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Common Kilka oil and its primary and secondary oxidative dynamics stabilized by different variants of clove essential oil

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SUMMARY: The objective of this study was to investigate the properties of clove essential oil extracted by different microwave-assisted methods and to evaluate its effects on the stability of common Kilka oil. Each of these methods was hypothesized to yield a clove essential oil that would have a distinguishable composition and effect when added to common Kilka oil by maintaining its oxidative stability. The oxidation of common Kilka oil was examined by accelerated oxidation using the active oxygen method and Rancimat test. The clove essential oil extracted by microwave-assisted hydrodistillation showed the highest induction period according to the active oxygen method (16.56 h) and the Rancimat induction period (3.64 h) in common Kilka oil and its antioxidant activity was comparable to that of BHT (16.59 h and 4.34 h, respectively) and tocopheryl acetate (16.30 h and 4.02 h, respectively). Furthermore, the microwave-assisted hydrodistillation method resulted in the amount of eugenol that exhibited the highest antioxidant capacity for preserving PUFA in common Kilka oil. Ultimately, clove essential oil can become an efficient natural antioxidant for the oxidative stability of common Kilka oil.

KEYWORDS: *Antioxidant activity; Clove essential oil; Common Kilka oil; Microwave; Oxidation*

RESUMEN: ***Aceite de Kilka común y su dinámica oxidativa primaria y secundaria estabilizada por diferentes variantes de aceite esencial de clavo.*** El objetivo de este estudio fue investigar las propiedades de los aceites esenciales de clavo que se extrajeron utilizando diferentes métodos asistidos por microondas y evaluar los efectos de estos aceites esenciales en la estabilidad del aceite de Kilka común. Se hipotetizó que cada uno de los métodos produce un aceite esencial de clavo que tendría una composición y un efecto distintivo cuando se agrega al aceite de Kilka común manteniendo su estabilidad oxidativa. La oxidación del aceite de Kilka común se determinó mediante oxidación acelerada utilizando el método de oxígeno activo y Rancimat. El aceite esencial de clavo extraído por hidrodestilación asistida por microondas logró en el aceite de Kilka común un período de inducción, mediante el método de oxígeno activo, más alto (16,56 h) y un período de inducción mediante Rancimat de 3,64 h y su actividad antioxidante fue comparable a la del BHT (16,59 h y 4,34 h, respectivamente) y a la del acetato de tocoferol (16,30 h y 4,02 h, respectivamente). Además, el método de hidrodestilación asistido por microondas influyó en la cantidad de eugenol que presentó una mayor capacidad antioxidante para preservar los PUFAs del aceite de Kilka común. Por último, el aceite esencial de clavo puede convertirse en un antioxidante natural eficiente para la estabilidad oxidativa del aceite de Kilka común.

PALABRAS CLAVE: *Aceite de Kilka común; Aceite esencial de clavo; Actividad antioxidante; Microondas; Oxidación*

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1. INTRODUCTION

The common Kilka (*Cluponella cultriventris*), the anchovy (*C. engraulifermis*), and the large-eyed Kilka (*C. grimmi*) are the most important fish in the Caspian Sea. Common Kilka is the most abundant species in the southern Caspian, comprising 97% of the total catch of clupeids there (Jorjani, 2014). It has been reported that fish oil has many health benefits such as reducing the risk of inflammatory and cardiovascular diseases (Kromhout *et al*., 2011; Wall *et al*., 2010). The quality of fish oil is primarily due to the presence of long-chain ω-3 polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Wang *et al*., 2011). However, unsaturated ω-3 PUFAs are highly prone to oxidation, leading to the development of free radicals, reactive aldehydes, and off-flavors, which ultimately reduce the shelf-life of fish oil (Jacobsen *et al*., 2008). Therefore, the use of potent antioxidants in fish oil is required in order to reduce oxidation. To that end, there is a considerable amount of focus on exploiting antioxidant compounds which can be found in medicinal plants (Golmakani *et al*., 2017a; Golmakani *et al*., 2018; Shahbazi *et al*., 2018).

Clove (*Syzygium aromaticum*) is a member of the *Myrtaceae* family and an important aromatic spice. The majority of compounds found in clove essential oil (CEO) are grouped as phenyl-propanoids, such as eugenol and eugenyl acetate, and sesquiterpenes such as β-caryophyllene and α-humulene (Chaieb *et al*., 2007; Guan *et al*., 2007). In this context, Gülçin (2011) reported that eugenol is capable of exhibiting a high antioxidant activity in a linoleic acid emulsion system. Furthermore, eugenol has a significant reducing power, can scavenge reactive oxygen species and reduce lipid peroxidation (Ogata *et al*., 2000). In addition, β-caryophyllene is known to have high radical scavenging activity (Mishra *et al*., 2013).

Hydrodistillation (HD) and steam distillation (SD) are two conventional methods for essential oil extraction. These methods are generally time consuming, degrade thermolabile compounds, and require large amounts of solvents for their performance (Wang and Weller, 2006). Recently, microwave-assisted extraction methods have become popular and more convenient compared to conventional extraction procedures (Mazidi *et al*., 2012). The variants of microwave-assisted extraction such as microwave-assisted hydro-distillation (MAHD) and microwave-assisted steam distillation (MASD) have proven to be successful methods for the extraction of essential oil (Chemat *et al*., 2012). It has been reported that the extraction time of essential oil in microwave-assisted extraction methods is shorter than the conventional methods. Furthermore, the vigor of radical scavenging activities, staged by the extracted essential oils, remains quite untouched when using the microwave radiation techniques (Mazidi *et al*., 2012).

