Short Note

Effect of thymoquinone and tocopherols on the oxidative stability of purified *Nigella sativa* oil

Semra TURAN [a]
Seyma DILMEN [a]
Mustafa KIRALAN [b]
Ayse G. OZAYDIN [c]
Gulcan OZKAN [d]
Mohamed Fawzy RAMADAN [e, f]

[a] Bolu Abant Izzet Baysal University
Faculty of Engineering, Department of Food Engineering, Bolu, Turkey

[b] Balıkesir University
Faculty of Engineering
Department of Food Engineering
Balikesir, Turkey

[c] Suleyman Demirel University,
YETEM Innovative Technology Application and Research Center, Isparta, Turkey

[d] Suleyman Demirel University
Faculty of Engineering
Department of Food Engineering
Isparta, Turkey

[e] Faculty of Agriculture
Biochemistry Department
Zagazig University, Egypt

[f] Deanship of Scientific Research
Umm Al-Qura University,
Makkah Kingdom of Saudi Arabia

(*) CORRESPONDING AUTHOR:
Prof. Dr. Mohamed Fawzy RAMADAN
Deanship of Scientific Research,
Umm Al-Qura University, Makkah,
Kingdom of Saudi Arabia
Tel: 00966597303044
Email: mhassanien@uqu.edu.sa

Received: August 22, 2019
Accepted: February 7, 2020

The effect of minor bioactive constituents (α-, γ-tocopherol and thymoquinone) and the binary and ternary mixtures of those constituents on the oxidative stability of purified black cumin oil (PBCO) was evaluated. PBCO [purified triacylglycerols (TAG)] was enriched with different concentrations of these bioactive compounds then subjected to an accelerated thermal oxidation tests in a Rancimat apparatus at 90°C and in an oven at 60°C. The induction period (IP) of purified TAG containing ternary mixtures of minor bioactive constituents was higher than that of control purified TAG. Peroxide value (PV), conjugated diene (K_{232}), *p*- anisidine value (AV) and polymer triglyceride content were measured during storage at 60°C. A ternary mixture of minor bioactive constituents was more effective to retard oil oxidation than a binary mixture. In addition, purified TAG containing individual and binary mixture with γ-tocopherol were more stable towards oxidation. During storage at 60°C, α-tocopherol disappeared rapidly followed by γ-tocopherol. The more resistant minor compound towards oxidation was thymoquinone wherein a slight decrease in its amount was observed at the end of storage experiment.

Keywords: *Nigella sativa*. Purified oil. Lipid oxidation. Polymer triglyceride. Thermal stability. Triacylglycerols

1. INTRODUCTION

Black cumin (*Nigella sativa*, family Ranunculaceae) is an annual herbaceous plant native to southwest Asia including Iran, India, and Pakistan. *Nigella sativa* seeds are used in several food products such as bread, cookies and some traditional dishes as well as in traditional medicine for culinary purposes [1-3]. The seeds contain several compounds including water, oil, proteins, carbohydrates, vitamins, and minerals [4].

The oil of *Nigella sativa* seeds is accounted for about 30% of seed weight [5]. Oil is generally extracted from seeds using the cold pressing method. Cold pressed black cumin seed oil (BCO) contain various bioactive compounds such as fatty acids, tocopherols, sterols, phenolics and colour pigments. Linoleic and oleic acids are the main unsaturated fatty acids and accounted for more than 80% of the total fatty acids in BCO [2, 6, 7]. BCO contains typical tocochromanols, like γ-tocopherol (938.5 μg/g), γ-tocotrienol (376.7 μg/g) and β-tocotrienol (284.3 μg/g), which exhibit strong antioxidant potential [7]. Thymoquinone is one of the dominant phenolics detected in BCO with antioxidant traits and therapeutic effects [8, 9].

BCO has a strong antioxidant activity and therefore the oil was successfully blended with some vegetable oils to improve the oxidative stability of these oils. Black cumin oil increased oxidative stability of corn oil [10], high linoleic sun-

flower oil [1, 11] and flaxseed oil [12] stored under thermal oxidation conditions at 60°C. The oxidative stability of black cumin oil is attributed to tocochromanols and phenolics especially thymoquinone. Besides, oxidation stability of purified black cumin oil (PBCO) was also investigated and compared with crude oil [13].

There are already published papers on the oxidation stability of black cumin oil as well as the chemical composition of the oil. However, to the best of our knowledge, there is no literature on the effects of minor compounds on oxidative stability of PBCO. The aim of this study was to examine the effect of minor constituents (thymoquinone and tocopherol isomers) on the oxidative stability of PBCO using accelerated oxidation in Rancimat and Schaal Oven test conditions.

2. MATERIALS AND METHODS

Commercial BCO used in this study was obtained from Neva & Sim Ltd. Co. (Istanbul, Turkey). The samples were stored at 4°C in the refrigerator until analysis.

2.1. COMPOSITION AND CHARACTERISTICS OF BCO

Free fatty acid content (FFA) (Ca 5a-40), peroxide value (PV) (Cd 8-53), *p*-anisidine value (AV) (AOCS Cd 18-90), specific absorbance values (K_{232} and K_{270}) (Ch 5-91) were determined in BCO using AOCS [14] official methods. The fatty acid composition was determined using Shimadzu GC-2010 plus gas chromatograph (GC) equipped with a flame ionisation detector (FID) and TR-CN 100 fused silica capillary column (Teknokroma, Spain) (60 m long, 0.2 µm film thickness and 0.25 mm i.d.) following methylation (Ce 2-66 in AOCS method). Helium was used as a carrier gas at a flow rate of 1 mL/min. 1 µL sample was injected to the column and split ratio was 1:50. The column temperature was set at 90°C for 5 min, then programmed at 240°C at 4°C/min and held at 240°C for 10 min. Fatty acid methyl ester (FAME) standard solution (37 FAME mix) was used to identify the peaks. Fatty acid composition of BCO was given in percentage proportions of FAME using the peak areas.

