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Evaluation of Sensory and *in Vitro* Cardio Protective Properties of Sardine (*Sardina pilchardus*): The Effect of Grilling and Brining*

Constantina Nasopoulou[1#], Evangelia Psani[1], Eleni Sioriki[1], Constantinos A. Demopoulos[2], Ioannis Zabetakis[1]

[1]Laboratory of Food Chemistry, Department of Chemistry, School of Sciences, National and Kapodistrian University of Athens, Athens, Greece; [2]Laboratory of Biochemistry, Department of Chemistry, School of Sciences, National and Kapodistrian University of Athens, Athens, Greece.
Email: #cnasopoulou@chem.uoa.gr, evapsa8@gmail.com, esioriki@gmail.com, demopoulos@chem.uoa.gr, izabet@chem.uoa.gr

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ABSTRACT

The aim of this study was to investigate the effect of grilling and brining on the sensory properties, the fillet fatty acid composition and the cardio-protective activity of sardine (*Sardina pilchardus*), studying the *in vitro* activity against Platelet-Activating-Factor (PAF) induced platelet aggregation. Sensory evaluation of grilled and brined sardine showed that grilled sardine had higher scores for the attributes: grilled fish, marine and fresh fish whereas brined sardine had higher scores for the attributes: salty, iodine, oily and bitter. Grilled sardine exhibited significantly increased fillet fatty acid content while the brined fish sample significantly decreased fatty acid levels. Polar lipids of all specimens (raw, grilled and brined) showed strong inhibitory activity against PAF action indicating that grilling and brining have not diminished the cardio-protective properties of sardine.

Keywords: *Sardina pilchardus*; Cardio-Protection; Sensory Evaluation; Grilling; Brining

1. Introduction

The benefits of a diet rich in fish on human health have been linked to ω-3 fatty acids' content, especially eicosapentaenoic acid (EPA; 20:5ω-3) and docosahexaenoic acid (DHA; 22:6ω-3) [1]. These fatty acids are reputed to have beneficial properties against cardiovascular diseases (CVDs) [2] by lowering blood pressure and serum triglycerides and improving endothelial function and plaque stability [3]. Apart from these ω-3 fatty acids it has been reported the presence of polar lipid compounds in raw and fried fish that posses *in vitro* antithrombotic properties [4] and *in vivo* anti-atherogenic activity [5].

Atherosclerosis is a chronic inflammatory condition with clinical end points of heart attack and stroke. PlateletActivating-Factor (PAF) [6] is a potent inflammatory phospholipid mediator, implicated in the mechanism of atherogenesis [7]. The protective role, against CVDs, of compounds that act as PAF agonists/inhibitors has been reported [8,9].

Fish is rarely eaten raw but is usually cooked in different ways or brined before consumption. During cooking, chemical and physical reactions take place that improve or impair the food nutritional value (e.g. digestibility is increased due to protein denaturation in food but the content of thermolabile compounds, fat-soluble vitamins or polyunsaturated fatty acids is often reduced) [10]. Cooking induces water loss in the food, that in turn increases its lipid content in most cases and only some fat is lost in the case of the fish richer in fat content.

The aim of the current study was to investigate the effect of grilling and brining of sardine (*Sardina pilchardus*) on the sensory properties, the fish fillet fatty acid composition and the *in vitro* biological activities of fish polar lipids to induce platelet aggregation or/and inhibit the PAF-induced platelet aggregation.

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#Corresponding author.

2. Materials and Methods

2.1. Reagents and Instruments

All reagents and solvents were of analytical grade purchased from Merck (Darmstadt, Germany). Fatty acid methyl ester standards bought individually, were of GC-quality and supplied by Sigma-Aldrich (St. Louis, MO, USA), as well as bovine serum albumin (BSA) and PAF.

Chromatographic material used for Thin Layer Chroma-tography (TLC) was silica gel G-60 supplied be Merck (Darmstadt, Germany) and polar lipid standards used for TLC was a mix standard of hen egg yolk supplied by Sigma-Aldrich (St. Louis, MO, USA). Platelet aggregation was measured in a Chrono-Log (Havertown, PA, USA) aggregometer (model 400-VS) coupled to a Chrono-Log recorder (Havertown, PA, USA).

2.2. Fish Sampling

Five kilograms (5 Kg) (one batch) of raw Greek sardines (*Sardina pilchardus*) were purchased from a local market and transported to the laboratory in ice. Individual weight fish was 21 ± 3.0 g. Raw fish were washed and filleted after fish head, scales, viscera, backbone, skin and tail were removed.

