SAMPLE PREPARATION METHODS AND PRE-HARVEST FACTORS INFLUENCING THE CONTENTS OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY IN PEPPERS

A Dissertation

by

HAE JIN BAE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Horticulture

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ABSTRACT

Sample Preparation Methods and Pre-harvest Factors Influencing the Contents of Bioactive Compounds and Antioxidant Activity in Peppers. (December 2011)

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Peppers are a rich source of diverse bioactive compounds with potential health-promoting properties. The levels of bioactive compounds and antioxidant activity can be affected by analytical methods, pre-harvest factors, and the quality of peppers. In order to understand the nutrient composition and antioxidant activity in peppers, determination of factors influencing the contents of bioactive compounds is important. The overall objectives were to determine the efficient conditions of sample preparation and the impact of pre-harvest factors affecting bioactive compounds and antioxidant activity.

Optimal extraction procedures were developed, and HPLC methods were validated for bioactive compounds in peppers. The highest flavonoids were extracted in ethanol, while myricetin was extracted using *N-N*-dimethylformamide. Optimized conditions for flavonoids were obtained during 3 h of extraction time and hydrolysis in 3 M HCl for 60 min at 95 °C. Capsaicinoids and ascorbic acid were simultaneously separated and extracted using a solvent mixture consisting of 3% metaphosphoric acid: ethanol (2:8) after 30 min of sonication. To determine the relationship between bioactive

compounds and antioxidant activities in pepper extracts from different solvent properties, bioactive compounds were analyzed, and the antioxidant activities were assayed by 2,2,-Diphenyl-1-picryl hydrazyl (DPPH), reducing power, and degradation of deoxyribose. Hexane extracts had the highest levels of capsaicinoids and carotenoids, while methanol extracts had the highest levels of flavonoids. Strong DPPH scavenging activity and reducing power were found in lipophilic extracts, while hydrophilic extracts were appropriate for inhibition of deoxyribose degradation. Variation in content of ascorbic acid, capsaicinoids, and flavonoids was evaluated at immature and mature stages of pepper cultivars in different locations over two years. Mature peppers contained the highest levels of capsaicinoids and ascorbic acid. Flavonoids were variable at different maturity stages. Interactions between pre-harvest factors and bioactive compounds were highly significant.

This study demonstrated the efficient sample preparation methods and simultaneous separation of bioactive compounds, which reduces analysis time and leads to reduced cost. The antioxidant properties were strongly associated with the concentration of bioactive compounds based on selective pepper extracts. The pepper quality can be improved by using appropriate pre-harvest conditions that increase the levels of bioactive compounds in peppers.

DEDICATION

This dissertation is dedicated to my father-in-law

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Peppers and health benefits

Peppers comprise diverse species in the genus *Capsicum* of the family Solanaceae. They are grown as perennial shrubs in appropriate climatic conditions in the southern United States and Central and South America. Five main domesticated pepper species are grown commercially: *Capsicum annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*. Fresh consumption of peppers increased from about 880,000 tons in 2002 to more than 1.04 million tons in 2006 (Lynch & McCarty, 2008). Peppers are a good source of vitamin C, capsaicinoid, flavonoids, other phenolics, and carotenoids (Kim et al., 2010). Flavonoids have been found to decrease the risk of inflammatory diseases and cancers with general intake of a few hundred mg per day (Hollman & Katan, 1999). Capsaicin and dihydrocapsaicin are responsible for the major pungent moieties in pepper and have shown antitumor activity in cell culture models (Surh, 2002). Capsaicin has been shown to antiperoxidative effects by inhibiting the generation of reactive oxygen species (Kogure et al., 2002). Ascorbic acid functions biological activities and prevents oxidative degradation.

This dissertation follows the style of *Food Chemistry*.

Ascorbic acid has potent ability to repair cellular damage and to affect the nervous system (Liang, Johnson & Jarvis, 2001; Salceda & Contreras-Cubas, 2007). The recommended daily allowance of vitamin C is about 90 and 75 mg/day for males and females, respectively (Ludke, Sharma, Bagchi & Singal).

Bioactive compounds in peppers

The word flavonoid is derived from the Latin word *flavus*, which indicates yellow color. Flavonoids are polyphenolic compounds found in higher vascular plants, particularly in flowers, leaves, and bark. They are especially abundant in fruits, grains, and nuts, more specifically, in the skins. Because of their antioxidant capacity, flavonoids have been consumed for their ability to decrease the risk of inflammatory diseases and cancers (Prasad, Phromnoi, Yadav, Chaturvedi & Aggarwal, 2010). Flavonoids have a basic structure of C_6-C_3-C_6 and contain several subclasses based on chemical structures (Crozier, Jaganath & Clifford, 2007), including flavonols and flavones found especially in peppers. Flavonoids in plants appear in the form of O-glycosides binding forms of sugar moieties (Hertog, Hollman & Venema, 1992b). Hydrolysis of glycosides to aglycones changes the quantity of flavonoids. An antioxidant capacity of aglycones is attributed to the catechol group in the B ring (Pietta, 2000). Flavonoids are synthesized in plants via the phenylpropanoid pathway. Phenylalanine is a precursor of flavonoid biosynthesis. The first key enzyme in phenylpropanoid pathway is $_{L}$-phenylalanine ammonia-lyase (PAL) (Barbero, Liazid, Palma & Barroso, 2008), and the first step in flavonoid synthesis is catalyzed by CHS

(chalcone synthase), resulting in different classes of flavonoids (C_{15}). It is possible that enhancing PAL increases accumulation of flavonoids.

Capsaicinoids are a group of pungent alkaloids that accumulate in the placenta region of pepper fruits. Capsaicinoids comprise five compounds including capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin I, and homocapsaicin II, and their content can increase or decrease inversely with the activity of peroxidase (Contreras-Padilla & Yahia, 1998). Capsaicin and dihydrocapsaicin, major pungent moieties, are derived from phenylalanine and fatty acid from valine (Blum et al., 2003). Genotype and environmental factors such as light, temperature, maturity, and location can affect the quality of capsaicinoids. For example, capsaicinoid metabolism is degraded when cellular disruption occurs during temperature oxidation (Kirschbaum-Titze, Hiepler, Mueller-Seitz & Petz, 2002).

Ascorbic acid functions as an antioxidant and free radical scavenger, preventing oxidative degradation. Ascorbic acid is a water-soluble vitamin, and can be infused in the body and easily eliminated. Ascorbic acid occurs in two forms: ascorbic acid and dehydroascorbic acid, an oxidized form of ascorbic acid (Gibbons, Allwood, Neal & Hardy, 2001). Dehydroascorbic acid is hydrolyzed irreversibly to 2,3-diketogulonic acid, so it exhibits little biological activity. Although ascorbic acid is a powerful antioxidant, its measurement always causes concern because it is easily oxidized and degraded. To maintain the stability of ascorbic acid, metaphosphoric acid as an optimum extraction solvent has been commonly used, and the quantification of ascorbic acid has been determined by HPLC methods (Odriozola-Serrano, Hernández-Jover & Martín-Belloso,

2007). Different amounts of ascorbic acid and antioxidant activities have been found in vegetables and fruits (Conforti, Statti & Menichini, 2007).

Analytical methods for bioactive compounds

To obtain optimum levels of bioactive compounds from food matrics, efficient and proper sample preparation is required. The quantitative levels of bioactive compounds can vary depending on extraction conditions and analysis methods. Although traditional methods are related to time consumption and degradation rate, most studies have not considered sample preparation procedures in particular. It is important to optimize sample extraction procedure and to determine appropriate analytical methods because unidentified or unextracted compounds still remain in foods like pepper. The natural components in peppers can be quantified by the HPLC and rapid extraction methods (Ertas, Özer & Alasalvar, 2007). In recent years, much research has focused on optimizing methodologies to quantify, identify, and separate bioactive compounds, because once sample procedures are standardized and confirmed, additional sample preparations for extraction provide a continuous source of data for analysis. Solvent extraction is the most widely used method because of its flexible applicability (Liu, Qi, Cao, Li, Li & Peng, 2008). Researchers found a number of flavonoid glycosides (Marín, Ferreres, Tomás-Barberán & Gil, 2004) and aglycones using one step for sample preparation. Barbero et al. reported that ultrasound-assisted extraction increased the content of capsaicinoids in 10 min using methanol (Barbero et al., 2008). It is known

that degradation of ascorbic acid can be expected from thermal heat treatments because of the accelerating oxidation of ascorbic acid to dehydroascorbic acid caused by heat.

Antioxidants in peppers

Antioxidants are considered the natural defense system of the human body. Antioxidants can protect lipids and proteins against oxidation induced by free radicals, and oxidative stress, which is increased by reactive oxygen species (Tykhomyrov, Nedzvetsky, Klochkov & Andrievsky, 2008). It is important to reduce reactive oxygen species in the body because epidemiological studies have shown that reactive oxygen species can cause cancer, DNA damage, and cardiovascular disease (Frohlich, McCabe, Arnold & Day, 2008; Honjo et al., 2008; Pelicano et al., 2009). Reactive oxygen species comprise two groups of free radicals and non-radicals. Superoxide (O_2^-) and hydroxyl radical (OH·) are included in free radical species, whereas non-radical species contain singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). The formation and activity of reactive oxygen species can be quenched by enzymatic and dietary antioxidants. Antioxidant enzymes include superoxide dismutase, GSH enzymes (glutathione peroxidase), and catalase. Superoxide dismutase targets superoxide radicals. Glutathione peroxidase and catalase can reduce hydrogen peroxide. Lipid peroxide can be broken down by glutathione peroxidase. Antioxidant enzymes can be obtained from dietary intake of vegetables including peppers. Since peppers contain bioactive compounds as chemical protectants, the antioxidant compounds in peppers are related to antioxidant

mechanisms with the ability to donate hydrogen atoms or electrons, to break the chain reaction of lipid peroxidation, and to reduce reactive oxygen species.

Pre-harvest factors influencing bioactive compounds

Bioactive compounds in peppers are commonly analyzed for quantification and separation. However, achieving consistent and accurate results is complicated because the content of bioactive compounds is affected by cultivar, year of harvest, stage of maturity, and environmental factors. To understand the relationship between levels of bioactive compounds and pre-harvest factors, the determination of plant and environmental factors is important. For example, the levels of ascorbic acid and capsaicinoids differed in paprika peppers during different maturity stages in various cultivars (Gnayfeed, Daood, Biacs & Alcaraz, 2001), while variable contents of flavonoids were observed in two types of bell peppers grown in different locations (Chassy, Bui, Renaud, Van Horn & Mitchell, 2006). One study found that environmental stress conditions (light, temperature, and fertilizer deficiencies) increased phenylalanine levels, which could increase flavonoid levels (Tan, 1980). Although pepper development was greater under high temperature and long irradiance in spring harvest season, the contents of antioxidant compounds can be variable in different cultivars under various environmental conditions (Martí et al., 2011).

Rationale

Peppers are considered excellent vegetable for functional food as they contain

antioxidant compounds including flavonoids, vitamin C, capsaicinoids, carotenoids, and other phenolics, which provide health benefits. The levels of bioactive compounds and their antioxidant activities are variable in pepper cultivars. However, careful consideration for extraction methods, solvent properties, and pre-harvest factors was warranted to ensure optimum extraction and detection of any given bioactive compounds to obtain maximum antioxidant activity. The overall hypothesis of this project was that optimized analytical methods increased different properties of major bioactive compounds and antioxidant activity in peppers, and that concentrations of bioactive compounds were strongly influence by pre-harvest conditions. The following research objectives were studied in order to test hypothesis.

Objectives

1. To determine the optimum extraction conditions and validation of an HPLC method for the separation and quantification of flavonoids from peppers.
2. To optimize the efficient conditions for simultaneous extraction and separation of capsaicinoids and ascorbic acid from pungent peppers.
3. To compare lipophilic and hydrophilic extracts on antioxidant activity and concentrations of bioactive compounds in pungent peppers.
4. To determine the extraction efficiency of solvent properties for antioxidant activity and the content of bioactive compounds in non-pungent peppers.
5. To evaluate the variation of bioactive compounds as affected by cultivars, maturity, and year of harvest in greenhouse-grown peppers.

6. To evaluate pre-harvest factors and their interaction on the levels of bioactive compounds in field-grown peppers.

CHAPTER II

EXTRACTION EFFICIENCY AND HPLC METHOD FOR FLAVONOID ANALYSIS FROM PEPPERS*

Introduction

Peppers are one of the most valuable vegetables, and consumption of peppers increased by 18% from 2002 to 2006 (Lynch et al., 2008). Peppers belong to the *Solanaceae* family and are grown as a perennial shrub in warm climatic zones of the world. Five main domesticated pepper species are grown commercially including *Capsicum annuum, C. baccatum, C. chinense, C. frutescens,* and *C. pubescens*. Peppers are a good source of several health-promoting compounds such as flavonoids, carotenoids, vitamin C, and capsaicinoids. Among the compounds, flavonoids are ubiquitous phytochemicals, which may be present at high levels in green, sweet, and hot peppers. Thus, many studies were focused on flavonoid levels in different peppers (Kim et al., 2010; Lin & Tang, 2007; Sgroppo & Pereyra, 2009; Sim & Sil, 2008). Flavonoids show high antioxidant and anticancer activities, which are determined by the presence of numbers of hydroxyl groups at a certain position and a double bond at a C_2-C_3 position.

* Reprinted with permission from "Extraction efficiency and validation of a HPLC method for the flavonoid analysis from peppers" by Haejin Bae, G.K. Jayaprakasha, John Jifon, Bhimanagouda S. Patil, 2012. *Food Chemistry*, 130, 751-758 © Elsevier.

Based on the relationship of structure and antioxidant-activity, myricetin was considered as the most powerful flavonoid (Gordon & Roedig-Penman, 1998; Lu, Papp, Fang, Rodriguez-Nieto, Zhivotovsky & Holmgren, 2006). Glycosides and aglycones of myricetin, quercetin, luteolin, kaempferol, and apigenin are found in peppers. Determination and quantification of flavonoid glycosides is challenging. Since some of the flavonoid glycosides are not available commercially, most researchers hydrolyze the glycosides to aglycones, which are quantified by HPLC. However, the quantitative variation of pepper flavonoids occurs with different sample extraction procedures, and is affected by extraction solvent, sample to solvent ratio, and extraction time. Therefore, it is vital to develop and optimize efficient extraction methods to produce real composition data and determine optimum levels of flavonoids. The objective of this study was to optimize the extraction conditions for the quantification of flavonoids using various combinations of extraction solvents, solvent ratios, extraction times, and hydrolysis conditions. Further, the samples were analyzed by the improved reverse phase HPLC method, and antioxidant potencies of solvent extracts were measured using in vitro methods.

Materials and methods

Instrumentation

Flavonoids were separated and quantified using Perkin Elmer HPLC (Salem, MA, USA) equipped with a LC-250 B pump, a Nelson 900 autosampler, and diode array detector 235C. TotalChrome Navigator Software (version 6.2.1) was used for the data

processing. The chromatographic condition was developed. The optimized separation method using C_{18} Phenomenex column (Torrance, CA, USA) Gemini series (250 × 4.6 mm i.d., 5 μm particle size) to identify myricetin, quercetin, luteolin, kaempferol, and apigenin. The chromatographic separation was performed with solvent A (0.03 M phosphoric acid in water) and B (MeOH) at a flow rate of 1 ml/min. The separated flavonoid peaks were identified by comparing the individual standards with the retention time. The optimum program elution used in this study was as follows: a linear gradient of 40-100% B (0-10 min), 100% B (12-15 min), and a linear gradient of 100-40% B (15-20 min). The column was equilibrated for 5 min before the next injection. The sample injection volume was 30 μl, and flavonoids were detected at 360 nm. Individual peaks in samples were compared and matched with mixed standard peaks of chromatograms. For the quantification, peak areas were calculated.

Materials

Ripe red paprika (*Capsicum annuum* L. cv. 'CA377') and yellow habanero (*Capsicum chinense* L. cv. 'TMH') peppers were used in this experiment. Paprika and habanero peppers were grown in a greenhouse at Texas A&M University in College Station, Texas. The soil less media (Pro Mix® BX, Premier Horticulture Inc., Quakertown, PA, USA) was used, and the pepper plants were applied by drip irrigation with greenhouse fertilizer solution. Whole peppers, excluding the pepper stalks, were chopped and ground for the analysis.

Chemicals

Myricetin, quercetin, luteolin, kaempferol, and apigenin were purchased from Sigma (St. Louis, MO, USA). HPLC grade dimethylformamide and methanol solvent was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Extraction procedure

Ripe paprika was used for the myricetin, quercetin, luteolin, and kaempferol analysis, whereas the ripe habanero peppers were used for the apigenin analysis. The ripe pepper (5 g) was homogenized with 40 ml of EtOH for 1 min, and extracted on a shaker for 3 h at room temperature. The extract solution (6 ml) was treated with 3 M HCl (3 ml) at 95 °C in a water bath for 1 h. The hydrolyzed sample was cooled to room temperature, and filtered through a 0.45 μm membrane.

Selection of extraction solvent

Paprika (5 g) was mixed with 40 ml of different extraction solvents of methanol (MeOH), ethanol (EtOH), N-N-dimethylformamide (DMF), DMF: EtOH (50:50, v/v), DMF: MeOH (50:50, v/v), and EtOH: water (80:20, v/v) into conical centrifuge tubes. The mixture was homogenized for 1 min by using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA), and placed on a shaker for 3 h at room temperature for extraction. The extract was filtered through a Whatman No. 1 filter paper. This extracted solution was treated with acid, and then the hydrolyzed extract was injected for HPLC analysis.

Homogenization time and ratio of sample to solvent

To test extraction efficiency of the flavonoids, homogenization time and different ratio of sample to solvent were investigated. The mixture of sample and solvent was homogenized for 1 min, 2 min, and 3 min to compare the quantification of flavonoids. To evaluate optimum volume of solvent, different ratios of sample to solvent (1:4, 1:6 and 1:8, v/v) were used. Thus, paprika (5 g) was homogenized with 20 ml, 30 ml, and 40 ml of EtOH. The homogenate was used for the hydrolysis and HPLC analysis.

Extraction time

For the complete extraction of flavonoids, 5 g of sample with 40 ml of EtOH were incubated for different periods of time (3 h, 6 h, 9 h, 12 h, 18 h, and 24 h). The samples were stirred at room temperature (23 °C). The extracts were hydrolyzed and injected into HPLC.

Optimization of acid hydrolysis

The concentration of HCl, hydrolysis time, and temperature were investigated to obtain all the flavonoids aglycones. Pepper (5 g) was homogenized with 40 ml of EtOH, and the mixture was extracted for 3 h. The extract solution was subjected to hydrolysis by HCl of 1 M, 2 M, 3 M, 4 M, 5 M, and 6 M. The hydrolyzed samples were cooled, filtered, and subjected to HPLC analysis. Similarly, 6 ml of extract solution was hydrolyzed with 3 M HCl in a water bath at 95 °C for different times (15, 30, 45, 60, 90, and 120 min). To observe the degradation of flavonoids, the extract solution (6 ml) and

3 ml of 3 M HCl was hydrolyzed at different temperatures in a water bath at 75, 85, and 95 °C, and samples were prepared as described above for the quantification.

Mass spectrometric analysis

The flavonoid peaks from paprika using EtOH were identified by HPLC analysis. Each flavonoid was confirmed by mass spectrometric analysis. ESI-MS analysis was performed on a QSTAR Pulsar quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Toronto, Canada). The extract solution was infused into the ESI ionization at a flow rate of 7μl/min with a syringe pump and analyzed in both the negative or positive ion mode. The analysis was performed using following instrumental settings: collision gas, nitrogen; curtain gas, scale 20 psi; ion spray voltage, 4500 V; declustering potential, 50 V; focusing potential, 20 V; declustering potential 2, 10 V; ion release delay, 11 ms, ion release width, 10; resolution ion energy, and 1; detector (MCP) 2150. Quercetin and kaempferol were analyzed by positive mode, and luteolin was analyzed in negative mode.

Method validation

<u>Specificity</u>

The specificity of the method was obtained by injecting the blank sample and the spiked sample. The specificity was to determine that the endogenous co-eluting components did not interfere with other constituents in the sample extract. No interfering peaks for determination of flavonoids were observed.

Recovery test

The accuracy of the method was assessed by performing the recovery test. The recovery study was conducted by adding known amount of flavonoid standards. Three different concentrations of quercetin (37.5, 75.0, and 150.0 µg), luteolin (5.8, 12.5, and 25.0 µg), and kaempferol (3.1, 6.2, and 12.5 µg) were added to paprika samples. The mixture was hydrolyzed and injected into HPLC. The percent recovery of each flavonoid from spiked samples was calculated as follows:

$$\% \text{ Recovery} = \frac{(\text{Amount of flavonoid after spiking} * 100)}{(\text{Original concentration of flavonoid} + \text{spiked amount})}$$

% RSD = (Standard deviation of flavonoid * 100)/ (Average content of flavonoid)

Precision

The precision of the intra-day and inter-day was evaluated by repeated injection. The intra-day experiment was obtained by six replicates for a day, and the inter-day was determined by six injections for 3 days for the retention time and the peak area. The precision was expressed as relative standard deviation (RSD, %).

Calibration curve

The calibration curves were plotted by peak area versus concentration of each flavonoid. To prepare the standard solution, myricetin (3.1, 6.2, 12.5, 25.0, and 50.0 µg/ml), quercetin (9.3, 18.7, 37.5, 75.0, and 150.0 µg/ml), luteolin (3.1, 6.2, 12.5, 25.0 and 50.0 µg/ml), kaempferol (3.1, 6.2, 12.5, 25.0, and 50.0 µg/ml) and apigenin (4.8, 9.7, 19.5, 39.0, and 78.0 µg/ml) were dissolved in methanol. The linear regression

equations were calculated as y = ax ± b, where x was concentration and y was the peak areas of each flavonoid. Linearity was established by the coefficient of determination (R^2).

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) was defined as the lowest concentration of sample determined by the analytical method to obtain the ratio of signal to noise (3:1). Limit of quantification (LOQ) as the lowest concentration of compounds was determined by injecting the known concentration of the diluted standards until the signal-to-noise ratio reached 10:1.

Robustness

The robustness of the method was evaluated by comparing the different flow rate (0.8 and 1.2 ml/min), mobile phase composition (0.025 M and 0.035 M of phosphoric acid in water), and columns using Gemini series, C_{18}, 250 × 4.6 mm i.d. 5 µm (Phenomenex, Torrance, CA, USA) and Spherisorb ODS2, C_{18}, 250 × 4.6 mm i.d. 5 µm (Waters, Milford, MA, USA).

Total phenolic content

The content of total phenolics from the paprika pepper was evaluated by Folin-Ciocalteu (FC) method (Jayaprakasha & Patil, 2007). The hydrolyzed sample (100 µl) was taken in a 15 ml tube, and volume was adjusted to 10 ml with water. Then, 500 µl of

diluted FC reagent was added and kept at room temperature (23 °C) for 10 min. Later, 1000 µl of saturated sodium carbonate was added and incubated at 23 °C for 20 min. The absorbance of blue color was measured at 760 nm using a 96 well plate in a KC-4 Microplate Reader (BioTek Instruments, Winooski, VT, USA). Catechin was used for a calibration graph. Total phenolics were expressed as µg of catechin equivalent/g of pepper.

DPPH assay

The hydrolyzed paprika extracts were used for scavenging of DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical (Jayaprakasha, Girennavar & Patil, 2008). The assay was conducted using a 96 well plate in the KC-4 Microplate Reader. DPPH (40 mg) was dissolved in methanol and made up to one liter. Standard ascorbic acid solution (0.15, 0.30, 0.45, 0.60, 0.75, 0.90, and 1.05 µg) was used for a calibration graph. The hydrolyzed pepper sample (10 µl) was pipetted into a 96 well plate. Then, the volume of each well was adjusted to 100 µl of MeOH and 180 µl of DPPH. Optical density of each well was measured at 515 nm for 30 min at 3 min interval. Three replications were performed to determine the antioxidant activity. The DPPH radical scavenging activity was expressed as µM of ascorbic acid equivalent/g of sample.

Reducing property assay

The reducing property of the hydrolyzed pepper sample was determined according to our published method. As a standard, 10 mg of ascorbic acid was dissolved

in 10 ml of metaphosphoric acid (3%). Different aliquots of the pepper sample (0.25, 0.5, 0.75, and 1 ml) were mixed with sodium phosphate (200 mM) up to 1.25 ml, and 1.25 ml of potassium ferricyanide (1%) was added. The mixture was incubated at 50 °C in a water bath for 20 min, and then 1.25 ml of trichloroacetic acid (10%) was added to the mixture. After vortexing all samples, sample aliquot (1 ml) was transferred to a new tube, and then, 1 ml of water and 0.5 ml of ferric chloride (0.1%) was added. Absorbance was measured at 700 nm. The higher absorbance indicated higher reducing property.

