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Research Article

Antioxidant Efficacy of Gamma-Oryzanol and Tocotrienol-Rich Fractions from Rice Bran in Chia Seed Oil under Accelerated Oxidation Conditions

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Abstract

The antioxidant efficacy of gamma-oryzanol rich fraction (OzF) and tocotrienol rich fraction (TRF) extracted from rice bran in retarding oxidation of chia seed oil (CSO) were evaluated and compared to butylated hydroxy toluene (BHT) during storage at 63 ℃. The total phenolic and flavonoid compounds in CSO and antioxidant activities of OzF and TRF were determined. OzF showed better metal chelating activities compared to TRF, whereas ABTS radical and superoxide anion scavenging activities were comparable for the both. The protective effects of aforesaid extracts in stabilizing CSO were tested by measuring some oxidation indices of oils related to primary and secondary oxidative compounds. Under storage conditions, the oxidative indices were increased faster in untreated oils than those of antioxidant rich fractions incorporated oils which indicate smaller release of oxidative products in later ones. However, the presence of OzF and TRF in CSO decreased the oxidation deterioration of polyunsaturated fatty acid measured by gas-liquid chromatography. In FTIR, the peak intensities in CSO were greatly altered compared to the rest samples during storage. Changes in color value, carotenoids and chlorophyll derivatives contents in CSO were greater than those of the remaining samples during oxidation time. Under the above oxidation conditions and in most cases studied herein, the antioxidant efficacy in stabilizing CSO followed the order: OzF + TRF > BHT > OzF > TRF. This study may open up new opportunities for further technological development in generating stable edible oils, which have potential applications in different types of processed foods.

Keywords: Chia Seed Oil, Fatty Acids, Rice Bran, Antioxidant Rich Fractions, Oxidative Stability.

1. Introduction

The lipid oxidation reactions in lipid matrices are noteworthy because of generating some volatile and non-volatile components that leads to generate unwanted flavor and different health problems such as cancer, diabetic, inflammation, hyperlipidemia etc. In addition, oxidation has economic implications as oxidized oils decrease consumer perceptions and decreases the value of oils, wherein quality defines price ranges [1-3]. Owing to several benefits to human health, the food materials containing polyunsaturated fatty acid (PUFA) are highly recommended. With the nature of multiple unsaturated carbon bonds, PUFAs are susceptible to oxidation that forms degradative products with reactive oxygen species [4]. Chia seed oil (CSO) extracted from chia seeds (*Salvia hispanica* L.) has gained popularity as a healthy food in recent years due to its high proportion of healthy PUFAs (79.80–82.83 %) consisting of mainly linoleic (17.12–19.91 %) and α-linolenic (59.81–65.72 %) acids leading to increased cultivation and commercialization [5]. At present, this seed oil is available in markets everywhere. CSO has been scientifically proven to have a wide range of therapeutic effects, including immune enhancement and managing many pathological conditions like dyslipidemia, diabetes, inflammation, hypertension, blood clotting, oxidative stress, laxative, antidepressant, anxiolytic, analgesic, and vision-related functions [6].

Despite being nutritionally favorable, higher amounts of PUFAs present in CSO result in lower oxidative stability [7]. How to improve oxidative stability of polyunsaturated oils is a major issue in lipid research. Owing to dual roles in food industry, antioxidants are gaining importance as lipid stabilizing agent and in preventive medicine as suppressor of higher oxidation. Of course, the roles of antioxidants are essentially and highly complicated in the dynamic network of oxidation. The antioxidant behavior of a given antioxidant system differed largely among lipid systems when different degrees of unsaturation are in place. The inappropriate use of high amount of antioxidative agent may result in pro-oxidant effect in the final product, which can possess negative impacts

on the food chain that includes human. Antioxidants incorporated with CSO in proper way can enhance its application in the food, pharmaceuticals and cosmetic industries by providing more stability during storage and processing. Owing to the harmful effects of synthetic antioxidants on human body, the food industries are desiring safe, healthy and easily available antioxidants from plant sources which do not require much prior testing. Indeed, natural antioxidants recovered from different plant matrices, demonstrate a potential effect, even better than synthetic antioxidants in some cases, to preserve oils from oxidation and to satisfy consumer demand for natural foods [8]. The brown layer of rice seeds known as rice bran generated from rice processing mill as a byproduct, is a reliable origin of phytonutrients as well as polyphenols which naturally act as antioxidants. This comprises 8–11 % of the rice grain and 8–23 % oil. The rice bran oil (RBO) contains 90 to 96 % lipid components (free fatty acids, waxes, di-, and triglycerides) and 3 to 5 % unsaponifiable materials like sterols, tocotrienols, triterpene alcohols and so on. By taking advantage of the powerful antioxidants such as γ-oryzanol, tocols, phytosterols, squalene etc. present in rice bran [9]; its crude extracts or isolated components may be considered as valuable natural additives which can enhance the oxidative stability of CSO. According to our previous work, the antioxidant-rich fractions such as gamma-oryzanol and tocotrienol rich fractions prepared from same rice bran sample, displayed strong antioxidant activities as evaluated by multiple antioxidant assays (DPPH radical scavenging assay, H_2O_2 scavenging activity, hydroxyl radical-scavenging activity etc.) [10]. To date, some studies have been conducted on the oxidation stability and physico-chemical characteristics of CSO in presence of antioxidants extracted from the various natural sources [7,11-13]. So far, no study has been conducted on how antioxidant rich fraction extracted from rice bran affects quality and oxidative stability of CSO under oxidative stress, which is the core objective in this study.

2. Materials and Methods

2.1. Materials

Chia seeds were procured from Dhaka, Bangladesh. The seeds were cleaned and dried to constant weight in shadow at ambient temperature. Then, the seeds were powdered by using a consumer-model electric grinder, and preserved at 4 ℃ in sealed polyethylene bag prior to analysis. Rice bran was obtained freshly from the milling process of grain industry. The bran was packed in a microwave-saved polyethylene bag, adjusted moisture at 21 % [14] and heated by a microwave oven (2450 MHz, output power 850 W) for 3 min to destroy seeds lipase. The bran was collected from the oven, cooled to ambient temperature, and preserved in polyethylene bags at –15 ℃ in a freezer. The study utilized various chemicals and reagents purchased from mainly Merck (Germany) and Sigma (USA).

2.2. Extraction of oil samples

The treated rice bran was subjected to rice bran oil (RBO) extraction using n-hexane as solvent (1:10, w/v) in an orbital shaking machine at ambient temperature for one hour. The RBO underwent filtration process for removing impurities and the residue was extracted twice using n-hexane at the same condition. The extracts were combined and filtered followed by evaporating solvent under low pressure at 45 ℃. The extraction of chia seed oil (CSO) was conducted with n-hexane from the chia seed, employing the same process mentioned above. The percent of yield of oils was calculated. The samples were stored in a freezer at –15 ℃.

2.3 Preparation of OzF and TRF from rice bran and RBO respectively

The gamma-oryzanol rich fraction (OzF) from stabilized bran was prepared following the process of Xu et al. [15]. The distilled water was added to the bran in a conical flask followed by the addition of ascorbic acid to the mixture. The whole mixture was subjected to vortexing and subsequent incubation at 60 ℃ for 30 min. Thereafter, the solvent system of isopropanol and hexane (50:50), was poured to the heated mixture, and the mixture vortexed for 30 seconds, and subjected to centrifugation at 200 g for 15 minutes. The organic phase was taken into a separating funnel. The residue was then centrifuged using same solvent. The organic phase was separated and added to the previous collection. Distilled water was poured to the separating funnel to facilitate the rinsing of the organic phase. After standing the funnel for 10 min, the water was removed. The rinsing process was subsequently repeated two times. Thereafter, the organic phase was taken into a flask. The extraction of the desired portion was dried under reduced pressure at 45 ℃.

