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Phytochemical Composition, Oxidative Stability, and Antioxidant Behavior of Roasted Expeller-Pressed Oils From Butternut Squash and Pumpkin Seeds

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ABSTRACT

Seed oils are increasingly valued for their nutritional quality and natural antioxidant content, yet their oxidative stability remains a critical quality concern. This study evaluated the phytochemical composition, antioxidant capacity, and oxidative stability of roasted butternut squash (*Cucurbita moschata*) (BSO) and pumpkin (*Cucurbita pepo*) seed oils (PSO). Major antioxidants, including tocopherols, tocotrienols, carotenoids, and phenolic compounds, were quantified, and antioxidant capacity was assessed. Oxidative stability indices were monitored during both accelerated storage and 20-week storage studies. Both oils were rich in γ-tocopherol and exhibited comparable oxygen radical absorbance capacity (ORAC) and total phenolic values. Five phenolic compounds were identified, including luteolin, tyrosol, vanillin, vanillic acid, and *trans*-cinnamic acid. Fatty acid analysis revealed that both oils were rich in unsaturated fatty acids, predominantly linoleic acid (57.14% in BSO and 48.54% in PSO) and oleic acid (18.34% in BSO and 33.38% in PSO), with relatively low levels of saturated fatty acids. Oxidation progressed slowly under dark storage, with peroxide and TBARS values remaining below the 20 meq O_2/kg regulatory limit for unrefined oils, indicating high intrinsic stability. Light exposure significantly accelerated oxidation, particularly in clear (90% UV light transmission) and emerald green bottles (60% UV light transmission), while amber bottles provided effective protection. Tocopherols and carotenoids showed progressive depletion during storage, displaying strong inverse correlations with oxidation indices. Total phenolic content measured by the Folin–Ciocalteu method, a nonspecific measure of total phenolics, remained stable over the 20-week storage. These findings demonstrate that roasted BSO and PSO possess favorable oxidative stability when appropriately packaged and stored below a temperature of 30°C and highlight their potential as value-added, antioxidant-rich specialty oils for functional food applications.

1 | Introduction

Pumpkin (*Cucurbita* spp.) is a globally cultivated agricultural commodity, valued not only as a food crop but also for its cultural, economic, and nutritional roles across many regions of the world. Worldwide production of pumpkins, including related squash and gourds, reached an estimated 27.8 million tonnes in 2022 [1], reflecting the crop's extensive cultivation and economic significance. Several *Cucurbita* species, such as *Cucurbita moschata* (butternut squash) and *Cucurbita pepo* L. (widely known as field pumpkin), are commercially cultivated for food processing, where the flesh is used in purees, soups, and baked goods. Because of these processing activities, substantial quantities of seeds are generated as a by-product. The seeds of

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C. pepo are characterized by a substantial oil content, typically ranging from 20% to 40% of the total seed mass, and are also abundant in bioactive compounds and unsaturated fatty acids. Seed oils upcycled from agricultural by-products, such as edible seeds, are of high interest to consumers because they are perceived as a healthy, safe, and natural product.

Pumpkin seed oil (PSO), especially when produced by specialty processes such as low-temperature mechanical pressing, has attracted interest due to its functional food attributes and associated antihyperglycaemic, cardioprotective, and chemoprotective effects [2], positioning it as a promising ingredient in health-oriented food markets. Valorization of pumpkin seeds by producing specialty oils not only creates new market opportunities for producers and processors but also addresses broader sustainability challenges within global food systems. Upcycling of processing by-products into high-value food ingredients enhances resource efficiency and reduces food loss and waste—an objective reflected in the United Nations Sustainable Development Goal 12: Responsible Consumption and Production [3].

The process of expeller pressing has gained prominence in the production of gourmet seed oils owing to its gentle methodology, which effectively preserves the inherent health-promoting constituents of the oils [4]. Oils derived from mechanical pressing are subjected to minimal thermal exposure (< 50°C) generated during the screw pressing process, and this technique does not incorporate any chemical treatments or solvents. Roasting of seeds prior to the screw pressing has been introduced prior to the expeller pressing step to enhance yield and impart distinctive aromatic qualities to the oil [5]. Roasting may augment the extractability of antioxidant phytochemicals and facilitate the generation of Maillard reaction products that could play a role in inhibiting lipid oxidation processes [6]. Nevertheless, owing to the unrefined characteristics of expeller-pressed seed oils, the presence of certain constituents, including metals or by-products of oxidative degradation, may accelerate lipid oxidation and adversely influence both the quality and shelf life of the oil [7].

While numerous studies have elucidated the nutritional composition of PSOs [8, 9], a limited number have rigorously scrutinized the role of intrinsic antioxidant systems in enhancing oxidative stability during storage under practical packaging and environmental conditions. Understanding these interrelationships is critical for converting compositional advantages into practical shelf life and quality benefits. Expeller-pressed seed oils, such as those derived from pumpkin seeds, are characterized by considerable concentrations of monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) as well as various antioxidant constituents [10]. This makes seed oils a suitable model for investigating the interactions and stability of natural antioxidants, specifically in terms of their role in mitigating oxidative deterioration. The oxidation process in oils may result in the generation of antinutritional elements or undesirable aromatic compounds [11]. Thus, an understanding of lipid oxidation phenomena in expeller-pressed oils, alongside the transformations undergone by their inherent antioxidants during storage, is crucial for maintaining both the nutritional and sensory attributes of these oils. This study was aimed at elucidating the behavior of the natural antioxidant constituents present in roasted expeller-pressed seed oils and establishing a correlation between the antioxidant compounds and the oxidative stability of the oils.

2 | Materials and Methods

2.1 | Reagents and Standards

Folin–Ciocalteu reagent; potassium iodide; (+)-6-hydroxy-2,5,7,8-tetramethylcromano-2-carboxylic acid (Trolox); sodium fluorescein; 2,2′-azobis (2-aminopropane) dihydrochloride (AAPH); randomly-methylated β-cyclodextrin (RMCD); β-/α-carotene; lutein; β-cryptoxanthin; zeaxanthin; vanillin; vanillic acid; tyrosol; luteolin; *trans*-cinnamic acid; and α-, γ-, and δ-tocopherols were obtained from Sigma-Aldrich (Allentown, Pennsylvania). Standards for tocotrienols were supplied by Santa Cruz Biotechnology (Santa Cruz, California). All standards were ≥ 96% purity. Unless otherwise stated, solvents were of analytical grade and were purchased from Fisher Scientific (Suwanee, Georgia).

2.2 | Extraction of Roasted Seed Oils

Roasted seed oils were provided by Stony Brook WholeHeartedFoods (https://www.wholeheartedfoods.com/), Geneva, New York. Seeds were harvested from fruit grown locally by Stony Brook's farmer partner, Martin Farms, in Brockport, New York. The pumpkin seeds were an oilseed pumpkin variety, *C. pepo* var. styriaca (commercial cultivar sourced from Johnny's Select Seeds of Fairfield, Maine), and butternut squash seeds were the Atlas hybrid variety of *C. moschata* (commercial cultivar sourced from Harris Seeds of Rochester, New York). Each variety was harvested and processed separately, with traceability records maintained by the producer to ensure botanical identity. Following fruit processing, seeds were manually separated from the pulp, washed with potable water to remove adhering flesh, and air-dried under controlled conditions prior to roasting. Briefly, dried butternut squash and pumpkin seeds underwent roasting for a duration of 30–35 min at a maximum temperature of 175°C. Expeller pressing was carried out at the company's processing facility located within Cornell University's New York State Agricultural Experiment Station and by trained personnel using a KOMET screw oil press (IBG Monforts Oekotec GmbH & Co., Germany) with a nominal processing capacity of approximately 5 kg/h, depending on seed type and operating conditions (Figure 1). The oil temperature during pressing was maintained below 50°C to minimize thermal degradation. The oils were allowed to settle for a minimum of 24 h, followed by coarse filtration. Subsequently, samples were transferred into amber glass bottles and immediately stored at −20°C until further analyses. For each oil type, two independent experimental replicates were produced under identical processing conditions, and all subsequent analyses were conducted using aliquots obtained from these batches.

