ANALYSIS OF THE OZONATION PRODUCTS OF FISH OIL WITH IR-SPECTROSCOPY AND GAS-LIQUID CHROMATOGRAPHY

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Abstract. The aim of the study was the IR spectroscopic and chromatographic analysis of products formed during the ozonation of fish oil. Fish oil samples were ozonized using an ozone therapy apparatus with an ozone destructor "Medozons - O3" (Nizhny Novgorod, Russia). The study of the chemical composition of fish oil ozonolysis products was performed on a Shimadzu IR Prestige 21 (Japan) infrared Fourier spectrophotometer in the region of wave numbers 4000 - 400 cm^{-1} in the form of liquid films in KBr, NaCl, or ZnSe windows. Chromatographic study of the composition of fatty acids was carried out on an Agilent 7890B gas chromatograph with a mass-selective detector 5977A. Qualitative and quantitative determination of propionic acid was carried out on the gas chromatographic complex "Chromosome GC – 1000". Chromatographic data processing was performed on the hardware and software complex "Chromatek-Analyst". The dynamics of IR spectra and quantitative composition of fatty acids (chromatographically) of the studied samples before and after ozonation were evaluated. The concentration of reactive oxygen species was controlled by iodometric titration according to the interstate standard GOST ISO 3900 – 2013. The acid number and saponification number of reaction products were determined by chemical methods. It was found that during the processing of fish oil, the reaction with ozone mainly proceeds locally along the ω–3 double bonds, leading to the formation of hydroperoxyesters and hydroperxy acids, as well as propionic acid. It is shown that the number of fragments of ω-6, ω-7 and ω-9 fatty acids practically does not change.

Keywords: IR – Fourier spectroscopy, gas chromatography, ozone, hydroperoxyesters, fatty ω-3 acids.

Introduction

Currently, ozone therapy technologies are successfully used for therapeutic purposes in various fields of medicine (dermatovenerology, gynecology, dentistry, and combustiology) (Cattel *et al.*, 2020; He *et al.*, 2012; Jiang *et al.*, 2019; Chemical encyclopedia, 1992; Knerel'man *et al.*, 2008). The production of medicinal preparations and cosmetic products for external action containing ozonated olive oil has already been implemented in many countries of the world (Russia, Ukraine, Italy, Cuba, Spain, Turkey, USA, Mexico, etc.) (Tirelli *et al.*, 2019; Mishina *et al.*, 2014). We have previously shown that the predominant role in ensuring the effect of these drugs is played not by ozonides, but by hydroperoxyesters and hydroperoxyacids, which are formed during ozonation of ω-9 fragments of oleates and oleic acid, respectively (Sen, 2020).

The clinical and economic validity of ozonide-containing drugs and cosmetic products is associated with a wide range of biological and sanogenetic effects of ozone. They include bactericidal action, antioxidant and antihypoxic properties, the ability to stimulate blood microcirculation. An important clinical effect of ozone therapy is a positive effect on the regenerative potential of cells and tissues due to the activation of the antioxidant system, as well as the intensification of aerobic metabolism (Corteselli *et al.*, 2019).

The use of fish oil as a substrate for ozonation is due to the fact that it contains a large amount of esterified ω-3 unsaturated acids (Zeng & Lu, 2018; Gordetsov *et al.*, 2019; Peretyagin *et al.*, 2007; Razumovskii & Zaikov, 1980). It is known that such features of the component composition of fish oil make it possible to widely use products based on it (Masan *et al.*, 2021; Peretyagin *et al.*, 2007;

Philibert *et al.*, 2019; Razumovskii & Zaikov, 1980), however, for full use, in-depth physico-chemical studies of fish oil ozonation products are required. As a result, the purpose of this study was the IR spectroscopic and chromatographic analysis of products formed during the ozonation of fish oil.

Materials and Methods

Fish oil samples were ozonized using an ozone therapy apparatus with an ozone destructor «Medozons – O3» (Nizhny Novgorod, Russia).