300 g of raw fish fillets-of the original sample of 5 kg were pooled together and that was the raw fish sample. Similarly 300 g of raw fish fillets of the original sample of 5 kg were pooled together and grilled-according to the following grilling procedure-and that was the grilled fish sample, while 300 g of raw fish fillets of the original sample of 5 kg were pooled together and immersed in brine solution according to the following brining procedure and that was the brined fish sample.

2.3. Grilling Procedure

Grilled fish fillets were prepared in a Black and Decker contact griller with its thermostat set at 217˚C. After the set temperature was attained the fish fillets were grilled for three minutes on each side.

2.4. Brining Procedure

Brine concentration was 26.5% (w/w) sodium chloride in distilled water. Brining performed at 20˚C for 3 h. This temperature is usually used in traditional salting of fish and the period of 3 h was used for salting at 20˚C to avoid fillet spoilage.

Raw fish fillets were covered by aluminum paper and were placed vertically-free lower edge surface in contact with brining solution-in order to allow unidirectional migrations of salt and moisture within the fillets. During the first two hours, the brine concentration was adjusted through renewing the brine solution each 30 min and then each hour in order to limit the external resistance to mass transfer [11].

2.5. Isolation of Fish Total Lipids, Total Polar and Total Neutral Lipids

Total lipids (TL) of raw, grilled and brined fish samples were extracted according to the Bligh-Dyer method [12] to obtain the aforementioned lipid fraction of each fish sample (raw, grilled or brined). For each extraction, a sample of 50 g of fish fillets was obtained by combining seven different fish fillets from each fish sample (raw, grilled or brined) and this sampling procedure was carried out in triplicates. One tenth of the TL was stored in sealed vials at −20˚C until Gas Chromatographic analysis. The rest was further separated into total polar lipids (TPL) and total neutral lipids (TNL) using the counter-current distribution method [13]. The TPL fractions (containing glycolipids and phospholipids) were assayed for PAF activities (*i.e.* for their ability to either induce washed platelet aggregation or inhibit the PAF-induced platelet aggregation).

2.6. Gas Chromatography Analysis

Fatty acid methyl esters of TL of raw, grilled and brined fish fillets were prepared using a solution 0.5N KOH in CH_3OH 90% and extracted with n-hexane. The fatty acid analysis was carried out using the internal standard method as described extensively by Nasopoulou [14]. The gas chromatographer used was a Shimadzu CLASS-VP (GC-17A) (Kyoto, Japan) equipped with a split/splitless injector and flame ionisation detector.

Separation of fatty acid methyl esters was achieved on an Agilent J&W DB-23 fused silica capillary column (60 mx 0.251 mm i.d., 0.25 μm; Agilent, Santa Clara California, USA). The oven temperature program was: 120˚C for 5 min, raised to 180˚C at 10˚C·min^{-1}, then to 220˚C at 20˚C·min^{-1} and finally isothermal at 220˚C for 30 min. The injector and detector temperatures were maintained at 220˚C and 225˚C, respectively. The carrier gas was high purity helium with a linear flow rate of 1 ml·min^{-1} and split ratio 1:50. Fatty acid methyl esters were identified using fatty acid methyl esters standards by matching retention times of the relative peaks.

2.7. Fractionation of TPL by TLC

The fractionation of TPL took place by TLC as described extensively by Nasopoulou [15]. Approximately 60.0 mg of TPL of each sample were applied to the TLC plates. A developing system consisting of chloroform:methanol: water 65:35:6 (v/v/v) was utilized for the separation of TPL. The obtained TPL were weighed, redissolved in 1

ml chloroform:methanol 1:1 (v/v) and stored at −20˚C.

2.8. Biological Assay

TPL and purified polar lipid fractions of raw, grilled and brined sardine obtained by the above TLC separations were tested for their biological activity according to the washed rabbit platelet aggregation assay [6]. 0.2 ml of each TPL sample corresponding to 7.12 mg TPL of raw sardine, 61.2 mg TPL of grilled sardine and 29.5 mg TPL brined sardine were redissolved in a final volume of 200 μL BSA (2.5 mg of bovine serum albumin, BSA, mL^{-1} of saline). The three solutions were further diluted in a volume of BSA by a factor of 10 and 4 μL of each diluted solution used in the biological assays. The amounts of TPL of raw, grilled and brined sardine that were present in the 4 μL aliquots were 1.42 μg, 12.3 μg and 5.89 μg, respectively. In a further experiment, approximately 60.0 mg of TPL of raw, grilled and brined sardine were fractionated by TLC and each TLC polar lipid fraction obtained was dissolved in 200 μL BSA; these aliquots were biologically assayed and the quantity (μg) of the each TLC polar lipid fraction used in the biological assay is given.

