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Effect of functional edible films and high pressure processing on microbial and oxidative spoilage in cold-smoked sardine (*Sardina pilchardus*)

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Abstract

The present experiment was an attempt to improve the shelf-life of cold-smoked sardine (*Sardina pilchardus*) using, singly or in combination, high pressure (300 MPa/20 °C/15 min) and gelatin-based functional edible films enriched by adding an extract of oregano (*Origanum vulgare*) or rosemary (*Rosmarinus officinalis*) or by adding chitosan. The uncoated muscle itself exhibited a certain level of antioxidant power (as measured by the FRAP method) ensuing from the deposition of phenols during smoking. Coating the muscle with the films enriched with the oregano or rosemary extracts increased the phenol content and the antioxidant power of the muscle, particularly when used in association with high pressure, due to migration of antioxidant substances from the film. The edible films with the added plant extracts lowered lipid oxidation levels (as measured by the peroxide and TBARS indices) and also, to a lesser extent, reduced microbial growth (total counts), whereas the gelatin–chitosan-based edible films lowered microbial counts (total counts, sulphide-reducing bacteria). Neither luminescent bacteria nor *Enterobacteriaceae* were detected in any of the batches. The combination of high pressure and edible films yielded the best results in terms of both preventing oxidation and inhibiting microbial growth.

Keywords: Edible film; High pressure; Cold-smoked sardine; Natural antioxidants; Chitosan

1. Introduction

Smoking has traditionally been used to preserve fish. Today, however, it is employed not so much as a preservation method but as a food processing technique to diversify the supply of available products and enhance added value. At the same time, consumers demand products with lower salt and smoke contents, and as a result the shelf-life of smoked products has been substantially shortened, being limited principally by microbial growth (Leroi, Joffraud, Chevalier, & Cardinal, 2001) and lipid oxidation. In recent years, studies have focussed on new storage methods aimed at extending the shelf-life and improving the quality of smoked fish products. Accordingly, high pressures have been employed of late to preserve various smoked fish species (Gómez-Estaca, Gómez-Guillén, & Montero, 2006; Lakshmanan, Miskin, & Piggott, 2005). Other possibilities that have not yet been used for smoked fish products include plant extracts (Giménez, Roncalés, & Beltrán, 2004, 2005; Mejholm & Dalgaard, 2002) and edible coatings (Sathivel, 2005; Stuchell & Krochta, 1995), which have yielded good results for fresh and frozen meats and fish. Furthermore, antioxidant extracts can be included in the formulations for edible coatings (Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004; Zivanovic, Chi, & Draughon, 2005) and hold out further promise.

A number of workers have characterized the compositions of a broad range of plant extracts and their antioxidant properties, closely related to their high phenol contents (Shahidi, Janitha, & Wanasundara, 1992; Zheng & Wang, 2001), as well as their antimicrobial properties

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against pathogens and spoilage organisms (del Campo, Amiot, & Nguyen-The, 2000; Hammer, Carson, & Riley, 1999; Lambert, Skandamis, Coote, & Nychas, 2001). Extracts of this kind have been applied directly to help preserve meat (Djenane, Sánchez-Escalante, Beltrán, & Roncalés, 2003; MacNeil, Dimick, & Mast, 1973) and refrigerated (Giménez et al., 2004, Giménez, Roncalés, & Beltrán, 2005; Mejholm & Dalgaard, 2002; Tassou, Drosinos, & Nychas, 1995) and frozen (Serdaroglu & Felekoglu, 2005; Vareltzis, Koufidis, Gavriililidou, Papavergou, & Vasiliadou, 1997) fish, and in most instances did exhibit the preliminary antioxidant and antimicrobial effects found earlier *in vitro*. In the case of smoked fish, plant extracts may also impart certain slight flavour notes that can be highly appreciated from the standpoint of taste.

Edible coatings can be prepared from a wide variety of raw materials, including polysaccharides, proteins and lipids (Gennadios, Hanna, & Kurth, 1997). Coatings may be applied directly to the foodstuff (Sathivel, 2005; Stuchell & Krochta, 1995), or they may be made into films that are then used to coat the surface of the food (Oussalah et al., 2004). The action of these coatings as a barrier to the passage of oxygen and water, thereby slowing oxidation reactions and retaining moisture, is the main mechanism used by coatings to enhance quality and extend storage life (Gennadios et al., 1997). Furthermore, adding plant extracts gives the coatings antimicrobial and antioxidant properties. Accordingly, the antimicrobial and/or antioxidant properties of a number of film-forming agents, such as chitosan, milk whey proteins and soy proteins, enriched with oregano, rosemary, pimento, tea, or garlic extracts, have been tested both *in vitro* and on such foodstuffs as sliced bologna and whole beef muscle (Chi, Zivanovic, & Penfield, 2006; Kim et al., 2006; Oussalah et al., 2004; Seydim & Sarikus, 2006). In this study, gelatin or a gelatin–chitosan mixture was used to make the edible films, because chitosan not only acts as a structural agent (Jeon, Kamil, & Shahidi, 2002) but also possesses antimicrobial (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001) and antioxidant (Xing et al., 2004) attributes. Oregano and rosemary were used as sources of natural antioxidants, inasmuch as their antimicrobial and antioxidant properties have been well documented (del Campo et al., 2000; Lambert et al., 2001).

