PHYTOCHEMICAL COMPOSITION AND OXIDATIVE STABILITY OF COLD-PRESSED BUTTERNUT SQUASH (*CUCURBITA MOSCHATA*) AND PUMPKIN (*CUCURBITA PEPO* L.) SEED OILS

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ABSTRACT

Pumpkin (*Cucurbita spp.*), a crop native to America, represents a profitable opportunity in the Northeast. In the US, production of pumpkin has expanded greatly particularly in Illinois, Michigan, Pennsylvania, New Jersey and New York. With the appreciable volume of production intended mainly for utilization of the flesh for food processing, it is also expected that significant amounts of seeds, as part of the processing wastes, are being generated. Pumpkin seeds have been reported to contain a significant amount of highly unsaturated oil. Therefore, utilization of pumpkin seeds for oil production may add value to this crop and could help reduce food-processing waste.

We studied the phytochemical composition, oxidative stability and behavior of the natural antioxidants of cold-pressed pumpkin (PSO) and butternut squash (BSO) seed oils. Experiments included quantification of carotenoid, tocopherol and phenolic content by HPLC analyses and determination of the effect of packaging light transmission properties and storage temperatures on the oxidative stability of the oils and the retention of their major antioxidant compounds.

The predominant carotenoid in BSO was β-carotene (21.8 mg/kg) while lutein+zeaxanthin (16.76 mg/kg) was the most abundant in PSO. α-carotene and β-cryptoxanthin were detected at lower concentrations but the latter was not detected in BSO. The total carotenoid content of PSO (32.4 mg/kg) was slightly lower than that of BSO (27.1 mg/kg). Significant concentrations of tocopherols (α, γ, and δ) were found with γ-tocopherol showing preponderance (>90%) in both oils. The total tocopherol

content of BSO (563 mg/kg) was not significantly different compared to that of PSO (553 mg/kg). Two isomers (γ and δ) of tocotrienol were also detected. The individual phenolics were tyrosol, luteolin, vanillin, vanillic acid, and trans-cinnamic acid. The oils were stable for at least 20 weeks when stored in amber bottles at temperature below 30^0C and we estimated a shelf-life for both oils of over 15 months based on accelerated storage test. Oxidation was most pronounced in clear bottles while green bottles provided only partial protection when exposed to 15W fluorescent light at 20^0C. The antioxidant compounds were better retained in amber bottles while significant reductions ($p<0.05$) were observed in clear and green bottles. The relative stability of the compounds was: γ-tocopherol > α-tocopherol; lutein > β-carotene. Phenolics exhibited only minor losses suggesting excellent stability of these compounds.

Because of their high content of natural antioxidants and good storage stability, cold-pressed butternut squash and pumpkin seed oils demonstrate potential for value-added uses such as production of specialty oils as natural sources of dietary antioxidants and in pharmaceutical and cosmetic applications.

BIOGRAPHICAL SKETCH

Restituto Tocmo was born on December 9, 1985, in Koronadal City, Philippines. He is the third among the six children of Mr. and Mrs. Hilario Tocmo. He finished his bachelor's degree in Food Technology in 2007 from the University of the Philippines Mindanao. In 2008, he joined the Department of Food Science and Chemistry of the same university as an Instructor. After two years of teaching, he realized the need to go to graduate school and started looking for graduate scholarships. In 2010, he obtained a scholarship from Ford Foundation International Fellowships Program and was accepted at Cornell University for a master's degree in food science. He was, at the same time, granted a graduate research assistantship thru Dr. Olga Padilla-Zakour, his academic adviser. His long-term goal is to become a professor and a researcher. Meanwhile, he wants to obtain a PhD. In March 2012, he was selected as a scholar to the Singapore International Graduate Award given by the government of Singapore. He will start his PhD in Food Science at the National University of Singapore in August 2012.

To my beloved family and friends.

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CHAPTER 1

INTRODUCTION

Agricultural Production and Utilization of Pumpkins and Butternut Squash

Pumpkin (*Cucurbita sp.*), a crop native to America, has offered a profitable opportunity in the recent years because of increasing demand due to enhanced popularity in Europe and the US of the halloween festivities and the more widespread use of pumpkin as food (Schäfer and Blanke 2012). Some cultivars of this crop commonly known as butternut squash (*Cucurbita moschata*) and 'pumpkin' (*Cucurbita pepo*) exhibit versatility as they are used for various agricultural purposes and food applications. In 2009, worldwide production of pumpkin amounted to 21.2 million tons per year (FAO, 2010). In Germany alone, pumpkin consumption increased by 60% (2003-2010) and its acreage by 33% (2005-2010) (FAO, 2010). In the US, production of pumpkin has expanded greatly particularly in Illinois, Pennsylvania, New Jersey, Michigan and New York with production volume reportedly reaching 1.06 billion pounds as of 2010 (USDA Economic Research Service). In New York State specifically, 71.8 million pounds of pumpkins was produced in 2011 with a farm value of $23.6 million, making pumpkins among the most valuable vegetable crops (USDA Economic Research Service). Moreover, Martin Farms in upstate New York has been producing locally grown butternut squash and processing them into packaged fresh cuts for retail distribution (www.martinfarms.com).

Pumpkins and butternut quash are sold mainly for food processing and for ornamental purposes, especially during the Halloween season. Some farms have developed value-added activities to boost sales. Because of its potential for making profits, pumpkin acreage has expanded greatly in recent years and competition in the pumpkin market has been increasing. With the appreciable volume of pumpkin production intended mainly for utilization of the flesh for food processing and for ornamental purposes, it is also expected that a substantial amount of seeds is being generated as part of the processing waste.

Pumpkin and Butternut Squash Seed Oils Production and Value

The seeds of pumpkins have been reported to contain a significant amount (20-40wt%) of highly unsaturated oil (Salgin and Korkmaz 2011; Ardabili and others 2011; Stevenson and others 2007). Therefore, utilization of pumpkin seeds for oil production may add value to this crop and could help reduce generation of food-processing wastes. Due to their high oil content, pumpkin seeds have become a well-appreciated source of oil in Congo (Nakavoua and others 2011), and are also popular as a source of salad oil in some southeastern European countries such as Slovenia, Croatia, Austria, and Hungary (Vujasinovic and others 2010). In the US, cold-pressed pumpkin and butternut squash seed oils are newly available in supermarkets especially in the Northeast. A company housed at the technology farm in Geneva, New York has been producing butternut squash (BSO) and pumpkin seed oils (PSO) for sale at a retail price ranging from $11.95-$13.95 per 187 mL of bottled oil (www.wholeheartedfoods.com). This price range commands a premium over other

oils suggesting good profitability of butternut squash and pumpkin seed oil production.

Pumpkin and Butternut Squash Seed Oils Composition

Several studies have shown cold-pressed pumpkin seed oil as a healthy natural product. In contrast to the traditional solvent extraction, cold pressing produces oils that are free from organic solvents. Although oil extraction with the use of a screw press yields relatively low percentage of oil compared to the traditional solvent extraction (Temilli 2009) or the more recent supercritical fluid extraction technology (Salgin and Kormaz 2011), cold-pressed oils retain their natural antioxidant composition and flavor compounds better compared to the other methods (Crews and others 2006). For instance, Parry and others (2006) reported that cold-pressed roasted pumpkin seed oil contains several carotenoid compounds including lutein (0.3 mg/kg), β-carotene (5.9 mg/kg) zeaxanthin (28.5 mg/kg) and cryptoxanthin (4.9 mg/kg). In another study, lutein and β-carotene were identified as the major carotenoids in pumpkin seed oil (Murkovic and Pfannhauser 2000). Carotenoids are known for their radical scavenging capacity and provitamin A activity, as well as for their important role in eye health (Rodriguez-Amaya and Kimura 2004; Kachik and others 1997). Significant amounts of tocopherols and tocotrienols (α, γ, δ) were also detected with γ-tocopherol comprising more than 78% of the total tocopherol content (Stevenson and others 2007; Parry and others 2006; Fruwirth and others 2003; Murkovic and Pfannhauser 2000). α-tocopherol is known as the most active in vivo antioxidant and may act as electron donor by giving up its hydrogen atom to lipid free radicals and as

scavenger of singlet oxygen (Krichene and others 2010, Kamal-Eldin and Appelqvist 1996). Some studies however, suggested that γ-tocopherol has greater antioxidant activity than the α-isomer (Wagner and others 2004; Jiang and others 2001). The higher γ-tocopherol (2 to 8 times higher than other isomers) content of pumpkin seed oil makes the oil more attractive from a nutritional perspective (Stevenson and others 2007). A few published articles reported at least five phenolic compounds including luteolin, vanillic acid, vanillin, and trans-cinamic acid, and tyrosol are present in cold-pressed pumpkin seed oils (Andjelkovic and others 2010). Siger and others (2008) and Tuberoso and others (2008) were able to detect p-coumaric acid, ferulic acid, and protocatechuic acid in cold-pressed oils from the seeds of various pumpkin varieties. Phenolic compounds are well known as powerful antioxidants, and act on mechanisms based on radical scavenging, hydrogen atom transfer and metal chelating attributes (Huang and others 2005). The presence of these healthful components makes the oil even more attractive from a nutritional and therapeutic point of view. Recent studies reported antihypertensive, cardioprotective and chemoprotective effects of pumpkin seed oil (El-Mosallammy and others 2012; Gossell-Williams and others 2006; Dvorkin and others 2002).

The unique sensory attributes of pumpkin seed oils and the beneficial components they contain have drawn the attention of researchers recently. A few studies on pumpkin seed oils have been conducted in different parts of the world however, published information is still scant and most researches have been conducted in locations other than the US. It is well accepted that growing conditions such as soil and temperature, genotype, post-harvest handling, and the variations in processing

conditions may significantly alter the chemical composition of food materials (Parry and others 2006). Therefore, it is important to study the phytochemical composition of locally sourced (US) and processed cold-pressed pumpkin seed oils to establish a benchmark for further investigations. In addition, there are no reports available on the natural antioxidant composition of cold-pressed oil from the seeds of butternut squash (*Cucurbita moschata*) grown in the US. Finally, there is dearth of information on the oxidative stability of cold-pressed pumpkin and butternut squash seed oils and the retention of their natural antioxidants during the expected shelf-life. Hence, the first part of this study was conducted to characterize locally produced cold-pressed pumpkin and butternut squash seed oils in terms of their tocopherol/tocotrienol, carotenoid, phenolic content, antioxidant capacity and certain quality attributes to obtain a holistic profile of the natural antioxidant composition and properties of these oils.

Oxidative Stability of Oils and Methods of Analysis

Oxidation of oils can lead to the production of antinutritional factors and undesirable flavor and aroma compounds (Lutterodt and others 2011). Therefore, knowledge of lipid oxidation in cold-pressed pumpkin and butternut squash seed oils and the changes that their natural antioxidants undergo at different storage conditions are fundamental to the preservation of nutritional and sensory qualities.

Numerous analytical methods have been routinely used to measure lipid oxidation in foods. These include a number of physical and chemical tests and instrumental analyses for measurement of various lipid oxidation parameters. The

Schaal Oven test has been widely used as a cost-effective and convenient way of conducting accelerated storage to determine oxidative stability of the oils. It has been established that 1 day of storage under this condition is equivalent to 1 month of storage at room temperature (Abou-Gharbia and others 1996). Conventionally, iodometric titration has been the preferred method to measure formation hydroperoxides reported as peroxide value (PV) (Nakavoua and others 2011). The thiobarbituric reactive species (TBARS) assay has also been commonly used to measure secondary lipid oxidation products (AOCS 1998). In the present study, we carried out Schaal oven test to determine the stability of pumpkin and butternut squash seed oil and estimate their shelf life.

With their significant levels of mono- and polyunsaturated fatty acids and antioxidant compounds, cold-pressed oil from pumpkins can be a good model to investigate the behavior of natural antioxidants in relation to their contribution to delaying oxidative changes. The second part of the present study was aimed to understand the behavior of the natural antioxidant composition of pumpkin and butternut squash seed oils and to establish a relationship between these compounds and the oils' oxidative stability. Specifically, we monitored changes undergone by carotenoids, tocopherols, phenolics and the oils' antioxidant capacity during storage employing different temperatures (20^0C and 30^0C) and packaging materials with different light transmission properties (clear, green and brown amber glass bottles) using cold-pressed oils from the seeds of two cultivars of pumpkin.

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CHAPTER 2

PHYTOCHEMICAL COMPOSITION AND OXIDATIVE STABILITY OF COLD-PRESSED BUTTERNUT SQUASH (*CUCURBITA MOSCHATA*) AND PUMPKIN (*CURCURBITA PEPO* L.) SEED OILS UNDER ACCELERATED STORAGE CONDITIONS

ABSTRACT

Cold-pressed butternut squash (*Cucurbita moschata,* BSO) and 'pumpkin' (*Cucurbita pepo,* PSO) seed oils were studied for their antioxidant content, oxidative stability and retention of natural antioxidants. An accelerated storage test (Schaal oven test) was conducted to monitor lipid oxidation and antioxidant compound retention over 15 days at 60^0C. Predominant carotenoids (mg/kg oil) were lutein+zeaxanthin (1.7-16.7) and β-carotene (11.1-21.8). Significant levels of tocopherols (α, γ, and δ) were detected with γ-tocopherol (>90% rel abundance) showing preponderance in both oils. Two isomers (γ and δ) of tocotrienol were also detected in both oils. The individual phenolics were tyrosol, luteolin, vanillin, vanillic acid, and trans-cinnamic acid with concentrations ranging from 0.07 to 2.25 mg/kg. The total phenolic content of BSO and PSO determined via Folin-Ciocalteu method were (1032 and 967 mg Gallic acid equivalents (GAE)/kg oil), respectively. The hydrophilic-ORAC values (μmol TE/g) of PSO (1.58) and BSO (1.37) were not significantly different ($p<0.05$). The lipophilic-ORAC values (μmol TE/g) were also essentially the same between PSO (0.48) and BSO (0.51). (Peroxide value (PV) and thiobarbituric acid reactive

species (TBARS) value of both oils were initially at acceptable levels and the rate of increase during the accelerated storage was low, indicating stability of the oils. Significant reduction ($p<0.05$) in α-tocopherol, γ-tocopherol, lutein and β-carotene ranging from 22 to 100% were observed toward the end of accelerated storage. Because of their high content of tocopherol and carotenoids and good storage stability, cold-pressed BSO and PSO demonstrate a potential for value-added uses such as natural sources of dietary antioxidants and for pharmaceutical and cosmetic applications.

Keywords: butternut squash, pumpkin, cold-pressed oil, carotenoids, tocopherols

Practical Application: Cold-pressed pumpkin seed oils are gaining popularity because of their nutritional benefits and unique sensory properties. Natural antioxidants derived from edible materials are in high demand amidst concerns about the safety of consumption of products with added synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydrosyanisole (BHA). Our results indicate that cold-pressed butternut squash and pumpkin seed oils are rich sources of natural antioxidants, which may protect the oils from lipid oxidation and make the oil highly viable from a nutritional perspective. As part of ongoing efforts to develop value-added utilization of fruit seeds, we were able to show the potential commercial value of pumpkin and butternut squash seed oils for utilization as functional foods and in cosmetic and pharmaceutical applications.

