

Effects of grilling on cholesterol oxide formation and fatty acids alterations in fish

Efeito do cozimento na formação de óxidos de colesterol e alteração da composição de ácidos graxos em peixes

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Abstract

Due to the adverse effects of the cholesterol oxidation products for the human health, the search of the occurrence and the quantification of these compounds in foods are considered of great importance. In this paper the effect of grilling in hake and sardine on cholesterol oxides formation and fatty acids alterations was investigated. The main fatty acids determined in both fishes were docosahexaenoic (DHA), oleic, eicosapentaenoic (EPA) and palmitoleic. The total lipids, fatty acids and cholesterol contents were decreased significantly ($p < 0.02$) after thermal treatment, with simultaneous increase of the cholesterol oxides contents. The cholesterol oxides determined in both species in the present study were: 19-hydroxycholesterol, 24(S)-hydroxycholesterol, 22(S) hydroxycholesterol, 25-hydroxycholesterol, 25(R)-hydroxycholesterol and 7-ketocolesterol. Besides the presence of the cholesterol oxides in raw fishes, there were a greater number of products resulting from the oxidation of cholesterol side chain, a fact rarely observed in foods.

Keywords: hake; sardine; fatty acids; cholesterol; effect of grilling; HPLC.

Resumo

Devido aos efeitos adversos dos produtos de oxidação do colesterol, a ocorrência e quantificação destes compostos em alimentos são consideradas de grande importância à saúde pública. O objetivo do presente trabalho foi verificar o efeito do cozimento em pescada e sardinhas na formação de óxidos de colesterol e alteração da composição de ácidos graxos. Os principais ácidos graxos determinados nas duas espécies de pescados foram: docosahexaenoico (DHA), oleico, eicosapentaenoico (EPA) e palmitoleico. Os teores de lipídios totais, ácidos graxos e colesterol diminuíram significativamente ($p < 0.02$) após o tratamento térmico, com simultânea elevação das concentrações dos óxidos de colesterol. Os óxidos determinados no presente estudo mostraram-se comuns as duas espécies de peixes: 19-hidroxicoolesterol, 24(S)-hidroxicoolesterol, 22(S)-hidroxicoolesterol, 25-hidroxicoolesterol, 25(R)-hidroxicoolesterol e 7-cetocolesterol. Além da presença de óxidos de colesterol em peixes frescos, observou-se um maior número de produtos originados a partir da oxidação da cadeia lateral do colesterol, fato raramente observado em alimentos.

Palavras-chave: pescada; sardinha; ácidos graxos; colesterol; efeito do cozimento; HPLC.

1 Introduction

Regular consumption of fish is recommended in view of its high content of omega-3 PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These compounds have been reported to prevent certain diseases, especially cardiovascular and inflammatory disorders, cancer, and stroke (DIN; NEWBY; FLAPAN, 2004).

Lipids can undergo alterations during food preparation with consequent decrease in the nutritional value. Lipid oxidation is one the major reactions affecting the fatty acid composition and cholesterol and originating cholesterol oxidation products (COPs) or cholesterol oxides. Cholesterol oxides are of clinical interest especially due to their association with atherogenesis, cytotoxicity, inflammatory process, rheumatic arthritis, mutagenesis, carcinogenesis, and degenerative diseases such as Parkinson and Alzheimer. COPs also impair the membranes

function, which results in the alteration of their permeability (SCHROEPFER, 2000).

The degree of oxide formation in food systems is related to processing temperature, heating time, storage conditions, level of activation, and packing conditions. The nature, proportion, and degree of unsaturation of fatty acids present in animal products will indicate the approximate susceptibility of cholesterol oxides formation, particularly in fish products rich in omega-3 PUFA (OHSHIMA, 2002).

Fish is normally grilled in a variety of ways before consumption. Grilling causes a protein denaturation, which can lead to the loss of antioxidant enzyme activity or the release of catalytically-active iron from metallo-proteins, disruption of cells membranes, which bring polyunsaturated fatty acids in contact with pro-oxidants, and thermal decomposition of hydroperoxides to pro-oxidants species, such as alkoxyl and

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hydroxyl radicals (HUR; PARK; JOO, 2007). These radicals will accelerate the chain reaction of lipid oxidation including cholesterol. Thus, grilling leads to significantly increased oxidation, as reflected by COPs formation.

