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The Effect of Neutral and Oxidized Lipids on Functionality in Hake (*Merluccius merluccius* L.): A Dimethylamine- and Formaldehyde-Forming Species During Frozen Storage

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ABSTRACT

*The effect of added cod liver oil and oxidized cod liver oil on protein solubility, apparent viscosity and emulsifying capacity was measured during frozen storage of vacuum packed minced hake (*Merluccius merluccius *L.), a species which forms dimethylamine (DMA) and formaldehyde (FA) during frozen storage.*

The results indicate that the samples with oxidized lipids added showed the best functionality during storage and the untreated control produced the worst.

The DMA content was found to be inversely related to the degree of oxidation of lipids as measured by the TBA index, suggesting that the apparent protective effect of unoxidized and oxidized cod liver oil on protein functionality is due to a lower rate of FA and DMA formation.

Electrophoretic studies indicated that differences existed in the types of interactions responsible for aggregation in the different treatments.

INTRODUCTION

During frozen storage, fish undergoes a number of changes, causing alterations to texture and flavour which determine its practical storage life. It is well known that the gadoid fishes, with low fat content, undergo alterations in texture and in functionality which can be attributed mainly to

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the action of formaldehyde (FA) on muscle protein. FA is formed during frozen storage by the breakdown of trimethylamine oxide (TMAO) into dimethylamine (DMA) and formaldehyde (Castell *et al.*, 1973).

The mechanisms of FA and DMA formation in frozen storage are not fully understood, evidence exists for both enzymatic (Amano & Yamada, 1964) and non-enzymatic (Spinelli & Dassow, 1982) mechanisms. It is accepted, however, that the decomposition of TMAO to DMA and FA occurs through oxidation–reduction pathways. It has been found that certain reducing agents, as well as processes such as vacuum packing or inert atmospheres, favour the formation of FA (Lundstrom *et al.*, 1981) while the presence of oxygen (Lundstrom *et al.*, 1981; Reece, 1983) or certain oxidizing agents (Harada, 1975; Spinelli & Kouri, 1979) inhibit the reaction. It has also been observed that, in some non-DMA-forming species, DMA is produced when they undergo processes such as canning (Hebard *et al.*, 1982).

It has been postulated that the stability of semi-fatty species, which do not form FA and DMA in large quantities during frozen storage, is due to the action of neutral lipids which, in proper concentration and location, can produce a protective effect on fish proteins. However, it has not been established how neutral lipids produce this action. Castell (1971) found that the decrease in extractable protein in several gadoid species is directly related to the DMA level formed during frozen storage and that those which produced the most DMA accumulated the least amount of free fatty acids (FFA). In studies, where the effect of lipids on FA forming species is studied, Stodolnik and Knasiak (1984) found that rheological properties improve in the presence of lipids and Swiniarska and Wlodarczyk (1987) observed a better protein solubility. The former authors suggested that the decrease in firmness may be due to a reduction in the action of FA on fish protein in the presence of neutral lipids. However, natural fish lipids tend to turn rancid during frozen storage; thus, the presence of oxidized lipids could mask the described protective effect on fish proteins because, as has been shown in model system studies, oxidized lipids have a detrimental action on fish proteins (Takama, 1974*a*,*b*).

There is a need to establish the interrelations between the different factors which accelerate or decelerate the denaturation and aggregation of proteins in order to fully know their action on fish muscle and thus be able to control detrimental changes (Shenouda, 1980). Therefore, the objective of this study was to observe the action of intact and rancid lipids on functionality in an FA- and DMA-forming species during frozen storage, in order to elucidate both the protecting and detrimental factors. For this purpose, we have studied the variation in protein functionality in minced muscle to which we added cod liver oil (CLO) or oxidized cod liver oil (OCLO). Also, both oxidative

rancidity and the formation of formaldehyde indirectly through the measure of DMA, have been determined to discover the influence of these parameters over protein functionality.

MATERIALS AND METHODS

Sample preparation

Hake (*Merluccius merluccius* L.) was caught on the Galician Shelf in March, 1985, 24 h prior to the beginning of the experiment, and kept on ice until its handling at the laboratory. Fifteen individuals, with a mean length of 60·8 cm and a mean weight of 1845 g after gutting, were used. The fish were headed, gutted, the kidneys were removed and the fish washed with iced water to remove blood, etc. The flesh was minced in a Baader model 694 apparatus using a drum with 3 mm diameter holes and kept below 7·5°C during subsequent handling.