INTRODUCTION

Pumpkin (*Cucurbita sp.*), a crop native to America, represents a profitable opportunity in the Northeast. Some cultivars of this crop commonly known as butternut squash (*Cucurbita moschata*) and 'pumpkin' (*Cucurbita pepo*) are used for various agricultural purposes as well as food applications. In the US, pumpkin production has expanded greatly particularly in Pennsylvania, New Jersey and New York, with production volume reportedly reaching 1.06 billion pounds as of 2010 (USDA Economic Research Service). In New York State specifically, 71.8 M pounds of pumpkins was produced as of 2011 with a farm value of $23.6 million making pumpkins among the most valuable vegetable crops (USDA Economic Research Service).

With the appreciable volume of production intended mainly for utilization of the flesh for food processing, it is also expected that a substantial amount of seeds is being generated as part of the processing waste. Pumpkin seeds have been reported to contain a significant amount (20-40%wt) of highly unsaturated oil (Salgin and Korkmaz 2011; Ardabili and others 2011; Stevenson and others 2007). Therefore, utilization of pumpkin seeds for oil production may add value to this crop and could help reduce food-processing waste. Due to their high oil content, pumpkin seeds have become a well-appreciated source of oil in Congo and are also popular as a source of salad oil in some southeastern European countries such as Slovenia, Croatia, Austria, and Hungary (Nakavoua and others 2011; Vujasinovic and others 2010). In the US, cold-pressed pumpkin seed oils are slowly gaining visibility in supermarkets and specialty oil stores especially in the Northeast.

A few studies have shown cold-pressed pumpkin seed oil to be a healthy natural product. In contrast to the traditional solvent extraction, cold pressing produces oils that are free from organic solvent. Although oil extraction with the use of screw press yields relatively low percentage of oil compared to the traditional solvent or the more recent extraction technology using supercritical fluid, the cold-pressed oil produced is well regarded for its excellent retention of antioxidant compounds and flavor (Salgin and Kormaz 2011; Temilli 2009; Crews and others 2006). For instance, Parry and others (2006) reported that cold-pressed roasted pumpkin seed oil contains several carotenoid compounds including lutein (0.3 mg/kg), β-carotene (5.9 mg/kg) zeaxanthin (28.5 mg/kg) and cryptoxanthin (4.9 mg/kg). In another study, lutein and β-carotene were identified as the major carotenoids in pumpkin seed oil (Murkovic and Pfannhauser 2000). Significant amounts of tocopherols and tocotrienols (α, γ, δ) were also detected with γ-tocopherol being the most abundant (more than 78% of the total tocopherol content (Stevenson and others 2007; Parry and others 2006; Fruwirth and others 2003; Murkovic and Pfannhauser 2000). A few published articles reported at least five phenolic compounds identified including luteolin, vanillic acid, vanillin, trans-cinamic acid, and tyrosol (Andjelkovic and others 2010). Siger and others (2008) and Tuberoso and others (2007) were able to detect p-coumaric acid, ferulic acid, and protocathechuic acid in cold-pressed oils from the seeds of various pumpkin varieties. The presence of these healthful components makes the oil even more attractive from a nutritional and therapeutic point of view. Recent studies reported antihypertensive, cardioprotective and chemoprotective effects of pumpkin seed oil (El-Mosallammy and others 2012; Gossell-Williams and others 2006; Dvorkin and others 2002).

The unique sensory attributes of pumpkin seed oils and the healthful components they contain have recently drawn the attention of researchers. A few studies on pumpkin seed oils have been conducted in different parts of the world however, published information is still scant and most is from studies conducted in locations other than the US. It is well accepted that growing conditions such as soil and temperature, genotype, post-harvest handling, and the variations in processing conditions may significantly alter the chemical composition of food materials (Parry and others 2006). Therefore, it is important to study the phytochemical composition of locally sourced (US) and processed cold-pressed pumpkin and butternut squash seed oils to establish a benchmark for further research. In addition, no report on natural antioxidant content is available for cold-pressed oil from the seeds of butternut squash (*Cucurbita moschata*) grown in the US. Finally, there is limited information on the oxidative stability of cold-pressed pumpkin and butternut squash seed oils and the retention of their natural antioxidants during storage. Hence, this study was conducted to characterize locally produced cold-pressed pumpkin and butternut squash seed oils in terms of their tocopherol/tocotrienol, carotenoid, phenolic content, antioxidant capacity and some quality attributes to obtain a holistic profile of the natural antioxidant composition and properties of these oils. The oxidative stability and the changes in the antioxidant content of the oils were also studied.

MATERIALS AND METHODS

Reagents and standards

Folin-Ciocalteu reagent, Potassium iodide (KI), (+)-6-hydroxy-2,5,7,8-tetramethylcromano-2-carboxylic acid (Trolox), sodium fluorescein, 2,2'- azobis(2-aminopropane) dihydrochloride (AAPH), randomyly-methylated β-cyclodextrin (RMCD), β- and α-carotene, lutein, β-cryptoxanthin, zeaxanthin, vanillin, vanillic acid, tyrosol, luteolin, trans-cinnamic acid, α-, γ-, δ- tocopherols were obtained from Sigma-Aldrich (Allentown, PA). Standards for tocotrienols were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All standards were ≥ 96% purity. Hexane, chloroform, 1-butanol, acetone, acetic acid, methanol, ethyl ether, 2-propanol, sodium chloride, 2-thiobarbituric acid (2-TBA) were analytical grade chemicals from Fisher Scientific Company Ltd (Suwanee, GA).

Extraction of cold-pressed oils

The oils used in this study were from the seeds of butternut squash (*Cucurbita moschata*) and pumpkin (*Cucurbita pepo* L.). Cold-pressed oils were obtained using a KOMET oil press (IBG Monforts Oekotec GmbH and Co, Napa, CA). Oils that come out of the press were at temperature below 50^0C to assure compliance with the regulation for cold-pressed oils (Joint FAO/WHO Food Standards Programme). The oils were allowed to settle for at least 24 h followed by coarse filtration. Samples were transferred into amber bottles and immediately stored at -20^0C. Experiments and subsequent analyses were immediately carried out after 2-3 days.

Accelerated storage test

An accelerated storage test (Schaal oven) (AOCS 1998) was carried out to evaluate the storage stability of the oil and the changes in their major antioxidant

compounds. Equal amounts of each oil were loaded into 60 mL loosely capped amber bottles and subjected to 15 days storage in an oven maintained at 60 $\pm$ 1^0C (Lab-line Instruments, Inc., Melrose, IL). These oils were subjected to monitoring of the evolution of peroxide values (PV) and thiobarbituric acid reactive species (TBARS) values as well as changes in tocopherol, carotenoid, total phenolic composition. A series of two bottles for each oil type was prepared and duplicate or triplicate samples were obtained from each bottle at scheduled intervals for analyses.

Determination of peroxide (PV) and thiobarbituric acid reactive species (TBARS) values

Iodometric method for the determination of peroxide value (PV) was performed according to AOCS Method Cd 8-53 (AOCS 1998). Oil samples (5mL) were dissolved in 30 mL solution of (2:3 v/v) Acetic acid-Chloroform and 0.5 mL of saturated KI solution was added. After thoroughly mixing, 30 mL of distilled H_2O was added and the mixture was titrated manually using a standardized sodium thiosulfate solution (0.01 mol/L). Results were expressed as milliequivalents of active oxygen (meq O_2)/kg of oil.

Thiobarbituric acid reactive species (TBARS) was determined according to AOCS Method Cd 19-90 (AOCS 1998). Oil samples (0.5g) were dissolved in 10 mL 1-butanol. Aliquot (5 mL) of this solution was transferred into a screw cap tube to which 5mL of freshly prepared 2-TBA solution was added. The solution was heated at 95^0C in a thermostated water bath. After 2 hr of heating and subsequent 15-min of cooling, absorbance was read at 532 nm using a Genesys 10S UV-Vis

spectophotometer (Thermo Fisher Scientific, Madison, WI). A standard curve was prepared using 1,1,3,3-tetramethoxypropane as the malondialdehyde (MDA) precursor. Results were expressed as mg MDA equivalents per kg oil.

Determination of total phenolics (TP)

Determination of TP was carried out using the Folin-Ciocalteu method (Singleton and Rossi 1965). Briefly, 2 g of oil was dissolved in 5 mL (100%) methanol and separation of the hydrophilic phase was done by centrifugation. This procedure was repeated two more times. An aliquot (200 μL) of the appropriately diluted methanolic extract was mixed with 250 μL of Folin-Ciocalteu reagent (diluted 10x from the original solution) and 3 mL of deionized distilled water was added. After 6 min, 750 μL of 20% sodium carbonate (Na_2CO_3) was added. After 2 h, absorbance was read at 750 nm using a Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, Madison, WI). Quantification was carried out using a standard curve of Gallic acid: $y= 124.2x + 0.006$; $R^2 = 0.999$. Experiments were carried out six times and results were expressed as mg Gallic acid equivalents (GAE) per kg oil.

Determination of hydrophilic- and lipophilic-ORAC values

The same methanolic extract from the TP determination was used for the determination of hydrophilic-ORAC (H-$ORAC_{FL}$) value. The microplate assay described by Prior and others (2003) was followed with slight modifications. The assay was carried out on a fluorescence microplate reader (Biotek Instruments, Inc., Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm set at a temperature of 37^0C. AAPH and fluorescein were used as the

radical generator and fluorescent probe, respectively. Each 96-well microplate contained 150 μL Fluorescein solution (0.004 μM), 25 μL AAPH solution (0.15 M), and 25 μL of appropriately diluted methanolic extract. A standard curve using Trolox (6.25 to 100 μM) was generated and results were expressed as μmol Trolox equivalents/mL (μmol TE/mL).

Extraction for the lipophilic-ORAC (L-ORAC$_{FL}$) value determination was based on the method of Prior and others (2003) with some modifications. Briefly, oil samples (2 g) were extracted in a 15-mL screw-cap tube with 2 x 10 mL of hexane. The hexane fractions were combined and dried using a rotavap. For the L-ORAC assay, the dried hexane extracts were diluted with 750 μL of a 7% RMCD solution (50% acetone/50% water, v/v). Any further dilution was with the 7% RMCD solution. The 7% RMCD solution was also used as a blank and to dissolve the Trolox standards. The same microplate assay as described in H-ORAC determination was carried out. For each type of oil, determination was carried out six times.

Analysis of tocopherol contents

Tocopherols and tocotrienols were analyzed by HPLC based on the method of Gliszczynska-Swiglo and Sikorska (2004) with slight modification. Oil samples (0.25-0.5 g) were dissolved in 15 mL of 2-propanol, filtered through a 0.20 μm PTFE syringe filter and analyzed in reversed phase HPLC system (Hewlett-Packard model 1100; Palo Alto, CA) equipped with a fluorescence detector. An Eclipse C18 (4.6 x 150 mm, 5 μm) column was used with methanol:acetonitrile:water (50:44:6, v/v/v) (Yang and others 2011) as the mobile phase. The system was operated isocratically

with an injection volume of 20 μL and a flow rate of 1.5 ml/min. Separations were carried out at 25^0C with the fluorescence detector excitation and emission wavelengths were set at 290 and 330 nm, respectively. A 5 min equilibration period was used between samples, requiring about 35 min/sample. Identification of compounds was based on retention times and UV absorption spectra while an external standard method was used for quantitation. The calibration curves ranged from (0.0025, 0.005, 0.01 0.02, 0.03, and 0.05 mg/mL, R^2= 0.9997) and prepared using 2-propanol.

Analysis of carotenoid composition

Carotenoids extraction was carried out following the procedure proposed by Ceron and others (2008) with slight modification. Samples (5 g of oil) were dissolved in 25 mL acetone and evaporated using a rotavap. The extract was redissolved in 25 mL pure ethyl ether and an equal volume of a solution of KOH in MeOH (4% w/v) was added for saponification. Saponification was performed to remove chlorophylls and some lipids and to help release carotenes from bound form (Rodriguez-Amaya and Kimura 2004). To stop the reaction and remove excess alkali, 2 mL of 10% (w/v) NaCl solution was added into the mixture. After evaporation of the ether phase, the remaining pigment was redissolved in 95% ethanol (stabilized with 30 ppm BHT), transferred to an amber vial, topped with N_2 gas and kept at -20^0C until further analyses.

The chromatographic analysis was based on the procedure described by Minguez-Mosquera and others (1992), as modified by Ceron and others (2007). The carotenoid extracts were filtered through a 0.45 μm PTFE syringe filter and injected

into the HPLC system (Hewlett-Packard model 1100; Palo Alto, CA) equipped with a photodiode array detector. The carotenoids were separated on an Eclipse C18 (4.6 x 150 mm, 5 μm) column. The solvent system composed of (A) water-methanol (2:8, v/v) and (B) acetone-methanol (1:1, v/v) and pigments were eluted at a rate of 1 mL/min at 25^0C according the following solvent gradient: 75% A/25% B at 0 min, 25% A/75% B at 8 min and remained isocratic until 10 min, 10% A/90% B at 18 min, 0% A/100% B at 23 min, and 75% A/25% B at 35 min. Chromatograms were acquired at 450 nm. Identification of peaks was based on retention times and respective UV spectra as compared to authentic carotenoid standards of lutein, α-carotene, β-carotene, zeaxanthin and β-cryptoxanthin supplied by Sigma-Aldrich (Milwaukee, WI). The analysis was carried out in triplicate and results were expressed in milligrams of compound per kilogram of oil (mg/kg).

Analysis of phenolic profile

Individual phenolic quantification was carried out by HPLC analyses based on the method described by Kim and Padilla-Zakour (2004) with some modifications. The same methanolic extract used in TP determination was appropriately diluted with methanol and filtered through a 0.45 μm PTFE filter. Separations were conducted on a reversed phase HPLC system (Hewlett-Packard model 1100; Palo Alto, CA) equipped with a photodiode array detector and Chemstation 7 for data collection. A 20 μL of sample was injected using a C18 reversed-phase Symmetry Analytical column (5 μm x 250 mm x 4.6 mm; Water Corp. Milford, MA) and detection was carried out at 280 nm. Mobile phases consisted of phase A (0.1% phosphoric acid in HPLC grade water)

and phase B (0.1% phosphoric acid in HPLC grade acetonitrile). The compounds were eluted at a flow rate of 1 mL/min at 25^0C according the following solvent gradient: 92% A/ 8% B at 0 min, 89% A/ 11% B at 4 min, 65% A/ 35% B at 25 min, 40% A/ 60% B at 30 min, 40% A/ 60% B at 40 min, 65% A/ 35% B at 45 min, 89% A/ 11% B at 50 min, 92% A/ 8% B at 55 min. Phenolics were identified and quantified based on their spectral characteristics and retention times as compared to external standards of tyrosol, luteolin, vanillic acid, vanillin, and trans-cinnamic acid.

Statistical analysis

Experiments were carried out in duplicate and triplicates and during analyses each measurement was repeated three to six times. Results were averaged and analyzed by ANOVA ($p<0.05$) and T-test using JMP® 9 (SAS Institute, Carey, NC). Differences among treatment means were detected by post-hoc comparison according to Tukey's HSD test ($p<0.05$).

RESULTS AND DISCUSSION

Tocopherol and tocotrienol content

Tocopherol and tocotrienols have four forms (α, β, γ, δ), which all together are designated as Vitamin E. They function as lipid-soluble antioxidants by trapping the hydroperoxide intermediate and stopping the autoxidation reaction, and are known to protect cell membranes from oxidation (Pinheiro-Sant'Ana and others 2011; Tuberoso and others 2007). Moreover, tocopherols have been recognized for their antiaging, anticancer, and antiatherosclerosis effects (Elfalleh and others 2011).