High pressure is known to delay the onset of spoilage of meat and fish by inhibiting microbial growth (Cheftel & Culioli, 1997), and for that reason it has been used with good results to help preserve a number of species, such as salmon, hake, octopus, shrimp and squid (Amanatidou et al., 2000; Hurtado, Montero, & Borderías, 2000, 2001; López-Caballero, Pérez-Mateos, Montero, & Borderías, 2000; Paarup, Sánchez, Peláez, & Moral, 2002). Nevertheless, application of high pressures to smoked fish is a recent development, and as a consequence the literature is limited (Gómez-Estaca et al., 2006; Lakshmanan & Dalgaard, 2004; Lakshmanan et al., 2005). Additionally, high pressure has yielded good results as a means of inactivating major pathogens present during the storage of smoked fish, such as *Listeria monocytogenes* (Lakshmanan & Dalgaard, 2004; Montero, Gómez-Estaca, & Gómez-Guillén, 2007).

The objective of this study was to enhance the microbiological and oxidative stability of cold-smoked sardine muscle during chilled storage by using, singly or in combination, an edible gelatin film-coating enriched with an oregano or a rosemary extract, a gelatin–chitosan film-coating, and/or high pressure.

2. Materials and methods

2.1. Salting and smoking

Sardine (*Sardina pilchardus*), caught during the month of March, were purchased at local markets. They were headed and gutted, cut into butterfly fillets, and washed in cold water. Salting was performed by immersion in brine (15 g NaCl/100 ml) at 20 °C for 10 min. The fillets were then rinsed under tap water and placed in the smoker [Eller model Micro 40 (Merino, Italy)]. They were first dried at 28 °C ± 1 °C for 12 min to remove excess moisture and then smoked using beech wood for 6 min, followed by drying for a further 50 min at the predetermined temperature, to achieve the desired final moisture content. The smoked sardine fillets (76.97% ± 0.21% moisture, 1.58% ± 0.21% fat, and 3.00% ± 0.10% salt in the muscle) were held under refrigeration (1 °C ± 1) overnight to allow time for diffusion of the salt and smoke components, after which they were skinned and sliced.

2.2. Preparation of the antioxidant extracts

Freeze-dried oregano (*Origanum vulgare*) and rosemary (*Rosmarinus officinalis*) were purchased at local markets. Quantities of 5 and 20 g, respectively, per 100 ml were mixed with distilled water that had been pre-warmed to 45 °C and extracted by continuous stirring in a warm water bath at 45 °C for 10 min. The aqueous extract thus obtained was filtered through Whatman no. 1 filter paper. Total phenol content and total reducing power (FRAP) of pure extracts were determined according to the methods described below. Results were: for phenol content 250 ± 1.01 and 4.7 ± 0.02 μg phenol/ml of extract for oregano and rosemary, respectively, and for antioxidant power (expressed as equivalent concentration of $FeSO_4 \cdot 7H_2O$ (mM)) 57.0 ± 0.1 and 92.9 ± 0.2 for measurements at 4 and 30 min, respectively, for oregano, and 10.1 ± 0.05 and 17.2 ± 0.1 for rosemary.

2.3. Formulation of the edible films

The oregano and rosemary film forming solutions (FFSs) were prepared using gelatin (from pigskin, Bloom 200/220, 4% w/v) and sorbitol and glycerol (15% of the gelatin each) as plasticizers. An oregano extract or a rosemary

extract was added to final concentrations of 1.25% and 20%, respectively, using distilled water to prepare the dilutions. These concentrations, which were selected according to previous experiments, were employed in an endeavour to obtain a similar phenol content and antioxidant power, according to determinations on pure extracts. The gelatin–chitosan FFS was prepared by mixing a 4% gelatin solution with a 3% solution of chitosan in 0.15 M acetic acid, in a proportion of 1:1 (v/v) to obtain a FFS containing 2% gelatin and 1.5% chitosan, and the pH was then adjusted to 5.5 with 1 M NaOH. Plasticizers were added to a concentration of 15% of the total gelatin and chitosan. All mixtures were warmed and stirred at 40 °C for 15 min to obtain a good blend, and the films were made by casting an amount of 40 ml over a plate of 144 cm^2 and drying at 45 °C in a forced-air oven for 15 h to yield a uniform thickness [100 μm ($P \leqslant 0.05$)] in all cases. Prior to coating the fish, films were conditioned over a saturated solution of KBr in desiccators for 3 d.