Statistical analysis

All experiments were performed using SAS statistical system 9.2 (SAS Institute, Cary, NC, USA) for the data analysis. The comparison of means was analyzed by Tukey's test. Data were presented as average and standard deviations. Significant differences were determined at the $P \leq 0.05$ level.

Results and discussion

Comparison of extraction solvents

To compare the efficiency of flavonoid extractions, various solvents (MeOH, EtOH, DMF, DMF: EtOH (50:50), DMF: MeOH (50:50), and EtOH: water (80:20)) were used (Table 1). Paprika contained high concentration of quercetin (460.42 μg/g), luteolin (91.40 μg/g), and kaempferol (50.17 μg/g) in EtOH, while lower levels (2.81-11.27 μg/g) of flavonoids were detected in extracts of DMF, DMF: EtOH (50:50), and

DMF: MeOH (50:50). For the extraction of pepper flavonoids, DMF was used along with other solvents, and myricetin was found in only the DMF extracts. Apigenin peak was detected in habanero, and the concentration ranged from 2.08 to 21.12 μg/g. The content of quercetin (5.43-15.11 μg/g) and kaempferol (5.28-10.65 μg/g) in habanero was lower than paprika. On the basis of total flavonoid results, it was found that EtOH was efficient solvent for the optimum extraction of quercetin, luteolin, kaempferol, and apigenin, whereas DMF was considered efficient solvent for extraction of a certain flavonoid like myricetin. Thus, EtOH was selected in further experiments for the analysis of quercetin, luteolin, and kaempferol because higher amounts of three flavonoids were consistently extracted in paprika. The flavonoid values were comparable to reported pepper flavonoids in the literature (Chassy et al., 2006; Kim, Ahn, Lee, Moon, Ha & Kim, 2011). In analytical chemistry, chemometrics was used to achieve the optimal HPLC conditions using the mathematical and statistical methods (Kulikov, Galat & Boichenko, 2009; Sivakumar, Manavalan, Muralidharan & Valliappan, 2007). However, the present study was focused on developing the optimum conditions for the extraction and validation of pepper flavonoids. The results (Table 1) demonstrated that various levels of flavonoids can be extracted and quantified using different solvents depending on the pepper species. MeOH, EtOH, and their aqueous solvent were most commonly used for extraction of polar flavonoids from peppers, as well as other vegetables (Nazzaro, Caliendo, Arnesi, Veronesi, Sarzi & Fratianni, 2009). In tea, fruit, and vegetable, DMF solvent was used for efficient flavonoid extraction (Turkmen, Sari & Velioglu, 2006; Wach, Pyrzynska & Biesaga, 2007). A few papers reported the

presence of myricetin in commercial bell peppers and chili peppers (Hertog, Hollman & Katan, 1992a; Miean & Mohamed, 2001).

Homogenization time, ratio of sample to solvent, and extraction time

In the sample preparation process, rapid extraction is desirable for the researchers to save time and degradation of flavonoids and. In this study, paprika with EtOH was homogenized for 1, 2, and 3 min for the extraction of flavonoids (Fig. 1A). No significant change from 1-3 min was observed in the levels of flavonoids. Thus, 1 min of homogenization was used in further studies. To improve flavonoid extraction from peppers, different ratios (1:4, 1:6, and 1:8) of sample to solvent were compared (Fig. 1B). The content of quercetin was significantly high at ratio of 1:8. The concentration of luteolin and kaempferol was not significantly affected by solvent volume. Thus, ratio of 1:8 (sample 5 g: solvent 40 ml) was the best and used in further experiments. Different extraction times (3, 6, 9, 12, 18, and 24 h) for the flavonoid concentration were tested (Fig. 1C). Maximum extraction of flavonoids was observed at 3 h, and flavonoid concentration was significantly decreased with longer extraction time. Thus, 3 h was selected for the efficient extraction time in further experiments. Previous studies reported that total flavonoids in habanero peppers were extracted for 2 h at a room temperature, and sweet peppers were extracted for 72 h at 0 ºC (Del Amor, Cuadra-Crespo, Varó & Gómez, 2009; Menichini et al., 2009). It was clear that different extraction time led to different amounts of soluble flavonoids.

Table 1

Extraction efficiency of flavonoids from the ripe peppers.

Pepper	Extraction solvent	Flavonoid concentration (μg/g) [a]					Total flavonoids
		Myricetin	Quercetin	Luteolin	Kaempferol	Apigenin	
Paprika	MeOH	nd	357.86 ± 4.71 b	59.15 ± 1.17 b	31.45 ± 0.87 c	nd	455.22
	EtOH	nd	460.42 ± 10.67 a	91.40 ± 4.08 a	50.17 ± 5.15 a	nd	621.89
	DMF	11.27 ± 0.99 a	85.24 ± 2.51 f	nd	6.29 ± 0.97 d	nd	107.28
	DMF:MeOH(50:50)	2.81 ± 0.75 c	205.06 ± 4.46 d	3.99 ± 1.15 c	6.99 ± 0.55 d	nd	225.77
	DMF:EtOH (50:50)	5.15 ± 0.21 b	166.18 ± 3.75 e	2.08 ± 0.27 c	8.50 ± 0.50 d	nd	186.65
	EtOH:Water (80:20)	nd	330.55 ± 21.02 c	59.15 ± 4.22 b	42.85 ± 4.50 b	nd	462.29
Habanero	MeOH	nd	12.43 ± 1.12 b	nd	5.28 ± 0.18 b	13.01 ± 0.72 c	30.72
	EtOH	nd	15.11 ± 1.15 a	nd	8.21 ± 0.50 a	21.12 ± 1.28 a	44.43
	DMF	nd	5.43 ± 1.02 d	nd	nd	nd	5.43
	DMF:MeOH(50:50)	nd	8.95 ± 0.91 cd	nd	nd	2.19 ± 0.35 d	11.14
	DMF:EtOH (50:50)	nd	10.32 ± 1.07 bc	nd	nd	2.08 ± 0.24 d	12.40
	EtOH:Water (80:20)	nd	11.57 ± 2.56 b	nd	10.65 ± 1.39 c	16.80 ± 2.12 b	39.02

[a] Values are means ± standard deviation of triplicate samples; nd, not detected.
Different alphabet letters denote significant difference ($P \leq 0.05$) in the same column.

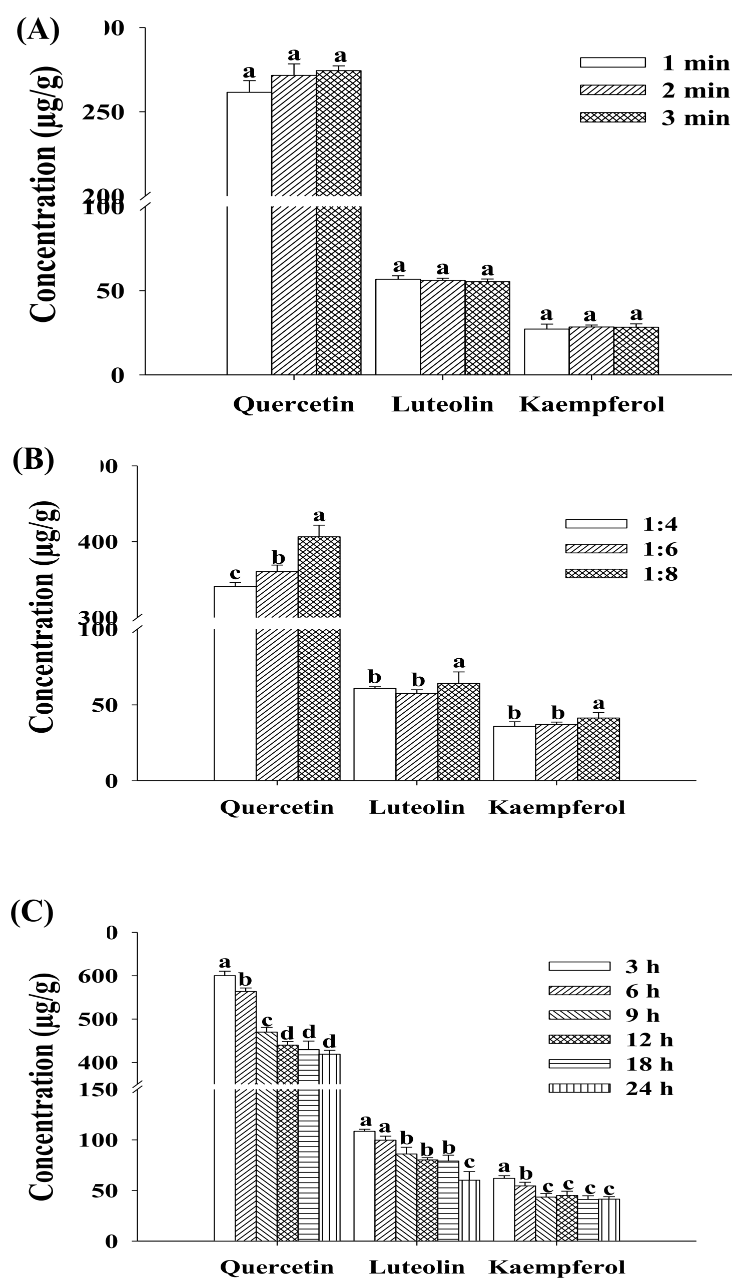

Fig. 1. Extraction of flavonoids from paprika using EtOH; (A) different homogenization time, (B) sample to solvent ratio, and (C) extraction time. Different alphabet letters denote significant difference ($P \leq 0.05$) within each flavonoid group.

Optimization of acid hydrolysis

Hydrolysis of flavonoid glycosides needs optimization of hydrochloric acid concentration, hydrolysis time, and temperature. EtOH extract was hydrolyzed with various concentrations of HCl (Fig. 2A). Quercetin content was the highest at hydrolysis with 3 M HCl and decreased in the order of acid 4 > 5 > 6 > 2 > 1 M HCl. Luteolin and kaempferol contents were high after hydrolysis with 6 M HCl. Moreover, the efficient hydrolysis time (15, 30, 45, 60, 90, and 120 min) was investigated (Fig. 2B). Quercetin levels were not significantly different after 45 min of hydrolysis time, and the highest content of quercetin was found after 60 min. The levels of luteolin and kaempferol were gradually increased from 15 min to 45 min of hydrolysis time. Fig. 2C shows the comparison of hydrolysis temperatures at 75, 85, and 95 ºC for the conversion of flavonoid glycosides to aglycones. The quercetin, luteolin, and kaempferol levels were significantly higher at 95 ºC than 75 and 85 ºC. It was possible that the high temperature accelerated the conversion of glycosides to aglycones. The results clearly suggested that higher concentrations of luteolin and kaempferol were obtained at higher acid concentrations and longer hydrolysis times at high temperature compared to quercetin. On the basis of the above results, we have used 3M HCl, at 95 °C for 60 min for the quantification of flavonoids in pepper species. The acid hydrolysis is a commonly used method for the conversion of glycosides to aglycone. Generally acid hydrolysis will be simple and convenient, as well as rapid and cost effective. It was reported that enzymatic hydrolysis showed the efficient conversion of glycosides to aglycones when α-glucosidase or β-glucosidase was involved. However, enzymatic hydrolysis requires a

longer time from 16 h to days, and may not complete the hydrolysis process (Bertino, Albro & Hass, 1987; De Marino et al., 2006; Higashiguchi, Nakamura, Hayashi & Kometani, 2006; Iorizzi et al., 2001).

Optimization of HPLC and mass spectrometric analysis

Flavonol (myricetin, quercetin, and kaempferol) and flavone (luteolin and apigenin) were detected in ripe paprika and habanero peppers. Considering separation of each flavonoid, the HPLC conditions were developed and for simultaneous determination. Separation was achieved by gradient mobile phase of phosphoric acid (0.03 M in water) and methanol. The developed HPLC method provided good separation of flavonoids, and the HPLC condition was applied to detect and separate flavonoids in pepper samples. Flavonoid glycosides of pepper crude extract were detected before hydrolysis (Fig. 3A). In paprika peppers, three flavonoids (quercetin, luteolin, and kaempferol) were detected in EtOH extract, and myricetin peak was detected in DMF (Fig. 3B and C). In habanero peppers, apigenin could be quantified (Fig. 3D). Further individual flavonoid peaks were confirmed by mass spectral analysis (Fig. 3E). The positive molecular ion showed at *m/z* 303.0246 for quercetin, and *m/z* 287.0416 for kaempferol, while luteolin was negative molecular ion at *m/z* 285.0445.

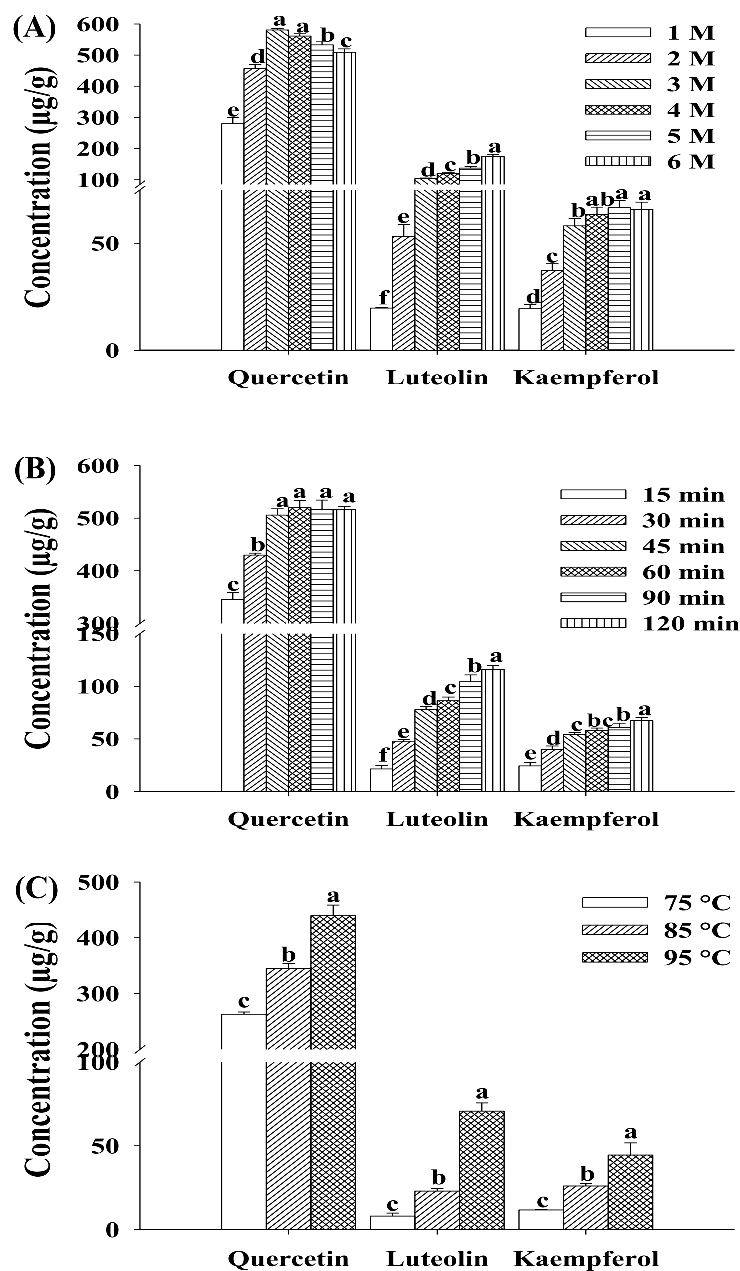

Fig. 2. Conversion of flavonoid glucosides to aglycones from paprika: (A) different concentrations of HCl, (B) hydrolysis time, and (C) temperature. Different alphabet letters denote significant difference ($P \leq 0.05$) within each flavonoid group.

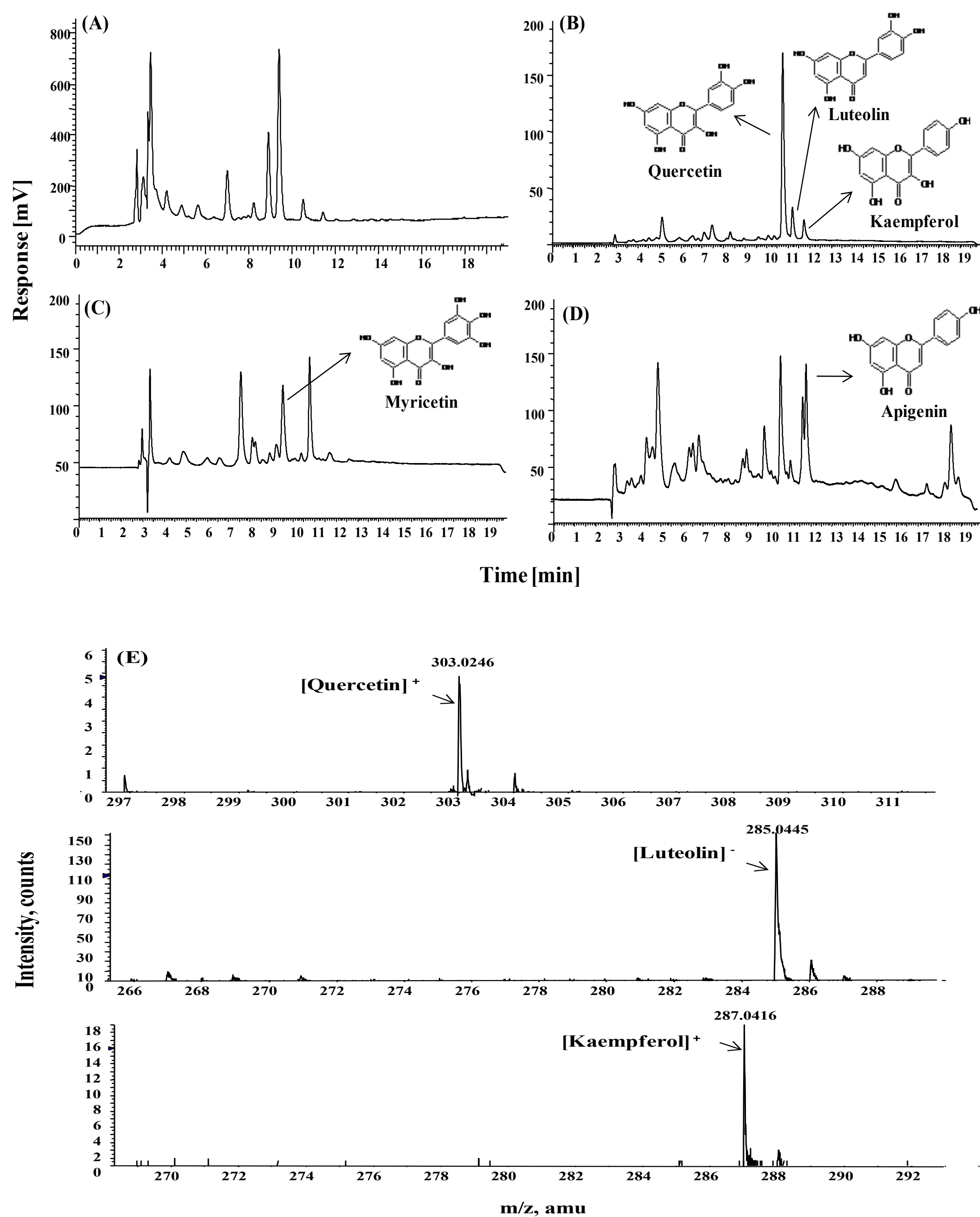

Fig. 3. Typical HPLC chromatograms of flavonoids for (A) EtOH extract from paprika before hydrolysis, (B) EtOH extract from paprika after hydrolysis, (C) DMF extract from paprika after hydrolysis, (D) EtOH extract from habanero after hydrolysis, and mass spectral analysis of (E) quercetin, luteolin, and kaempferol.

Method validation

In order to evaluate the accuracy of analytical methods, a recovery study of EtOH extracts from paprika peppers was conducted by adding each spike level (low, medium, and high) of standards. The mean recovery was 103.35-124.98% for quercetin, 92.34-111.07% for luteolin, and 96.33-98.97% for kaempferol (Table 2). Certain recovery percentages higher than 100% could be explained by the interference of a sample matrix. Relative standard deviation (RSD) determines the accuracy and stability of methods. The %RSD of average recovery was 1.3% for quercetin, 1.72% for luteolin, and 1.76% for kaempferol. For the instrumental precision, intra-day (on the same day) and inter-day (on the three different days) precision was determined. The intra-day and inter-day precisions (RSD) were less than 0.39% and 4.51% for the retention times and the peak areas (Table 3). The calibration curve for the linearity between five concentrations of each flavonoid (myricetin, quercetin, luteolin, kaempferol, and apigenin) and corresponding peak areas with developed HPLC methods were constructed. Linear ranges from 0.03 to 1.50 μg showed good correlation ($R^2 > 0.99$) in the concentration ranges. The limit of detection (LOD) and the limit of quantification (LOQ) were established for the sensitivity. The LOD and LOQ values ranged from 0.01 to 0.02 μg and 0.02 to 0.04 μg, respectively, for the flavonoid compounds (Table 4). The robustness of the method was assessed with the modification of several parameters such as flow rate, phosphoric acid concentration in solvent A (water) of mobile phase, and column (Table 5). The low RSD values indicated that the HPLC system was suitable. The observed variation confirmed the robustness of the analysis system.

Table 2

Recovery study of quercetin, luteolin, and kaempferol from paprika.

Flavonoids	Concentration in sample (µg) [a]	Standard added (µg)	Recovery (µg)		Recovery (%)	RSD (%)
			Expected	Actual [a]		
Quercetin	340.37 ± 10.37	37.50	377.87	390.52 ± 4.82	103.35	1.23
	340.37 ± 10.37	75.00	415.37	519.12 ± 8.74	124.98	1.68
	340.37 ± 10.37	150.00	490.37	544.97 ± 5.35	111.13	0.98
Luteolin	43.03 ± 1.07	5.80	48.86	45.12 ± 0.78	92.36	1.73
	43.03 ± 1.07	12.50	55.53	51.28 ± 1.22	92.34	2.37
	43.03 ± 1.07	25.00	68.03	75.56 ± 0.81	111.07	1.07
Kaempfer	16.27 ± 1.37	3.13	19.40	19.20 ± 0.34	98.97	1.77
	16.27 ± 1.37	6.25	22.52	21.70 ± 0.11	96.33	1.82
	16.27 ± 1.37	12.50	28.77	27.88 ± 0.77	96.89	1.69

[a] Values are means ± standard deviation of triplicate samples.

Table 3

Intra-day and inter-day precision for flavonoids.

Compounds	Intra-day (n=6, RSD%)						Inter-day (n=3, RSD%)	
	Day 1		Day 2		Day 3			
	Rt[a]	PA[b]	Rt	PA	Rt	PA	Rt	PA
Myricetin	0.33	3.24	0.20	2.68	0.21	3.28	0.39	2.43
Quercetin	0.22	2.77	0.27	2.86	0.17	2.04	0.34	2.90
Luteolin	0.19	3.07	0.19	2.64	0.22	2.83	0.37	4.51
Kaempferol	0.12	2.74	0.19	1.61	0.19	1.87	0.31	3.51
Apigenin	0.13	3.21	0.18	2.81	0.20	3.02	0.26	2.57

[a] Rt is RSD(%) of retention time
[b]PA is RSD(%) of peak area

Table 4

Linear regression equation, linear range, LOD, and LOQ.

Compounds	Regression equation (y=ax+b)[a]	R^2 [b]	Linear range (µg)	LOD[c] (ng)	LOQ[d] (ng)
Myricetin	y = 2099.2x - 7.444	0.9954	0.09 - 1.50	15	38
Quercetin	y = 2662.7x + 20.688	0.9990	0.09 - 1.50	15	38
Luteolin	y = 2685.9x - 2.7222	0.9992	0.03 - 0.50	9	19
Kaempferol	y = 3277.8x - 37.583	0.9984	0.03 - 0.50	9	19
Apigenin	y = 3020.1x + 12.616	0.9990	0.05 - 0.78	15	38

[a] x = the concentration of the compound (µg/ml); y = peak area
[b] R^2= coefficient of determination
[c] LOD: Limit of detection
[d] LOQ: Limit of quantification

Table 5

Robustness study with the variable conditions.

Compounds	Flow rate (ml/min)		Phosphoric acid (M)[a]		Column	
	0.8	1.2	0.025	0.035	Gemini	Spherisorb
Myricetin	0.32 [b]	0.41	0.53	1.35	0.22	0.67
Quercetin	0.08	0.49	0.33	1.07	0.26	0.44
Luteolin	0.08	0.40	0.28	0.88	0.26	0.42
Kaempferol	0.12	0.29	0.22	0.75	0.18	0.37
Apigenin	0.10	0.33	0.22	0.74	0.20	0.36

[a] Phosphoric acid concentration in mobile phase A (water)
[b] Values indicate %RSD of retention time

Total phenolics, DPPH, and reducing property

To evaluate the relationship between extraction of polyphenols and antioxidant activity, the hydrolyzed extracts were analyzed for total phenolics, DPPH, and reducing property. In Fig. 4A, the content of total phenolics ranged from 0.91 to 1.38 mg/g as catechin equivalent. The EtOH extract showed the maximum (1.38 mg of catechin equiv/g) phenolics followed by EtOH: water (80:20) (1.30 mg of catechin equiv/g), while DMF had the lowest phenolics (0.91 mg of catechin equiv/g). The levels of total phenolics of the EtOH extract in this study were higher than the presented in previous study (Antonious, Lobel, Kochhar, Berke & Jarret, 2009).