Figures 1a and 1b present the typical chromatograms of BSO and PSO, respectively. The order of elution for the specific mobile phase used was as follows: δ-tocopherol (17.4 min), γ- tocopherol (22.4 min), and α-tocopherol (28.6 min). According to literature plant oils do not contain β-tocopherol or contain it in relatively low amounts as compared to γ- tocopherol (Gregory 1996; Crawley 1993). Moreover, several studies were unable to detect β-tocopherol in pumpkin seed oils; hence, we did not include this compound in the determination (Nyam and others 2009; Stevenson and others 2007; Fruwirth and others 2003). Two isomers of tocotrienols were identified (chromatogram not shown) with elution order as follows: δ-tocotrienols (6.8 min), γ-tocotrienols (8.4 min).

The tocopherol and tocotrienol content of PSO and BSO and their relative abundance are shown in Table 1. Significant amounts of tocopherol were observed in both oils with BSO showing slightly higher value (563 mg/kg) compared to PSO (535 mg/kg). Only δ- and γ- tocotrienols were detected in both oils with values ranging from 0.8 to 2.8 mg/kg and 4.5 to 5.8 mg/kg, respectively. In general, the quantities of tocotrienols in both oil types were significantly lower relative to the levels of tocopherol. Both oils showed preponderance of γ-tocopherol (more than 90% of total tocopherol), followed by α-tocopherol (29.0-31.2 mg/kg), and δ-tocopherol (12.3-15.4 mg/kg). Similarly, γ-tocotrienol dominates in both oils comprising 67% to 84% of the total tocotrienol.

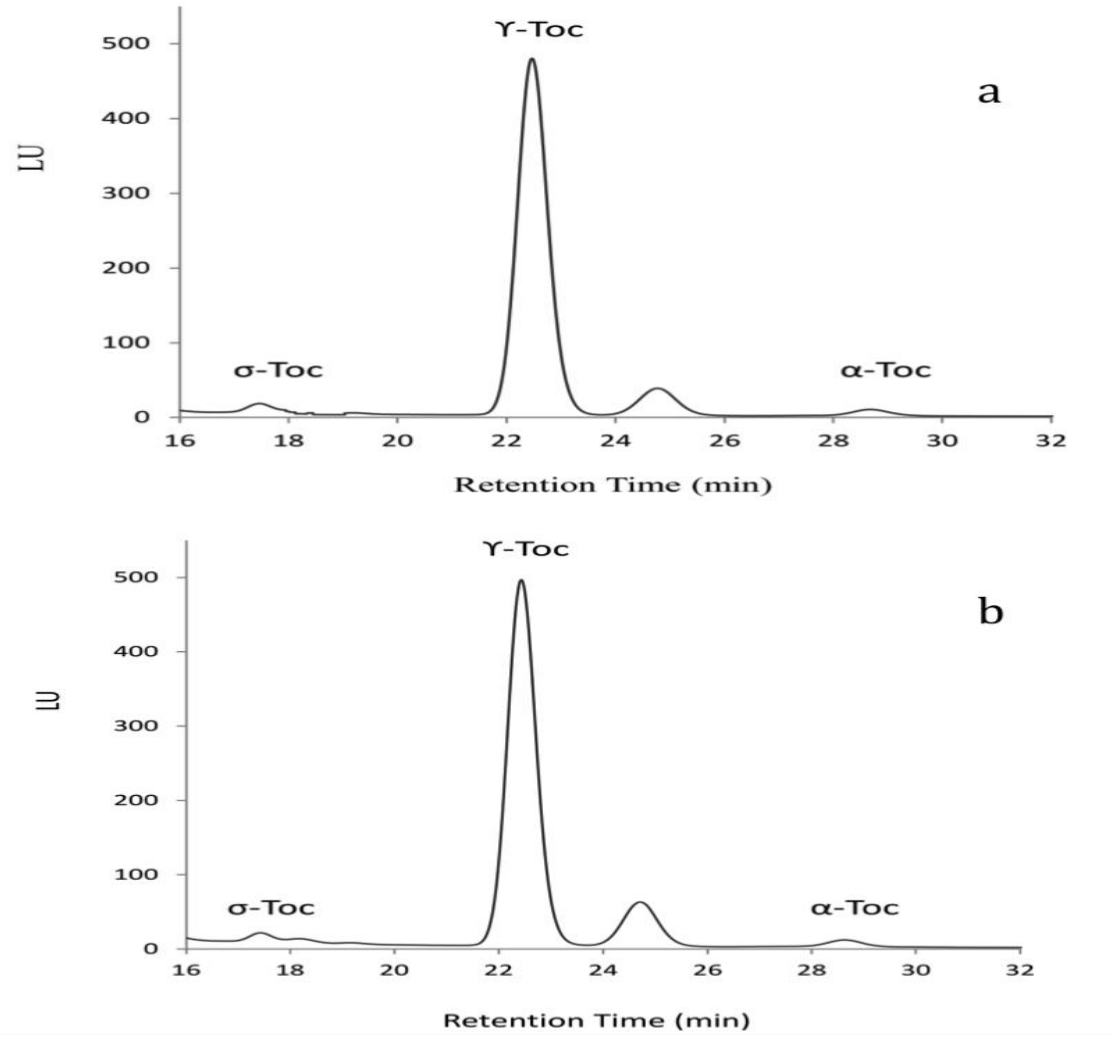

Figure 1. Typical chromatograms of tocopherols from (a) pumpkin seed oil (PSO) and (b) butternut squash seed oil (BSO)

The percent relative abundance of tocopherols and tocotrienols showed a similar trend in both oils as follows: γ-tocopherol > α-tocopherol > δ-tocopherol and γ-tocotrienols > δ-tocotrienols, respectively. Our results were in agreement with those reported in the literature (Tuberoso and others 2007; Stevenson and others 2007), although variability within each seed oil variety is possible and can be explained by factors including genotype, growing conditions, and others (Parry and others 2006).

Table 1. Peak assignments, retention times, concentrations and relative abundance of tocopherols and tocotrienols in cold-pressed pumpkin seed (PSO) and butternut squash (BSO) seed oils[a]

Assignment	Retention time (min)	BSO (mg/kg)	Relative abundance (%)	PSO (mg/kg)	Relative abundance (%)
Tocopherol					
δ-tocopherol	17.4	12.2 ± 1.0^{A}	2.2	15.4 ± 0.4^{A}	2.9
γ-tocopherol	22.4	519.0 ± 3.0^{A}	92.3	490.0 ± 18^{A}	91.7
α-tocopherol	28.6	31.2 ± 2.4^{A}	5.5	29.0 ± 3.7^{A}	5.4
Total		563 ± 3.0^{A}		535 ± 18^{A}	
Tocotrienol					
δ-tocotrienol	8.4	2.8 ± 0.4^{A}	32.4	0.80 ± 0.02^{B}	15.4
γ-tocotrienol	6.8	5.8 ± 1.6^{A}	67.6	4.5 ± 0.3^{A}	84.6
α-tocotrienol	9.8	n.d	-	n.d	-
Total		8.6 ± 1.6^{A}		5.3 ± 0.3^{B}	

[a]All results are reported as means of three replicates ± standard deviation. Different letters between columns PSO and BSO represent significant differences ($p<0.05$).
n.d: not detected

These results provide useful information for industrial application of BSO and PSO as good sources of Vitamin E. High levels of tocopherols and tocotrienols may also contribute to better stability of the oils against oxidation.

Carotenoid Content

The carotenoid content of PSO and BSO are of interest because, like tocopherols, they are lipid-soluble compounds known as potent antioxidants. Figures 2a and 2b show the typical chromatograms for the carotenoid content of PSO and BSO, respectively. Detection of individual carotenoids was made at 450 nm and identification was achieved by comparing retention times and absorption spectra with those of authentic standards. Five carotenoids were identified, however, the HPLC method used was not able to separate lutein and zeaxanthin, giving exactly the same elution time. Lutein is isomeric to zeaxanthin, having an identical chemical formula, differing only in the placement of double bonds (Britton and others 1998). The commonly used C18 reversed-phase HPLC columns are well suited for separation of carotenoids, however, their selectivity is inadequate for the separation of the structural isomers of carotenoids such as lutein and zeaxanthin (Gilmore and others 1991). Therefore, it is reasonable to report this peak as lutein+zeaxanthin. However, we based the quantitation on the standard curve constructed for lutein, as this is one of the major carotenoids in pumpkins and butternut squash, as previously reported (Kurz and others 2008; Hidaka and others 1987). In order of elution, these were lutein+zeaxanthin (9.6 min), β-cryptoxanthin (17.3 min), α-carotene (26.3 min) and β-carotene (26.7 min).

Table 2 presents the concentration and percent relative abundance of the individual carotenoids in BSO and PSO. The major carotenoid compounds in PSO were

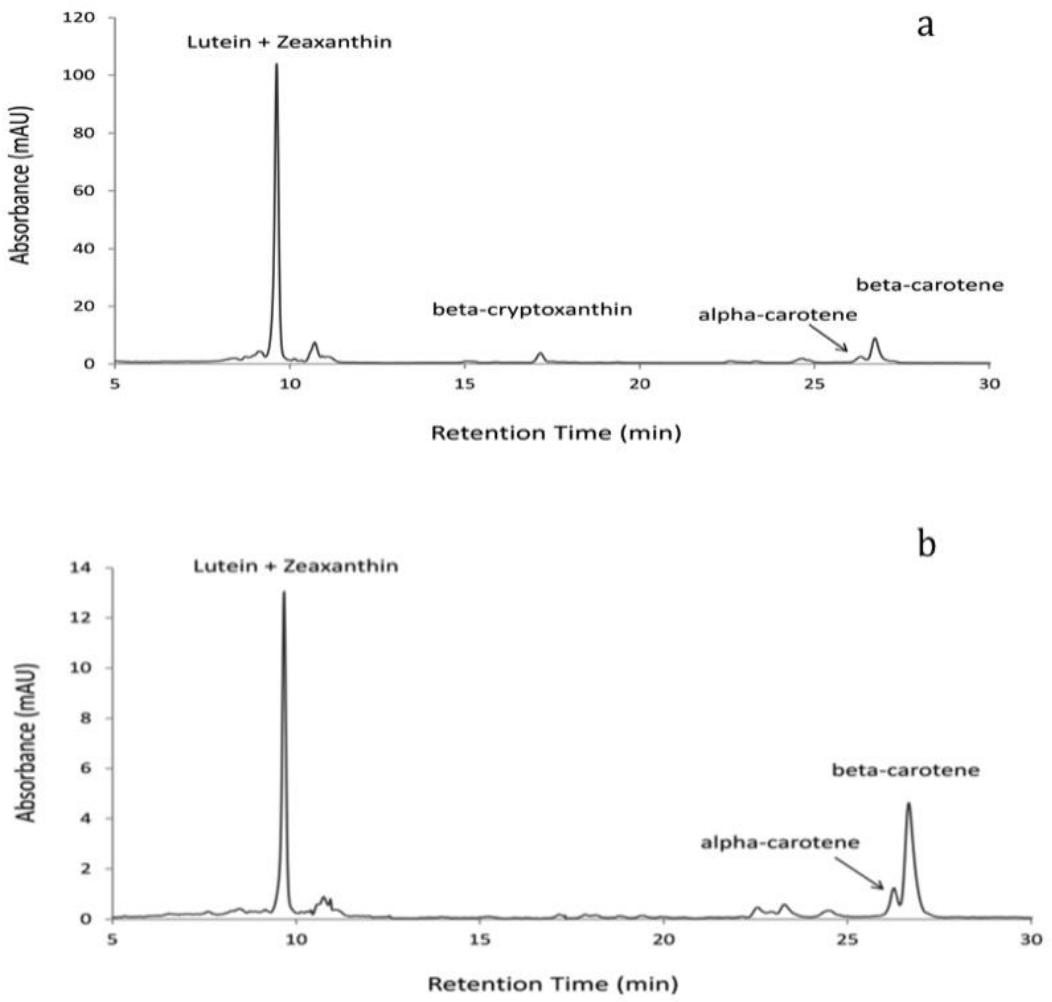

Figure 2. Typical chromatograms of individual carotenoids from (a) pumpkin seed oil (PSO) and (b) butternut squash seed oil (BSO)

lutein+zeaxanthin (16.8 mg/kg) comprising >50% of the total carotenoid content followed by β-carotene (11.1 mg/kg). Relatively lower concentrations of β-cryptoxanthin (0.7 mg/kg) and α-carotene (3.8 mg/kg) were also detected. The major carotenoid in BSO was β-carotene (21.8 mg/kg) comprising 80% of the total carotenoid content while lutein+zeaxanthin (1.7 mg/kg) comprised only 6.2% of the total carotenoid content. The α-carotene (3.59 mg/kg) in BSO was comparable to that of PSO. β-cryptoxanthin however, was not detected in BSO. Our

Table 2. Peak assignments, retention times, concentration, and relative abundance of carotenoids in cold-pressed pumpkin (PSO) and butternut squash (BSO) seed oils

Assignment	Retention time (min)	BSO (mg/kg)	Relative abundance (%)	PSO (mg/kg)	Relative abundance (%)
Lutein+zeaxanthin	9.6	1.7 ± 0.2^{A}	6.2	16.8 ± 0.6^{B}	51.7
β-cryptoxanthin	17.3	n.d	-	0.70 ± 0.03	2.1
α-carotene	26.3	3.6 ± 0.2^{A}	13.3	3.80 ± 0.04^{A}	11.8
β-carotene	26.7	21.8 ± 1.1^{A}	80.5	11.1 ± 1.0^{B}	34.4
Total		27.1 ± 0.9^{A}		32.4 ± 1.1^{A}	

[a]All results are reported as means of three replicates ± standard deviation. Different letters between columns PSO and BSO represent significant differences ($p<0.05$).
n.d: not detected

results for PSO were comparable to those of Parry and others (2006) who reported similar carotenoid compounds (with the exception of α-carotene) in roasted pumpkin seed oil. However, they reported zeaxanthin (28.52 mg/kg) as the most abundant carotenoid in the pumpkin seed oil. There are only a few published reports on the carotenoid content of cold-pressed pumpkin seed oils. In fact, the carotenoid content of oils from butternut squash seeds sourced in the Northeastern part of United States is reported here for the first time. There are however, a few publications on carotenoid contents of butternut pumpkin puree. For instance, Provesi and others (2011) reported a comparable content of β-carotene (19.45 mg/kg) and lutein (0.59 mg/kg) in *C. moschata* from Brazil. Our result for BSO was also comparable to that of Kurz and others (2008) and Hidaka and others (1998) who reported β-carotene and lutein as the major carotenoids in *C. moschata* flesh.

Phenolic profile and H- and L-ORAC values

Phenolic compounds have a great deal of influence on the stability, sensory, and nutritional characteristics of oils (Quites and others 2002; Koski and others 2003). Phenols are important for their ability to quench radicals responsible for lipid oxidation and their applications for promotion of health (Nyam and others 2009; Siger and others 2008). There are only a few published papers on the phenolic content of cold-pressed pumpkin seed oils and most of these studies report only the total phenolic (TP) content of the oil obtained by the Folin-Ciocalteu method. The TP content of BSO (1032 mg GAE/kg oil was slightly higher than that of PSO (967 mg GAE/kg oil) (Table 3). Our values agree with those of Parry and others (2006) who obtained a TP value of 980 mg GAE/kg oil for roasted pumpkin seed oils.