Some authors reported the presence of cholesterol oxides in processed fish (AL-SAGHIR et al., 2004; OHSHIMA et al., 1996; SALDANHA; BRAGAGNOLO, 2008; SAMPAIO et al., 2006; SHOZEN et al., 1995), however, information about the presence of these compounds in fresh fishes is scarce. Thus, the objective of this study was to verify the alterations in the fatty acid composition and formation of cholesterol oxides in raw and grilled popular fish species consumed in Brazil.

2 Materials and methods

2.1 Chemicals

Cholesterol and cholesterol oxides standards 19-hydroxycholesterol (19-OH), 20- α -hydroxycholesterol (20 α -OH), 22 $^{\circ}$ R-hydroxycholesterol (22R-OH), 24 $^{\circ}$ S-hydroxycholesterol (24S-OH), 22 $^{\circ}$ S-hydroxycholesterol (22S-OH), 25-hydroxycholesterol (25-OH), 5,6 α -epoxycholesterol (α -EP), 5,6 β -epoxycholesterol (β -EP), 25 $^{\circ}$ R-hydroxycholesterol (25R-OH), 7-ketocholesterol (7-keto), 7 β -hydroxycholesterol (7 β -OH), and 7 α -hydroxycholesterol (7 α -OH) were purchased from Sigma (Milford, MA, USA) and Steraloids (Newport - RI, USA). Nonadecanoic methyl ester was purchased from Sigma (St. Louis - MO, USA) and the fatty acids standard mixture was purchased from SupelcoTM 37 (FAME Mix 18919, Bellefonte, PA, USA). The purity of the standards varied from 95 to 98 %. HPLC grade *n*-hexane and 2-propanol were obtained from Mscience (Darmstadt, Germany), and all other analytical grade solvents were obtained from Merck (Darmstadt, Germany).

2.2 Samples

Fresh Brazilian sardines (*Sardinella brasiliensis*) and Atlantic Hake Fillets (*Merluccius hubbsi*) were purchased from local market in the city of Sao Paulo. One kg of each fish was washed and divided into 2 lots; half was grilled and the other half was analyzed raw. The sardines were eviscerated and filleted. The hake fillets were thawed and cut lengthwise. The fishes were grilled under 175 °C for two minutes on each side until reach an internal temperature of 75 ± 1 °C. The temperature was monitored manually using a digital calibrated thermometer (Traceable Long-Stem, Friendswood, TX, USA). The samples were ground and homogenized in a multi-processor (Mega Master, RI 3170, 500 W, Sao Paulo, Brazil) to obtain homogeneous dough. The experiment and the analyses were carried out in duplicate (n = 4).

2.3 Methods

The moisture content was determined according to Association of Official Analytical Chemists (2000). The lipids were extracted and determined according to Bligh and Dyer (1959) method.

The lipids (25 mg) obtained by Bligh and Dyer (1959) method were converted into methyl esters according to

Joseph and Ackman (1992) to determine the fatty acids using a gas chromatograph (Varian 3400CX, Walnut Creek, CA, USA), equipped with a split injector (1:50) and a flame ionization detector, and a workstation. The chromatographic separation was achieved on a fused silica CP-SIL 88 capillary column 100 m × 0.25 mm i.d., 0.20 μ m film thickness (Chrompack, Middelburg, The Netherlands) according to procedures and conditions previously reported (SALDANHA; BRAGAGNOLO, 2007). The temperature program was: initial temperature, 120 °C (8 minute) followed by 15 °C/minute up to 160 °C (zero minute), 4 °C/minute up to 195 °C (12 minute), and 10 °C/minute up to the final temperature of 230 °C (25 minute); injector and detector temperatures 280 °C. The equipment used hydrogen as carrier gas at a flow rate of 1 mL/minute and nitrogen as the make-up gas at 30 mL/minute. Retention times of FAME standards were used to identify chromatographic peaks of the samples. The fatty acid contents were calculated using the nonadecanoic methyl ester as the internal standard.