The DPPH radical scavenging activity is commonly used for evaluating antioxidant activity. The radical scavenging activity of paprika extracts was analyzed by the DPPH assay, and the results were presented as ascorbic acid equivalents. The radical scavenging activities were not significantly different in EtOH (35.07 μM of ascorbic acid equiv/g), MeOH (35.61 μM of ascorbic acid equiv/g), and EtOH: water (80:20)

(35.35 μM of ascorbic acid equiv/g), while the activities in DMF and DMF combination solvent were low (12.58-15.38 μM of ascorbic acid equiv/g). The result was similar to that of the DPPH value in MeOH extract of red peppers (Sun, Xu, Wu, Janes, Prinyawiwatkul & No, 2007). The present data implied that flavonoids extracted from EtOH and MeOH contained higher phenolics, and that the phenolics transfer proton to scavenge free radicals to obtain a stable end product.

Further, the reducing abilities of paprika extracts were measured using the potassium ferricyanide method (Fig. 4B). The reducing properties of all pepper extracts were enhanced by an increase of the concentrations of extracts. The MeOH extract showed higher reducing property than the EtOH extract. The high activity of reducing property was in order as follows, MeOH > EtOH: water (80:20) > EtOH > MeOH: DMF (50:50) > EtOH: DMF (50:50) > DMF. The order of extracts for reducing property was similar to DPPH activity. The results were correlated between total phenolics and antioxidant activities. However, in other studies, *C. annuum* L. var. *acuminatum* and other pepper cultivars did not show the relationship between total phenolics and antioxidant activity (Deepa, Kaur, George, Singh & Kapoor, 2007).

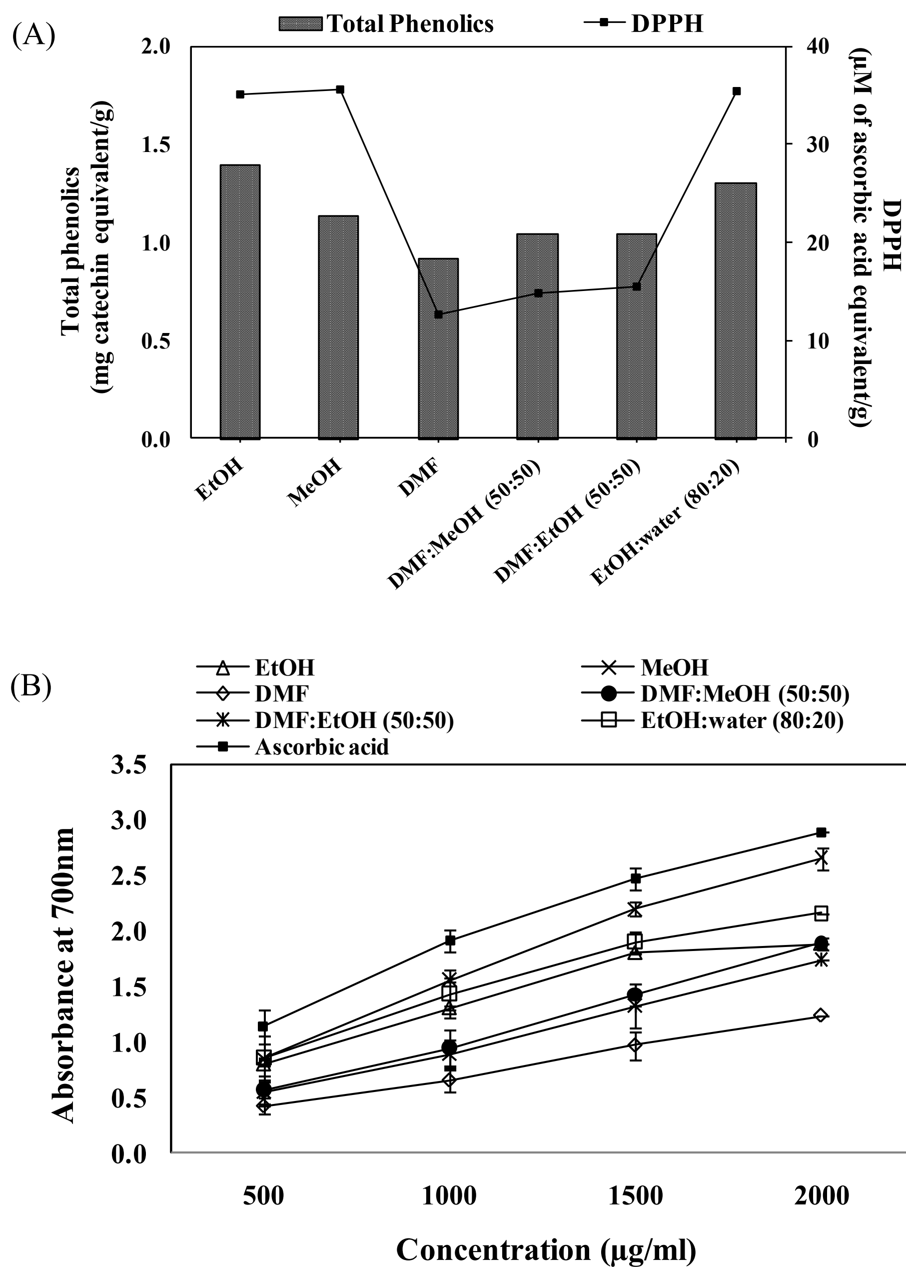

Fig. 4. Determination of (A) total phenolic content and DPPH scavenging radical activities of paprika extracts, and (B) reducing property of different extracts of paprika at various concentrations.

CHAPTER III

SIMULTANEOUS EXTRACTION AND SEPARATION OF CAPSAICINOIDS AND ASCORBIC ACID

Introduction

Peppers are commonly used in many countries as part of a daily diet, as well as in food preparation. Considering the presence of health promoting bioactive compounds such as capsaicinoids, ascorbic acid, carotenoids, and flavonoids, the nutritional value of pepper has been widely studied (Srinivasan, 2005). In pungent peppers, capsaicinoids and ascorbic acid (Fig. 5) are the most abundant components.

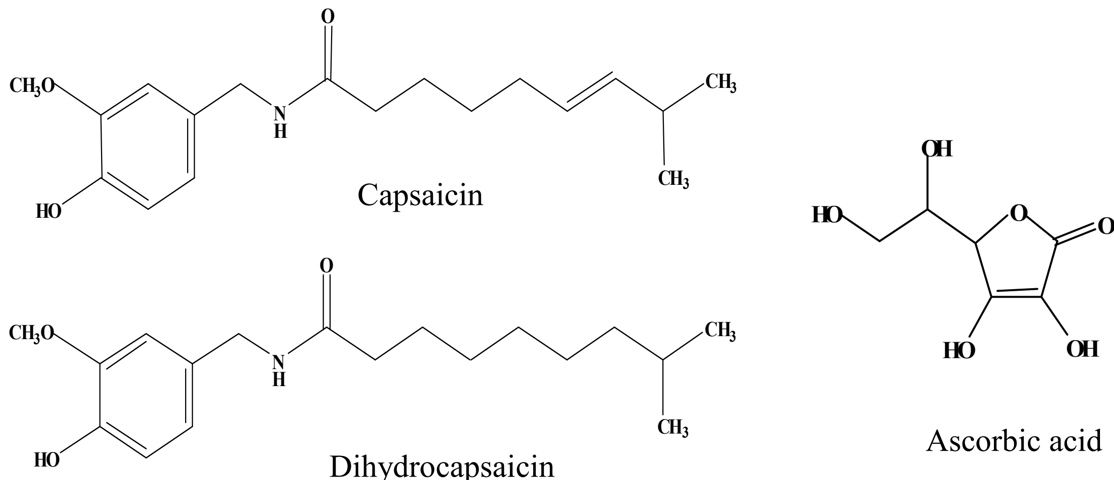

Fig. 5. The structures of capsaicin, dihydrocapsaicin, and ascorbic acid quantified in the present study.

Among capsaicinoid compounds, capsaicin and dihydrocapsaicin are the most pungent capsaicinoids, while other capsaicinoids such as nonivamide, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin are relatively less pungent compounds (Reilly, 2001). Several studies showed anti-cancer and anti-proliferative effects of capsaicinoids (Babbar, Chanda & Bley, 2010; Malagarie-Cazenave, Olea-Herrero, Vara, Morell & Díaz-Laviada, 2011) and ascorbic acid (Perrone et al., 2009). Therefore, development of rapid, reliable, and cost effective methods are critical for the analysis of capsaicinoids and ascorbic acid.

In continuation of developing extraction methods for the quantification of bioactive compounds from fruits and vegetable, the present study was conducted to extract and determine the levels of capsaicinoids and ascorbic acid simultaneously from peppers. Different extraction-assisted methods, such as ultrasound, supercritical fluid, and microwave techniques, were developed for isolation of capsaicinoids and ascorbic acid. The ultrasound-assisted method was used for the extraction of capsaicinoids using methanol or ethanol from minute to hour (Boonkird, Phisalaphong & Phisalaphong, 2008; Choi, Suh, Kozukue, Kozukue, Levin & Friedman, 2006). While the supercritical fluid method was used to extract capsaicinoids (Fernández-Ronco, Ortega-Noblejas, Gracia, De Lucas, García & Rodríguez, 2010), this method was expensive to be used for the routine analysis. Although the microwave-assisted method increased the extraction rate using energy (Barbero, Palma & Barroso, 2006), aqueous solvents were required to obtain efficient extraction (Fuentes, Báez & Reyes, 2006). Unlike capsaicinoids, the extraction-assisted methods for ascorbic acid were not commonly applied (Vega-Gálvez,

Lemus-Mondaca, Bilbao-Sáinz, Fito & Andrés, 2008) because ascorbic acid is not stable during the extraction process. To avoid degradation or oxidation of ascorbic acid, dilute metaphosphoric acid was most commonly used for extraction (Alvarez-Parrilla, de la Rosa, Amarowicz & Shahidi, 2010; Burini, 2007). Topuz and Ozdemir (Topuz & Ozdemir, 2007) reported the quantification of carotenoids, capsaicinoids and ascorbic acid in peppers cultivars. However, samples were extracted and quantified separately. Capsaicinoids and ascorbic acid were also analyzed using colorimetry (Gibbs & O'Garro, 2004; Singh, Singh, Deka, Sanwal, Patel & Verma, 2011), capillary electrophoresis (Liu, Chen, Liu, Deng, Duan & Tan, 2010; Wu, Guan & Ye, 2007), and LC-MS techniques (Frenich, Torres, Vega, Vidal & Bolanos, 2005; Zhang, Hu, Sheng & Li, 2010). While the colorimetric methods are cost effective for the quantification of capsaicinoids and ascorbic acid, separation and quantification of individual capsaicinoids and ascorbic acid is not possible. Furthermore, capillary electrophoresis required the least amount of organic solvent and small quantity of samples for the analysis, but it showed poor sensitivity (Simpson Jr, Quirino & Terabe, 2008). While metaphosphoric acid is a good solvent for extraction of ascorbic acid, Randall et al (Randall, Pippen, Potter & McCready, 1975) successfully extracted ascorbic acid using 5 % meta-phosphoric acid and ethanol. Therefore, reversed phase HPLC methods were developed for the independent separation and quantification of capsaicinoids and ascorbic acid.

Capsaicinoids and ascorbic acid analysis are common study because of the ubiquitous compounds in peppers. However, until now, no attempt has been made for simultaneous separation and extraction of capsaicinoids and ascorbic acid. In this study,

simultaneous extraction of capsaicinoids and ascorbic acid in pepper samples using 3% metaphosphoric acid and ethanol was conducted. Various extraction conditions including extraction solvents, solvent ratios, and extraction times, and HPLC methods for simultaneous separation and detection of capsaicinoids and ascorbic acid were optimized for the quantification. To best of our knowledge, this is first report on simultaneous extraction and analysis of capsaicinoids and ascorbic acid from peppers.

Materials and methods

Instrumentation

A Perkin Elmer (Salem, MA, USA) HPLC system consisting of a LC-250 B pump, a Nelson 900 autosampler, and diode array detector 235C was used. The analysis was performed on a C_{18} Gemini column (250 × 4.6 mm i.d., 5 µm particle size; Phenomenex, Torrance, USA) with gradient mobile phase of solvent A (0.03 M of phosphoric acid in water) and solvent B (MeOH) at a flow rate of 1 ml/min. The gradient program was used for the separation of compounds as follows: 0% B (0-5 min), 0-100% B (5-12 min), 100% B (12-15 min), and 100-0% B (15-20 min). Capsaicinoids and ascorbic acid were simultaneously separated and detected at 282 and 254 nm, respectively. The data was processed using TotalChrome Navigator software (version 6.2.1).

Chemicals and sample material

Capsaicin and dihydrocapsaicin were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-Ascorbic acid was purchased from Mallinckrodt (Paris, KY, USA). HPLC

grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Mature serrano peppers (*Capsicum annuum* L. cv. 'Tuxtlas') were harvested at a greenhouse, Texas A&M University in College Station, Texas. Whole peppers, excluding the pepper stalks, were chopped and ground for the analysis.

Extraction solvents and ratio of sample to solvent

The pepper sample (5 g) was homogenized with 40 ml of solvent for 30 sec using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA). Five solvents of 3% metaphosphoric acid (3% MPA), ethanol (EtOH), 3% MPA:EtOH (8:2, v/v), 3% MPA:EtOH (1:1, v/v), and 3% MPA:EtOH (2:8, v/v) were used to extract capsaicinoids and ascorbic acid. The homogenate was sonicated (Cole-Parmer 8893, Cole-Parmer Instrument Company, USA) for 30 min, and centrifuged (Marathon 16KM, Fisher Scientific, Fair Lawn, NJ, USA) at 7500 rpm for 10 min. The supernatant was filtered through a 0.45 μm membrane filter and injected into HPLC. For the extraction efficiency of capsaicinoids and ascorbic acid, different ratios of sample to solvents (1:3, 1:4, 1:6, and 1:8) were compared. The pepper sample (5 g) was homogenized with 15, 20, 30, and 40 ml of 3% MPA: EtOH (2:8, v/v). The extracts were sonicated for 30 min, centrifuged, and filtered for HPLC analysis. Values shown are mean ± SD from three independent experiments.

Sonication and extraction time

The pepper sample (5 g) was homogenized with 40 ml of 3% MPA: EtOH

(2:8, v/v). The homogenate was sonicated for different time (15, 30, 45, and 60 min). Then, samples were further extracted at various times (30 min, 3 h, 6 h, 12 h, and 24 h) in a shaker. Finally, the extracts were centrifuged and filtered before HPLC analysis.

Recovery study

The recovery study was evaluated by spiking known concentration of standard capsaicinoids and ascorbic acid to the pepper samples. Three different concentrations of capsaicin (4.17, 14.30, and 23.80 µg/ml), dihydrocapsaicin (8.03, 10.70, and 13.37 µg/ml), and ascorbic acid (12.60, 25.20, and 50.40 µg/ml) were used for spiking study. After adding standards to samples, the pepper sample (5 g) was homogenized with 40 ml of 3% MPA: EtOH (2:8, v/v). The mixture was sonicated, centrifuged, and filtrated for HPLC analysis.

Calibration curve, LOD, LOQ, and precision

The calibration curves of capsaicinoids and ascorbic acid were constructed by serial dilution of different concentrations and measurement of the peak areas. Standards of capsaicin (7.8, 15.6, 31.2, 62.5, 125, and 250 µg/ml), dihydrocapsaicin (3.4, 6.8, 13.7, 27.5, 55, and 110 µg/ml), and ascorbic acid (15.6, 31.2, 62.5, 125, 250, and 500 µg/ml) were prepared. The regression equation was calculated in the form of $y = ax \pm b$, where x was concentration, and y was the peak areas of compounds. The linearity was established by the coefficient of determination (R^2).

Limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting serial diluted standard solutions, obtaining the ratio of signal to noise (3:1) for LOD, and signal-to-noise ratio (10:1) for LOQ. The precision of the HPLC condition was evaluated by the intra-day (7 injection/a day), and inter-day (consecutive 5 days) injection of standards. The precision was expressed as relative standard deviation (RSD, %) of retention time.

Statistical analysis

All experiments were performed using SAS statistical system 9.2 (SAS Institute, Cary, NC, USA) for the data analysis. The comparison of means was analyzed by Tukey's test. Data were presented as average and standard deviations. Significant differences were determined at the $P \leq 0.05$ level.

Results and discussion

Extraction solvents

Five solvents of 3% metaphosphoric acid (MPA), ethanol (EtOH), 3% MPA: EtOH (8:2), 3% MPA: EtOH (1:1), and 3% MPA: EtOH (2:8) were used for the extraction efficiency of capsaicinoids and ascorbic acid (Fig. 6A). Capsaicin content was not significantly different in EtOH, 3% MPA: EtOH (2:8), and 3% MPA: EtOH (1:1). The highest level of dihydrocapsaicin was extracted in EtOH, followed by 3% MPA: EtOH (2:8). Following is the order of solvents used for extraction of ascorbic acid: 3% MPA (2130.33 μg/g), 3% MPA: EtOH (8:2) (2119.26 μg/g), 3% MPA: EtOH (1:1)

(2113.00 μg/g), and 3% MPA: EtOH (2:8) (2109.60 μg/g). Although water-based solvents were not commonly used for capsaicinoid analysis, extraction efficiency of target compounds was increased using small amounts of water (Barbero et al., 2008). Based on our results, 3% MPA: EtOH (2:8) was a better combination of solvents for efficient extraction of capsaicinoids and ascorbic acid simultaneously, reducing the analysis time, which in turn leads to reduced costs for routine analysis.

Volume of solvent

Different ratios (1:3, 1:4, 1:6, and 1:8, w/v) of sample to solvent were compared to determine the optimum extraction efficiency. Fig. 6B showed the maximum amounts of capsaicinoids (412.61 μg/g) and ascorbic acid (2785.93 μg/g) were extracted from the pepper samples using a sample to solvent ratio of 1:8 while 1:3 extracted lowest anoumts. While Topuz and Ozdemir (Topuz et al., 2007) used 1:6 and 1:4 ratios of sample to solvent for the extraction of capsaicinoids and ascorbic acid, respectively, extarction and analysis was perfromed idependenlty. The present study clearly demonstrated that solvent volume is crtical for the maximum extarction of capsaicinoids and ascorbic acid.

Sonication and extraction time

Sonication is one of the key parameter for better extraction of capsaicinoids and ascorbic acid. Pepper samples were sonicated for 15, 30, 45, and 60 min for extraction, and capsaicinoids and ascorbic acid showed variable concentrations following different

sonication time (Fig. 7A). The content of capsaicinoids was the highest (391.38 µg/g) at 30 min of sonication time. Low levels of capsaicinoids and ascorbic acid were observed, when the sonication time was shorter or longer than 30 min. Ascorbic acid was significantly higher at 30 min (2719 µg/g), followed by 45 min, and 60 min. This study cleary demonstarted that the levels of ascorbic acid were not fully extracted within 15 min of sonication.

To improve the extraction efficiency of capsaicinoids and ascorbic acid, different extraction time (30 min, 3 h, 6 h, 12 h, and 24 h) in a shaker was compared, and the results were presented in Fig. 7B. Concentrations of capsaicinoids and ascorbic acid were significantly higher at 30 min while the levels were decreased or degraded during 3-6 h up to 10-13%, and the content was further decreased up to 19% for 12-24 h of extraction time. The extraction efficiency of ascorbic acid was reduced in the order of 3 h (18 %) > 6 h (22%) > 12 h (24%) > 24 h (26%). The degradation rate of ascorbic acid was faster than that of capsaicinoids. Previous study reported maximum capsaicinoids at 60 min of extraction using acetonitrile with dry sample to solvent ratio of 1:33 (Karnka, Rayanakorn, Watanesk & Vaneesorn, 2002). In another study, ascorbic acid was extracted from dry pepper powder by shaking for 15 min with 3 % metaphosphoric acid using a 1:100 ratio of sample to solvent (Daood, Kapitány, Biacs & Albrecht, 2006). In the present study, the maximum amount of capsaicinoids and ascorbic acid were simultaneously extracted in 30 min.

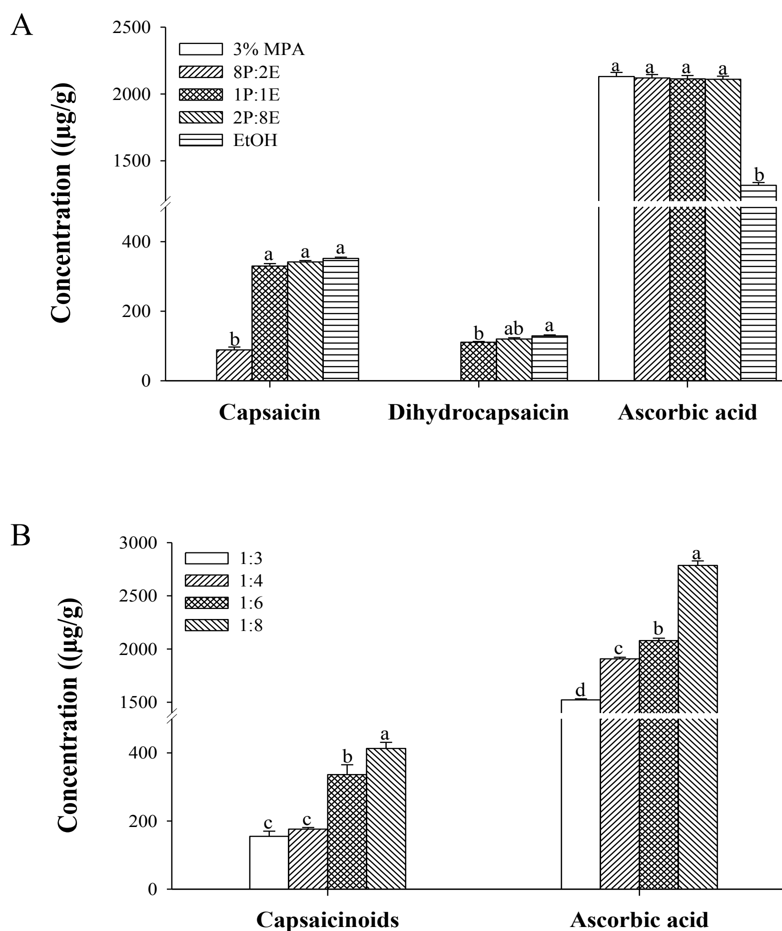

Fig. 6. Extraction efficiency of capsaicinoids (capsaicin, dihydrocapsaicin), and ascorbic acid. (A) Different extraction solvents: 3% MPA, 8P:2E, 1P:1E, 2P:8E, and EtOH. (B) Ratio of sample to solvent using 2P:8E. Different alphabet letters denote significant difference ($P \leq 0.05$) within each group of capsaicinoids and ascorbic acid. Abbreviation: 3% MPA(3% metaphosphoric acid), 8P:2E (3% metaphosphoric acid: EtOH=8:2), 1P:1E (3% metaphosphoric acid: EtOH=1:1), 2P:8E (3% methaphosphoric acid: EtOH=2:8), and EtOH (ethanol). Values shown are mean ± SD from three independent experiments.

