EVALUATION OF THE OXIDATIVE THERMAL STABILITY OF FISH OIL WITH THE ADDITION OF PUMPKIN SEED OIL OR ROSEMARY EXTRACT

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ABSTRACT

The present study investigates the thermal stability of the commercial fish oil, rich in unsaturated fatty acids, and compares the sensory properties of pure fish oil with a mixture of fish oil and added rosemary extract (RE), as well as roasted and unroasted pumpkin seed oil, at a concentration of 5 %. All samples were monitored using FTIR spectroscopy to measure the specific absorptivity of conjugated dienes (CDs) and conjugated trienes (CTs), as well as the peroxide value. Additionally, GC/FID was employed to evaluate the oxidative degree of the fish oil and compare the antioxidative effect of roasted pumpkin seed oil, specifically in comparison to rosemary extract. For this purpose, the oil stability was optimized by comparing the oxidation levels of fish oils exposed to range of temperature, including 23°C, 50°C, 70°C, 90°C, and 110°C. This was done in the presence of a low percent of rosemary extract, unroasted pumpkin seed oil (UPSO), and roasted pumpkin seed oil (RPSO). Based on the obtained results, a clear difference is observed in the blended samples, particularly when roasted pumpkin seed oil is used. This difference is evident in the ultraviolet chemical parameters, fatty acid profile, and most notably in the optimized FTIR vibrational bands. The ratios of area peaks such as 3444/2854, 1745/2854 and 3010/2854 are considered important parameters for monitoring the chemical changes and lipid stability. All the chemical parameters confirm the possibility of enhancing the stability of fish oil by blending it with healthy pumpkin seed oil. The composition of pumpkin seed oil increases the stability of fish oil. Consequently, it is evident that pumpkin seed oil, known for its high healthy benefits, can successfully be used to improve the thermal and oxidative stability of fish oil lipids. The principal component analysis (PCA) was used to define clusters, which revealed a wide range of both chemically changed and unchanged samples. The application of FTIR spectroscopy as an alternative method provides excellent parameters for easy operation, affordability, and ecological considerations, making in an efficient tool for controlling the quality of edible oils.

Keywords: principal component analysis (PCA), conjugated diene, conjugated triene, natural extract, thermal oxidation.

INTRODUCTION

Edible oils contain triglycerides, which are fatty acid triesters, constituting approximately 95 % of their composition [1]. They also contain smaller quantities of free fatty acids and other components such as phospholipids, tocopherols, antioxidants etc. Triglycerides found in fish oil are composed of polyunsaturated fatty acids, notably eicosatetraenoic acid (ETA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These fatty acids offer significant health benefits to humans and play important physiological roles such as reduction of cardiovascular morbidity and mortality, cancer prevention, hypertension, etc.

However, these triglycerides may exhibit instability and susceptibility to oxidation due to the presence of double bonds in their chemical structure [2].

Omega-3 fatty acid present in fish oil are highly susceptible to oxidation, and various methodologies have been employed to stabilize them and prevent this process [3]. Additional antioxidants can be applied to minimize lipid peroxidation, such as incorporating antioxidants into fish by-products during the production process of fish oils. Some commonly used antioxidants in fish oil include BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), propyl gallate, TBHQ (tert-butylhydroquinone), tocopherol, and ascorbyl palmitate. However, there are limited reports on the application of natural products with antioxidative effects in preventing lipid peroxidation [4]. Adding fish oil to food products enhances their nutritional value, but at the same time, it reduces lipid stability, especially during storage [5].

The unsaturation level in lipids has the potential to undergo changes or oxidation, resulting in various oxidized compounds, such as hydroperoxides, which are considered to be highly toxic [6]. The initial stage of lipid oxidation involves the formation of hydroperoxides, which are primary oxidized compounds. These intermediates further decompose, leading to the formation of oxidized secondary compounds, such as ketones, aldehydes, esters, and alcohols route of the reactions are presented in (Fig. 1) [7 - 9].

The incorporation of fish oil in food products should be approached with caution due to the presence of long-chain unsaturated fatty acids. When exposed to air, light, and high temperatures, these fatty acids can undergo lipid oxidation, leading to the formation of off-flavor compounds in the food.

This can occur during storage and microencapsulation process, particularly during spray-drying, which is the commonly employed technique in the industry [10 - 12]. To address this issue, a small amount of natural oils, such as rosemary extract and pumpkin seed oil (both roasted and unroasted), were utilized. Studies have reported the chemical changes in pumpkin seeds during the roasting process and their positive impact on enhancing lipid stability, likely resulting from the formation of antioxidative compounds during roasting [13]. The roasting process of pumpkin seeds triggers various chemical reactions, leading to the formation of compounds with potent antioxidant and protective properties. These compounds contribute to the increased oxidative stability of polyunsaturated fatty acids [14].