2.4. High pressure processing

The fish samples were packed in sealed bags (Cryovac BB4L, Barcelona, Spain) and pressurized at 300 MPa/20 °C/15 min. Pressurization took place in a pilot high-pressure unit (ACB 665, GEC Alsthom, Nantes, France). The temperature of the immersion medium (distilled water) was regulated by a thermocouple connected to programmed thermostatting equipment (model IA/2230 AC, INMASA, Barcelona, Spain). The pressure was increased by 2.5 MPa/s and, after pressurization, samples were depressurized back to atmospheric pressure and recovered in around 2 s. Following the pressure processing the bags were opened so that the pressurized batches could be stored under the same no-vacuum packaging conditions as the non-pressurized batches.

In preparation for the different experimental treatments tested, the fish slices were placed between two layers of edible film so that all sample slices were coated on both sides with one of the films. In all, there were six batches (two pressurized) testing various combinations of conditions, (i) batch C, smoked but uncoated; (ii) batch O, smoked and coated with the oregano extract-enriched film; (iii) batch R, smoked and coated with the rosemary extract-enriched film; (iv) batch Q, smoked and coated with the chitosan-enriched film; (v) batch CHP, smoked and pressurized, uncoated; (vi) batch OHP, smoked, coated with the oregano extract-enriched film, and then pressurized. All batches were stored chilled at 5 °C in clean bags.

2.5. Determination of the peroxide and the free fatty acids indices

Lipid extraction was according to the method of Bligh and Dyer (1959). The peroxide index was assayed according to UNE standard 55-023-73 (1973), and the results were expressed in milliequivalents of peroxides per kg of fat. The free fatty acid (FFA) determination employed the method recommended by the A.O.C.S. (1955), and the results were expressed as percentage free oleic acid. All determinations were performed at least in duplicate.

2.6. Determination of the 2-thiobarbituric acid index

A modified version of the method of Vyncke (1970) was employed, incubating at 20 °C for 15 h instead of heating. A standard curve was prepared using 1,1,3,3-tetraethoxypropane as by Botsoglou et al. (1994). The results were expressed as mg of malonaldehyde per 100 g of fat. All determinations were performed at least in triplicate.

2.7. Total phenol determination

The method described by Cardinal et al. (2004) was used, and the results were expressed as mg of phenol per kg of muscle. All determinations were performed at least in triplicate.

2.8. Antioxidant power measurement: FRAP method

The ferric reducing/antioxidant power (FRAP) assay was used as a measure of the reducing ability of the muscle, following the method of Benzie and Strain (1996). The method is based on increased absorbance at 595 nm due to formation of the tripyridyltriazine (TPTZ)-Fe(II) complex in the presence of tissue reducing agents. Absorbance was read after 4 and 30 min. The results were expressed as the concentration of $FeSO_4 \cdot 7H_20$ mM per mg of muscle, based on a standard curve for ferrous iron prepared in advance. All determinations were performed at least in triplicate.

2.9. Total volatile basic nitrogen

The total volatile basic nitrogen (TVBN) determination was effected according to the method of Antonacopoulos and Vyncke (1989). The results were expressed as mg of N per 100 g of muscle. All determinations were performed at least in triplicate.

2.10. Microbiological assays

An amount of 25 g of muscle per batch was mixed with 225 ml of buffered 0.1% peptone water (Oxoid, Basingstoke, UK) in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK). This mixture was homogenized in a Stomacher blender (model Colworth 400, Seward, London, UK) for 1 min, and appropriate dilutions were prepared for the following microorganism counts: (i) total bacteria [APC (aerobic plate counts)] on 1% NaCl Iron Agar (Scharlau, 01584) spread plates, incubated at 15 °C for 3 d according to the method described by Gram, Trolle, and Huss (1987), as modified by Dalgaard, Mejholm, Christiansen, and Huss (1997); (ii) H_2S-reducing organisms, likewise on 1% NaCl

Iron Agar spread plates, incubated at 15 °C for 3 d; (iii) luminescent bacteria, again on 1% NaCl Iron Agar spread plates, incubated at 15 °C for 5 d, with counts being effected in a dark room; (iv) *Enterobacteriaceae*, on double-layer Violet Red Bile Glucose (VRBG) Agar (Oxoid) pour plates, incubated at 30 °C for 48 h (Anonymous, 1999). All microbiological counts were performed in duplicate, and the results were expressed as the log of the number of colony-forming units per g (log CFU/g) of muscle.