The following lots were obtained from the minced fish:

HN: minced hake to which 3% cod liver oil (CLO) was added using a Hobbart homogenizer model N-SO 6 for 1 min on position 2.

HO: lot prepared as lot HN, but to which the CLO added was previously oxidized under conditions indicated below.

HC: minced hake, no lipids added. Homogenized under the same conditions described for avoiding differences in handling. This lot was considered the control lot.

The lots obtained were packaged on trays containing approximately 600 g, frozen in a horizontal plate freezer until the thermal centre temperature reached −18°C, vacuum packed and stored at −18°C for 360 days.

Cod liver oil

The CLO used was purchased at a local supplier (Riesgo) and did not contain phospholipids, butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) in appreciable quantities.

Oxidation of cod liver oil

Dried compressed air was bubbled through CLO at 37°C until a peroxide value (POV) of 800 meq/kg was reached.

Analysis

Crude protein (AOAC num. 24024, 1975), moisture content (AOAC num. 24003, 1975), ash (AOAC num. 18021, 1975), pH (Vyncke, 1981), crude fat (Bligh & Dyer, 1959, modified by Knudsen *et al.*, 1985), free formaldehyde (Nash, 1953) and disc-SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) of the natural actomyosin (Kawashima *et al.*, 1973) were determined when required.

DMA (Dyer & Mounsey, 1945), TBA (Lemon, 1975), percentage of soluble protein (PS/PT) (Ironside & Love, 1958), apparent viscosity (η app) (Borderias *et al.*, 1985*a*) and emulsifying capacity (EC) (Tejada *et al.*, 1987) were analyzed periodically throughout storage.

POV (UNE, 1973) and TBA number (Sinhuber & Yu, 1977) were measured in the CLO and OCLO added.

Statistical analysis

A one way analysis of variance (Guenther, 1964) of the variables studied periodically was performed using a CDC CYBER 180/855 computer applying a BMDP 1V program. The degree of significance was established for $P < 0{\cdot}05$. Likewise, correlations were established among the variables determined periodically. The degree of significance of the correlations was obtained from tables (Lamotte, 1981).

RESULTS AND DISCUSSION

Tables 1 and 2 show the proximate analyses and pH of the minced hake and the initial conditions of the lipids added. Table 3 shows the analysis of variance of the variables studied periodically for the different storage times.

TABLE 1
Initial Analyses

Lot	*Crude protein* (%)	*Crude fat* (%)	*Moisture* (%)	*Ash* (%)	*pH*
HC	16·96	0·98	81·45	1·24	6·53
HN	16·71	3·98	79·17	1·15	6·50

HC = Control lot.
HN = 3% CLO added.

TABLE 2
Initial Analyses of Lipids Added to the Minced Hake

	Peroxide value (POV) (*meq/kg*)	*Free fatty acids* (*FFA*) (%)	*TBA value* (*μmol MA/100 g*)
CLO	10·5	0·36	472
OCLO	810	0·49	10·9

CLO = Cod liver oil.
OCLO = Oxidized cod liver oil.

TABLE 3
Analysis of Variance of the Variables Studied for the Different Storage Times and Conditions

Variable	*Lots*	*Days*					
		0	*28*	*56*	*88*	*161*	*255*
DMA	HC	*a/x*	*b/x*	*c/x*	*c/x*	*d/x*	*e/x*
	HN	*a/y*	*b/y*	*c/y*	*d/y*	*e/y*	*f/y*
	HO	*a/z*	*b/z*	*c/z*	*c/z*	*d/z*	*e/z*
TBA value	HC	*a/x*	*b/x*	*c/x*	*b/x*	*d/x*	*e/x*
	HN	*a/y*	*b/y*	*c/y*	*d/y*	*e/y*	*f/y*
	HO	*a/z*	*b/z*	*c/z*	*d/z*	*d/z*	*e/z*
% Protein solubility	HC	*a/x*	*b/x*	*c/x*	*d/x*	*e/x*	*e/x*
	HN	*a/x*	*b/y*	*c/y*	*d/y*	*e/y*	*e/y*
	HO	*a/x*	*b/y*	*c/y*	*d/y*	*d/z*	*e/xy*
Apparent viscosity	HC	*a/x*	*b/x*	*c/x*	*d/x*	*d/x*	*d/x*
	HN	*a/y*	*b/y*	*c/x*	*d/y*	*e/x*	*e/x*
	HO	*a/x*	*b/z*	*c/y*	*d/z*	*e/y*	*e/y*
Emulsifying capacity	HC	*a/x*	*b/x*	*c/x*	*c/x*	*d/x*	*d/x*
	HN	*a/xy*	*b/y*	*cd/x*	*c/y*	*d/y*	*e/x*
	HO	*a/y*	*b/y*	*c/y*	*d/z*	*e/z*	*d/y*
Homogenate protein concentration	HC	*a/x*	*a/x*	*a/x*	*b/xz*	*a/xz*	*c/x*
	HN	*ad/x*	*a/xy*	*d/xy*	*a/y*	*b/xz*	*c/y*
	HO	*a/x*	*b/y*	*b/y*	*c/zy*	*d/x*	*e/z*