The study of the chemical composition and structure of ozonolysis products containing ROS was performed on a Shimadzu IR Prestige 21 (Japan) infrared Fourier spectrophotometer in the region of wave numbers 4000 - 400 cm^{-1} in the form of liquid films in windows made of KBr, NaCl, or ZnSe. ROS was identified by comparing the IR spectra of samples of the studied substances before and after ozonation. The concentration of ROS was also determined by the method of iodometric titration according to the Interstate standard GOST ISO 3960 – 2013 (Grechkaneva *et al.*, 2018).

Chromatographic study of the composition of fatty acids was carried out on an Agilent 7890B gas chromatograph with a mass-selective detector 5977A. Capillary column 60 m long, inner diameter 0.25 mm, film thickness 0.25 microns with a fixed phase – 5% phenylmethylpolysiloxane. The regime in accordance with ASTM D5974 «Standard method for the quantitative determination of the content of fatty and resin acids in the products of fractionation of tallow oil». Sample preparation was carried out according to GOST 31665-2012, item 6.

The laboratory sample was thoroughly mixed. (0.1 + 0.02) g of the product was taken into a test tube and dissolved in 2.0 cm^3 hexane. 0.1 ml of methanol solution of potassium hydroxide was added to the resulting solution, the tube was closed with a stopper and intensively mixed for 2 minutes. Then, for 5 minutes, the solution was settled to separate the glycerin. The top layer containing methyl esters was filtered through a paper filter. In the presence of fatty acids with the number of carbon atoms less than 8 in the mixture of methyl esters, filtration was replaced by centrifugation.

The qualitative and quantitative composition of fatty acids, obtained by saponification of fish oil before and after ozonation was determined by gas–liquid chromatography (GC) on the Chromos GC – 1000 gas chromatographic complex equipped with both a flame ionization detector and a capillary column with a polyethylene glycol phase modified with nitrophthalic acid (ZB FFAP 50 m × 0.32 m × × 0.5 μm). The analysis was carried out at the following parameters: column temperature – 150 °C, evaporator – 200 °C, detector – 150 °C; flow division – 10:1.

Results

We have previously shown that as a result of ozonation of olive oil, namely oleates (i.e., triacylglycerides of oleic acid), which are part of it, hydroperoxyesters or hydroperoxic acids are formed in large quantities. (Sen, 2020). It is worth noting that as a result of ozonation, the formation of ROS in high concentrations is observed.

When ozonating fish oil (RYE) under similar technological conditions, it was assumed that the treatment of oleates would give a similar positive result. However, analysis of the data obtained using IR spectroscopy and GC methods showed that ozone reacted mainly by the ω-3 double bonds of linoleic, eicosantrienoic, eicosanpentaenoic (EPA), docosapentaenoic (DPA) and docosahexaenoic (DHA) residues. This fact is confirmed by their total decrease in concentration by 4-5 times compared to the initial level and the appearance of a large amount of propionic acid as one of the main reaction products. Its concentration increased 4.5-6 times, respectively. At the same time, ω-6, ω-7 and ω-9 double bonds of fatty acids in esterified and free form (linoleic, oleic, palmitoleic, heptadecene) were oxidized less actively. Their concentration during ozonation practically did not change, and the amount of carboxylic acids remained within 0.1–0.7 mass percent (for comparison, propionic acid has

19.2%) (Table 1, 2). Based on the above, due to a decrease in the concentration of ω-3 acids, the relative concentration of marginal fatty acids increases: myristic, palmitic and stearic (Table 2). At the same time, the possibility of direct conversion of unsaturated acids into limiting ones (for example, oleic acid into stearic acid) should be completely excluded due to the absence of hydrogen in the reaction system. Thus, it became necessary to set up additional experiments related to the search for short-chain fatty acids, the hypothetical formation of which is assumed during the ozonation of ω-3 double bonds of fatty acids.

So, if during the ozonation of oleates, nonanoic and pelargonic acids were previously found as one of the main products (Sen, 2020), then propionic acid prevails during the ozonation of RYE (Fig. 1).