Tocopherols were analysed following the AOCS Official Method Ce 8-89 [14] with minor modification [(950 µL of mobile phase (heptane: tetrahydrofuran, 95:5, v/v) was added to 50 µL oil sample)] using Prominence 2005 HPLC (Shimadzu Tokyo, Japan) with a SIL–20AC Prominence and RF-10AXL Fluorescence detector, set for excitation at 295 nm and emission at 330 nm. The column was a normal-phase Luna Silica (250 × 4.6 mm; 5 m). Of each sample, 10 µL was injected and separated by mobile phase consisted of heptane: tetrahydrofuran (95:5, v/v) at a flow rate of 1.2 mL/min. The tocopherols were quantified using external calibration for each isomer separately. The amounts of tocopherols in the samples were given in mg/kg using the calibration curve of each tocopherol isomer.

Aliquots of oil (2.5 g) were dissolved in *n*-hexane (2.5 mL) and mixed with 2.5 mL of methanol in 50 mL centrifuge tube in a vortex. After centrifugation at 4000 rpm for 3 min, the hydroalcoholic extracts were separated from the lipid phase and the extraction was repeated twice. The combined hydroalcoholic extracts were evaporated in a rotary evaporator at 40°C under vacuum, then the oil was dissolved in 1 mL methanol. The total phenolic content (TPC) and thymoquinone content in BCO samples were determined using HPLC according to the modified method of Karacabey and Mazza [15] and Caponio et al. [16], respectively. HPLC analysis was performed using a Shimadzu HPLC system equipped with an automatic injector, a column oven, and a diode array detector (DAD). The analysis was carried out at 30°C on an Agilent Eclipse XDB-C18 column (250×4.6 mm, 5 µm) in isocratic mode at a constant flow rate of 0.8 mL/min. Injection volume of the sample was 20 µL and the mobile phase consisted of methanol: 3% acetic acid in water (50:50, v/v).

2.2 PREPARATION OF PBCO (PURIFIED TAG)

Cold pressed black cumin oil (BCO) was purified according to Karabulut et al. [17] using activated carbon adsorption and alumina column chromatography treatments to obtain a purified TAG.

2.3 PREPARATION OF OIL SAMPLES ENRICHED WITH TOCOPHEROLS AND THYMOQUINONE

It was determined that BCO used in this study contain 10 mg/kg α-tocopherol, 0.5 mg/kg β-tocopherol, 67 mg/kg γ-tocopherol and 0.5 mg/kg δ-tocopherol. The major phenolic compound, thymoquinone was 1082 mg/kg in BCO. Because of the content of β-tocopherol and δ-tocopherol in the BCO was very low, only pure α- and γ-tocopherols were added to the purified TAG and in a combination of thymoquinone. The addition amount was selected according to levels of these compounds found in BCO. Before addition to PBCO, pure tocopherol isomers were prepared in *n*-hexane while thymoquinone was prepared in methanol. For the Rancimat assay, these solutions were added to 3 g of PBCO (purified TAG) with a micropipette. They were placed in an ultrasonic water bath for 1 min for dissolution. Subsequently, nitrogen was flushed into the enriched oil to remove the solvent. In the Schaal Oven test, these additives were added to 50 g of purified TAG. After the flushing of nitrogen gas into the enriched TAG, they were stored at -18°C until Rancimat analysis and oxidation test were performed.

Tocopherols and thymoquinone were added to purified oil to obtain the following formulations:

- Purified TAG (control)
- Purified TAG + BHT (100 mg/kg)

- ¿ Purified TAG+ thymoquinone (1082 mg/kg)
- ¿ Purified TAG+ α-tocopherol (10 mg/kg)
- ¿ Purified TAG+ γ- tocopherol (67 mg/kg)
- ¿ Purified TAG+ α-tocopherol (10 mg/kg) + thymoquinone (1082 mg/kg)
- ¿ Purified TAG+ γ-tocopherol (67 mg/kg) + thymoquinone (1082 mg/kg)
- ¿ Purified TAG+ α-tocopherol (10 mg/kg) + γ-tocopherol (67 mg/kg) + thymoquinone (1082 mg/kg)
- ¿ Cold pressed black cumin oil (BCO)

2.4. ACCELERATED OXIDATION TESTS

2.4.1. Rancimat test

The induction periods of PBCO mixed with thymoquinone and/or tocopherol isomers were carried out with the Rancimat apparatus (Metrohm, Herisau, Switzerland). The oil samples (3 g) were placed in the Rancimat apparatus at 90°C at an air flow rate of 10 L h^{-1}.

2.4.2. Schaal oven test

Fifty grams of sample was weighted in 50 mL glass bottles and kept 10 days at 60°C in an oven. Samples were examined at 24 h intervals by collecting the same bottles upon the particular period. The oxidative stability of the samples was evaluated by polymer triglyceride, PV, AV and K_{232}. Besides, the amount of thymoquinone and tocopherol isomers were determined during storage. Experiments were set up in two repetitions for each tested sample.

2.5. DETERMINATION OF POLYMER TRIGLYCERIDES

The amount of polymer triglyceride in the samples taken in the oxidation test was determined according to Gertz [18] and AOCS Official Method Cd 22-91 [14]. The results were given as a percentage.

2.6. STATISTICAL ANALYSIS

Oxidation test experiments were performed in two replicates. The results were given as mean ± standard deviation. The results were statistically evaluated using the Minitab 17 Statistical Software (v17.3.1) package program. The difference between the group means was determined according to variance analysis technique (ANOVA) and independent samples *t*-test (p <0.05).