The aggregatory biological activity of each lipid fraction to induce platelet aggregation was expressed as height (cm) of the aggregation curve. The inhibitory activity was expressed as % inhibition of platelet aggregation caused by PAF at a final concentration of 10^{-9} M.

2.9. Sensory Evaluation

An untrained panel of 10 assessors (six females, four males), aged 21 - 42 years, was recruited from the University of Athens. They were subjected to training in order to be able to identify taste, aftertaste, odour and give scores for each attribute. The tests for training and monitoring the assessors as well as for the right selection were duo-trio [16], triangle [17] and ranking tests [18]. Solutions for identification of the basic senses of taste-sweet, salty, bitter and sour-were used. Concentrations of the solutions were varied in proportion to the threshold of each ingredient [19]. Five products, enclosed in dark bottles (seaweed, shells, sea salt, fish oil and tincture of iodine) were used for training the subjects to different odours. The basic training programme was carried out during 10 sessions of 45 min each. During the seven succeeded sessions the lexicon was created, using four samples of grilled and brined sardines different from the samples that were used for the purpose of the experiment. These samples were used as a frame of reference in order to develop terminology based on the attribute differences. The final list was determined so that all sensory descriptors were comprehensive and understood by all assessors. This list comprised of seventeen attributes (**Table 1)**. During the final step of training (five sessions), panelists practiced the use of descriptors, using four samples of grilled and brined sardines different from the samples that were used for the purpose of the experiment, as explained above.

The fish fillets were grilled just before the sensory analysis for three minutes on each side in a Black and Decker contact griller as described above. Fish brining performed as described above and the excess of brine was washed off before evaluation. Portions of approximately 30 g of grilled and brined fish samples were served to each panelist on plastic plates. Grilled and brined sardine samples were sequential monadic, random allocated, coded with three-digit random numbers [19] and assessed twice over a 2 week period in different orders, balanced for order presentation in the session and for interference effect of the previous sample [20]. Water and plain biscuits were supplied to all panelists to wash

Table 1. Attributes (taste, aftertaste, odour) and their descriptors[a] used during sensory analysis for testing of the grilled and brined sardine samples based on the lexicon developed by assessors; p values produced from ANOVA analysis, represent the variance of the assessors' responses for each attribute given specific descriptors.

Attributes	Descriptors	p *values*
Sweet (taste)	Sugar	3.29E−02
Salty (taste)	Sodium ions	4.93E−05
Astringent (taste)	Quince	9.57E−04
Fatty (taste)	Honey	2.24E−03
Rich (taste)	Full Mouthfeel	6.96E−02
Marine (taste)	Fish/shellfish/mussels	7.19E−01
Fresh fish (taste)	Fresh grilled fish	1.25E−05
Sweet (aftertaste)	Sugar	5.32E−03
Bitter (aftertaste)	Caffeine and similar bitter substances	8.56E−02
Oily (aftertaste)	Olive oil	1.24E−04
Lasting (aftertaste)	Garlic or hot pepper	4.35E−03
Iodine (aftertaste)	Tincture of iodine	2.39E−01
Fresh fish (odour)	Fresh fish	6.87E−03
Sea complex (odour)	Shells	3.97E−05
Grilledfish (odour)	Grilled fish	9.12E−04
Fish oil (odour)	Cod liver oil	4.18E−02
Marine (odour)	Seaweed	3.05E−01

[a]Each descriptor gives information about the standard compound (or the implied characteristic) used in the training of the assessors.

out their mouth during assessment of samples. The assessors marked each sample of grilled and brined fish for all the seventeen attributes on a paper based continuous scale from 0 (lowest) to 10 (highest). All attributes were scored at ambient temperature and the rooms had natural light.

2.10. Statistical Analysis

All experimental analysis was carried out in triplicates and all results were expressed as mean value ± SD. One-way analysis of variance (ANOVA) was used in order to find the significant differences between raw and grilled samples.

Differences were considered to be statistically significant when p value was less than 0.05. The data were analysed using a statistical software package (PASW 18 for Windows, SPSS Inc., Chicago, IL, 17 USA).