So far, many studies have focused on the antioxidant activity of different herbs and spices in edible oils. For instance, Olmedo *et al*., (2018) reported that *Aloysia triphylla* and *Minthostachys mollis* essential oils were effective in reducing sunflower oil oxidation. Golmakani *et al*., (2018) showed that common Kilka oil (CKO) oxidation can be reduced by *Ocimum sanctum* essential oil nearly as much as it can be reduced by the synthetic BHA. One report claimed that *Zataria multiflora* essential oil can reduce the oxidation of virgin olive oil to the same extent that BHT can. *Zataria multiflora* essential oil proved to be more effective than β-carotene (Golmakani *et al*., 2017a).

This study was designed to evaluate the quality of CEO when extracted by different methods (i.e. HD, SD, MAHD, and MASD) and to assess its effects on the oxidative stability of CKO in comparison to the effects produced by synthetic BHT, natural α-tocopherol and β-carotene, and semi-natural tocopheryl acetate, which are all considered to be effective antioxidants. The assessments were performed with the help of Rancimat and AOM (active oxygen method). In addition, changes that may occur in the fatty acid profile of CKO samples were investigated during accelerated storage.

2. MATERIALS AND METHODS

2.1. Materials

Dried clove buds (*Syzygium aromaticum*) were purchased from a local market, Shiraz, Iran. The genus and species of the plant were confirmed by elite taxonomists from the Herbarium of Biology Department, Shiraz University, Shiraz, Iran. The CKO was provided by the Pars Kilka Company (Babolsar, Iran). The α-Tocopherol, β-carotene, tocopheryl acetate, and BHT were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) and Merck (Darmstadt, Germany).

2.2. CEO extraction

Four different extraction methods were used in order to extract the CEO: two conventional methods (HD and SD) and two relatively new methods (MAHD and MASD). Briefly, 40 g of the dried clove buds were processed by HD with a Clevenger-type apparatus. The extraction was performed in the presence of water (400 mL) and took 4 h. The ratio of clove-to-water was 1:10 (w/w). The SD method was similar to HD, but the clove was placed in a glass column, of which the bottom and top parts were connected to a water flask and a condenser, respectively. The structures of MAHD and MASD were similar to HD and SD, respectively. However, a domestic microwave oven (ME3410W, Samsung, Malaysia) was used for MAHD and MASD instead of the Electromantle heater of HD and SD. The microwave operated at a frequency of 2.45 GHz with a maximum output power of 1000 W for 80 min. The extracted CEOs were then dehydrated with anhydrous sodium sulfate, collected in amber-colored vials, and were kept at -18 °C until further experiments were performed (Golmakani and Moayyedi, 2015; Golmakani *et al*., 2017b).

2.3. Gas chromatography analysis of CEOs

The chemical composition of CEO was determined by a gas chromatography system (Agilent technologies 7890A, Santa Clara, CA) coupled to a mass spectrometer (Agilent Technologies 5975C, Santa Clara, CA), operating at 70 eV ionization energy, 0.5 s/scan, and at the mass range of 35-400 amu. The HP-5MS capillary column was characterized by 5% Phenyl Polysilphenylene-siloxane, 30m length, 0.25mm internal diameter and 0.25 µm film thickness (Agilent Technologies, Santa Clara, CA) (Golmakani *et al*., 2017a).

2.4. Physicochemical properties and fatty acid composition of CKO

2.4.1. Fatty acid composition by gas chromatography

To determine fatty acid profiles, a gas chromatography/flame ionization detector (B420A, BEIFEN, China) was used. The film thickness, length and inner diameter of the BPX70 GC column were 0.25 mm, 60 m, and 0.25 mm, respectively. The stationary and carrier phases were Polymer Biscyanopropylsiloxane silphenylene and nitrogen gas, respectively (Keramat and Golmakani, 2016).

2.4.2. Peroxide value (PV)

The PV was quantified according to the Official Method (Cd 8-53) of American Oil Chemists' Society and was expressed as meq O_2 per kg oil (AOCS, 2000).

2.4.3. Free fatty acid content

Reported as a percentage of oleic acid, the free fatty acid content was measured according to the AOCS official method (Cd 3d-63) (AOCS, 2000).

2.4.4. Density measurement

The density of CKO was measured following the AOCS official method (Cc10a- 25) whereby a pycnometer was used (AOCS, 2000).

2.4.5. Solid fat content (SFC)

Measuring the CKO was assisted by Nuclear Magnetic Resonance (NMR, Bruker, NMF 100, Karlsruhe, Germany). Prior to NMR analysis, the samples were melted at 100 °C and then cooled to 80 °C for 5 min. After that, the temperature was decreased to 60 °C and held for 5 min. Finally, the samples were rapidly cooled to 0 °C and t held at this temperature for 1 h. The samples were stabilized at 0, 5, 10, 20, 25, and 30 °C and were kept for 30 min at each temperature prior to NMR analysis *(Nejadmansouri et al*., 2016).

2.4.6. Refractive index

The refractive index of CKO was determined using a refractometer (RX7000a; Atago, Japan) at 20 °C.

