Effect of a flaxseed (*Linum usitatissimum*) soaking treatment on the frozen storage stability of mackerel (*Scomber scombrus*) fillets

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ABSTRACT

The effect of a flaxseed (*Linum usitatissimum*) soaking on the development of rancidity in frozen mackerel (*Scomber scombrus*) was studied. Fresh mackerel fillets were soaked in an aqueous flaxseed extract for 20 min and then kept frozen (-20°C) for up to 7 months. A parallel experiment with no-soaked fillets was carried out under the same conditions. The development of rancidity was measured by biochemical (free fatty acids, peroxides, conjugated dienes and trienes, secondary oxidation products, fluorescent and browning compounds and lipoxygenase activity) and sensory (general aspect, odour and colour) analyses. An inhibitory effect of the soaking treatment on rancidity development was observed according to the peroxide content and the formation of fluorescence and browning. A lower lipoxygenase activity was detected at 1 month in the soaked fillets; after this, no differences were obtained between either type of sample, whose activities at month 7 were negligible. According to the sensory analyses, non-soaked fillets had fair quality at 1 month and were rejectable at 3 months, while the soaked ones were still of good quality at 1 month and rejectable at 5 months. According to the present results, soaking in an aqueous flaxseed extract could be useful for inhibiting the development of rancidity in fatty fish fillets.

Keywords: Mackerel, fillets, frozen storage, flaxseeds, soaking, rancidity, shelf life

Running Title: Flaxseed soaking and frozen mackerel rancidity

INTRODUCTION

Most marine species give rise to products of great economic importance in many countries. Fatty fish is attracting considerable attention because of the positive role of marine lipids in human nutrition and health^{1,2}; great efforts are therefore being made to commercialise such species in the frozen state^{3,4}. During frozen storage, enzymatic and non-enzymatic rancidity is known to strongly influence the shelf life of fatty fish products. Thus, the presence of pro-oxidant molecules in fish muscle^{5,6} has been shown to deteriorate the highly unsaturated lipid composition⁷.

One abundant fatty fish species in both North Atlantic coasts is Atlantic mackerel (*Scomber scombrus*) belonging to the *Scombridae* family⁸. Although it is recognised as a healthy food because it is a good source of high-quality nutrients, in particular ω 3 fatty acids^{9,10}, it has been reported to have a poor frozen shelf life¹¹. In this sense, previous research has unveiled an important endogenous pro-oxidant activity^{12,13} and quality loss during frozen storage¹⁴ and further processing¹⁵.

To extend the lag phase as long as possible, and hence delay lipid oxidation, considerable attention is now being given to the use of natural antioxidants^{16,17}. Recent efforts have focused on the positive role of the antioxidant molecules present in plant extracts. Thus, successful applications have been carried out in marine oils^{18,19}, minced fish^{20,21} and fish fillets^{22,23}.

One such plant products is flaxseed (*Linum usitatissimum*, L.), which has been reported to be an important functional food because of its cancer prevention activity²⁴, cardiovascular disease reduction²⁵ and pro-inflammatory mediator inhibition²⁶. Flaxseed has been shown to be a natural source of major plant food phytochemicals such as flavonoids, coumarins and phenolic acids, and providing an interesting lignan content ($>500\mu\text{g g}^{-1}$), such

as secoisolariciresinol diglycoside^{27,28}. Thus, an important antioxidant behaviour has been recognised for this plant²⁸⁻³⁰. With a view to assessing the different compounds present in flaxseeds, extractions with different polar solvents and solvent mixtures such as ethanol³¹, ethanol:1,4-dioxane (1:1)³² and methanol-acetone-water³³ have been carried out. Further, the analysis of the water extract pointed to the important presence of different glycosides (linamarin and lotaustralin, namely) supporting an important antioxidant behaviour^{34,35}.

In a preliminary paper, the authors treated minced herring with ground flaxseed and the mixture was kept under frozen conditions; an inhibition of lipid oxidation was concluded as a result of this flaxseed treatment³⁶. The present work addressed mackerel (*Scomber scombrus*) traded as a frozen product. Filleted fish was soaked in an aqueous extract of flaxseed to prolong its lipid stability during frozen (-20°C) storage and hence the shelf life and consumer acceptance. A water extract was employed because of the mentioned presence of glycosides and the convenience for trading purposes of employing such kind of extract system. During the present experiment, lipid damage was monitored for up to 7 months by biochemical and sensory indices. Results obtained after the soaking treatment are compared with those determined in parallel in non-soaked fillets that were kept frozen under the same conditions.