Rosemary extract has been widely employed as an antioxidant in fish oil and other edible oils to safeguard the highly sensitive polyunsaturated fatty acids from thermal oxidation, changes in unsaturation level, and other unidentified chemical alterations [15 - 19]. The optimal concentration of rosemary extract has been determined to enhance its preventive effect [15 - 19]. Additionally, pumpkin seed oil, which also contains polyunsaturated fatty acids, has been utilized in combination with fish oil to prevent oxidation [20].

During the roasting process at temperatures above 100°C, pumpkin seed undergo substantial chemical changes, leading to the production of oil with a pronounced antioxidant capacity. These changes probably occur as a result of lipid peroxidation, streak degradation and, Maillard reactions and the interrelations between them, which are able to produce volatile compounds [21].

Despite this, there is a lack of published research on the protective effect of pumpkin seed oil, both before and after seed roasting, on the lipid stability of fish oil. The objective of this study was to investigate the impact of natural rosemary extract and pumpkin seed oil (both unroasted and roasted) on the thermal stability of fish oil, especially EPA, ETA, and DHA. Molecular spectroscopy techniques such as FTIR and UV/Vis were employed, in addition to the standard peroxide value method for the determination of the oil oxidation degree level. The fatty acid profile was continuously monitored using GC/FID.