The TRF was prepared from the extracted RBO using methanol as solvent [16]. In brief, methanol was added to the RBO, the solution was mixed using magnetic stirring for 1 h. The methanolic layer contained the desired TRF, was then collected. Finally, the methanol was removed under vacuum at 45 ℃, allowing for the recovery of the TRF.

Bemvenuti et al. [17] and Bardhan et al. [18] reported to selectively enrich the corresponding antioxidative components i.e., γ -oryzanol and tocotrienol from rice bran matrices, thereby establishing the methods of Xu et al. [15] and Siddiqui et al. [16] respectively. As the extracts may also contain other polyphenols, these are named as γ-oryzanol rich fraction and tocotrienol rich fraction consecutively rather than purified individual compounds in this study.

2.4 Estimation of bioactive compounds in chia seed oil

Total phenolic compounds (TPC): The estimation of TPC was carried out using Folin-Ciocalteau's reagent [19]. In this regard, 2.5 mL of the FCR (diluted to 10 times) and 2 mL of 75 g/L Na_2CO_3 were added together with 0.5 mL of test sample. The solution was subsequently cooled after incubation for 5 min at 50 ℃; the absorbance of the solution recorded at 760 nm. Gallic acid was used as a standard to express the TPC content in CSO as mg gallic acid equivalent per gram of oil (mg GAE/g oil).

Total flavonoid compounds (TFC): TFC was measured

following the process of Wu and Ng [20]. An aliquot (0.5 mL) of oil was taken into a test tube that contained 2.2 mL distilled water, 0.15 mL 5 % $NaNO_2$, and 0.15 mL 10 % $AlCl_3$. The mixture was allowed to react for 6 min followed by adding 2.0 mL 4 % NaOH. The resulting solution was allowed to stand for 15 min at ambient temperature; the absorbance of the final solution was recorded at 510 nm. Catechin was used as a standard to determine the flavonoid content as mg catechin equivalent per gram of oil (mg CE/g oil).

2.5 Antioxidant activities of OzF and TRF

ABTS scavenging test: First, 7 mM ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] solution was prepared using distilled water. The $ABTS^{\bullet+}$ was synthesized by the reaction of ABTS solution with 2.45 mM $K_2S_2O_8$ at a ratio of 1:0.5. The solution was allowed to react at ambient temperature in absence of light for 12–16 h. Subsequently, ethanol was added to ABTS solution until an absorbance of the diluted solution reached to 0.7 at 734 nm. To 100 µL of sample, 1 mL of ABTS solution was added and mixed well. The reaction mixture was incubated in the dark at ambient temperature for 30 min; the absorbance of the prepared solution computed at 734 nm [21]. The scavenging ability was determined by using equation (1).

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (1)$$

Where A stands for the absorbance.

Superoxide anion scavenging activity: The scavenging of superoxide ($O_2^{\bullet-}$) radical anion was assessed following the method of Lalhminghlui et al. [22]. A reaction mixture was prepared taking 2 mL alkaline dimethyl sulfoxide (DMSO), 0.6 mL sample and 0.2 mL of nitro blue tetrazolium chloride (NBT) (1 mg/mL of solution in DMSO) and diluted to final volume 2.8 mL. The absorption was computed at 560 nm against the corresponding blank solution. The blank contains pure DMSO in lieu of alkaline DMSO. The superoxide anion scavenging activity was calculated using Equation (2).

$$\text{Scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (2)$$

Where A stands for the absorbance.

Metal chelating ability: The capability of the OzF or TRF to chelate the ferrous ion (Fe^{2+}) was estimated as per Sharma et al. [23]. A solution was prepared taking 0.5 mL sample, 1.6 mL distilled water, and 0.05 mL $FeCl_2$ (2 mM). Following 30 seconds interval, 0.1 mL of ferrozine (5 mM) was mixed with to it. In this regard, stable magenta complex soluble in water, was formed by the reaction of ferrozine with Fe^{2+}. After 10 min of incubation at ambient temperature, the absorbance of the Fe(II)-ferrozine was determined at a wavelength of 562 nm. The chelating ability was determined using Equation (3).

$$\text{Chelating rate (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (3)$$

Where A stands for the absorbance.

2.6 Preparation of oil samples

γ-Oryzanol rich fraction (OzF), tocotrienol rich fraction (TRF) and BHT were added separately to the CSO at 200 ppm concentrations, marked as CO, CT and CB respectively. In addition, a mixture of 100 ppm OzF and 100 ppm TRF was applied to the oil sample to examine the combined activity of these antioxidant rich fractions, designed as COT. The 200 ppm concentrations were achieved on a w/w basis (mg /kg oil) by incorporating 200 mg of each antioxidative fraction (OzF, TRF or BHT) per kg of CSO. Each additive was homogeneously mixed into the oil using magnetic stirring for sufficient time. For the COT group, 100 mg of OzF and 100 mg of TRF were combined. The dosage of 200 ppm was chosen based on the standard legal limits defined by global regulatory bodies (such as Codex Alimentarius) for commercial synthetic antioxidants like BHT in food oils and to ensure relevant comparison between the natural fractions (OzF, TRF) and the standard commercial antioxidant at its maximum allowable legal efficacy while avoiding excessive antioxidant loading that may exert pro-oxidant effects at higher concentrations [7, 24, 25]. Oil samples were placed in glass beakers and stored at 63 ± 1 ℃ for 21 consecutive days following the Schaal oven test, a widely used accelerated oxidation method for evaluating the oxidative stability of edible oils and lipid-containing systems [26-28]. Temperatures in the range of 60–65 ℃ are commonly employed because they substantially accelerate autoxidation reactions while avoiding the severe thermal degradation mechanisms associated with high-temperature processing or frying conditions. Although a direct conversion between accelerated and real-time storage is not universally applicable due to differences in lipid composition and storage environments, the Schaal oven test is broadly recognized as a practical model for predicting relative oxidative stability and long-term storage behavior of edible oils under ambient conditions. The samples were periodically collected at 0, 7, 14 and 21-day intervals for subsequent analyses.

2.7 Analysis of oil sample

Oxidation parameters: The oxidation parameters such as free acidity (FFA, method Ca 5a-40), peroxide value (PV, method Cd 8-53) and thiobarbituric acid value (TBA, method Cd 19-90) were estimated following the American Oil Chemists' Society official AOCS, [29] methods. The PORIM [30] methods were followed to determine specific extinctions (method p2.15) at 233 nm and 269 nm and *p*-anisidine value (*p*-AV, method p2.4) using UV-Vis spectrophotometer (T 60, PG Instruments, Leicestershire, UK). Total oxidation values (TOTOX) were calculated using equation (4)[31].