3. RESULTS AND DISCUSSION

3.1. COMPOSITION AND CHARACTERISATION OF BCO

Some properties of BCO and PBCO are given in Table I. The initial FFA, PV, K_{232}, K_{270}, AV of BCO samples were 6.1%, 16.3 meq O_2/kg, 2.76, 0.56 and 1.78, respectively, while these values in PBCO were significantly (p<0.05) decreased to 0.08%, 5.3 meq O_2/kg, 1.36, 0.06 and 0.08, respectively. This decrease could be related to purification. During the purification process, most of the compounds including tocopherols, carotenoids, FFA, mono- and diglycerides, peroxides and some of the secondary oxidation products removed from crude oil. The recorded results for FFA, PV, K_{232} and K_{270} values were found lower than the findings reported for BCO [6, 19]. However, the results in FFA, PV and K_{232} values were higher than those reported by Gharby et al. [2] and Kostadinović Veličkovska et al. [20]. The AV of oil was higher than those presented by Symoniuk et al. [21]. A significant (p<0.05) decrease was also observed in total tocopherols, thymoquinone and TPC after purification process. The total tocopherol content was lower than those reported by Sultan et al. [22] who found 361.71 mg/kg of total tocopherols. However, the results for total tocopherols agreed with some data published by Matthaus and Özcan [23] who reported a range of 9.15 mg/100 g to 27.92 mg/100 g of total tocopherols. The main fatty acid in BCO and its triglyceride was linoleic acid (18:2n-6) 55.07%-56.16% followed by oleic acid (18:1n-9) at a level of 27.1%-25.9% and palmitic (16:0) acid at 11.48%-11.60% of total fatty acids, respectively. The results were similar to those presented by Lutterodt et al. [24] and Gharby et al. [2]. Although the fatty acid composition of BCO changed slightly after purification process, this change was found as significant (p<0.05).

The initial TPC and tocopherol composition in oils are shown in Table II. BCO contained the highest level of

Table I - Composition and characterization of BCO and PBCO[a]

	Cold press black cumin oil (BCO)	Purified black cumin oil (PBCO)
Free fatty acid content (% oleic acid)	6.1±0.40	0.08±0.01
Peroxide value (meq O_2/kg)	16.3±1.2	5.3±0.7
K_{232}	2.76±0.23	1.36±0.18
K_{270}	0.56±0.11	0.06±0.02
p-Anisidine value	1.78±0.92	0.08±0.03
Total tocopherol content (mg/kg)	78.72±0.10	0.09±0.01
Total phenolic content (mg/kg, HPLC)	293.57±14.31	*N.D.
Thymoquinone content (mg/kg)	1082.54±0.24	7.19±0.23
Fatty acids (%)		
Miristic acid (C14:0)	0.14±0.0	0.12±0.0
Palmitic acid (C16:0)	11.60±0.01	11.48±0.0
Stearic acid (C18:0)	2.99±0.01	3.1±0.0
Oleic acid (C18:1n9c)	25.9±0.02	27.1±0.0
Linoleic acid (C18:2n6c)	56.16±0.02	55.07±0.0
γ-linolenic acid (C18:3n6)	0.31±0.0	0.21±0.0
Linolenic acid (C18:3n3)	0.64±0.01	0.60±0.0
cis-11,14-eicosadienoic acid (C20:2)	2.26±0.0	2.32±0.0

[a]Mean ±standard deviation; *N.D., not determined

Table II - Phenolic compounds and tocopherol profile of BCO[a]

	Content (mg/kg)
Phenolic compound	
p-Hydroxybenzoic acid	0.80 ± 0.04
Vanilin	0.04 ± 0.00
Sinapinic acid	1.05 ± 0.05
Benzoic acid	1.79 ± 0.08
Quercetin	1.05 ± 0.11
Luteolin	1.04 ± 0.07
Apigenin	16.09 ± 0.59
Thymoquinone	1082.5 ± 0.24
Tocopherol	
α-tocopherol	10.13 ± 0.15
β- tocopherol	0.57 ± 0.01
γ- tocopherol	67.45 ± 0.07
δ- tocopherol	0.58 ± 0.01

[a] Mean ± standard deviation

Table III - Induction periods of BCO and PBCO[a]

	Induction period (h)	Protection factor
TAG (PBCO)[b]	2.66 ± 0.51d	1.0
TAG+BHT	14.23 ± 1.37b	5.3
TAG+T	4.44 ± 0.14cd	1.7
TAG+ *α*-TOC	5.18 ± 0.04cd	1.9
TAG+ *γ*-TOC	12.39 ± 0.0b	4.7
TAG+ *α*-TOC +T	6.44 ± 0.15c	2.4
TAG+ *γ*-TOC +T	14.92 ± 1.53b	5.6
TAG+ *α*-TOC+ *γ*-TOC +T	15.36 ± 0.23b	5.8
BCO	19.73 ± 0.25a	-

[a] Mean ±standard deviation

[a-d] Values within each column with different letters are different ($p < 0.05$).

[b] TAG (PBCO), purified black cumin oil; BCO, cold pressed black cumin oil; T, thymoquinone; α-TOC, α-tocopherol; γ-TOC, γ-tocopherol; BHT, butylated hydroxytoluene.

thymoquinone (1082.54 mg/kg). The other seven phenolic compounds found at lower concentrations. Thymoquinone content was higher than that of 3.48-8.73 mg/kg detected in six different batches of cold-pressed BCO [24], but it was lower than that of 460 mg/100 g detected in the BCO [11].