Cholesterol and cholesterol oxides were determined simultaneously (SALDANHA et al., 2006) and extracted as follows: in a flask with screw cap (70 mL, previously dried with N₂ to prevent the formation of artifacts), a sample weighing 2 g was treated with a 4 mL of a 50% aqueous solution of KOH plus 6 mL of ethanol to perform saponification at room temperature for 22 hours in the dark. For the extraction of the unsaponifiable matter, 5 mL of distilled water and 10 mL of hexane were added to the samples, which were then shaken and the hexane fraction was separated. The extraction with 10 mL of hexane was repeated three times (total of 4 extractions). Subsequently, the solution was dried in a rotary evaporator (Tecnalise, Sao Paulo, SP, Brazil). The residue was dissolved in 5 mL of hexane, transferred to a screw top flask, dried under N₂, diluted with 1 mL of mobile phase, filtered through a 22 μ m filter (Millipore, Maryland, MD, USA), and injected into the HPLC system (SALDANHA et al., 2006).

HPLC-UV-IR analysis was carried out on a Shimadzu liquid chromatograph (Kyoto, Japan) equipped with on-line UV-visible (SPD-10 AV_{vp}) and refractive index (RID-10AV_{vp}) detectors, rheodyne injector with a 20 μ L loop, a tertiary solvent delivery system (LC-10_{vp}), oven heated column at 32 °C (CTO-10_{vp}), and a computer software (CLASS LC-10). The chromatography separation was achieved on a 4 μ m Nova Pack CN HP (300 × 3.9 mm, Waters, Milford, MA, USA) normal-phase column, preceded by a 4 μ m Hypersil BDS CN (7.5 × 4.6 mm, Alltech, Deerfield, IL, USA) guard column according to procedures and conditions previously reported (SALDANHA et al., 2006). The mobile-phase was *n*-hexane:2-propanol (97:3, v/v) at a flow rate of 1 mL/minute and an analysis time of 30 minutes.

The quantification was done by external standardization with a concentration range from 0.3 to 70 μ g.mL⁻¹ for the oxides and from 0.2 to 1.8 mg.mL⁻¹ for the cholesterol. In order to confirm the identity of cholesterol and cholesterol oxides in the sardine samples, these samples were analyzed using HPLC with atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS). The mass spectrometer used was a Qtrap (Applied Biosystems, Concord, Ontario, Canada) with a QqQ

(linear ion trap) configuration (SALDANHA et al., 2006). The cholesterol and epimeric 5,6 epoxides were identified using a refractive index detector for two reasons: better resolution of cholesterol and because the epoxides do not absorb at UV wavelengths. The other oxides were quantified using an UV detector at 210 nm. The detection limits (signal-to-noise ratio of 3) were 0.04 $\mu\text{g}\cdot\text{g}^{-1}$ for 19-OH and 20 α -OH; 0.06 $\mu\text{g}\cdot\text{g}^{-1}$ for 22(R)-OH, 24(S)-OH, 25(R)-OH and 25-OH; 0.07 $\mu\text{g}\cdot\text{g}^{-1}$ for 22(S)-OH, 7 β -OH and 7 α -OH; 0.01 $\mu\text{g}\cdot\text{g}^{-1}$ for 7-keto and cholesterol; and 0.18 $\mu\text{g}\cdot\text{g}^{-1}$ for epimeric 5,6 epoxides. The recovery varied between 95 to 103% for all cholesterol oxides.

2.4 Statistical analysis

All data were subjected to analysis of variance (ANOVA). The comparison of the means between raw and grilled fish was carried out by Tukey's multiple range tests with $P < 0.02$. Statistical analysis was performed using the Origin 5.0 for Windows.

3 Results and discussion

3.1 Moisture, fat, and cholesterol contents

Data of moisture, lipids, and cholesterol for the different fishes obtained in this study are shown in Table 1.