2.3 | Analysis of Total Phenolic Content (TPC)

Determination of total phenolic (TP) was carried out using the Folin–Ciocalteu method [12] as previously described [13]. Briefly, 2 g of oil was dissolved in 5 mL of methanol, and the separation

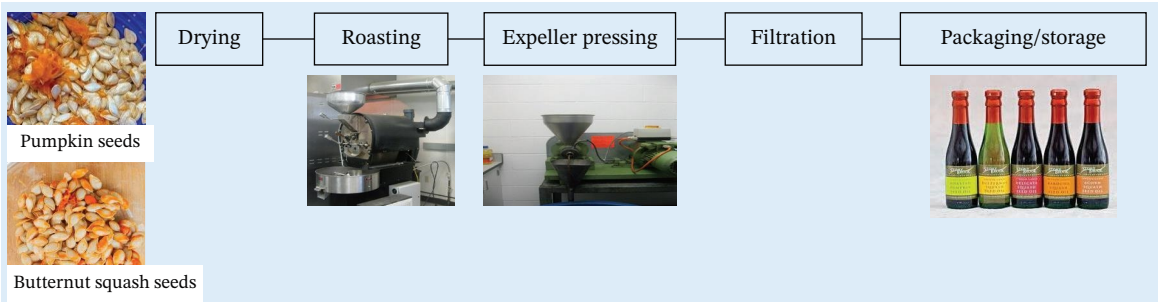

FIGURE 1 | Overview of the production steps of expeller-pressed roasted seed oils.

of the hydrophilic phase was done by centrifugation. This procedure was repeated two more times. An aliquot (200 μL) of the 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, Wisconsin). Quantification was carried out using a standard curve of gallic acid: $y = 124.2x + 0.006$; $R^2 = 0.999$. Results were expressed as milligram gallic acid equivalents (GAE) per kilogram of oil.

2.4 | 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 et al. [14] was followed with some modifications [13]. The assay was carried out on a fluorescence microplate reader (Biotek Instruments Inc., Winooski, Vermont) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm set at a temperature of 37°C. AAPH and fluorescein were used as the radical generator and fluorescent probe, respectively. Each well in a 96-well microplate contains 150 μL fluorescein solution (0.004 mM), 25 mL AAPH solution (0.15 M), and 25 μL of appropriately diluted methanolic extract. A standard curve using Trolox (6.25–100 μM) was generated, and results were expressed as millimoles of Trolox equivalents per gram (micromoles of Trolox equivalents per gram). For the lipophilic ORAC (L-$ORAC_{FL}$) value determination, oil samples (2 g) were extracted twice in a 15-mL screw-cap tube with 10 mL of hexane. The hexane fractions were combined and dried using a rotary evaporator. Dried hexane extracts were diluted with 750 mL 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.

2.5 | HPLC Analysis of Phenolics

Individual phenolic quantification was carried out by HPLC analyses based on a method described previously [15] with some modifications [13]. Methanolic extracts were appropriately diluted with methanol and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter. Separations were conducted on a reversed-phase HPLC system (Hewlett-Packard model 1100; Palo Alto, California) equipped with a photodiode array detector (PDA). Samples (10 μL) were injected using a C18 reversed-phase symmetry analytical column (250 × 4.6 mm, 5 μm; Water Corp., Milford, Massachusetts), 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°C according to 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, and 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.

2.6 | HPLC Analysis of Tocopherols

Tocopherols and tocotrienols were analyzed by HPLC based on a previous method [16] with some modifications [13]. 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 a reversed-phase HPLC system (Hewlett-Packard model 1100; Palo Alto, California) equipped with a fluorescence detector. An Eclipse C18 (4.6 × 150 mm, 5 μm) column was used with methanol:acetonitrile:water (50:44:6, $v/v/v$) 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°C, with the fluorescence detector excitation and emission wavelengths set at 290 and 330 nm, respectively. Identification of compounds was based on retention times and UV absorption spectra, while an external standard method was used for quantification.

2.7 | HPLC Analysis of Carotenoids

Carotenoid extraction and chromatographic analysis were carried out following a previously reported method [17] with slight modifications [13]. Samples (5 g of oil) were dissolved in 25 mL of acetone and evaporated using a rotary evaporator. The extract was redissolved in 25 mL pure ethyl ether, and an equal volume of KOH in MeOH (4% w/v) solution was added for saponification. Saponification is a preparatory step performed to hydrolyze carotenoid esters and remove coextracted lipids and other interfering compounds, thereby improving extraction efficiency, chromatographic separation, and detection of carotenoids in complex oil matrices [18]. To stop the reaction and remove

excess alkali, 2 mL of 10% (w/v) NaCl solution was added to the mixture. After evaporation of the ether phase, the remaining pigment was redissolved in 95% ethanol (stabilized with 30 ppm butylated hydroxytoluene), transferred to an amber vial, flushed with nitrogen (N_2), and kept at −20°C. The carotenoid extracts were filtered through a 0.45 μm PTFE syringe filter and injected into an HPLC system (Hewlett-Packard model 1100; Palo Alto, California) equipped with a PDA. Carotenoids were separated on an Eclipse C18 (4.6 × 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°C according to 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. Results were expressed in milligrams of compound per kilogram of oil (milligrams per kilogram).

2.8 | Measurement of Initial Oxidation and Quality Indices

The American Oil Chemists' Society (AOCS) Official Methods [19] were used to determine peroxide value (PV, Method Cd 8-53) expressed as milliequivalents of active oxygen per kilogram of oil, free fatty acid value (FFA, Method Ca 5a-40) expressed as a percentage of oleic acid, acid value (AV, Method Cd 3d-63) expressed as milligrams of potassium hydroxide per gram, and thiobarbituric acid reactive species (TBARS, Method Cd 19-90) expressed as milligrams of malondialdehyde per kilogram of oil. Briefly, the reaction mixture was heated to promote formation of the MDA-TBA complex, and the absorbance was measured spectrophotometrically at 532 nm. Quantification was performed using a calibration curve prepared with a malondialdehyde standard [20]. Experiments were performed in triplicate, and results were reported as mean values ± standard deviation.

2.9 | Determination of Fatty Acid Composition

Oil samples were analyzed for fatty acids as described in detail previously [21]. Briefly, oils were dissolved in heptane, and fatty acid methyl esters (FAME) were prepared by treatment with boron trifluoride (BF_3) and methanol (Sigma Chemical Co., St. Louis, Missouri). FAME was analyzed by gas chromatography using a BPX-70 column (60 m × 0.32 × 0.25 μm) with H_2 as the carrier gas and with a temperature-programmed oven. An equal weight standard (GLC reference standard GLC-68A, Nuchek Prep, Elysian, Minnesota) was used to measure response factors, which were applied to the raw data. FAME mass spectral assignments were confirmed by conversion of the FAME to picolinyl ester derivatives according to previous methods [22, 23], followed by GC/MS analysis and comparison to literature spectra as previously demonstrated [21]. FAME concentrations were expressed as percent weight per weight of the total FAME detected.

2.10 | Oxidative Stability Experiments

2.10.1 | Accelerated Storage Test

An accelerated storage test (Schaal oven) [19] was carried out to evaluate oil oxidative stability and antioxidant retention [13]. Briefly, equal amounts of each oil were loaded into 60-mL amber bottles with rubber-lined plastic caps and subjected to a 15-day storage in an oven maintained at 60°C ± 1°C (Lab-Line Instruments, Inc., Melrose, Illinois). Monitoring of the evolution of PV (Method Cd 8-53) [19] and TBARS (Method Cd 19-90) [19], as well as changes in tocopherol, carotenoid, and TPC, was carried out. A series of two bottles (independent experimental replicates) for each oil type was prepared, and triplicate analytical measurements were obtained from each bottle at scheduled intervals.