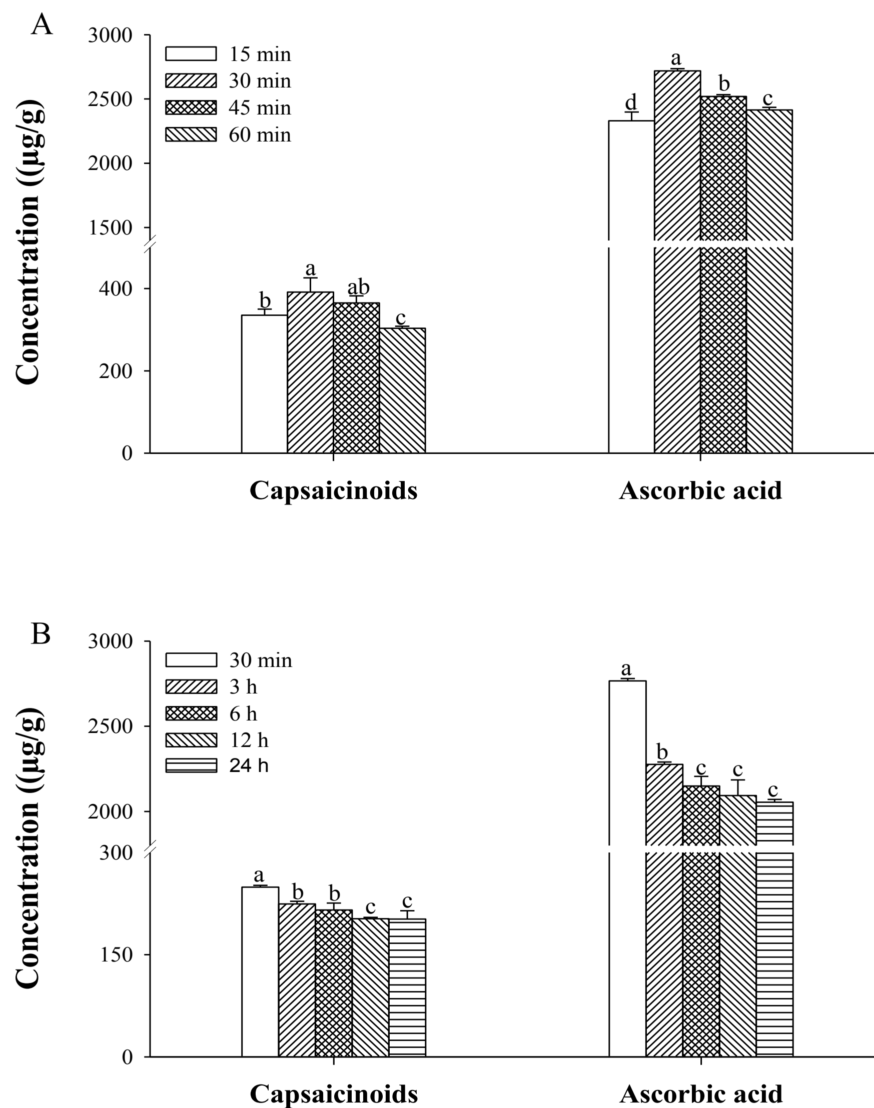

Fig. 7. Levels of capsaicinoids and ascorbic acid using 2P:8E (3% metaphosphoric acid:EtOH=2:8) at (A) sonication time and (B) extraction time. Different alphabet letters denote significant differences ($P \leq 0.05$) within each group of capsaicinoids and ascorbic acid. Values shown are mean ± SD from three independent experiments.

Development of HPLC method

Capsaicin, dihydrocapsaicin, and ascorbic acid were simultaneously extracted maximum with 3% metaphosphoric acid: EtOH (2:8), and the peaks were determined by the HPLC method (Fig. 8). The method was applied to separate and quantify capsaicinoids and ascorbic acid using the mobile phase of phosphoric acid (0.03 M) and methanol within 20 min. The separated peaks of capsaicinoids and ascorbic acid were detected in HPLC at 282 nm and 254 nm, respectively. Previous studies reported the quantification of capsaicinoids and ascorbic acid independently (Schweiggert, Carle & Schieber, 2006), while in the present study, simultaneous separation and detection of capsaicinoids and ascorbic acid was accomplished using an optimum combination of solvents. The developed method provided good separation and quantification of capsaicinoids and ascorbic acid in pepper varieties.

Recovery study

The recovery study was carried out by adding three different concentrations of each standard. The % recovery ranged from 96.21% to 104.97% for capsaicin, 98.44% to 108.71% for dihydrocapsaicin, and 97.01% to 98.83% for ascorbic acid (Table 6). It is clear from our experiment that recovery of capsaicinoids was very high, and in some cases, recovery was greater than 100%. It might be because either maximum capsaicinoids from sample matrix were extracted or extraction efficiency was high for the extractable capsaicinoids. In other studies, recovery of capsaicinoids also showed over 100% (Hartman, 1970; Perkins et al., 2002). The %RSD for recovery ranged from

0.26% to 2.16%, and 0.28 to 1.64% for capsaicinoids and ascorbic acid, respectively. The low RSD values indicated that the proposed method can be reproducible. The recovery data supported the reliability and accuracy of the newly developed analytical method.

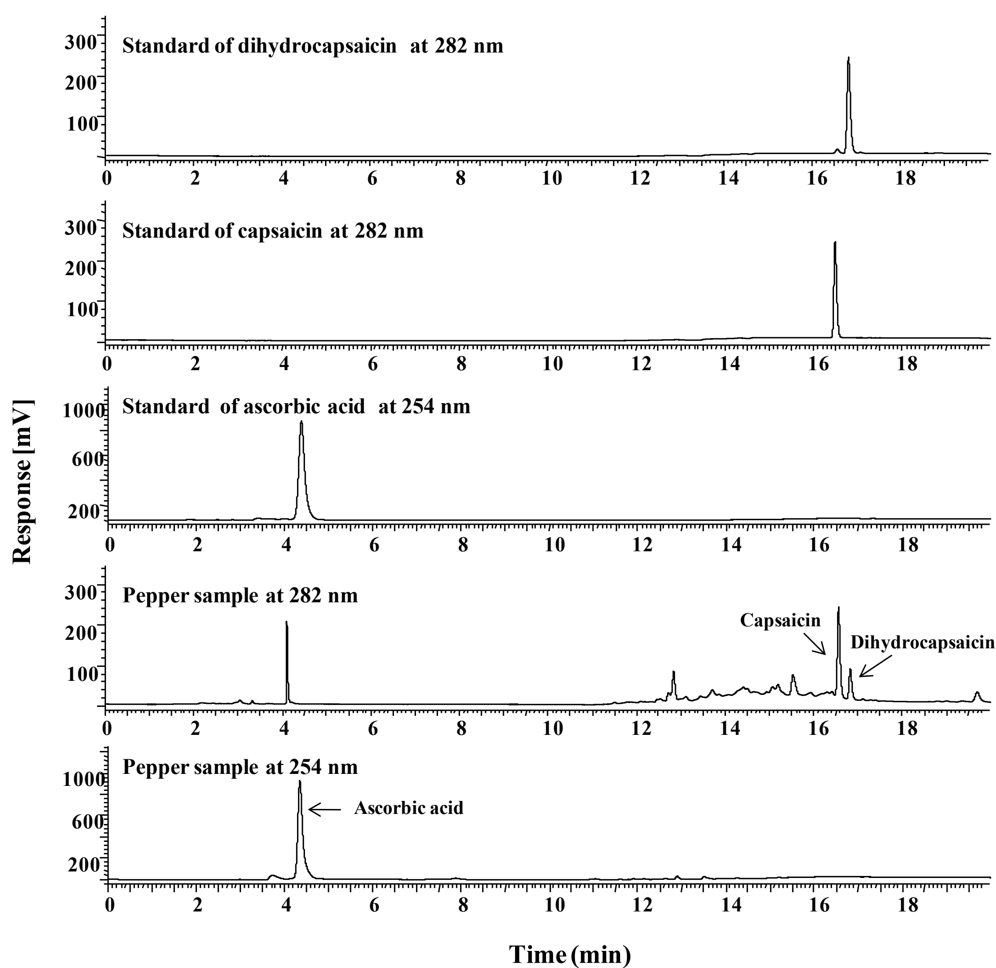

Fig. 8. Simultaneous HPLC separation for the standards of dihydrocapsaicin, capsaicin, and ascorbic acid, and the pepper sample at 282 nm and 254 nm.

Table 6

Recovery study of capsaicin, dihydrocapsaicin, and ascorbic acid from peppers.

Compounds	Standard added (µg)	Recovery (µg)		Recovery (%)	RSD (%)
		Expected	Actual [a]		
Capsaicin	4.17	101.82	99.28 ± 0.12	96.21	0.38
	14.30	111.95	121.24 ± 0.14	104.97	0.26
	23.80	121.45	126.78 ± 0.21	102.38	0.49
Dihydrocapsaicin	8.03	51.34	51.64 ± 0.58	100.59	1.13
	10.70	54.01	53.16 ± 0.44	98.44	0.83
	13.37	56.68	62.00 ± 1.34	108.71	2.16
Ascorbic acid	12.60	507.99	492.79 ± 8.10	97.01	1.64
	25.20	520.59	513.08 ± 3.24	98.56	0.63
	50.40	545.79	539.38 ± 1.53	98.83	0.28

[a] Values are means ± standard deviation of triplicate samples.

Calibration curve, LOD, LOQ, and precision

The linear regression equations were determined by concentrations of each compound and peak areas for capsaicin, dihydrocapsaicin, and ascorbic acid. Good linearity and correlation coefficients ($R^2 > 0.99$) were obtained for each analyte (Table 7). Limits of detection (LOD) for capsaicin, dihydrocapsaicin, and ascorbic acid were 0.24 µg, 0.21 µg, and 0.26 µg, respectively. Limit of quantification (LOQ) is the lowest concentration of analyte, which can be measured by the developed method. LOQ for capsaicin, dihydrocapsaicin, and ascorbic acid were 1.95 µg, 1.72 µg, and 3.91 µg, respectively. The precision under the developed HPLC system was evaluated by inter-day and intra-day injection of capsaicin, dihydrocapsaicin, and ascorbic acid (Table 7). Intra-day repeatability ranged from 1.16% to 1.98% for capsaicinoids, and was 0.86%

for ascorbic acid. For the reproducibility, inter-day (5 days) ranged from 1.30% to 1.34% for capsaicinoids, and was 2.93% for ascorbic acid. Low RSD values (< 3%) suggested good precision of this method developed for simultaneous extraction and analysis of capsaicinoids and ascorbic acid in peppers.

Table 7

Regression equation, linear range, limits of detection (LOD), limits of quantification (LOQ), and precision data of extracted capsaicin, dihydrocapsaicin, and ascorbic acid.

Compounds	Regression equation (y = ax ± b)	R^2	Linear (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Intra-day RSD (%)	Inter-day RSD (%)
Capsaicin	y = (430.32)x + 8.8939	0.9998	1.95 - 62.50	0.24	1.95	1.16	1.34
Dihydrocapsaicin	y = (427.78)x + 6.7018	0.9993	1.72 - 55.00	0.21	1.72	1.98	1.30
Ascorbic acid	y = (1869.7)x + 14.023	0.9981	3.91 - 62.50	0.26	3.91	0.86	2.93

CHAPTER IV

EVALUATION OF SOLVENT PROPERTY AFFECTING ANTIOXIDANT ACTIVITY AND EXTRACTION OF BIOACTIVE COMPOUNDS IN PUNGENT PEPPERS

Introduction

The oxygen molecule is essential to human life, providing respiration as well as APT energy. Reactive oxygen species (ROS) refer to free radicals involving the oxygen element. Epidemiological studies showed that ROS could cause cancer, DNA damage and cardiovascular diseases (Frohlich et al., 2008; Honjo et al., 2008; Pelicano et al., 2009; Wiseman & Halliwell, 1996). The formation of ROS can be quenched by consuming fruits and vegetables due to selective bioactive compounds (Girard-Lalancette, Pichette & Legault, 2009). Pungent peppers such as habanero, cayenne, jalapeno, and serrano, contain flavonoids, carotenoids, vitamin C, vitamin E, and alkaloids which play important roles in human health. In other studies, antioxidant activities in peppers were determined using radical scavenging activity (Conforti et al., 2007), inhibition of lipid peroxidation (Menichini et al., 2009), and metal chelating activity (Cíz, Cízová, Denev, Kratchanova, Slavov & Lojek, 2010). Capsaicinoids and carotenoids exhibited anticancer (Aggarwal, Kunnumakkara, Harikumar, Tharakan, Sung & Anand, 2008; Cui, Lu, Bai, Shi, Zhao & Zhao, 2007; Hwang, Lee, Shin & Park, 2009) and antioxidant activity (Anandakumar, Kamaraj, Jagan, Ramakrishnan, Vinodhkumar & Devaki, 2008; Johnson, 2009; Matsufuji, Nakamura, Chino & Takeda,

1998). Flavonoids have been shown to act as antioxidants, and possess anti-inflammatory (Loke et al., 2008), anti-allergic (Seelinger, Merfort & Schempp, 2008), anti-viral (Liu, Wang, Lee, Wang & Du, 2008), and anti-bacterial effects (Hong, Landauer, Foriska & Ledney, 2006). A previous study showed the antioxidant activity of pepper extracts due to the presence of bioactive compounds, such as polyphenols, carotenoids, capsaicinoids, and ascorbic acid (Alvarez-Parrilla et al., 2010; Hervert-Hernández, Sáyago-Ayerdi & Goñi, 2010). However, research concerning the relationship between bioactive compounds and antioxidant activity of hydrophilic and lipophilic extracts of peppers is limited. Therefore, the objective of the present study was to evaluate the antioxidant activity of pepper extracts by comparison of DPPH, reducing power, and deoxyribose degradation methods and their correlation with bioactive compounds in different solvent polarities.

Materials and methods

Chemicals

Sodium phosphate, potassium ferricyanide, trichloroacetic acid, ferric chloride, 2,2-Diphenyl-1-pricrylhydrozyl, ascorbic acid, butylated hydroxyltoluene, 2-deoxy-D-ribose, and thiobarbituric acid, quercetin, myricetin, luteolin, kaempferol, apigenin, β-carotene, capsaicin, and dihydrocapsaicin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Capsanthin was purchased from ChromaDex (Irvine, CA, USA). Folin-Ciocalteu was from Biomedicals (Illkirch, France). Hydrogen peroxide (30% aqueous solution) was obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA).

HPLC grade methanol and tert-butyl methyl ether were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Plant materials

Cayenne (*Capsicum annuum* cv. 'CA408' and 'Mesilla'), jalapeno (*C. annuum* cv. 'Ixtapa'), and serrano (*C. annuum* cv. 'Tuxtlas') were grown in a field of Texas A&M University AgriLife Research Center (Weslaco, Texas, USA). Mature peppers excluding stalks were homogenized, freeze dried, and stored at -80 °C until further use.

Extraction

Freeze-dried pepper sample (20 g) was extracted in a Soxhlet extractor using hexane for 8 h. The hexane extract was concentrated under vacuum, freeze dried, and stored at -20 °C until further analysis. Similarly the same sample material was extracted successively with ethyl acetate, acetone, methanol (MeOH) and MeOH: water (80:20, v/v) for 8 h each, concentrated and stored at -20 °C.

Sample preparation

A known concentration (5 mg/ml) of each extract was dissolved in acetone or MeOH containing water, and used for antioxidant assays as well as quantification of bioactive compounds. All samples were passed through a 0.45 µm filter prior to HPLC analysis.

Determination of capsaicinoids

The HPLC system (Elmer, Salem, MA, USA) contained a C_{18} Gemini column (250 × 4.6 mm ID), a Nelson 900 autosampler, and a photo-diode array detector 235C. TotalChrome Navigator Software (version 6.2.1) was used for the data processing. Capsaicinoids were detected at 282nm. The gradient elution with solvent A (0.03 M phosphoric acid in water) and solvent B (methanol) was used as follows: 0% B (0-5 min), 0-100% B (5-12 min), 100% B (12-15 min), and 100-0% B (15-20 min) at a flow rate of 1 ml/min. The standard capsaicin (1.95, 3.90, 7.81, 15.62, 31.25, and 62.50 µg/ml) and dihydrocapsaicin (1.72, 3.43, 6.87, 13.75, 27.50, and 55.00 µg/ml) was prepared to construct a calibration curve.

Quantification of capsanthin and β-carotene

Capsanthin and β-carotene were determined using Elmer HPLC (Salem, MA, USA) analysis with a C_{30} YMC carotenoid column (150 × 4.6 mm ID, 3 µm particle size), a Nelson 900 autosampler, and a diode array detector was set at 450 nm. The gradient mobile phase comprised of (A) MeOH and (B) tert-butyl methyl ether with a flow rate of 0.8 ml/min. Carotenoids were eluted as follows, 0-80% B (0-15 min), 100% B (15-20 min), and 100-0% B (20-25 min). Standard solution of capsanthin (5.6, 11.2, 22.5, 45.0, and 90.0 µg/ml) and β-carotene (5.6, 11.2, 22.5, 45.0, and 90.0 µg/ml) were used for the calibration curve. The concentrations of capsanthin and β-carotene were calculated by regression equations and by the application of dilution factor.

Determination of flavonoids

Four flavonoids were determined by HPLC according to our recent publication (Bae, Jayaprakasha, Jifon & Patil, 2012). Calibration graphs of standard quercetin (9.3, 18.7, 37.5, 75.0, and 150.0 μg/ml), luteolin (3.1, 6.2, 12.5, 25.0 and 50.0 μg/ml), kaempferol (3.1, 6.2, 12.5, 25.0, and 50.0 μg/ml) and apigenin (4.8, 9.7, 19.5, 39, and 78 μg/ml) were prepared. For the analysis of total flavonoid aglycones hydrolysis, 6 ml of extracts (5 mg/ml) were treated with 3 ml of 3 N HCl, heated at 95 °C in a water bath for 1 h. The hydrolyzed solution was cooled to room temperature and filtered through a 0.45 μm membrane and analyzed by HPLC. The eluent with 0.03 M phosphoric acid in water (A) and MeOH (B) was used for the separation with a flow rate of 1 ml/min. The gradient was as follows: 40-100% B (0-10 min), 100% B (10-15 min), and 100-40% B (15-20 min). The sample injection volume was 30 μl, and flavonoids were detected at 360 nm.

Total phenolics

Total phenolics were determined by the Folin-Ciocalteu (FC) method using catechin as a standard. An aliquot (100 μl) of the sample or standards was placed in a test tube and the volume adjusted to 10 ml with water. Then, 500 μl of a diluted FC reagent with water (50:50, v/v) was added to all tubes. After 10 min, 1000 μl of sodium carbonate was added, and the mixture was incubated for 20 min. Absorbance of the resulting blue color was measured at 760 nm in a 96-well microplate using a Microplate

Reader (Model KC-4, BioTek Instruments, Winooski, VT, USA). Total phenolics were expressed as mg of catechin equivalents/g of extract.

2,2,-Diphenyl-1-picrylhydrazyl (DPPH)

The DPPH radical scavenging activity was performed to measure antioxidant activity. DPPH (0.1 mM) radical solution was prepared by dissolving 40 mg DPPH in 1000 ml of MeOH. Ascorbic acid (150 µg/ml) was used as a standard for comparison purpose. Aliquots (20 µl) of extracts were pipetted into 96-well microplates, and the total volume of each well adjusted to 100 µl with MeOH. DPPH solution (180 µl) was added into wells, and the absorbance was measured at 515 nm for 30 min at a 3 min interval. Radical scavenging activity was expressed as inhibition percentage.

Reducing power

The reducing power of pepper extracts was measured according to previously reported method (Jayaprakasha et al., 2008). Reducing power was calculated from the calibration graph of ascorbic acid and expressed as µg/ml of extracts. Aliquots of sample extracts were mixed with 200 mM of sodium phosphate and the volume was adjusted to 1.25 ml with water, followed by addition of 1.25 ml of 1% potassium ferricyanide. Tubes were incubated for 20 min at 50 °C, and then 1.25 ml of 10% trichloroacetic acid was added and vortexed. An aliquot (1 ml) was mixed with 1 ml of water and 0.5 ml of 0.1% ferric chloride, and then thoroughly vortexed. The absorbance was measured at 700 nm against a blank. Increased absorbance indicates higher reducing power. The

reducing power was calculated using reducing power of ascorbic acid, and expressed as ascorbic acid equivalents (μg/g).

Degradation of deoxyribose assay

The assay was performed to determine the inhibition of deoxyribose decomposition induced by hydroxyl radicals (Burits, Asres & Bucar, 2001), and the method was modified as follows. Pepper extracts of hexane, acetone, MeOH, and MeOH: water (80:20) were dissolved in phosphate buffer (0.1 M, pH 7.4) to make concentration (0.625 mg/ml) of extracts, while ethyl acetate extracts were dissolved in a small quantity of acetone and mixed with buffer to obtain concentrated extracts because ethyl acetate extracts were not easily dissolved in buffer solution. All reagents were freshly prepared. An aliquot (500 μl) of the sample was mixed with 200 μl of 20 mM deoxyribose in buffer, 200 μl of 100 mM $FeCl_3$:1 mM EDTA (50:50, v/v), 500 μl of 10 mM H_2O_2, and 100 μl of 2 mM ascorbic acid. The mixture was vortexed and incubated at 37 °C for 1 h. Then, 1 ml of 10% trichloroacetic acid and 1 ml of 1% thiobarbituric acid in 0.05 N NaOH was added to the reaction mixture. The solution was incubated at 100 °C for 20 min and cooled to 25 °C. Absorbance was measured at 532 nm. Butylated hydroxyltoluene (BHT) was used as standard. The results were expressed as the inhibition of deoxyribose degradation using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Control optical density} - \text{sample optical density} \times 100}{\text{Control optical density}}$$

Statistical Analysis

Data were analyzed with the SAS statistical system 9.2 (SAS Institute, Cary, NC, USA), and the comparison of means was analyzed using a Tukey's test. Significant differences were determined at the $P \leq 0.05$ level.

Results and discussion

Pepper extracts

The yields of pepper extracts using hexane, ethyl acetate, acetone, methanol (MeOH), and MeOH: water (80: 20, v/v) were summarized in Table 8. MeOH extraction gave maximum yields (25.67-34.67%), whereas minimum yields (0.67-2%) were observed in acetone extraction in the pepper cultivars. All extracts were analyzed for capsaicinoids, carotenoids, and flavonoids by HPLC. The different levels of capsaicinoids (capsaicin and dihydrocapsaicin), carotenoids (capsanthin and β-carotene) and flavonoids (quercetin, luteolin, kaempferol, and apigenin) were determined in four pepper samples (Fig. 9).

Table 8

Yields (g/100g of extract) of pepper extracts obtained by Soxhlet extraction.

Type	Cultivar	Solvents used for extraction				
		Hexane	Ethyl acetate	Acetone	MeOH	MeOH:water (80:20)
Cayenne	CA408	3.00	3.33	0.67	34.67	6.67
Cayenne	Mesilla	3.67	1.00	1.00	29.00	4.00
Jalapeno	Ixtapa	4.00	2.67	2.00	33.67	6.33
Serrano	Tuxtlas	3.33	2.67	1.33	25.67	6.33

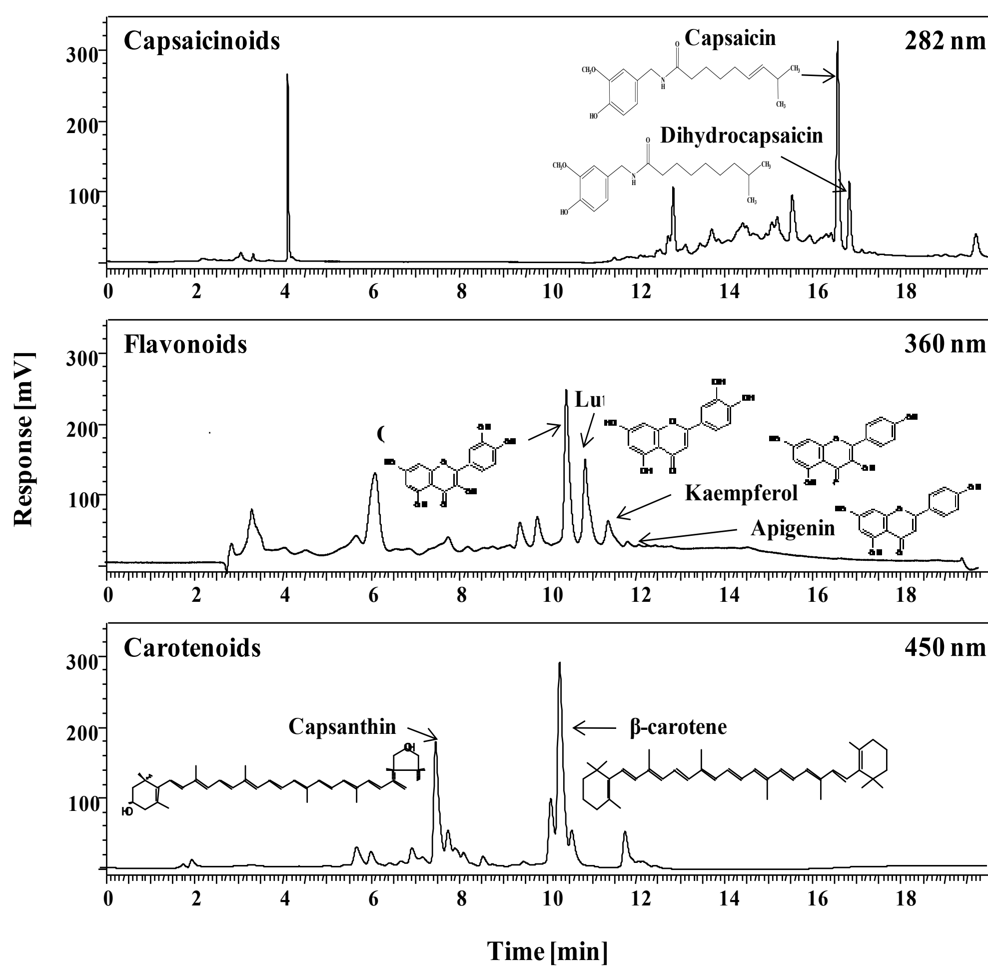

Fig. 9. HPLC chromatograms of capsaicinoids (capsaicin and dihydrocapsaicin) and carotenoids (capsanthin and β-carotene) from hexane extracts of jalapeno peppers, and flavonoids (quercetin, luteolin, kaempferol, and apigenin) from acetone extract of serrano pepper.

