed effect may be due to the presence of iron or copper ions in combination with ascorbic acid, which has been shown to promote carotene bleaching (Kanner et al.,

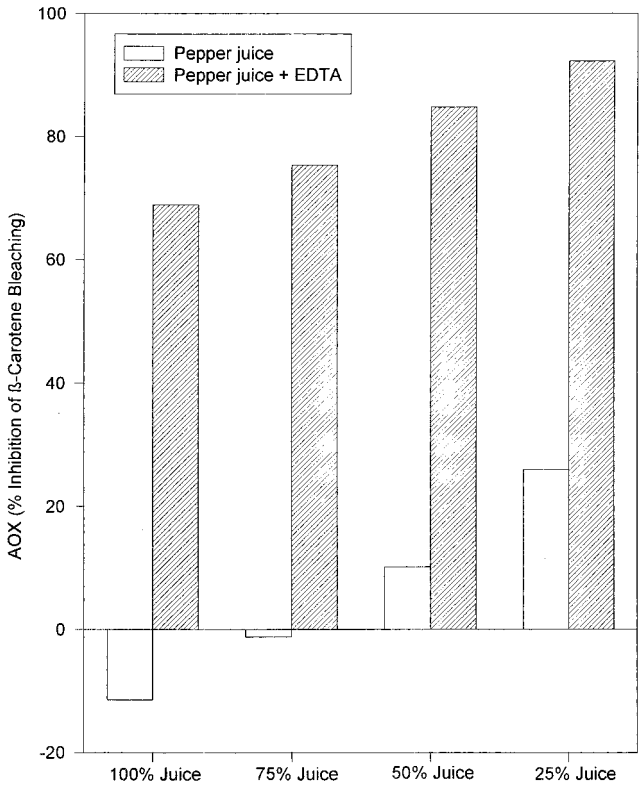

Figure 3. AOX of yellow bell pepper juice extracts as influenced by serial dilution and addition of 500 mg/L EDTA.

1977; Kanner and Budowski, 1978). In an effort to determine the effect of water-soluble analytes on AOX of peppers, we performed serial dilutions on preboiled pepper juice to determine if the pro-oxidant effect could be eliminated by simple dilution (Figure 3). A pro-oxidant effect was observed with 100% juice, whereas AOX incrementally increased up to 26% when the juice was diluted 4-fold. Additional dilutions (up to 32-fold) of the juice resulted in a maximum AOX of 49% at a 16-fold dilution (data not shown). Addition of the metal chelator EDTA (500 mg/kg) to identical pepper extracts also resulted in increased AOX, causing the 100% juice sample to exhibit a 69% AOX that increased to >92% after a 4-fold dilution. The increase in AOX due to both dilution and the presence of EDTA indicated that pro-oxidant components were incrementally diminished in the system. Kanner and Mendel (1976) also observed an inhibitory effect of EDTA on carotene bleaching in a β-carotene−linoleate model system, using paprika extracts. Our results demonstrate that pro-oxidant metals and other pro-oxidant compounds significantly interfere with the accurate measurement of AOX in biological systems. Because the pepper types analyzed in this study exhibited relatively high AOX values at a 6-fold dilution, it was presumed that dilution of water-soluble components coupled with metal chelation by the high level of phenolic acids present (Kono et al., 1998) were important factors influencing AOX.

Relationships between in Vitro and in Vivo Results. Regression analysis from the in vitro model systems implied a negative relationship to AOX with increasing concentrations of quercetin and luteolin, confirming in vivo results obtained from analysis of the pepper cultivars (Table 5). Experimentally, the actual AOX values of the models were always the same or higher when caffeic or ascorbic acids were added to quercetin and luteolin, thus excluding an antagonistic or pro-oxidant effect between the compounds. Observations from analysis of the pepper cultivars showed that AOX, ascorbic acid, and phenolic compounds increased with maturity while flavonoids decreased. As noted with the model systems, levels of ascorbic and phenolic acids above their terminal threshold did not significantly increase AOX values under the conditions of the assay. Therefore, the increase in AOX observed as the pepper types matured could not be attributed to the additional synthesis of antioxidant compounds. Due to the apparent competitiveness between reducing compounds, as demonstrated in the model systems, the decrease in flavonoids with maturity resulted in increased AOX in the pepper extracts. One exception to this trend was observed with Mesilla, in which the flavonoids increased with maturity and AOX decreased, fully supporting the observations of the model systems. Tabasco was another exception; a positive correlation to AOX was observed with luteolin, but only in the presence of ascorbic acid. Because Tabasco did not contain an appreciable amount of quercetin, the antioxidant response paralleled that of the model system, in which luteolin and ascorbic acid demonstrated an additive effect on AOX values. Generally, little information is available concerning the interactions among various antioxidants present in fruits and vegetables that appear to be important considerations affecting their AOX capacity.

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