Several other studies report values of pumpkin seed oils that are notably different. For instance, Fruwirth and others (2003) and Andjelkovic and others (2010) reported lower TP values of 29 mg GAE/kg and 24.7 to 50.9 mg GAE/kg, respectively. TP values are difficult to compare due to the lack of standardization of method and the influence of interfering substances (Prior and others 2005). Aside from differences in extraction procedure, variations in the assay used may significantly influence results (Andjelkovic and others 2010). For example, Siger and others (2008) and Haiyan and others (2007) reported TP values of 2.46 mg Caffeic acid equivalent/100g and 15.9 mg CAE/kg, respectively. The use of different standards to quantify TP makes it difficult to compare TP values.

The individual phenolic content of BSO and PSO are given in Table 3. Data shown were from chromatograms (not shown) obtained at 280 nm, which represent a compromise wavelength for detection of phenols. There was little difference in the phenolic content of the oils. PSO showed a slightly higher content (2.96 mg/kg) of total phenolic compounds compared to BSO (2.46 mg/kg) however, only two phenols were identified namely, trans-cinnamic acid comprising 75.6% of the total phenolic content, tyrosol (0.73 mg/kg) making up the rest. On the other hand, five phenolic compounds were detected in BSO, identified as trans-cinnamic acid (0 07 mg/kg), (0.4 mg.kg), vanillic acid (0.97 mg/kg), tyrosol (0.87 mg/kg) and luteolin (0.16 mg/kg) (Table 3). It is notable that trans-cinnamic acid is significantly higher ($p<0.05$) in PSO compared to BSO. Our results for PSO were comparable to those of Tuberoso and others (2007) who identified a much lower amount of trans-cinammic acid (1.0 mg/kg). However, they detected p-coumaric acid at a concentration of 1.8 mg/kg,

Table 3. Phenolic profile, hydrophilic and lipophilic ORAC (H- and L-ORAC) values of cold-pressed pumpkin (PSO) and butternut squash (BSO) seed oils[a]

	BSO (mg/kg)	Relative abundance (%)	PSO (mg/kg)	Relative abundance (%)
trans-cinnamic acid	0.07 ± 0.01^{A}	2.8	2.25 ± 0.30^{B}	75.6
vanillin	0.40 ± 0.20	16.2	n.d	-
vanillic acid	0.97 ± 0.40	39.3	n.d	-
tyrosol	0.87 ± 0.02^{A}	35.24	0.73 ± 0.20^{A}	24.4
luteolin	0.16 ± 0.05	6.45	n.d	-
Total	2.46 ± 0.50^{A}		2.96 ± 0.10^{A}	
TP[b] (mg GAE/kg)	1032 ± 37^{A}		967 ± 28^{A}	
L-ORAC value (μmol TE/g)	0.51 ± 0.01^{A}		0.48 ± 0.04^{A}	
H-ORAC value (μmol TE/g)	1.58 ± 0.20^{A}		1.37 ± 0.30^{A}	

[a]All results are reported as means of three replicates ± standard deviation. Different letters between columns PSO and BSO represent significant differences ($p<0.05$).
[b]TP: total phenolics expressed as gallic acid equivalents
n.d: not detected

which we did not identify in the present study. Our results are also in agreement with those of Anjelkovic and others (2010) who reported the presence of tyrosol, vanillin, vanillic acid, and luteolin in seed oils from different varieties of Slovenian pumpkin at concentrations ranging from 0.20 to 17.69 mg/kg. In another study, cold-pressed pumpkin seed oil from Poland was reported to contain protocatechuic acid, ferulic acid and p-coumaric acid (Siger and others 2008). Nyam and others (2009) also reported presence of gallic acid, p-hydroxybenzoic acid, syringic acid and caffeic acid in pumpkin seed oil from Malaysia. These compounds were not detected in the present study however factors including growing conditions, varietal differences, post-harvest handling of seeds, and processing conditions may explain these differences (Parry and others 2006). In general, cold-pressed oils were found to have slightly higher phenolic content than that of refined oils. Haiyan and others (2007) showed that cold-pressed pumpkin, avocado camellia, soybean, and sesame seed oils contain higher amounts of phenolics than their refined counterparts. Phenolic compounds may be removed during the refining stage of a conventional oil processing procedure.

The H-ORAC values of PSO (1.58 μmol/g oil) and BSO (1.37 μmol TE/g oil) were essentially the same (Table 3). The value we obtained for PSO was comparable to that reported by Parry and others (2006) (1.1 μmol TE/g oil). The L-ORAC values of PSO and BSO are reported here for the first time. L-ORAC value for BSO (0.51 μmol TE/g oil) was essentially the same as in PSO (0.48 μmol TE/g oil). Other published values for antioxidant capacity of cold-pressed oils are measured using different methods. For instance, Tuberoso and others (2007) and Anjelkovic and others (2010) both measured the antioxidant capacity of oils via the DPPH assay at a

range of 0.08-0.95 mmol Trolox equivalent/L. The antioxidant capacity of cold-pressed PSO and BSO were found to be lower than those reported for cold-pressed boysenberry, red raspberry, blueberry, and marionberry seed oils (78, 49, 36, and 17 μmol TE/g oil, respectively).

Oxidative stability under accelerated storage

Lipid oxidation is the major factor that contributes to the deterioration of quality and nutritional value of edible oils. Oxidized lipids can further accelerate the deterioration of the sensory quality of oils leading to the development of off-flavors and loss in nutritional content and thus the oil's consumer acceptance (Lutterodt and others 2011). Since the BSO and PSO used in the present study were not refined, the oils may contain components (degradative oxidation products, metals, etc) that can decrease their stability (Vujasinovic and others 2010). For this reason, it is important to monitor lipid oxidation during storage.

The initial PV and TBARS values of the oils were low. The evolution of these oxidation indices as a function of storage time is presented in Figures 3a and 3b. Results show that the rate of oxidation of both oils was slow. PV did not exceed the maximum limit of 20 meq O_2/kg for unrefined oils (EU Regulation 1989/2003) suggesting excellent stability of the studied oils.

Retention of natural antioxidants

To evaluate retention of the antioxidant compounds in the oils and to account for the excellent stability of the oils, we monitored changes in two major carotenoid compounds (lutein and β-carotene), and two major tocopherol compounds (α and γ) as

lipid oxidation progressed. We postulated that the action of antioxidant substances naturally present in the oils could help retard the oxidation process contributing to the stability of the oils.

Figures 4a-4d present the changes in the four major antioxidants in BSO and PSO during accelerated storage. α-tocopherol showed the highest depletion (68 and 100% for PSO and BSO, respectively) whereas γ-tocopherol was found to be more stable, showing a relatively lower percent depletion (21 and 29% for PSO and BSO, respectively) (Table 4). Our results were in agreement with those of Yi and others (2011) who also found faster depletion of α-tocopherol than γ-tocopherol during storage of palm olein/fish oil. It is well known that α-tocopherol is the most biologically active isomer, however, it may not be as good an antioxidant in vitro as γ-tocopherol (Tasan and others 2011; Nyam and others 2009). Therefore, oils that have higher levels of γ-tocopherol could have better oxidative stability. Significant decrease in carotenoids was also observed. Degradation of lutein (42%) and β-carotene (44-48%) showed a similar behavior in both oils (Table 4). According to Stahl and Sies (2003), carotenoids react with radicals generated in the process of lipid peroxidation by trapping chain-carrying peroxyl radicals.

It has been established that 1 day of storage under Schaal oven condition is equivalent to 1 month of storage at room temperature (Abou-Gharbia and others 1996). Therefore, it can be estimated that the shelf-life of both BSO and PSO was at least 15 months when stored in the dark. Moreover, results of the antioxidant retention under accelerated conditions (60^0C in the dark) suggest significant retention of lutein,

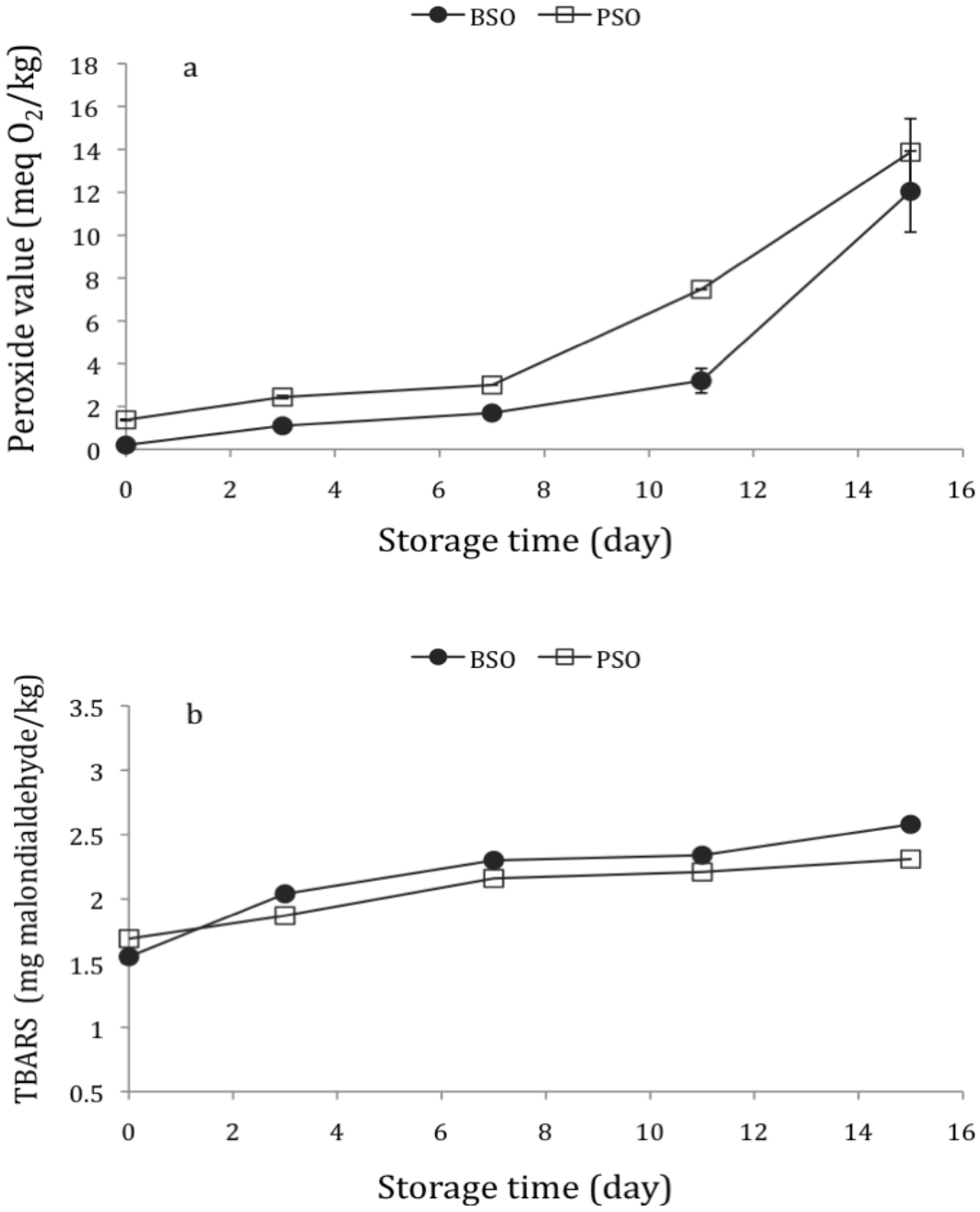

Figure 3. Evolution of hydroperoxides (PV) and thiobarbituric acid reactive species (TBARS) values in cold-pressed butternut squash (BSO) and pumpkin (PSO) seed oils under accelerated storage at 60^0C in the dark.

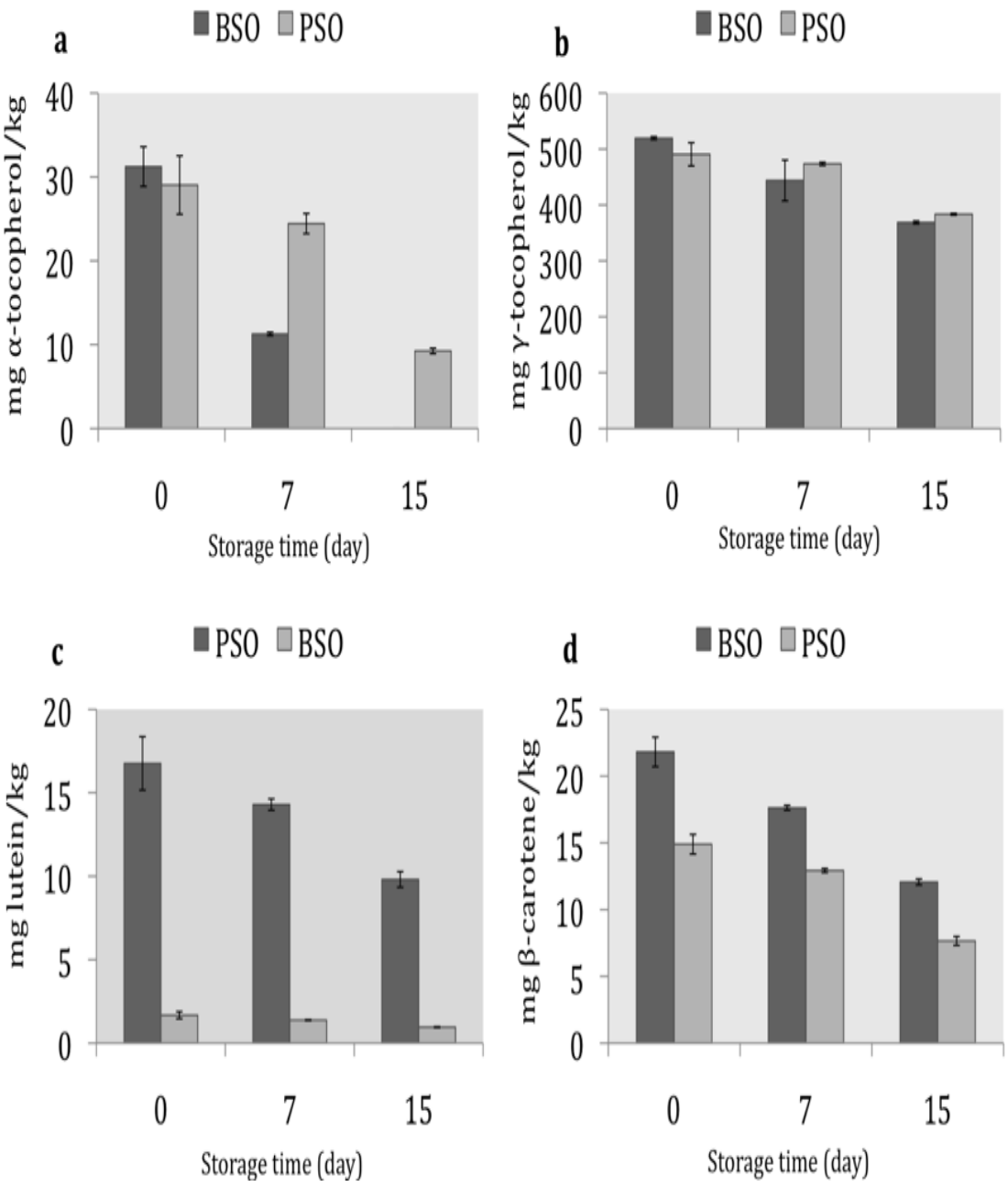

Figure 4. Changes in the major carotenoid and tocopherol content of cold-pressed butternut squash (BSO) and pumpkin (PSO) seed oils under accelerated storage at 60^0C in the dark. Error bars represent one standard deviation.