Levels of capsaicinoid, carotenoids, and flavonoids

The present study demonstrated variation of capsaicinoid concentration in four different cultivars of pepper samples. Levels of capsaicinoid extraction were observed in

the following order of solvents: hexane > EtOAC > acetone > MeOH. Capsaicinoids were not found in MeOH: water (80: 20) extract. The maximum amount of capsaicin and dihydrocapsaicin were extracted in hexane, ranging from 35.12 to 2495.37 μg/g and 16.76 to 1016.07 μg/g, respectively (Table 9). The highest level of capsaicinoids was found in Ixtapa, while the lowest amount was found in Tuxtlas pepper. Previous studies reported that peppers were extracted in solvents of hexane, dichloromethane, ether, acetone, ethanol, and methanol to obtain highest levels of capsaicinoids (Peña-Alvarez, Ramírez-Maya & Alvarado-Suárez, 2009; Saha, Hedau, Kumar, Mahajan & Gupta, 2010; Thapa, Skalko-Basnet, Takano, Masuda & Basnet, 2009). The present study further confirms the use of hexane for extraction of capsaicinoids. Carotenoid (capsanthin and β-carotene) levels were determined using high performance liquid chromatography (HPLC) with a C_{30} column and the results were presented in Table 9. Since carotenoids are lipophilic in nature, the different rates of carotenoids were extracted according to solvent efficiency. The highest amount of carotenoids (722.09 μg/g) was found in hexane extracts, and the lowest amount (31.26 μg/g) was observed in acetone. It is well known that hydrophilic (polar) solvent is poor solvent property for extraction of carotenoids due to the lipophilic property. Tuxtlas had the highest carotenoid content while Mesilla had the lowest. Table 10 depicted the levels of flavonoids in four different pepper cultivars using five solvents. Hydrophilic pepper flavonoids were extracted to the maximum using MeOH. The highest levels of flavonoids were found in Tuxtlas (59.21 μg/g) and Mesilla (57.15 μg/g). Generally, polar solvents were used for flavonoid extraction and quantification from fresh

materials, while in the present study, wide range of polar solvents were used for the extraction. The results of the current study were similar to previous studies, in which the content of polyphenols was higher in acetone than MeOH from aromatic plants. Moreover, total flavonoids were extracted more in 80% acetone than ethanol from various types of peas (Proestos & Komaitis, 2008; Xu & Chang, 2007).

Total phenolics and DPPH

Total phenolics and DPPH radical scavenging activity were shown in Table 11. Total phenolics were higher in ethyl acetate extracts, ranging from 36.43 to 68.89 mg of catechin equivalents/g, except acetone extract in CA408 (65.92 mg of catechin equivalents/g). Total phenolics are commonly extracted with methanol or ethanol. In this study, total phenolics were higher in ethyl acetate than in methanol or aqueous methanol. The extractions were isolated using a Soxhlet extractor. Folin-Ciocalteu reagent react with non-phenolic compounds, and thus, the final total phenolics could be overestimated (Ferreira, Aires, Barreira & Estevinho, 2009) from the data based on the HPLC analysis (Table 10). Antioxidant activity by DPPH scavenging activity varied significantly with each extract (Table 11). Hexane extracts of the peppers exhibited the highest inhibition of DPPH radical scavenging activity (79.56 – 95.06 %), while DPPH scavenging activity was variable in the extracts of ethyl acetate, acetone, MeOH, and MeOH: water (80:20).

Table 9

The content of capsaicinoids and carotenoids in solvent extracts of pepper cultivars.

Pepper cultivar	Solvents used for extraction	Capsaicinoids (µg/g) [a]			Carotenoids (µg/g) [a]		
		Capsaicin	Dihydrocapsaicin	Total	Capsanthin	β-carotene	Total
CA408	Hexane	nd	83.79 ± 0.27	83.79	149.50 ± 1.72	298.58 ± 0.67	448.08 a
	Ethyl acetate	nd	nd	nd	90.43 ± 2.33	223.00 ± 0.57	313.43 b
	Acetone	nd	nd	nd	5.36 ± 0.09	13.54 ± 0.94	18.91 c
	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:water (80:20)	nd	nd	nd	nd	nd	nd
Mesilla	Hexane	391.41 ± 6.46	157.89 ± 6.44	549.31 a	7.16 ± 0.85	24.10 ± 0.71	31.26 a
	Ethyl acetate	14.19 ± 0.32	3.85 ± 0.18	18.03 b	3.27 ± 0.05	8.40 ± 0.29	11.67 b
	Acetone	3.95 ± 0.28	nd	7.44 c	1.18 ± 0.12	6.07 ± 0.89	7.25 c
	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:water (80:20)	nd	nd	nd	nd	nd	nd
Ixtapa	Hexane	2495.37 ± 4.22	1016.07 ± 5.91	3511.45 a	196.27 ± 14.86	273.20 ± 22.61	469.46 a
	Ethyl acetate	691.63 ± 7.07	343.68 ± 19.78	1035.31 b	34.45 ± 7.34	292.26 ± 40.31	326.71 b
	Acetone	354.37 ± 22.68	171.25 ± 19.41	525.62 c	3.02 ± 0.04	3.26 ± 0.57	6.28 c
	MeOH	18.57 ± 3.09	nd	18.57 d	nd	nd	nd
	MeOH:water (80:20)	nd	nd	nd	nd	nd	nd
Tuxtlas	Hexane	35.12 ± 0.14	16.76 ± 0.55	51.88 a	189.10 ± 1.58	532.98 ± 5.94	722.09 a
	Ethyl acetate	7.99 ± 0.55	3.45 ± 0.95	11.44 b	64.97 ± 4.83	35.06 ± 0.96	100.03 b
	Acetone	3.46 ± 0.12	4.70 ± 0.89	8.16 b	20.24 ± 0.17	15.32 ± 0.87	35.56 c
	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:water (80:20)	nd	nd	nd	nd	nd	nd

[a] Values are means ± standard error of triplicate samples; nd: not detected.

Table 10

The content of flavonoids (μg/g)[a] in solvent extracts of pepper cultivars.

Cultivar	Solvents used for extraction	Quercetin	Luteolin	Kaempferol	Apigenin	Total flavonoids
CA408	Hexane	nd	nd	nd	nd	nd
	Ethyl acetate	1.90 ± 0.62	2.40 ± 0.08	nd	10.81 ± 0.32	15.11 ± 1.14 b
	Acetone	4.00 ± 0.12	5.25 ± 0.09	0.94 ± 0.04	0.39 ± 0.02	10.58 ± 0.10 b
	MeOH	nd	26.86 ± 3.01	nd	nd	26.86 ± 3.01 a
	MeOH:water(80:20)	nd	2.33 ± 0.05	nd	nd	3.11 ± 0.07 c
Mesilla	Hexane	nd	nd	nd	nd	nd
	Ethyl acetate	6.37 ± 0.51	1.72 ± 0.07	4.23 ± 0.16	nd	12.32 ± 0.53 b
	Acetone	4.20 ± 0.23	4.56 ± 0.12	0.27 ± 0.09	2.16 ± 0.04	11.19 ± 0.42 b
	MeOH	57.15 ± 3.74	nd	nd	nd	57.15 ± 3.74 a
	MeOH:water (80:20)	nd	3.92 ± 0.06	nd	nd	3.92 ± 0.06 b
Ixtapa	Hexane	nd	nd	nd	nd	nd
	Ethyl acetate	2.90 ± 0.09	nd	2.16 ± 0.09	nd	5.06 ± 0.05 c
	Acetone	nd	3.86 ± 0.17	4.72 ± 0.45	1.97 ± 0.05	10.56 ± 0.44 b
	MeOH	nd	nd	nd	18.25 ± 1.72	18.25 ± 1.72 a
	MeOH:water (80:20)	nd	1.20 ± 0.08	0.87 ± 0.03	nd	2.76 ± 0.15 d
Tuxtlas	Hexane	nd	nd	nd	nd	nd
	Ethyl acetate	24.27 ± 0.59	1.27 ± 0.02	15.41 ± 0.37	nd	40.95 ± 0.84 b
	Acetone	10.51 ± 0.31	3.17 ± 0.04	1.66 ± 0.06	2.22 ± 0.06	17.57 ± 0.24 c
	MeOH	17.76 ± 2.39	41.45 ± 4.67	nd	nd	59.21 ± 7.05 a
	MeOH:water (80:20)	1.16 ± 0.00	4.88 ± 0.05	nd	nd	9.82 ± 0.22 d

[a] Values are means ± standard error of triplicate samples; nd: not detected.

Table 11

Total phenolic content and DPPH scavenging activity in solvent extracts of pepper cultivars.

Pepper type	Cultivar	Solvents used for extraction	Total phenolics [a] (mg catechin equivalents/ g of extract)	DPPH Inhibition (%)[a]
Cayenne	CA408	Hexane	0.00	85.80 ± 0.83 a
		Ethyl acetate	62.61 ± 3.69 a	49.43 ± 1.13 b
		Acetone	65.92 ± 0.84 a	49.67 ± 3.75 b
		MeOH	49.03 ± 2.12 b	45.81 ± 0.30 b
		MeOH:water (80:20)	35.66 ± 2.07 b	45.11 ± 0.27 b
Cayenne	Mesilla	Hexane	0.00	79.56 ± 1.10 a
		Ethyl acetate	51.63 ± 2.02 a	29.97 ± 2.62 c
		Acetone	28.22 ± 1.12 b	29.33 ± 1.33 c
		MeOH	30.31 ± 2.40 b	40.68 ± 1.99 bc
		MeOH:water (80:20)	24.81 ± 1.66 b	44.78 ± 0.22 b
Jalapeno	Ixtapa	Hexane	0.00	95.06 ± 2.23 a
		Ethyl acetate	36.43 ± 1.97 a	27.09 ± 0.73 b
		Acetone	23.41 ± 1.39 b	22.53 ± 0.35 c
		MeOH	32.48 ± 1.84 a	18.45 ± 0.32 d
		MeOH:water (80:20)	32.40 ± 1.15 a	22.74 ± 0.35 c
Serrano	Tuxtlas	Hexane	0.00	92.35 ± 0.40 a
		Ethyl acetate	68.89 ± 3.69 a	25.54 ± 0.76 e
		Acetone	53.33 ± 2.79 b	39.95 ± 0.99 d
		MeOH	30.89 ± 2.64 c	59.25 ± 0.48 b
		MeOH:water (80:20)	29.26 ± 2.92 c	48.76 ± 0.47 c

[a] Values are means ± standard error of triplicate samples.
Different alphabet letters denote significant difference ($P \leq 0.05$) in the same column.

Comparing our results in polar extracts (18.45-59.25%), Yuan reported 8.31% for DPPH inhibition in an ethanol extract of dried chilli pepper (Lu, Yuan, Zeng & Chen, 2011), whereas Ranilla's result for 95% ethanol extract in dried paprika pepper showed 61-73% DPPH inhibition (Ranilla, Kwon, Apostolidis & Shetty, 2010).

Reducing power and degradation of deoxyribose

Reducing power was based on the reaction with potassium ferricyanide, and indicated electron transfer ability to reduce ferric to ferrous. Reducing power was increased with concentration and showed different potential antioxidant activity in lipophilic and hydrophilic extracts (Fig. 10). Hexane extracts of CA408, Ixtapa, and Tuxtlas pepper cultivars possessed strong reducing power, whereas high reducing power was found using ethyl acetate extract of Mesilla. The reducing power in hexane and ethyl acetate extracts were proportional to the content of capsaicinoids and carotenoids (Table 9). The results indicated that the amount of extractable components from peppers was associated with the property of reducing power. Furthermore, non polar and mid-polar extracts showed higher reducing power than polar extracts.

Degradation of deoxyribose can be increased by the hydroxyl radical induced by hydrogen peroxide, and is inhibited by hydroxyl radical scavengers. In order to determine scavenging effects in pepper extracts, inhibition of deoxyribose degradation was measured (Fig. 10). Extracts of ethyl acetate, acetone, MeOH, and MeOH: water (80:20) prevented deoxyribose degradation, and inhibition of deoxyribose damage was not significantly different from their respective extracts. The results indicated that pepper extracts has ability to scavenge hydroxyl radicals, equating to high inhibition in various extract concentration. This assay may not be a preferred method to measure the antioxidant activity in hexane extracts because the extract could accelerate deoxyribose damage and induce pro-oxidant activity. Furthermore, this study demonstrated that the

antioxidant activity can be attributed not only specific compounds but also type of solvents used for extraction of peppers.

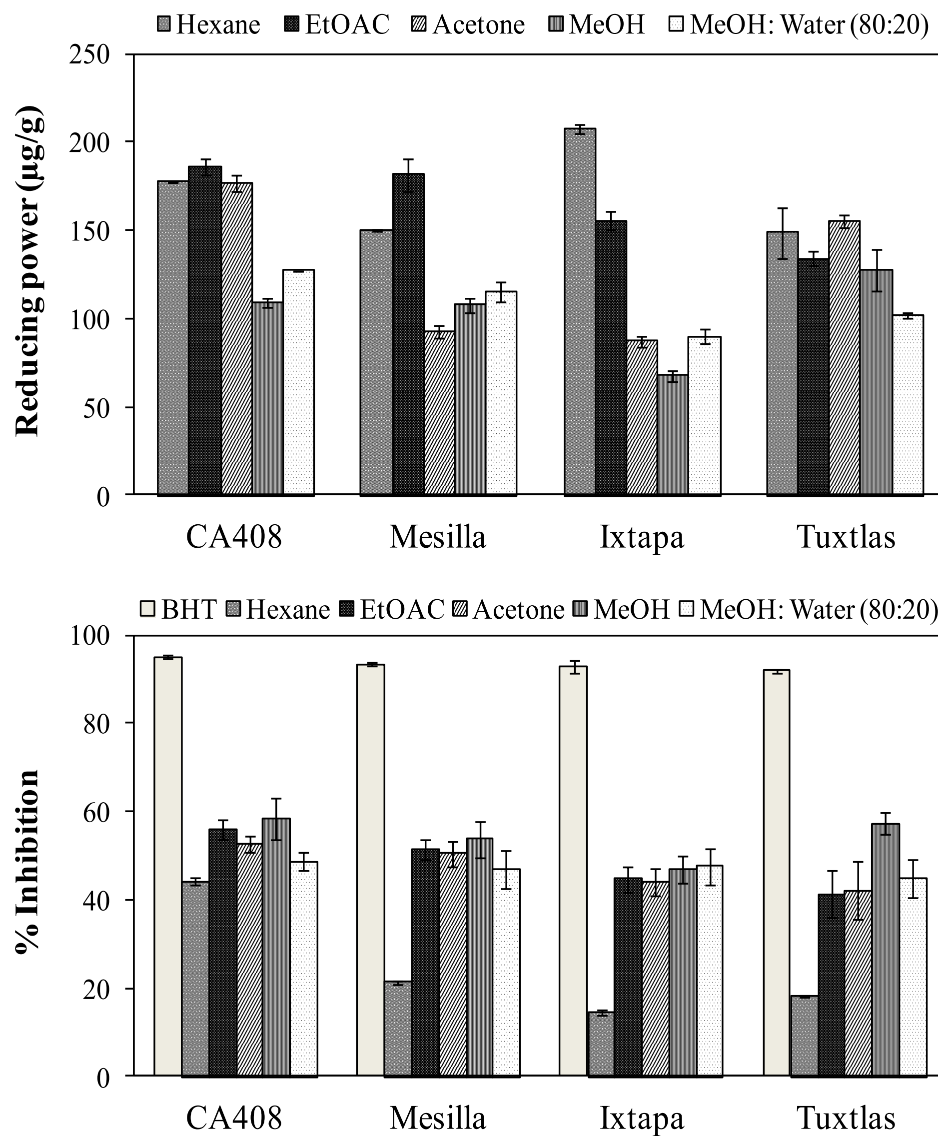

Fig. 10. Reducing power (µg ascorbic acid equivalents/g) and inhibition of deoxyribose degradation (%) of pepper extracts at different concentrations.

Correlations

It was worthwhile to observe various relationships using different assays to determine whether the content of major bioactive compounds in the extracts could reflect antioxidant potential. Correlations between the major compounds and the antioxidant activities of the extracts were shown in Table 12. Positive correlation between lipophilic and hydrophilic compounds and DPPH radical scavenging activity was observed. Similarly, carotenoids and total phenolics were positively correlated with reducing power and deoxyribose degradation. DPPH radical scavenging activity was positively correlated with reducing power, but negatively correlated with deoxyribose degradation.

Table 12

Pearson's correlation coefficients of bioactive compounds and antioxidant activity.

	DPPH	Reducing power	Deoxyribose degradation
Capsaicinoids	0.89**	0.83**	-0.75**
Carotenoids	0.78**	0.68**	-0.71**
Flavonoids	0.64**	ns	0.80**
Total phenolics	0.94**	0.86**	0.69**

*, ** Significant at $P \leq 0.05$ and 0.001, respectively; ns: not significant.

CHAPTER V

DETERMINATION OF EXTRACTION EFFICIENCY FOR THE LEVELS OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY IN NON-PUNGENT PEPPERS

Introduction

In recent years, awareness of the benefits of functional foods and the interest in the discovery of natural antioxidants has risen exponentially (Block, 2005; Block, 2009). Certain compounds, which may be associated with high antioxidant activity, have been identified from many plants. Bioactive compounds such as ascorbic acid, carotenoids, flavonoids, and phenolic compounds occur naturally in many foods, particularly fruits and vegetables such as peppers (Wahyuni, Ballester, Sudarmonowati, Bino & Bovy, 2011). The specific biological activity such as antioxidant, anti-viral, or anti-bacterial activity of fruits and vegetables appears to depend on the diversity as well as concentrations of bioactive compounds (Dillard & Bruce German, 2000; Gigante et al., 2003; Ramful, Tarnus, Aruoma, Bourdon & Bahorun, 2011; Sun, Chu, Wu & Liu, 2002). It is important to identify cultivars that have potential bioactive compounds, which are responsible for antioxidant activity. This information is valuable to breeding and improvement programs aimed at enhancing the functional properties of foods. Quantifying the levels of bioactive compounds is complicated by the fact that different food matrices can bind and inactivate compounds of interest, thereby yielding inaccurate

estimates of concentration (Mithen, Dekker, Verkerk, Rabot & Johnson, 2000; Weiss, Decker, McClements, Kristbergsson, Helgason & Awad, 2008).

Use of appropriate extraction solvents can influence the accuracy of concentration estimates of bioactive compounds (Bettaieb Rebey et al., 2011; Chung, Ji, Canning, Sun & Zhou, 2010). Bioactive compounds and antioxidant activity are directly related with solvent properties such as lipophilic and hydrophilic solvents (Menichini et al., 2009; Sun et al., 2007), and their respective polarity. Carotenoids being lipophilic are dissolved in non-polar solvents, while flavonoids are extracted more in polar solvents due to their hydrophilic properties. In this study, different polar solvent extracts were used to evaluate antioxidant activity in peppers. The 2,2,-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity is one of the most widely used assays to determine antioxidant activity of peppers. Reducing power has been tested to measure the reducing ability of antioxidants in specific concentrations of hydrophilic and lipophilic sample extracts. In a deoxyribose degradation assay, hydroxyl radicals generated by hydrogen peroxide, heat, and acid damage deoxyribose, so that antioxidants in the sample matrix can inhibit this degradation of deoxyribose. Therefore, it is important that the relationship between solvent properties and antioxidant activity of bioactive compounds needs to be established. The present study was undertaken to determine extraction efficiency of five different solvents for the bioactive compounds from four pepper cultivars. The antioxidant activities of the various pepper extracts were tested by DPPH, reducing power, and deoxyribose degradation methods. The active bioactive compounds were identified and quantified by HPLC. The objective of this research was to evaluate

antioxidant activities using DPPH, reducing power, and degradation of deoxyribose, and the results were correlated with total phenolics, carotenoids, and flavonoids.

Materials and methods

Chemicals

Folin-Ciocalteu was from Biomedicals (Illkirch, France). 2,2,-Diphenyl-1-picrylhydrazyl, potassium ferricyanide, trichloroacetic acid, ferric chloride, 2-deoxy-D-ribose, and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide was obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). HPLC grade methanol and tert-butyl methyl ether were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Plant materials

Habanero (*Capsicum chinense* cv. 'TMH'), jalapeno (*C. annuum* cv. 'TMJ'), and paprika (*C. annuum* cvs. 'PA137' and 'B58') non-pungent peppers were grown in a greenhouse at the Vegetable and Fruits Improvement Center of Texas A&M University at College Station, Texas. The peppers were planted in spring and harvested in summer when peppers were fully matured and ripe.

Sample preparation

The mature peppers were ground, stored at -80 °C, and freeze dried. The freeze-dried samples (20 g) were extracted in Soxhlet extractors using hexane, ethyl acetate,

acetone, methanol (MeOH), and MeOH: water (80:20, v/v) successively. Extractions were continued for 10 h in each solvent. The extracts were concentrated using a rotary evaporator, and the yields of the freeze-dried extracts were calculated.

HPLC instrumentation and conditions

Carotenoids from pepper extracts were quantified by HPLC. The Elmer HPLC (Salem, MA, USA) system with a C_{30} YMC Carotenoid column (150 × 4.6 mm ID, 3 µm, particle size), a Nelson 900 autosampler, and photo-diode array detector was set at 450 nm. The mobile phase consisted of MeOH (A) and tert-butyl methyl ether (B) with a flow rate of 0.8 ml/min. Gradient elution was as follows: 0–80% B (0–15 min), 100% B (15 - 20 min), and 100–0% B (20–25 min). Since capsanthin and β-carotene are commonly detected in peppers, the sum of capsanthin and β-carotene were used for content of carotenoids.

Flavonoids were determined by using the same HPLC system with a C_{18} Gemini column (250 × 4.6 mm ID, 5 µm particle size) at 360 nm (Bae et al., 2012). The eluent with 0.03 M phosphoric acid in water (A) and MeOH (B) was carried out at a flow rate of 1 ml/min. The gradient was as follows: 40-100% B (0-10 min), 100% B (10-15 min), and 100-40% B (15-20 min). For the quantification of flavonoid aglycones, flavonoid hydrolysis was performed. A 6 ml aliquot of the extraction was mixed with 3 ml of 3 M HCl, and the mixture was kept at 95 °C in a water bath for 1 h. The hydrolyzed solution was cooled to room temperature (23 °C) and filtered through a 0.45 µm membrane before HPLC analysis.

Total phenolic content

The concentration of total phenolics in extracts was determined using the Folin-Ciocalteu (FC) method. The hydrolyzed sample (100 µl) was adjusted to 10 ml with water. Diluted FC reagent (500 µl) was added and the sample kept at room temperature. After 10 min, 1000 µl of sodium carbonate was added to the mixture and it was incubated at 23 °C for 20 min. The absorbance of blue color was measured at 760 nm using a 96-well plate in a KC-4 Microplate Reader (BioTek Instruments, Winooski, VT, USA). Catechin was used for a calibration graph. Total phenolics were expressed as µg of catechin equivalent/g of pepper.

Determination of antioxidant activity

<u>DPPH (2,2,-Diphenyl-1-picrylhydrazyl) radical scavenging</u>

The DPPH (0.1 mM) radical solution was prepared by dissolving 40 mg DPPH in 1000 ml of MeOH. Standard ascorbic acid solution (0.15, 0.30, 0.45, 0.60, 0.75, 0.90, and 1.05 µg) was used for a calibration graph. All the test sample extracts were prepared in concentration of 5 mg/ml. Aliquots (10 and 20 µl) of the extracts were pipetted into a microplate, and the volume of each well was adjusted to 100 µl with MeOH. The DPPH solution (180 µl) was added to all wells, and absorbance was measured at 515 nm for 30 min at a 3 min interval. The DPPH radical scavenging activity was calculated from the calibration curve of inhibition against standard concentration.

Reducing power

The reducing power of the extracts was determined according to our published method (Jayaprakasha et al., 2008). Different concentrations (0.25, 0.50, 0.75, and 1.00 µg/ml) of the pepper extracts were mixed with sodium phosphate (200 mM) until the volume reached 1.25 ml, and then 1.25 ml of potassium ferricyanide (1%) was added to the mixture. After incubation at 50 °C in water bath for 20 min, 1.25 ml of trichloroacetic acid (10%) was added. The mixture (1 ml) was combined with 1 ml of water and 0.5 ml of ferric chloride (0.1%). Absorbance was measured at 700 nm, a high absorbance indicating strong reducing power.