According to Fig. 1, the active oxygen form (ROS) is hydroperoxyester (GPE), which, according to researchers (Chemical encyclopedia, 1992), has sufficient thermodynamic stability due to the formation of intra- and intermolecular hydrogen bonds between UN groups and ester groups –C=O. GPE is determined using the peroxide number index and the IR spectrometry method. Thus, the peroxide number of fish oil before ozonation is 11.17, and after ozonation – 103.4. These data show that the composition of ROS is chemically stable, however, over time their concentration decreases (by 20% within a month).

At the same time, the value of the acid number (k.h.) before and after ozonation indicates that the native fat contains only 1-2% of free acids (k.h. 1.94), while the products of the ozonation reaction have an acid number of 20.82 (an increase of 10 times is recorded). k.h. during storage (within 2-3 months) of ozonated fat increases. These changes also correspond to Fig. 1 and are justified by the accumulation of low molecular weight acids (mainly propionic) as a result of the oxidation of their double bonds.

When analyzing the data of the Table 2 it should be noted that the initial composition of RYE (in our experiment, RYE-1 and RYE-2) does not significantly affect the dynamics of changes in the concentrations of saturated and unsaturated acids. So, if the total mass percentage of ω-3 acids in the composition of RJ-1 before ozonation was 36.68%, then after ozonation it was 7.48% (a decrease of 4.9 times is recorded), and in RJ-2 the results are as follows: before ozonation – 52.61%, after ozonation – 14.41% (a decrease is recorded 3.7 times). At the same time, the amount of propionic acid during ozonation of RJ-1 increases by 6.5 times, and RJ-2 – by 4.7 times. At the same time, the relative concentrations of ω-6, ω-9, and ω-7 acids either do not change or increase. It should be borne in mind that in the process of ozonolysis, the participation of these fragments of molecules is negligibly small. In general, the total percentage of unsaturated fatty acids in RYE-1 before ozonation was 68.2%, after ozonation 38.8% (a decrease of 1.8 times), in RYE -2 – 76.8 and 47.6, respectively (a decrease of 1.7 times). For saturated acids, the opposite trend was observed: for RJ -1 before ozonation, 28.4%, after ozonation – 57.8% (an increase of 2 times), for RJ-2 before ozonation, 12.9%, after ozonation – 38.3% (an increase of 2.9 times).

The analysis of IR spectra of reaction mixtures (Fig. 2) also confirms the theoretical positions. The following absorption bands were found in the IR spectra, cm^{-1}: 3485 - 3468 (ν OOH), 1743 -1738 (ν C=O in peroxyesters and lipids [8]), 1160 (ν C-O of triglycerides), 1101 (ν COO), 885 (ν O-O, shoulder) (GPE), 1040 (ν O-O of ozonide [2, 4, 16]).

It should be noted that the peak with a maximum of 3012 cm^{-1} (ν =CH) present in the initial substance practically passed into a small shoulder, and 720 cm^{-1} (out-of-plane deformation vibrations of C-H in cis isomers [10]) passed into 723 cm^{-1} with a significant decrease in intensity. By the method of subtraction of IR spectra [4,13,20], the main absorption peaks of GPE were confirmed and refined (Fig 2b, c), cm^{-1}: 3480 (ν OOH), 1739 (ν C=O), 1103 (ν COO). Thus, the main carrier of ROS in this case is hydroperoxy derivatives of lipids of the GPE type (Sen, 2020).

Table 1

Experimental data of GC chromatograms for short–chain carboxylic acids

Initial unsaturated residues	Carboxylic acids formed after ozonation	FISH OIL-1 % by weight			FISH OIL-2 % by weight		
		Before ozonation	After ozonation	1 month after ozonation	Before ozonation	After ozonation	1 month after ozonation
ω 3	Propionic (C_3)	2.94	19.2		3.75	14.8	
ω 6	Nylon (C_6)	–	–	–	0.10	–	0.28
ω 9	Nonanova (C_9)	–	0.15	–	0.19	0.71	

Table 2

Experimental data of GC chromatograms of compositions «Fish oil-1» (RJ-1), «Fish oil-2» (RJ-2)