3. Results and Discussion

3.1. TL, TPL and TNL Content of Raw, Grilled and Brined Sardine Samples

TL of the three fish samples (*i.e.* raw, grilled and brined) were extracted and separated into TNL and TPL. The obtained amounts of TL, TPL and TNL expressed as g (100 g fish tissue)$^{-1}$ are given in **Table 2**. Grilled and brined sardine samples exhibited statistically elevated TL levels in comparison to raw sardine TL levels (**Table 2**), data that is in accordance with the literature [21,22]. This is probably due to the fish tissue water loss during cooking [21] and brining [11] which leads to increased lipid content. The statistically increased TL content of grilled and brined sardine was mainly attributed to the increased TPL content.

3.2. Fatty Acid Content of Raw, Brined and Grilled Fish Fillet Samples

Raw sardine fillet showed high contents of palmitic (16:0), steatic (18:0), eicosapentaenoic (EPA; 20:5ω-3) and docosahexanoic acid (DHA; 22:6ω-3) (**Table 3**).

Table 2. TL expressed as g (100 g fish tissue)$^{-1}$ [mean ± SD (n = 3)] and percentages of TPL and TNL content in raw, grilled and brined sardine (*Sardina pilchardus*) fillets.

Fish sample	TL (g/100 g fish fillets)	TPL (% TL)	TNL (% TL)
Raw sardine	0.75 ± 0.04^{a}	52.1	47.9
Grilled sardine	2.62 ± 0.13^{b}	84.9	15.1
Brined sardine	2.74 ± 0.14^{b}	58.4	41.6

^{a,b}indicates significantly different values ($p < 0.05$) between TL content of raw and grilled and of raw and brined fish samples, according to Anova analysis.

Table 3. Fatty acid profile of raw, grilled and brined sardine (*Sardina pilchardus*) fillets TL, expressed as mg (kg fish tissue)$^{-1}$ [mean + SD (n = 3), p < 0.05].

Fatty acids	Raw sardine	Grilled sardine	Brined sardine
14:0	16.6 ± 1.01^{a,b}	23.5 ± 0.20^{a}	45.9 ± 9.82^{b}
16:0	446 ± 6.38^{a,b}	478 ± 2.52^{a}	360 ± 6.19^{b}
16:1 (ω-7)	21.2 ± 0.42^{a,b}	35.5 ± 0.59^{a}	27.3 ± 4.89^{b}
18:0	110 ± 1.17^{a,b}	131 ± 5.06^{a}	48.4 ± 2.62^{b}
18:1 cis (ω-9)	72.5 ± 1.06^{a}	88.8 ± 4.21^{a}	n.d.
18:1 trans (ω-9)	39.7 ± 0.65^{a,b}	65.3 ± 0.22^{a}	96.7 ± 0.33^{b}
18:2 (ω-6)	n.d.	n.d.	n.d.
18:3 (ω-3)	n.d.	n.d.	n.d.
20:4 (ω-6)	18.1 ± 2.92^{a}	10.4 ± 0.69^{a}	n.d.
20:5 (ω-3)	357 ± 5.53^{a,b}	451 ± 6.99^{a}	62.5 ± 2.95^{b}
22:5 (ω-3)	n.d.	n.d.	n.d.
22:6 (ω-3)	389 ± 39.19^{b}	421 ± 36.1	480 ± 26.5^{b}
Total SFA	573 ± 8.56^{a,b}	632 ± 7.78^{a}	454 ± 18.6^{b}
Total ω-7 FA	21.2 ± 0.42^{a,b}	35.5 ± 0.59^{a}	27.3 ± 4.89^{b}
Total ω-9 FA	112 ± 1.71^{a,b}	154 ± 4.43^{a}	96.7 ± 0.22^{b}
Total MUFA	133 ± 2.13^{a,b}	190 ± 5.02^{a}	124 ± 5.11^{b}
Total ω-3 PUFA	746 ± 44.7^{a,b,†}	872 ± 43.1^{a,†}	543 ± 2.4^{b}
Total ω-6 PUFA	18.1 ± 2.92^{a,†}	10.4 ± 0.69^{a,†}	n.d.
ω-6/ω-3	0.024 ± 0.003^{a}	0.012 ± 0.002^{a}	0

^{a}indicates significantly different values within the same row between raw and grilled fish fillet samples ($p < 0.05$), according to ANOVA analysis. ^{b}indicates significantly different values within the same row between raw and brined samples ($p < 0.05$), according to ANOVA analysis. †Indicates significantly different values within the same column between the same fish samlpe ($p < 0.05$), according to ANOVA analysis. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; n.d.: not detected.

These findings are in agreement with those obtained by other researchers [22,23]. In all sardine samples (raw, brined, grilled) polyunsaturated fatty acids (PUFAs) were the most abundant fatty acid class followed by saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) (**Table 3**). The ω-3 fatty acid content was significant higher than that of ω-6 fatty acids (**Table 3**). These results emphasize the high quality of sardine fillet-fat from a cardio-protective point of view [24].