The moisture contents of the raw fishes were found to be 81.3 ± 2.0 in sardine and $79.4 \pm 0.2 \text{ g}\cdot 100 \text{ g}^{-1}$ in hake. Similar levels were found in the grilled fishes: 72.1 ± 1.0 in sardine and $71.8 \pm 0.5 \text{ g}\cdot 100 \text{ g}^{-1}$ in hake samples. The results revealed that the total lipids varied between 7.7 ± 0.7 and $9.2 \pm 0.2 \text{ g}\cdot 100 \text{ g}^{-1}$ (dry weight basis) for both raw and grilled fishes. After grilling, significant differences ($p < 0.02$) were observed only in hake. The values obtained in the present study were similar to other studies of the same authors (SALDANHA; BRAGAGNOLO, 2007, 2008; SALDANHA; BENASSI; BRAGAGNOLO, 2008) and to the study of Méndez and González (1997), but they were lower than the observed by Luzia et al. (2003). Nowadays it is known that the lipid components of fish can vary according to the month of capture, the season, and the particular area of the sea. The mean values of cholesterol content were 214 ± 2 and $327 \pm 3 \text{ mg}\cdot 100 \text{ g}^{-1}$ (dry weight basis) in the raw and grilled fishes. Higher values were determined in sardine samples. Grilling affected the cholesterol by causing a significant decrease in the evaluated fishes, similar to that found in other studies (MÉNDEZ; GONZÁLEZ, 1997; OHSHIMA et al., 1996; OHSHIMA, 2002,

Table 1. Moisture ($\text{g}\cdot 100 \text{ g}^{-1}$), total lipid ($\text{g}\cdot 100 \text{ g}^{-1}$ dry basis) and cholesterol ($\text{mg}\cdot 100 \text{ g}^{-1}$ dry basis) levels in raw and grilled sardine and hake samples.

	Sardine		Hake	
	Raw	Grilled	Raw	Grilled
Moisture	81.30 ± 2.0^A	72.10 ± 1^B	79.40 ± 0.2^a	71.80 ± 0.5^b
Total lipids	8.50 ± 0.6^A	8.00 ± 0.7^A	9.15 ± 0.2^a	7.70 ± 0.7^b
Cholesterol	327.00 ± 2.7^A	253.00 ± 2.0^B	249.30 ± 0.9^a	214.00 ± 2.2^b

^{A,B} Mean and relative standard deviation (RSD) of the four analyses in sardine (two batches in duplicate); ^{a,b} Mean and relative standard deviation (RSD) of the four analyses in hake (two batches in duplicate); and means in the same letter do not differ significantly ($p > 0.02$).

OSADA et al., 1993; SALDANHA; BRAGAGNOLO, 2007, 2008; SALDANHA; BENASSI; BRAGAGNOLO, 2008).

3.2 Fatty acid composition

Table 2 shows the fatty acid contents in raw and grilled sardine and hake samples reported as $\text{g}\cdot 100 \text{ g}^{-1}$ of the oil, as well as the total amount of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA).

Sardines and hake had similar fatty acid patterns. The main fatty acids found in all of the analyzed fishes were docosahexaenoic (DHA, 22:6n3), oleic (18:1), palmitic (16:0), and eicosapentaenoic (EPA, 20:5n3). The fatty acids profile was quite similar to the data reported by other authors in sardine (CANDELA; ASTIASÁRAN; BELLO, 1997, 1998; GARCÍA-ARIAS et al., 2003; SALDANHA; BENASSI; BRAGAGNOLO, 2008), and hake samples (CANDELA; ASTIASÁRAN; BELLO, 1997; MÉNDEZ; GONZÁLEZ, 1997; SALDANHA; BRAGAGNOLO, 2007, 2008).

In the raw samples the SFA, MUFA, and PUFA accounted for approximately 16, 21, and 38 $\text{g}\cdot 100 \text{ g}^{-1}$ in sardine, respectively, and 23, 29, and 30 $\text{g}\cdot 100 \text{ g}^{-1}$ in hake samples, respectively. The DHA and EPA were the predominant PUFA accounting for 16.7-18 and 6.4-11.4 $\text{g}\cdot 100 \text{ g}^{-1}$, respectively, of the total FA for the two fish species.