Table 4. Loss (%) in the concentrations of lutein, β-carotene, α-tocopherol and γ-tocopherol in cold-pressed butternut squash (BSO) and pumpkin seed (PSO) oils after accelerated storage (15 days at 60^0C in the dark)

	BSO (%)	R^2	PSO (%)	R^2
Lutein	42.2 ± 1.3	0.99	41.5 ± 2.8	0.97
β-carotene	44.7 ± 1.0	0.93	48.6 ± 2.2	0.99
γ-tocopherol	29.0 ± 0.5	1.00	21.8 ± 0.3	0.87
α-tocopherol	100	0.97	68.2 ± 1.1	0.91

β-carotene and γ-tocopherol (>50%) in either BSO or PSO even after 15 months. Retention of α-tocopherol, however, was observed to be significantly low with retention values of 0% and 32% for BSO and PSO, respectively. This result suggests that α-tocopherol is the least stable among the major antioxidant compounds monitored.

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CHAPTER 3

OXIDATIVE STABILITY AND BEHAVIOR OF THE NATURAL ANTIOXIDANTS OF COLD-PRESSED PUMPKIN (*CUCURBITA PEPO* L.) AND BUTTERNUT SQUASH (*CUCURBITA MOSCHATA*) SEED OILS

ABSTRACT

The effect of packaging transmission light properties (clear, green and amber glass bottles) and storage temperature (20 and 30^0C) on the oxidative stability of cold-pressed pumpkin (*Cucurbita pepo* L.) and butternut squash (*Cucurbita moschata*) seed oils and the behavior of their major antioxidants were studied as a function of storage time (0-20 weeks). Analyses included monitoring of peroxide (PV), thiobarbituric acid reactive species (TBARS), and quantification of the major antioxidant compounds. Oxidation was most pronounced in clear bottles while green bottles provided only partial protection to the oils. The antioxidant compounds were better retained in amber bottles while significant reductions ($P<0.05$) were observed in clear and green bottles. The relative stability of the compounds was: γ-tocopherol > α-tocopherol; lutein > β-carotene. Total phenolic content was found to be stable. Results highlight the superiority of using amber over green and clear bottles in protecting cold-pressed oils against oxidative deterioration and in retaining their antioxidant composition.

Key Words: Pumpkin seed oil, Butternut squash seed oil, Oxidative stability, Carotenoids, Tocopherols

INTRODUCTION

Pumpkin (*Cucurbita sp.)* is a main agricultural produce in the United States. In 2010, 1.06 billion pounds of this crop were produced in the US, with farm value amounting to $117M (*1*). Recently, much attention has been focused on pumpkin seeds as a way of utilizing food-processing byproducts and waste. The seeds from some cultivars commonly known as 'pumpkin' (*Cucurbita pepo* L.) and butternut squash (*Cucurbita moschata*) contain oil that varies from 40-60wt% (*2-4*). Aside from their unique sensory attributes, pumpkin seed oils are also known to contain significant levels of natural antioxidants and have been implicated in providing many health benefits (*5-7*).

Previous studies revealed that pumpkin seed oil is highly unsaturated, with fatty acid (FA) composition predominantly composed of 43-56% linoleic acid and 20-42% oleic acid (*8, 9*). Fruwirth and Hermetter (*10*) reported that Styrian pumpkin seed oil contains 45% of polyunsaturated FA, 36% of monounsaturated FA and 18% of saturated FA. Levels of linolenic acid and other highly unsaturated fatty acids are low making the oil less prone to oxidation (*4*). Significant amounts of natural antioxidants have also been reported in cold-pressed pumpkin seed oils, consisting mainly of carotenoids, tocopherols and polyphenols (*4, 11, 12*). The most abundant carotenoids found in pumpkin seed oils were β-carotene, which is known for its radical scavenging capacity and provitamin A activity (*13*) and lutein, which has been implicated for its important role in eye health (*14*). Several studies also found significant amounts of tocopherols (*4, 12, 15, 16*) one of which is α-tocopherol known as an active in vivo antioxidant that may act as electron donor by giving up its hydrogen atom to lipid free

radicals and as scavenger of singlet oxygen (*17, 18*). Some studies however, suggested that γ-tocopherol has greater antioxidant activity than the α-isomer (*19, 20*). In this case, the higher γ-tocopherol (2 to 8 times higher than other isomers) content of pumpkin seed oil makes the oil more attractive from a nutritional perspective (*4*). Phenolic compounds in pumpkin seed oils are relatively lower in concentration, some of which may act as antioxidants on mechanisms based on radical scavenging, hydrogen atom transfer and metal chelating attributes (*21*).

The industrial production of pumpkin seed oil has recently employed cold pressing in an effort to preserve natural healthful components and to avoid governmental restrictions and consumer concerns about the safety of the use of organic solvents in the traditional solvent extraction method (*2*). Cold-pressed oils receive only minimal heat ($<50^0$C) generated during the course of screw pressing and the process does not involve any chemical treatment (*22*). The resulting oil is then coarsely filtered at room temperature to remove particulates. Recently, roasting of the seeds prior to screw pressing has been practiced to improve yield and impart unique aroma to the oil (*22*). Moreover, roasting of the seeds may increase the antioxidant capacity of the oil due to the increased extractability of phytochemicals as a result of cellular deformations which take place during the process, and the formation of Maillard reaction products (MRPs) which may contribute to terminating lipid oxidation reactions (*23*). Because of this simple process, cold-pressed oils maintain their natural composition and may be a better source of antioxidant compounds and other healthful components (*24*). However, due to the oils' unrefined nature, the

presence of components (metals, oxidative degradation products, etc) with pro-oxidant activities may promote lipid oxidation thus lowering the shelf life of the oil (*8*).

With their significant levels of mono- and polyunsaturated fatty acids and antioxidant compounds, cold-pressed oil from pumpkin and butternut squash seeds is a good model to investigate the behavior of natural antioxidants in relation to their contribution to delaying oxidative changes. Oxidation of oils can lead to the production of antinutritional factors and undesirable flavor and aroma compounds (*25*). Therefore, knowledge of lipid oxidation in cold-pressed pumpkin oils and the changes that its natural antioxidants undergo under different storage conditions are fundamental to the preservation of the nutritional and sensory qualities of these oils. The present study aims to understand the behavior of the natural antioxidant composition of pumpkin seed oils and to establish a relationship between these compounds and the oils' oxidative stability. Specifically, the present study aims to monitor changes undergone by carotenoids, tocopherols, phenolics and the oils' antioxidant capacity during storage employing different temperatures (20^0C and 30^0C) and packaging materials with different light transmission properties (clear, green and amber glass bottles), using cold-pressed oils from the seeds of two cultivars of pumpkin.

MATERIALS AND METHODS

Reagents and standards. Potassium hydroxide (KOH), Potassium iodide (KI), (+)-6-hydroxy-2,5,7,8-tetramethylcromano-2-carboxylic acid (Trolox), sodium fluorescein, 2,2'- azobis(2-aminopropane) dihydrochloride (AAPH), Folin-Ciocalteu reagent, β-

and α-carotene, lutein, β-cryptoxanthin, zeaxanthin, p-coumaric acid, vanillin, vanillic acid, tyrosol, luteolin, trans-cinnamic acid, α-, γ-, δ- tocopherols were obtained from Sigma-Aldrich (Allentown, PA). All standards were ≥ 96% purity. Standards for tocotrienols were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Hexane, chloroform, 1-butanol, acetone, acetic acid, methanol, ethyl ether, 2-propanol, sodium chloride, 2-thiobarbituric acid (2-TBA) were analytical grade chemicals from Fisher Scientific Company Ltd (Suwanee, GA).

Oil Extraction and Oxidative Stability Experiments. Raw and dried seeds were roasted for 30-35 minutes at a temperature of up to 175^0C. Cold-pressed oils were extracted from the roasted seeds using a KOMET oil press (IBG Monforts Oekotec GmbH and Co, Napa, CA) of capacity 8-10 kg/hr. The oil that comes out from the press was monitored to not exceed 50^0C (*26*). The oils were collected in stainless steel vessels and coarsely filtered after 48 h without further refining. Samples were collected in amber bottles, topped with N_2 gas, capped and immediately stored at -20 ^{0}C until further analyses. Samples were collected from two different extraction batches.

To study the effect of storage temperature, oil samples from each extraction batch were placed in amber bottles leaving a half of an inch headspace, topped with N_2 and sealed with screw-type rubber-lined cap of negligible permeability to oxygen. Containers were stored in two separate thermostatic chambers in the absence of light using two temperatures: 20^0C and 30^0C. To study the effect of packaging light transmission property (%T in the UV range), clear (90%T) and green (60%T) glass

bottles (Waterloo Container, Waterloo, NY) covered with a natural cork with plastic top of capacity 180 mL were chosen to simulate a commonly used packaging material to store and commercially distribute cold-pressed pumpkin seed oils. Containers were filled and stored in a temperature-controlled (20^0C) chamber with constant exposure to an artificial light (15 W fluorescent lamp). For each sample, a series of two bottles were prepared. All samples were held in the above conditions for a period of 20 weeks. Starting at time zero (0) and at an interval of 4 weeks thereafter, duplicate or triplicate samples from each bottle were periodically removed from the chamber for scheduled analyses.

Initial Oxidation and Quality Indices. The American Oil Chemists' Society (AOCS) Official Methods (*27*) were used to determine peroxide value (PV, Method Cd 8-53) expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2/kg); free fatty acid value (FFA, Method Ca 5a-40) expressed as percentage of oleic acid; Acid value (AV, Method Cd 3d-63) expressed as % oleic acid; and thiobarbituric acid reactive species (TBARS, Method Cd 19-90) value expressed as milligrams of malondialdehyde per kilogram of oil (mg malondialdehyde/kg). Experiments were carried in duplicate or triplicate and results were reported as average $\pm$ standard deviation.

Tocopherol Analyses. The chromatographic method for the analysis of tocopherol was based (with some modifications) on the method of Gliszczynska-Swiglo and Sikorska (*31*). Samples of BSO and PSO were weighed (0.5-1.0 g) and dissolved in 10

mL of 2-propanol and filtered through a 0.20 um PTFE syringe filter and analyzed using reversed phase HPLC system (Hewlett-Packard model 1100; Palo Alto, CA) equipped with a fluorescence detector set at 290 nm emission and 330 nm excitation wavelengths. Separation was performed at 25^0C using an Eclipse C18 (4.6 x 150 mm, 5 um) column with a mobile phase consisting of methanol:acetonitrile:water (50:44:6, v/v/v) (*32*). Injection volume was 20 uL and the flow rate was 1.5 mL/min. Identification of compounds was based on retention times and UV absorption spectra and quantitation was carried out based on calibration curves using authentic standards of α- and γ-tocopherols. Stock and working solutions (0.0025, 0.005, 0.01 0.02, 0.03, and 0.05 mg/mL, R^2= 0.9997) were prepared using 2-propanol. The analyses were carried out in duplicate.

Carotenoid Analyses. Changes in lutein and beta-carotene contents were determined by HPLC after an analytical extraction procedure based (with some modifications) on the procedure proposed by Ceron et al. (*28*). Samples (5 g of oil) were dissolved in 25 mL pure ethyl ether. An equal volume of a solution of KOH in MeOH (4% w/v) was added for saponification. The mixture was vortexed for 30 s and allowed to stand under nitrogen at 0^0C in the dark. To stop the reaction and remove excess alkali, 4 mL of 10% (w/v) NaCl solution was added into the mixture. Separation of phases was done using a separatory funnel. The ether phase was evaporated and the remaining pigment was redissolved in 95% ethanol (stabilized with 30 ppm BHT), sealed in an amber vial, topped with N_2 gas and kept at -20^0C until further analyses.

The chromatographic method was based on the procedure described by

Minguez-Mosquera et al. (*29*), as modified by Ceron et al. (*30*). The sample redissolved in ethanol and filtered through a 0.20 μm PTFE membrane was subjected into a liquid chromatography using a reversed phase HPLC system (Hewlett-Packard model 1100; Palo Alto, CA) equipped with a photodiode array detector. Separation was performed using an Eclipse C18 column (4.6 x 150 mm, 5 μm). The eluents used were (A) water-methanol (2:8, v/v) and (B) acetone-methanol (1:1, v/v). The pigments were eluted at a rate of 1 mL/min at 25^0C according the following solvent gradient: 75% A/25% B at 0 min, 25% A/75% B at 8 min and remained isocratic until 10 min, 10% A/90% B at 18 min, 0% A/100% B at 23 min, and 75% A/25% B at 35 min. Detection was at 450 nm and 470 nm. Results from three determinations were expressed in milligrams of compound per kilogram of oil (mg/kg).

Analysis of Total Phenols (TP). Total phenolic content was determined using the Folin-Ciocalteu method (*33*). Samples (2 g) of oil were added with 5 mL of methanol in a screw-cap centrifuge tube. The mixture was vortexed for 30 seconds and the emulsion was separated using a Sorvall RC-5B Refrigerated Superspeed (DuPont Instrument, CT) centrifuge at 6000 x g rpm for 6 minutes. The hydrophilic layer was collected and the extraction was repeated another two times. The extract was appropriately diluted and placed in an amber bottle, topped with N_2, sealed and stored at -20^0C until further analysis. For the TP assay, methanolic extract (200 μL) was placed in a test tube, and 250 μL of Folin-Ciocalteu reagent (diluted ten times from the original reagent) was added. After six minutes, 750 μL of 20% sodium carbonate (Na_2CO_3) and 3 mL water were added. Absorbance at 750 nm was determined using a

Genesys 10S UV-Vis spectophotometer (Thermo Fisher Scientific, Madison, WI) after 2 h. Calibration was performed using Gallic acid and a calibration curve was obtained: y= 124.2x + 0.006; R^2 = 0.9997 in the range 0-100 mg/L). Experiments were carried out in 6 replicates and data were expressed as mg Gallic acid equivalents per kg oil (mg GAE/kg).

Radical Scavenging Capacity. To determine the peroxyl radical scavenging capacity of the oils, methanolic extracts were subjected to ORAC assay based on a modified $ORAC_{FL}$ method (*34*). The assay was carried out on a Synergy HP fluorescence microplate reader (Biotek Instruments, Inc., Winooski, VT) with Fluorescein as the fluorescent probe. AAPH was used as the radical generator and a standard curve was generated using Trolox at concentration ranging 6.25 to 100 μM. Each 96-well microplate contained 150 μL Fluorescein solution (0.004 μM), 25 μL AAPH solution (0.15M), and 25 μL of appropriately diluted methanolic extract. Flourescence reading was taken every 1 minute for 1 h (485 nm excitation and 520 nm emission). All dilutions were carried out using 75 mM K_3PO_4 buffer (pH 7.4). ORAC values were expressed as μmol Trolox equivalents per gram oil (μmol TE/g). Experiments were carried out in triplicate.

Statistical Analysis. Experiments were conducted in duplicates and triplicates and differences in means were detected using analysis of variance (ANOVA) and T-tests via JMP® 9 (SAS Institute, Carey, NC). Posthoc comparison of means was conducted using Tukey's HSD test. Statistical significance was defined at $P \leq 0.05$. Results were

reported as mean ± standard deviation.