$$\text{TOTOX} = 2\text{PV} + p\text{-AV} \quad (4)$$

Free fatty acids: The oil sample was weighed accurately and taken into a conical flask where it was dissolved in previously neutralized 50 ml ethanol as solvent. Afterward, 2-3 drops of phenolphthalein solution were added to the solution as indicator. The sample was titrated against a standard sodium hydroxide solution with constant shaking vigorously until the appearance of the first permanent pink color. The percentage of free fatty acids (FFA) was calculated using equation (5)

$$\text{FFA (\% as Oleic acid)} = \frac{28.2 \text{xNxV}}{\text{W}} \quad (5)$$

Where,

N = Normality of NaOH solution

V = Volume of NaOH solution in ml

W = Weight of sample (oil) in gram

Peroxide value: A given amount of oil sample was weighed

accurately into a conical flask followed by the addition of 30 ml of glacial acetic acid - chloroform (3:2) mixture. The flask was swirled gently until the sample was dissolved. Then 0.5 ml of saturated potassium iodide solution was added to the solution with a graduated pipette and the solution was allowed to stand with occasional shaking for 1 min. Thirty milliliter of distilled water was added. The solution was titrated against standard sodium thiosulfate ($Na_2S_2O_3$) solution from burette until the yellow color was disappeared. Half milliliter starch indicator was added and titration was continued with constant shaking. The thiosulfate solution was added drop wise until the blue color just disappeared. A blank test was also conducted. The peroxide value (PV) was calculated using equation (6).

$$PV = \frac{(V_s - V_b) x N x 1000}{W} \quad (6)$$

Where,

V_s = Volume of sodium thiosulfate solution in the sample titration (in ml)

V_b = Volume of sodium thiosulfate solution in the blank titration (in ml)

N = Normality of sodium thiosulfate solution

W = Weight of sample in gram used in the test portion.

para-Anisidine value (p-AV): The oil sample was weighed accurately and poured into a 25 ml volumetric flask and isooctane was added up to the mark of the flask in order to dissolve the sample. The spectrophotometer was turned to zero at 350 nm using the solvent. The absorbance (A_b) of the sample was measured at 350 nm in the sample cell. Five milliliter of oil solution was pipetted into one test tube and exactly 5 ml of solvent into a second test tube. One milliliter of the p-anisidine reagent was added to each tube. The test tubes were shaken to homogenize the solution and the reagent. Finally, the solution was allowed to stand for 10 minutes and the absorbance (A_s) at 350 nm of the solution in the first test tube in the sample cell was measured using the solution from the second test tube as a blank in the reference cell. The p-AV was calculated using equation (7).

$$p\text{-}AV = \frac{25x(1.2A_s - A_b)}{W} \quad (7)$$

Where,

A_s = Absorbance of sample solution after reaction with p-anisidine reagent

A_b = Absorbance of sample solution before the addition of p-anisidine reagent

W = Weight of sample in gram.

Total oxidation (TOTOX) value: *para*-Anisdine value is often used in the industry in conjunction with peroxide value to calculate the so-called total oxidation (TOTOX) value. The TOTOX value of the oil sample was calculated using Holm's equation (4).

Thiobarbituric acid value: The sample was weighed and poured into a 25 ml conical flask and dissolved with 1-butanol. Five milliliter of the sample solution was taken into a dry test tube and 5 ml Thiobarbituric Acid (TBA) reagent (prepared by dissolving 200 mg of TBA in 100 ml 1-butanol) was added to it. Test tubes were shaken thoroughly and were placed in a thermostat at 95 ℃. After 2 hours the test tube was removed from the thermostat and cooled in running tap water for about 10 minutes at room temperature. Similarly, a reagent blank was prepared using solvent in place of sample. Absorbance of the reaction solution in a 1 cm cell was measured at 530 nm using distilled water in the reference cell. TBA value was calculated by using equation (8).

$$TBA\ Value = \frac{50x(A - B)}{W} \quad (8)$$

Where,

A = Absorbance of the test solution

B = Absorbance of the reagent blank

W = Weight of the oil in test portion, in milligram.

Color value, carotenoids and chlorophyll derivatives contents: To ascertain the color value of the samples, the absorbance of a 5.0 % (w/v) solution of oil in chloroform was recorded at 420 nm and 520 nm. The absorbance of oil sample diluted in cyclohexane was recorded at 470 nm and 670 nm for determining concentrations of carotenoid and chlorophyll in ppm using same UV-Visible spectrophotometer following the methods expressed elsewhere [32].

Fatty acids profile: Fatty acids composition (FAC) was estimated as fatty acid methyl esters (FAME) using gas chromatography (PerkinElmer, Clarus 950, USA) furnished with capillary column (0.25 mm i.d × 60 m × 0.2 μm) after transesterification of the oils according to PORIM [30] test method p3.4. The operating conditions were: carrier He at 25.3 psig and at 2.0 mL/min; oven temperature 120 ℃ increased by 4 ℃/min to 240 ℃; injection temperature 12 ℃; FID detector temperature 250 ℃. The flow rates of air and hydrogen gas were maintained at 450.0 and 50.0 mLmin-1 in the detector. The fatty acids were quantified taking the peak area of each component in an individual sample run.

Fourier Transform Infrared (FTIR) spectroscopy: FTIR analysis for the oils and also for antioxidant rich fractions were carried out by FTIR spectrophotometer (IR Affinity - 1S, Shimadzu Corporation, Japan) furnished with a high-sensitivity pyroelectric detector (deuterated L-alanine doped triglycine sulphate). The samples were analyzed directly in neat form without solvent dilution. A thin film of the sample was mounted uniformly on a transparent sodium chloride crystal cell prior to spectral acquisition. Since the same sample loading procedure and crystal configuration were used throughout the study, the optical path length was maintained effectively constant for comparative spectral analysis among samples. The data were analyzed using Lab Solution software tool. The spectra were recorded in the range of 850 to 4000 cm^{-1} with a resolution of 4 cm^{-1} using 16 acquisitions. The background spectrum was subtracted to correct for the interference caused by water vapor. To minimize humidity-related artefacts, the instrument was maintained with the automatic dehumidifier. The FTIR spectral absorbance was measured.

2.8 Statistical analysis

All data were expressed as the mean ± standard deviation (SD) of three independent experimental replicates. The normality of data and homogeneity of variances were verified using the Shapiro-Wilk and Levene's tests respectively to ensure the assumptions of ANOVA were met. A One-way Analysis of Variance (ANOVA) was carried out to evaluate significant

differences among the experimental groups at each storage time. Post-hoc pairwise comparisons were performed using Duncan's multiple range test, with statistical significance defined at $p < 0.05$.

3. Results and Discussion

3.1 Extraction yield and bioactive phytochemicals

The solvent extract of chia seeds yielded 25.66 % CSO, which is appreciably less than the value 27 % reported by Souza et al. [33]. The rice bran contained 15.32 % RBO which was slightly lower than 17.08 % as reported by Ali et al. [34]. Phenolic or flavonoids compounds as potent antioxidants may slow down the oxidation process of fatty foods by various mechanisms including scavenging free radicals, chelating transition metal ions, and quenching $^1O_2$ [35]. In this research, the CSO contained 2.11 mg GAE/g of TPC, similar to the value 2.00 mg GAE/g for the cold-pressed CSO reported by Oliveira-Alves *et al.*, [36]. TFC value in CSO computed in the current study, was 1.60 mg CE/g that is much higher than that of 0.08 mg CE/g reported by Ali *et al.*, [34] for RBO. Mutlu *et al.*, [37] determined total flavonoids in CSO and reported to be 150.00 µg QE/mL which is not comparable with the present value.

3.2 Antioxidant activities of OzF and TRF

Both OzF and TRF exhibited a concentration-dependent increment of ABTS free radical scavenging activities (Figure 1). The maximum activities for OzF and TRF at 500 µg/mL were found to be 78.29 % and 79.23 % in the current study. Lalhminghlui and Jagetia [22] evaluated ABTS free radical potency for various extracts of *Schima wallichii* and stated that activities increased with increasing extract concentrations.