2.10.2 | Twenty-Week Storage Study

To study the effect of storage temperature (Figure 2), oil samples from each extraction batch were placed in amber bottles, leaving a half-inch headspace. The headspace was flushed with N_2, and the bottles were sealed with a screw-type rubber-lined cap of negligible permeability to oxygen [13]. Containers were stored in two separate thermostatic chambers in the absence of light using two temperatures: 20°C and 30°C. To study the effect of packaging light transmission property (percentage transmission in the UV range), clear (90% T) and emerald green (60% T) glass bottles (180 mL, Waterloo Container, Waterloo, New York) were covered with

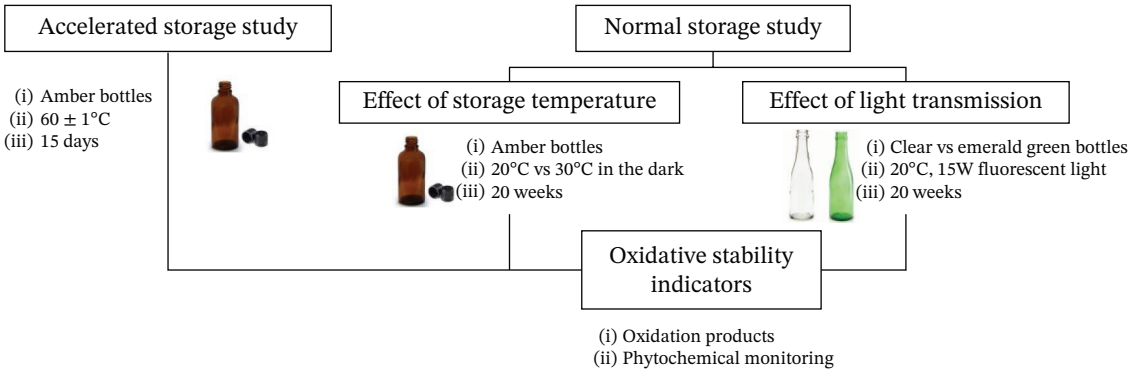

FIGURE 2 | Overview of the storage studies and oxidative stability monitoring.

natural cork. The natural cork was intentionally selected to simulate realistic commercial packaging conditions for cold expeller-pressed oils, where both light exposure and oxygen ingress contribute to oxidative changes. Oils were stored in a temperature-controlled (20°C) chamber with constant exposure to an artificial light (15 W fluorescent lamp). For each oil type, two independent experimental replicates were subjected to the respective storage conditions. All samples were held under the above conditions for 20 weeks. Starting at time zero and at 4-week intervals thereafter, triplicate analytical measurements were obtained from each independent experimental replicate for scheduled analyses. Monitoring of the evolution of PV (Method Cd 8-53) [19] and TBARS (Method Cd 19-90) [19], as well as changes in tocopherol, carotenoid, and TPC, was carried out.

2.11 | Statistical Analysis

Experiments were conducted using either independent experimental replicates or analytical replicates, as specified for each analysis. For storage studies, two independent experimental replicates (separate bottles) were prepared for each condition, and triplicate analytical measurements were performed from each bottle at each sampling point. For compositional analyses, measurements were conducted in triplicate (analytical replicates) unless otherwise stated. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, California, United States). Differences in means were evaluated using Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test. Statistical significance was defined at $p < 0.05$. For all statistical comparisons, the independent experimental replicate (n = number of bottles or batches) was considered the unit of analysis, while analytical replicates were used to estimate measurement variability. Pearson's correlation analysis was conducted using GraphPad Prism and considered to be significant at a value of $p < 0.05$.

3 | Results and Discussion

3.1 | Physicochemical Characteristics of Expeller-Pressed Oils

Freshly produced butternut squash oil (BSO) and PSO revealed predominantly similar initial physical and chemical properties (Table 1). The FFA concentrations of both oils remained within the maximum threshold of 2.0% for unrefined oils as per Commission Regulation (EC) No. 251/2011, with BSO exhibiting a lower initial concentration (0.40% oleic acid) in contrast to PSO (1.01% oleic acid). The minimal FFA levels observed in the fresh oils were likely generated during the seed preparation process preceding expeller pressing. The initial AV was lower for BSO (1.18 mg of KOH/g oil) in comparison to PSO (2.55 mg of KOH/g oil). An elevated AV indicates a higher degree of lipolysis, possibly resulting from enzymatic actions within the seeds during upstream preparation stages [24]. Both oils exhibited low initial PV (1.46 and 1.96 meq O_2/kg) and TBARS values (1.21 and 1.52 mg MDA/kg). These minimal concentrations of oxidation products in fresh oils can be ascribed to their generation during the preprocessing handling of the seeds.

TABLE 1 | Initial physicochemical characteristics of expeller-pressed butternut squash (BSO) and pumpkin (PSO) seed oils subjected to oxidative stability tests*.

	BSO	PSO
Peroxide value (meq O_2/kg)	1.46 ± 0.00^a	1.96 ± 0.60^b
TBARS* (mg MDA/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)	0.59 ± 0.00^a	1.31 ± 0.10^b
Color		
L^*	38.08 ± 0.28^a	25.27 ± 0.10^b
a^*	5.11 ± 0.04^a	2.46 ± 0.18^b
b^*	21.09 ± 0.27^a	0.17 ± 0.07^b

Note: Different letters between columns represent significant differences ($p < 0.05$).
Abbreviations: MDA, malondialdehyde; meq, milliequivalents; TBARS, thiobarbituric acid reactive species.
*All results are reported as means of three replicates ± standard deviation.

3.2 | TPC, Phenolic Compounds, and Antioxidant Capacity

A slightly higher TPC was observed in BSO in comparison to that of PSO (Table 2). Several studies ([25, 26]; G. [27]) have documented varying TP values in PSO, which can be attributed to factors impacting TP assay results, such as the presence of substances (e.g., small amounts of proteins) that could interfere with the assay and differences in assay procedure. In the case of PSO, only two phenolic compounds were detected, namely *trans*-cinnamic acid (accounting for 75.6% of the overall detectable phenolic content) and tyrosol, whereas in BSO, five compounds were identified, including vanillin, vanillic acid, *trans*-cinnamic acid, luteolin, and tyrosol. *Trans*-cinnamic acid was significantly higher in PSO compared to BSO. The phenolic compounds identified were similar to those reported in earlier research [26, 28], but additional compounds found in previous studies, such as p-coumaric acid, protocatechuic acid, caffeic acid, ferulic acid, syringic acid, gallic acid, and p-hydroxybenzoic acid, were not found in our samples.

Limited detection of phenolics in the samples may be attributed to several factors. First, the roasting of seeds prior to expeller pressing may have contributed to the limited number of detected phenolics, as thermal processing can lead to the degradation or transformation of heat-sensitive phenolic compounds, as well as interactions with Maillard reaction products that may hinder their extraction and detection [29, 30]. The expeller pressing process may have further contributed to the limited recovery of phenolics, as many phenolic compounds, especially polar phenolics, tend to remain in the residual press cake rather than partitioning into the oil phase [31, 32]. Mechanical extraction is less efficient than solvent-based methods in recovering bound or matrix-associated phenolics [33]. In oil matrices, the high abundance of triacylglycerols and other

TABLE 2 | Phenolic compounds and hydrophilic and lipophilic ORAC (H- and L-ORAC) values of expeller-pressed pumpkin (PSO) and butternut squash (BSO) seed oils*.

	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	nd	—
Vanillic acid	0.97 ± 0.40	39.3	nd	—
Tyrosol	0.87 ± 0.02^{a}	35.24	0.73 ± 0.20^{a}	24.4
Luteolin	0.16 ± 0.05	6.45	nd	—
Total	2.46 ± 0.50^{a}		2.96 ± 0.10^{a}	
TP** (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}	

Note: Different letters between columns PSO and BSO represent significant differences ($p < 0.05$).
Abbreviation: nd, not detected.
*All results are reported as mean values of three replicates ± standard deviation.
**TP: total phenolics expressed as gallic acid equivalents (GAE).

nonpolar components can interfere with the extraction of phenolic compounds, as coexisting lipids influence solvent partitioning [34]. Additionally, differences in varietal origin, agronomic conditions, seed storage, and oil processing techniques may further contribute to the variability in phenolic profiles observed across studies [35–37].