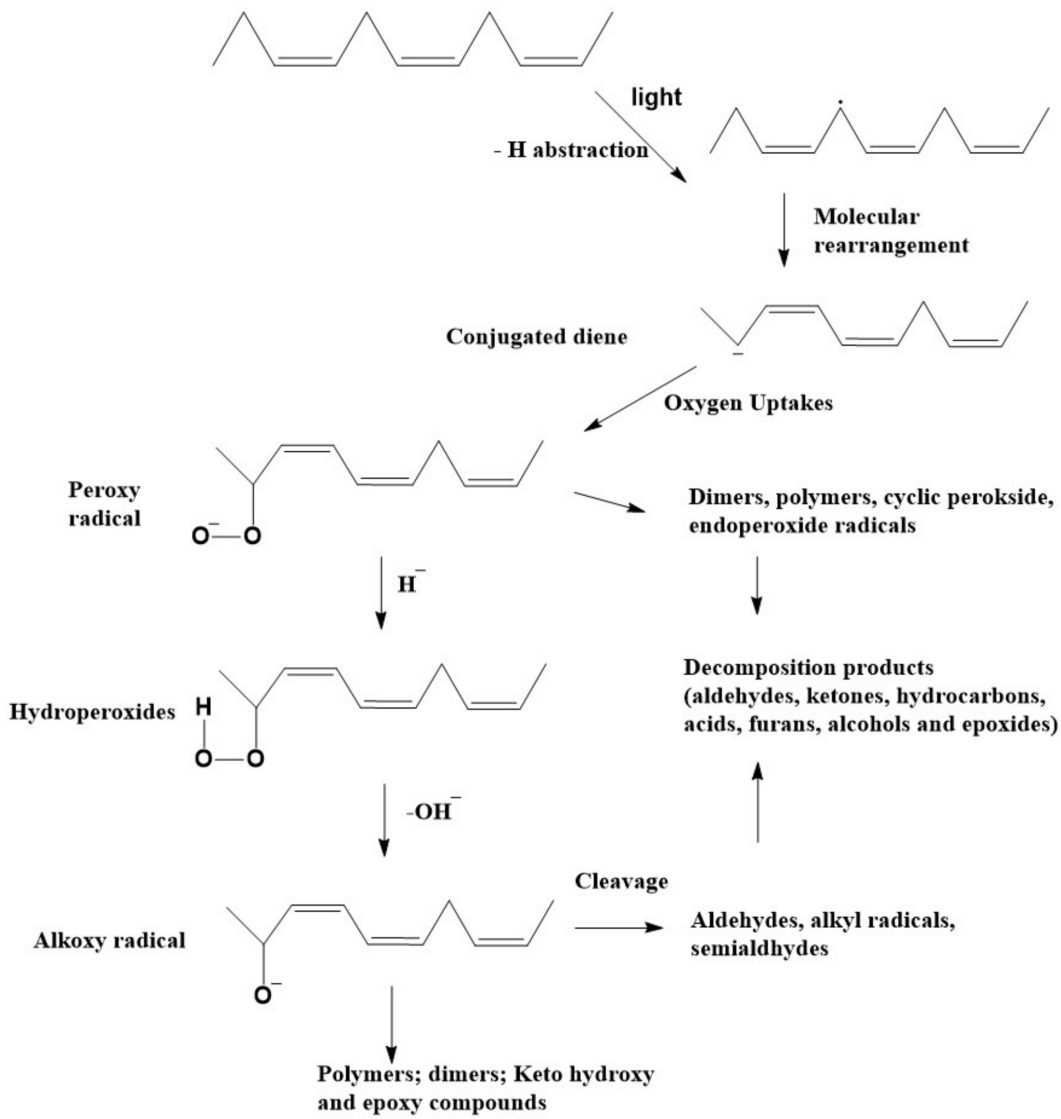

Fig. 1. Routes of lipid oxidation and formation of different oxidized.

EXPERIMENTAL

Material and reagents

Pure natural tissue fish oil without any added tocopherols was purchased at a local pharmacy (Prishtina, Kosovo). Pumpkin seed samples were provided from the Peja region in the west of Kosovo from varieties Cucurbita Pepo L. (without shell). Rosemary extract (RE) (Type HT-125), with a chemical composition of 6.1 % carnosic acid and 1.0 % carnosol was provided by Kalsec Inc. (Kalamazoo, MI).

The reagents used were n-hexane, boron trifluoride (14 % BF_3 w/w dissolved in methanol), acetone, anhydrous cyclohexane (99.5 % purity, A.R. grade, Merck Darmstadt Germany). Sodium thiosulphate, sodium sulfate anhydrous, diethyl ether, toluene, chloroform, methanol, potassium iodide, and acetic acid were of A.R. grade and purchased from Merck, Darmstadt Germany.

A 7 % BF_3 solution was prepared from a commercial 14 % BF_3 solution dissolved in methanol. A standard solution of fatty acid methyl ester (FAME, C4:0-C24:0 certified reference material CRM) was purchased from Analytical Supelco, USA.

Preparation of natural oils and extracts

The preparation of natural oils and extracts involved the roasting of pumpkin seeds for 70

minutes at a maximum temperature of 130°C [22]. The oil was extracted from both roasted (RPSO) and unroasted pumpkin seed (UPSO) using a screw cold press (Koçmaksan, KMS10, Izmir, Turkey) [23]. The extracted oil was then stored at -18°C until the blending procedure and analysis of the prepared formulation.

Thermal oxidative stability tests

For the thermal oxidative stability tests, three different mixtures were prepared:

- Pure natural fish oil;
- Fish oil with the addition of 5 % unroasted pumpkin seed oil (UPSO);
- Fish oil with the addition of 5 % roasted pumpkin seed oil (RPSO);
- Fish oil with the addition of 5 % rosemary extract (RE).

The thermal oxidative stability of fish oil mixtures (prepared fish oil formulations) was compared to that of pure natural fish oil. All samples were heated from 23°C to 110°C in each selected temperature for interval of 2 hours.

The thermal oxidative stability of all samples was analyzed by determining the fatty acid profile using GC/FID and FTIR spectroscopy, as well as measuring the peroxide value and the levels of conjugated diene and triene using UV/Vis spectroscopy.

Fatty acid analysis by GC/FID

The fatty acid methyl esters (FAME) mixture solution was dissolved in 1 mL n-heptane and injected into the GC-FID. The FAME mixture, used as certified reference material (CRM), was employed to identify all fatty acids in the samples by comparing the retention times and peak areas with the methyl ester C11:0 used as an internal standard.

The methylation of fatty acids was carried out following the official method AOAC 996.06 [24], according to the following procedure: The extracted fat is dissolved in chloroform-diethyl ether mixture and then is evaporated to dryness in 40°C water bath under nitrogen stream. 2.0 mL 7 % BF_3 reagent, and 1.0 mL toluene are added. The vial is heated in oven 45 min at 100°C and after adding of 5.0 mL H_2O, 1.0 mL hexane, and 1.0 g Na_2SO_4, the resulting samples are directly injected into a GC/FID for determination of fatty acids.

The analysis of fish oil composition before and after heat treatment was performed using a GC-FID system (Agilent Technologies 7890BA GC System, USA). The capillary column utilized for fatty acid separation was SP2560 (100 mx 0.25 mm with 0.25 μm film, Agilent Technologies, USA) as the stationary phase, and helium was used as the carrier gas with a flow rate of 1.4 mL.

The split injection mode (200:1) was used to insert 1 μL of each sample at 250°C (injector temperature), with the detector at 300°C. The applied temperatures of the GC/FID oven system are shown in Table 1. All samples were analyzed three times using GC-FID, and the results were calculated using Chemstation Software. The results were presented as percentage (%) of the total fatty acid content.