2.11. Statistical analysis

One-way and two-way analyses of variance were carried out. The SPSS® computer programme (SPSS Inc., Chicago, IL, USA) was used, and differences between pairs of mean values were evaluated by the Tukey-B test using a 95% confidence interval.

3. Results and discussion

3.1. Phenol content and antioxidant activity

Table 1 summarizes the results for the total phenols and the antioxidant power of the cold-smoked sardine muscle. Various compounds in the smoke are deposited in the muscle during smoking. These include phenols, whose antioxidant properties have been documented (Daun, 1969). Batch CHP had a higher phenol content than had batch C, leading to higher antioxidant power values, although differences were not significant ($P \leqslant 0.05$) due to the fact that O and OHP batches presented very much higher values than did the others. The higher values recorded on CHP batch could be due to the greater diffusion of the phenolic compounds under high pressure, which contributes to a certain breakdown of muscle structure (Montero & Gómez-Guillén, 2005).

Applying a complex film to a foodstuff can result in migration of compounds from the film into the food (Chi et al., 2006), and in consequence batches O, and OHP had significantly higher ($P \leqslant 0.05$) levels of phenols in the muscle, from the films, and hence they also had higher antioxidant activity levels. Comparing the results of antioxidant power for the batches coated with the oregano and rosemary extract-enriched films revealed similar values, despite the latter having much lower phenol values ($P \leqslant 0.05$). This is indicative of quantitative and qualitative differences in the nature of the phenolic compounds present in the extracts from these two plants. Comparing batches O and OHP, in turn, showed higher total phenols and higher total antioxidant power ($P \leqslant 0.05$) in the latter batch, attributable to the release of higher levels of antioxidant compounds from the film to the muscle as a consequence of pressurization.

The batches coated with the chitosan-enriched film differed hardly at all from the control batch, but in fact no increase in the total phenols was to be expected. Unlike what happen in the case of the plant extracts, the antioxidant power of chitosan is due, not to its reducing power, but rather to its ability to prevent the formation of reactive oxygen species (ROS) via metal chelation (Kamil, Jeon, &

Table 1
Total phenols (mg phenol/kg muscle) and total antioxidant activity by the FRAP method (mM $FeSO_4 \cdot 7H_2O$/mg muscle) in the different batches of cold-smoked sardine during storage: control (C), coated with oregano extract-enriched film (O), coated with rosemary extract-enriched film (R), coated with chitosan-enriched film (Q), pressurized control (CHP), coated with oregano extract-enriched film and pressurized (OHP)

Batch	FRAP 4 min				FRAP 30 min				Phenols			
	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20
C	34.3	27	26.3	28.0	59.1	47.4	50.4	53.5	17.7	15.1	15.5	12.1
	(5.8)	(1.4)	(3)	(3.7)	(8.2)	(2.3)	(5.2)	(5.8)	(1.6)	(0.4)	(1.4)	(0.5)
	ax	bx	bx	bx	ax	bx	abx	abx	ax	axy	ay	bx
	137	124	135	108	274	254	284	204	209	143	223	177
O	(23.8)	(15.1)	(12.4)	(11.2)	(48)	(35.5)	(18.7)	(24.8)	(36.1)	(4.6)	(12.4)	(26.1)
	az	abz	azwv	bw	az	az	aw	bw	az	bw	aw	abw
	131	141	145	138	197	210	215	211	26.0	26.8	34.7	34.6
R	(13.9)	(19)	(10.3)	(17.6)	(19.7)	(30.5)	(12.9)	(21.5)	(2.8)	(4.3)	(1.8)	(1.0)
	az	az	av	av	aw	aw	av	aw	ay	az	by	bz
	41.3	23.6	19.1	21.4	62.3	43.3	38.2	42.8	20.7	11.1	11.2	12.1
Q	(4.5)	(1.4)	(3.5)	(1.8)	(7)	(2.5)	(5.3)	(2.1)	(1.5)	(1.0)	(0.9)	(0.3)
	ax	bx	by	by	ax	bx	by	by	ax	bx	bx	bx
	55.1	41.1	42.3	37.5	86.8	67.2	73.5	66.9	21.2	19.0	20.8	18.2
CHP	(6.8)	(5.9)	(2.9)	(3)	(11.3)	(7.5)	(4.4)	(2.4)	(1.8)	(0.2)	(0.3)	(1.1)
	ay	by	bz	bz	ay	by	bz	bz	ax	aby	abz	by
	174	165	130	150	314	336	272	296	369	234	260	273
OHP	(13.2)	(22.8)	(13.6)	(13.2)	(27.4)	(36.6)	(28.4)	(22)	(20.7)	(19.1)	(19.0)	(18.8)
	aw	aw	bw	abv	abv	bv	aw	abv	aw	bv	bv	bv