Acids	FISH OIL-1 (% by weight)			FISH OIL-2 (% by weight)	
	Before ozonation	After ozonation	1 month after ozonation	Before ozonation	After ozonation
Saturated					
Palmitic	16.72	33.24	47.40	8.18	18.22
Myristic	6.33	15.42	20.68	0.50	1.38
Stearic	3.97	6.94	10.00	3.37	
Margarine	0.85	1.11	–	–	6.77
Pentadecane	0.43	1.11	–	–	0.13
Eicosan	–	–	–	0.80	1.95
Total %	28.36	57.82	78.08	12.85	38.28
Unsaturated					
ω 3					
Docosahexaenoic	18.28	2.51	0.40	18.41	2.75
Eicosapentaenoic	11.86	2.23	0.90	6.85	10.42
Linolenic	2.65	1.57	0.20	–	0.14
Docozapentaenoic	2.19	0.32	0.30	6.24	0.73
Eicosatriene	1.70	0.85	–	1.11	0.37
Total %	36.68	7.48	1.80	52.61	14.41
ω 6					
Linoleic	1.79	1.65	3.50	2.22	2.35
Eicosatetraene	–	–	–	4.69	1.39
Eicosadiene	–	–	–	0.80	0.35
Total %	1.79	1.65	3.50	7.71	4.09
ω 9					
Oleic	18.29	19.95	5.10	12.49	22.35
Eicosene	2.57	0.47	–	3.48	3.92
Total %	20.86	20.42	5.10	15.97	26.27
ω 7					
Palmitoleic	7.87	9.29	4.10	0.41	2.79
Hexadecene	1.02	–	–	0.08	0.07
Total %	8.89	9.29	4.10	0.49	2.86
Total % Unsaturated acids	68.22	38.84	14.50	76.78	47.63

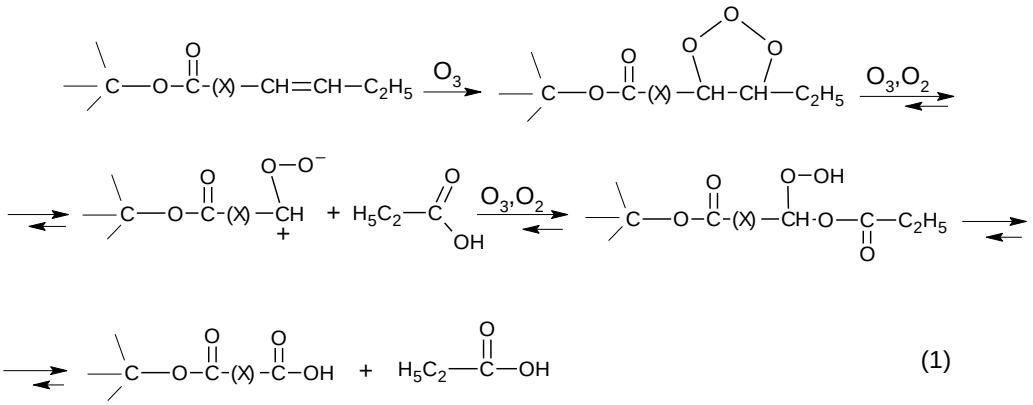
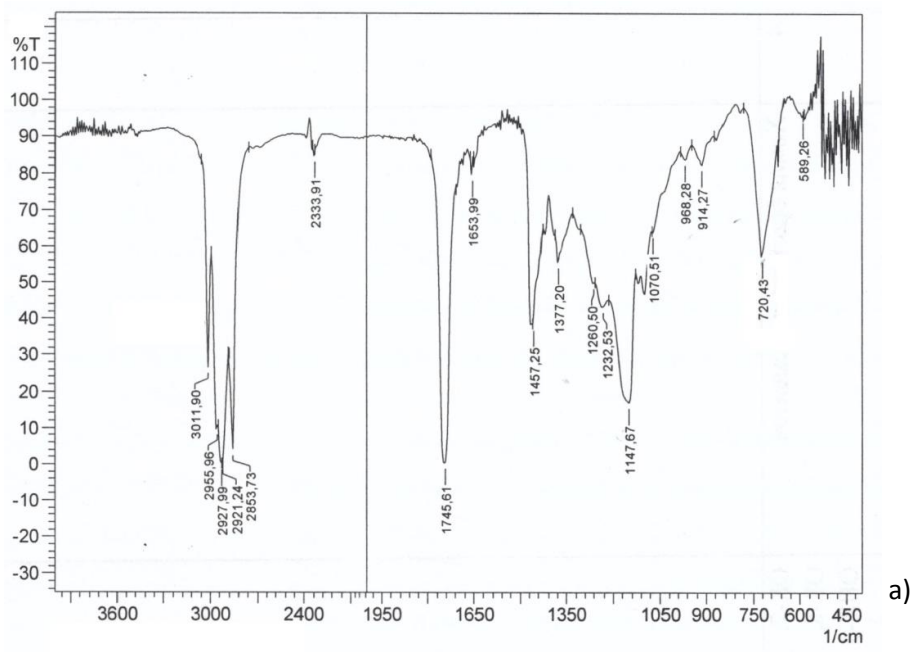