FTIR spectroscopy

FTIR spectra of the studied samples were acquired using a Shimadzu FTIR-Iraffinity-1 spectrometer equipped with a DLATGS detector and calcium fluoride (CaF_2) FTIR cuvette. IR spectra measurements were performed in the spectral range from 4000 to 1000 cm^{-1} with a resolution of 4 cm^{-1}. A drop of oil was placed on the CaF_2 window, and after each scan, the CaF_2 window was cleaned with acetone.

Peroxide value

The peroxide value is a chemical parameter used to determine the total peroxides present in the oil

Table 1. Gradient temperature program for GC-FID analysis.

Parameters	Rate, °C min^{-1}	T, °C	Hold time, min	Run time, min
Initial		70	1	1
Ramp 1	5	100	2	9
Ramp 2	10	175	2	18.5
Ramp 3	3	220	5	38.5

samples by measuring iodine released from potassium iodide (KI). The oil sample was dissolved in a mixture of acetic acid and chloroform and then saturated with KI. The resulting solution was titrated with sodium thiosulphate [25].

Conjugation indices

The determination of the conjugated dienes (CDs) and trienes (CTs) was performed using the following procedure: the oil samples were dissolved in anhydrous cyclohexane at a concentration of 1 %, and the absorbance was measured using a UV-Vis spectrophotometer at 232 nm and 270 nm [26].

Statistical analysis

Statistical analyses were conducted using the software OriginPro 2016 Sr2 by Originlab Corporation. The software was utilized for performing principal component analysis (PCA) on all samples analyzed using FTIR spectroscopy. The experimental data were analyzed using ANOVA, and mean comparisons were conducted using Tukey's test. A significance level of $p < 0.05$ was considered statistically significant. All measurements were performed in triplicate, and the values were presented as mean ± SD (standard deviation).

RESULTS AND DISCUSSION

Determination of fatty acid composition

Chemical changes of the fatty acids present in pumpkin oil were monitored before and after roasting at two recommended temperatures: 100°C and 130°C [22]. Table 2 presents the fatty acid composition of the same pumpkin seed oil before and after roasting. The results show a minor difference in the level of saturated fatty acids. However, the unsaturated fatty acids undergo changes in their levels after the roasting process, especially polyunsaturated fatty acid (18:3) n-3, which exhibit the highest increase at a roasting temperature of 130°C. This can be attributed to the chemical transformations that occur in the seeds during roasting, particularly at higher temperatures, resulting in the formation of numerous oxidized products [21].

Fatty acid composition is a useful parameter for understanding the chemical composition of triglycerides [27]. During this study, thermal stability of fatty acids as a crucial part of triglycerides was monitored. The concentration of most saturated fatty acids did not change appreciably during the thermal treatment. Thus, only some of the polyunsaturated fatty acids, characterized by high sensitivity to oxidation, were monitored. The results are presented in Table 3.

It can be seen that without thermal treatment, minor differences exist between them due to the lack of monitored unsaturated fatty acids in three different selected additives of the fish oil. During thermal treatment, pure fish oil shows changes in the concentration of all three monitored fatty acids. In contrast, in the presence of 5 % of additives in the fish oil, the level of transformation was statistically insignificant ($p > 0.05$). Rosemary extract and pumpkin seed oil extracted from roasted seeds exhibited high protective activity. However, the unroasted seed oil showed the lowest protective activity towards the fish oil.

GC/FID determines the free fatty acids after esterification; however, it is not clear whether the chemical conversions during the heat treatment changed their percentage or if other chemical components were generated. If there has been a change

Table 2. The fatty acid composition of pumpkin seed oil determined by GC/FID.

Fatty acids (% w/w)	Without roasting	Roasted 100°C	Roasted 130°C
C14:0	0.101	0.094	0.094
C16:0	11.464	11.231	11.53
C16:1	0.118	0.108	0.147
C18:0	9.689	9.733	9.721
C18:1	36.541	36.812	37.316
C18:2	41.33	41.275	40.360
C18:3 (n-3)	0.133	0.131	0.183
C20:0	0.529	0.525	0.555

Table 3. Chemical composition of three polyunsaturated fatty acids in fish oil samples.