Different letters in each row (*a*, *b*, *c*) and in each column (*x*, *y*, *z*) indicate significant differences ($P < 0{\cdot}05$).
HC = Control lot.
HN = 3% CLO added.
HO = 3% OCLO added.

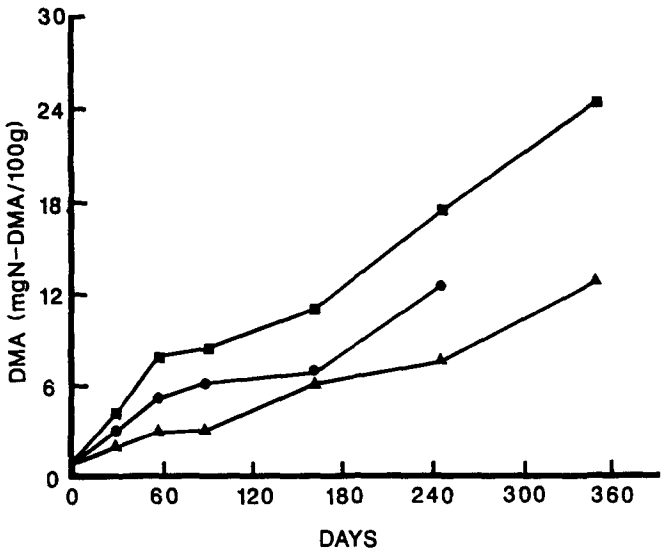

Fig. 1. Formation of dimethylamine (DMA) during frozen storage (−18°C) of minced hake. HC (■), control lot; HN (●), 3% CLO added; HO (▲), 3% OCLO added.

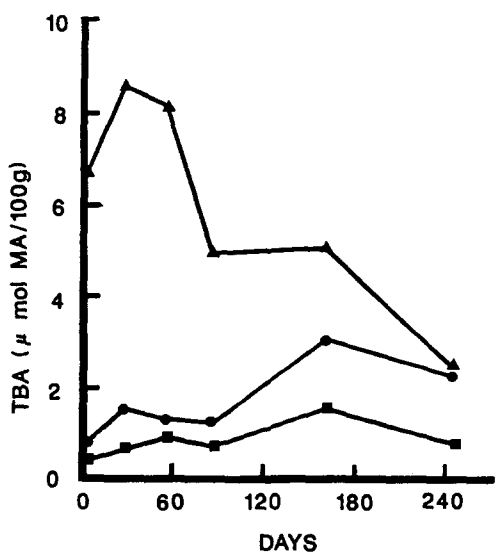

Fig. 2. 2-Thiobarbituric acid value (TBA) during frozen storage (−18°C) of minced hake. HC (■), control lot; HN (●), 3% CLO added; HO (▲), 3% OCLO added.

Dimethylamine

DMA values are shown in Fig. 1. The greatest DMA formation occurred in the control samples, followed by the CLO- and OCLO-added samples, respectively. In order to corroborate this fact and to rule out possible interference in the technique used for DMA determination, free formaldehyde was measured at 348 days, giving values of 34·7, 21·8 and 11·5 mgFA/100 g for the samples, respectively, supporting the observations found with DMA.