Tocopherol content of BCO is also given in Table II. The content of γ-tocopherol was the highest, with 67.45 mg/kg, followed by α-tocopherol at a concentration of 10.13 mg/kg. However, δ- and β-tocopherol were detected in lower amounts in the oil (0.58 mg/kg and 0.57 mg/kg, respectively). γ-tocopherol was found as the main tocopherol isomer in BCO and its value was lower than that of black cumin oil (938.5 µg/g) reported by Hassanien et al. [25].

3.2. ACCELERATED OXIDATION EXPERIMENTS

3.2.1 Induction period (IP)

The induction periods of BCO, purified triglycerides blended with tocopherols and thymoquinone used for the accelerated oxidation experiments are reported in Table III. The higher IP was recorded for BCO as 19.73 h, while lower IP was recorded for PBCO (2.66 h). IP of TAG significantly ($p<0.05$) increased with the addition of α-, γ-tocopherols and thymoquinone. When the effects of tocopherols and thymoquinone were individually compared, γ-tocopherol was the most effective antioxidant. The IP of TAG enriched with γ-tocopherol was 12.39 h, however, the lower IP was recorded for TAG enriched with thymoquinone (4.44 h). Concerning the results of the tocopherol homologs, the similar behaviour was seen in the reports of Wagner and Elmadfa [26] who reported that γ-tocopherol was more effective in the prolongation of the shelf life of olive oil and linseed oil than α-tocopherol. On the other hand, the lower stability of α-tocopherol than γ-tocopherol at higher temperatures could be explained that α-tocopherol is consumed first, followed by γ-tocopherols due to higher reactivity of α-tocopherol [27].

When minor bioactive constituents were added together, the highest IP value was observed in oil sample spiked with α-tocopherol/γ-tocopherol /thymoquinone (15.36 h). Usage of thymoquinone with tocopherols caused a slight increase in IP values compared with tocopherols. In addition, when TAG incorporated with a synthetic antioxidant (BHT) at 100 mg/kg, IP value of this sample was close to TAGs containing γ-tocopherol/thymoquinone and α-tocopherol/γ-tocopherol/thymoquinone ($p>0.05$).

3.2.2. Schaal oven test

Hydroperoxide is the primary product of lipid oxidation. Therefore, the determination of PV can be used as an oxidative index for the early stage of lipid oxidation [13]. The effects of minor bioactive constituents and BHT on PV of PBCO stored under Schaal oven conditions are presented in Table IV. PV of PBCO containing tocopherols and/or thymoquinone significantly increased during 10 days of oven test ($p<0.05$). Moreover, PV of PBCO increased from 11.0 to 234.9 meq/kg, and PV increased from 21.1 to 33.2 meq/kg in BCO at the end of storage. Similarly, Ramadan and Mörsel [13] reported that crude oils extracted by *n*-hexane had lower PV than that of stripped oils over the 21 days of storage period and the peroxides accumulated in the stripped oils to high levels.

Until the 7th day of storage, the minimum PV observed in PBCO incorporated with BHT ($p<0.05$). Among purified TAG containing individual bioactive constituents, the effectiveness of antioxidants decreased during 7 days (at 60°C) in the following order: TAG+γ-TOC > TAG+T > TAG+α-TOC. When investigating the antioxidant effects of tocopherols under thermal storage at 60°C, γ-tocopherol was more stable, and it consumed more slowly than α-tocopherol. These findings agree with data reported earlier [28, 29]. Purified TAG con-

Table IV - Changes in PV in BCO and PBCO stored under Schaal oven conditions at 60°C

Storage period (day)	TAG[a]	TAG+BHT	TAG+T	TAG+ α-TOC	TAG+ γ-TOC	TAG+ α-TOC +T	TAG+ γ-TOC +T	TAG+ α-TOC+ γ-TOC +T	BCO
Zero	11.0±1.7bG	9.1±0.9bF	19.9±0.9aG	9.7±0.0bF	10.4±0.8bG	18.7±0.8aC	20.1±0.6aD	21.6±0.0aF	21.1±2.8aB
1	24.8±0.6aF	9.1±0.9eF	21.0±0.8abG	15.8±1.7cdEF	12.8±0.9deFG	18.8±0.8bcC	20.6±0.2bD	21.8±1.6abF	18.9±0.8bcB
2	30.9±0.2aEF	13.4±1.6eE	28.4±0.6bcFG	30.5±1.8abDEF	22.6±2.6cdEFG	25.0±0.8bcdC	26.6±1.3bcD	23.8±0.9cdEF	20.0±2.6dB
3	46.3±1.7aE	14.0±0.9dDE	39.1±1.7bEF	39.0±1.5bCDEF	27.9±0.0cEF	25.4±3.4cC	28.0±0.2cD	30.4±1.8cDE	18.2±1.8dB
4	58.6±1.9aD	17.6±0.8dCD	48.5±3.3abDE	51.0±1.4abCDE	38.4±0.8bcDE	30.0±8.4cdC	38.7±3.4bcCD	34.6±0.5cD	20.0±0.6dB
5	65.4±0.7aD	18.7±0.7bC	58.4±1.3aCD	61.0±0.3aCD	45.4±10.1abCD	42.7±15.7abBC	38.4±12.8abCD	43.9±1.6abC	20.6±0.2bB
6	80.1±0.7aC	20.7±1.8dC	67.2±1.8abC	75.7±2.0aBC	58.2±0.0bcC	49.4±11.0cBC	49.9±3.1cBC	48.7±3.7cC	23.7±0.8dB
7	101.9±4.4aB	25.0±0.8dB	87.7±3.6abB	101.9±1.2aB	76.0±0.5bcB	68.9±13.0bcB	59.2±4.2cB	57.8±1.0cB	20.7±0.1dB
10	234.9±7.1aA	37.2±0.0eA	158.5±9.8bA	244.7±29.2aA	186.0±6.0bA	149.8±4.2bcA	113.0±3.4cdA	100.3±2.1dA	33.2±4.2eA

[a] Mean ±SD (standard deviation). TAG, purified black cumin oil; BCO, cold pressed black cumin oil; T, thymoquinone; α-TOC, α-tocopherol; γ-TOC, γ-tocopherol; BHT, butylated hydroxytoluene.
[a-e] Means within each row with different superscripts are significantly different (p <0.05)
[A-D] Means within each column with different superscripts are significantly different (p <0.05)

taining α-tocopherol/γ-tocopherol/thymoquinone had significantly (p <0.05) lower PV than purified TAG enriched with a mixture of α-tocopherol/ thymoquinone and γ-tocopherol/thymoquinone at the end of storage.