Grilled fish sample exhibited significant increased PUFAs' levels, whereas brined fish samples significant decreased (**Table 3**). The most abundant ω-6 fatty acid in raw and grilled specimens, was arachidonic acid (ArA; 20:4ω-6) where the most abundant ω-3 fatty acids were

DHA and EPA (**Table 3**). Grilled sardine sample showed significant increased ω-3 fatty acids' content, especially EPA, when compared to raw fish sample, probably as a consequence of the water loss that occurs during grilling (**Table 3**). A similar trend in sardine (*i.e.* increased levels of ω-3 fatty acids during grilling) has been reported [22]. In addition oven-baked sardines (20 min at 200˚C) rich in ω-3 PUFAs, especially EPA and DHA, has also been reported [21]. The same trend has also been observed in eel fillets, where grilled and microwave-cooked eel fillets were found to have the highest levels of EPA [25]. However, in catfish fillets, boiling, baking and grilling were found to marginally affect the fatty acid levels; some fatty acids (20:0; 22:0; 14:1ω-5 and 22:1ω-9) were not detected in raw fillets but they were found at low levels after these heating treatments [26]. Nevertheless grilling decreased the ω-6 content of the fish.

It was found that brining caused a significant decrease in the levels of some fatty acids, especially EPA and ArA, probably due to the effect of salt. Sodium chloride has been shown to catalyse lipid oxidation in muscle tissues, fish being one of them [27]. Chloride ion can be converted to a radical via a mechanism as observed with myeloperoxidase. It could then be added directly to a double bond or abstract a hydrogen [28]. Alternatively, the Na^+ species may replace the iron from a cellular complex via an ion exchange reaction. The displaced iron may then participate in the initiation of lipid peroxidation.

During grilling, statistically significant increases were observed at the levels of total SFAs and total MUFAs of grilled sardine when compared to raw sardine (**Table 3**). On the contrary, brining led to a statistically significant decrease of the levels of SFAs and MUFAs, probably due to the effect of salt. Also grilling and brining affected statistically significant the ratio of ω-6/ω-3. The highest ratio was observed in raw sardine (0.024), followed by grilled sardine (0.012) and brined sardine (0) (**Table 3**). This could be explained as a result of the statistically significant decrease during grilling and brining of the levels of ArA.

3.3. Biological Activity of Sardine's TPL and TLC Polar Lipid Fractions

Some food lipid constituents have been found to exhibit either agonistic (induce platelet aggregation) or inhibitory effect on PAF-induced biological activity. When they act as agonists of PAF they act as PAF (induce platelet aggregation) but they are thousands times less active than PAF thus they are practically acting as PAF-inhibitors, whereas when they exhibit PAF-inhibitory effect they block PAF activity, acting as PAF-inhibitors, as well. PAF agonists (with aggregatory activity) have been found to have better *in vivo* antiatherogenic activity than PAF-inhibitors [5,29]. Therefore, the detection of lipids (TPL or TLC polar lipid fractions) that exhibit aggregatory and/or inhibitory actions is a strong indication that these lipids are biologically active compounds against PAF and consequently against atherogenesis.

With these in mind, a comparison of the biological activities of TPL and TLC polar lipid fractions of all fish samples (raw, grilled and brined) follows. The biological activities of TPL of all fish samples against washed rabbit platelets aggregation were studied for whether they induced platelet aggregation (PAF agonists) or inhibited the PAF-induced platelet aggregation (PAF inhibitors).

According to **Table 4**, all three TPL samples (1.42 μg of raw, 12.3 μg of grilled and 5.89 μg of brined TPL, respectively) have shown 95% - 100% inhibition of the biological activity against PAF. Thus, it could be suggested that compounds with strong inhibitory activity against PAF were present in TPL of raw, grilled and brined sardine but grilled and brined fish TPL were about 8 and 4 times, respectively, less biologically active, against PAF, than raw TPL.