MATERIALS AND METHODS

Flaxseeds extract preparation

A mixture consisting of 25.2g flaxseeds and 15 litres of water was stirred for 15 min in an isothermal room (4°C). It was then centrifuged (3000g; 4°C) and the supernatant was employed immediately to soak the fish fillets.

Raw fish, sampling, and processing

Fresh mackerel (*Scomber scombrus*) (54 specimens) were obtained at a local market. From the time of the catch until arrival at our laboratory, the fish were kept on ice. All fish collected for the present study were males. Their gonads were at the 5th /6th stage of Maier's scale of gonad maturity.

The fish were carefully dressed and filleted by hand without separating the skin. Some fillets (prepared from 6 specimens) were taken as starting material and analysed immediately. Other fillets (prepared from 24 specimens) were packaged directly in individual polyethylene bags and frozen at -80°C . The remaining fillets (also prepared from 24 specimens) were soaked in the above-mentioned aqueous extract of flaxseeds ($7.5 \text{ g flaxseeds kg}^{-1} \text{ fish}$) in an isothermal room at 4°C . After 20 min, the fish were removed, packaged in individual polyethylene bags and frozen at -80°C . After 24 hours at -80°C , all fish were placed at -20°C ; no thin glaze on the surface of the fillets was produced. Sampling was undertaken at 1, 3, 5 and 7 months of frozen storage at -20°C and on the starting material.

For all types of sample, three different independent groups were considered, such that analyses were carried out in triplicate ($n=3$) to accomplish the statistical study. Fillets from two specimens were considered for each sampling point. Once the fish had been subjected to sensory analysis, the muscle was separated, homogenised and divided into smaller portions to carry out the different biochemical analyses.

Water and lipid analyses

Water contents were determined by the weight difference between homogenised fish muscle (1-2 g) and after 24 hr at 105°C . Results were calculated as $\text{g water kg}^{-1} \text{ muscle}$.

Lipids were extracted by a chloroform-methanol mixture (2:1) according to Linko³⁷. Quantification results are expressed as $\text{g total lipids kg}^{-1} \text{ wet muscle}$.

Indices of lipid damage

Free fatty acid (FFA) contents were determined in the chloroform-methanol lipid extract according to the Lowry and Tinsley³⁸ method, based on complex formation with cupric acetate-pyridine. Results were obtained spectrophotometrically at 715 nm and are expressed as g FFA kg⁻¹ lipids.

The content of hydroperoxides and secondary oxidation compounds was determined in the organic and aqueous phases, respectively, resulting from the lipid extraction³⁷ according to the method of Schmedes and Hølmer³⁹ and both are expressed as mg malondialdehyde kg⁻¹ muscle. In this method, lipid peroxides are cleaved to malondialdehyde by ferric chloride and made to react with TBA reagent, absorbance being measured spectrophotometrically at 532 nm; secondary oxidation products react with TBA to obtain TBA-reactive substances (TBARS).

Conjugated dienes (CD) and trienes (CT) were determined spectrophotometrically (232 nm and 267 nm, respectively)⁴⁰ in the chloroform-methanol lipid extract obtained from the above Linko³⁷ method. The CD and CT contents were expressed as absorption coefficients (AC), according to the formula⁴⁰: $AC = A / w$, where **A** is the absorbance reading and **w** denotes the lipid mass (g) per 100 ml of the final solution used for measurement.

Formation of interaction compounds

Interaction compounds (tertiary oxidation compounds) produced between oxidised lipids and nucleophilic compounds present in the fish muscle (free amino acids, peptides, proteins, aminated phospholipids) were followed by the formation of fluorescence and browning^{41,42}.