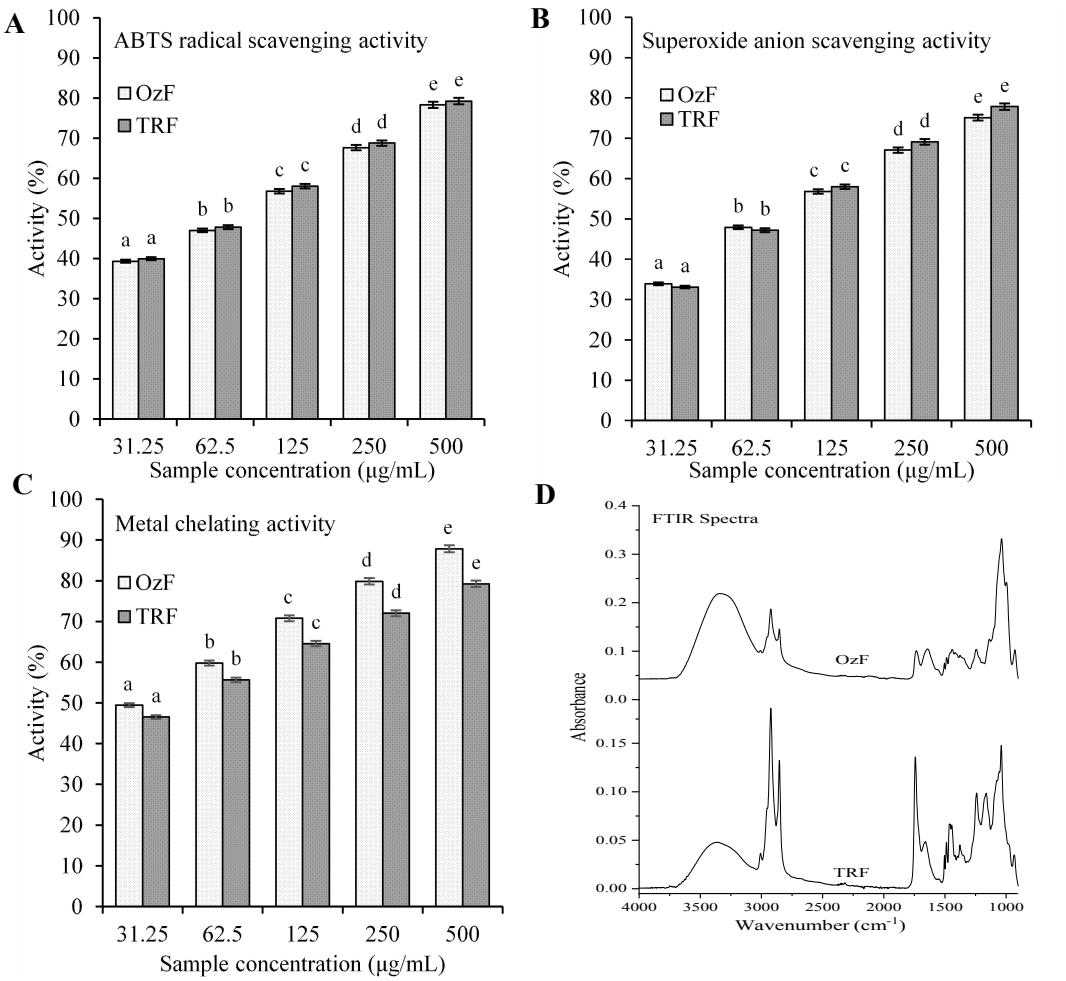


Figure 1. (A) ABTS radical, (B) superoxide anion scavenging, (C) metal chelating activities and (D) FTIR spectra of OzF and TRF.. Each value is the mean ± standard deviation of triplicate determinations. Values within each sample with different letters on bar are significantly different ($p < 0.05$).

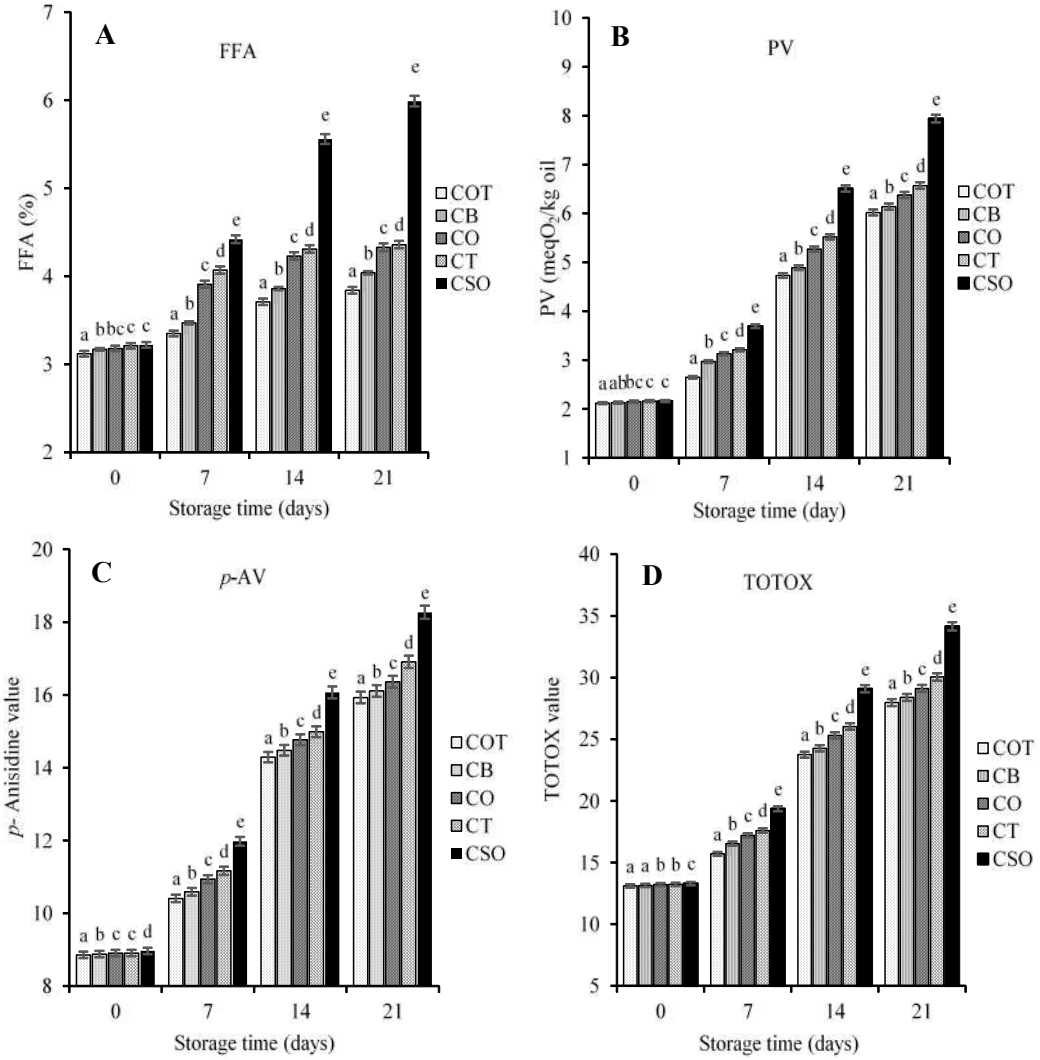


Figure 2. Changes of (A) FFA, (B) PV, (C) p-AV and (D) TOTOX values of untreated and antioxidant treated chia seed oils during storage at 63 ℃. Each value is the mean ± standard deviation of triplicate determinations. Values in each storage time grouping with different letters on bar are significantly different ($p < 0.05$). COT- chia seed oil containing 100 ppm OzF and 100 ppm TRF; CB- chia seed oil containing 200 ppm BHT; CO- chia seed oil containing 200 ppm OzF; CT- chia seed oil containing 200 ppm TRF; CSO- chia seed oil.