The fact that the TPL of all three samples of sardines (raw, grilled and brined) have shown strong PAF-inhibitory activities has prompted us to further fractionate the TPL of all three fish samples and study their biological properties. Therefore, approximately 60.0 mg TPL of raw, grilled and brined sardine fillets were further separated by preparative TLC (**Figure 1**) and the lipid

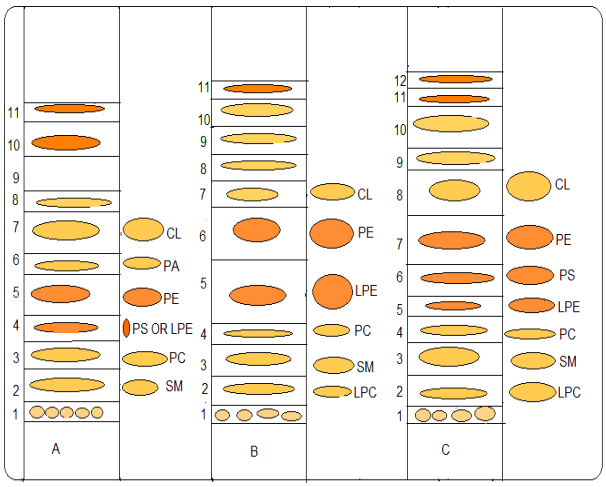


Figure 1. Typical profile of TPL separation of the fish samples on preparative TLC. A: raw sardine B: grilled sardine C: brined sardine, L-PC: lyso-phosphatidylcholine, SM: sphingomyelin, PC: phosphatidylcholine, PI: phosphatidylinositol, PS: phosphatidylserine, L-PE: lyso-phosphatidylethanolamine, PE: phosphatidylethanolamine, PA: phosphatidic acid, CL: cardiolipin. The developing system used for the separation of total polar lipids was chloroform:methanol:water 65:35:6 (v/v/v).

Table 4. *In vitro* biological activities, against platelet aggregation, of TPL and TLC polar lipid fractions of raw, grilled and brined sardine (*Sardina pilchardus*).

Lipid samples	Raw sardine sample			Grilled sardine sample			Brined sardine sample		
	Amount (μg) added in the cuvette	Aggregation (cm)[a]	Inhibition (%)[b]	Amount (μg) added in the cuvette	Aggregation (cm)[a]	Inhibition (%)[b]	Amount (μg) added in the cuvette	Aggregation (cm)[a]	Inhibition (%)[b]
TPL	1.42	-	100	12.3	-	100	5.89	-	95
TLC polar lipid fractions									
1	109	-	100	109	-	82	100	-	54
2	109	-	100	109	-	70	100	8.1	-
3	109	20.1	-	109	-	100	100	-	100
4	109	-	100	109	15.8	-	100	11.3	-
5	109	-	100	11	-	89	100	-	55
6	109	18.5	-	109	-	44	100	-	100
7	27	20.2	-	27	-	64	100	-	53
8	109	-	100	11	-	90	100	-	100
9	109	-	100	109	-	69	100	-	75
10	109	-	100	27	-	90	100	-	100
11	109	-	100	109	-	80	100	-	94
12	-	-	-	-	-	-	100	-	25

[a]The aggregation was expressed as cm of the height of the platelet aggregation curve. The quantity (μg) of the lipid fractions used in the biological assay is given. [b]The inhibition was expressed as % percentage of the aggregation caused by PAF at a final concentration (in the cuvette) of 10^{-9} M.

fractions obtained were tested for their ability to induce washed rabbit platelet aggregation or inhibit the PAF induced platelet aggregation. The quantity (in μL of raw TPL) was chosen in order to obtain 100% inhibition of PAF and the same quantity of grilled and brined TPL was assayed, in order to compare the biological activities of the individual TLC fractions of the three different fish samples.

The aggregation and inhibition values of each TLC polar lipid fraction from all samples are shown in **Table 4**. In order to keep **Table 4** as simple as possible, it should be noticed that the numbers of the TLC fractions do not correspond to the same Retardation factor (R_f). Thus, the TLC lipid fractions need to be compared in both **Figure 1** and **Table 4.**

The majority of TLC polar lipid fractions of raw, grilled and brined sardine fillets exhibited inhibitory activity (**Table 4**). As mentioned above, the TLC polar lipid fractions of raw sardine exhibited the maximum inhibitory activity and that was compared to the corresponding biological activities that equal amounts of TLC polar lipid fractions of grilled and brined exhibit. The observed inhibition of raw sardine was attributed to almost all TLC polar lipid fractions (1, 2, 4, 5, 8, 9, 10 and 11) apart from lipid fractions 3, 6 and 7 that exhibited aggregatory activities (**Figure 1** and **Table 4**). TLC polar lipid fractions of grilled and brined sardine also exhibited inhibitory activities that could be attributed to almost all TLC polar lipid fractions. The only exceptions were observed for lipid fraction 4 of grilled samples and fractions 2 and 4 of brined samples that exhibited aggregatory activity. TLC polar lipid fraction 3 of raw sardine and lipid fraction 4 of grilled and brined sardine have similar R_f values to that of phosphatidylcholine (PC) (**Figure 1**). PC does not have aggregating properties but oxidized-PC, which has a similar R_f value to that of PC, may have PAF-like activity [30].