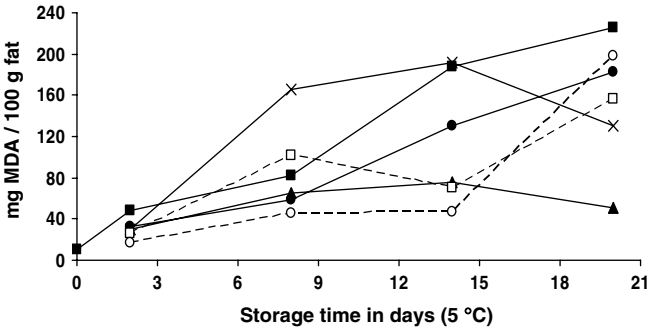
Results are the mean values with the standard deviation in brackets. Different letters (a, b, c) represent significant differences between sampling dates for a given batch. Different letters (w, x, y, z) represent significant differences between batches on the different sampling dates.

Shahidi, 2002), heme iron playing a particularly important role and being deemed to be one of the main factors causing oxidation in fish because of its catalytic effects (Igene, King, Pearson, & Gray, 1979; Rhee, Ziprin, & Rhee, 1979). Some researchers have also reported that chitosan is capable of scavenging ROS directly (Xing et al., 2004; Xue, Yu, Hirata, Terao, & Lin, 1998). The FRAP method is not able to disclose either of these antioxidant mechanisms.

Only minor fluctuations in phenol content were observed in the muscle during storage. On the whole there was a slight tendency for the phenol content to decrease in the uncoated batches (C and CHP), perhaps because of consumption of the phenols by oxidation reactions or because of slight exudation losses. There were differences in the behaviour of the coated batches, though in both batches, O and R, phenols were released at the beginning of storage. Levels held steady ($P \leqslant 0.05$) with slight fluctuations in the batches coated with the oregano extract-enriched film (O and OHP), whereas they increased about midway through storage in batch R. This is suggestive of a gradual release of antioxidant components mediated by the rosemary-enriched film. Oussalah et al. (2004) assessed the availability of antioxidants carried in films in beef muscle stored for seven days, and they observed increasing availability at first, after which concentrations levelled off or decreased slightly, depending on the source of the antioxidant employed. Chi et al. (2006) enriched chitosan-based films with the essential oil of oregano and reported diffusion of the active components, primarily carvacrol, into the food (sliced bologna). In contrast, the phenol content of the muscle decreased over one week in batch Q, possibly because of scavenging of phenols by the film at the start of storage through an exchange of components between the fish muscle and the film. On the other hand, levels then held constant until the end of storage.

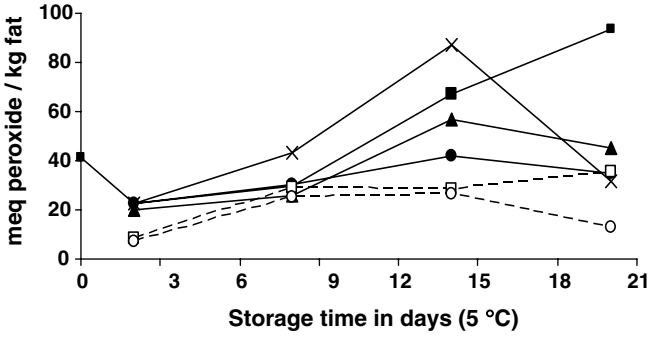
3.2. Lipid oxidation

The TBARS index (Fig. 1) revealed initial oxidation of the smoked sardine to be low on day 0 and then to increase progressively as storage advanced (batch C). Coating with edible films enriched with an oregano or a rosemary extract lowered the oxidation rate, particularly in the case of the latter film. By comparison, the gelatin–chitosan film did not exhibit any protective effect. The peroxide index (Fig. 2) pointed to similar results, with increasing levels of oxidation in the control batch and lower levels in the batches coated with the films enriched with the oregano or rosemary extract. The high levels recorded on day 0 were attributable to smoking, during which large quantities of peroxides are formed (Beltrán & Moral, 1991). However, after two days, the peroxides had broken down into secondary oxidation products, as evidenced by the higher TBARS index values (Fig. 1). Use of natural plant extracts to prevent lipid oxidation in fish has been described in the literature. Giménez et al. (2004, 2005) used rosemary



	Batch	Storage time in days 0	2	8	14	20
■—	C	a	bx	cx	dx	ew
●—	O	-	ay	bx	cy	dzw
▲—	R	-	azy	bx	bz	cx
×—	Q	-	azy	by	bx	cy
·□·	CHP	-	azy	bx	bz	cyz
·○·	OHP	-	az	ax	az	bzw