Degradation of deoxyribose

The modified deoxyribose degradation method was used to determine the inhibition of deoxyribose decomposition induced by the hydroxyl radicals. Different concentrations (5.00, 2.50, 1.25, and 0.63 mg/ml) of the each extract were prepared in phosphate buffer (0.1 M, pH 7.4). All reagents were freshly prepared. Extract (500 µl) was mixed with 200 µl of deoxyribose in buffer (20 mM), 200 µl of $FeCl_3$ (100 mM): EDTA (1 mM) (50:50, v/v), 500 µl of H_2O_2 (10 mM), and 100 µl of ascorbic acid (2 mM). The mixture was incubated at 37 °C in a water bath for 1 h, and then mixed with 1 ml of trichloroacetic acid (10%) and 1 ml of thiobarbituric acid (1%) in 0.05 M NaOH. The prepared reaction solution was heated at 100 °C for 20 min and cooled to room temperature. Absorbance was measured at 532 nm, and the degradation of deoxyribose was expressed as the inhibition (%).

Statistical analysis

Data were analyzed with SAS 9.2 statistical software package (SAS Institute, Cary, NC, USA), and means were compared using Tukey's test for significant differences. Pearson correlation coefficients were calculated, and significant difference was determined at the 95% probability level.

Results and discussion

Total phenolics, carotenoids, and flavonoids

Four cultivars of peppers were extracted using five solvents, and the extracts were concentrated to obtain the yields (Table 13). The highest yield was obtained in MeOH extracts from all pepper cultivars, while the lowest yields were extracted in acetone with the exception that ethyl acetate provided the lowest yield in paprika peppers. Total phenolics in five extracts were shown in Table 14. The levels of phenolics in various pepper extracts ranged from 36.71 to 73.57 mg of catechin equivalents (CE)/g for TMH, from 26.98 to 39.61 mg of CE/g for TMJ, from 28.53 to 37.21 mg of CE/g for PA137, and from 30.39 to 37.44 mg of CE/g for B58. The levels of total phenolics were the highest in TMH using acetone. It is interesting to note that phenolics were extracted not only with methanol and 80% methanol but also with ethyl acetate and acetone. The levels of phenolics in each extract varied in each pepper. Thondre et al. (Thondre, Ryan & Henry, 2011) analyzed total phenolics using different extraction solvents including 70% acetone, 70% methanol, and 70% ethanol, respectively. They found that aqueous acetone had superior total phenolics than other extraction solvents. Additionally, Ornelas-Paz et

al reported that total phenolics were extracted using 80% MeOH, and the content was 2307.8 μg of gallic acid equivalents/g in habanero and 2549.7 μg in jalapeno boiled peppers (Ornelas-Paz et al., 2010).

Table 13

Yields of pepper extracts obtained by Soxhlet extraction.

Type	Cultivar	Solvents used for extraction	Yields (g/100g of extract)
Habanero	TMH	Hexane	1.33
		Ethyl acetate	1.67
		Acetone	0.67
		MeOH	33.33
		MeOH:water (80:20)	5.33
Jalapeno	TMJ	Hexane	4.00
		Ethyl acetate	2.67
		Acetone	2.00
		MeOH	33.67
		MeOH:water (80:20)	6.33
Paprika	PA137	Hexane	4.67
		Ethyl acetate	2.00
		Acetone	1.33
		MeOH	27.00
		MeOH:water (80:20)	4.67
Paprika	B58	Hexane	6.67
		Ethyl acetate	1.00
		Acetone	1.33
		MeOH	37.00
		MeOH:water (80:20)	3.67

The concentrations of carotenoids and flavonoids were measured in pepper extracts (Table 14). The contents of carotenoids depended on the type of solvent used to extract peppers. The highest levels of carotenoids were observed in hexane extracts

followed by ethyl acetate or acetone. B58 had the highest content of carotenoids (628.83 µg/g), while TMH had the lowest content (47.24 µg/g) in hexane extracts. It was found that lipophilic (non-polar) solvents were appropriate for extracting carotenoids.

Table 14

The content [a] of total phenolics, carotenoids and flavonoids in solvent extracts of pepper cultivars.

Cultivar	Solvent extraction	Total phenolics (mg of catechin equivalents/ g of extract)	Carotenoids (µg/g of extract)	Flavonoids (µg/g of extract)
TMH	Hexane	0	47.24 ± 0.08 a	nd
	Ethyl acetate	45.27 ± 2.05 b	4.83 ± 0.01b	11.89 ± 0.47 b
	Acetone	73.57 ± 2.87 a	nd	6.83 ± 0.13 bc
	MeOH	36.71 ± 0.27 c	nd	24.90 ± 4.16 a
	MeOH:water(80:20)	39.77 ± 0.89 c	nd	23.33 ± 0.95 a
TMJ	Hexane	0	103.71 ± 2.23 a	nd
	Ethyl acetate	39.61 ± 1.63 a	21.90 ± 0.21 b	7.67 ± 0.19 bc
	Acetone	34.19 ± 2.25 a	11.49 ± 0.65 bc	13.41 ± 0.65 b
	MeOH	27.05 ± 0.78 a	nd	32.44 ± 5.30 a
	MeOH:water(80:20)	26.98 ± 1.09 a	nd	8.28 ± 0.14 bc
PA137	Hexane	0	354.77 ± 1.77 a	nd
	Ethyl acetate	34.11 ± 0.62 a	13.41 ± 1.41 b	9.85 ± 0.35 b
	Acetone	34.65 ± 1.24 a	24.99 ± 0.61 b	2.30 ± 0.13 c
	MeOH	37.21 ± 0.31 a	nd	27.06 ± 1.47 a
	MeOH:water(80:20)	28.53 ± 0.93 b	nd	2.03 ± 0.11 c
B58	Hexane	0	628.83 ± 1.56 a	nd
	Ethyl acetate	33.57 ± 1.94 a	4.18 ± 0.11 b	40.58 ± 1.23 b
	Acetone	34.88 ± 2.02 a	7.22 ± 0.09 b	37.66 ± 0.25 b
	MeOH	30.39 ± 1.01 a	nd	152.24 ± 2.22 a
	MeOH:water(80:20)	37.44 ± 0.08 a	nd	4.66 ± 0.04 c

[a] Values are means ± standard error of triplicate samples; nd: not detected.

The large variance in carotenoid content reflected the fact that paprika-type peppers (PA137 and B58) contained more carotenoids than habanero (TMH) and jalapeño-type (TMJ) cultivars, suggesting that the levels of carotenoids were influenced by genetic variation of peppers. Different levels of flavonoids such as quercetin, luteolin, kaempferol, and apigenin were found in pepper extracts. While flavonoids were not detected in hexane extracts, the remaining four solvents extracted differential levels of flavonoids in all pepper cultivars. The highest levels of flavonoids were extracted in MeOH solvents, and the flavonoids showed approximately a 47-fold difference between lowest and highest concentrations.

Antioxidant activity

Different extraction solvents showed variable antioxidant activity due to their selective extraction ability for bioactive compounds from pepper extracts. Hexane extracts exhibited the highest DPPH radical scavenging activity (75.30-86.66%), while MeOH extracts resulted in the lowest radical scavenging activity (26.93-50.11%) in all pepper cultivars at 100 ppm (Fig. 11). In our results, non-polar and mid-polar solvents were effective to determine DPPH radical scavenging activity. It is possible that more carotenoids were extracted in hexane (Table 14). Therefore, DPPH scavenging activity was higher in hexane than in other extracts, which was consistent with a previously reported study (Jiménez-Escrig, Jiménez-Jiménez, Sánchez-Moreno & Saura-Calixto, 2000). In a study, regarding the relationship between carotenoids and DPPH, the mature tomatoes showed the highest DPPH radical scavenging activity due to high contents of

carotenoids in diethyl ether and water solvents (Guil-Guerrero & Rebolloso-Fuentes, 2009). In another study, carotenoids extracted in hexane were low content, but showed a high DPPH radical scavenging activity in bambangan (*Mangifera pajang* Kosterm.) peel (Khoo, Prasad, Ismail & Mohd-Esa, 2010).

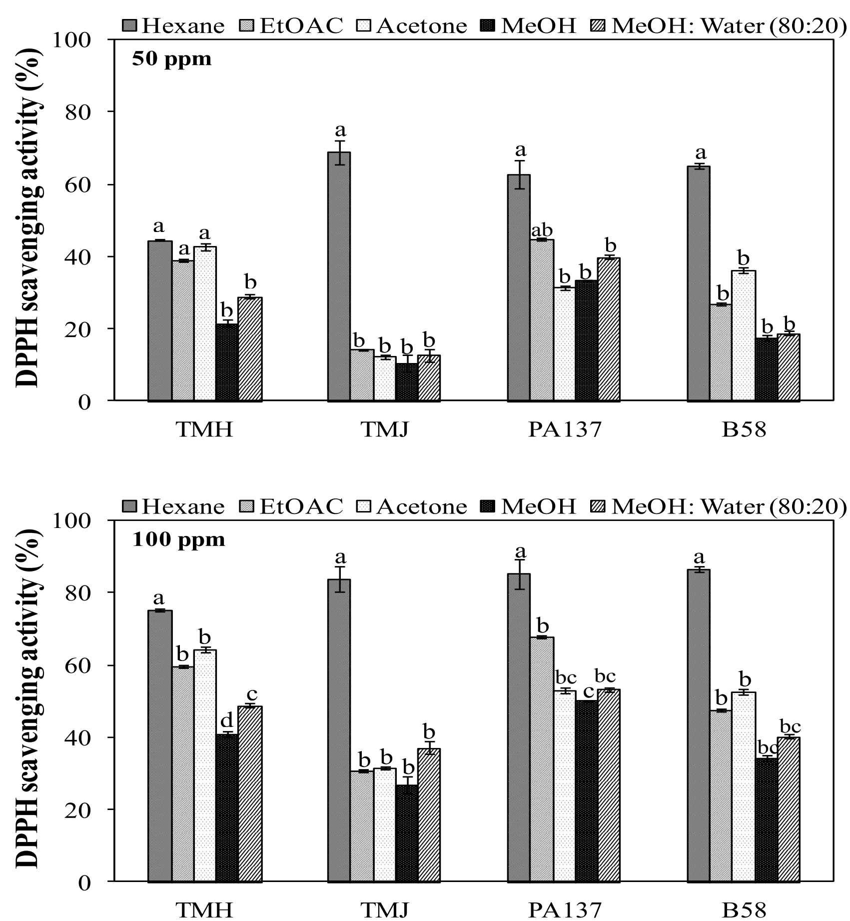

Fig. 11. DPPH radical scavenging activity of various pepper extracts from four cultivars at different concentrations. The values are expressed as mean ± SD of three independent experiments.

On the contrary, carotenoids did not show any activity of DPPH radical scavenging in carrot and tomato juice when methanol: tetrahydrafuran (1:1) was used (Müller, Fröhlich & Böhm, 2011) or showed lower radical scavenging activity in fruit *Canarium odontophyllum* when hexane: acetone: ethanol (70:15:15) was used (Prasad, Chew, Khoo, Yang, Azlan & Ismail, 2011).

Reducing power was tested at different concentrations of pepper extracts in five solvents (Fig. 12). Pepper extracts showed high antioxidant activity for the reduction of ferrous in all pepper extracts. Reducing power of extracts increased with concentration. Ethyl acetate extracts showed strong reducing power in TMJ and PA137, while acetone extracts had the highest activity in TMH and B58. The results demonstrate that reducing power may due to the presence of total phenolics from pepper extracts, and total phenolics could have the ability to act as reducing agents. Previous studies reported a strong correlation between reducing power and total phenolics in various species of peppers (Barreira, Ferreira, Oliveira & Pereira, 2008; Ferreira, Baptista, Vilas-Boas & Barros, 2007; Odabasoglu et al., 2004). The increase of absorbance at 700 nm indicated increased reducing power. In our results, absorbance greater than 2.0 supported the highest reducing power in pepper extracts. Sim and Sil (Sim et al., 2008) reported that various antioxidant activities in aqueous EtOH extracts of pepper showed similar reducing powers at the concentration of 500 and 1000 μg/ml.

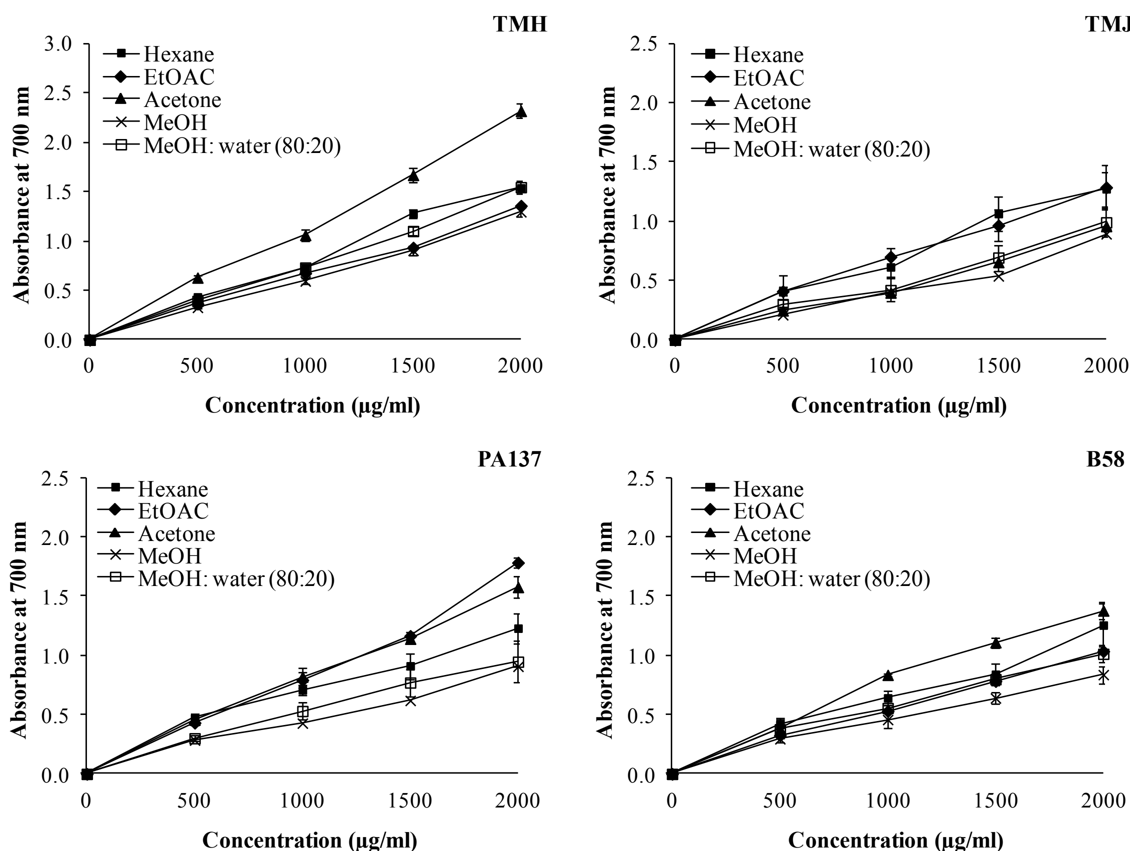

Fig. 12. Reducing power of pepper extracts from hexane, ethyl acetate, acetone, methanol, and methanol: water (80: 20) extracts in pepper cultivars at different concentrations. The values are expressed as mean ± SD of three independent experiments.

In the deoxyribose degradation assay, hydroxyl radicals are generated by the presence of hydrogen peroxide, ascorbate, ferric, and EDTA. Since deoxyribose is damaged by the radicals and degraded, inhibition (%) of deoxyribose degradation was measured in the presence of pepper extracts. The results were shown in Fig. 13. MeOH extracts exhibited the maximum scavenging activity, with inhibition values ranging from

47.42 to 52.61%. The lowest inhibition of deoxyribose degradation was observed in MeOH: water (80:20) extracts. In a previous study, the author used various extract concentrations (0-16.8 mg/ml), and obtained >95% inhibition (Oboh & Rocha, 2007). These findings indicate that the antioxidant activity of pepper extracts was strongly influenced by the type of extraction solvents, which selectively extract certain bioactive compounds.

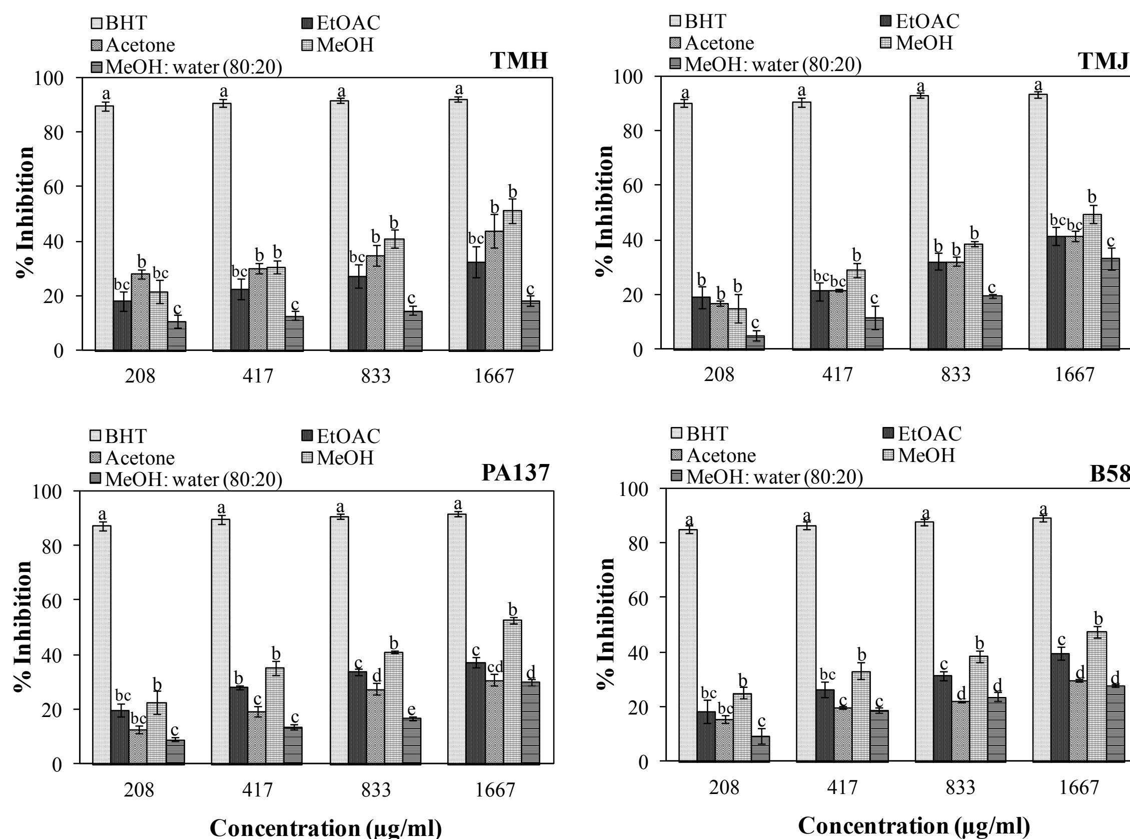

Fig. 13. Inhibition of deoxyribose degradation by various pepper extracts at different concentrations. The values are expressed as mean ± SD of three independent experiments.

Correlations

In order to further explain the antioxidant activity in pepper extracts, the correlation between antioxidant activities and extracted major bioactive compounds was investigated (Table 15). The DPPH results showed a positive correlation with total phenolics (r=0.66), carotenoids (r=0.79), and flavonoids (r=0.85). In addition, reducing power and inhibition of deoxyribose degradation was also significantly correlated to flavonoids and total phenolics. Interestingly, the levels of carotenoids were not correlated with reducing power activity and inhibition of deoxyribose degradation. This may be due to the fact that lipophilic carotenoids did not react effectively in hydrophilic assays such as the reducing power and deoxyribose inhibition assays. The findings in this study were supported by Serrano et al. (Serrano, Zapata, Castillo, Guillén, Martínez-Romero & Valero, 2010), who demonstrated that hydrophilic antioxidant activity was strongly correlated to total phenolics and lipophilic antioxidant activity was highly correlated to carotenoids in sweet peppers.

Table 15

Pearson's correlation coefficients of antioxidant activities, total phenolics, carotenoids, and flavonoids.

	DPPH	Reducing power	Deoxyribose degradation
Total phenolics	0.66^{*}	0.72^{**}	0.81^{**}
Carotenoids	0.79^{**}	ns	ns
Flavonoids	0.85^{**}	0.77^{**}	0.83^{**}

* Significant at $P < 0.05$; ns: not significant.

CHAPTER VI

IMPACT OF CULTIVAR, YEAR, MATURITY AND THEIR INTERACTIONS WITH BIOACTIVE COMPOUNDS IN GREENHOUSE-GROWN PEPPERS

Introduction

Research related to health promoting compounds obtained from various vegetables attracts public attention because of the diversity of naturally occurring compounds. Among the commonly consumed vegetables, peppers (*Capsicum* spp.) contain an array of bioactive compounds including ascorbic acid, capsaicinoids, flavonoids, carotenoids, capsinoids, and capsiconinoids. Accumulated evidence suggested that these bioactive compounds may impart antioxidant activity, reduce the risk of certain cancers, and protect against cardiovascular disease (Archer & Jones, 2002; Rimm, Katan, Ascherio, Stampfer & Willett, 1996). Variation in the nutritional property and the levels of bioactive compounds in peppers is due to the different genotype, maturity, and growing season (year) (Chassy et al., 2006; Crosby, 2008; Pandjaitan, Howard, Morelock & Gil, 2005). A previous study reported that the levels of ascorbic acid and capsaicinoids were higher in mature peppers than in immature peppers (Deepa et al., 2007), while flavonoids decreased as peppers ripened (Marín et al., 2004). Peppers grown in a greenhouse could reduce variation in the content of bioactive compounds because of controlled environmental conditions. One study found that vegetables produced in a greenhouse showed better quality and yield than those grown in a field (Rodriguez, Shaw & Cantliffe, 2007). Since pre-harvest factors play an important role in

the contents of bioactive compounds, it is critical that studies involving these factors need to be investigated. Although bioactive compounds of peppers grown in a greenhouse were previously studied (Navarro, Flores, Garrido & Martinez, 2006), this study did not include combination of pre-harvest factors. The current study was to determine the effects of cultivar, maturity, and harvesting time on the major bioactive compounds of peppers under a controlled environment of a greenhouse. Therefore, the objective of this study was to determine the variation of bioactive compounds in eight cultivars, maturity, and year in greenhouse-grown peppers.

Materials and methods

Pepper cultivars and growing conditions

Eight pepper cultivars were grown in a greenhouse at the Vegetable and Fruit Improvement Center, Texas A&M University (College Station, TX) in April 2008 and 2009. The following non-pungent cultivars, habanero (*Capsicum. chinense* L. cv. 'TMH'), jalapeño (*C. annuum* L. cv. 'TMJ'), and paprika (*C. annuum* L. cv. 'PA137' and 'B58'), and pungent cultivars, cayenne (*C. annuum* L. cv. 'CA408' and 'Mesilla'), jalapeño (*C. annuum* L. cv. 'Ixtapa'), and serrano (*C. annuum* L. cv. 'Tuxtlas') were grown in soilless media (Pro Mix® BX, Premier Horticulture Inc., Quakertown, PA, USA), and automatically irrigated twice a day. Average temperature during pepper maturity was 34.4/23.3 °C (high/low) in 2008 and 36.1/25.0 °C (high/low) in 2009. Solar radiation levels were 20.78 and 20.40 (MJm^2) in 2008 and 2009, respectively. During both years, peppers were harvested in July at the immature (green) stage, and in August

at the mature (fully colored) stage. The pepper fruits, excluding stalks, were chopped, ground, and then stored at -80 °C until analysis.

Ascorbic acid and capsaicinoid analysis

Each pepper sample (5 g) was homogenized with 40 ml of solvent mixture of 3% meta-phosphoric acid and ethanol (8:2) for extraction of ascorbic acid and capsaicinoids. The homogenate was sonicated for 30 min for extraction. The extracts were centrifuged and filtered through a 0.45 μm membrane before HPLC injection. The Perkin Elmer HPLC system (Salem, MA, USA) included a LC-250 B pump, a Nelson 900 autosampler, and diode array detector 235C. The analysis was performed on a C_{18} Gemini column (250 × 4.6 mm i.d., 5μm particle size, Phenomenex, Torrance, CA, USA), with the gradient mobile phase of solvent A (0.03 M of phosphoric acid in water) and solvent B (methanol). The gradient program was as follows: 0% B (0-5 min), 0-100% B (5-12 min), 100% B (12-15 min), and 100-0% B (15-20 min). The column was equilibrated for 5 min before the next injection. The flow rate was 1 ml/min, and detection was performed at 254 and 282 nm for ascorbic acid and capsaicinoids, respectively.