The highest activity for chloroform extract was found at 350 µg/mL, whereas ethanol and aqueous extracts exhibited highest ABTS inhibitory effect at 60 and 100 µg/ml, respectively. The γ -oryzanol and tocotrienol rich extracts inhibited superoxide generation and their superoxide anion scavenging activities increased with increasing sample concentration (Figure 1). At low concentration (31.25 µg/mL), both OzF and TRF displayed around 33 % activity. However, at 500 µg/mL, OzF and TRF showed 75 % and 78 % activities, respectively. Ferrozine has the ability to form complex with Fe^{2+} ions which is known as metal chelation. The formation of such complex (which has a distinct color) is interrupted in presence of chelator that results in reduction in the intensity of the coloration. Consequently, the capability of the chelating agent may be quantified by determining color reduction. According to figure 1, the Ferrous-ferrozine complex formation was interrupted by the OzF and TRF which indicates the presence of chelating capability of the samples. The chelating activity ranged from 49.44 % and 46.53 % at 31.25 µg/mL to 87.85 % and 79.21 % at 500 µg/mL for samples OzF and TRF, respectively. This study demonstrates that TRF and OzF possess remarkable iron (II) chelating capabilities, indicating the presence of polyphenols with potent iron chelating properties. From the present studies, it is clear that, at low concentrations, the ABTS and superoxide anion scavenging activity of OzF and TRF are almost equivalent. However, OzF showed more metal chelating activity than that of TRF, perhaps due to structural feasibility of gamma-oryzanol.

Moreover, the absorption bands in FTIR spectra obtained from OzF and TRF correspond to specific functional groups within the molecules, are shown in figure 1. Both spectra

showed similar, broad features characteristic of complex organic molecules. The distinctive bands reflecting the information about the structure of polyphenols were found in the regions 3500−3000 cm^{-1} (characteristic of the O−H stretching vibration), 3000−2800 cm^{-1} (attributed to C−H stretch), 1600−1650 cm^{-1} and 1500 cm^{-1} (corresponded to C=C stretching in aromatic rings), 1000−1250 cm^{-1} (C−O stretching) and fingerprint region 1400–900 cm^{-1} (C−O, C−C, and bending vibrations) recognized in both the fractions [38]. The hydroxyl functionality is an integral part of most phenolics such as polyphenols, tocols, oryzanols and flavonoids, to impart antioxidant activity. In addition, the peak near 1700−1750 cm^{-1} was assigned to –C=O stretching vibration in carbonyl compounds, which may be characterized by the presence of high content of polyphenol in the extracts [39]. While the major functional groups appear similar, there are noticeable differences in the relative intensities and fine structure of the peaks. The O−H (around 3300 cm^{-1}) band is noticeably broader and more intense in OzF compared to TRF. This implies that, OzF likely contains a higher proportion of hydroxyl-containing compounds. On the other hand, in TRF C−H stretching peaks (2920−2850 cm^{-1}) are relatively more intense compared to the O−H band than in the OzF spectrum. This suggests a relatively higher content of aliphatic or hydrocarbon components (long fatty chains) in TRF compared to OzF. This is a common feature of lipid soluble polyphenols due to the long chain on the molecule [40]. The FTIR spectra suggest that, OzF appears to be richer in polar, hydroxylated compounds, while TRF has a relatively higher content of non-polar, aliphatic components.

3.3 Analysis of oil sample

3.3.1 Oxidation parameters

The free acidity (FFA) of each sample was increased with increasing the storage time due to decomposition of oil glycerides into FFA (Figure 2). The increment of FFA concentration for untreated CSO was greater compared to that of the antioxidative agent treated samples during the oxidation period. After 21 days storage, the concentration of FFA was greatest ($p < 0.05$) in CSO (5.99 %) compared to others (3.84 – 4.36 %). These results are in accordance with previous findings reported by Tavakoli et al. [25], who observed lower increment in acid value in soybean oil blended with tocotrienol-rich unsaponifiable matters prepared from kolkhoung hull oil. As can be seen in figure 2, the generation of unstable oxidation compounds estimated by peroxide value (PV) which was increased faster in untreated samples during the oxidation period. The increase in PV during oxidation process is likely due to the formation of hydroperoxides which results from peroxidation of unsaturated fatty acids by free radicals [41]. The sample COT had the lowest concentration of hydroperoxide (6.02 meq O_2/kg) than that of fresh CSO (7.94 meq O_2/kg) at the end of 21 days storage which is supposed to be due to the antioxidative potency of the antioxidant rich fractions such as TRF and OzF. Tavakoli et al. [25] also found the highest AV in soybean oil after 32 hours frying, while the lowest values were correlated with the soybean oil containing 200 meq O_2/kg of tocotrienol-rich unsaponifiable matters prepared from kolkhoung hull oil. The present data indicates that, the antioxidant rich fractions and synthetic BHT were effective in retarding hydroperoxides development in CSO. The storage conditions concurrently enhanced the *p*-anisidine values (*p*-AV) for all oils with increase in oxidation time (Figure 2). The increment of *p*-AV during storage might be happened due to the degradation of hydroperoxides to non-volatile carbonyls which results in the development of rancid odor of oil. The increment was lower significantly ($p < 0.05$) in treated oils at each storage period, which might be due to their contents of exogenous antioxidative substances, such as TRF and OzF. However, this increment was low at later phase, whereas high at earlier phase of oxidation time. After 21 days storage, the *p*-AVs attained to 15.93, 16.11, 16.36, 16.91 and 18.27 for COT, CB, CO, CT and CSO samples, respectively. The total oxidation (TOTOX) values indicate the total concentration of oxidation products that indicates better idea of the progressive oxidation degradation of lipids. As furnished in figure 2, the TOTOX values in treated and untreated oils increased as oxidation progressed, and significant differences ($p < 0.05$) in the samples observed after 21 days of storage. Hossen *et al.*, [42] reported similar increasing trend in TOTOX values during storage at 62 ℃ of oil samples extracted from microwaved black cumin seed. The admixing of antioxidative agents with oil samples reduced the formation rate of oxidative products as indicated by TOTOX in CSO during storage. The changes in thiobarbituric acid (TBA) level of antioxidant-enriched-fraction containing CSOs were greatly lower ($p < 0.05$) than that of fresh CSO (Figure 3). Before storage, the TBA values of COT, CB, CO, CT and CSO samples were 0.62, 0.62, 0.64, 0.65 and 0.65, and after 21 days, the values were raised correspondingly to 0.84, 0.86, 0.98, 1.08 and 1.19, respectively. In addition, a sharp increase in TBA values was detected at the earlier stage of storage followed by a decrease; this can be due to volatilization of secondary oxidation products or their break down that also realized by Hossen et al. [42] during oxidation at 62 ℃ of oil samples from microwaved black cumin seed. Changes in the specific extinction at 232 and 269 nm related to the alteration of the conjugated dienes and trienes which are generated for the oxidative degradation of PUFAs, are shown in figure 3. Ultraviolet absorptions at 232 and 269 nm increased significantly for all oils during the accelerated storage periods. However, addition of antioxidant rich fractions decelerated conjugated dienes and trienes formation in oil samples. The levels of conjugated dienes and trienes after 21 days of storage at 63 ℃ were however greatest in CSO (3.18 at 232 nm and 0.70 at 269 nm) and lowest in COT (2.57 at 232 nm and 0.55 at 269 nm). In addition, in all samples, the levels of conjugated trienes were lower than that of dienes which was proved by the higher values of ultraviolet absorption at 232 nm. A similar trend was reported in the literatures, for frying media canola, soybean and palm olein [43] and for pumpkin oil [44]. In this project, the combined antioxidant rich fractions COT prepared from rice bran inhibited the oxidation products' formation more efficiently than BHT ($p < 0.05$).