2.5. Oxidative stability of CKO

CEOs were added to the CKO at 1000 ppm concentration. The β-carotene, α-tocopherol, tocopheryl acetate and BHT were added to the CKO at 100 ppm concentration. For the control, a pure sample was used without any antioxidant.

2.5.1. Active oxygen method (AOM)

The AOM analysis was carried out according to the AOCS official method (Cd 12-57). Briefly, 20 mL portions of CKOs were poured into the reaction tube and placed in a constant-temperature heater which kept the temperature constant at 97.8 ± 0.2 °C. The total flow rate was adjusted to 2.33 mL/min for each tube. The AOM induction period (IP) (i.e. the time required to reach a PV of 100 meq O_2/kg oil) was calculated according to the standard method of AOM (AOCS, 2000).

The protection factor (PF) was calculated according to eq. (1).

$$PF=\frac{\text{AOM IP}_a}{\text{AOM IP}_c} \quad \text{eq. (1)}$$

Where AOM IP_a is the AOM induction period (IP) of the CKO samples containing antioxidants (BHT, β-carotene, α-tocopherol, tocopheryl acetate and CEO) and AOM IP_c is the AOM IP of the control sample (Hraš *et al*., 2000).

2.5.2. Rancimat method

The oxidative stability was estimated by measuring the Rancimat IP, using a rancimat 743 apparatus (Metrohm, Switzerland) according to the AOCS method (Cd 12b-92). The tests were carried out with 3.0 ± 0.1 g of CKO. All samples were studied at 80 °C. The temperature of the conductivity tube was kept constant at 21 °C and the air flow rates of motion were set at 20 L/h for each experiment. The Rancimat IP was printed automatically by the apparatus software with a precision of two decimals (AOCS, 2000).

2.6. Statistical analysis

All experiments were performed in triplicate. The results were reported as the mean value ± standard deviation. Analysis of variance (ANOVA) was performed using the SPSS software (ver. 22, IBM, New York, NY), and the Duncan's multiple range test was used so as to compare the data with the mean values. A *P*-value of < 0.05 was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Gas chromatography analysis of CEO

The chemical compositions of the CEOs extracted by HD, SD, MAHD, and MASD showed variations (Table 1). Even though there were similarities among the compositions of the CEOs extracted by the different methods, the relative concentrations of the identified compounds were apparently different.

According to Table 1, phenylpropanoids such as eugenol (78.44-88.14%) and eugenyl acetate (8.11-18.26%) were the main components of the CEOs. Vanin *et al*., (2014) reported that eugenol and eugenyl acetate are capable of exhibiting high antioxidant activity. Also, Mishra *et al*., (2013) reported that the antioxidant activity of eugenol tends to be higher than that of other phenolic compounds in essential oil - compounds such as thymol and carvacrol.

In the current study, the CEOs obtained by the SD-based methods contained lower percentages of eugenol, and higher amounts of eugenyl acetate. The CEOs obtained by HD-based methods had higher amounts of eugenol and lower eugenyl acetate. The eugenol/eugenyl acetate ratios in CEOs obtained by the HD and MAHD methods (8.59 and 10.87, respectively) were higher than those obtained by the SD and MASD methods (4.56 and 4.54, respectively). This may be due to the hydrolysis of eugenyl acetate and its conversion to eugenol when HD-based methods were employed (Golmakani *et al*., 2017b). The CEO obtained by MAHD contained the highest percentage of eugenol (88.14%). Similar to our findings, Golmakani *et al*., (2017b) reported that MAHD is capable of extracting CEO with the highest percentage of eugenol, whereas the CEO extracted by the SD reportedly contained the lowest percentage of eugenol.

3.2. Physicochemical properties and fatty acid composition of CKO

According to Table 2, the values relating to the refractive index, density, PV, and acid content of CKO were 1.4711, 0.934 g/mL, 2.02 meq O_2/kg, and 1.134 mg KOH/g, respectively, at the beginning of the experiment. The PV and acid value were lower than the maximum permitted level recommended by Codex (10 meq O_2/kg for PV and 4 mg KOH/g for acid value) (Codex Alimentarius, 2015).

The SFC of CKO decreased from 4.29 to 0%. This decrease occurred parallel to the increase in temperature from 0 to 30 °C. Nejadmansouri *et al*., (2016) also reported that the SFC of fish oil decreased from 11.96 to 2.48% by increasing the temperature from 0 to 25 °C.

TABLE 1. Chemical composition of clove essential oil obtained by different extraction methods