Flavonoid analysis

Each pepper sample (5 g) was homogenized with 40 mL of ethanol and *N,N*-dimethylformamide for extraction of quercetin, luteolin, kaempferol, apigenin, and myricetin. The homogenate was extracted by shaking for 3 h at room temperature. For the hydrolysis process, 6 ml of extract was mixed with 3 ml of 3N HCl, and heated at 95 °C

in a water bath for 1 h. Flavonoids were separated on a C_{18} Gemini column (250 × 4.6 mm i.d., 5 μm) following a gradient program using solvent A (0.03 M of phosphoric acid in water) and solvent B (methanol). The gradient elution was as follows: a linear gradient of 40-100% B (0-10 min), 100% B (12-15 min), and a linear gradient of 100-40% B (15-20 min). Flavonoids were detected at 360 nm with a flow rate of 1 ml/min.

Total phenolics and DPPH (2,2-Diphenyl-1-picrylhydrazyl)

Total phenolics were evaluated by the modified Folin-Ciocalteu (FC) method using catechin as a standard (Jayaprakasha et al., 2007). An aliquot (100 μl) of a sample was taken in a test tube, and distilled water was added to 10 ml. Folin-Ciocalteu reagent with water (50:50, v/v) was prepared, and 500 μl of the diluted FC reagent was added to the test tubes. After 10 min at room temperature, 1000 μl of sodium carbonate was pipetted into the tubes, and the mixture was incubated for 20 min. Absorbance of the reacting blue color was measured at 760 nm. Total phenolics were expressed as mg of catechin equivalents/g of fresh weight sample.

The DPPH assay was followed according to the recently modified procedure. The DPPH solution (40 mg) was prepared with 1000 ml of methanol. Ascorbic acid was dissolved in 3% metaphosphoric acid to make a standard stock solution (150 μg/ml). An aliquot (10 μl) of the extract was pipetted into a 96-well microplate. The volume of each well was adjusted to 100 μl with MeOH, and then 180 μl of DPPH solution was added. Absorbance of the discoloration (from purple to yellow) was measured against methanol as a blank using a microplate reader (Model KC-4, BioTek Instruments, Winooski, VT,

USA) at 515 nm for 30 min. The radical scavenging activity was expressed as percentage inhibition of DPPH.

Statistical analysis

This study was carried out in a completely randomized design. SAS statistical program 9.2 (SAS Institute, Cary, NC, USA) was used for the experimental analysis. The effects of cultivar, maturity, year, and their interaction on levels of bioactive compounds were tested by analysis of variance. Comparisons among treatments were calculated using Duncan's multiple-range test. The probability level of $P \leq 0.05$ was set for significance.

Results and discussion

Ascorbic acid analysis

The ascorbic acid was determined in pepper cultivars at different maturity stages in 2008 and 2009 (Table 16). In immature peppers, the maximum level of ascorbic acid (1373 µg/ml) was found in B58 in both years while the lowest levels were observed in TMH (273.07 µg/ml) and Tuxtlas (195.51 µg/ml) during 2008 and 2009 respectively. As peppers matured, ascorbic acid increased up to 2517.19 (PA137) and 2817.18 µg/ml (B58) in 2008 and 2009, respectively, while the lowest levels were observed in TMJ. The content of ascorbic acid increased from 27 to 81% in 2008, and 61 to 85% in 2009 in the matured peppers. Ascorbic acid concentration among cultivars showed significant variation during growing years and ripening stages ($P \leq 0.05$) (Table 19). In general,

ascorbic acid levels of mature peppers were considerably higher than those of the immature fruits (Osuna-Garcia, Wall & Waddell, 1998; Pérez-López, del Amor, Serrano-Martínez, Fortea & Núñez-Delicado, 2007a). The reason for increasing ascorbic acid could be related to strong light intensity during growth since ascorbic acid synthesis occurs through photosynthesis (Lee & Kader, 2000). Our data showed that solar energy rate during development of peppers ranged from 20.43 to 20.78 MJm^2 in 2008, and 19.80 to 20.40 MJm^2 in 2009 (TexasET, 2008-2009). In comparing growing years, the variation of ascorbic acid was up to 22% for immature peppers and 4% for mature peppers between 2008 and 2009. The pepper cultivars used in this study contained higher concentrations of ascorbic acid (195.51-2817.18 µg/g), compared to ascorbic acid content (152 and 1550 µg/g) reported in other studies (Serrano et al., 2010; Topuz et al., 2007).

Capsaicin and dihydrocapsaicin analysis

Capsaicin and dihydrocapsaicin content differed among CA408, Mesilla, Ixtapa, and Tuxtlas pungent peppers (Table 17). Capsaicinoids among the cultivars (C) were significantly influenced by year-to-year (Y) and maturity (M) variation (Table 19). Significant interactions of C × Y, C × M, Y × M, and C × Y × M were observed. Capsaicinoids were not detected in habanero-type TMH and jalapeño-type TMJ because these cultivars were bred for markets preferring non-pungent pepper cultivars. Presence or absence of capsaicinoids is due to the different alleles of the *Pun1* gene locus, which is responsible for pungency (Stewart, Mazourek, Stellari, O'Connell & Jahn, 2007). The major pungent compound in cayenne and jalapeno peppers was capsaicin, which was

present in higher levels than dihydrocapsaicin. By contrast, dihydrocapsaicin was higher than capsaicin in serrano (Tuxtlas).

Table 16

Ascorbic acid content of pepper cultivars at different stages of maturity in 2008 and 2009.

Year	Type	Cultivar	Ascorbic acid (µg/g of fresh weight) [a]	
			Immature	**Mature**
2008	Habanero	TMH	273.07 ± 7.91 g	1446.68 ± 37.22d
	Cayenne	CA408	655.56 ± 2.36 c	2305.30 ± 34.18 b
	Cayenne	Mesilla	569.01 ± 5.85 d	1439.05 ± 28.28 d
	Jalapeno	Ixtapa	375.20 ± 22.59 e	1190.60 ± 23.03 e
	Jalapeno	TMJ	318.72 ± 11.90 f	782.03 ± 19.59 f
	Paprika	PA137	1040.40 ± 2.26 b	2517.19 ± 13.26 a
	Paprika	B58	1373.00 ± 13.69 a	1885.84 ± 21.94 c
	Serrano	Tuxtlas	367.65 ± 9.45 e	1301.82 ± 4.21 de
2009	Habanero	TMH	479.06 ± 3.95 d	1512.17 ± 16.80 d
	Cayenne	CA408	583.54 ± 4.49 c	2535.64 ± 22.71 b
	Cayenne	Mesilla	302.98 ± 33.40 e	1274.76 ± 9.84 e
	Jalapeno	Ixtapa	290.21 ± 32.49 e	905.32 ± 6.46 f
	Jalapeno	TMJ	271.72 ± 4.27 e	693.51 ± 0.97 g
	Paprika	PA137	814.78 ± 24.44 b	2458.35 ± 40.58 c
	Paprika	B58	929.18 ± 17.64 a	2817.18 ± 16.16 a
	Serrano	Tuxtlas	195.51 ± 3.18 f	1324.71 ± 24.67 e

[a] Values are means ± standard deviation of triplicate samples.
The same alphabet letter within a column in a year is not significantly different at $P \leq 0.05$ using Duncan's multiple-range test.

In our study, another jalapeno-type (Ixtapa) and cayenne (Mesilla) peppers contained the highest capsaicinoids. However, previous study showed that capsaicinoid content was higher in serrano peppers than jalapeno (Ornelas-Paz et al., 2010). It is possible that the growing location of previous study, Mexico, and our current study location, USA, may have major influence on the content of capsaicinoids. The levels of capsaicinoids were

between 20.80 (CA408) and 230.16 μg/g (Ixtapa) in immature peppers, and between 93.77 μg/g (CA408) and 338.86 μg/g (Ixtapa) in mature peppers. Capsaicinoid content increased in matured peppers. It is possible that capsaicin synthase, a key enzyme is enhanced by high temperatures. The enzyme increases during pepper maturity as the fruits were exposed to high temperatures during July and August. Therefore, capsaicinoids increased in mature peppers (Kim, Park, Lee & Kim, 2009). Our data support this explanation because environmental temperatures were higher in mature stages (high temp; 34.4 and 36.1 °C) than immature stages (low temp; 23.3 and 25.0 °C) in both years.

Flavonoid analysis

The concentrations of quercetin, luteolin, kaempferol, apigenin, and myricetin were quantified in pepper cultivars during maturation over two years (Table 18). Flavonoids were significantly different between cultivars, years, and maturity variations (Table 19). A highly significant interaction between growing years and maturity among pepper cultivars was observed ($P \leq 0.05$). Quercetin, luteolin, kaempferol, apigenin, and myricetin were detected among pepper cultivars. Myricetin content was comparable to the reported levels in commercial chili peppers (12 μg/g and < 1 μg/g) (Bahorun, Luximon-Ramma, Crozier & Aruoma, 2004; Hertog et al., 1992b). In our results, paprika-type cultivars had the highest levels of total flavonoids. The levels of flavonoids varied during maturity stages depending on cultivar and year. Menichini et al. found that the content of flavonoids was higher in immature peppers than in mature peppers

(Menichini et al., 2009). Therefore, our results indicated that flavonoid contents depend on pepper cultivars and maturity stage.

Table 17

Content of capsaicinoids (capsaicin and dihydrocapsaicin) for four pungent peppers at different stages of maturity in 2008 and 2009.

Year	Type	Cultivars	Concentration (µg/g of fresh weight) [a]		
			Capsaicin	**Dihydrocapsaicin**	**Total**
2008	**Immature**				
	Cayenne	CA408	75.13 ± 4.19 b	16.43 ± 1.29 c	91.55 ± 4.50 b
	Cayenne	Mesilla	80.92 ± 3.98 b	17.06 ± 1.64 c	97.98 ± 6.02 b
	Jalapeno	Ixtapa	165.48 ± 0.46 a	64.67 ± 4.89 a	230.16 ± 4.97 a
	Serrano	Tuxtlas	39.10 ± 1.48 c	47.81 ± 3.02 b	86.91 ± 4.26 b
2008	**Mature**				
	Cayenne	CA408	79.66 ± 3.22 bc	37.36 ± 5.55 c	117.02 ± 1.82 b
	Cayenne	Mesilla	95.56 ± 3.93 b	27.66 ± 3.62 c	123.22 ± 4.85 b
	Jalapeno	Ixtapa	217.52 ± 5.50 a	121.34 ± 4.05 a	338.86 ± 2.24 a
	Serrano	Tuxtlas	49.28 ± 4.42 c	66.24 ± 2.36 b	115.52 ± 4.72 b
2009	**Immature**				
	Cayenne	CA408	20.80 ± 0.71 d	nd	20.80 ± 0.36 d
	Cayenne	Mesilla	149.45 ± 3.10 a	71.98 ± 1.08 a	221.43 ± 3.91 a
	Jalapeno	Ixtapa	115.84 ± 0.87 b	53.95 ± 0.46 c	169.79 ± 0.87 b
	Serrano	Tuxtlas	47.54 ± 0.64 c	57.16 ± 0.50 b	104.70 ± 0.68 c
2009	**Mature**				
	Cayenne	CA408	53.70 ± 0.60 d	40.06 ± 1.63 c	93.77 ± 1.26 d
	Cayenne	Mesilla	211.72 ± 6.03 a	114.58 ± 3.90 a	326.31 ± 7.20 a
	Jalapeno	Ixtapa	169.00 ± 8.04 b	113.51 ± 0.49 a	282.52 ± 0.68 b
	Serrano	Tuxtlas	81.29 ± 3.02 c	87.47 ± 5.44 b	169.03 ± 5.00 c

[a] Values are means ± standard deviation of triplicate samples. The same letter within a column and a year is not significantly different using Duncan's multiple-range test.

Total phenolics and DPPH

Total phenolics (µg of catechin equivalents/g) and DPPH radical scavenging activity were determined in pepper cultivars at different maturity stages (Fig. 14). The highest total phenolics were found in cayenne-type Mesilla at immature (1.32 mg/g) and mature stages (2.69 mg/g). Total phenolics were significantly different at different maturity stages, as well as during separate growing years. Since total phenolics increased as peppers matured, antioxidant activity was measured to determine its relationship with the content of bioactive compounds. Higher DPPH radical scavenging activity was observed with increased total phenolics. The results indicated that the content of total phenolics positively influenced antioxidant activity relative to the maturity of the peppers.

Table 18

Variation of flavonoid levels (µg/g of fresh weight) [a] of pepper cultivars at different stages of maturity in 2008 and 2009.

Maturity (Year)	Cultivars	Quercetin	Luteolin	Kaempferol	Apigenin	Myricetin	Total
Immature (2008)	TMH	nd	2.09 ± 0.06	2.61 ± 0.06	2.87 ± 0.20	nd	7.58 ± 0.24d
	CA408	nd	2.27 ± 0.12	1.05 ± 0.04	nd	nd	3.31 ± 0.12 e
	Mesilla	11.71 ± 0.69	14.05 ± 0.72	nd	nd	nd	25.76 ± 1.39 a
	Ixtapa	nd	3.03 ± 0.05	1.43 ± 0.06	nd	nd	4.46 ± 0.10 e
	TMJ	nd	4.75 ± 0.12	2.25 ± 0.15	1.48 ± 0.12	nd	8.47 ± 0.26 d
	PA137	6.72 ± 0.38	3.96 ± 0.21	nd	nd	nd	10.69 ± 0.44 c
	B58	3.33 ± 0.14	4.14 ± 0.31	6.85 ± 0.21	nd	nd	14.32 ± 0.45 b
	Tuxtlas	6.81 ± 0.28	3.86 ± 0.43	nd	nd	nd	10.66 ± 0.58 c
Mature (2008)	TMH	nd	2.27 ± 0.14	6.38 ± 0.08	nd	nd	8.65 ± 0.13 c
	CA408	2.04 ± 0.36	3.09 ± 0.41	1.80 ± 0.13	nd	0.92 ± 0.22	7.85 ± 0.16 c
	Mesilla	0.96 ± 0.20	5.25 ± 0.55	nd	3.10 ± 0.21	2.94 ± 0.10	12.25 ± 0.21 b
	Ixtapa	2.72 ± 0.36	7.03 ± 0.43	2.89 ± 0.40	nd	nd	12.65 ± 0.67 b
	TMJ	nd	3.18 ± 0.15	2.60 ± 0.05	nd	nd	5.78 ± 0.16 d
	PA137	3.72 ± 0.14	1.15 ± 0.10	nd	nd	1.89 ± 0.12	6.78 ± 0.16 c
	B58	2.70 ± 0.51	3.94 ± 0.42	nd	3.37 ± 0.22	4.01 ± 0.09	14.02 ± 0.81 a
	Tuxtlas	1.16 ± 0.28	2.27 ± 0.23	nd	2.09 ± 0.04	nd	5.52 ± 0.47 d
Immature (2009)	TMH	1.88 ± 0.25	nd	0.90 ± 0.11	6.69 ± 0.67	nd	9.47 ± 0.79 d
	CA408	5.97 ± 0.58	2.00 ± 0.23	1.99 ± 0.33	nd	nd	9.97 ± 0.74 d
	Mesilla	22.93 ± 1.08	19.12 ± 0.40	4.65 ± 0.19	nd	2.08 ± 0.09	48.78 ± 1.47 a
	Ixtapa	4.31 ± 0.21	nd	5.92 ± 0.26	nd	nd	10.23 ± 0.33 d
	TMJ	nd	2.31 ± 0.30	3.80 ± 0.15	nd	nd	6.10 ± 0.28 e
	PA137	34.50 ± 2.24	4.57 ± 0.35	3.93 ± 0.11	nd	nd	43.00 ± 1.94 b
	B58	22.46 ± 0.32	7.67 ± 0.25	4.34 ± 0.25	nd	nd	34.47 ± 0.30 c
	Tuxtlas	9.32 ± 0.27	nd	2.14 ± 0.19	nd	nd	11.46 ± 0.19 d
Mature (2009)	TMH	1.72 ± 0.12	nd	nd	nd	nd	1.72 ± 0.12 f
	CA408	3.74 ± 0.77	7.14 ± 0.59	6.25 ± 0.26	nd	7.21 ± 0.07	24.35 ± 1.11 d
	Mesilla	21.76 ± 0.93	10.21 ± 1.04	6.41 ± 0.12	nd	5.86 ± 0.20	44.25 ± 0.95 c
	Ixtapa	nd	3.16 ± 0.25	1.40 ± 0.17	nd	nd	4.56 ± 0.32 f
	TMJ	7.23 ± 0.41	2.70 ± 0.54	1.99 ± 0.16	nd	nd	11.93 ± 1.04 e
	PA137	47.41 ± 1.66	21.32 ± 0.72	4.89 ± 0.47	nd	13.00 ± 0.47	86.63 ± 2.82 a
	B58	30.72 ± 1.22	20.26 ± 0.54	nd	nd	7.61 ± 0.10	58.58 ± 1.62 b
	Tuxtlas	8.13 ± 0.40	1.37 ± 0.17	nd	nd	nd	9.51 ± 0.46 e

[a] Values are means ± standard deviation of triplicate samples; nd, not detected

Table 19

Analysis of variance for ascorbic acid, capsaicinoids, flavonoids, and total phenolics in eight pepper cultivars at different maturity stages in 2008 and 2009.

Variable	Ascorbic acid			Capsaicinoids			Flavonoids			Total phenolics		
	df	MS [a]	F	df	MS	F	df	MS	F	df	MS	F
Cultivar	7	5627865.85	1087.84**	3	143539.75	270.53**	7	3997.38	824.88**	7	529601.86	110.61**
Year	1	37696.99	7.29**	1	15595.03	29.39**	1	12313.45	2540.93**	1	2180664.15	455.44**
Maturity	1	57781185.3	11168.9**	1	108200.81	203.93**	1	593.50	122.47**	1	15607834.83	3259.79**
C × Y	7	161557.44	31.23**	3	66560.91	125.45**	7	2612.57	539.12**	7	213507.44	44.59**
C ×M	7	1139851.24	220.33**	3	5405.49	10.19**	7	544.77	112.42**	7	588748.58	122.96**
Y × M	1	577269.98	111.58**	1	11175.20	21.06**	1	1193.65	246.32**	1	227338.72	47.48**
C×Y×M	7	371526.40	71.81**	3	1615.86	3.05**	7	555.28	114.58**	7	134227.61	28.03**

** Significant at $P \leq 0.001$

[a] MS; Mean Square

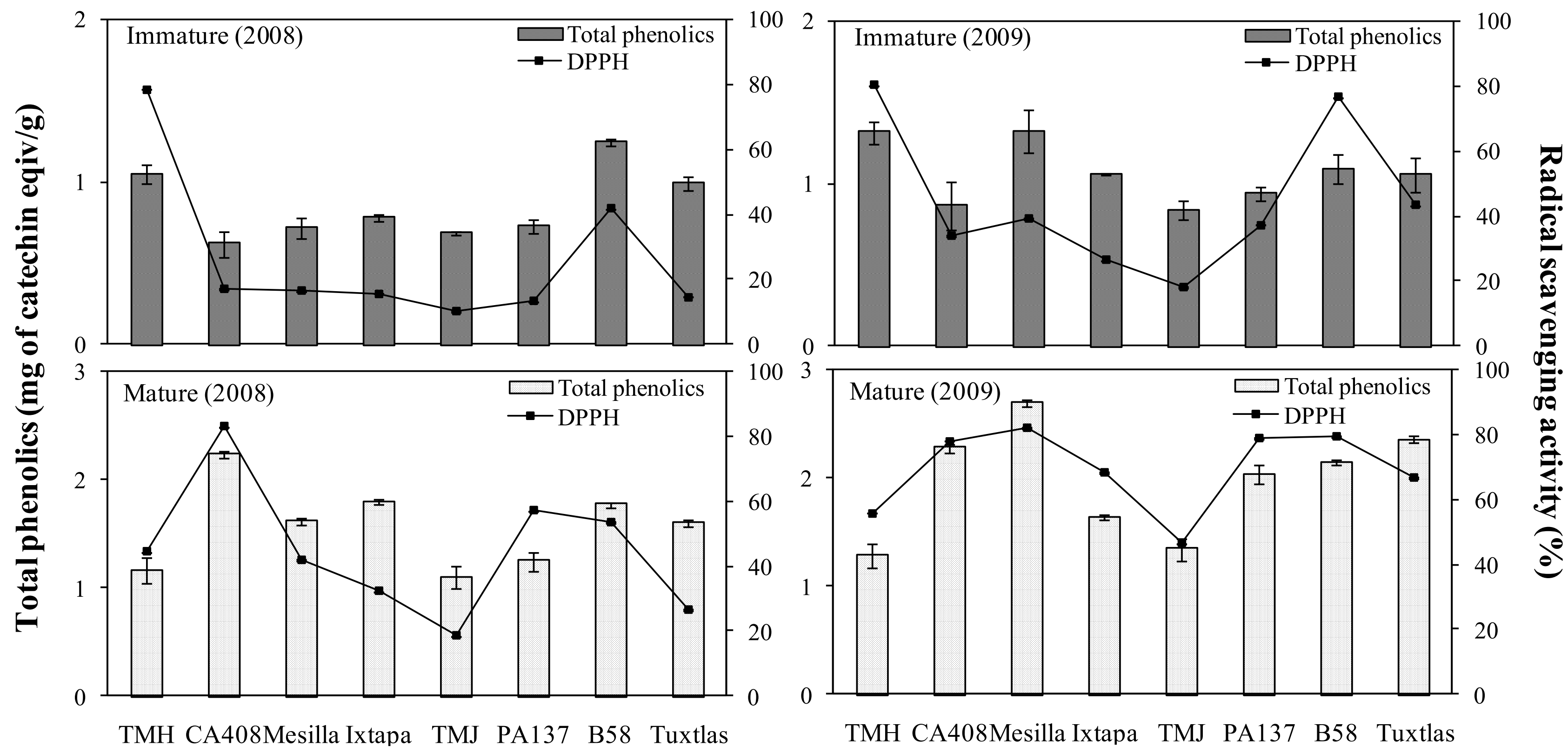

Fig. 14. Total phenolics (mg of catechin equivalents/g of fresh weight) and DPPH radical scavenging activity (%) of pepper cultivars at different maturity stages in 2008 and 2009.

CHAPTER VII

IMPACT OF PRE-HARVEST FACTORS AND THEIR INTERACTIONS WITH BIOACTIVE COMPOUNDS IN FIELD-GROWN PEPPERS

Introduction

Peppers are an important vegetable commodity, widely produced and consume all over the world (FAO, 2009). In the US, Florida and California produce the most fresh peppers (Lynch et al., 2008). Peppers are known for their health promoting properties due to the presence of bioactive compounds (Navarro et al., 2006; Pérez-López, López-Nicolas, Núñez-Delicado, Amor & Carbonell-Barrachina, 2007b; Willcox, Willcox, Todoriki & Suzuki, 2009). More recently, bioactive compounds such as ascorbic acid, capsaicinoids, and phenolics have been linked to disease prevention and wellbeing. These compounds have been associated with decreased risk of inflammatory and cardiovascular diseases among others (Heiss, Keen & Kelm, 2010; Sim et al., 2008; Surh, 2002; Williams, Spencer & Rice-Evans, 2004).

The content of bioactive compounds in peppers is influenced by various pre-harvest factors such as genotype, location, growing year, and maturity. The levels of ascorbic acid, capsaicinoids, and phenolic compounds differed considerably in various pungent and sweet peppers (Deepa et al., 2007; Wahyuni et al., 2011), and in growing locations due to environmental factors (Lee, Crosby, Pike, Yoo & Leskovar, 2005). Other studies reported that content of ascorbic acid, flavonoids, and total phenolics in peppers were influenced by the growing season (Chassy et al., 2006; Deepa, Kaur, Singh

& Kapoor, 2006), and stage of maturity (Matsufuji, Ishikawa, Nunomura, Chino & Takeda, 2007; Pérez-López et al., 2007a). As pepper fruits develop, they often go through various color changes, transitioning from green to red, orange, or yellow (Huh et al., 2001). These color changes as a fruit reaches maturity are linked to synthesis of pigment bioactives as well as non-pigment secondary compounds. Furthermore, previous studies suggested that variation in the content of bioactive compounds could be attributed to cultural strategies or environmental factors such as fertilization (Flores, Navarro, Garrido, Rubio & Martínez, 2004), light intensity (Marín, Gil, Flores, Hellín & Selma, 2008), and temperature (Vega-Gálvez et al., 2008). Few studies have considered the combinations of these pre-harvest factors and their interaction effects on bioactive compounds in various pepper cultivars. The present study was focused on the effects of four pre-harvest factors on the composition of bioactive compounds in peppers. Specially, this study evaluated the influence of genotype, location, growing year, maturity, and their interactions on the concentration of ascorbic acid, capsaicinoids, flavonoids, and total phenolics of eight cultivars of peppers.