Fatty acid composition (%)	Pure fish oil	Fish oil with 5 % UPSO	Fish oil with 5 % RPSO	Fish oil with 5 % RE
	Before thermal treatment			
DHA	14.27 ± 0.2[a]	13.42 ± 0.2[a]	13.21 ± 0.2[a]	13.31 ± 0.2[a]
EPA	3.58 ± 0.2[a]	3.22 ± 0.2[a]	3.34 ± 0.1[a]	3.18 ± 0.1[a]
ETA	6.54 ± 0.2[a]	6.12 ± 0.2[a]	6.15 ± 0.1[a]	6.06 ± 0.2[a]
	Heated temperature 100˚C			
DHA	13.60 ± 0.2[a]	13.17 ± 0.2[a]	13.15 ± 0.2[a]	13.29 ± 0.2[a]
EPA	3.14 ± 0.2[a]	2.98 ± 0.2[a]	3.3 ± 0.1[a]	3.22 ± 0.1[a]
ETA	6.12 ± 0.1[a]	5.97 ± 0.1[a]	6.11 ± 0.2[a]	6.11 ± 0.2[a]
	Heated temperature 120˚C			
DHA	13.13 ± 0.2[a]	12.91 ± 0.1[a]	13.09 ± 0.1[a]	13.25 ± 0.1[a]
EPA	2.83 ± 0.2[a]	2.89 ± 0.2[a]	3.26 ± 0.2[a]	3.16 ± 0.2[a]
ETA	5.83 ± 0.1[b]	5.89 ±0 .2[a]	6.14 ± .2[a]	6.06 ± 0.2[a]

Different lowercase letters (a and b) indicate a significant difference between the mean values in a row ($p \leq 0.05$).

in amounts, it is critical to know what chemical forms they are converted into and whether they have been oxidized during the heat treatment. It is unclear what their chemical pathways are.

The identification of the oxidized chemicals obtained is made difficult using GC equipment due to the need of standard compounds and complex matrix containing many unknown compounds. In addition, there are many instrumental limitations and difficulties with GC method, which limit the understanding of all the details of chemical transformations in the fish oil. For this purpose, spectroscopic methods such as FTIR and UV/Vis are recommended. They both present several advantages, such as ease of instrumental operation, low cost, simplicity, time savings, and not requiring toxic or expensive reagents [28].

FTIR spectroscopy

The use of infrared vibrational spectroscopy is one possibility to detect the first, second, and third stages of oxidation in the final and initial stages of oxidation of edible oils, indicating their intensity, intensity ratio, and shift position [29]. Upon closer examination of the spectra of the different samples no significant differences ($p > 0.05$) were observed in the FTIR spectra. To obtain relative quantitative information about individual molecule systems, intensity ratios of area can be utilized. The use of intensity ratios helps overcome any experimental limitations, such as variations in the volume of the sample analysis [30].

Identification of primary oxidized compounds

In general, the infrared spectra in the region of 3200 cm^{-1} - 3700 cm^{-1} correspond to the hydroxyl group. This region allows for the detection of hydroperoxides, which are among the first compounds formed during lipid peroxidation, based on their vibrational band investigations [34]. The vibration of hydroperoxides has been reported to occur precisely at 3444 cm^{-1} while the overtone of carbonyl group from triglyceride compounds appears near 3470 cm^{-1}.

When the hydroperoxide vibrational band is identifiable, it can be used as the ratio 3444/2854,

Table 4. FTIR spectral band assignments of lipids.

Wavenumber (cm^{-1})	Vibrational Assignment
3470	Overtone of the carbonyl group of triglycerides [31]
3444	Hydroperoxide [31]
3010	=C-H *(cis)* symmetric stretch [32]
2925 and 2854	C-H asymmetric and symmetric stretch. aliphatic vibrations [33]
1745	C=O stretching vibrations of carbonyl group in Triglycerides [32]

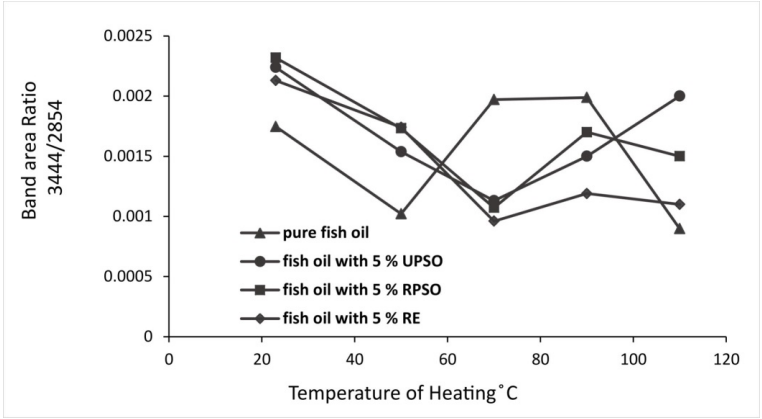

Fig. 2. Ratio values of the band area changes.

which serves as a useful indicator for hydroperoxide compounds. As shown in Fig. 2, there is a noticeable trend of changes in pure fish oil that are completely different from that observed in fish oil with additives.