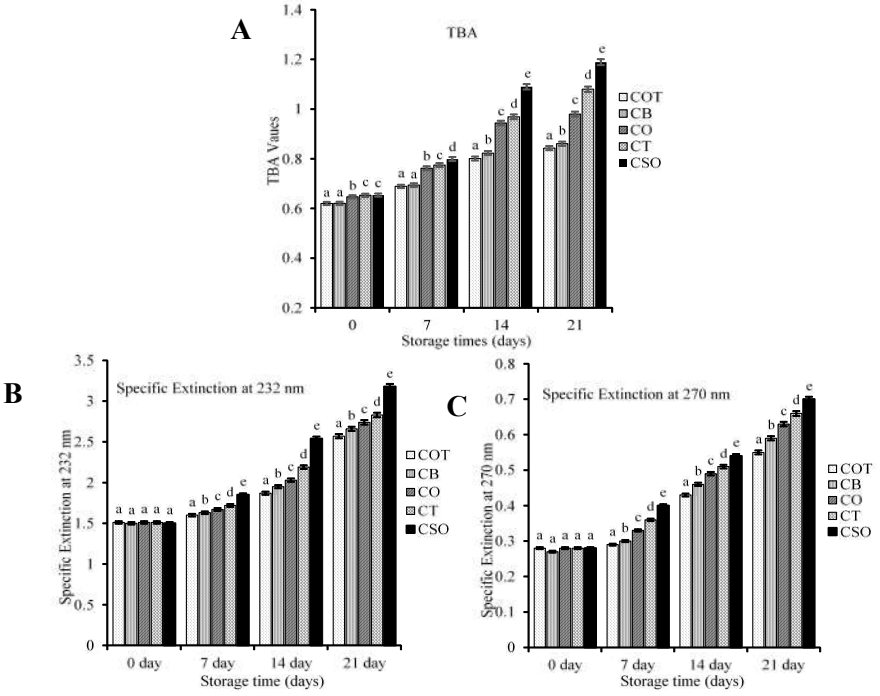


Figure 3. Changes in (A) Thiobarbituric Acid (TBA) Values, (B) Specific Extinction at 232 nm (K232), and (C) Specific Extinction at 270 nm (K270) of Untreated and Antioxidant-Treated Chia Seed Oils during Storage at 63 °C. Each value is the mean ± standard deviation of triplicate determinations. Values in each storage time grouping with different letters on bar are significantly different ($p < 0.05$). COT- chia seed oil containing 100 ppm OzF and 100 ppm TRF; CB- chia seed oil containing 200 ppm BHT; CO- chia seed oil containing 200 ppm OzF; CT- chia seed oil containing 200 ppm TRF; CSO- chia seed oil.

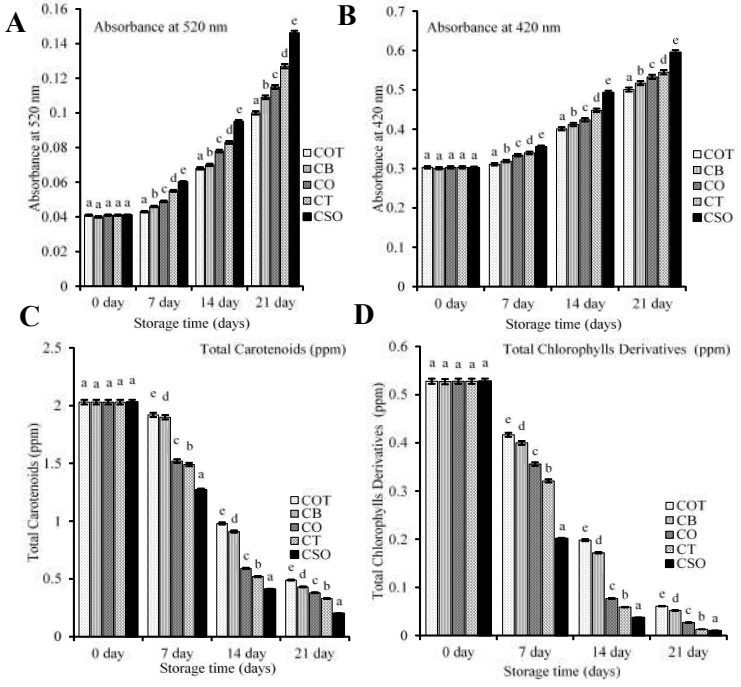


Figure 4. Figure 4. Changes in (A) Absorbance at 520 nm, (B) Absorbance at 670 nm, (C) Total Carotenoid Content, and (D) Total Chlorophyll Derivatives of Untreated and Antioxidant-Treated Chia Seed Oils during Storage at 63 °C. Each value is the mean ± standard deviation of triplicate determinations. Values in each storage time grouping with different letters on bar are significantly different ($p < 0.05$). COT- chia oil containing 100 ppm OzF combined with 100 ppm TRF; CB- chia oil containing 200 ppm BHT; CO- chia oil containing 200 ppm OzF; CT- chia oil containing 200 ppm TRF; CSO- chia seed oil.

Table 1. Fatty acid composition (%) of chia seed oils before storage.

Fatty acids	Oil samples				
	CSO	Antioxidant rich fraction incorporated oil samples			
		CT	CO	CB	COT
Palmitic Acid (C16:0)	7.42±0.15^{a}	7.50±0.15^{a}	7.44±0.15^{a}	7.46±0.15^{a}	7.51±0.15^{a}
Stearic Acid (C18:0)	2.47±0.40^{a}	2.81±0.40^{b}	2.87±0.40^{c}	2.75±0.40^{b}	2.84±0.40^{bc}
Oleic Acid (C18:1)	9.79±0.75^{d}	9.37±0.75^{b}	9.67±0.75^{cd}	9.60±0.75^{c}	9.19±0.75^{a}
Linoleic Acid (C18:2)	19.37±0.35^{b}	19.08±0.35^{a}	19.10±0.35^{a}	19.10±0.35^{a}	19.02±0.35^{a}
Linolenic Acid (C18:3)	61.00±0.50^{a}	61.23±0.50^{ab}	61.00±0.50^{a}	61.10±0.50^{a}	61.43±0.50^{b}
∑Saturated fatty acid	9.89	10.31	10.31	10.21	10.35
∑Monounsaturated fatty acid	9.79	9.37	9.67	9.60	9.19
∑Polyunsaturated fatty acid	80.37	80.31	80.10	80.20	80.45

Each value is the mean ±standard deviation of triplicate determinations. Values with different letters within a row for each fatty acids were significantly different ($p < 0.05$). COT- chia seed oil containing 100 ppm OzF and 100 ppm TRF; CB- chia seed oil containing 200 ppm BHT; CO- chia seed oil containing 200 ppm OzF; CT- chia seed oil containing 200 ppm TRF; CSO- chia seed oil.