Fig. 1. Thiobarbituric acid reactive substances (TBARS) in cold-smoked sardine (CSS): control CSS (batch C), oregano extract-enriched film-coated CSS (batch O), rosemary extract-enriched film-coated CSS (batch R), gelatin–chitosan film-coated CSS (batch Q), high pressure-processed CSS (batch CHP), and oregano extract-enriched film-coated, high pressure-processed CSS (batch OHP) during chilled storage (5 °C). Results have been expressed as mg of malonaldehyde (MDA) per 100 g of fat. Different letters (a, b, c) represent significant differences between sampling dates for a given batch. Different letters (x, y, z) represent significant differences between batches on the different sampling dates.



	Batch	Storage time in days 0	2	8	14	20
■—	C	a	bx	cx	dx	ew
●—	O	-	ay	bx	bz	cx
▲—	R	-	azy	by	bx	cy
×—	Q	-	azy	by	bx	cy
·□·	CHP	-	azy	bx	bz	cyz
·○·	OHP	-	az	ax	az	bzw

Fig. 2. Peroxide value for the different batches (as in Fig. 1) during chilled storage (5 °C). Results have been expressed as meq of peroxides per kg of fat. Different letters (a, b, c) represent significant differences between sampling dates for a given batch. Different letters (x, y, z) represent significant differences between batches on the different sampling dates.

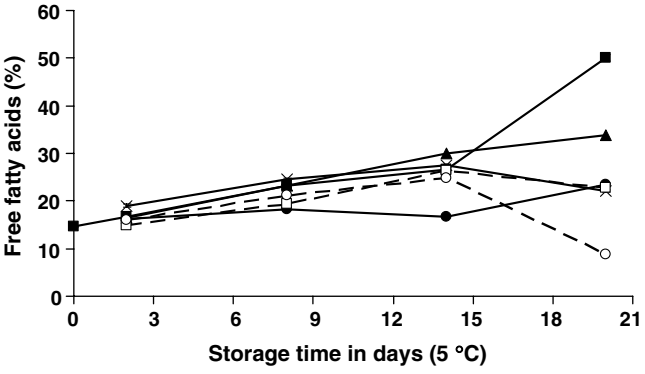
extract to help preserve gilt-head sea bream (*Sparus aurata*) and salmon (*Salmo salar*) fillets and found that lipid oxidation was slowed. Serdaroglu and Felekoglu (2005) and Vareltzis et al. (1997) also reported delayed lipid oxidation when rosemary extract was used as a preservative in the frozen storage of sardine and mackerel mince. Tsimidou, Papavergou, and Boskou (1995) studied the antioxidant activity of oregano on mackerel oil and found greatly slowed lipid oxidation rates similar to the rate obtained using rosemary.

There are very few reports dealing with edible films containing natural plant extracts and, what is more, most of those that have been published focus on the physical, chemical and/or antimicrobial properties of the films, not on their antioxidant effects when applied to foodstuffs. Oussalah et al. (2004) developed milk whey protein-based films enriched by adding the essential oil of oregano or pimento. In any case, the antioxidant potential of such films notwithstanding, they did not record any protective effect against oxidation in beef muscle, unlike the findings reported here. The reason for this difference might be ascribable to the differing natures of meat and fish lipids, given that fish lipids are known to be more highly unsaturated and accordingly more vulnerable to oxidation; and indeed, in their study Oussalah et al. (2004) reported low levels of oxidation. Furthermore, the antioxidant extracts were also different in nature, in that we used an aqueous extract while Oussalah et al. (2004) used a lipophilic extract. Chi et al. (2006) reported diffusion of the active components from the film (chitosan enriched with essential oil of oregano) to the foodstuff (sliced bologna), but they did not carry out any assessment of oxidative stability.

Unlike the case of the batches coated with the films enriched with the oregano or rosemary extracts, oxidation levels in the batch coated with the gelatin–chitosan film were similar to or higher than those in the control batch, even though other workers (Jeon et al., 2002) have reported lower TBARS contents over storage in chitosan-coated herring and cod samples than in uncoated samples. The differences may have been due to the substrate used in that study, namely, fresh muscle, which is more readily oxidized than is smoked muscle, thereby giving rise to more appreciable differences. Additionally, scavenging of phenols by the film, as mentioned in the preceding section, may have boosted oxidation rates compared with the control batch. Similarly, López-Caballero, Gómez-Guillén, Pérez-Mateos, and Montero (2005) failed to find changes in TBARS levels in lean fish patties coated with chitosan.