Phenolic compounds are a main component with profound influence on the stability of dietary oils owing to their ability to halt or reduce lipid peroxidation by free radical quenching [38, 39]. Antioxidant properties attributed to phenolics are commonly measured using various antioxidant capacity assays [40], including the ORAC assay. The antioxidant capacity of both oils (expressed as H- and L-ORAC values) was comparable despite variations in their individual phenolics. Our results were consistent with other studies reporting considerable antioxidant capacity values in expeller-pressed PSO ([41]; G. [27]). To our knowledge, there are no previous reports on the L-ORAC values for BSO. The antioxidant capacity of expeller-pressed oils or seed oils in general has been reported using various chemical antioxidant assays [35, 41, 42], making comparison difficult.

A notable discrepancy was observed between the TPC determined by the Folin–Ciocalteu assay (~1000 mg GAE/kg) and the sum of individual phenolic compounds quantified by HPLC (~2.5–3.0 mg/kg). This difference can be attributed to the nonspecific nature of the Folin–Ciocalteu method, which measures total reducing capacity rather than phenolics exclusively. In lipid matrices such as edible oils, various compounds, including tocopherols and lipid oxidation products, can react with the Folin reagent, leading to an overestimation of phenolic content [43–45]. Furthermore, antioxidant capacity assays (e.g., ORAC) and Folin-based measurements may be influenced by oxidation-derived species and free radicals present in oils, particularly during storage, which can contribute to assay responses independent of phenolic compounds [42, 46]. In addition, melanoidins and other Maillard reaction products formed during roasting possess strong reducing capacity and may contribute to the elevated values obtained using the Folin–Ciocalteu assay [5, 47]. In contrast, HPLC provides a more selective quantification of individual phenolics, which explains the substantially lower values observed. Therefore, TPC values obtained from Folin assays in oil systems should be interpreted with caution, as they may reflect the cumulative reducing capacity of multiple compounds rather than true phenolic content.

3.3 | Tocopherol and Tocotrienol Contents

Tocopherols and tocotrienols, collectively referred to as vitamin E compounds, exist predominantly as α-, β-, γ-, and δ-isomers. However, not all isomers exhibit equivalent vitamin E activity, with α-tocopherol being the most biologically active form in humans. Despite this, all tocopherols and tocotrienols function as lipid-soluble antioxidants, capable of inhibiting lipid peroxidation by scavenging free radicals and interrupting autoxidation reactions [48]. Three tocopherol isomers (δ, γ, and α) were detected (Table 3 and Figure S1), all of which were higher in BSO than in PSO. Our HPLC method did not resolve β-tocopherol as a distinct peak. This is likely due to the coelution of β- and γ-tocopherol on reversed-phase C18 columns [49], which limits their chromatographic separation under typical conditions. As a result, β-tocopherol may be present but not individually quantifiable, and the detected peak is predominantly attributed to γ-tocopherol. This limitation has been reported in previous studies analyzing tocopherols in vegetable oils [27, 50]. γ-Tocopherol was the predominant tocopherol in both oils, comprising > 90% of the total tocopherol content. δ-Tocotrienols and γ-tocotrienols were also detected, with the latter comprising 67%–84% (4.5–5.8 mg/kg) of the total tocotrienol contents of both oils. This is consistent

TABLE 3 | Peak assignments, retention times, concentrations, and relative abundance of tocopherols and tocotrienols in expeller-pressed pumpkin seed (PSO) and butternut squash (BSO) seed oils*.

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	nd	—	nd	—
Total		8.6 ± 1.6^{a}		5.3 ± 0.3^{b}	

Note: Different letters between columns PSO and BSO represent significant differences ($p < 0.05$).
Abbreviation: nd, not detected.
*All results are reported as mean values of three replicates ± standard deviation.

TABLE 4 | Peak assignments, retention times, concentration, and relative abundance of carotenoids in expeller-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	nd	—	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}	

Note: Different letters between columns PSO and BSO represent significant differences ($p < 0.05$).
Abbreviation: nd, not detected.
*All results are reported as mean values of three replicates ± standard deviation.

with a previous study that detected 6.9 mg/kg of γ-tocotrienols in roast pumpkin seeds from the Slovenian *C. pepo* L. variety "Slovenska golica" [51]. However, a recent study did not detect γ-tocotrienols in *C. moschata* expeller-pressed seed oil from Brazil [52]. Quantitatively, both oils exhibited the following trend of tocopherol and tocotrienol relative abundance: γ-tocopherol > α-tocopherol > δ-tocopherol and γ-tocotrienols > δ-tocotrienols. Our results were consistent with recent studies that showed a similar trend in compound abundance in PSO, especially for tocopherols [53–55].

3.4 | Carotenoid Content

Like tocopherols, carotenoids are equally important secondary metabolites present in seed oils owing to their potent antioxidant effects. PSO had five detectable carotenoids, including lutein + zeaxanthin, α-carotene, β-cryptoxanthin, and β-carotene (Table 4 and Figure S2). BSO contained four carotenoids, with β-cryptoxanthin undetectable in the samples tested. The HPLC separation method we adapted was unable to resolve lutein and zeaxanthin into separate peaks. Lutein and zeaxanthin are isomeric compounds, possessing identical chemical formulas that differ only in the placement of double bonds [56]. While suitable for separating most carotenoids, commonly used reversed-phase C18 HPLC columns are unable to separate structural isomers of carotenoids such as lutein and zeaxanthin. For this reason, we deemed it reasonable to report the corresponding peak as lutein + zeaxanthin, basing quantification on a lutein standard curve, given a previous study's findings showing lutein as a major carotenoid in the flesh of pumpkins and butternut squash [57].

Lutein + zeaxanthin comprised > 50% of the total carotenoid in PSO, which also contained β-carotene, β-cryptoxanthin, and α-carotene at relatively lower concentrations. Contrastingly, β-carotene comprised greater than 80% of total carotenoids in BSO, with lutein + zeaxanthin comprising only 6.2% of the total value. Our results were comparable to recent studies that reported the same carotenoids in PSO. To our knowledge, there are no previous reports on the carotenoid content of BSO produced

from butternut squash seeds sourced from the Northeastern part of the United States. Although given the presence of carotenoids in *C. moschata* puree (Ninčević Grassino), it is expected that BSO contains similar carotenoids.

3.5 | Fatty Acid Contents

Unsaturated fatty acids, particularly linoleic (C18:2) and oleic (C18:1) acids, were predominant in both oils (Table 5), with the former comprising 57.14% in BSO and 48.54% in PSO. In contrast, PSO exhibited a higher oleic acid content (33.38%) compared to BSO (18.34%). Among saturated fatty acids (SFAs), palmitic acid (C16:0) was the dominant component, with a notably higher concentration in BSO (17.95%) compared to PSO (11.36%). Stearic acid (C18:0) was present at comparable levels in both oils (~5%), while other SFAs were detected only in minor amounts (< 1%).

TABLE 5 | Fatty acid contents (percentage) of expeller-pressed butternut squash (BSO) and pumpkin seed (PSO) oils.