No.	Compound	Retention time (min)	Retention index	Releative peak area (%)			
				HD*	SD	MAHD	MASD
1	Benzaldehyde	7.5	893	0.07±0.02^{a}	0.03±0.00^{a}**	0.05±0.02^{a}	0.05±0.03^{a}
2	2-Nonanone	11.5	988	0.04±0.00^{b}	0.03±0.02^{b}	0.08±0.02^{a}	0.03±0.00^{b}
3	Benzyl acetate	13.1	1087	0.05±0.01^{ab}	0.06±0.01^{ab}	0.10±0.03^{a}	0.04±0.00^{b}
4	Ethyl benzoate	13.6	1084	0.17±0.00^{b}	0.28±0.01^{a}	0.19±0.04^{b}	0.26±0.01^{a}
5	Methyl salicylate	16.7	1082	0.07±0.00^{a}	0.05±0.01^{a}	0.07±0.01^{a}	0.06±0.01^{a}
6	Chavicol	18.9	1179	0.03±0.01^{ab}	0.07±0.03^{a}	0.02±0.01^{b}	0.01±0.01^{b}
7	Eugenol	22.6	1276	86.49±1.94^{a}	78.56±0.84^{b}	88.14±2.8^{a}	78.44±0.59^{b}
8	(E)-Caryophyllene	25.1	1374	1.97±0.53^{a}	2.40±0.19^{a}	2.33±1.05^{a}	1.97±0.70^{a}
9	α-Humulene	26.1	1373	0.84±0.32^{a}	0.96±0.08^{a}	0.76±0.47^{a}	0.67±0.32^{a}
10	Eugenyl acetate	28.3	1470	10.07±1.13^{b}	17.24±0.62^{a}	8.11±1.18^{b}	18.26±0.53^{a}
11	Caryophyllene oxide	31.0	1467	0.09±0.03^{b}	0.17±0.02^{a}	0.07±0.02^{b}	0.14±0.93^{ab}
12	Benzyl benzoate	36.7	1692	0.10±0.04^{a}	0.14±0.02^{a}	0.07±0.02^{a}	0.06±0.04^{a}
Eugenol/Eugenyl acetate				8.59	4.56	10.87	4.54

*HD, hydro-distillation; MAHD, microwave-assisted hydro-distillation; SD, steam-distillation; MASD, Microwave- assisted steam-distillation.
**Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's test; Degree of significance: $P < 0.05$

The fatty acid composition of CKO is presented in Table 2. The major SFA and MUFA were palmitic acid and oleic acid, respectively. DHA was the most abundant PUFA and EPA was the second major PUFA in CKO. Our results on the major fatty acids of CKO are in agreement with previous reports (Golmakani *et al*., 2017a; Hosseini *et al*., 2019a). Here, the PUFA/SFA ratio in the CKO (0.99) was higher than the minimum level recommended by the UK Department of Health (0.45) (HMSO, 1994) which indicates that CKO is highly susceptible to deterioration by oxidation. The ω-3/ω-6 ratio in CKO was 10.99. Similarly, Hosseini *et al*., (2018) reported that the ω-3/ω-6 ratio in CKO was 7.62.

3.3. Oxidative stability of CKO

3.3.1. Active oxygen method (AOM).

The PVs of CKO samples were worthy of documentation during accelerated storage (Figure 1). The PVs of all CKO samples increased at the early stages of the storage period. Even though the PV of the control group began to decrease after 12 h, the PVs of the samples containing antioxidants began to decrease after 18 h. The PV of the control sample increased much faster and to a higher level than that of the CKO samples as they contained antioxidants and reached 107.93 meq O_2/kg after 12 h. In going beyond the mentioned time, and especially after 18 h, the PVs of samples which contained CEOs extracted by HD, SD, MAHD and MASD reached 89.94, 82.18, 80.20, and 88.45 meq O_2/kg, respectively (Figure 1a). This indicated that the CEOs can delay the rate of hydroperoxide formation. The CEO extracted by MAHD functioned more effectively in reducing the PV of CKO, compared to the function of CEOs extracted by the HD, SD and MASD methods ($P < 0.05$). This may be attributed to the presence of higher amounts of eugenol in the CEO extracted by the MAHD method. Also, Golmakani *et al*., (2017b) reported that a higher antioxidant activity of CEO extracted by HD-based methods is a result of higher eugenol contents. The antioxidant activity of phenolic compounds comes from their chemical structure and reducing functions. They quench the singlet oxygen, neutralize free radicals and chelate transitional metals (Hosseini *et al*., 2019b). It has been reported that eugenol can prevent the creation of iron and OH radicals, two known chemical agents which contribute to lipid peroxidation (Nagababu *et al*., 2010).

TABLE 2. Physicochemical properties and fatty acid profile of common Kilka oil

Characteristic	Amount
Refractive index (20 °C)	1.4711±0.0001*
Density (20 °C)	0.934±0.004
Peroxide value (meq O_2/kg oil)	2.02±0.08
Acid value (mg KOH/g oil)	1.13±0.01
Free fatty acid (% oleic acid)	0.57±0.01
Solid fat content (%)	
at 0 °C	4.29±0.01
at 5 °C	2.48±0.01
at 10 °C	2.21±0.01
at 20 °C	1.90±0.01
at 25 °C	1.29±0.01
at 30 °C	0.00±0.00
Fatty acid (%)	
Myristic acid (C14:0)	4.86±0.07
Palmitic acid (C16:0)	20.39±0.05
Palmitoleic acid (C16:1 ω-7)	6.95±0.01
Stearic acid (C18:0)	3.78±0.04
Oleic acid (C18:1 ω-9 cis)	28.88±0.34
Linoleic acid (C18:2 ω-6 cis)	2.05±0.01
α-Linolenic acid (C18:3 ω-3)	2.12±0.02
Arachidic acid (C20:0)	3.16±0.03
Docosatetraenoic acid (C22:4 ω-6)	0.57±0.01
Eicosapentaenoic acid (C20:5 ω-3)	8.43±0.26
Docosapentaenoic acid (C22:5 ω-3)	0.56±0.01
Docosahexaenoic acid (C22:6 ω-3)	18.26±0.23
SFA (saturated fatty acid)	32.18
UFA (unsaturated fatty acid)	67.82
MUFA (monounsaturated fatty acid)	35.83
PUFA (polyunsaturated fatty acid)	31.99
PUFA/SFA	0.99

* Mean ± SD (n=3).