Table 2. Changes in saturated, monounsaturated, and polyunsaturated fatty acids of chia seed oil samples during heating at 63 ℃.

Sample		Storage time (Day)	SFA	MUFA	PUFA	P/S
CSO		0	09.89 (100)	09.79 (100)	80.37 (100)	8.2
		14	13.01 (131.54)	10.22 (104.39)	76.77 (95.52)	6.0
		21	16.02 (161.98)	10.33 (105.51)	73.65 (91.63)	4.6
Antioxidant rich fraction incorporated oil samples	CT	0	10.31 (100)	09.37 (100)	80.31 (100)	7.8
		14	12.11 (117.45)	10.22 (109.07)	77.66 (96.70)	6.5
		21	14.17 (137.43)	10.76 (114.83)	75.07 (93.47)	5.3
	CO	0	10.31 (100)	09.67 (100)	80.10 (100)	7.8
		14	10.52 (102.03)	10.83 (111.99)	78.65 (98.18)	7.5
		21	14.22 (137.92)	10.72 (110.85)	75.07 (93.72)	5.3
	CB	0	10.21 (100)	09.60 (100)	80.20 (100)	7.9
		14	12.31 (120.56)	09.46 (98.54)	78.23 (97.54)	6.4
		21	13.14 (128.69)	10.24 (106.66)	76.61 (95.52)	5.8
	COT	0	10.35 (100)	09.19 (100)	80.45 (100)	7.8
		14	11.46 (110.72)	9.42 (102.50)	79.12 (98.34)	7.0
		21	12.20 (117.87)	9.80 (106.63)	78.00 (96.95)	6.4

Number in parenthesis is relative % of saturated, monounsaturated and polyunsaturated fatty acids based on the initial saturated, monounsaturated and polyunsaturated fatty acids content before heating. P/S- ratio of polyunsaturated to saturated fatty acids. Each value is the mean of triplicate determinations. COT- chia seed oil containing 100 ppm OzF and 100 ppm TRF; CB- chia seed oil containing 200 ppm BHT; CO- chia seed oil containing 200 ppm OzF; CT- chia seed oil containing 200 ppm TRF; CSO- chia seed oil.

3.3.2 Changes in color value, carotenoids and chlorophyll contents

The visual indication of color is an expedient method for assessing the quality of vegetable oil. In the present work, a notable transformation was observed in the hue of the CSO, which shifted from yellow to a deep reddish tone upon oxidation (Figure 4). The absorbance values in all samples detected as 0.30 at 420 nm and 0.04 at 520 nm, were increased significantly ($p< 0.05$) during oxidation time, and these increments were greater in CSO (0.59 at 420 nm and 0.14 at 520 nm) after 21st day of storage. Hossen et al. [42] stated that the absorbance at 420 nm enhanced markedly ($p< 0.05$) during storage at 62 ℃ of seed oil extracted from microwave irradiated black cumin seed; these increments detected to be higher in the untreated oils. Visually, color of the CSO samples initially appears yellow (absorbance at 420 nm), then after heat treatment it turns to reddish brown (absorbance 520 nm). However, the darkening of oil color might be attributed to the PUFA degradation. As storage time increases, the formation of ketones, dienoic acids, and elevated levels of peroxides contribute to the darkening of the

oil [45]. Thus, the present results lend support to earlier findings, which indicated an increase in the color values of oils during heating of mixed canola cooking oils [46]. Owing to improper storage, the edible oils are exposed to light, heat, and oxygen. In such a way, edible oils are oxidized that occurs dissipation of its carotenoids and chlorophyll derivatives resulting into a change in color.

This exposure may also influence the scent and taste of fat. As illustrated in figure 4, the data revealed the concentration of carotenoids and chlorophyll decreased with increasing storage time. Specifically, carotenoids content in CSO reduced from 2.03 to 0.20 ppm and chlorophyll content from 0.53 to 0.01 after 21 days of oxidation. The values in fresh CSO were much lower than the values 5.41 ppm for carotenoids and 4.66 ppm for chlorophyll reported by Bodoira et al. [13] for same seed oil and 2.4 ppm for carotenoids and 2.7 ppm for chlorophyll reported by Borello and Domenici [47] for blend cultivar of olive oil. According to Ghafoor *et al.*, [48] chia seed oil contained 3.66 ppm carotenoids that was reduced to 0.83 ppm after seed roasting at 180 ℃. However, the present value was lower compared to literature values which might be due to the different methods of determination. In addition, factors including climate, soil, irrigation, geographic location, postharvest handling and processing are well known to affect the composition of chia seeds [49]. After 21 days of oxidation, lowest decline was detected for both pigments in COT.

3.3.3 Changes in fatty acids profile

The fatty acids of plant oils play a vital role for the determination of the extent of its oxidation stability. The changes in the fatty acids concentration can indicate their stabilities, physical properties and nutritional properties. The major fatty acids of untreated CSO were C18:3 (61.00 %), C18:2 (19.37 %), C18:1 (9.79 %) and C16:0 (7.42 %) while C18:0 present in concentrations less than 3 % (Table 1). These two PUFAs comprised about 80 % of the total fatty acids in CSO, which differ slightly from previously reported data (79 %) [50]. Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in fresh CSO accounted 9.89, 9.79 and 80.37 % of the total fatty acids, respectively. The FAC remained almost unchanged upon antioxidant treatment. As shown in table 2, the relative content of PUFA was decreased, while that of SFA or MUFA increased in oil samples during oxidation at 63 ℃, probably due to PUFA degradation. But the change in fatty acids concentration was lower in antioxidant treated oils than that found in untreated ones during storage. A similar trend was found by Sunil et al. [24] during storage at 37 ℃ of sunflower oil incorporated with oryzanol concentrate and purified oryzanol. The highest reduction of PUFA was computed in fresh CSO (8.37 %) and lowest in COT (3.05 %) after 21 days of storage. Hossen et al. [42] also found the same trend during storage at 62 ℃ of oil samples from microwaved black cumin seed. Thus, the significantly slower rate of decreasing the percentage of PUFA in antioxidant containing oils during accelerated storage probably indicates the protection by antioxidant agents against lipid oxidation. In addition, the oil samples incorporated with antioxidants, exhibited lowest ratio of polyunsaturated to saturated fatty acids (P/S) change indicating the oxidative reactions progressed more rapidly in fresh CSO than the remaining samples during heating.

3.3.4 Changes in FTIR spectra

A great deal of information on the oxidative state of oils may be achieved by considering the frequency and absorbance of several bands of infrared spectra. An increase or decrease in some wave regions was noted in the present work. However, only the regions that are related to certain oil oxidation products, were evaluated. Figure 5 illustrates the significant spectral changes in CSO samples subjected to oxidative exposure to 63 ℃. The detected functional groups responsible for IR absorption peak: 3008 cm^{-1} [stretching =C-H(cis)], 2928 cm^{-1} [asymmetric stretching –C-H(-CH_2)], 2854 cm^{-1} [symmetric stretching –C-H(-CH_2)], 1744 cm^{-1} [stretching –C=O], 1464 cm^{-1} [bending –C-H(CH_2)], 1377 cm^{-1} [bending –CH_2], 1238, 1160 and 1098 cm^{-1} [stretching –C-O ester] [51]. The intensity of cis-double bond at 3008 cm^{-1} (shoulder) suffered a gradual increase during heating. The observed increase can be attributed to the formation of free radicals during oxidation, which then leads to the primary oxidation of unsaturated fatty acids.