Fatty acid	BSO	PSO
Myristic acid (14:0)	0.13 ± 0.00	0.13 ± 0.02
Pentadecanoic acid (15:0)	0.04 ± 0.01	0.02 ± 0.01
Palmitic acid (16:0)	17.95 ± 0.14[a]	11.36 ± 0.31[b]
Palmitoleic acid (16:1)	0.19 ± 0.00	0.14 ± 0.01
Heptadecanoic acid (17:0)	0.08 ± 0.01	0.06 ± 0.01
Heptadecenoic acid (17:1)	0.03 ± 0.00	0.02 ± 0.01
Stearic acid (18:0)	4.84 ± 0.07	5.06 ± 0.08
Oleic acid (18:1)	18.34 ± 0.05[a]	33.38 ± 0.23[b]
Linoleic acid (18:2)	57.14 ± 0.13[a]	48.54 ± 0.07[b]
Linolenic acid (18:3)	0.32 ± 0.00	0.16 ± 0.01
Arachidic acid (20:0)	0.32 ± 0.01	0.42 ± 0.01
Eicosenoic acid (20:1)	0.07 ± 0.00	0.11 ± 0.01
Behenic acid (22:0)	0.14 ± 0.08	0.17 ± 0.04
Lignoceric acid (24:0)	0.05 ± 0.01	0.07 ± 0.04

Note: All results are reported as mean values of three replicates ± standard deviation. Different letters between values (PSO vs. BSO) represent significant differences ($p < 0.05$). Values without superscript letters are not significantly different ($p < 0.05$).

MUFAs were mainly represented by oleic acid, with minor contributions from palmitoleic (C16:1), heptadecenoic (C17:1), and eicosenoic (C20:1) acids. The higher MUFA content in PSO may confer improved oxidative stability relative to more PUFA-rich oils, as MUFAs are less susceptible to lipid peroxidation. PUFAs were dominated by linoleic acid, with only trace amounts of linolenic acid (C18:3) detected (0.32% in BSO and 0.16% in PSO). The low levels of linolenic acid are advantageous from a stability standpoint, as this fatty acid is highly prone to oxidation.

Overall, the fatty acid profiles of both oils align with previously reported compositions of PSOs, which are typically rich in linoleic and oleic acids with relatively low levels of SFAs [10, 54, 58]. This contributes to the recognized nutritional value of PSO, including potential cardioprotective effects [59]. The higher linoleic acid content in BSO suggests greater susceptibility to oxidative degradation, whereas the higher oleic acid content in PSO may contribute to enhanced oxidative stability. These compositional differences likely play a role in the observed variations in oxidative behavior during storage discussed in subsequent sections.

3.6 | Oxidative Stability During Storage

We assessed the oils' oxidative stability using both accelerated conditions (Schaal oven test) and a 20-week storage study (Figure 2), evaluating oxidation through parameters such as PV and TBARS. PV is a measure of primary oxidation product evolution, while TBARS represents the evolution of secondary breakdown products such as carbonyl compounds (aldehydes and ketones) [60]. Under accelerated storage conditions, a slow oxidation rate was observed in both oils (Figure 3a,b), with PV not exceeding the maximum limit of 20 meq O_2/kg for unrefined oils (Codex [61]), indicating excellent oxidative stability. Accelerated oxidation tests such as the Schaal oven method (60°C–65°C) are commonly used to simulate

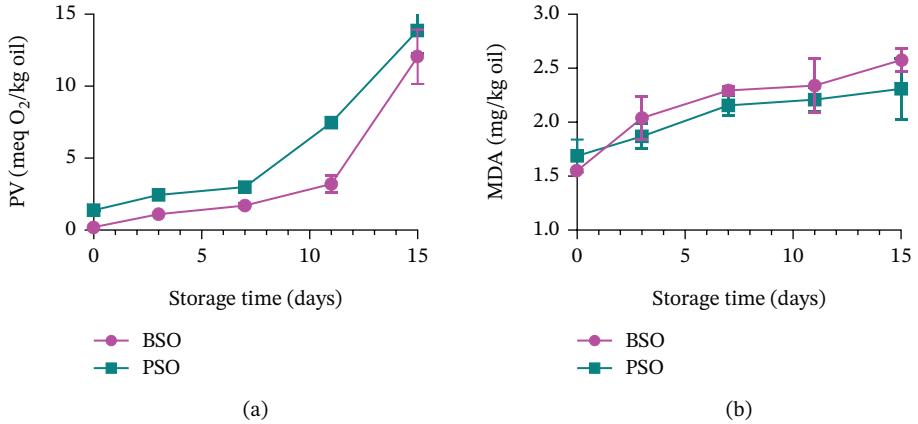

FIGURE 3 | Changes in oxidative stability indices of the oils during an accelerated storage study. (a) Peroxide value (PV) changes during accelerated storage at 60°C in the dark. (b) Changes in thiobarbituric acid reactive substances (TBARS) during accelerated storage at 60°C in the dark. Data are presented as mean values from two independent experiments (with triplicate samples from each experiment analyzed across all samples collected at the specified sampling intervals). Error bars represent standard deviations.

long-term storage at ambient conditions. Lipid oxidation rates increase exponentially with temperature and are commonly described by Arrhenius kinetics or Q_{10} relationships, whereby reaction rates increase approximately two- to three-fold for every 10°C rise [62, 63]. Consequently, recent studies have used the approximation that 1 day of storage under Schaal oven conditions (65°C) is equivalent to 4 weeks of storage at room temperature (28°C ± 2°C) [64, 65]. Therefore, the shelf life of BSO and PSO can be estimated to be at least 15 months, provided the oils are stored in the dark.

In the 20-week storage study, oxidative changes under both temperature-controlled and light-exposed conditions are presented in Figure 4. Specifically, Figure 4a shows the evolution of PV for oils stored in amber bottles under dark conditions at 20°C and 30°C, while Figure 4b presents PV changes for oils stored at 20°C under continuous fluorescent light in clear (90% transmission) and emerald green (60% transmission) glass bottles. Corresponding secondary oxidation products (TBARS) under these conditions are shown in Figure 4c (temperature study) and Figure 4d (light exposure study), respectively.

Under dark conditions (Figure 4a), a gradual increase in hydroperoxides was observed, with similar PV values at 20°C and 30°C by Week 20, indicating that both oils remained within acceptable oxidation limits. Similar to those observed in the accelerated study, PV values at both temperatures (after 20 weeks) were below the 20 meq O_2/kg maximum limit (Codex [61]), indicating oxidative stability of the oils. Our results were consistent with findings of recent studies that demonstrated good oxidative stability of oils stored at temperatures below 30°C [42, 66].

In contrast, under light exposure (Figure 4b), rapid PV evolution was observed, particularly in PSO, demonstrating the pronounced effect of light-induced oxidation. PV values peaked earlier under light exposure (Week 8 for PSO and Week 16 for BSO), followed by a decline corresponding to the formation of secondary oxidation products, as reflected in TBARS values (Figure 4c,d). Oils stored in clear and green bottles exceeded the regulatory PV limit more rapidly than those stored in the dark, indicating that light exposure had a stronger impact on oxidation than temperature differences within the tested range. Although green bottles provided partial light protection compared to clear bottles, the effect was limited, suggesting that partial light transmission reduction alone is insufficient to effectively prevent photooxidation. These findings highlight that both packaging type (light transmission properties) and storage conditions (light vs. dark, temperature) play critical roles in determining oxidative stability. Furthermore, the observed