Tocopheryl acetate, α-tocopherol, and β-carotene reduced the PV of CKO to a similar extent that BHT did after 8 h of storage (Figure 1b). After 12 h, however, tocopheryl acetate, α-tocopherol, and β-carotene were less effective in their functions than BHT was. Also, Keramat *et al.*, (2017) reported that β-carotene and BHT were equally effective in reducing the PV of virgin olive oil after 21 days of storage. However, β-carotene showed a pro-oxidant activity after 42 days of storage. They concluded that the differences among the antioxidant activities of different antioxidants can be clearly determined at the latter stages of oxidation. The effect of BHT in reducing the PV of CKO was similar to that of the CEO extracted by the MAHD method. After 12 h of storage at 97.8 °C, BHT and the CEO extracted by the MAHD method reduced the PV of CKO by 31.49 and 25.69%, respectively. Furthermore, Golmakani *et al.*, (2018) reported that *O. sanctum* essential oil is able to inhibit the oxidation of CKO, quite similar to the action of BHA.

The AOM induction period and protection factor of the CKO samples are presented in Table 3. There was no significant difference ($P < 0.05$) among the AOM IP values of CKO samples containing the different variants of CEOs. Among different CEO samples, the one extracted by MAHD showed the highest PF. The PFs of CKO samples containing BHT and tocopheryl acetate showed no significant differences compared to the PF of the CEO extracted by MAHD. The AOM IPs of the samples containing the MAHD-extracted CEO, BHT, and tocopheryl acetate were 1.53, 1.54 and 1.51 times higher than that of the control sample. This indicates that the CEO extracted by MAHD can replace the function of BHT. Furthermore, α-tocopherol was observed to be less effective than tocopheryl acetate in extending the AOM IP of CKO. A better performance of tocopheryl acetate was observed, which increased the AOM IP of CKO more sharply than the action of *α*-tocopherol. This can be due to the fact that the esters of *α*-tocopherol become less susceptible to degradation at elevated temperatures. According to Wüstenberg *et al.*, (2011), the esterification of natural antioxidants can improve their chemical stability, oxidative stability, and heat tolerance, especially in systems which are characterized by the presence of lipids. Here, the AOM IPs and PFs of CKO samples containing β-carotene and *α*-tocopherol were similar to those which contained CEOs extracted by the HD, SD, and MASD methods.

3.3.2. Rancimat method

The role of any antioxidant in retarding the formation of secondary oxidation products in the CKO can be monitored by the Rancimat method.

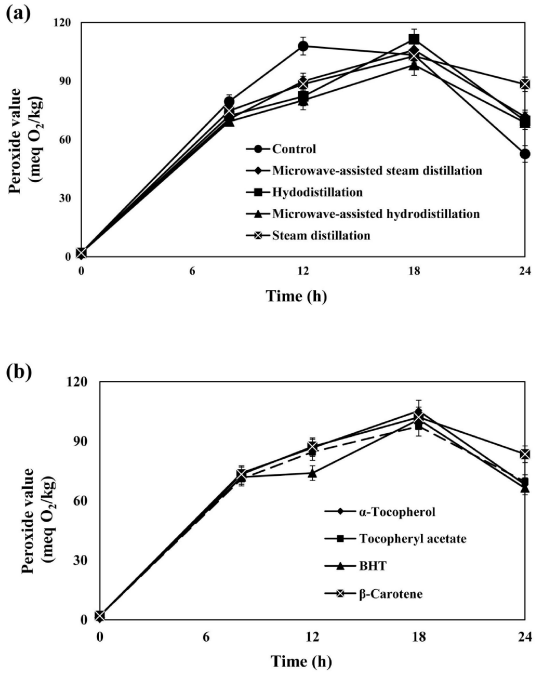

FIGURE 1. Changes in peroxide values of common Kilka oil samples containing (a) different variants of clove essential oil and (b) natural, semi-natural, and synthetic antioxidants during accelerated storage at 97.8 °C (Mean ± standard deviation; Number of replicates for each analysis: 3).

The Rancimat IP of the CKO samples was automatically recorded and taken as the breaking point of the plotted curves (i.e. the intersection point of the two extrapolated parts of the curve). Per antioxidant, the Rancimat IP values of the CKO samples varied when quantified by the Rancimat method (Figure 2). The Rancimat IP of the control sample was significantly lower (2.79 h) which can be attributed to the presence of high amounts of PUFAs in the CKO, hence the susceptibility to oxidation. All antioxidants that were used herein proved to be effective in increasing the Rancimat IP of CKO in comparison to the control. In this regard, Viuda-Martos *et al*., (2010) reported that oregano, thyme, clove, sage, and rosemary essential oils were effective in increasing the Rancimat IP of lard. Here, CKO samples that contained CEOs experienced increases in their Rancimat IP values, especially when the CEO had higher amounts of eugenol. For example, the Rancimat IP was extended by 30.11% in the case of the CKO sample enriched with the CEO extracted by MAHD (which had a eugenol content of 88.14%). On the other hand, the Rancimat IP was extended by 18.28% in the case of the sample containing the CEO extracted by MASD (which had a eugenol content of 78.44%). This shows a slower oxidation rate in samples with higher eugenol contents. The CEOs extracted by the HD and SD methods increased the Rancimat IP of CKO by 22.22 and 20.43%, respectively. BHT and tocopheryl acetate were the most effective antioxidants in reducing the production of secondary oxidation products in CKO. Accordingly, BHT and tocopheryl acetate increased the Rancimat IP of CKO by 55.55 and 44.08%, respectively. The Rancimat method revealed that the Rancimat IP values of samples containing CEO (extracted by MAHD) were significantly lower than those of samples enriched with the BHT and tocopheryl acetate. However,