The oxidative effect of high pressure has been well documented, both in fresh (Cheftel & Culioli, 1997) and smoked muscle. For instance, Lakshmanan et al. (2005) reported an appreciable increase in TBARS levels during chilled storage of pressurized (300 MPa) cold-smoked salmon. In the study reported here, in contrast, pressurization did not result in lipid oxidation as measured by the TBARS and peroxide indices (Figs. 1 and 2), and the pressurized batches remained quite stable at levels below that for the control batch throughout storage under the same no vacuum-packaging conditions. This was particularly true for coated batch OHP, which nonetheless underwent a sharp increase in levels in week 3. Pressurization may enable phenolic compounds to diffuse into the muscle more readily, thereby promoting interaction between the phenols and the lipids and thus accounting for the lower oxidation index values. The lower oxidation index levels are also consistent with the higher phenol concentrations and the higher degree of antioxidant activity recorded. In a previous study on smoked dolphinfish (Gómez-Estaca et al., 2006), no appreciable oxidative differences were found as a consequence of the effect of pressurization or during storage, and the antioxidant effect was ascribed to the phenols acquired during smoking. Discussing the combined action of natural antioxidants and high pressure, Montero, Giménez, Pérez-Mateos, and Gómez-Guillén (2005) noted that phenol-based antioxidants, such as rosemary and quercetin extracts, protected pressurized homogenized fish muscle from lipid oxidation.

Percentage FFAs is a measure of the degree of lipid hydrolysis, hydrolyzed lipids being more susceptible to oxidation. The percentage FFAs increased progressively in all the batches (Fig. 3) as storage advanced, a finding attributed to the action of lipases. Batch O was an exception, with levels holding steady for the first two weeks. There were no significant differences ($P \leqslant 0.05$) between the pressurized batches (CHP and OHP) and the control batch (C) in the first two weeks, which indicated that the pressure



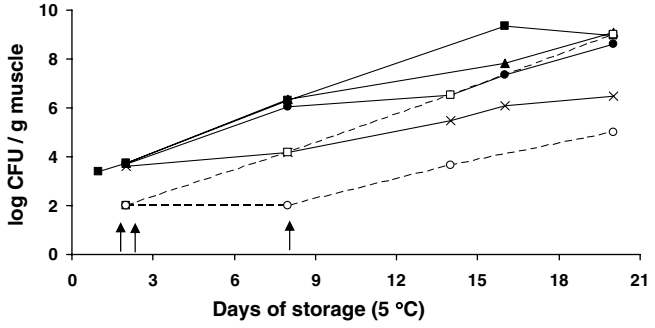
Batch	Storage time in days				
	0	2	8	14	20
C	a	bxy	cw	dyz	fv
O	-	axy	bx	ax	cz
R	-	axy	bw	cw	dw
Q	-	ay	bv	cz	dy
CHP	-	ax	by	cyz	dyz
OHP	-	axy	bz	by	cx

Fig. 3. Free fatty acids in the different batches (as in Fig. 1) during chilled storage. Results have been expressed as percentage oleic acid. Different letters (a, b, c) represent significant differences between sampling dates for a given batch. Different letters (x, y, z) represent significant differences between batches on the different sampling dates.

level applied failed to inactivate the lipase enzymes. Seyderhelm, Boguslawski, Michaelis, and Knorr (1996) found lipase enzymes to be stable at pressures up to 600 MPa, and He, Adams, Farkas, and Morrissey (2002) likewise did not observe inhibition of lipase activity in oysters pressurized at 200–300 MPa. However, by day 20, a significant decrease in FFAs was recorded in the pressurized batch coated with the oregano extract-enriched film (batch OHP), and this finding was consistent with the observed decrease in the peroxide index value (Fig. 2) and the pronounced increase observed in the TBARS index (Fig. 1), though in all cases values were lower than for the control batch.

3.3. Microbial spoilage

Initial total microorganism counts were 10^3 CFU/g, increasing to 10^9 CFU/g on day 20 in batch C (Fig. 4). Coating the fish with films enriched with an oregano or a rosemary extract slowed microbial growth by 1.99 and 1.54 log cycles, respectively, on day 16, though by day 20 counts had recovered to levels similar to those recorded in the control batch, in which growth reached the stationary phase. The antimicrobial effects of plant extracts on a wide range of microorganisms have been amply described (Dorman & Deans, 2000; Hammer et al., 1999), with oregano and rosemary being two plants that have been particularly well documented (del Campo et al., 2000; Lambert et al., 2001). As a consequence, plant extracts have been used to preserve meat and fish products, both for their antimicrobial effects and for their antioxidant effects. Mejholm and Dalgaard (2002) tested, in fish, the antimicrobial effects of nine essential oils against *Photobacterium phosphoreum*, which has been identified as one of the most important fish spoilage bacteria, and found oregano and cinnamon to have strong antibacterial effects, capable of extending shelf-life when applied to naturally contaminated fresh cod fillets packed in a modified atmosphere. In contrast, Giménez et al. (2004, 2005) did not observe any reduction in microbial counts when a rosemary extract was added to chilled gilt-head sea bream (*S. aurata*) or salmon (*S. salar*) fillets likewise packed in a modified atmosphere.