RESULTS AND DISCUSSION

Initial Composition and Physicochemical Characteristics. The initial physical and chemical characteristics of cold-pressed butternut squash (BSO) and pumpkin seed (PSO) oils are summarized in Table 1. BSO has a lower initial free fatty acid (FFA) value (0.40% oleic acid) compared to PSO (1.01% oleic acid). The FFA values of both oils are within the 2.0% maximum limit according to the regulation for unrefined oils (*35*). The observed FFA values were probably formed during the preparation of the seeds prior to cold pressing. Initial acid value (AV) was observed to be low for both oils with BSO having a significantly lower ($p<0.05$) value (1.18 mg of KOH/g of oil) compared to PSO (2.55 mg of KOH/g of oil). The higher acid value indicates a higher degree of lipolysis due to enzymatic activity in the seeds during preparation (*36*). Both BSO and PSO have low initial peroxide value (PV, 1.46 and 1.96 meq O_2/kg oil, respectively) and thiobarbituric acid reactive species (TBARS) values (1.21 and 1.52 mg malondialdehyde/kg oil, respectively). The small amounts of oxidation products detected can be attributed to the formation of hydroperoxides and subsequently TBARS during pre-process handling of the seeds.

Significant concentrations of lutein and β-carotene were detected in cold-pressed BSO and PSO (Table 1). In BSO, β-carotene (21.8 mg/kg) was found to be significantly ($p<0.05$) higher than lutein (1.7 mg/kg). The opposite was observed in PSO, in which lutein (21.3 mg/kg) was detected at a significantly higher ($p<0.05$) concentration than β-carotene (14.9 mg/kg). The concentration of these two major

carotenoids in BSO and PSO was higher than that reported by Parry et al. (*12*). Values obtained in this study were also higher than those reported for cold-pressed red raspberry, blueberry, marionberry, and boysenberry seed oils (12.5–30.0 μmol/kg) (*37*). These results indicate that cold-pressed oils from roasted butternut squash and pumpkin seeds are good dietary sources of carotenoids, especially lutein and β-carotene.

Table 1. Initial chemical characteristics of cold-pressed butternut squash (BSO) and pumpkin (PSO) seed oils[a]

	Butternut squash	Pumpkin
Peroxide value (meq O_2/kg)	1.46 ± 0.00 a	1.96 ± 0.60 b
TBARS[b] (mg malondialdehyde/kg)	1.21 ± 0.10 a	1.56 ± 0.10 b
Acid value (mg of KOH/g)	1.18 ± 0.10 a	2.60 ± 0.10 b
Free fatty acid (% oleic acid)	0.40 ± 0.00 a	1.00 ± 0.10 b
β-carotene (mg/kg)	21.8 ± 1.1 a	14.9 ± 3.2 b
lutein (mg/kg)	1.70 ± 0.2 a	21.3 ± 1.6 b
α-tocopherol (mg/kg)	31.2 ± 2.2 a	29.0 ± 3.2 a
γ-tocopherol (mg/kg)	519.5 ± 2.5 a	490 ± 21 a
Total phenolics (mg GAE/kg)	1051 ± 40 a	943 ± 16 a
ORAC value (μmol TE/kg)	1.70 ± 0.4 a	1.60 ± 0.5 a

[a]All results are reported as means of three replicates ± standard deviation. Different letters between columns represent significant differences ($p<0.05$).
[b]Thiobarbituric acid reactive species.

The initial concentration of γ- and α-tocopherol did not vary significantly between PSO and BSO. Predominant amounts of γ-tocopherol (490-519 mg/kg), approximately 92% of the total tocopherol contents and 17 times higher than α-tocopherol (29.0-31.2 mg/kg), were observed in both oils (Table 1). Our data was comparable to that of Stevenson et al. (*4*) and Parry et al. (*12*). The TPC of BSO (1051

mg GAE/kg) did not significantly ($p<0.05$) differ from that of PSO (943 mg GAE/kg, Table 1). TPC for PSO agrees with that of Parry et al. (*12*) who used a similar extraction solvent (100% methanol).

The peroxyl radical scavenging capacity, measured as oxygen radical absorbance capacity (ORAC) values, of PSO (1.6 μmol TE/g oil) and BSO (1.7 μmol TE/g oil) were not significantly different ($p<0.05$). The ORAC values of the studied oils were comparable to those obtained by Parry et al. (*12*) (1.1 μmol/g oil).

Stability During Storage. Several parameters can be used to describe the oxidative stability of oils. In this study, we monitored the evolution of PV and TBARS value as suitable indices for monitoring the progress of oxidation (*38*). PV is used to measure formation of primary oxidation products while TBARS value measures the evolution of secondary deterioration products such as carbonyl compounds (aldehydes and ketones) that could negatively affect the smell and taste of edible oils (*39*).

The formation of hydroperoxides started immediately (Figures 1a and 1b) and subsequent formation of secondary products (Figure 2a and 2b) was also observed. There was no significant difference in the PV and TBARS values of oils observed between 20^0C and 30^0C at week 20. PV of PSO was slightly higher than that of BSO probably due to the higher initial PV of the former. The PV (Figure 1a) and TBARS values (Figure 2a) of the oils stored at both temperatures did not exceed the maximum value for unrefined oils of 20 meq O_2/kg (*40*) after 5 months of storage indicating a low rate of lipid oxidation. A similar result was reported by Pristouri et al. (*41*) who found that olive oils did not exceed the PV limit until 12 months of storage at 13^0C

and 22^0C in the dark, however at 35^0C, PV exceeded the limit after 9 months. Our results were also in agreement with those of Nakavoua et al. (*42*) who found that pumpkin seed oils kept well at an average temperature of 30^0C. Our results suggest that, taking into account only the effect of storage temperature, PSO and BSO must be stored at temperatures 30^0C and below in the dark to delay the progress of oxidative deterioration and for the oils to reach a shelf life of at least 5 months.

In the case of oils stored in clear and green glass bottles exposed to constant 15W fluorescent light at 20^0C, a rapid increase in PV from time 0 (1.9 meq O_2/kg) to week 8 (30.4 meq O_2/kg) and from time 0 (1.5 meq O_2/kg) to week 16 (27.2 meq O_2/kg) was observed in PSO and BSO, respectively (Figure 1b). After reaching peak values at weeks 8 and 16, PV started to decrease until week 20, which indicates the breakdown of hydroperoxides into secondary products as evidenced by the observed increase in TBARS (Figures 2a and 2b). PSO stored in clear and green bottles exceeded the minimum PV limit after 8 weeks of storage. PSO in clear bottles had a slightly higher PV at week 8 however not significantly different ($p<0.05$) than those stored in green bottles indicating a slightly higher rate of PV evolution in the former. The use of green bottles did not demonstrate a significantly better protective effect against oxidation as compared to clear bottles, and only gave a shelf life of 8 weeks to PSO. BSO stored in green bottles (Figure 1b) exceeded the PV limit only after 16 weeks while those stored in clear bottles exceeded the said limit after 12 weeks thus the use of green bottles extended the shelf life of BSO up to 4 weeks more as compared to clear bottles. This result further indicates that the use of green bottles, which is now being commercially practiced

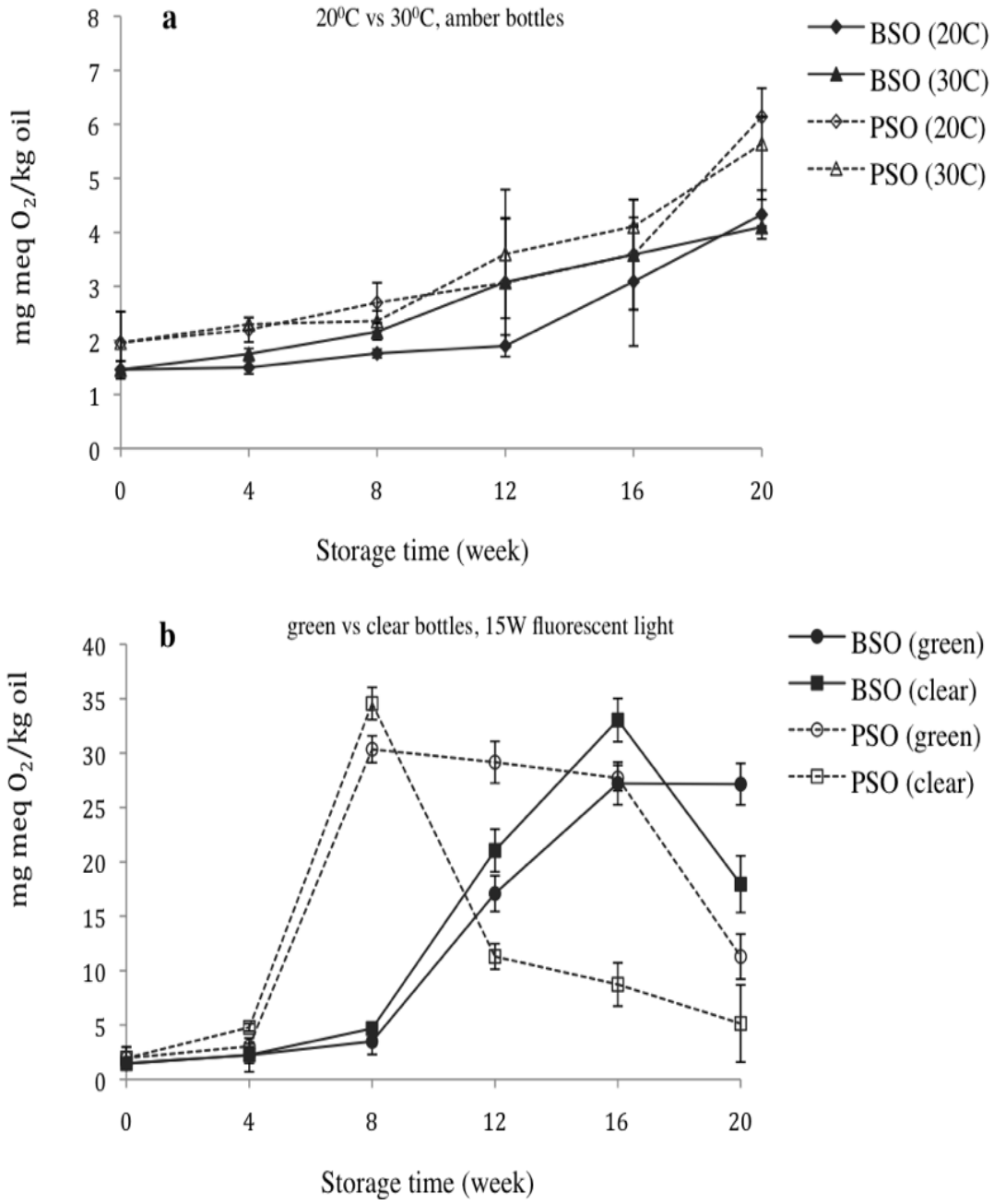

Figure 1. Evolution of hydroperoxides (PV) in cold-pressed butternut squash (BSO) and pumpkin (PSO) seed oils under different storage conditions for 20 weeks. Error bars represent one standard deviation.

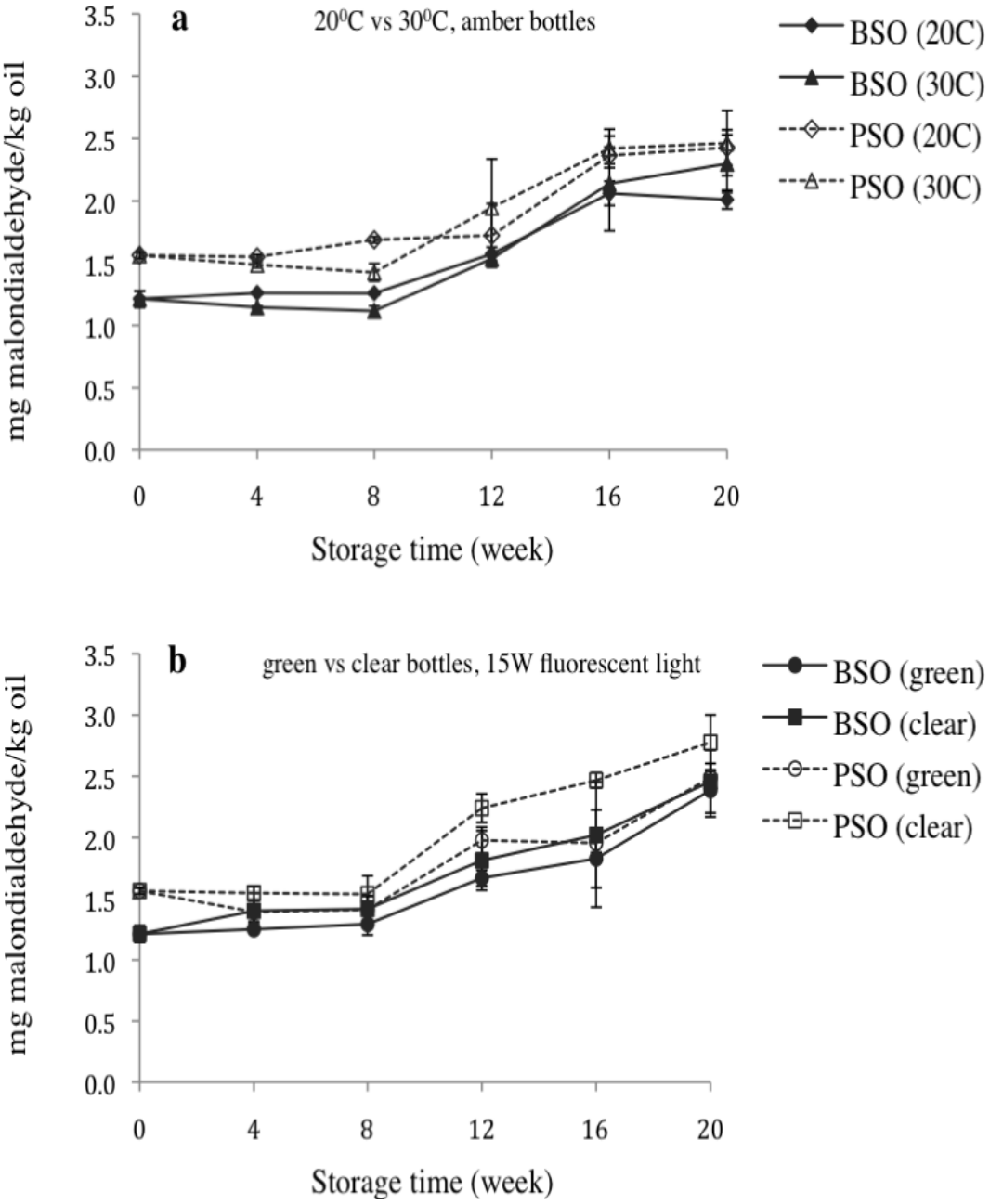

Figure 2. Changes in thiobarbituric acid reactive species (TBARS) values of cold-pressed of butternut squash (BSO) and pumpkin (PSO) seed oils under different storage conditions for 20 weeks. Error bars represent one standard deviation.

provides only partial protection to PSO and BSO while complete protection from light by using amber glass bottles (at 20^0C) gives the oils a shelf life of >5 months. Our result is comparable to that of Pristouri et al. (*41*) who found that olive oils stored in PET + aluminum foil gave longer shelf life of 9 months compared to a shelf life of 3 months for oils stored in PET + UV blocker. The significantly higher rate of lipid oxidation observed in oils exposed to light during the first few weeks of storage was consistent with that of Poiana et al. (*35*) who reported that the oxidation process in the first period of storage of grape seed oil was more affected by daylight than by temperature. In the present study, we did not take into account the possible effect of the differences in permeability of the packaging material to oxygen, however it could be possible that the use of cork with a plastic top in the green and clear bottles allows oxygen to slowly permeate resulting in a significantly faster rate of oxidation compared to oils stored in amber bottles with rubber-lined screw caps. Other sources of oxygen could be the initial amount present as dissolved oxygen or in the headspace (*41*), however given the fact that the amount of oil and thus the headspace was the same in all containers, it is logical to assume that the concentration of oxygen was equal in all samples.