In detail, raw sardine TLC lipid fractions 3, 6, and 7 exhibited aggregatory properties while in grilled and brined sardine only the analogous TLC fractions 4 exhibited aggregatory properties. The analogous TLC fraction of 7 of raw sardine lipids, in grilled and brined sardine exhibited inhibitory properties (**Figure 1** and **Table 4**).

Grilled sardine exhibited either similar or reduced inhibitory activity in comparison to raw sardine. TLC lipid fractions 1, 5, 8, 9, 10 and 11 showed reduced inhibitory

activity in comparison to the analogues TLC polar lipid fractions of raw sardine (**Figure 1** and **Table 4)**.

Similarly, brined sardine exhibited either similar or reduced inhibitory activity in comparison to raw sardine. Lipid fractions 1, 5, 7, 9 and 12 of brined sardine were found to have reduced inhibitory activity whereas lipid fractions 3, 6, 10 and 11 showed similar inhibitory activity in comparison to analogues TLC polar lipid fractions of raw sardine (**Figure 1** and **Table 4**).

When comparing grilled and brined sardine, their nutritional value (with emphasis against atherogenesis) seems to be similar since lipid fractions 1 and 5 of brined fish, exhibited reduced inhibitory activity in comparison to grilled sardine whereas lipid fractions 8, 10 and 11 of brined fish exhibited increased inhibitory activity in comparison to grilled sardine (**Figure 1** and **Table 4**).

The existence of PAF antagonists in various foodstuffs is of major importance for their nutritional value, considering the importance of platelet activation and thrombosis in cardiovascular diseases. Moreover, protective intervention studies against atherogenesis have shown that only specific PAF inhibitors [31], fish polar lipids [5], olive oil polar lipids [32] and statins [33] are able to reduce atherogenesis *in vivo*.

3.4. Sensory Analysis

The scope of this analysis was to detect possible differences between samples that, *a priori*, are of good quality. The panelists scored the grilled and brined sardine samples using values between zero (0) and ten (10) (the lowest "0", the highest "10") for attributes describing taste, aftertaste and odour characteristics and the obtained scores are outlined in **Table 5**.

The assessors indicated statistically significant organoleptic differences among grilled and brined sardines. More specific brined sardine exhibited more salty taste ($p = 0.001$) (**Table 5**) in comparison to grilled sardine, as it was expected, while grilled sardine was evaluated as more sweet ($p = 0.017$) and more fresh ($p = 0.001$) (**Table 5**) when compared to brined sardine. The sensation of aftertaste of brined sardine was statistically more intensive for the attributes bitter ($p = 0.001$) and lasting ($p = 0.002$) (**Table 5**) in comparison to grilled sardine. On the other hand grilled sardine was found to be more sweet aftertaste ($p = 0.023$) (**Table 5**) than brined one. Finally the attributes of odour fresh fish and grilled fish had significant higher scores for grilled sardine when compared to the ones for brined sardine (**Table 5**). The sensory profiles of grilled and brined sardine in terms of taste, aftertaste and odour are given in **Figure 2**, where the scores of each fish (either grilled or brined) for the different attributes used are given in the form of spider-web plots.

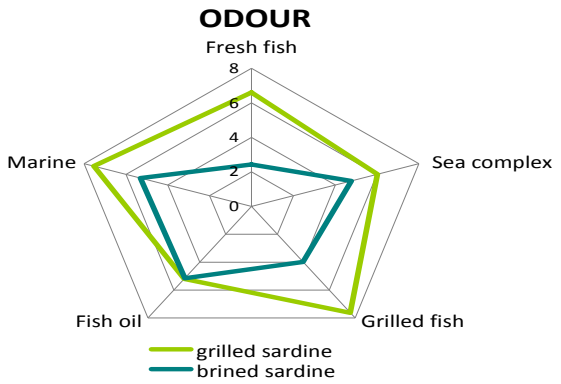
Table 5. Attribute score data of grilled and brined sardines after fish samples evaluation by ten trained assessors. The values are average scores of three replicates with statistical deviation of 99% confidence level; *p* values of each attribute produced from ANOVA analysis.