Table V summarises the effects of minor bioactive constituents on the formation of CD in (PBCO). Absorption at 232 nm is related to the formation of primary oxidation products [13]. Therefore, K_{232} values of oil samples showed a pattern in good agreement with that of the PV. Conjugated diene contents of the oil samples increased significantly (p<0.05) with the increase in storage time, due to the formation of conjugated dienes [13]. K_{232} value of BCO slightly increased to 4.9, whereas K_{232} value of PBCO was determined as 27.0 at the end of oven test. The lowest K_{232} values were observed for TAG enriched with BHT followed by BCO during 7 days of storage. When compared with oils containing individual bioactive constituents, γ-tocopherol was found to be more effective in retarding the formation of oxidation up to 7th days. After this day, K_{232} value of oil enriched with thymoquinone increased to 22.6 which was lower than those of α- or γ-tocopherol containing PBCO. During the storage period, purified TAG spiked with a mixture of thymoquinone, α-tocopherol and γ-tocopherol exhibited the lowest K_{232} value among α-, γ-tocopherol or thymoquinone incorporated PBCO. Besides, γ-tocopherol addition with thymoquinone had a lower K_{232} value than α-tocopherol incorporated with thymoquinone on the 7th and 10th day.

Table VI shows the changes in AV and polymer triglyceride content during the storage at 60°C for 10 days. AV measures the unsaturated aldehydes (principally 2-alkenals and 2,4-dienals) in oils [13]. AV of PBCO samples significantly increased with the storage period (p<0.05), whereas AV of BCO did not change significantly (p>0.05). While AV of PBCO significantly (p<0.05) increased from 0.4 to 16.4, polymer triglyceride contents of BCO was 1.44% at the end of 10 day. During storage, the lowest AV and polymer triglyceride contents were observed in BCO and purified TAG enriched with BHT. Individual use of minor bioactive constituents retarded the increase in AV and polymer triglyceride content. Especially, γ-tocopherol caused lower values than α-tocopherol during storage at 60°C.

The similar changes in AV of TAG was observed by Lampi et al. [28] who emphasised that tocopherols significantly (p<0.05) retarded the formation of secondary products on rapeseed oil TAG according to AV measurements. Binary mixtures of minor bioactive compounds decreased AV and polymer triglyceride content compared with individual usage of these compounds. Besides, ternary blend provided higher protection against oxidation than the individual and binary use of minor bioactive constituents.

Table V - Changes in CD (K_{232}) in BCO and PBCO stored under Schaal oven conditions at 60°C

Storage period (day)	TAG[a]	TAG+BHT	TAG+T	TAG+ α-TOC	TAG+ γ-TOC	TAG+ α-TOC +T	TAG+ γ-TOC +T	TAG+ α-TOC+ γ-TOC +T	BCO
Zero	1.9±0.01cG	1.7±0.03cC	2.4±0.01bG	1.9±0.08cH	1.8±0.13cG	2.2±0.03bD	2.4±0.09bF	2.3±0.05bF	2.9±0.07aCD
1	3.9±0.23aF	1.8±0.05dC	2.5±0.13bcFG	2.5±0.11bcH	2.1±0.03cdG	2.2±0.08cdD	2.2±0.05cdF	2.3±0.03bcdF	2.8±0.08bCD
2	5.0±0.16aE	2.1±0.05fC	3.4±0.10cF	4.0±0.01bG	3.2±0.13cdF	2.3±0.20fD	2.8±0.21deEF	2.8±0.05eEF	2.7±0.06eD
3	6.0±0.10aE	2.3±0.09fC	4.5±0.18cE	5.3±0.07bF	4.1±0.12cE	2.7±0.39efD	3.2±0.16deDE	3.3±0.10dDE	2.9±0.13deCD
4	7.3±0.09aD	2.1±0.04eC	5.1±0.20bcDE	6.3±0.12abE	5.0±0.12bcD	2.9±0.81deD	3.7±0.08cdD	3.7±0.04cdD	2.4±0.15deD
5	8.1±0.18aD	2.1±0.28eC	6.0±0.10bcD	7.1±0.04abD	5.5±0.17cD	3.1±0.11deD	3.8±0.11dD	3.8±0.11dD	2.4±0.17eD
6	16.1±0.59aC	3.5±0.10dB	12.9±0.40bC	13.8±0.61aC	10.9±1.10bC	6.3±0.14cC	6.6±0.07cC	6.4±0.04cC	3.7±0.01dBC
7	17.1±0.51aB	3.8±0.06dB	13.7±1.30bB	15.7±0.10aB	11.7±0.63bB	9.5±0.31cB	8.8±0.35cB	8.3±0.12cB	3.9±0.25dB
10	27.0±0.23aA	5.9±0.28gA	22.6±0.40cA	27.6±0.15aA	25.0±0.32bA	21.2±0.15dA	15.8±0.14eA	14.2±0.08fA	4.9±0.06gA