Fluorescence formation (Perkin-Elmer LS 3B) at 327/415 nm and 393/463 nm was studied as described elsewhere^{43,44}. The relative fluorescence (RF) was calculated as follows:

$RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission pair, and F_{st} is the fluorescence intensity of a quinine sulphate solution ($1 \mu\text{g ml}^{-1}$ in $0.05 \text{ M H}_2\text{SO}_4$) at the corresponding wavelength. The fluorescence ratio (FR) was measured in the chloroform-methanol lipid extract, according to the following calculation: $FR = RF_{393/463\text{nm}} / RF_{327/415\text{nm}}$.

Brown colour formation (BCF) was determined at 420 nm in the chloroform-methanol lipid extract according to Labuza and Massaro⁴⁵. Results were calculated using the following formula⁴⁶: $BCF = B \times V / w$, where B is the absorbance reading, V denotes the volume (ml) of the sample and w is the mass (mg) of the lipid sample.

Lipoxygenase activity

Lipoxygenase was extracted with 10.0 mM phosphate buffer (pH 7.0), according to the Harris and Tall⁴⁷ method, modified by Stodolnik and Samson⁴⁸. Enzyme activity was expressed according to the malondialdehyde content ($\text{nmol malondialdehyde mg}^{-1} \text{ protein}$) produced after 24 h incubation of an enzyme extract – hydro-lipid emulsion mixture^{48,49}.

Sensory analyses

Sensory analyses were conducted by a taste panel consisting of five experienced judges, according to the guidelines presented in Table 1⁵⁰. Four categories were ranked: highest quality (E), good quality (A), fair quality (B) and rejectable quality (C). Sensory assessment of the fish included the following parameters: general aspect, odour and colour.

At each sampling time, the different fish fillets were thawed and then analysed in the same session. The fish fillets were served to the panel members in the individual polyethylene bags where they had been kept frozen and were scored individually.

Statistical analyses

Data from the different biochemical indices were subjected to the one-way ANOVA method ($p < 0.05$)⁵¹; comparison of means was performed using a least-squares difference (LSD) method. The Spearman test ($p < 0.05$) for non-parametric correlations was performed in the case of sensory assessments⁵¹.

RESULTS AND DISCUSSION

Water and lipid contents

The water content in the muscle of the mackerel fillets ranged between 690 and 725 g kg⁻¹; the lipid content ranged between 80 and 145 g kg⁻¹. In the present case, mackerel was captured in the period of highest lipid content^{9,10}. Differences in both constituents (water and lipids) may be explained as a result of individual fish variations, and not of frozen storage or soaking treatment. Comparison of the current results with those from previous research showed a lower water content than leaner fish species (blue whiting, hake and cod)^{43,44} in agreement with a common inverse ratio between water and lipid matter⁵².

Lipid hydrolysis

Since mackerel is a fatty fish species, the values of the FFA contents of the initial material on a lipid basis (Table 2) were found to be lower than in the case of medium fat (horse mackerel)⁵³ and lean (blue whiting, cod, haddock)^{43,44} fish species. The current mean values showed (Table 2) a gradual increase with storage time for both the soaked and non-soaked samples. The soaking treatment produced a higher FFA value at 1 and 5 months, although at the end of the experiment no differences were obtained. Examining the extent of

lipid hydrolysis was deemed important for the study because of the development of an important degree of lipid hydrolysis previously obtained in mackerel during frozen storage^{11,15,54} and also because of the strong influence of free fatty acids in lipid oxidation.

Free fatty acids oxidise more rapidly than phospholipids and triglycerides, mainly because they are smaller molecules and thus provide greater accessibility to oxygen and other pro-oxidant molecules^{55,56}. Further, a pro-oxidant effect of free fatty acids on the remaining lipid matter has been observed and explained on the basis of a catalytic effect of the carboxyl group on the formation of free radicals due to the decomposition of hydroperoxides^{55,56}. However, molecules resulting from the aminated phospholipid (phosphatidylethanolamine, phosphatidylserine) hydrolysis have shown an important antioxidant activity that has been attributed not only to the side-chain amino group, but also to the cooperative effect of the hydroxy group in the side chain^{57,58}.