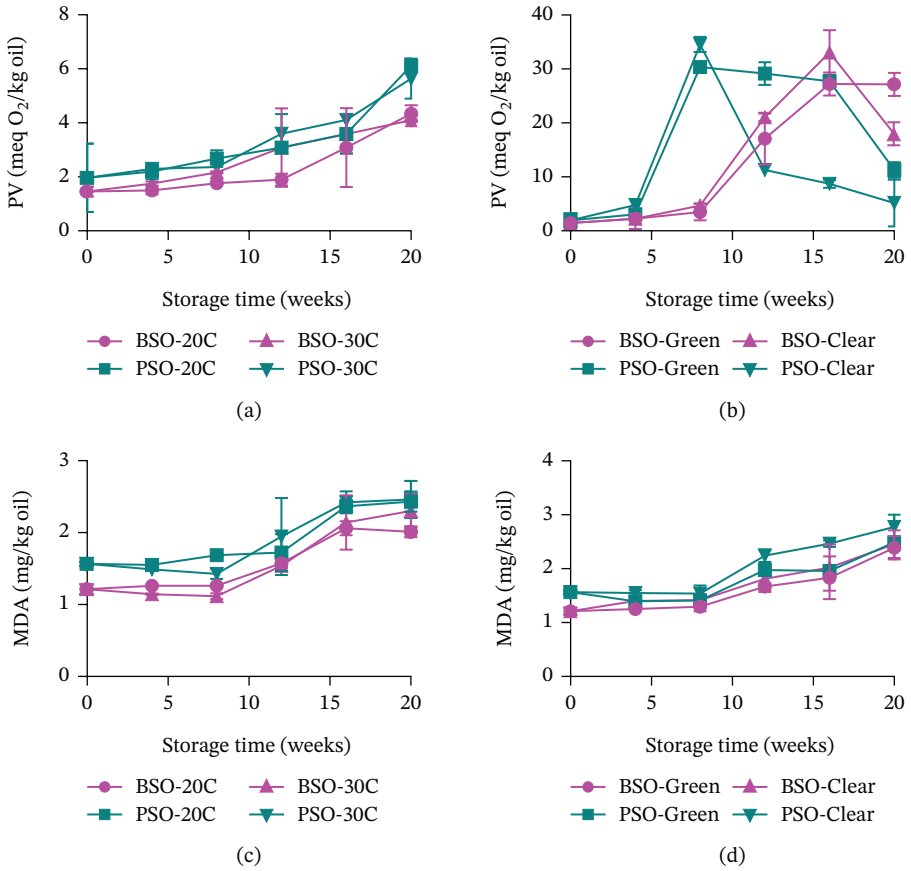

FIGURE 4 | Changes in oxidative stability indices of the oils during a 20-week storage study. (a) Effect of storage temperature on peroxide value (PV) under dark storage in amber bottles. (b) Effect of packaging light transmission on PV under light exposure at 20°C in clear (90%T) and green (60%T) bottles. (c) Effect of storage temperature (20°C vs. 30°C) on thiobarbituric acid reactive substances (TBARS) under dark storage conditions. (d) Effect of packaging light transmission on TBARS at 20°C in clear (90%T) and green (60%T) bottles. Data are presented as mean values from two independent experiments (with triplicate samples from each experiment analyzed across all samples collected at the specified sampling intervals). Error bars represent standard deviations.

differences in oxidative stability of the oils stored in the same colored-glass bottles suggest that, aside from the protection conferred by the packaging material, the intrinsic antioxidant contents of the oils significantly play a role in the oils' oxidative stability. Our results showed that, to more effectively protect expeller-pressed oils from photooxidation, oils must be stored in amber glass bottles and tightly sealed.

It should be noted that the present study primarily evaluates oxidative stability through oxidation indices (PV and TBARS) and changes in antioxidant constituents, without direct assessment of fatty acid composition during storage, which represents a limitation of the study. The observed differences in fatty acid composition between freshly produced BSO and PSO (Table 5) may also contribute to their oxidative behavior during storage. BSO exhibited a higher linoleic acid (C18:2) content compared to PSO, indicating a greater proportion of PUFAs, which are generally more susceptible to lipid peroxidation due to the presence of bis-allylic hydrogen atoms [8, 9, 67]. In contrast, the relatively higher oleic acid (C18:1) content in PSO suggests a greater proportion of MUFAs, which are comparatively more resistant to oxidative degradation. Based solely on fatty acid composition, BSO would therefore be expected to exhibit lower oxidative stability than PSO. However, the oxidative stability results indicate that BSO demonstrated comparable or, in some cases, improved resistance to oxidation. This suggests that the effects of fatty acid composition are modulated by the presence of endogenous antioxidants, particularly tocopherols and carotenoids, which may play a dominant role in determining overall oxidative stability in these expeller-pressed oils [68].

3.7 | Behavior of Natural Antioxidants During Storage

Besides the effect of the different storage conditions discussed above, protection against oxidation conferred by antioxidant phytochemicals may explain the observed differences in the oils' oxidative stability at different storage conditions. To better understand the role of the natural antioxidative components in the seed oils, we quantitatively monitored changes in their major phytochemical contents under both accelerated and 20-week storage studies.

3.7.1 | Changes in Major Tocopherols

For oils stored in clear or green bottles with constant exposure to fluorescent light, α-tocopherol (Figure 5a) was undetectable after 8 weeks, suggesting that this compound was quickly used up. Significant reductions in γ-tocopherol (Figure 5c) were also observed, suggesting that it was also being used up, although to a lesser degree than α-tocopherol (Table 6). Our results were consistent with those of a recent study that showed depletion of α-tocopherol at a much faster rate than γ-tocopherol in corn oils [69]. This can be explained by the relatively low O–H bond dissociation energy and lower redox potential of α-tocopherol, which means that it is a stronger hydrogen donor. This property makes it an effective chain-breaking antioxidant but also renders it susceptible to oxidation during radical scavenging [69, 70]. The decreases in both α- and γ-tocopherol showed modest-to-strong negative correlations with the formation of primary (PV) and secondary (TBARS) products (Table 7). In BSO, γ-tocopherol had a highly significant negative correlation with both PV and TBARS in both packaging materials, indicating that it is being depleted as oxidation products are generated. Contrastingly, for oils stored at 20°C and 30°C, α-tocopherol was largely unchanged up to Week 16 and only decreased by 14%–23% at Week 20 (Figure 5b and Table 6). γ-Tocopherol remained essentially constant (7% reduction at week 20) under both storage temperatures (Figure 5d and Table 6). The correlations between α- and γ-tocopherol and the oxidation indices were weaker in oils stored in amber bottles at either 20°C or 30°C than those exposed to light (Table 7). This is clearly due to compound levels remaining unchanged for most of the 20-week storage.

Although it is widely acknowledged that α-tocopherol represents the most biologically active isomer, its efficacy as an antioxidant in vitro is inferior to that of γ-tocopherol, which possesses the ability to form γ-tocopherol diphenyl dimer, augmenting its reactivity [71]. This suggests that oils with higher contents of γ-tocopherol may exhibit better oxidative stability. Therefore, the slightly higher γ-tocopherol content in BSO may partially explain its better oxidative stability than PSO. α-Tocopherol may function as a pro-oxidant when present in elevated concentrations, a phenomenon that can be ascribed to the generation of the tocopheroxyl [72]. Given that PSO contains high levels of C18:1 and C18:2 unsaturated FA [27, 73], α-tocopherol may enhance oxidation during the initial stages of storage (as demonstrated by its faster decomposition, Figure 5a) through the formation of α-tocopheroxyl radicals involved in secondary radicals [74]. The same trends in stability of α-tocopherol and γ-tocopherol were observed during the accelerated storage study, with the former depleting more rapidly (Figure S3).

3.7.2 | Changes in Major Carotenoids

The concentrations of lutein and β-carotene, two major carotenoids in the oils, were monitored over the 20-week study. For oils contained within green or clear bottles and subjected to fluorescent light exposure, a notable reduction (> 50%, Table 6) in β-carotene concentration was recorded by Week 8, followed by further gradual reductions (Figure 5e). At Week 20, β-carotene levels diminished by 74%–82% (Table 6). There were no significant differences in percentage losses between oils stored in green versus clear bottles, suggesting a negligible protective effect afforded by partial light transmission blocking on β-carotene stability. Substantial reductions in β-carotene concentrations (21%–69%) (Figure 5f and Table 6) were similarly noted in both oils maintained at 20°C and 30°C within amber bottles. Under these conditions, the depletion of β-carotene is markedly lower in comparison to oils exposed to fluorescent light, implying that photooxidation exerts a more profound impact on the stability of β-carotene. For both BSO and PSO, the depletion of β-carotene at the end of the storage study was significantly more pronounced at 30°C than at 20°C (Table 6), indicating that β-carotene is also susceptible to variations in storage temperature.