The amount of $\omega 3$ polyunsaturated fatty acids in the raw sardines ($30.18 \pm 0.2 \text{ g}\cdot 100 \text{ g}^{-1}$) and hake ($25.42 \pm 0.3 \text{ g}\cdot 100 \text{ g}^{-1}$) were much higher than $\omega 6$ (7.97 ± 0.2 in sardine and $4.70 \pm 0.1 \text{ g}\cdot 100 \text{ g}^{-1}$ in hake). As referred in previous research reports, marine fishes contain higher amounts of $\omega 3$ than $\omega 6$ fatty acids, fact that has been connected, among other factors, to the type of their diet, which varies in different marine regions (LAVANIEGOS; LOPEZ-CORTES, 1997).

Significant differences ($p < 0.02$) in the total amount of SFA, MUFA, and PUFA were observed among the raw and grilled fishes. Grilling the fishes resulted in a decrease in the total amount of fatty acids in the order of 14.6% in sardine and 15.2% in hake. The content of EPA and DHA after grilling was significantly reduced in the range of 16.2 to 31.8% in EPA and 21.6 to 28.3 % in DHA. Unsaturated fatty acids are far more heat-labile since their instability increases with the degree of unsaturation. In the presence of oxygen, PUFA degradation occurs more readily and undergoes pronounced oxidative effects (LITTLE; ARMSTRONG; BERGAN, 2000). PUFA contents in aquatic species were demonstrated to decrease after heat treatment (CANDELA; ASTIASÁRAN; BELLO, 1997; OHSHIMA et al., 1996; SALDANHA; BRAGAGNOLO, 2007, 2008; SALDANHA; BENASSI; BRAGAGNOLO, 2008).

Possible mechanisms for the changes occurring in the heat treatment are the leaching of fat-soluble molecules out of the food and oxidation reactions (LITTLE; ARMSTRONG; BERGAN, 2000). These mechanisms are also responsible for the changes in the fat and cholesterol.

Although many authors estimate the potential nutritive value of fishes on the basis of per cent content of PUFA, it would be better to draw such conclusions using mass units,

Table 2. Fatty acids composition (g.100 g⁻¹ of oil) in raw and grilled sardine and hake samples.