Fig. 1. The proposed scheme is confirmed by experimental data obtained using the GC method



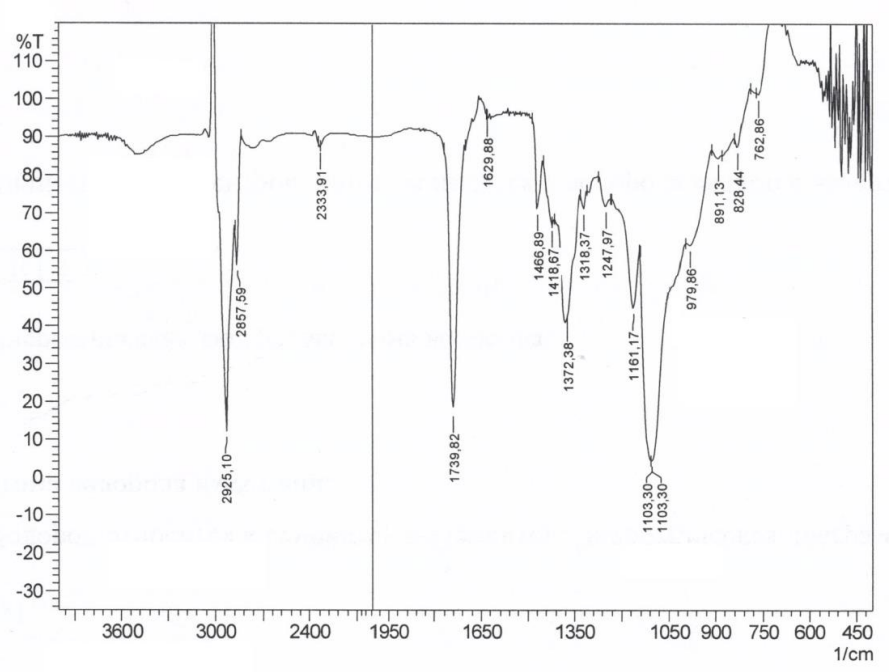


Fig. 2. The analysis of the IR spectra of reaction mixtures: a) – the fish oil before ozonation; b) – the fish oil after ozonation; c) – subtraction of IR spectra b and a

Conclusions

The main active form of oxygen formed during the ozonation of fish oil are hydroperoxy derivatives of lipids obtained by the predominant oxidation of ω-3 double bonds of ester residues, and ozonides are contained only in the form of impurities. Ozonation of the ω-6, ω-7 and ω-9 double bonds of fatty acids that are part of fish oil is difficult due to steric factors and the presence of enzymatic components in it. Ozonated fish oil retains the function of a carrier of ROS for 3–4 months. When stored, it records a slow decrease in the peroxide number and an increase in the acid number, which allows it to be used as a basis for the creation of medicinal products with biocidal and energy-stimulating properties that will be relevant in dentistry, dermatology and cosmetology, to combat viral infections and their consequences. It is convenient to monitor the dynamics of chemical transformations involving ROS using the methods of GC and IR spectroscopy.

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