Oils contained within green and transparent bottles showed notable reductions in lutein concentrations, ranging from 33% to

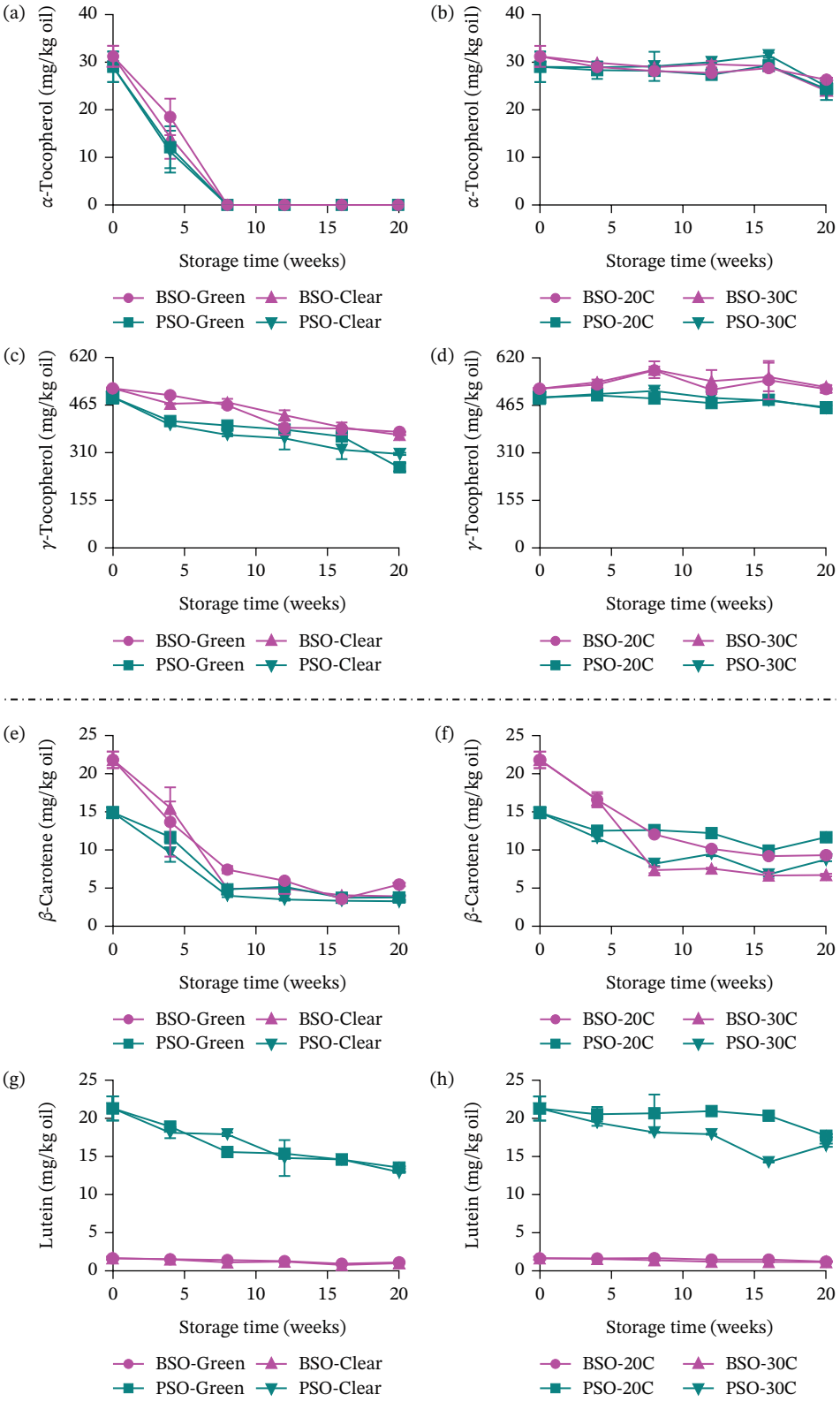

FIGURE 5 | Changes in major antioxidant phytochemicals in the oils during a 20-week storage study. (a) Effect of packaging material light transmission property on α-tocopherol. (b) Effect of storage temperature on α-tocopherol. (c) Effect of packaging material light transmission property on γ-tocopherol. (d) Effect of storage temperature on γ-tocopherol and storage temperature on major tocopherols in the oils. (e) Effect of packaging material light transmission property on β-carotene. (f) Effect of storage temperature on β-carotene. (g) Effect of packaging material light transmission property on lutein. (h) Effect of storage temperature on lutein. Data are presented as mean values from two independent experiments (with triplicate samples from each experiment analyzed across all samples collected at the specified sampling intervals). Error bars represent standard deviations.

TABLE 6 | Percentage loss in the concentrations of the major antioxidants of expeller-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°C, amber bottles	57.2 ± 2.3	26.6 ± 2.3	15.7 ± 0.1	0.1 ± 2.5
	30°C, amber bottles	69.2 ± 7.1	30.3 ± 7.1	23.1 ± 0.0	1.8 ± 1.1
	20°C, green bottles, 15 W fluorescent light	74.9 ± 6.4	32.7 ± 2.3	100	27.6 ± 0.6
	20°C, clear bottles, 15 W fluorescent light	82.0 ± 0.7	38.8 ± 7.1	100	25.9 ± 0.7
PSO	20°C, amber bottles	12.7 ± 5.8	14.9 ± 5.8	15.9 ± 3.1	7.1 ± 1.9
	30°C, amber bottles	34.7 ± 4.8	21.7 ± 0.8	14.0 ± 2.5	6.5 ± 1.0
	20°C, green bottles, 15 W fluorescent light	71.9 ± 0.3	35.0 ± 5.8	100	32.3 ± 2.8
	20°C, clear bottles, 15 W fluorescent light	75.5 ± 1.7	37.8 ± 4.8	100	45.3 ± 1.9

TABLE 7 | Pearson's correlation coefficients between the monitored antioxidants and oxidative indices in butternut squash (BSO) and pumpkin seed (PSO) as affected by packaging type or storage temperature.

Oil type	Packaging type	Antioxidant	PV	TBARS	Temperature	PV	TBARS
BSO	Clear	α-Tocopherol	−0.648	−0.675	20°C	−0.689	−0.583
		γ-Tocopherol	−0.809*	−0.975*		−0.183	−0.188
		β-Carotene	−0.705	−0.727		−0.672	−0.768
		Lutein	−0.836*	−0.760		−0.932**	−0.842*
		TP	0.866*	0.899*		0.782	0.157
	Green	α-Tocopherol	−0.682	−0.615	30°C	−0.778	−0.727
		γ-Tocopherol	−0.940**	−0.867**		−0.019	−0.228
		β-Carotene	−0.773	−0.670		−0.811*	−0.593
		Lutein	−0.955*	−0.807*		−0.958**	−0.816*
		TP	0.820*	0.897*		0.705	0.074
PSO	Clear	α-Tocopherol	−0.474	−0.578	20°C	−0.851	−0.470
		γ-Tocopherol	−0.209	−0.786		−0.912*	−0.704
		β-Carotene	−0.443	−0.707		−0.180	−0.594
		Lutein	0.023	−0.906*		−0.949**	−0.755
		TP	−0.771	0.412		0.568	0.397
	Green	α-Tocopherol	−0.775	−0.463	30°C	−0.473	−0.193
		γ-Tocopherol	−0.269	−0.818		−0.881*	−0.887*
		β-Carotene	−0.792	−0.653		−0.546	−0.622
		Lutein	−0.689	−0.685		−0.742	−0.812*
		TP	−0.457	0.548		0.184	0.672

Abbreviations: TBARS, thiobarbituric acid reactive substances; TPs, total phenolics.
*Correlation is significant at the $p < 0.05$ level.
**Correlation is significant at the $p < 0.01$ level.