TABLE 3. Oxidative stability of common Kilka oil samples during accelerated storage at 97.8 °C

Sample	Active oxygen method induction period (h)	Protection factor
Control	10.80 ± 0.56^{c*}	1.00 ± 0.00^{c}
HD**	15.56 ± 0.81^{ab}	1.44 ± 0.08^{b}
SD	15.38 ± 1.69^{ab}	1.42 ± 0.01^{b}
MAHD	16.56 ± 0.94^{a}	1.53 ± 0.01^{a}
MASD	15.59 ± 0.81^{ab}	1.44 ± 0.00^{b}
β-Carotene	15.57 ± 0.90^{ab}	1.44 ± 0.01^{b}
α-Tocopherol	15.28 ± 0.87^{ab}	1.41 ± 0.01^{b}
Tocopheryl acetate	16.30 ± 0.94^{a}	1.51 ± 0.01^{a}
BHT	16.59 ± 0.94^{a}	1.54 ± 0.01^{a}

* Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's test; Degree of significance: $P < 0.05$.

**HD, hydro-distillation; MAHD, microwave-assisted hydro-distillation; SD, steam-distillation; MASD, Microwave- assisted steam-distillation.

through the AOM assay, no significant differences were found between the AOM IP of samples containing CEO (extracted by MAHD) and those containing BHT and tocopheryl acetate. A strong and significant correlation was not found between the AOM IP values of CKO samples through the AOM and their Rancimat IP values obtained by the Rancimat method (R^2 of 0.48). These results may be related to the fact that the Rancimat method measures the changes in electrical conductivity caused by the generation of volatile compounds by thermal oxidation (Velasco *et al*., 2004). Thus, in CKO samples containing CEO, the volatile compounds in the CEO could possibly be measured as secondary oxidation products through the Rancimat method. The Rancimat method is not a reliable method for measuring the antioxidant activity of CEO.

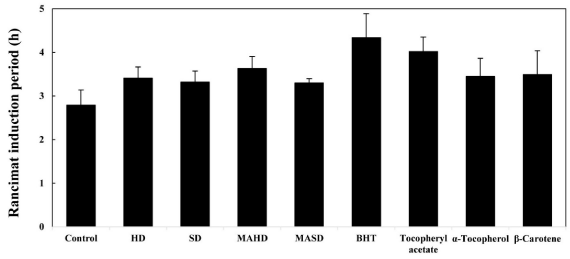

FIGURE 2. Rancimat induction period of common Kilka oil samples containing clove essential oils extracted with hydrodistillation (HD), steam distillation (SD), microwave-assisted hydrodistillation (MAHD), and microwave-assisted steam distillation (MASD) (Mean ± standard deviation; Number of replicates for each analysis: 3).

The α-tocopherol and β-carotene increased the Rancimat IP of CKO by 22.94 and 25.09%, respectively, indicating that they were less effective than BHT, tocopheryl acetate, and CEO extracted by MAHD. Similarly, a previous study showed that BHT, *Zataria multiflora* essential oil, and *Thymus vulgaris* essential oil were more effective than α-tocopherol and β-carotene in reducing virgin olive oil oxidation (Keramat and Golmakani, 2016; Keramat *et al*., 2017).

3.3.3. Changes in fatty acid composition of CKO samples

There were significant differences among the fatty acid profiles of the CKOs before AOM (untreated) and after AOM (Table 4). The PUFA content of all CKO samples, particularly EPA and DHA, decreased under accelerated oxidation conditions. Regarding the control group, the amount of PUFA in the CKO was 31.11%, but it decreased to 17.13% at the end of the accelerated storage period. Furthermore, the amounts of EPA and DHA of the control sample were reduced by 49.32 and 49.97%, respectively, in comparison to the untreated CKO. Natural and synthetic antioxidants markedly reduced the loss in PUFA in CKO samples during accelerated storage. CEOs extracted by HD, SD, MAHD, and MASD methods reduced the PUFA contents in the CKO samples by 40.63, 47.03, 29.67, and 42.46%, respectively. On the other hand, the use of BHT, tocopheryl acetate, α-tocopherol, and β-carotene in CKO samples reduced their PUFA contents by 42.14, 40.50, 39.18, and 39.15, respectively. The CEO extracted by MAHD was the most effective antioxidant, followed by β-carotene and α-tocopherol. The effects of BHT and tocopheryl acetate, as revealed by the AOM assay, were similar to the effect of CEO extracted by MAHD, and they were better than the effects of β-carotene and α-tocopherol. Nonetheless, BHT and tocopheryl acetate were less effective when compared to the CEO extracted by MAHD, and also when compared to β-carotene and α-tocopherol, regarding the preservation of the PUFA in CKO. Similar to our findings, Luther *et al*., (2007) reported that although the extract of black raspberry seed flour was more effective than the extract of Chardonnay grape seed flour in preserving the PUFA of fish oil, the extract of grape seed flour exhibited a stronger capacity for reducing the overall lipid oxidation in fish oil. These results indicate that the capacity of an antioxidant for preserving a selected fatty acid may not be the same as its ability to reduce the overall lipid oxidation of an oil sample. Here, the CEOs extracted by the HD and SD methods protected the PUFAs of the CKO to the same extent that the BHT and tocopheryl acetate did. Similarly, Golmakani *et al*., (2018) showed that the PUFA contents of soybean oil samples containing *O. sanctum* essential oil were similar to the PUFA contents of samples containing BHA during storage at both 45 and 60 °C.