The trend of changes needs to be understood in correlation with the formation of hydroperoxides, which are known as primary oxidized compounds and their subsequent conversion into secondary oxidized compounds.

Hydroperoxide compounds are also present in fresh samples, but during thermal treatment where the temperature is raised up to 50°C, these unstable compounds decrease in concentration due to their conversion into other compounds.

After heating up to 50°C, this ratio rapidly starts to increase, indicating lipid peroxidation. As a result, more hydroperoxides are rapidly formed until 70°C. From 70°C to 90°C, the studied ratio decreases, indicating the conversion of hydroperoxides into other secondary oxidized compounds.

However, this trend is not observed in fish oil samples containing 5 % of additives. In this case, until 70°C, the intensity ratio of 3444/2854 decreased, indicating that a minor portion of the hydroperoxides present in fresh samples undergoes slight conversion into other compounds. Lipid peroxidation was observed at temperatures higher than 70°C, but to a lesser extent compared to pure fish oil. At temperatures above 90°C, the primary oxidized compounds were transformed into other secondary oxidized compounds.

Rosemary extract has been found to exhibit higher activity [35] compared to roasted pumpkin seed oil and unroasted pumpkin seed oil in protecting lipids in fish oil from peroxidation. This difference in activity can be attributed to their distinct chemical composition. The results indicate significant chemical changes in pumpkin seed oil after the roasting process.

Identification of secondary oxidized compounds

The second step in the peroxidation of triglyceride molecules involves their transformation into other compounds known as secondary oxidized compounds, such as aldehyde, ketones, alcohols, esters, etc.

All these compounds contain a carbonyl functional group. The vibrational frequencies of these compounds are very close to that of the carbonyl group, and their bands overlap significantly. To overcome this drawback, the intensity ratio at 1745/2854 can be used as an indicator of lipid stability.

The observed changes in the studied intensity ratio of the pure fish oil are completely different from those in the fish oils with additives. Triglyceride exhibit rapid show chemically changes starting from a heating temperature of 50°C, which is correlated with the formation of hydroperoxide at the same temperature point.

The spectra of the samples containing 5 % of additives (Fig. 3) are similar, indicating that the additives effectively preserve the triglyceride from oxidation. The conversion of the additives was observed to begin at temperatures higher than 70°C.

Identification of cis unsaturation in triglyceride molecules

Hydroperoxide are initially formed through the oxidation of double bonds, which involves the breaking of cis unsaturated double bonds (=C-H) at

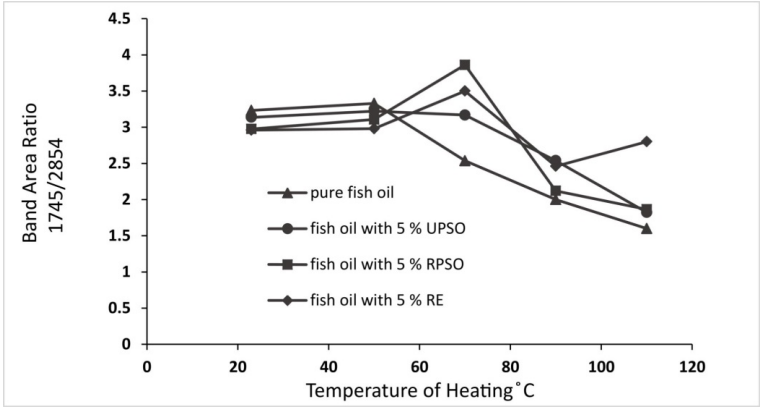

Fig. 3. Ratio values of the band area changes.

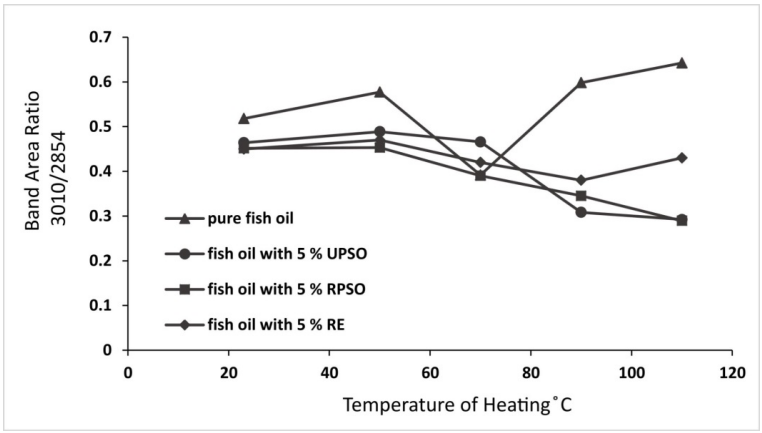

Fig. 4. Ratio values of the band area changes.