2-Thiobarbituric acid index

Figure 2 shows the TBA values. The sharp decrease in the TBA index of the OCLO-treated samples was assumed to be due to the secondary lipid oxidation products being degraded (Maleki, 1974) or reacting with proteins (Buttkus, 1967; Crawford *et al.*, 1967; Nair *et al.*, 1986) in such a way that its effective concentration and thus its measure, decrease. The variability in all the samples was thought to be due to the formaldehyde produced during frozen storage, interfering in the TBA technique (Careche & Tejada, 1988). In general, the values found, in each of the samples, were significantly different for each storage period (Table 3), but probably do not directly reflect the state of lipid oxidation in the fish mince.

Functional properties: solubility, viscosity and emulsifying capacity

The percentage of soluble protein (Fig. 3) did not initially show significant differences between samples (Table 3). A gradual decrease in solubility is observed during the first month in the control sample followed by a sharp fall during the second month to reach low values from the third month of frozen storage, with slight changes during the following period. Samples with neutral (HN) and oxidized (HO) lipids added in general do not show significant differences between them during frozen storage. Solubility of these samples is different from that of the control, being lower in the first month and higher in the remainder of the storage period although, as in the control lot, solubility decreases at a much lower rate after the third month of study.

Apparent viscosity (Fig. 4) of the control sample decreases sharply during the first month of storage until reaching, after 3 months, values so low that they do not register on the measurement scale under the experimental conditions employed. The behaviour of the samples with neutral (HN) and oxidized (HO) lipids is different, since sample HN starts out with values lower than the control becoming higher in the first month of storage, after

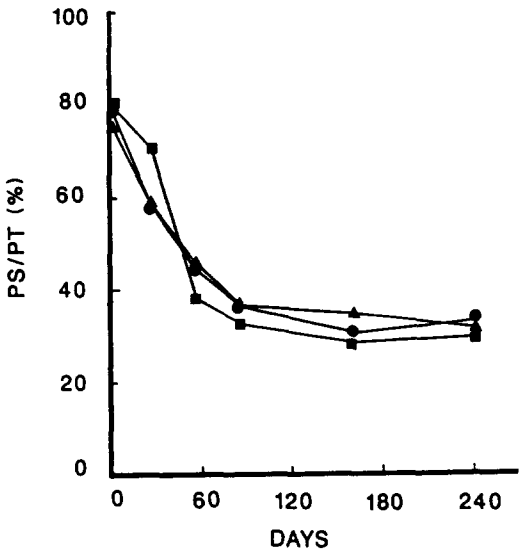

Fig. 3. Percentage protein solubility (PS/PT) during frozen storage (−18°C) of minced hake. HC (■), control lot; HN (●), 3% CLO added; HO (▲), 3% OCLO added.

which it does not show significant differences with respect to the control (Table 3). Up to 161 days of storage, the sample with oxidized lipids added has values higher than the control lot (Table 3) although the decrease in viscosity is also sharp. These results differ from the detrimental effect of lipid oxidation products on isolated actomyosin described by Takama (1974*a*,*b*).

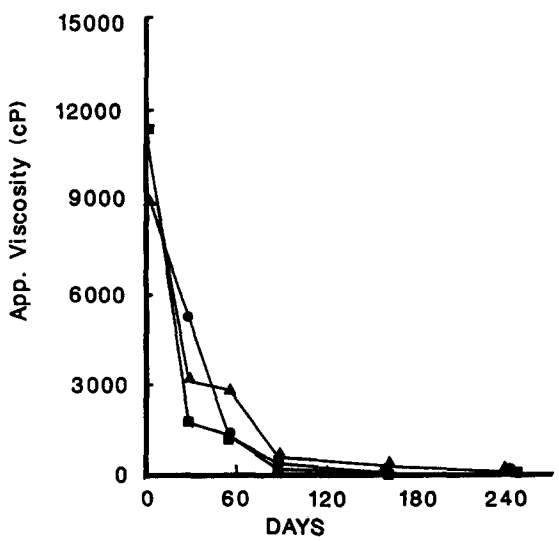

Fig. 4. Apparent viscosity (η_{app}) during frozen storage (−18°C) of minced hake. HC (■), control lot; HN (●), 3% CLO added; HO (▲), 3% OCLO added.