4. CONCLUSIONS

In this study, CEOs were extracted by four methods, namely HD, SD, MAHD and MASD. The effects of these CEOs were evaluated on CKO oxidation in comparison to the function of synthetic (BHT), natural (α-tocopherol and β-

carotene), and semi-natural (tocopheryl acetate) antioxidants. CEOs and other natural or synthetic antioxidants that were used here caused increases in the oxidative stability of CKO when analyzed through the AOM assay and the Rancimat method. Among the four types of CEOs, the CEO extracted by MAHD showed the best performance in increasing the AOM IP and Rancimat IP of the CKO, and this can be attributed to the higher yield of eugenol obtained by this extraction method. The role of CEO in reducing CKO oxidation was similar to the functional strength of BHT and tocopheryl acetate. Furthermore, CEO performed better than α-tocopherol and β-carotene. Specifically, the CEO extracted by MAHD was stronger than BHT and tocopheryl acetate in preserving the PUFA of CKO. In conclusion, CEO extracted by MAHD can be suggested as a suitable natural antioxidant for improving the oxidative stability of CKO and for preserving its nutritional value during storage.

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TABLE 4. Fatty acid composition (%) of common Kilka oil as a function of different variants of clove essential oil during accelerated storage at 97.8 °C

Fatty acid	Untreated oil	Control	HD*	SD	MAHD	MASD	β- Carotene	α- Tocopherol	Tocopheryl acetate	BHT
C8:0	0.00±0.00^{g**}	0.01±0.00$^{g\ de}$	0.01±0.00^{e}	0.02±0.00^{b}	0.01±0.00^{f}	0.02±0.00^{a}	0.02±0.00^{b}	0.01±0.00^{cd}	0.01±0.00^{c}	0.01±0.00^{c}
C10:0	0.01±0.00^{g}	0.01±0.00$^{g\ f}$	0.02±0.00^{cde}	0.02±0.00^{b}	0.02±0.00^{ab}	0.01±0.00^{e}	0.02±0.00^{cde}	0.02±0.00^{bc}	0.02±0.00^{de}	0.02±0.00^{a}
C12:0	0.07±0.01^{ab}	0.07±0.00$^{g\ b}$	0.07±0.00^{b}	0.07±0.00^{ab}	0.07±0.00^{b}	0.07±0.00^{b}	0.07±0.00^{ab}	0.07±0.00^{b}	0.07±0.00^{ab}	0.08±0.00^{a}
C13:0	0.10±0.00^{bcd}	0.10±0.01$^{g\ cd}$	0.10±0.01^{d}	0.13±0.01^{a}	0.08±0.00^{e}	0.11±0.01^{b}	0.09±0.01^{d}	0.08±0.00^{e}	0.11±0.01^{bc}	0.10±0.01^{bcd}
C14:0	4.74±0.09^{d}	5.25±0.26$^{g\ bcd}$	5.36±0.27^{abc}	5.27±0.26^{abcd}	4.92±0.25^{cd}	5.30±0.27^{abcd}	5.42±0.27^{abc}	5.33±0.27^{abcd}	5.53±0.28^{ab}	5.69±0.28^{a}
C15:0	0.88±0.01^{b}	1.10±0.10$^{g\ a}$	1.07±0.05^{a}	1.11±0.06^{a}	1.04±0.05^{a}	1.19±0.05^{a}	1.11±0.06^{a}	1.12±0.06^{a}	1.08±0.05^{a}	1.09±0.05^{a}
C16:0	19.77±0.25^{a}	25.62±1.28$^{g\ a}$	23.92±1.20^{a}	25.93±1.30^{a}	24.37±1.22^{a}	25.51±1.28^{a}	24.91±1.25^{a}	24.23±1.27^{a}	24.31±1.22^{a}	24.58±1.23^{a}
C16:1ω-7	6.75±0.08^{b}	8.08±0.40$^{g\ a}$	7.60±0.37^{a}	8.09±0.04^{a}	7.60±0.38^{a}	7.98±0.40^{a}	7.73±0.39^{a}	7.55±0.38^{a}	7.73±0.39^{a}	7.80±0.39^{a}
C17:0	0.54±0.00^{d}	0.69±0.03$^{g\ a}$	0.60±0.03^{cd}	0.66±0.03^{ab}	0.61±0.03^{c}	0.67±0.03^{ab}	0.67±0.03^{ab}	0.60±0.03^{cd}	0.61±0.03^{bc}	0.66±0.03^{abc}