Materials and methods

Plant materials

Peppers (*Capsicum* spp.) were grown in Uvalde and Weslaco fields at the Texas A&M University AgriLife Research Centers in Texas during the spring growing seasons of 2008 and 2009. The eight pepper cultivars were habanero (*Capsicum chinense* cv. 'TMH'), cayenne (*Capsicum annuum* cvs. 'CA408' and 'Mesilla'), paprika (*Capsicum*

annuum cvs. ‘PA137’ and ‘B58’), jalapeño (*Capsicum annuum* cvs. ‘Ixtapa’ and ‘TMJ’), and serrano (*Capsicum annuum* cv. ‘Tuxtlas’). Pepper seeds were sown in multicell polystyrene trays filled with root media (Pro Mix® BX, Premier Horticulture Inc., Quakertown, PA, USA). Transplants were field transplanted in single lines spaced 1.0 m apart with plants spaced 30.5 cm apart in April 2008 and 2009. Irrigation and fertigation was done through a drip irrigation system following standard practices for the regions. Growing conditions were as described in Table 20. Data were the average of two year replications for pH and fertilizer treatment. Climatic data, temperature, rainfall, and solar radiation was recorded through the Texas ET network, Texas AgriLife Extension Service (TexasET, 2008-2009). The experiments followed completely randomized design, with three replications. Thirteen plants per cultivar were planted in each replication. Once the pepper fruits in the Uvalde and Weslaco fields were developed, they were randomly harvested in June for immature (unripe) peppers and in July for mature (ripe) peppers. At the immature stage, pepper fruits were green, and then fruit colors changed from green to red and yellow at the mature stage. After harvesting, all peppers fruits were bagged and stored at -80 °C until analysis.

Table 20

Soil and average climatic conditions at Uvalde and Weslaco during pepper growth (April - August) in 2008 and 2009.

Location	Soil type	pH	mg/kg			Year	Temp. ˚C (min/max)	Rain fall (cm)	Solar radiation (MJ/m^2)
			NO_3-N	P	K				
Uvalde	Silty clay loam	7.7	39.0	56.0	698.0	2008	21.9/ 35.1	84.3	21.49
						2009	22.8/ 35.6	62.0	22.94
Weslaco	Sandy loam	8.2	27.8	51.2	624.0	2008	23.9/ 33.3	438.15	24.97
						2009	24.4/ 34.4	77.0	32.76

Ascorbic acid and capsaicinoid analysis

Each pepper sample (5 g) was homogenized with 40 ml of 3% metaphosphoric acid: ethanol (20:80), and then extracted for 30 min of sonication. The sonicated extract was filtrated with a 0.45 µm membrane for HPLC analysis. Ascorbic acid and capsaicinoids were detected in the same HPLC conditions using a C_{18} Gemini column (250 × 4.6mm i.d., 5µm particle size, Phenomenex, Torrance, CA, USA) with a mobile phase of 0.03M phosphoric acid in water (A) and methanol (B). The gradient elution consisting of solvent A (0.03 M phosphoric acid in water) and solvent B (methanol) was as follows: 0% B (0-5 min), 0-100% B (5-12 min), 100% B (12-15 min), and 100-0% B (15-20 min), at a flow rate of 1 ml/min. Ascorbic acid and capsaicinoids were detected at 254 nm and 282 nm, respectively, for 20 min.

Flavonoid analysis

A pepper sample (5 g) was homogenized with 40 ml of ethanol, and the homogenate was extracted for 3 h in a shaker. The extract was then filtered and prepared for hydrolysis. The extract (6 ml) and 3N HCl (3 ml) were mixed and heated at 95 °C for 1 h, and then the hydrolyzed solution was filtered with a 0.45 µm membrane for aglycone analysis in HPLC using a C_{18} Gemini column (250 × 4.6 mm i.d., 5 µm particle size, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.03 M phosphoric acid (A) and methanol (B) at a flow rate of 1ml/min. Gradient elution was as follows: a linear gradient of 40-100% B (0-10 min), 100% B (12-15 min), and a linear

gradient of 100-40% B (15-20 min). Flavonoid peaks were detected at 360 nm, identified by comparing the individual standard peaks, and quantified using a standard curve.

Total phenolics

Total phenolics were determined by the Folin-Ciocalteu (FC) method using catechin as a standard. An aliquot (100 µl) of the sample or standards was placed in a test tube and the volume adjusted to 10 ml with water. Then, 500 µl of a diluted FC reagent with water (50:50, v/v) was added to all tubes. After 10 min, 1000 µl of sodium carbonate was added, and the mixture was incubated for 20 min. Absorbance of the resulting blue color was measured at 760 nm in a *96-well* microplate using a Microplate Reader (Model KC-4, BioTek Instruments, Winooski, VT). Total phenolics were expressed as mg of catechin equivalents/g of extract.

Statistical analysis

Experiments were set up in a completely randomized design. The effects of cultivar, location, maturity, and year on ascorbic acid, capsaicinoids, flavonoids, and total phenolics were determined by two-way and three-way analysis of variance using the SAS statistical program 9.2 (SAS Institute, Cary, NC, USA). Duncan's multiple-range test was used to determine the differences between means at a significance level of 5% ($P \leq 0.05$).

Results and discussion

The content of ascorbic acid, capsaicinoids, flavonoids, and total phenolics from pepper cultivars according to location, year, and maturity stage was presented in Tables 21, 22, 23, and 24, respectively. A summary of the analysis of variance for cultivar, location, year, and maturity, as well as their two-way and three-way interactions were shown in Table 25.

Ascorbic acid content

Differences in ascorbic acid content among cultivars were observed, and the contents of ascorbic acid were significantly higher in mature peppers than in immature peppers (Table 21). Mature paprika peppers contained the highest levels of ascorbic acid (1443.28 μg/g) among pepper cultivars and the values were comparable to previous studies, in which ascorbic acid levels varied in fresh peppers, from 344 μg/g in chili peppers to 649 μg/g in red peppers (Bahorun et al., 2004; Topuz et al., 2007). Significant differences of ascorbic acid between years seemed to be due to temperature and light intensity (Table 20). Ascorbic acid was higher in Weslaco than Uvalde for most cultivars except for B58 in both years and TMH in one year. In other studies, the variation in content of ascorbic acid across locations was evident, with 1882 μg/g in red peppers grown in Chile (Vega-Gálvez et al., 2009), and only 885 and 1074 $\mu g\ g^{-1}$ in green and red peppers, respectively, in Portugal (Castro et al., 2008). The results of the studies in Portugal and Chile indicated that the nitrogen content of soil in different locations affected the content of ascorbic acid in peppers.

Table 21

Ascorbic acid content (μg/g of fresh weight)[a] of pepper cultivars at immature and mature stages at Uvalde and Weslaco in 2008 and 2009.

Cultivar	Location	2008		2009	
		Immature	Mature	Immature	Mature
TMH	Uvalde	285.53 ± 4.54 a	522.52 ± 0.71 a	138.06 ± 5.20 b	440.24 ± 3.92 b
	Weslaco	118.98 ± 3.93 b	392.33 ± 6.61 b	366.77 ± 4.38 a	615.94 ± 1.63 a
CA408	Uvalde	231.65 ± 1.05 b	956.38 ± 18.73 b	240.53 ± 2.35 b	964.50 ± 2.60 b
	Weslaco	322.51 ± 5.22 a	1150.50 ± 4.30 a	474.06 ± 13.92 a	1398.67 ± 19.75 a
Mesilla	Uvalde	132.93 ± 0.67 a	1014.45 ± 2.96 a	194.05 ± 4.58 a	319.97 ± 5.44 b
	Weslaco	126.06 ± 2.22 b	1018.13 ± 8.44 a	191.35 ± 6.93 a	441.59 ± 15.44 a
Ixtapa	Uvalde	83.57 ± 4.56 b	428.55 ± 5.04 b	73.10 ± 2.47 a	151.31 ± 0.92 b
	Weslaco	109.41 ± 4.78 a	449.29 ± 5.17 a	76.77 ± 1.15 a	395.14 ± 2.62 a
TMJ	Uvalde	74.41 ± 3.32 a	271.51 ± 1.16 a	83.25 ± 5.15 b	196.13 ± 0.79 a
	Weslaco	80.51 ± 0.85 a	271.55 ± 2.89 a	127.38 ± 1.10 a	172.86 ± 8.43 a
PA137	Uvalde	389.49 ± 10.66 a	933.56 ± 13.94 a	229.85 ± 7.49 b	1165.70 ± 5.79 b
	Weslaco	404.23 ± 11.76 a	1013.41 ± 9.88 a	504.52 ± 10.57 a	1443.28 ± 13.72 a
B58	Uvalde	446.38 ± 4.15 a	1340.39 ± 6.42 a	153.51 ± 4.51 a	1293.60 ± 20.22 a
	Weslaco	118.32 ± 5.19 b	628.71 ± 14.99 b	85.47 ± 2.74 b	615.87 ± 8.51 b
Tuxtlas	Uvalde	122.15 ± 11.34 a	224.72 ± 5.62 b	79.35 ± 2.02 b	326.39 ± 2.00 b
	Weslaco	124.74 ± 1.23 a	434.97 ± 1.41 a	105.70 ± 2.87 a	483.32 ± 1.45 a

[a] Values are means ± standard deviation of triplicate samples.
The same letter within a column and a year is not significantly different at $P \leq 0.05$ using Duncan's multiple-range test.

In our analysis, the NO_3nitrogen content of soil at Weslaco (27.8 mg/kg) was lower than the nitrate-nitrogen content at Uvalde (39.0 mg/kg). The results showed that increasing NO_3 content might decrease ascorbic acid concentration in peppers. In this respect, our results are consistent with a previous study of peppers in Spain (Flores et al., 2004). Another environmental factor, solar radiation intensity, could influence the concentration of ascorbic acid. Since light intensity was stronger in Weslaco (24.97 – 32.76 MJ/m^2) than in Uvalde (21.49 - 22.94 MJ/m^2), ascorbic acid levels were higher in most Weslaco-grown cultivars. Our results for the relationship between light intensity and ascorbic acid content agreed with the previously reported data (Lee et al., 2000). In our combined analysis of variance according to the F-test, the significance of the two-way and three-way interactions was shown (Table 25). The main effects of cultivar, location, year, maturity, and their interactions were significant for ascorbic acid.

Capsaicinoid content

The content of capsaicinoids, major pungent compounds, in peppers grown in two locations is shown in Table 22. The highest content of capsaicinoids was observed in mature Ixtapa (316.81 μg/g) and Mesilla (357.24 μg/g) in 2008 and 2009, respectively. Concentrations of capsaicinoids were higher in peppers grown at Weslaco than in Uvalde, with differences of 23-36 % in 2008 and 5-78% in 2009. The differences between locations could be due to a response to environmental conditions. Capsaicinoids are found in the pepper placenta, which increases in size as the pepper grows and matures. Therefore, the content of capsaicinoids was higher in mature peppers than in

immature peppers in this study. Similar results were found by Yaldiz et al., who reported that capsaicin content was affected by maturity and temperature (Yaldiz, Ozguven & Sekeroglu, 2010). Regarding the relationship between environmental stress and capsaicin content, one study showed that water stress was a main factor in regulating plant metabolism, and capsaicin levels in particular increased in conditions of low water supply (Estrada, Pomar, Díaz, Merino & Bernal, 1999). Aza-González et al indicated that genotype and environmental factors strongly affected capsaicin content although molecular events were not clear during capsaicinoid biosynthesis (Aza-González, Núñez-Palenius & Ochoa-Alejo, 2011). Our results implied that mature peppers grown in Weslaco under higher temperatures and sandy soils would contain less water than growing under moderate temperatures and silty-clay soils, conditions that also may influence dehydration and eventually triggered their capsaicinoid concentration. Pepper cultivar, location, growing year, and maturity, as well as the interactions of these factors, significantly affected the content of capsaicinoids (Table 25). It is important to evaluate interaction of cultivar with location, maturity, and year in order to identify methodologies for developing new pepper breeding lines in the future.

Flavonoids and total phenolics

Variable contents of flavonoids were observed among cultivars at different maturity (Table 23). Changes in flavonoid contents in peppers grown at the Uvalde and Weslaco fields were observed between 2008 and 2009. Our results showed that mature paprika PA137 had significantly higher flavonoid contents than other cultivars.

Table 22

Capsaicinoid content (µg/g of fresh weight) [a] of pepper cultivars at immature and mature stages at Uvalde and Weslaco in 2008 and 2009.

Location	Cultivar	2008		2009	
		Immature	Mature	Immature	Mature
CA408	Uvalde	30.65 ± 2.47 b	73.98 ± 1.39 b	52.46 ± 2.13 a	153.42 ± 1.39 a
	Weslaco	48.98 ± 0.60 a	96.13 ± 1.09 a	53.44 ± 1.12 a	145.90 ± 4.99 a
Mesilla	Uvalde	88.70 ± 2.66 b	86.20 ± 4.99 b	64.76 ± 2.19 b	79.53 ± 0.60 b
	Weslaco	121.10 ± 5.79 a	132.22 ± 6.71 a	214.63 ± 1.28 a	357.24 ± 6.24 a
Ixtapa	Uvalde	175.94 ± 6.78 a	232.25 ± 5.87 b	74.98 ± 2.24 b	84.93 ± 3.87 b
	Weslaco	178.16 ± 6.82 a	316.81 ± 8.78 a	211.84 ± 6.37 a	423.75 ± 14.42 a
Tuxtlas	Uvalde	39.04 ± 2.24 a	80.30 ± 2.17 a	26.47 ± 1.05 b	82.71 ± 4.26 b
	Weslaco	32.22 ± 3.34 a	51.34 ± 0.96 b	42.85 ± 0.84 a	113.25 ± 4.55 a

[a] Values are means ± standard deviation of three independent experiments.
The same letter within a column and a year is not significantly different at $P \leq 0.05$ using Duncan's multiple-range test.

Table 23

Flavonoid content (µg/g of fresh weight)[a] of pepper cultivars at immature and mature stages at Uvalde and Weslaco in 2008 and 2009.

Cultivar	Location	2008		2009	
		Immature	Mature	Immature	Mature
TMH	Uvalde	68.43 ± 0.70 a	62.50 ± 0.70 a	12.78 ± 0.79 b	26.03 ± 0.67 a
	Weslaco	28.12 ± 0.62 b	21.08 ± 0.75 b	37.74 ± 1.05 a	6.52 ± 0.80 b
CA408	Uvalde	11.14 ± 0.27 b	60.84 ± 0.24 a	8.50 ± 0.23 a	15.44 ± 0.89 a
	Weslaco	21.23 ± 1.59 a	16.53 ± 1.26 b	4.21 ± 0.27 b	10.53 ± 0.45 b
Mesilla	Uvalde	63.67 ± 1.19 a	56.87 ± 0.44 a	13.82 ± 0.54 b	44.81 ± 1.41 a
	Weslaco	36.38 ± 0.59 b	58.16 ± 0.67 a	78.91 ± 0.43 a	45.46 ± 1.06 a
Ixtapa	Uvalde	13.89 ± 0.57 a	6.70 ± 0.59 b	5.19 ± 0.28 b	6.15 ± 0.63 b
	Weslaco	4.26 ± 0.32 b	10.61 ± 0.62 a	15.74 ± 0.42 a	19.39 ± 0.16 a
TMJ	Uvalde	5.23 ± 0.30 a	6.22 ± 0.33 b	5.33 ± 0.25 a	4.65 ± 0.30 a
	Weslaco	3.56 ± 0.30 b	36.71 ± 0.26 a	5.21 ± 0.12 a	3.58 ± 0.73 b
PA137	Uvalde	106.11 ± 1.76 a	139.45 ± 2.70 a	61.41 ± 1.51 a	107.55 ± 0.77 a
	Weslaco	32.24 ± 1.34 b	50.82 ± 1.21 b	55.02 ± 0.50 b	111.33 ± 5.61 a
B58	Uvalde	65.48 ± 2.70 a	138.75 ± 1.05 a	29.19 ± 0.63 b	65.77 ± 0.52 b
	Weslaco	9.84 ± 0.48 b	39.89 ± 0.39 b	62.67 ± 0.35 a	92.75 ± 0.88 a
Tuxtlas	Uvalde	9.37 ± 0.22 a	69.64 ± 0.33 a	7.88 ± 0.16 b	23.69 ± 0.22 a
	Weslaco	8.78 ± 0.34 a	40.80 ± 0.18 b	10.58 ± 0.36 a	20.90 ± 1.53 b

[a] Values are means ± standard deviation of three independent experiments.
The same letter within a column and a year is not significantly different at $P \leq 0.05$ using Duncan's multiple-range test.

Flavonoid levels showed large variations at immature and mature stages among pepper cultivars. Flavonoid contents were higher in peppers grown in Uvalde than those grown in Weslaco due to higher rain-fall in Weslaco in 2008 (Table 20). The levels of flavonoids were higher in immature peppers grown in Weslaco, compared to the peppers grown in Uvalde in 2009 due to the strong light intensity in Weslaco in 2009, while the mature peppers contained similar or a little higher in Uvalde. Lee et al. showed similar results for flavonoid content in peppers grown in two locations (Lee et al., 2005). Strong light intensity might influence active photosynthesis, which enhances phenylpropanoid synthesis. In the biosynthesis process, high phenylalanine ammonialyase is directly linked to high level of flavonoids, which is enhanced by high light intensity and limited nitrogen (Olsen et al., 2009). Pepper cultivars differed significantly in flavonoid content between immature and mature stages, and variation among cultivars was a much greater factor than location, year, or maturity (Table 25). However, the significant interactions of the four factors of cultivar, location, year, and maturity showed that flavonoid synthesis is impacted by interactions of genotype and environment.

Total phenolics in pepper cultivars depended on location, year, and maturity (Table 24). Total phenolics were quantified for pepper cultivars grown in Uvalde and Weslaco at immature and mature stages, and were 1.06-1.18 times higher at mature stages than immature in both years. Previous results were consistent with our results (Materska & Perucka, 2005). The content of total phenolics was higher in Weslaco field-grown peppers than those grown in Uvalde in 2008. However, in 2009, total phenolics were higher in Uvalde-grown immature peppers, while Weslaco produced higher

phenolics levels in mature peppers. Interestingly, patterns of total phenolics content differed noticeably with patterns of flavonoids, indicating that various phenolic compounds could be differentially affected by the same environmental conditions. The F values of cultivar, location, year, and maturity, as well as the effects of their interaction, were presented in Table 25. All of the pre-harvest factors significantly affected the content of total phenolics in peppers, with maturity having the greatest influence. Two-way and three-way interactions were significant, but no interactive effect was observed between year and maturity.

Table 24

Total phenolic content (mg catechin equivalents/g of extract)[a] of pepper cultivars at immature and mature stages at Uvalde and Weslaco locations in 2008 and 2009.

Cultivar	Location	2008		2009	
		Immature	Mature	Immature	Mature
TMH	Uvalde	0.49 ± 0.04 b	0.49 ± 0.03 b	0.51 ± 0.03 b	0.65 ± 0.02 b
	Weslaco	0.70 ± 0.01 a	0.64 ± 0.01 a	0.69 ± 0.01 a	0.60 ± 0.01 a
CA408	Uvalde	0.41 ± 0.03 b	0.65 ± 0.02 a	0.68 ± 0.02 a	0.72 ± 0.02 a
	Weslaco	0.68 ± 0.02 a	0.65 ± 0.02 a	0.58 ± 0.04 b	0.68 ± 0.01 b
Mesilla	Uvalde	0.50 ± 0.01 b	0.61 ± 0.02 a	0.64 ± 0.02 a	0.67 ± 0.01 a
	Weslaco	0.59 ± 0.01 a	0.62 ± 0.02 a	0.67 ± 0.02 a	0.66 ± 0.02 a
Ixtapa	Uvalde	0.45 ± 0.01 b	0.59 ± 0.01 b	0.62 ± 0.01a	0.59 ± 0.01 b
	Weslaco	0.52 ± 0.01 a	0.74 ± 0.01 a	0.52 ± 0.04 b	0.89 ± 0.04 a
TMJ	Uvalde	0.36 ± 0.03 b	0.45 ± 0.05 a	0.47 ± 0.05 a	0.57 ± 0.03 b
	Weslaco	0.45 ± 0.02 a	0.52 ± 0.03 a	0.42 ± 0.03 a	0.92 ± 0.01 a
PA137	Uvalde	0.47 ± 0.04 a	0.58 ± 0.02 b	0.60 ± 0.02 a	0.79 ± 0.01 a
	Weslaco	0.49 ± 0.04 a	0.83 ± 0.01 a	0.51 ± 0.04 b	0.69 ± 0.01 b
B58	Uvalde	0.55 ± 0.03 a	0.83 ± 0.01 a	0.87 ± 0.01 a	0.72 ± 0.01 b
	Weslaco	0.55 ± 0.01 a	0.79 ± 0.01 b	0.57 ± 0.03 b	0.91 ± 0.01 a
Tuxtlas	Uvalde	0.46 ± 0.02 b	0.79 ± 0.03 a	0.82 ± 0.03 b	0.65 ± 0.01 b
	Weslaco	0.58 ± 0.02 a	0.62 ± 0.03 b	0.59 ± 0.02 a	0.82 ± 0.01 a

[a] Values are means ± standard deviation of three independent experiments.
The same letter within a column and a year is not significantly different at $P \leq 0.05$ using Duncan's multiple-range test.

Table 25

Significance of main effects and their interactions for ascorbic acid, capsaicinoids, flavonoids, and total phenolics of pepper cultivars grown in two locations in two years.

	Ascorbic acid		Capsaicinoids		Flavonoids		Total phenolics	
Main effects	df	*F* value	df	*F* value	df	*F* value	df	*F* value
Cultivar	7	995.82**	3	648.35**	7	748.60**	7	55.17**
Location	1	6.16*	1	565.79**	1	213.16**	1	46.10**
Year	1	28.58**	1	69.09**	1	165.83**	1	160.50**
Maturity	1	8178.47**	1	580.66**	1	550.19**	1	353.57**
Two-way interactions								
Cultivar × Location	7	176.60**	3	152.09**	7	82.50**	7	9.28**
Cultivar × Year	7	106.45**	3	232.35**	7	23.53**	7	4.63**
Cultivar × Maturity	7	341.10**	3	10.38**	7	94.09**	7	17.34**
Location × Year	1	118.22**	1	347.86**	1	756.15**	1	29.33**
Location × Maturity	1	4.40*	1	35.98**	1	64.30**	1	25.00**
Year × Maturity	1	22.81**	1	25.07**	1	38.95**	1	3.38ns
Three-way interactions								
Cultivar × Location × Year	7	20.14**	3	127.58**	7	101.76**	7	5.41**
Cultivar × Location × Maturity	7	61.07**	3	15.37**	7	19.70**	7	13.38**
Cultivar × Year × Maturity	7	129.53**	3	59.03**	7	18.22**	7	8.72**
Location × Year × Maturity	1	5.94*	1	5.77*	1	3.57ns	1	84.97**

*Significant at $P \leq 0.05$, **$P \leq 0.001$, ns: not significant

CHAPTER VIII

SUMMARY AND CONCLUSION

Peppers are found to be a rich source of certain bioactive compounds such as ascorbic acid, capsaicinoids, flavonoids, carotenoids, and phenolics and show high antioxidant activity. The levels of bioactive compounds and antioxidant activity vary considerably due to sample preparation methods, extraction solvent property, and pre-harvest conditions.

Sample preparation methods have been optimized to extract higher levels of bioactive compounds. The present study demonstrated the optimum extraction conditions for bioactive compounds, and the HPLC methods were validated for instrumental precision and sensitivity to quantify and separate flavonoids, capsaicinoids, and ascorbic acid. Five flavonoids were separated and quantified as aglycones of myricetin, quercetin, luteolin, kaempferol, and apigenin. Capsaicinoids and ascorbic acid were simultaneously extracted and separated. The efficient extraction conditions for the optimum levels of the major compounds in peppers were developed using appropriate extraction solvent, solvent ratio, and extraction time.

The concentrations of capsaicinoid, carotenoids, flavonoids, and total phenolics were highly dependent on the nature of solvent used to extract the compounds from pepper fruits. Carotenoids and flavonoids were the highest in hexane and methanol extracts, respectively. The pepper extracts also varied significantly with the highest activity of DPPH and reducing power being recorded from capsaicinoids and carotenoids

in lipophilic extracts. For the deoxyribose degradation assay, pepper extracts showed the highest inhibition in methanol extracts due to the presence of flavonoids. These observations demonstrated that solvent properties can significantly influence estimates of specific bioactive compounds in different peppers, and impact the concentration of bioactive compounds. It showed that estimates of the antioxidant activity of pepper extracts depended not only on solvent property and pepper cultivar, but also on the nature of the assay utilized.

Variation of bioactive compounds in peppers can be influenced by genetic variation, maturity, year of harvest, and environmental factors. Interactions of cultivar, maturity, and year were highly significant for variation of ascorbic acid, capsaicinoids, flavonoids, and total phenolics. Maturity had the highest effect on the content of ascorbic acid and capsaicinoids, while cultivar and location effects were greater than other factors on flavonoids. The results in the present study can be used for the rigid separation and quantification of flavonoids, capsaicinoids, ascorbic acid, and carotenoids in commercial peppers for the accurate determination of bioactive compounds.

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