The resulting primary oxidative products contain *cis* and conjugated double bonds, as was previously observed in the auto-oxidation of oleic and linoleic acids [52]. As the oxidation proceeded, the intensities of the bands at 2928 and 2854 cm^{-1} increased. This observation can be attributed to the advanced oxidation state in oils under investigation [53]. Increase in peak intensity at 1744 cm^{-1} refers to the changes observed at the C=O during the storage period. This transformation is related to the deterioration of hydroperoxides and the generation of saturated aldehydes or other compounds including alcohols, ketones, acids, and esters in further secondary oxidation [54]. Carbonylic compounds are the main secondary products of oxidation formed when hydroperoxide decomposes. The presence of more carbonylic compounds is indicated by a higher intensity at 1744 cm^{-1} [55].

The intensity of a weak peak at 1465 cm^{-1} increases with the storage time. The saturated acyl groups present in the sample are indicated by the peak observed at 1160 cm^{-1}. A similar trend was computed for the peaks at 3008, 2928, 2854, 1745, 1465, and 1165 cm^{-1} by Hossen *et al.*, [42] during oxidation at 62 ℃ of oil samples from microwaved black cumin seed. The peak intensities (absorbencies) of crude CSO were greatly altered compared to pretreated oils during the storage indicating the impact of incorporating antioxidant-rich-extracts on oxidation of lipids.

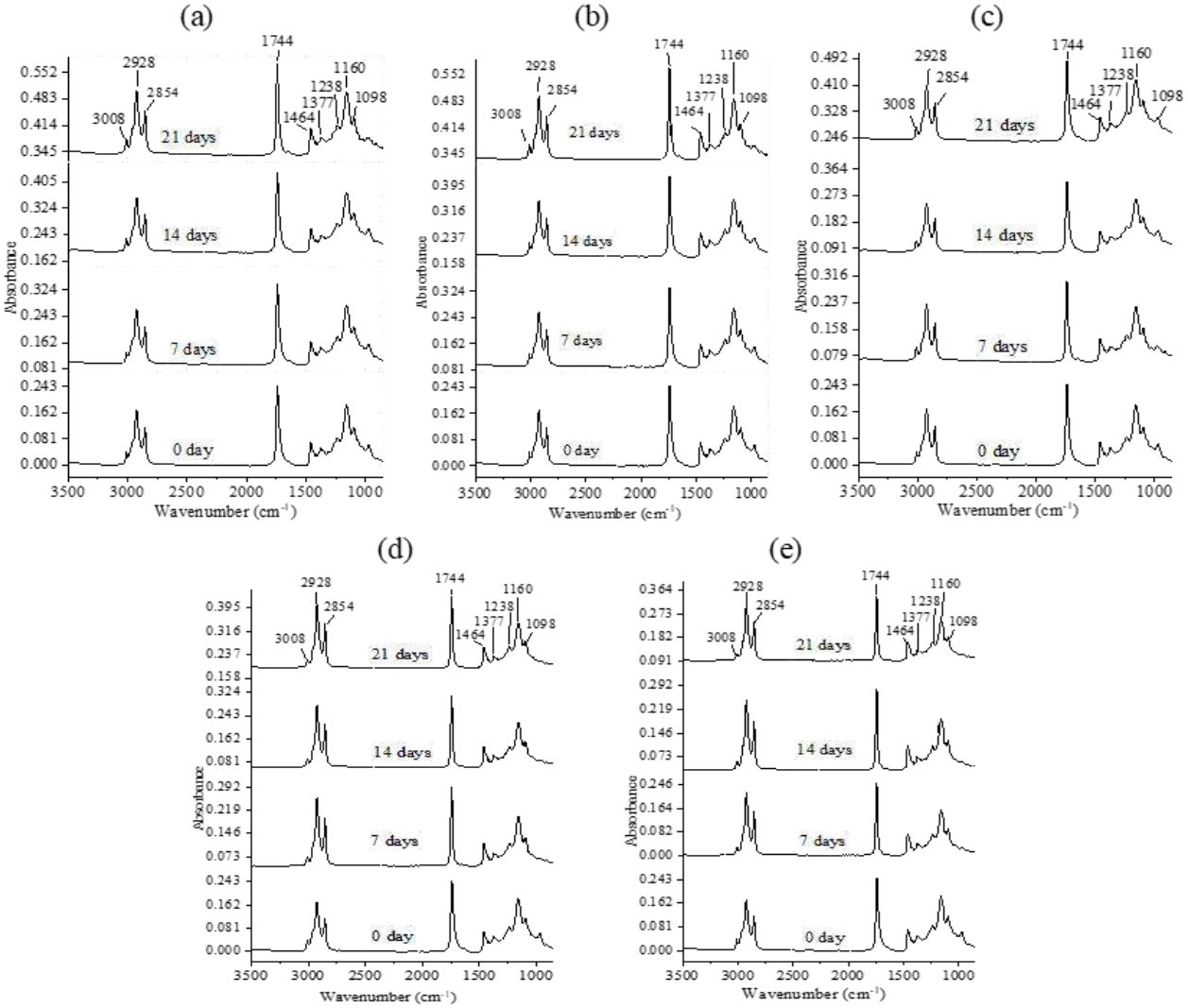


Figure 5. Changes of FTIR spectra of (a) crude chia oil, (b) chia oil containing 200 ppm TRF, (c) chia oil containing 200 ppm OzF, (d) chia oil containing 200 ppm BHT, (e) chia oil containing 100 ppm OzF and 100 ppm TRF during storage at 63 ℃.

4. Conclusion

The present results reflect the significant effects of admixing antioxidant rich fractions extracted from rice bran with CSO on the oxidative stability and compositional changes in CSO. γ- Oryzanol rich fraction (OzF) showed strong ability to act as an oxidation-inhibitor as compared to tocotrienol rich fraction (TRF). Oxidative parameters indicate lower tendency to form oxidative products in the antioxidant rich oils than those in fresh CSO during storage. During storage at 63 ℃, the oils become oxidized with degradation of PUFA and formation of some undesired harmful compounds. However, the slower oxidation rate of PUFA in antioxidative agent blended oils probably provided protection by antioxidative agents against oil oxidative process. With a few exceptions, the FTIR spectra of the oils displayed similar spectral characteristics of the functional group region indicating similar compounds present in the oils after storage period. However, the intensities of the bands varied with the samples, indicating unequal levels of oxidation degradative products in the samples. After 21 days of storage, the difference in the quality of CSO incorporated with a mixture of 100 ppm OzF and 100 ppm TRF, was remarkable compared to rest oil samples. The data indicate that, while both fractions suppress lipid peroxidation, their individual efficacies vary over time. The obtained results suggest a possible synergistic interaction between γ- oryzanol and tocotrienol rich fractions in inhibiting the lipid oxidation of CSO, rather than the clear dominance of a single extract. This work presents that rice bran can serve as an important source of natural antioxidants with potent antioxidant activity. In addition, the analyzed results will help small entrepreneurs and farmers in improving the quality of underutilized CSO for better marketability.

Author contributions

Aktarun Nahar: Conceptualization, investigation, methodology, data curation, formal analysis, visualization, writing – original draft, writing – review & editing, validation. M. Abbas Ali: Conceptualization, resources, methodology,

writing – review & editing, supervision. Jewel Hossen: Visualization, writing - review & editing. M. Ahasanur Rabbi: writing - review & editing. All authors read and approved the final manuscript.

Ethical approval

Not applicable.

Conflicts of Interest

The authors report no conflicts of interest.

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Data availability statement

Data are available from the corresponding author upon reasonable request.

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