[a] Mean±SD (standard deviation). TAG, purified black cumin oil; BCO, cold pressed black cumin oil; T, thymoquinone; α-TOC, α-tocopherol; γ-TOC, γ- tocopherol
[a-e] Means within each row with different superscripts are significantly different (p <0.05)
[A-D] Means within each column with different superscripts are significantly different (p <0.05)

Table VI - Changes in AV and polymer triglyceride content (%) of BCO and PBCO stored under Schaal oven conditions at 60°C

	Storage period (day)	TAG[a]	TAG+BHT	TAG+T	TAG+ α-TOC	TAG+ γ-TOC	TAG+ α-TOC +T	TAG+ γ-TOC +T	TAG+ α-TOC+ γ-TOC +T	BCO
AV	0	0.4±0.0bD	0.4±0.0bC	0.4±0.0bD	0.4±0.0bC	0.4±0.0bC	0.4±0.0bD	0.4±0.0bC	0.4±0.0bC	1.7±0.4aA
	3	1.8±0.0aC	0.9±0.1cB	1.3±0.2abcCD	1.7±0.1abBC	1.0±0.1bcC	0.9±0.3cCD	0.7±0.1cBC	0.8±0.1cC	1.8±0.4abA
	5	2.7±0.2aC	1.0±0.0eB	1.7±0.0cBC	2.1±0.0bBC	1.2±0.2deC	1.1±0.2deC	1.4±0.1cdeBC	1.5±0.0cdB	1.5±0.1cdA
	7	4.8±0.2aB	0.9±0.1dB	2.8±0.0bB	4.4±0.6aB	2.5±0.1bcB	2.1±0.1bcB	1.0±0.1dB	1.4±0.1cdB	1.4±0.5cdA
	10	16.4±0.6aA	2.0±0.1eA	7.5±0.6bcA	15.8±2.1aA	9.7±0.5bA	5.5±0.1cdA	2.5±0.4deA	2.4±0.3eA	1.0±0.0eA
Polymer triglyceride content (%)	0	0.0±0.0aD	0.0±0.0aB	0.0±0.0aC	0.0±0.0aB	0.0±0.0aC	0.0±0.0aC	0.0±0.0aC	0.0±0.0aB	0.0±0.0aB
	3	0.07±0.01aCD	0.01±0.0efB	0.03±0.0cC	0.05±0.01bB	0.03±0.0cdC	0.01±0.0deC	0.02±0.0deC	0.01±0.0defB	0.0±0.0fB
	5	0.16±0.01aC	0.02±0.0deAB	0.08±0.01cBC	0.13±0.0bB	0.07±0.01cBC	0.04±0.01dC	0.04±0.0dC	0.04±0.0dB	0.0±0.0eB
	7	0.32±0.01aB	0.05±0.0efAB	0.19±0.0bB	0.30±0.03aB	0.16±0.0bcB	0.11±0.02cdB	0.08±0.0deB	0.08±0.01deB	0.0±0.0fB
	10	1.44±0.06aA	0.08±0.03dA	0.64±0.08bcA	1.36±0.31aA	0.79±0.05bA	0.56±0.03bcA	0.29±0.02cdA	0.26±0.05cdA	0.04±0.01dA

[a] Mean±SD (standard deviation). TAG, purified black cumin oil; BCO, cold pressed black cumin oil; T, thymoquinone; α-TOC, α-tocopherol; γ-TOC, γ- tocopherol; BHT, butylated hydroxytoluene.
[a-e/a-f] Means within each row with different superscripts are significantly different (p <0.05)
[A-D] Means within each column with different superscripts are significantly different (p <0.05)

Table VII The remaining α-, γ-tocopherols and thymoquinone in BCO and PBCO stored under Schaal oven conditions at 60°C

	Zero	1	3	5	7	10
α-tocopherol						
TAG+α-TOC[a]	10.41±0.13a	2.07±0.11b	0.02±0.00c			
TAG+α-TOC + T	8.81±0.19a	7.22±0.98a	5.61±1.99ab	1.08±1.52b		
TAG+α-TOC+ γ-TOC +T	9.32±0.16a	5.70±1.23b	2.67±0.79bc	0.70±0.05c	0.03±0.00c	0.05±0.00c
BCO	10.13±0.15d	19.20±0.41a	19.46±0.55a	16.14±0.69ab	14.59±1.41bc	11.69±0.82cd
γ-tocopherol						
TAG+γ-TOC	55.54±6.05aA	56.75±5.71aB	43.99±2.79abC	34.91±5.08bC	0.07±0.00cC	0.04±0.00cC
TAG+γ-TOC+T	66.75±1.89aA	47.44±9.58bB	53.19±1.47abBC	49.36±0.11bBC	0.16±0.03cC	0.45±0.01cC
TAG+α-TOC+ γ-TOC+T	55.36±6.31aA	57.80±5.09aB	62.57±5.08aB	53.68±5.18aB	35.43±0.74bB	12.15±0.92cB
BCO	67.45±0.07cA	127.45±3.54aA	132.68±0.13aA	124.30±2.75aA	136.32±7.39aA	109.54±1.76bA
Thymoquinone						
TAG+T	960.5 ± 2.4aA	812.9 ± 73.0aB	773.0 ± 13.7aB	848.2 ± 25.5aAB	549.9 ± 23.5aC	501.4 ± 37.3bC
TAG+α-TOC + T	960.5 ± 2.4bA	812.2 ± 17.6aB	787.3 ± 13.6aB	864.5 ± 11.8aAB	496.1 ± 45.2aC	508.6 ± 31.0bC
TAG+γ-TOC + T	960.5 ± 2.4bA	805.3 ± 48.5aB	690.2 ± 33.9aBC	633.2 ± 39.6aCD	612.7 ± 46.3aCD	523.7 ± 16.8abD
TAG+α-TOC+ γ-TOC +T	960.5 ± 2.4bA	775.7 ± 36.3aAB	868.3 ± 130.6aAB	675.5 ± 93.7aAB	619.4 ± 76.5aB	595.0 ± 44.3abB
BCO	1082.5 ± 0.2bA	802.9 ± 60.3aAB	740.4 ± 89.4aAB	684.0 ± 156.9aAB	726.1 ± 197.4aAB	635.0 ± 14.9aB