39% (Figure 5g and Table 6). The degree of depletion was marginally greater in transparent bottles, indicating a partial protective role of the colored containers. For oils stored in amber bottles and subjected to temperatures of 20°C and 30°C, lutein levels remained relatively stable until Week 12 but subsequently started to decline at Week 16 (Figure 5h). The extent of depletion

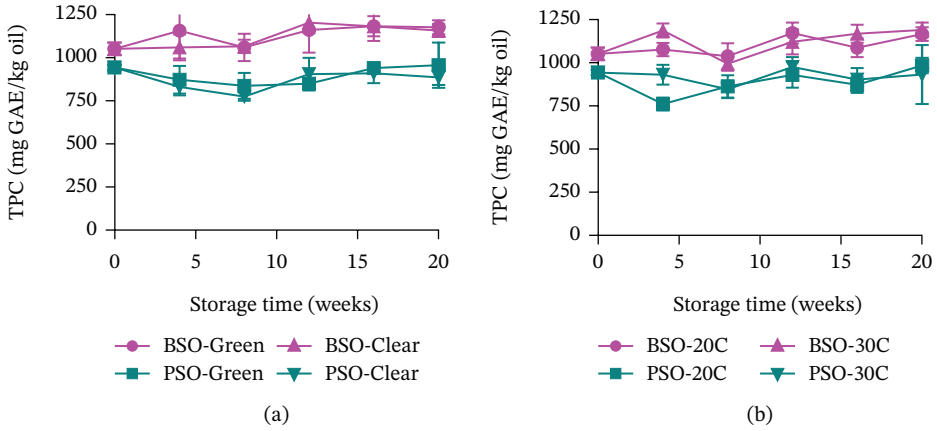

FIGURE 6 | Changes in total phenolic content (TPC) of the oils during a 20-week storage study. (a) Effect of packaging material on light transmission. (b) Effect of storage temperature. Data are presented as mean values from two independent experiments (with triplicate samples from each experiment analyzed across all samples collected at the specified sampling intervals). Error bars represent standard deviations.

varied from 15% to 30% for both oils, with a more pronounced depletion observed at 30°C. Collectively, the findings from both experimental conditions imply that expeller-pressed oils will most effectively preserve their β-carotene and lutein contents when these oils are stored in amber bottles, which exhibit 0% light transmission in the ultraviolet spectrum, in contrast to the 60% light transmission characteristic of emerald green bottles [75]. Furthermore, the reduction in lutein concentrations in both oils was, in general, less pronounced than that of β-carotene, suggesting that β-carotene possesses higher reactivity and may function as a more effective antioxidant compared to lutein. This observation aligns with prior observations indicating that carotenes (such as β-carotene) are more effective in quenching radicals than xanthophylls (such as lutein), because the long, nonpolar conjugated polyene chains in carotenes allow efficient electron/energy transfer and stabilization of carotenoid radical intermediates, whereas oxygen substituents in xanthophylls could introduce polarity and modify conjugation, generally lowering radical-quenching efficiency [76, 77]. In terms of stability, lutein was generally found to be more stable than β-carotene (Table 6), irrespective of the type of oil and the conditions under which it was stored.

In general, the observed reduction of β-carotene and lutein suggests that these compounds are being utilized as lipid oxidation advances. In fact, moderate to robust correlations between β-carotene or lutein and the oxidative parameters (PV and TBARS) were noted (Table 7). Carotenoids scavenge the chain-propagating peroxyl radicals produced during lipid peroxidation [78] and are effective radical scavengers in conditions of low oxygen concentration, which was the case in oils stored in nonpermeable amber glass bottles. However, after its initial antioxidant effect, β-carotene can exhibit pro-oxidant behavior when oxygen concentrations are high [79]. Bottles were sealed with natural cork to simulate commonly used commercial packaging conditions for cold expeller-pressed oils. While cork is known to be permeable to oxygen, its use was intentional to reflect realistic storage environments in which both light exposure and oxygen ingress contribute to oxidative processes. It is therefore acknowledged that the oxygen permeability of cork closures may have facilitated oxygen diffusion into the headspace and oil, potentially accelerating oxidation and influencing the measured oxidative stability parameters during storage [80, 81]. Nonetheless, this approach provides a more representative assessment of oil behavior under typical commercial and consumer storage conditions. The difference in the time required for PV to exceed the regulatory maximum (16 weeks for BSO vs. 8 weeks for PSO) may partly be due to the higher β-carotene content in the former (Table 4). It has been reported that synergism between carotenoids and tocopherols results in enhanced antioxidant effects and oxidative stability in foods [82, 83]. Based on this, BSO, which contains high levels of α-tocopherol and a significantly higher content of β-carotene than PSO, is expected to have higher oxidative stability, which is consistent with the observations of the present study.

3.7.3 | Changes in TPs

In all cases, TP (Figure 6a,b) remained constant in both oils, indicating excellent storage stability of phenolic acids in the oils, especially at lower temperatures. This observation is consistent with findings from recent published work showing stability of phenolic compounds and antioxidant capacity indices (e.g., DPPH radical scavenging) during oil storage at ambient or lower temperatures [84–86]. It has been shown that polar phenolics are the main active compounds responsible for the antioxidant capacity of seed oils [87, 88]. Therefore, the phenolic compounds in the expeller-pressed oils (Table 1), which were all polar phenolics, may play a role in protecting the oils against oxidative degradation.

4 | Conclusion

In conclusion, expeller cold-pressed BSO and PSO contained natural antioxidants, particularly tocopherols, which contributed to their oxidative stability when stored in amber bottles at temperatures of 30°C or below. Although carotenoids and phenolics were present, their concentrations were comparatively low and may have played a more limited role in overall antioxidant protection. Both oils showed low rates of oxidation in the absence of light, with an estimated shelf life of at least 15 months when stored in the dark under accelerated storage

conditions. Light exposure markedly accelerated oxidation, highlighting the critical role of packaging, with partial light transmission blockade not translating into significant protection against lipid oxidation. Differential depletion patterns of tocopherols and carotenoids demonstrated their active involvement in oxidation processes, with both compounds showing strong inverse correlations with oxidation indices. One limitation of the study was the shorter duration (20 weeks) of the storage study conducted. A future much longer storage study (up to 2 years) would provide a more complete picture of the oxidative stability of the oils. Overall, the results underscore the importance of natural antioxidant systems and appropriate packaging in preserving expeller-pressed oil quality and support the potential of BSO and PSO as stable, value-added sources of dietary antioxidants for functional food and pharmaceutical applications.

Author Contributions

Conceptualization: R.T. and O.I.P.Z. Methodology: R.T. and O.I.P.Z. Formal analysis: R.T. Investigation: R.T. Data curation: R.T. Writing—original draft preparation: R.T. Writing—review and editing: R.T. and O.I.P.Z. Visualization: R.T. Project administration: O.I.P.Z. Funding acquisition: O.I.P.Z.

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Disclosure

The data presented in this manuscript have been presented as a thesis and are accessible via the following link: https://ecommons.cornell.edu/server/api/core/bitstreams/e2f6da94-66a3-4832-9bc9-31c6bb1f2200/content.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data will be made available upon request from the authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Supporting Information.** Figure S1: Typical chromatograms of tocopherols from expeller-pressed seed oils. The HPLC method is described in detail in Section 2.6 of the paper. (a) Representative chromatograms of tocopherols in pumpkin seed oil (PSO). (b) Representative chromatograms of tocopherols in butternut squash seed oil (BSO). The quantification data is presented in Table 3. Figure S2: Typical chromatograms of carotenoids from expeller-pressed seed oils. The HPLC method is described in detail in Section 2.7 of the paper. (a) Representative chromatograms of carotenoids in pumpkin seed oil (PSO). (b) Representative chromatograms of carotenoids in butternut squash seed oil (BSO). The quantification data is presented in Table 4. Figure S3: Changes in major antioxidant contents during an accelerated storage study. The study details are described in Section 2.9 of the paper. (a) Changes in α-tocopherol. (b) Changes in γ-tocopherol. (c) Changes in lutein. (d) Changes in β-carotenoid. Data are presented as mean values from two independent experiments (with triplicate samples from each experiment analyzed across all samples collected at the specified sampling intervals). Error bars represent standard deviations.