3010 cm^{-1}. This peak was used in combination with the stretching vibration of aliphatic saturated –C-H bonds because, logically, during oxidation, unsaturated bonds are transformed into saturated bonds. In this point of view, the 3010/2854 ratio was applied as a valuable and quantitatively better indicator for monitoring the changes in the cis olefinic bond [34]. As shown in Fig. 4, the studied ratio for pure fish oil follows a different trend compared to the samples with additives. The ratio of pure fish oil starts to decrease at temperatures above 50°C and rapidly increases at 70°C. The observation can be explained by the first stage of oxidation, which involves hydroperoxide formation and their subsequent transformation into various complex secondary oxidized compounds. The samples containing additives, when heated at the same temperature, exhibited a similar trend of decreasing the studied intensity ratio, but at higher temperatures. The samples with additives demonstrated greater resistance due to the preserving activity of the 5 % added oil or extract.

Peroxide value

Fig. 5 presents the peroxide value for all studied samples. The peroxide number of fish oil was higher, and it remained higher after heating at 70˚C and higher temperatures, in comparison to the three oil samples with additives, confirming their preserving activity.

Oil oxidation using conjugated diene and triene

The increased UV absorption of K232 and K270, in conjunction with conjugated diene and triene formation, is proportional to oxygen uptake and results from peroxide formation. Therefore, both parameters can be used as relative measurements of lipid oxidation [36].

Three fish oil samples, each with 5 % additives

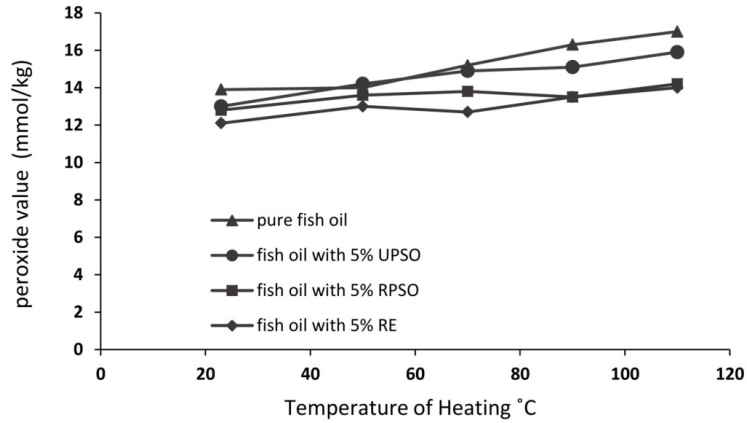

Fig. 5. Peroxide values of the fish oil samples.

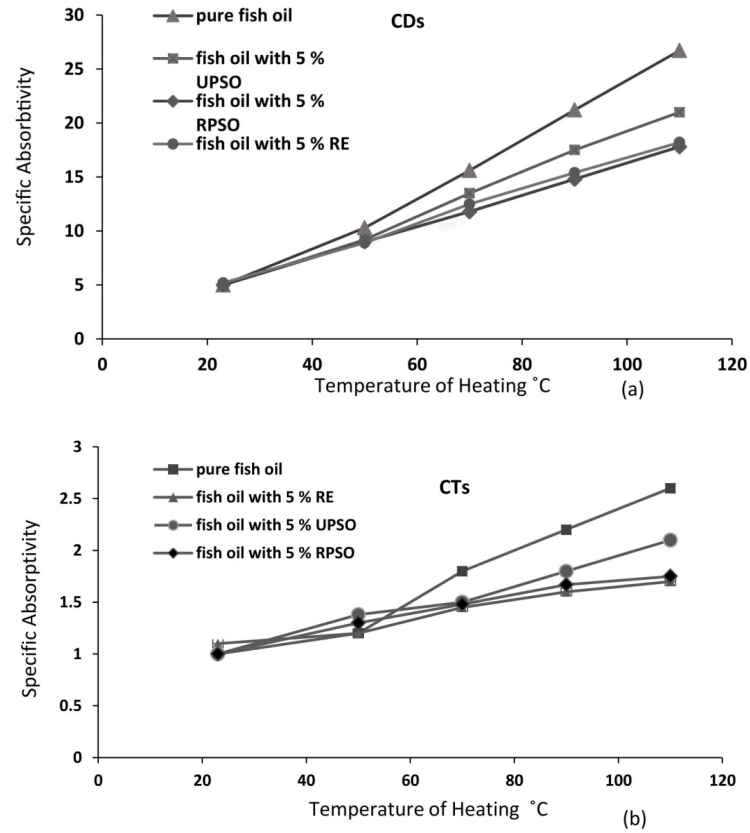

Fig. 6. Changes in the specific absorptivity of CDs (a) and CTs (b) obtained from pure fish oil and other fish oil samples with UPSO, RPSO and RO during the heat treatment.