C18:0	3.68±0.08 ^{d}	4.53±0.23$^{g\ bc}$	5.02±0.26^{a}	4.99±0.25^{a}	4.06±0.20^{cd}	5.13±0.26^{a}	5.06±0.25^{a}	4.94±0.25^{a}	4.83±0.24^{ab}	4.77±0.24^{ab}
C18:1ω-9	28.12±0.18^{c}	33.71±1.69$^{g\ ab}$	33.38±1.67^{ab}	33.74±1.69^{ab}	31.16±1.56^{bc}	33.26±1.68^{ab}	33.32±1.62^{ab}	33.29±1.66^{ab}	33.66±1.68^{ab}	33.78±1.69^{ab}
C18:2ω-6	1.99±0.04^{a}	1.86±0.09$^{g\ a}$	2.10±0.11^{a}	1.94±0.10^{a}	2.01±0.01^{a}	1.94±0.10^{a}	1.92±0.10^{a}	2.01±0.10^{a}	1.66±0.08^{b}	1.99±0.10^{a}
C18:3ω-3	2.06±0.02^{a}	1.65±0.08$^{g\ bcd}$	1.69±0.08^{cd}	1.66±0.08^{cd}	1.87±0.09^{b}	1.63±0.08^{cd}	1.72±0.09^{cd}	1.78±0.09^{bc}	1.71±0.09^{cd}	1.66±0.08^{cd}
C20:0	3.07±0.01^{a}	2.17±0.11$^{g\ cd}$	2.04±0.01^{cd}	2.01±0.10^{d}	2.47±0.12^{b}	1.97±0.10^{de}	2.13±0.11^{cd}	2.23±0.11^{c}	2.11±0.11^{cd}	2.08±0.10^{cd}
C21:0	0.39±0.10^{d}	0.41±0.02$^{g\ de}$	1.33±0.07^{a}	0.39±0.02^{de}	0.71±0.03^{b}	0.34±0.02^{ef}	0.32±0.02^{f}	0.60±0.03^{c}	0.41±0.02^{de}	0.42±0.02^{de}
C22:0	0.21±0.05^{b}	0.18±0.01$^{g\ cd}$	0.16±0.01^{d}	0.16±0.01^{d}	0.20±0.01^{bc}	0.16±0.01^{d}	0.18±0.01^{cd}	0.18±0.01^{cd}	0.18±0.01^{cd}	0.17±0.01^{cd}
C20:5ω-3	8.19±0.29^{a}	4.15±0.21$^{g\ de}$	4.76±0.24^{cd}	4.26±0.21^{ef}	5.69±0.28^{b}	4.19±0.21^{ef}	4.93±0.25^{c}	4.76±0.24^{cd}	4.87±0.24^{c}	4.69±0.23^{cd}
C23:0	0.03±0.00^{g}	0.04±0.00$^{g\ ef}$	0.05±0.00^{de}	0.05±0.00^{bc}	0.04±0.00^{ef}	0.05±0.00^{ab}	0.05±0.00^{de}	0.04±0.00^{def}	0.05±0.00^{cd}	0.04±0.00^{f}
C22:4ω-6	0.55±0.00^{a}	0.26±0.01$^{g\ c}$	0.36±0.02^{cde}	0.33±0.02^{e}	0.46±0.02^{b}	0.33±0.02^{e}	0.37±0.02^{cd}	0.42±0.02^{b}	0.35±0.02^{cde}	0.34±0.02^{de}
C24:0	0.48±0.01^{e}	0.80±0.04$^{g\ a}$	0.74±0.04^{abc}	0.79±0.04^{a}	0.70±0.03^{cd}	0.80±0.04^{a}	0.74±0.04^{abc}	0.71±0.04^{bcd}	0.70±0.03^{bcd}	0.66±0.03^{d}
C24:1ω-9	0.02±0.00^{e}	0.04±0.00$^{g\ ab}$	0.03±0.00^{d}	0.04±0.00^{ab}	0.03±0.00^{d}	0.04±0.00^{ab}	0.04±0.00^{bc}	0.03±0.02^{cd}	0.04±0.00^{ab}	0.03±0.00^{d}
C22:5ω-3	0.54±0.00^{a}	0.32±0.02$^{g\ cd}$	0.38±0.02^{cde}	0.39±0.02^{cde}	0.44±0.02^{b}	0.37±0.02^{def}	0.39±0.02^{cd}	0.34±0.02^{cde}	0.41±0.02^{bc}	0.35±0.02^{ef}
C22:6ω-3	17.77±0.47^{a}	8.89±0.45$^{g\ b}$	9.19±0.46^{bc}	7.91±0.39^{d}	11.44±0.57^{a}	8.95±0.45^{c}	9.60±0.48^{bc}	9.58±0.48^{bc}	9.53±0.48^{bc}	8.96±0.45^{c}
PUFA	31.11±0.81^{a}	17.13±0.86^{cde}	18.47±0.92^{c}	16.48±0.82^{de}	21.88±1.09^{b}	17.39±0.84^{cde}	18.93±0.95^{c}	18.92±0.95^{c}	18.51±0.93^{c}	18.00±0.90^{cd}
UFA	66.00±1.64^{a}	58.96±4.12^{b}	59.49±4.14^{b}	58.35±3.44^{b}	62.68±4.14^{ab}	58.68±4.02^{b}	59.19±3.94^{b}	59.80±4.21^{b}	59.94±4.24^{b}	59.60±4.32^{b}

*HD, hydro-distillation; MAHD, microwave-assisted hydro-distillation; SD, steam-distillation; MASD, Microwave- assisted steam-distillation.

**Mean ± standard deviation; Number of replicates for each analysis: 3; Statistical test: ANOVA and multiple comparison of means using Duncan's test; Degree of significance: $P < 0.05$.

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