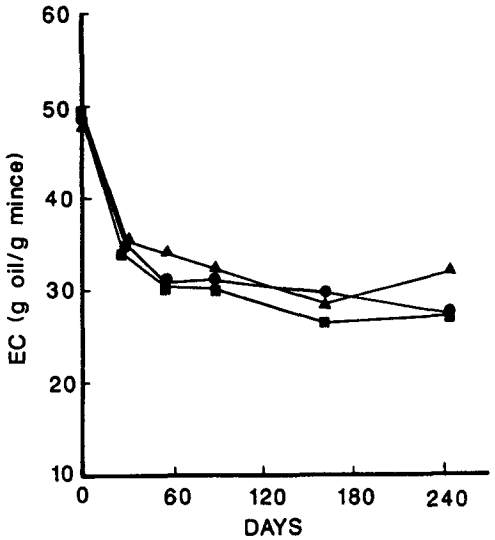

Fig. 5. Emulsifying capacity (EC) during frozen storage ((− 18°C) of minced hake. HC (■), control lot; HN (●), 3% CLO added; HO (▲), 3% OCLO added.

With respect to emulsifying capacity (Fig. 5) the greatest percentage of variation occurs in the first 2 months of storage. Although the three samples start off with similar values, the one with oxidized lipids (HO) shows values significantly higher than the control throughout the storage period (Table 3), while in general the sample with neutral lipids added (HN) shows values slightly higher than those of the control sample.

The total protein of the homogenate used to measure viscosity and emulsifying capacity (Table 4) had similar values for the three samples throughout all the controls except at 245 days. This may be due to the fact that, in this species, at the end of the storage period, the muscle fibres do not

TABLE 4
Homogenate Protein Concentration (mg/g) during Frozen Storage (− 18°C) of Minced Hake

Lots	*Days*					
	0	*28*	*56*	*88*	*161*	*245*
HC	32·6	32·5	33·5	28·9	32·1	11·0
HN	31·5	31·1	32·1	31·1	30·7	12·7
HO	32·3	30·5	31·0	29·8	33·2	19·2

HC = Control lot.
HN = 3% CLO added.
HO = 3% OCLO added.

TABLE 5
Correlations between Percentage of Protein Solubility (PS/PT), Apparent Viscosity (η_{app}), Emulsifying Capacity (EC) and Dimethylamine (DMA) in the Minced Hake Lots

		PS/PT	η_{app}	*EC*
HC	η_{app}	0·814**	—	—
	EC	0·877**	0·984**	—
	DMA	−0·835**	−0·736*	−0·809**
HN	η_{app}	0·974**	—	—
	EC	0·930**	0·950**	—
	DMA	−0·803**	−0·812**	−0·822**
HO	η_{app}	0·966**	—	—
	EC	0·926**	0·973**	—
	DMA	−0·830**	−0·774*	−0·739*

HC = Control lot.
HN = 3% CLO added.
HO = 3% OCLO added.
** = $P < 0{\cdot}001$.
.* = $P < 0{\cdot}01$.

break down completely during the blending and were retained by the cheese cloth when filtering before measuring (Tejada *et al.*, 1987). In this way, after 245 days, the values of emulsifying capacity (Fig. 5) could be the resultant of both the decrease in EC due to protein modification in frozen storage (Tejada *et al.*, 1987) and the increase in EC due to the dilution of protein in the homogenate (Borderias *et al.*, 1985*b*).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Figures 6(a,b) and 7(a,b) show the electrophoretic patterns of the natural actomyosin isolated from the control sample, no lipids added (HC), cod liver oil (HN)- and oxidized cod liver oil (HO)- treated samples in presence (Fig. 6) and absence (Fig. 7) of 5% β-mercaptoethanol, at the beginning (a) and after 4 months of frozen storage (b). Table 6 shows the myosin heavy chain/actin peak area ratios in the different samples at the beginning and after 4 months of frozen storage (−18°C). In presence of β-mercaptoethanol, the control sample (HC) and that with neutral lipids (HN) did not show appreciable differences in the two periods studied, while in the sample with oxidized lipids (HO) the ratio decreased because the band corresponding to the myosin heavy chain has a tendency to disappear during the storage time. This was more apparent when studied in the absence of β-mercaptoethanol.