The differences observed in the rate at which the oxidation progressed in PSO and BSO stored under similar storage conditions clearly suggest a difference in stability of these oils as affected by factors other than temperature and packaging material light transmission properties. These differences could also be due to the presence of prooxidant pigments such as protochlorophylls and protopheophytin in PSO, which could enhance lipid oxidation in the presence of light. Chlorophylls

absorb light, become excited and subsequently transfer this excess energy to ground state triplet oxygen, which in turn form the excited state singlet oxygen that could readily react with free fatty acids (*43*). Differences in the content of natural antioxidants and their behavior over time could also be important factors to consider in assessing the oxidative stability of the oils.

Behavior of Natural Antioxidants. Besides the effect of the different storage conditions discussed previously, contribution of the natural antioxidants may explain differences in the oxidative stability of these oils (*44*). It may be expected that as lipid oxidation progresses, the oils' natural antioxidants are also being progressively depleted (*17*). To better understand the role of the endogenous antioxidative system in PSO and BSO, we monitored the changes undergone by their major phytochemical components mainly carotenoids, tocopherols and total phenolics. Together, these compounds are assumed to constitute the main endogenous antioxidative system of the oils.

Changes in Major Tocopherol Content. Our results (Table 1) and other previously reported studies (*4, 12*) on tocopherol content of pumpkin seed oils confirmed the significant levels of this compound in PSO and BSO with the γ-tocopherol showing preponderance over other Vitamin E isomers. Tocopherols in oil seeds are potent antioxidants that could protect the oils from oxidation by trapping hydroperoxide intermediates thus increasing the shelf life of oils (*15, 45*).

Changes in the γ- and α-tocopherol concentrations in PSO and BSO over 20 weeks of storage are shown in Figures 3a–d. In the case of BSO and PSO stored in clear and green bottles with constant exposure to artificial fluorescent light (15W), complete depletion of α-tocopherol (Figure 3a) was observed after 8 weeks of storage in both conditions suggesting that α-tocopherol reacted and was being used up quickly to protect the oils against oxidation. A similar observation was reported by Krichene et al. (*17*) who found that α-tocopherol in olive oil stored in both closed and open bottles was rapidly depleted during medium temperature accelerated storage suggesting the antioxidant activity of this tocopherol isomer. Our results are in agreement with those of Yi et al. (*46*) who found faster depletion of α-tocopherol than γ-tocopherol during storage of palm olein/fish oil. A significant decrease ($p<0.05$) in γ-tocopherol (Figures 3c and 3d) throughout the storage time was observed suggesting that this Vitamin E isomer was being used up and, therefore, contributes to delaying the progress of oxidation. While both a- and γ-tocopherol were depleted over the storage period, we observed that the latter was more stable or was being used up at a much lower rate than the former. In both oils a decrease ranging from 26% to 45 % from initial concentration was observed in γ-tocopherol after 20 weeks as opposed to the complete depletion of α-tocopherol (Table 2) at week 8.

For oils placed in amber bottles and stored at 20^0C and 30^0C, α-tocopherol concentration in both oil types remained essentially constant up to week 16 and showed a significant ($p<0.05$) decrease (14-23%) only at week 20 (Figure 3b).

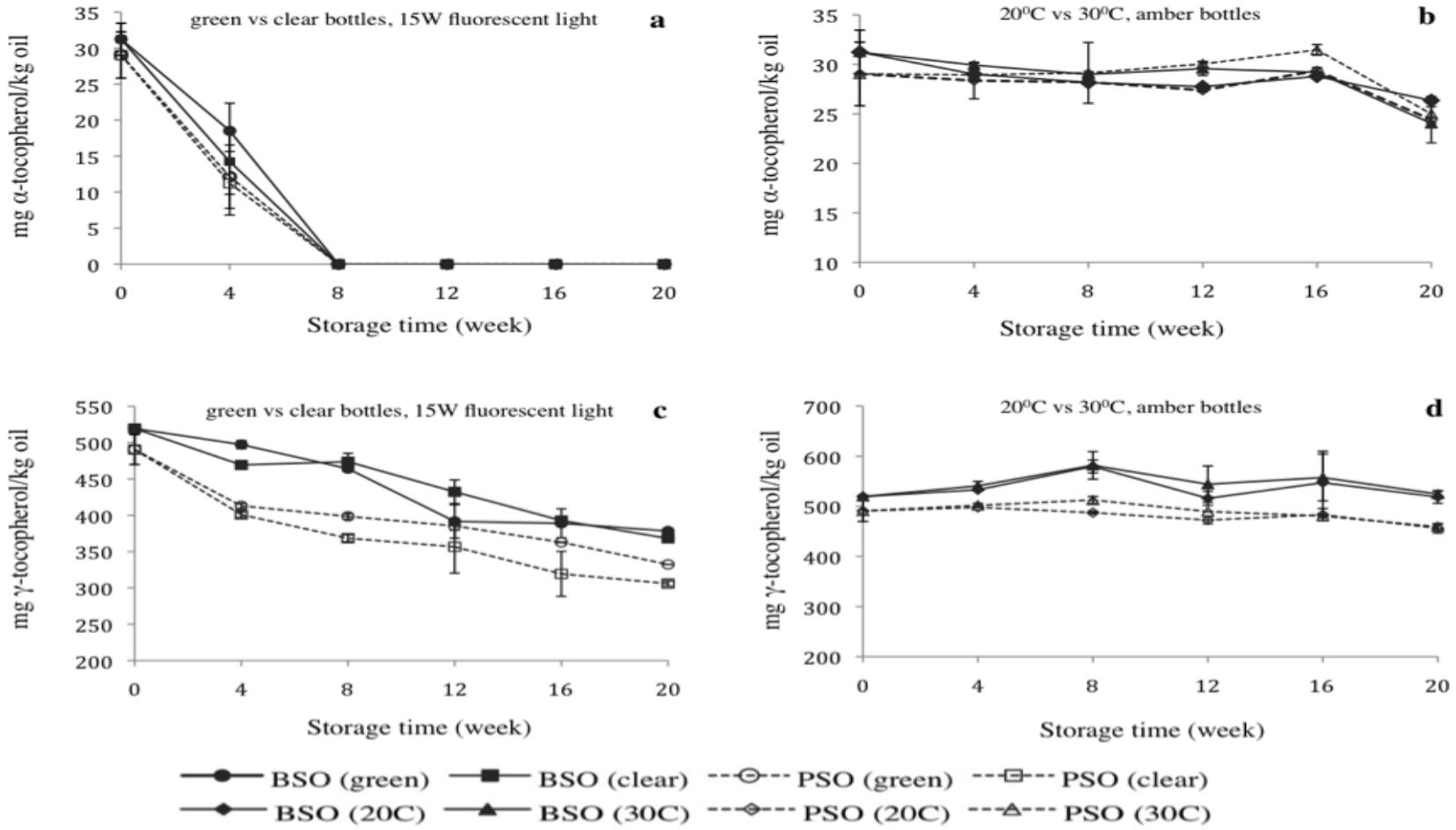

Figure 3. Changes in α- and γ-tocopherol content of cold-pressed butternut squash (BSO) and pumpkin (PSO) seed oils under different storage conditions for 20 weeks. Error bars represent one standard deviation.

This result further suggests that the rate of depletion of α-tocopherol could be associated with the rate of oxidation of the oils. Indeed, in all cases, the depletion of α-tocopherol correlated well (r=-0.68 to -0.85) with the PV and TBARS suggesting the apparent relationship between the depletion of α-tocopherol and the progress of oxidation, thus the antioxidant role of α-tocopherol. A similar behavior was observed on γ-tocopherol in both oils. The concentration of γ-tocopherol remained essentially constant under both storage temperatures throughout the 20-week storage period, showing only a minimal decrease of up to 7% (Table 2). While it is known that α-tocopherol is the most biologically active isomer, it may not be as good an antioxidant in vitro as γ-tocopherol (*47, 48*). According to Goh et al. (*49*), the formation of γ-tocopherol diphenyl dimer enhances the reactivity γ-tocopherol. Therefore, oils that have higher levels of γ-tocopherol could have better oxidative stability. The good correlation (r=-0.71 to -0.97) between γ-tocopherol and the oxidation indices (PV and TBARS) suggests that this compound is a major contributor to the antioxidant stability of PSO and BSO. However, Murkovic and Pfannhauser (*16*) observed an opposite relationship between tocopherols and the induction time of pumpkin seed oils obtained by Rancimat method. They observed that neither α- nor γ-tocopherol showed an influence on the oxidative stability of the pumpkin seed oil, instead a strong pro-oxidant property of the former at concentrations above 1 mg/g oil was observed, significantly reducing the induction time. According to Cillard et al. (*50*), α-tocopherol can act as prooxidant at high concentrations, which is attributed to the formation of tocopheroxyl radical (*51*). Moreover, in highly

Table 2. Percentage (%) loss in the concentrations of the major antioxidants of cold-pressed butternut squash (BSO) and pumpkin seed (PSO) oils after 20 weeks at different storage conditions

Oil	Storage condition	β-carotene % loss	lutein % loss	α-tocopherol % loss	γ-tocopherol % loss
BSO	20^0C, amber bottles	57.2 ± 2.3	26.6 ± 2.3	15.7 ± 0.1	0.1 ± 2.5
	30^0C, amber bottles	69.2 ± 7.1	30.3 ± 7.1	23.1 ± 0.0	1.8 ± 1.1
	20^0C, green bottles, 15W fluorescent light	74.9 ± 6.4	32.7 ± 2.3	100.0	27.6 ± 0.6
	20^0C, clear bottles, 15W fluorescent light	82.0 ± 0.7	38.8 ± 7.1	100.0	25.9 ± 0.7
PSO	20^0C, amber bottles	12.7 ± 5.8	14.9 ± 5.8	15.9 ± 3.1	7.1 ± 1.9
	30^0C, amber bottles	34.7 ± 4.8	21.7 ± 0.8	14.0 ± 2.5	6.5 ± 1.0
	20^0C, green bottles, 15W fluorescent light	71.9 ± 0.3	35.0 ± 5.8	100.0	32.3 ± 2.8
	20^0C, clear bottles, 15W fluorescent light	75.5 ± 1.7	37.8 ± 4.8	100.0	45.3 ± 1.9

polyunsaturated oils α-tocopherol is regarded as a poor antioxidant as it can become a chain-carrier by regenerating peroxyl radicals (16). Therefore, since the linolenic acid and other highly unsaturated fatty acids are low in either PSO or BSO, we do not associate the rapid depletion of α-tocopherol to a potential prooxidant activity of this compound. Instead, the observed higher rate of depletion of α-tocopherol compared to γ-tocopherol could partially be explained, aside from being sacrificially used up to delay oxidation, in terms of the stability of these compounds. Clearly, α-tocopherol is less stable than γ-tocopherol, resulting in the rapid depletion of the former under similar storage conditions.

Changes in Major Carotenoid Compounds. Previous studies found that lutein and β-carotene are the major carotenoids in pumpkin seed oil (*12, 16*). Our results (Table 1) confirmed this finding however, differences between the studied oils in terms of these individual carotenoids were observed with lutein showing preponderance in PSO while β-carotene was significantly ($p<0.05$) higher in BSO. To establish the influence of these two major carotenoids on the oxidative stability of the oils and to study their behavior under different storage conditions, we monitored changes in their concentration over 20 weeks of storage.

Figures 4a and 4b present the changes in the concentration of β-carotene under the different experimental conditions described previously. For both PSO and BSO stored in green and clear glass bottles and exposed to fluorescent light, a significant decrease in β-carotene ($p<0.05$), following almost a linear trend, was observed from

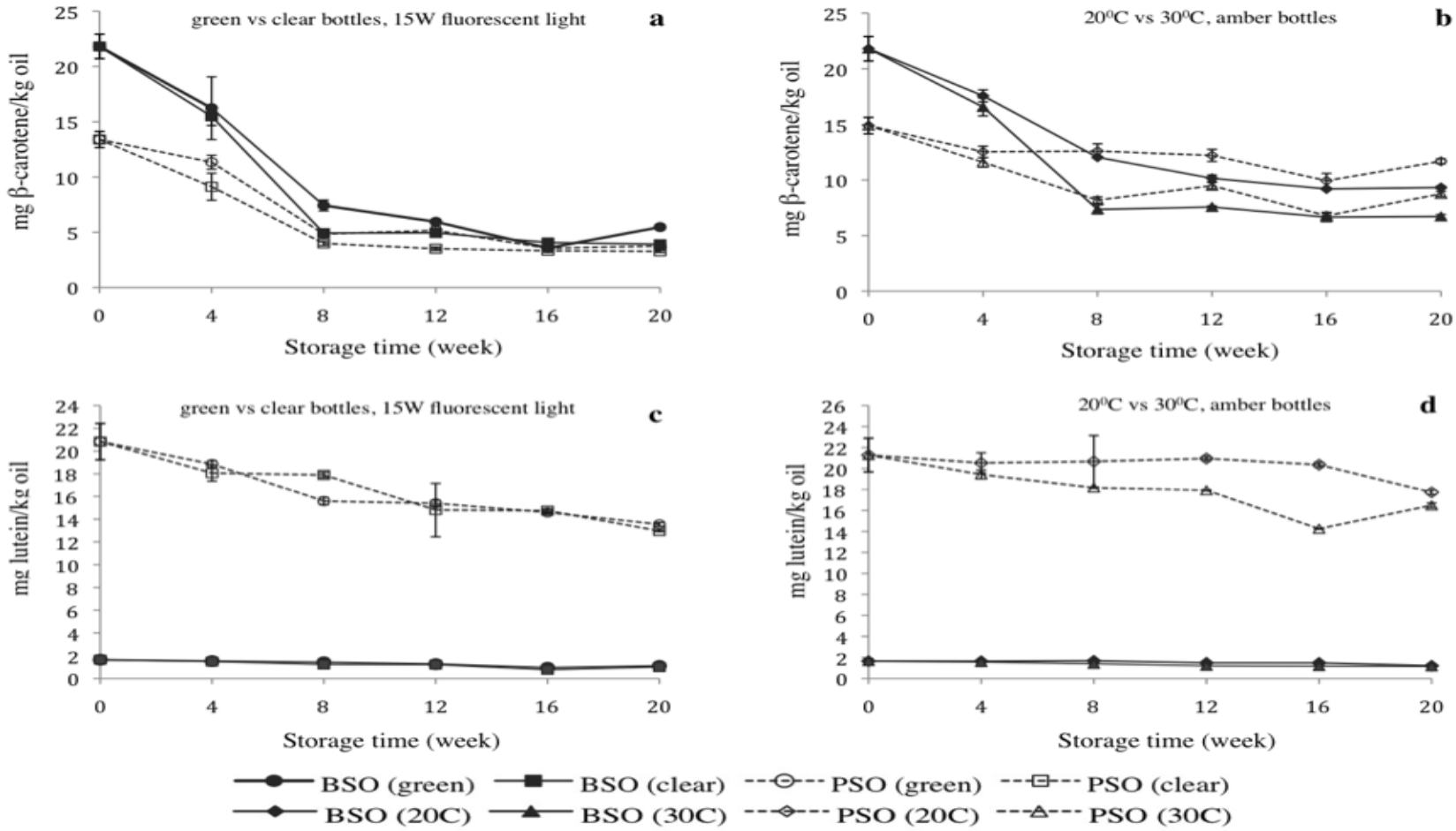

Figure 4. Changes in lutein and β-carotene content of cold-pressed butternut squash (BSO) and pumpkin (PSO) seed oils under different storage conditions for 20 weeks. Error bars represent one standard deviation.

time 0 to week 8 and incremental decrease was observed thereafter. During the first 8 weeks of storage, the β-carotene content of both oil types placed in green bottles showed >50% decrease. At week 20 the β-carotene content of both oils was in the range of 3.28-5.47 mg/kg indicating 74-82% depletion (Table 2). No significant difference ($p<0.05$) in terms of percentage loss at the end of the storage period was found between oils stored in green and clear bottles indicating a minimal protective effect of the former on the β-carotene content of the oils. On the other hand, significant decreases ($p<0.05$) in β-carotene concentrations ranging from 21% to 69% (Table 2) were observed in both oils stored at 20^0C and 30^0C in amber bottles. Under these conditions however, β-carotene depletion is significantly lower ($p<0.05$) compared to values obtained in oils exposed to 15W fluorescent light. We explain this difference in terms of the protective effect of using amber bottles against photooxidation. For both oils, the loss of β-carotene at the end of the storage period was significantly higher ($p<0.05$) at 30^0C than at 20^0C (Table 2) indicating the sensitivity of β-carotene to changes in storage temperature.