[a] Mean±SD (standard deviation). TAG, purified black cumin oil; BCO, cold pressed black cumin oil; T, thymoquinone; α-TOC, α-tocopherol; γ-TOC, γ- tocopherol; BHT, butylated hydroxytoluene.
[a-d/a-c/a-b] Means within each row with different superscripts are significantly different (p <0.05)
[A-C/A-D] Means within each column with different superscripts are significantly different (p <0.05)

The change in the content of minor constituents (α-, γ-tocopherol and thymoquinone) in oil samples is given in Table VII.

Tocopherol isomers in PBCO and BCO oil samples decreased significantly during storage (p<0.05). α-Tocopherol was consumed sharply in purified TAG samples enriched with α-tocopherol and α-tocopherol/thymoquinone after 3 and 5 days of storage, respectively (p<0.05). The degradation of α-tocopherol was slow in TAG containing ternary blends of minor bioactive constituents and BCO during storage. α-Tocopherol content of BCO changed between 10.13 to 19.46 mg/kg during thermal oxidation. It was believed that the bound and free thymoquinone and other antioxidant compounds found in the BCO caused regeneration of α-tocopherol. The degradation rate of γ-tocopherol was slower compared to α-tocopherol during storage. Binary and especially ternary mixtures of γ-tocopherol increased the remaining of γ-tocopherol in TAG samples. The amount of γ-tocopherol in PBCO containing ternary bioactive compounds was 12.15 mg/kg at the end of 10 days. As a result, decrease in both α- and γ-tocopherols was observed at the minimum level during storage at 60°C and these results agree with Kiralan et al. [11]. The change in tocopherol isomers are in accordance with the study of Wagner et al. [30] who reported that the loss of α-tocopherol was faster than γ-tocopherol in TAG-in-water emulsion at 40°C. The most stable constituent was thymoquinone and the loss of this compound was very slow during storage at 60°C. This stable behaviour for thymoquinone was observed in the study of Kiralan et al. [11].

4. CONCLUSION

This study showed that minor bioactive compounds play a major role in oxidation stability of BCO. α- and γ-tocopherols and thymoquinone retarded oil oxidation under accelerated conditions. γ-tocopherol was a more effective antioxidant than α-tocopherol isomer. Besides, the use of γ- tocopherol with other bioactive constituents enhanced oxidation stability of PBCO. Results indicated that γ-tocopherol and thymoquinone are better antioxidants under thermal conditions and contribute more to the oxidative stability of BCO. Other minor compounds (thymoquinone isomers, tocotrienols, sterols and carotenoids) which have antioxidant activity should be evaluated in order to find the reason of high oxidative stability of cold pressed BCO.

Acknowledgement
Authors thank Scientific Research Projects Fund of Bolu Abant Izzet Baysal University (Turkey) for providing fund support to the project under grant contract number 2017.04.1131. The authors also thank the employees of the YENIGIDAM Research Centre of Bolu Abant Izzet Baysal University for their help for the HPLC and GC-FID analysis and Prof. Dr. Ihsan Karabulut for his help for the Rancimat analysis.

REFERENCES

[1] M.F. Ramadan, Healthy blends of high linoleic sunflower oil with selected cold pressed oils: Functionality, stability and antioxidative characteristics. Ind. Crops Prod. *43,* 65-72 (2013)

[2] S. Gharby, H. Harhar, D. Guillaume, A. Roudani, S. Boulbaroud, M. Ibrahimi, M. Ahmad, S. Sultana, T.B. Haddah, I. Chafchaouni-Moussaoui, Z. Charrouf, Chemical investigation of Nigella sativa L. seed oil produced in Morocco. J. Saudi Soc. Agri. Sci. *14*(2), 172-177 (2015)

[3] W. Kooti, Z. Hasanzadeh-Noohi, N. Sharafi-Ahvazi, M. Asadi-Samani, D. Ashtary-Larky, Phytochemistry, pharmacology, and therapeutic uses of black seed (*Nigella sativa*). Chinese J. Nat. Med. *14*(10), 732-745 (2016)

[4] M.S. Al-Jassir, Chemical composition and microflora of black cumin (Nigella sativa L.) seeds growing in Saudi Arabia. Food Chem. *45*(4), 239-242 (1992)

[5] M. Ashraf, Q. Ali, Z. Iqbal, Effect of nitrogen application rate on the content and composition of oil, essential oil and minerals in black cumin (*Nigella sativa* L.) seeds. J. Sci. Food Agric. *86*(6), 871-876 (2006).

[6] M. Kiralan, G. Özkan, A. Bayrak, M.F. Ramadan, Physicochemical properties and stability of black cumin (Nigella sativa) seed oil as affected by different extraction methods. Ind. Crops Prod. *57*, 52-58 (2014)

[7] M. Rudzińska, M.M. Hassanein, A.G. Abdel-Razek, K. Ratusz, A. Siger, Blends of rapeseed oil with black cumin and rice bran oils for increasing the oxidative stability. J. Food Sci. Technol. *53*(2), 1055-1062 (2016)

[8] F. Forouzanfar, B.S.F. Bazzaz, H. Hosseinzadeh, Black cumin (*Nigella sativa*) and its constituent (thymoquinone): A review on antimicrobial effects. Iran. J. Basic Med. Sci. 17(12), 929 (2014)