TABLE 6
SDS-PAGE Electrophoresis: Myosin Heavy Chain/Actin Peak Areas Ratio in Frozen Stored (−18°C) Minced Hake

	β-mercaptoethanol added		*No β-mercaptoethanol added*	
	0 days	*4 months*	*0 days*	*4 months*
HC	2·1	2·2	2·0	1·9
HN	2·2	2·1	2·8	2·2
HO	2·2	1·0	3·1	0·4

HC = Control lot.
HN = 3% CLO added.
HO = 3% OCLO added.

The results indicate that differences exist between the types of interactions responsible for the aggregation of proteins in the presence or absence of oxidized lipids although they do not lead to a further decrease in the functional properties in this sample. The type of interactions involved in the sample with oxidized lipids added seems to be mainly due to covalent links rather than secondary forces. These results are in agreement with those of Takama (1974*a*,*b*). Although the contribution of covalent links cannot be discarded in the control sample, there seem to be a high contribution of non-covalent forces, which were broken with the SDS used in the electrophoresis. These results agree with those of other authors (Owusu-Ansah & Hultin, 1987).

Correlations

High inverse correlations are found between the functional properties studied, solubility, viscosity, emulsifying capacity and the DMA (Table 5), even in the HO lot in which a detrimental effect of oxidized lipids on protein can be expected. However, the correlations with the TBA value were, in all cases, below 95%.

Taking the results as a whole, we consider that the slightly higher functionality of the sample with neutral lipids added concurs with the protective effect of these lipids described by other authors (Stodolnik & Knasiak, 1984; Swiniarska & Wlodarczyk, 1987). However, we have not found equivalent studies in minced fish with oxidized lipids added and, although in model systems a deteriorating effect of lipid oxidation products on proteins has been described (Takama, 1974*a*,*b*), it has not been confirmed by our results. On the contrary, we have found an apparent protective effect by

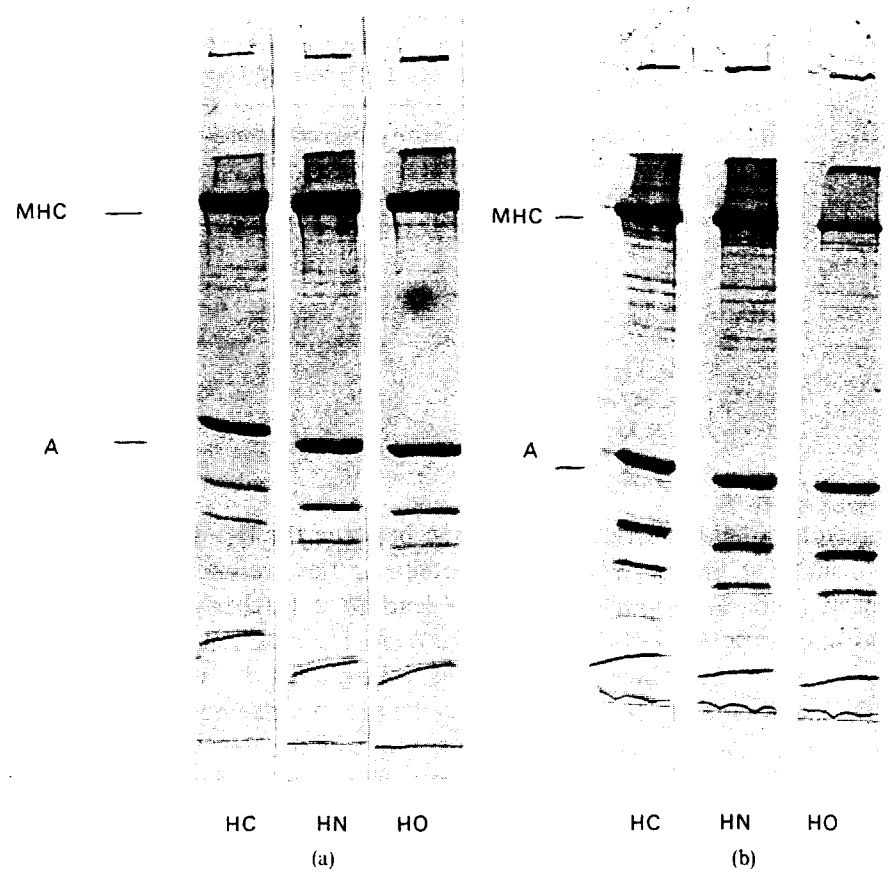

Fig. 6. SDS-polyacrylamide gel electrophoresis (10%) (SDS-PAGE) of the natural actomyosin of minced hake. Samples treated for electrophoresis with 5% β-mercaptoethanol. (a) at 0 days, (b) after 120 days of frozen storage ($-18^{\circ}C$). HC, control lot; HN, 3% CLO added; HO, 3% OCLO added. MHC = myosin heavy chain; A = actin.

oxidized lipids on protein functionality when comparing with the control lot. This behaviour we think is due to the lower rate of DMA and FA formation in the lots with lipids added.