Fatty acids	Sardine		Hake	
	Raw	Grilled	Raw	Grilled
Lauric 12:0	-	-	0.69 ± 0.01 ^a	0.50 ± 0.02 ^b
Myristic 14:0	3.08 ± 0.8 ^A	2.94 ± 0.1 ^A	4.10 ± 0.2 ^a	3.66 ± 0.2 ^b
Pentadecylic 15:0	0.40 ± 0.01 ^A	0.35 ± 0.03 ^A	0.91 ± 0.03 ^a	0.74 ± 0.1 ^b
Palmitic 16:0	9.19 ± 0.1 ^A	8.86 ± 0.2 ^B	13.57 ± 0.4 ^a	13.30 ± 0.4 ^a
Margaric 17:0	0.43 ± 0.02 ^A	0.34 ± 0.01 ^A	0.27 ± 0.05 ^a	0.20 ± 0.03 ^a
Stearic 18:0	1.89 ± 0.05 ^A	1.61 ± 0.3 ^A	1.91 ± 0.6 ^a	1.61 ± 0.2 ^a
Arachidic 20:0	0.59 ± 0.01 ^A	0.55 ± 0.02 ^A	0.63 ± 0.02 ^a	0.52 ± 0.03 ^a
Behenic 22:0	0.31 ± 0.04 ^A	0.26 ± 0.03 ^A	0.69 ± 0.05 ^a	0.58 ± 0.01 ^a
Lignoceric 24:0	0.19 ± 0.03 ^A	0.14 ± 0.03 ^A	0.66 ± 0.08 ^a	0.55 ± 0.02 ^a
∑ SFA	16.08 ± 0.1 ^A	15.05 ± 0.09 ^B	23.43 ± 0.2 ^a	21.66 ± 0.09 ^b
Myristoleic 14:1 ω9	0.38 ± 0.02 ^A	0.31 ± 0.05 ^A	0.41 ± 0.01 ^a	0.34 ± 0.02 ^a
Palmitoleic 16:1 ω7	5.58 ± 0.3 ^A	4.16 ± 0.5 ^B	8.84 ± 0.8 ^a	7.65 ± 0.6 ^b
Margaroleic 17:1 ω7	0.33 ± 0.01 ^A	0.25 ± 0.01 ^A	2.22 ± 0.4 ^a	1.93 ± 0.1 ^a
Oleic 18:1 ω9	13.40 ± 0.8 ^A	12.00 ± 0.6 ^B	15.78 ± 1 ^a	14.59 ± 1 ^b
Gadoleic 20:1 ω11	0.19 ± 0.01 ^A	0.14 ± 0.02 ^A	0.59 ± 0.02 ^a	0.50 ± 0.03 ^a
Erucic 22:1 ω9	0.48 ± 0.02 ^A	0.36 ± 0.02 ^A	0.42 ± 0.01 ^a	0.30 ± 0.05 ^b
Nervonic 24:1 ω6	0.76 ± 0.03 ^A	0.62 ± 0.03 ^B	0.67 ± 0.02 ^a	0.53 ± 0.02 ^b
∑ MUFA	21.12 ± 0.2 ^A	17.84 ± 0.2 ^B	28.93 ± 0.3 ^a	25.84 ± 0.3 ^b
Linoleic 18:2 ω6	0.83 ± 0.06 ^A	0.69 ± 0.01 ^B	0.63 ± 0.00 ^a	0.54 ± 0.00 ^a
α-Linolenic 18:3 ω3	0.91 ± 0.05 ^A	0.87 ± 0.00 ^A	0.29 ± 0.00 ^a	0.22 ± 0.00 ^a
γ-linolenic 18:3 ω6	3.80 ± 0.5 ^A	3.40 ± 0.1 ^A	1.50 ± 0.3 ^a	1.31 ± 0.1 ^a
Eicosatrienoic 20:3 ω3	1.12 ± 0.2 ^A	0.92 ± 0.02 ^A	-	-
Arachidonic 20:4 ω6	3.34 ± 0.2 ^A	2.39 ± 0.2 ^B	2.57 ± 0.05 ^a	2.31 ± 0.01 ^a
EPA 20:5 ω3	11.42 ± 0.5 ^A	9.57 ± 0.3 ^B	6.42 ± 0.3 ^a	4.38 ± 0.3 ^b
DHA 22:6 ω3	16.73 ± 0.3 ^A	13.12 ± 0.2 ^B	18.71 ± 0.8 ^a	13.42 ± 0.7 ^b
∑ PUFA	38.15 ± 0.2 ^A	30.96 ± 0.1 ^B	30.12 ± 0.2 ^a	22.18 ± 0.2 ^b
Elaidic 18:1 <i>tr</i> ω9	0.39 ± 0.01 ^A	0.62 ± 0.03 ^B	0.25 ± 0.00 ^a	0.32 ± 0.00 ^a
Linolelaidic 18:2 <i>tr</i> ω6	0.72 ± 0.01 ^A	0.84 ± 0.01 ^A	0.41 ± 0.02 ^a	0.53 ± 0.01 ^a
∑ trans	1.11 ± 0.01 ^A	1.46 ± 0.02 ^B	0.66 ± 0.01 ^b	0.85 ± 0.00 ^a
∑ FA	76.46 ± 0.1 ^A	65.31 ± 0.1 ^B	83.14 ± 0.2 ^a	74.03 ± 0.1 ^b
∑ ω3	30.18 ± 0.2 ^A	24.48 ± 0.1 ^B	25.42 ± 0.3 ^a	18.02 ± 0.3 ^b
∑ ω6	7.97 ± 0.2 ^A	6.48 ± 0.08 ^B	4.70 ± 0.1 ^a	4.16 ± 0.00 ^b
ω3/ω6	3.78 ± 0.2 ^A	3.77 ± 0.09 ^A	5.40 ± 0.2 ^a	4.33 ± 0.1 ^b
EPA+DHA	28.15 ± 0.4 ^A	22.69 ± 0.3 ^B	25.13 ± 0.3 ^a	17.8 ± 0.3 ^b
PUFA/SFA	2.37 ± 0.1 ^A	2.05 ± 0.1 ^B	1.28 ± 0.2 ^a	1.02 ± 0.1 ^b