Lipid oxidation

Oxidation of lipids to hydroperoxides occurred during storage in both kinds of samples, and gradually increased up to month 5 (Figure 1). After this time, no differences ($p > 0.05$) were obtained for control samples, and a content decrease ($p < 0.05$) for the soaked ones was observed. Comparison of both kinds of fillet led to the conclusion that soaking treatment significantly inhibited ($p < 0.05$) hydroperoxide formation along the 3-7 month period.

Higher CD values ($p < 0.05$) were obtained in the 1-7 month period for the soaked samples (Figure 2). However, no regular trend for either type of sample was observed during the experimental period. It has been reported that CD formation can be used successfully as a lipid damage assessment for the initial stages of oxidation⁵⁹. However, in advanced oxidation

stages CD have been shown to be relatively unstable and capable of interacting with other constituents⁶⁰, such that their analysis would not be a valuable tool for assessing lipid damage.

The mean values obtained from the CT analysis (Figure 3) of the control samples revealed a continuous increase with storage time; this was especially marked at 7 months. The soaked fillets showed a decrease in the CT content ($p<0.05$) at 1 month, followed by a gradual increase up to the end of the experiment. Comparison of both kinds of samples revealed that the soaking treatment led to a lower ($p<0.05$) value at 1 month, but a higher value ($p<0.05$) at 5 and 7 months. It may be concluded that this index is not suitable for lipid damage assessment in the present experiment since relatively unstable molecules are involved⁶⁰.

A continuous increase in secondary lipid oxidation compounds (Figure 4) was obtained for both kinds of samples up 5 months, followed by a decrease ($p<0.05$) at the end of the experiment. Significant differences between both kinds of samples were only obtained at 1 month, in the sense that the soaking treatment inhibited ($p<0.05$) the formation of secondary oxidation compounds.

Formation of interaction compounds

In the case of the non-soaked fish, detection of fluorescent compounds revealed an increase at 3 months that was followed by no change up to the end of the experiment (Table 2). However, in the case of the soaked samples, no significant increases were observed along the whole of the storage time. Comparison of both kinds of samples revealed a protective effect of the soaking treatment since lower FR values were obtained for the soaked samples in the 3-7 month period.

The development of browning (polymerisation products from Schiff bases) (Table 2) followed an increasing trend along the storage time in the case of the non-soaked samples; significant increases were obtained at 1 and 5 months. On considering the soaked fish, an

increase was observed at 1 month, followed by no changes up to the end of the experiment. Comparison of both kinds of samples revealed an inhibitory effect of the soaking treatment at 5 and 7 months.

Lipoxygenase activity

Both kinds of samples provided an increase ($p < 0.05$) in lipoxygenase activity at 1 month, followed by a gradual decrease up to the end of the experiment (month 7), when negligible activity values were detected (Figure 5). The soaking effect only provided activity differences at 1 month, when a higher lipoxygenase activity ($p < 0.05$) was observed for the control samples.

An increase in lipoxygenase activity in the first month of frozen storage has already been observed⁶¹ for Baltic herring muscle when stored at -25°C . This increase was explained as better lipoxygenase extraction from cell structures after frozen storage release from cell membranes. Application of washing treatments on fish products has been shown to provide the opportunity to remove some water-soluble pro-oxidant compounds^{62,63}. Accordingly, the lower ($p < 0.05$) lipoxygenase activity found in the present experiment at 1 month for the soaked fillets can be assumed to be the result of the washing effect during the soaking treatment.

Previous research has shown the presence in mackerel muscle of pro-oxidant compounds that may catalyse lipid oxidation^{12,47}. In this sense, Saeed and Howell¹³ reported that 12-lipoxygenase was the main promoter of the oxidation of mackerel lipids and that it could be partially inhibited by synthetic and natural antioxidants, although it was active even at -70°C .

Sensory analysis

The sensory analysis (general aspect, odour and colour) uncovered (Table 3) a decrease in acceptance with storage time. Good Spearman correlations of the three sensory attributes with storage time were observed ($r^2 = 0.84\text{--}0.96$) for both kinds of products. The Spearman correlations of the three sensory attributes with the biochemical indices were also studied. In all cases, the best results were obtained by comparison with peroxide detection ($r^2 = 0.82\text{--}0.97$) and FFA formation ($r^2 = 0.71\text{--}0.90$).