The changes in lutein content are shown in Figures 4c and 4d. For oils stored in green and clear bottles, significant ($p<0.05$) reductions ranging from 33% to 39% were observed in both oils at the end of the storage period (Table 2). In both oils, percent depletion was slightly higher in clear bottles indicating a partial protective effect of using green on the lutein content of the oils. However, no significant difference in percent loss ($p<0.05$) was observed between oil types suggesting a similar behavior of lutein in both oils. In the case of oils in amber bottles stored at 20^0C and 30^0C, lutein concentration in both oil types remained essentially

constant up to week 12 and started to decrease at week 16. Percent depletion ranged from 15% to 30% for both oils with higher depletion observed at 30^0C as expected. Our data clearly show that β-carotene and lutein are better preserved when PSO and BSO are stored in amber bottles, which obviously protect the oils from oxidation due to direct exposure to light. The use of green bottles imparts only a partial protection to the carotenoid content of PSO and BSO. Moreover, the decrease in the concentration of lutein in both oils was, generally, lower than that of β-carotene under most of the experimental conditions indicating that β-carotene is more reactive and could be a better antioxidant than lutein. This observation is consistent with that of Miller et al. (*52*) who demonstrated that carotenes (i.e β-carotene) are more efficient quenchers of $ABTS^+$ radicals than xanthophylls (i.e lutein), with the exception of β-cryptoxanthin as influenced by the presence of functional groups with increasing polarities such as carbonyl and hydroxyl groups as well as by the number of conjugated double bonds. In terms of stability, lutein, in general was observed to have better stability than β-carotene as evidenced by the significantly lower ($p<0.05$) percentage of depletion of the former (in almost all cases except at 20^0C, in amber bottles). It has been shown that β-carotene is more susceptible to degradation than lutein (*53*).

In general, the observed depletion of β-carotene and lutein indicates that these compounds are being used up as lipid oxidation progresses suggesting their antioxidant property. Indeed, good correlations ($r=-0.70$ to -0.96) between β-carotene, lutein and the oxidative indices (PV and TBARS) were observed. According to Stahl and Sies (*54*), carotenoids react most efficiently with radicals generated in the process

of lipid peroxidation by trapping chain-carrying peroxyl radicals. Our results, however, are in contrast to those of Yi et al. (*46*) who suggested that loss in carotenoid content of red palm olein mixtures is independent of lipid oxidation and therefore carotenoids did not act as antioxidants. This discrepancy could be due to the different conditions used (air tight amber glass bottles vs air-permeable 10 ml polypropylene tubes). Carotenoids are effective as radical scavengers under conditions of low oxygen concentration (*55*), which was the case in oils stored in nonpermeable amber glass bottles as observed in the present study. The significant difference ($p<0.05$) in the evolution of lipid peroxidation in oils stored in clear and green glass bottles sealed with natural cork as opposed to those stored in amber bottles with rubber lined plastic caps could be explained by the oxygen permeability of the cork. The initial antioxidant activity of β-carotene is followed by a prooxidant action at high oxygen concentration (*53*). Moreover, the discrepancy in the rate at which PV exceeded the maximum regulatory limit between BSO (16 weeks) and PSO (8 weeks) could be partly due to the higher content of β-carotene in the former (Table 1). It has also been reported that β-carotene in combination with α-tocopherol provide a higher antioxidative capacity in a membrane system than β-carotene or α-tocopherol alone (*56*). BSO, which has both α-tocopherol and a significantly higher ($p<0.05$) content of β-carotene than PSO, would be expected to have higher oxidative stability, as was observed in the present study.

Changes in Total Phenolic Content and ORAC Values. The use of the Folin-Ciocalteu method to estimate the total phenolic content of a food sample has several

drawbacks. This assay suffers from inconsistencies in reported phenol values due to variations in the assay conditions used by different researchers, differences in the extraction conditions and the interference of other compounds (*35*). However, in order to enable comparison of data from previous studies, we used the assay to monitor the influence of the naturally-occurring polyphenolics in BSO and PSO on the oxidative stability of the oils.

The changes in the concentration of TP in BSO and PSO expressed as mg GAE/kg oil are shown in Figures 5a and 5b. In all cases, no significant changes ($p<0.05$) were observed in the TP values of either oil after 20 weeks of storage indicating excellent storage stability of these compounds even under prolonged conditions. Our results agree with those reported by Pacheco-Palencia et al. (*57*) who found no significant decline in the concentrations of phenolic compounds in Acai oils after 10 weeks of storage at 20^0C and 30^0C. Our data are also in agreement with those of Pacheco-Palencia et al. (*58*) who observed no significant decrease in non-anthocyanin polyphenolics of acai fruits during heating at elevated temperature (80^0C for up to 60 min).

Similarly, in all experimental conditions, the ORAC values did not significantly decrease (data not shown). This observation was clearly associated with the behavior of the TP observed suggesting that the phenolic content of BSO and PSO, which are responsible for the antioxidant capacity measured as ORAC values, do not contribute significantly to delaying oxidation of the oils.

There are only a few publications that correlate TP with the oxidative stability of pumpkin seed oils. Some of them disagree with the findings of the others. Results

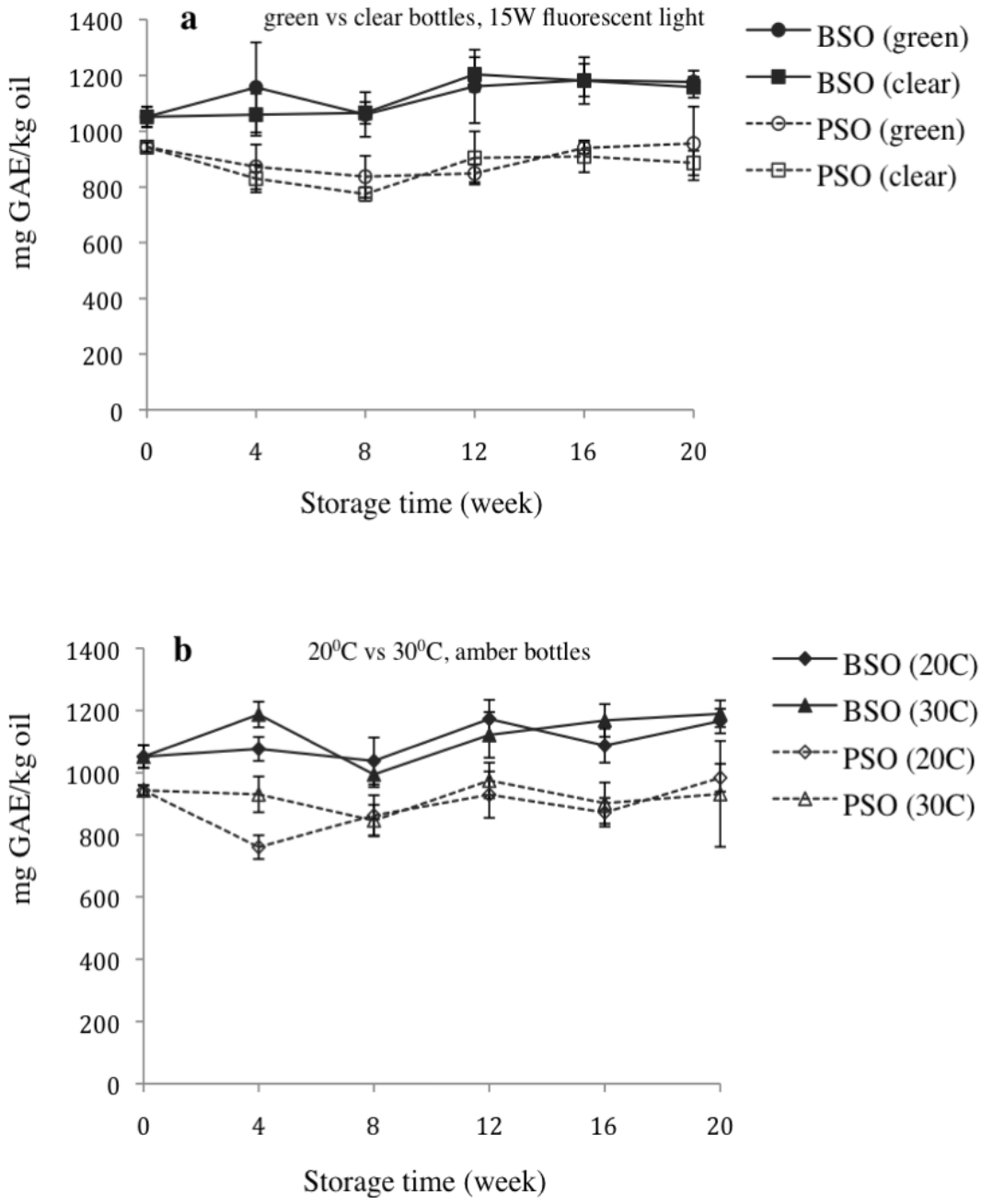

Figure 5. Changes in total phenolic content of cold-pressed BSO butternut squash (BSO) and pumpkin (PSO) seed oils under different storage conditions for 20 weeks. Error bars represent one standard deviation.

of the present study suggest that, since TP content of both oils remained essentially constant after week 20, polyphenols make no significant contribution to protecting PSO and BSO against oxidative deterioration. Our results are in agreement with those of Andjelkovic et al. (*59*) who found a low correlation (r=0.36) between polyphenolic content and the oxidative stability index (OSI) of pumpkin oils. In contrast, Fruwirth et al. (*60*) suggested that in pumpkin seed oil, 59% of the antioxidant capacity of the oil is due to polar phenolics while only 41%is contributed by tocopherols. However, other natural bioactive compounds with antioxidant activities such as carotenoids must be taken into account in assessing the protective effect of the natural antioxidants present in pumpkin oils.

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CHAPTER IV

CONCLUSION

The analysis of the natural antioxidant composition of cold-pressed pumpkin and butternut squash seed oils in this study provided us baseline information on the potential of these novel oils as functional foods and in pharmaceutical applications. We showed that cold-pressed BSO and PSO are good sources of antioxidant compounds, especially tocopherols and carotenoids. Interestingly, the two oils were different in terms of the concentrations of individual carotenoids. In PSO, lutein+zeaxanthin comprised more than 50% of the total carotenoid content while β-carotene was 80% in BSO. The tocopherol content of both oils was, however, very similar with γ-tocopherol as the most abundant (>90%) tocopherol isomer. The carotenoid and tocopherol contents detected in the oils were in agreement with those previously reported (Anjelkovic and others 2010; Stevenson and others 2007; Parry and others 2006; Fruwirth and others 2003). Variations in the concentration of the individual compounds can be attributed to some factors, which include differences in growing conditions, the inherent genetic differences, post-harvest handling, and processing conditions (Parry and others 2006).

Deterioration of oils as they age, especially highly unsaturated and unrefined oils, is an inevitable process. Presence of prooxidants and other external factors such as temperature, exposure to UV light, and so forth initiate lipid oxidation quickly after pressing the oils. Therefore, it is important to determine the stability of cold-pressed oils to gain knowledge applicable for their marketing and distribution. In this study,

we showed that the oils, provided proper storage conditions, may last for more than 15 months, showing only minimal oxidation under accelerated conditions (60^0C in the dark). We observed that cold-pressed PSO and BSO should be stored at temperatures below 30^0C and should, ideally, be contained in bottles with similar light transmission properties as amber bottles (0% transmission in the UV range) for maximum protection from oxidation. Moreover, we were able to demonstrate the effect of light transmission properties of green, clear and amber bottles as to the protection they give to cold-pressed oils. Amber bottles depicted excellent protection against oxidation in oils stored at either 20 or 30^0C. Green bottles, while they give partial protection to the oils, were found not ideal for use to distribute cold-pressed BSO and PSO. Rapid progress of oxidation was observed when the oils were stored in either green or clear bottles suggesting the minimal and insignificant protection they give to the oils. The oils exceeded the maximum limit for unrefined oils of 20 meq O_2/kg oil very quickly (after 8 and 12 weeks for PSO and BSO, respectively) when stored in either green or clear bottles and exposed to 15W fluorescent light. Our results are in agreement with previous studies that also showed the minimal protection that UV filters impart to oils (Pristouri and others 2010; Poiana and others 2009).

Aside from the effect brought about by external factors such as temperature and light, we observed a difference in the oxidative stability of BSO and PSO when exposed to light with BSO showing greater stability than PSO. This might be due to the differences in the natural antioxidants composition of these oils, particularly in their carotenoid content. Our findings on the behavior of the major carotenoids and tocopherols in PSO and BSO suggest that in the advancement of the oxidation process

these compounds contribute to delaying the oxidation process by acting as sacrificial antioxidants until they are oxidized and lose their antioxidant potential (Akoh 1994). Notable differences in the retention and depletion of the major antioxidants were observed. α-tocopherol was less stable than γ-tocopherol. In terms of carotenoids, lutein showed greater stability than β-carotene. With the exception of α-tocopherol, all the other three compounds showed good retention (>50%) after 15 days of storage under Schaal oven test. As has been established, 1 day under Schaal oven conditions is assumed to be equal to 1 month under normal storage conditions (Abou-Gharbia and others 1996). Taking this as a basis and confirmed by the storage studies conducted, our results suggest that after 15 months of storage under normal ambient temperature conditions, with good protection from light, BSO and PSO still retain significant amounts (>50%) of γ-tocopherol, β-carotene and lutein.

Synergistic action of the various naturally-occurring antioxidants in PSO and BSO is possible, however, this concept must be further studied. Potential prooxidant action of carotenoids, tocopherol and pigments such as chlorophyll also needs to be further elucidated. Results of our study affirm that presence of antioxidant compounds in cold-pressed oils is a novel and important criterion for quality control with respect to the storage stability and shelf life of these oils.

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