^{A,B}Mean and relative standard deviation (RSD) of the four analyses in sardine (two batches in duplicate); ^{a,b}Mean and relative standard deviation (RSD) of the four analyses in hake (two batches in duplicate); and means in the same letter do not differ significantly ($p > 0.02$).

e.g., quantity of EPA+DHA content in a fish dish, or, in the other words, the quantity of fish dish to be consumed by a individual to obtain the daily quantity of the two essential PUFA recommended by WHO (GLADYSHEV et al., 2007), which is 1 g per day. The grilled fishes contained between 0.5 and 0.39 g EPA+DHA .100 g⁻¹ product. Thus, our results indicate that consuming approximately 180 g of sardine or 260 g hake

daily would be sufficient to obtain the recommended amount of EPA+DHA.

The ω3/ω6 ratio in the raw and grilled fishes varied from 3.77 and 5.40, and according to current WHO recommendations, the daily ratio ω3/ω6 in total human diet should be no higher than 0.2 (VUJKOVIC et al., 1999). The values found in the present study are higher than the recommended values, but it

should be taken into account that in most part of other foods the $\omega 3/\omega 6$ ratio is lower than necessary.

3.3 Cholesterol oxides

The cholesterol oxide contents, calculated on a dry weight basis are shown in Table 3. Two oxides, 7-keto and 19-OH were originated from the main chain and four, 24(S)-OH, 22(S)-OH, 25(R)-OH, and 25-OH, from the side chain. Similar results were reported in other studies by the same authors (SALDANHA; BRAGAGNOLO, 2007, 2008; SALDANHA; BENASSI; BRAGAGNOLO, 2008).

Nevertheless, in many previous studies (OHSHIMA et al., 1996; OHSHIMA, 2002; TAI; CHEN; CHEN, 2000) it was reported that 7-keto, 7 β -OH, 7 α -OH, and the epimeric epoxides were predominant in the total of COP in fish and 25-OH was the only side chain COP found in seafood products (OHSHIMA, 2002). Even so, under the chromatographic conditions used in the present study, it was possible to separate and to identify a large number of oxides originated from the oxidation of lateral chain, which are normally not determined in food products.

The raw sardine samples showed higher total oxide amounts ($20.62 \pm 0.4 \mu\text{g}\cdot\text{g}^{-1}$) than those of the hake samples ($8.79 \pm 1.3 \mu\text{g}\cdot\text{g}^{-1}$), and the amounts of COP found in the different analysed samples were probably correlated with a remarkable differences in the chemical composition, mainly cholesterol and total of PUFA contents among the fish species. This difference depends on several factors including the region and the season of fishery and sex and age of the fish (LUZIA et al., 2003).

19-hydroxycholesterol was the main contributor of COP in the raw sardine samples, being the most abundant fraction in these fish ($15.18 \pm 1.2 \mu\text{g}\cdot\text{g}^{-1}$). In the raw hake samples, higher content of 25-OH ($4.49 \pm 0.2 \mu\text{g}\cdot\text{g}^{-1}$) followed by 19-hydroxycholesterol ($2.16 \pm 0.1 \mu\text{g}\cdot\text{g}^{-1}$) was observed. The origin of the 19-OH in food systems has not been elucidated although in many studies this oxide was found in mutton meat (KOWALE et al., 1996), buffalo meat (RAO et al., 1996) and fish samples (SALDANHA; BRAGAGNOLO, 2007; SALDANHA; BRAGAGNOLO, 2008; SALDANHA; BENASSI;

BRAGAGNOLO, 2008). Either the metabolic production in fish or the proximity of position 19 with double bond between C_5 and C_6 making the C_{19} more susceptible to the attack of the peroxy radicals could be considered two likely ways for the formation of this oxide.