Non-soaked fillets proved to have fair quality at month 1 and were rejectable at 3 months. The soaked fish was still of good quality at 1 month and rejectable at 5 months. A preservation of quality and a more prolonged shelf life were obtained as a result of the soaking treatment. In both cases, the limiting factors proved to be odour and colour, closely related to the development of rancidity.

FINAL REMARKS

According to biochemical indices related to lipid damage (hydroperoxides, fluorescence and browning) and to sensory assessment (general aspect, odour and colour), the present results on mackerel fillets indicate that the development of rancidity can be slowed and hence that quality preservation after freezing and frozen storage could be obtained by means of a previous treatment consisting of soaking the fillets in an aqueous flaxseed extract. This soaking treatment would include a washing effect of the fish muscle that removes part of the water-soluble pro-oxidant compounds (namely enzymes and heme-proteins)^{62,63} and would also incorporate into the fish tissue water-soluble compounds (linamarin and lotaustralin, namely) present in flaxseed, that have already been reported as having an

antioxidant behaviour^{34,35}. As a result, a commercially profitable increase in shelf-life is obtained.

Previous research on frozen storage of fatty fish has shown that the development of endogenous rancidity is the main cause of quality loss^{3,12}. Among the different enzymatic systems available, lipoxygenase has attracted a great deal of attention in the case of fatty fish (sardine, mackerel and herring)^{5,13,48}. In the present results, the reduction in lipoxygenase activity due to the soaking treatment could be observed at 1 month, in agreement with the removal effect of water washing during soaking^{62,63}.

Industrial requirements are always related to the search for valuable and practical treatments that may lead enhancing commercial possibilities. In this sense, the use of aqueous extracts and solutions is considered most suitable. Although a previous work has already reported the presence of different kinds of glycosides in the aqueous flaxseed extract supporting an antioxidant behaviour³⁴, a complementary study should be carried out in order to elucidate the different compounds extracted under the current water extract conditions and to assess their possible role in the inhibition of rancidity.

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FIGURE LEGENDS

Figure 1: Hydroperoxide determination (mg malondialdehyde kg^{-1} muscle) during frozen storage of non-soaked and soaked mackerel fillets*

* Bars denote standard deviation of means (n=3).

Figure 2: Conjugated diene determination (absorption coefficient) during frozen storage of non-soaked and soaked mackerel fillets*

* Bars denote standard deviation of means (n=3).

Figure 3: Conjugated triene determination (absorption coefficient) during frozen storage of non-soaked and soaked mackerel fillets*

* Bars denote standard deviation of means (n=3).

Figure 4: Determination of secondary oxidation compounds (mg malondialdehyde kg^{-1} muscle) during frozen storage of non-soaked and soaked mackerel fillets*

* Bars denote standard deviation of means (n=3).

Figure 5: Lipoxygenase activity assessment (nmol malondialdehyde mg^{-1} protein) during frozen storage of non-soaked and soaked mackerel fillets*

* Bars denote standard deviation of means (n=3).

TABLE 1

Scale employed to evaluate the quality of frozen mackerel fillets

Attribute	E (highest quality)	A (Good quality)	B (Fair quality)	C (Rejectable quality)
General aspect (dryness, myotome breakdown, white spots)	Strongly hydrated; totally adhered myotomes; absence of white spots	Still hydrated; myotomes adhered; absence of white spots	Slightly dry; myotomes adhered in groups; small white spots	Dry; myotomes totally separated; white spots
Odour	Shellfish	Weakly shellfish	Slightly sour and incipient rancidity	Sharply sour and rancid
Colour	Strongly pink	Still pink	Slightly pale	Yellowish

TABLE 3

Sensory acceptance* during frozen storage (months) of non-soaked and soaked mackerel fillets

Frozen Storage Time (months)	Attribute					
	General Aspect		Odour		Colour	
	Non-soaked	Soaked	Non-soaked	Soaked	Non-soaked	Soaked
1	B	A	A	A	B	A
3	B	A	C	B	C	B
5	C	B	C	C	C	C
7	C	C	C	C	C	C

* Category marks according to Table 1. The same starting material was employed for non-soaked and soaked conditions, showing category E for all the attributes.