(rosemary extract and pumpkin seed oil), show differences when compare to pure fish oil without any additives. These differences indicate a lower specific absorptivity, as shown in the conjugated diene analysis (Fig. 6(a)) and the conjugated triene analysis (Fig. 6(b)). This means that they exhibited resistance to the oxidation process, particularly the rosemary extract and the roasted pumpkin seed oil, showing greater resistance to the oxidative thermal effect. This can be explained by the different chemical compositions of the pumpkin seed oil after undergoing the roasting process, which has an impact on the lipid stability of the pumpkin seed oil [13].

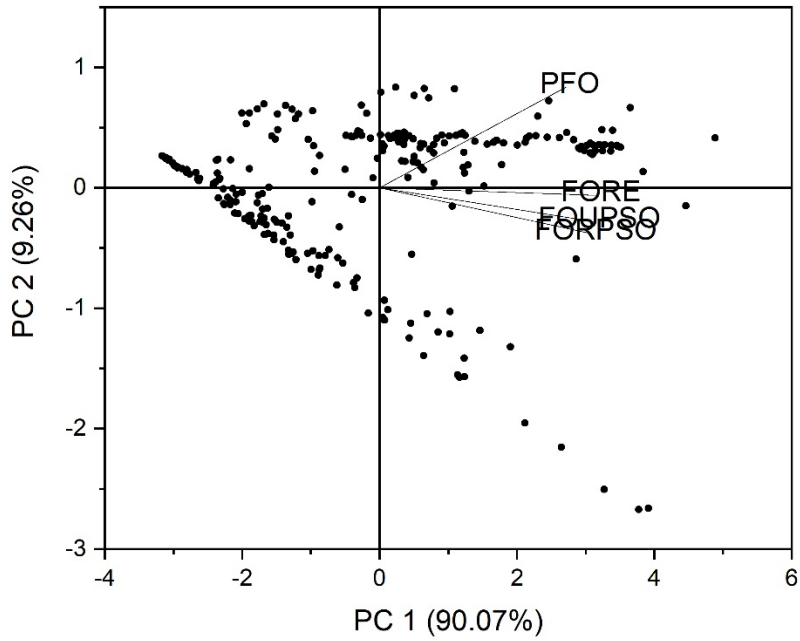

Fig. 7. PCA score plots of FTIR spectra obtained from pure fish oil (PFO), fish oil with Rosemary extract (FORE) and fish oil with unroasted (FOUPSO) and roasted pumpkin seed oil (FORPSO).

Principal component analysis

PCA is a chemometric technique that converts the original large scale of parameters into a smaller number of different parameters called principal components (PCs). As shown in Fig. 7, PCA is capable to differentiating the oils based on their distinct infrared spectra after thermal treatment. This differentiation is further confirmed by the maximum variation values (PC1 + PC2 = 99.33).

As observed in Fig. 7, the three fish oil samples with 5 % additions are clustered on the negative side of PC2, while the pure fish oil is located on the positive side of the PC2. Furthermore, all the fish oil samples with and without additions are clustered together on the positive side of PC1. The two last samples, composed of unroasted and roasted pumpkin seed oil, are very close to each other in terms of their PC1 values.

Based on these results, it can be concluded that the chemistry of pure fish oil changed slightly during the thermal treatment compared to the three other samples, which were also exposed to the same thermal treatment. However, all samples containing additions remained chemically unchanged. This suggests that only 5 % of the added oils contribute to high protective effect.

CONCLUSIONS

The results obtained from peroxide value, UV/Vis, and FTIR spectroscopy revealed significant chemical changes ($p < 0.05$) in the heat-treated fish oil samples due to lipid peroxidation. This difference was particularly observed in the three samples with additives, where their lipid peroxidation was prolonged at higher heated temperatures. This effect can be attributed to the additives present at a very low level (5 %), which play a crucial role in protecting the lipids from further oxidation and enhancing their thermal stability. Moreover, the addition of pumpkin seed oil to fish oil not only contributes to its health benefits but also improves its overall quality.

The limitations of these blended oils arise when considering their application in higher thermal processes, as the chemical components of pumpkin seed oil tend to degrade at temperatures exceeding 130°C. For future research, it is recommended to explore the application of fish oil in natural food products, such as dairy products, to enhance their nutritional value. However, it is advised to add roasted pumpkin seed oil as an addition ingredient to increase the stability of the fish oil during storage.

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