These inverse relationships between the degree of lipid oxidation (Fig. 2) and DMA formation (Fig. 1) suggest the existence of an interference mechanism between the reactions of lipid oxidation and of degradation of TMAO and would explain the values of DMA found by Castell (1971) for species with different free fatty acid contents. Thus, any agent able to decrease the formation of FA in the fish muscle should extend its functionality for a longer period of time. Our results also suggest that the protective role attributed to neutral lipids in semi-fatty and fatty species could be due to an inhibition of FA formation, and not only to a direct

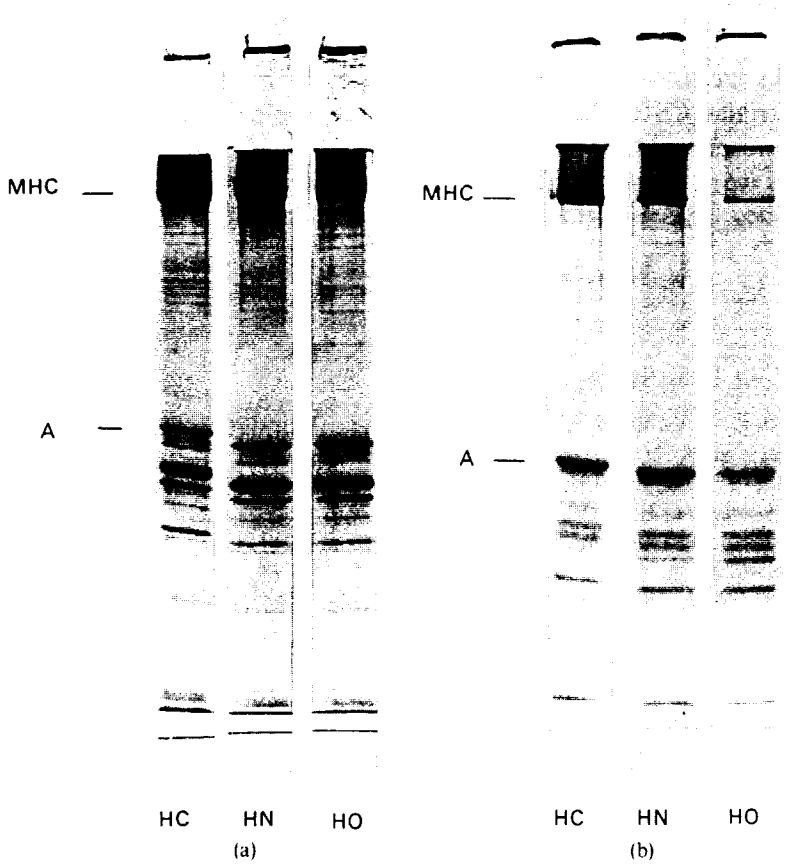

Fig. 7. SDS-polyacrylamide gel electrophoresis (10%) (SDS-PAGE) of the natural actomyosin of minced hake. Samples treated for electrophoresis without β-mercaptoethanol, (a) at 0 days, (b) after 120 days of frozen storage (-18 °C). HC, control lot; HN, 3% CLO added; HO, 3% OCLO added. MHC = myosin heavy chain; A = actin.

protective effect of neutral lipids on proteins. In fact, studies made by other authors, in which it was found that neutral lipids protect fish from deterioration, were mainly made in DMA- and FA-forming species in frozen storage.

The fact that we have found a lower formation of DMA and thus of FA when the lots are more oxidized leads us to consider the role of oxygen in the inhibition of the TMAO degradation from a new perspective.

Work is in progress on establishing the role of added lipids in systems with isolated proteins or in other species in which deterioration during frozen storage cannot be attributed to FA action in order to demonstrate the existence of an interference mechanism between the reactions of lipid oxidation and degradation of TMAO as has been postulated in this paper.

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