Attributes	Score Average ± SD		*p* values (grilled vs. brined fish samples attribute score data)
	Grilled fish samples	Brined fish samples	
Taste			
Sweet	3.5 ± 1.7	1.5 ± 1.7	0.017
Salty	4.4 ± 1.9	9.6 ± 0.5	0.001
Astringent	3.7 ± 2.4	5.4 ± 1.9	0.104
Fatty	4.9 ± 2.9	5.2 ± 3.1	0.853
Rich	5.4 ± 2.6	6.5 ± 2.5	0.346
Marine	6.4 ± 2.5	6.0 ± 1.7	0.696
Fresh fish	7.4 ± 2.1	3.4 ± 2.5	0.001
Aftertaste			
Sweet	4.1 ± 2.2	1.7 ± 1.0	0.023
Bitter	2.0 ± 2.0	6.9 ± 3.0	0.001
Oily	3.9 ± 2.7	5.5 ± 3.8	0.304
Lasting	5.7 ± 2.7	9.2 ± 1.2	0.002
Iodine	4.6 ± 3.4	5.8 ± 3.3	0.445
Odour			
Fresh fish	6.6 ± 2.4	2.4 ± 1.7	0.001
Sea complex	6.0 ± 2.0	4.8 ± 3.3	0.320
Grilled fish	7.6 ± 1.8	4.0 ± 2.0	0.001
Fish oil	5.2 ± 3.1	5.2 ± 3.2	0.972
Marine	7.5 ± 2.1	5.3 ± 2.8	0.058

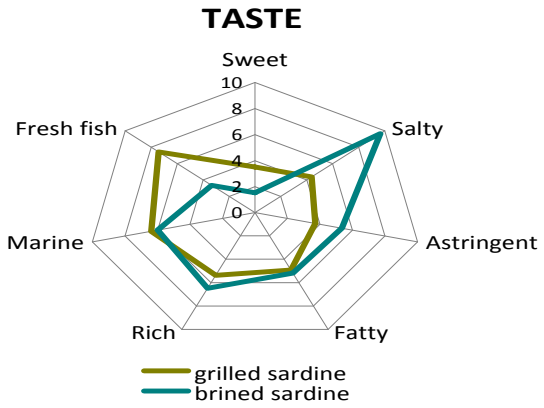
Significantly different attribute score data were found between grilled and brined sardines, according to ANOVA analysis ($p < 0.05$).

4. Conclusion

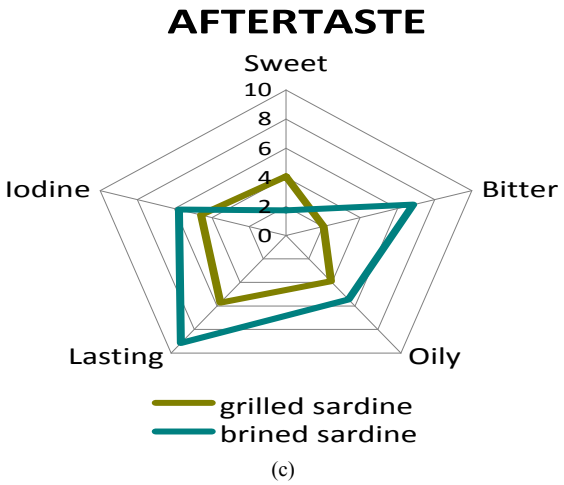
The obtained results underline the nutritional value of sardine against atherogenesis suggesting that its cardio-protective properties are not diminished during grilling and brining; a positive result given the fact that sardine is mainly consumed as grilled or brined. Therefore, the commercial choice between grilled and



(a)



(b)



(c)

Figure 2. Comparison of sensory profiles of grilled and brined sardine. (a) Odour attributes scores; (b) Taste attributes scores; (c) Aftertaste attributes scores.

brined sardine should not only be based on terms of nutritional value as both processed fish have high nutritional value but it should also be based on which taste and odour attributes the consumers prefer.

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Abbreviations

ArA, arachidonic acid;
BSA, bovine serum albumin;
CL, cardiolipin;
CVDs, cardiovascular diseases;
DHA, docosahexaenoic acid;
EPA, eicosapentaenoic acid;
L-PC, lyso-phosphatidylcholine;
L-PE, lyso-phosphatidyle-thanolamine;
MUFAs, monounsaturated fatty acids;
PAF, Platelet-Activating-Factor;
PA, phosphatidic acid;
PC, phosphatidylcholine;
PE, phosphatidylethanolamine;
PI, phosphatidylinositol;
PS, phosphatidylserine;
PUFAs, polyunsaturated fatty acids;
SFAs, saturated fatty acids;
SM, sphingomyelin;
TLC, thin layer chromatography;
TL, total lipids;
TNL, total neutral lipids;
TPL, total polar lipids.