[9] H. Bakhshabadi, H. Mirzaei, A. Ghodsvali, S.M. Jafari, A.M. Ziaiifar, The influence of pulsed electric fields and microwave pretreatments on some selected physicochemical properties of oil extracted from black cumin seed. Food Sci. Nutr. *6*(1), 111-118 (2018)

[10] M.F. Ramadan, K.M.M. Wahdan, Blending of corn oil with black cumin (*Nigella sativa*) and coriander (*Coriandrum sativum*) seed oils: Impact on functionality, stability and radical scavenging activity. Food Chem. *132*(2), 873-879 (2012)

[11] M. Kiralan, M. Ulaş, A. Özaydin, N. Özdemır, G. Özkan, A. Bayrak, M.F. Ramadan, Blends of cold pressed black cumin oil and sunflower oil with improved stability: A study based on changes in the levels of volatiles, tocopherols and thymoquinone during accelerated oxidation conditions. J. Food Biochem. *41*(1), e12272 (2017)

[12] S. Hamed, G.A. Abo-Elwafa, Enhancement of oxidation stability of flax seed oil by blending with stable vegetable oils. J. Appl. Sci. Res. *8*(10), 5039-5048 (2012)

[13] M.F. Ramadan, J.-T. Mörsel, Oxidative stability of black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.) and niger (*Guizotia abyssinica* Cass.) crude seed oils upon stripping. Euro. J. Lipid Sci. Technol. *106*(1), 35-43 (2004)

[14] AOCS, Official and recommended methods of the American Oil Chemist's Society, American Oil Chemist's Society Press, Champaign, IL (2000)

[15] E. Karacabey, G. Mazza, Optimization of Solid-Liquid Extraction of Resveratrol and Other Phenolic Compounds from Milled Grape Canes (*Vitis vinifera*). J. Agric. Food Chem. *56*(15), 6318-6325 (2008)

[16] F. Caponio, V. Alloggio, T. Gomes, Phenolic compounds of virgin olive oil: Influence of paste preparation techniques. Food Chem. *64*, 203-209 (1999)

[17] I. Karabulut, A. Topcu, C. Akmil-Basar, Y. Onal, A.M. Lampi, Obtaining butter oil triacylglycerols free from β-carotene and α-tocopherol via activated carbon adsorption and alumina-column chromatography treatments. J. Amer. Oil Chem. Soc. *85*(3), 213-219 (2008)

[18] C. Gertz, Determination of polymerized triglycerides content in deep-frying fats and oils. Euro. J. Lipid Sci. Technol. *103*(2), 114-116 (2001)

[19] M. Kiralan, S.S. Kiralan, Changes in volatile compounds of black cumin oil and hazelnut oil by microwave heating process. J. Amer. Oil Chem. Soc. *92*(10), 1445-1450 (2015)

[20] S. Kostadinović Veličkovska, L. Brühl, S. Mitrev, H. Mirhosseini, B. Matthäus, Quality evaluation of cold-pressed edible oils from Macedonia. Euro. J. Lipid Sci. Technol. *117*(12), 2023-2035 (2015)

[21] E. Symoniuk, K. Ratusz, E. Ostrowska-Ligęza, K. Krygier, Impact of selected chemical characteristics of cold-pressed oils on their oxidative stability determined using the rancimat and pressure differential scanning calorimetry method. Food Anal. Meth. *11*(4), 1095-1104 (2018)

[22] M.T. Sultan, M.S. Butt, F.M. Anjum, A. Jamil, S. Akhtar, M. Nasir, Nutritional profile of indigenous cultivar of black cumin seeds and antioxidant potential of its fixed and essential oil. Pak. J. Bot. *41*(3), 1321-1330 (2009)

[23] B. Matthaus, M.M. Özcan, Fatty acids, tocopherol, and sterol contents of some Nigella species seed oil. Czech J. Food Sci. *29*(2), 145-150 (2011)

[24] H. Lutterodt, M. Luther, M. Slavin, J.J. Yin, J.

Parry, J.M. Gao, L.L. Yu, Fatty acid profile, thymoquinone content, oxidative stability, and antioxidant properties of cold-pressed black cumin seed oils. LWT-Food Sci. Technol. *43*(9), 1409-1413 (2010)
[25] M.M. Hassanien, A.G. Abdel-Razek, M. Rudzińska, A. Siger, K. Ratusz, R. Przybylski, Phytochemical contents and oxidative stability of oils from non-traditional sources. Euro. J. Lipid Sci. Technol. *116*(11), 1563-1571 (2014)
[26] K.H. Wagner, I. Elmadfa, Effects of tocopherols and their mixtures on the oxidative stability of olive oil and linseed oil under heating. Euro. J. Lipid Sci. Technol. *102*(10), 624-629 (2000)
[27] H. Yoshida, G. Kajimoto, S. Emura, Antioxidant effects of δ-tocopherols at different concentrations in oils during microwave heating. J. Amer. Oil Chem. Soc. *70*(10), 989-995 (1993)
[28] A.M. Lampi, L. Kataja, A. Kamal-Eldin, P. Vieno, Antioxidant activities of α-and γ-tocopherols in the oxidation of rapeseed oil triacylglycerols. J. Amer. Oil Chem. Soc. *76*(6), 749-755 (1999)
[29] R.E. King, D.B. Min, S.C. Min, Study of α-, γ-, and δ-tocopherols in the oxidative stability of lard. Food Sci. Biotechnol. *20*(3), 817-822 (2011)
[30] K.H. Wagner, B. Isnardy, I. Elmadfa, γ-and δ-tocopherols are more effective than α-tocopherol on the autoxidation of a 10% rapeseed oil triacylglycerol-in-water emulsion with and without a radical initiator. Euro. J. Lipid Sci. Technol. *106*(1), 44-51 (2004)