The formation of cholesterol oxidation products increased significantly ($p < 0.02$) after grilling for all samples evaluated. The increase of total oxides was about 110 and 120% for sardine and hake, respectively. The most affected oxides by the heat treatment were the 25-OH and 24(S)-OH in both samples. Normally, in food systems the major oxide found is 7-keto, which is also considered an indicative of oxidation. However, in this study, 7-keto was not observed in the fresh hake, only in the grilled samples. Heat oxidation in cholesterol rich food systems is a dynamic reaction (KIM; NAVAR, 1991; NAVAR et al., 1991), which depends on the amount of cholesterol present, the type and severity of the heat treatment, and the presence of free radicals in the sample. Several studies on the generation of COP during heat treatment of fish have been published (AL-SAGHIR et al., 2004; OHSHIMA, 2002; SAMPAIO et al., 2006), and many COP formations through grilling have been reported (OHSHIMA et al., 1996; SALDANHA; BRAGAGNOLO, 2007, 2008; SALDANHA; BENASSI; BRAGAGNOLO, 2008; SHOZEN et al., 1995). The nature, proportion, and degree of unsaturation of fatty acids present in fish product systems will indicate the approximate susceptibility of those products to oxidative deterioration. According to Al-Saghir et al., (2004) the higher degree of unsaturation, the higher the lipid oxidative process, and the more COP are formed. The COPs are formed in the majority of processed foods containing cholesterol, and heating is one of the major causes of their synthesis (TAI; CHEN; CHEN, 2000). Considering the amounts of PUFA found in the fresh fishes studied and the temperature of grilling, the increase of oxides formation after thermal treatment can be justified.

4 Conclusion

It is evident from a consideration of the results obtained in this study that the oxidation of cholesterol was stimulated by the presence of PUFA and that grilling favored the COP formation. The samples of sardine and hake showed great polyunsaturated fatty acids levels, and these results indicate that consuming approximately 180 g of sardine or 260 g hake daily is sufficient to obtain the recommended amount of EPA+DHA. However, higher amounts of COP were formed. In addition, a more systematic analysis of heated fishes is necessary since there is a trend in the consumption of fish due the positive effects of its long chain PUFA regarding cardiovascular diseases. The physiological implication of ingesting COP also deserves further studies.

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Table 3. Cholesterol oxides levels ($\mu\text{g}\cdot\text{g}^{-1}$, dry basis) in raw and grilled sardine and hake samples.

COPs	Sardine		Hake	
	Raw	Grilled	Raw	Grilled
19-OH	15.18 ± 1.2^B	27.69 ± 1.3^A	2.16 ± 0.1^b	3.84 ± 0.3^a
24(S)-OH	1.24 ± 0.2^B	3.45 ± 0.6^A	0.37 ± 0.03^b	1.19 ± 0.1^a
22(S)-OH	1.37 ± 0.3^B	2.29 ± 0.6^A	0.96 ± 0.05^b	1.53 ± 0.7^a
25-OH	1.17 ± 0.2^B	6.48 ± 0.1^A	4.49 ± 0.2^b	10.84 ± 1.0^a
25(R)-OH	0.35 ± 0.04^B	0.69 ± 0.01^A	0.81 ± 0.01^b	1.29 ± 0.3^a
7-keto	1.31 ± 0.6^B	2.68 ± 0.3^A	-	0.66 ± 0.1^a
Total	20.62 ± 0.4^B	43.28 ± 0.4^A	8.79 ± 0.1^b	19.35 ± 0.4^a

^{A,B}Mean and relative standard deviation (RSD) of the four analyses in sardine (two batches in duplicate); ^{a,b}Mean and relative standard deviation (RSD) of the four analyses in hake (two batches in duplicate); means in the same letter do not differ significantly ($p > 0.02$).

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