	Batch	Days of storage 0	2	8	14	16	20
■—	C	a	ay	bz	-	cz	cz
—●	O	-	ay	bz	bz	cy	cz
—▲	R	-	ay	bz	-	cy	dz
—×	Q	-	ay	ay	by	bx	by
···□···	CHP	-	ax	by	cz	-	dz
···○···	OHP	-	ax	ax	bx	-	cx

Fig. 4. Aerobic plate counts (15 °C) for the different batches (as in Fig. 1) during chilled storage (5 °C). Results have been expressed as log CFU per g of muscle. Arrows indicate levels below the detection threshold. Different letters (a, b, c) represent significant differences between sampling dates for a given batch. Different letters (x, y, z) represent significant differences between batches on the different sampling dates.

The literature is more meagre and more recent on the subject of adding functional extracts to edible films. Seydim and Sarikus (2006) prepared various whey protein-based edible films enriched with essential oils obtained from oregano, rosemary or garlic and tested their antimicrobial activity against various bacterial pathogens in an agar medium. The oregano-enriched film exhibited the strongest antibacterial properties, followed by the garlic-enriched film. Conversely, the rosemary-enriched film did not display any antibacterial attributes at all. Oussalah et al. (2004) tested the antibacterial effects of milk protein-based edible films containing essential oil from oregano or pimento against *Escherichia coli* O157:H7 and *Pseudomonas spp*. in preserving whole beef muscle. They found the film that contained the added oregano extract to be the most effective, achieving reductions of around 1 log unit for each of these bacterial species at the end of storage compared with uncoated samples. Kim et al. (2006) tested the antimicrobial activity of edible soy protein isolate films enriched with green tea extract against several types of bacteria, including food pathogens, on agar media and found *Staphylococcus aureus* to be inhibited at concentrations of 4%. Other workers (Zivanovic et al., 2005) combined the properties of a chitosan film with the essential oil of oregano and observed it to be efficacious against both *L. monocytogenes* and *E. coli* O157:H7. In that study, of several film formulations used to coat the surface of inoculated bologna slices, pure chitosan by itself displayed bactericidal activity, but higher activity was achieved by chitosan film enriched with oregano extract.

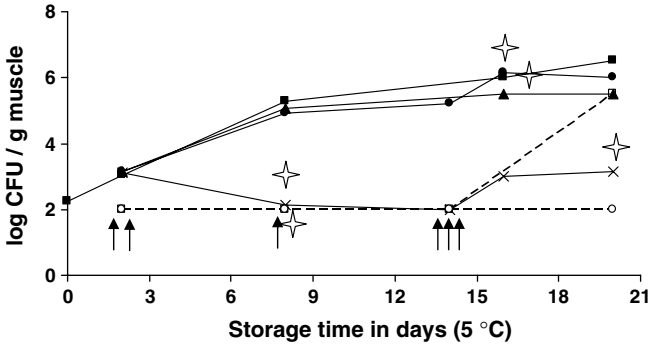
The gelatin–chitosan film exhibited better antimicrobial properties than did the oregano and rosemary-enriched films, yielding aerobic plate counts (APCs) some 2–3 log cycles lower over the course of the entire storage period. López-Caballero et al. (2005) reported APCs about 2 log cycles lower in cod patties coated with a gelatin–chitosan blend after storage for 11 days. Jeon et al. (2002) studied the stability of chitosan-coated herring and cod and recorded reductions of up to 3 and 2 log cycles, respectively, in total counts in the coated batches as compared to the control batches after chilled storage for 12 days. Chen, Liau, and Tsai (1998) applied chitosan to help preserve oysters and found reduced growth of coliform, *Pseudomonas*, *Aeromonas*, and *Vibrio* species that extended

shelf-life by 4–7 days, depending on the type of chitosan employed. The mechanism of action of chitosan appears to involve altering the membrane of Gram-negative bacteria, increasing its permeability and thereby leading to cell death (Helander et al., 2001). In addition, it acts as a barrier to oxygen and in that way inhibits the growth of aerobic microorganisms (Jeon et al., 2002).

High-pressure processing, in turn, resulted in counts below the detection limit (Fig. 4) in batches CHP and OHP (day 2). From that point on, levels rose quickly in batch CHP to about 10^9 CFU/g at the end of storage, similar to the level in batch C (unpressurized). In any case, throughout storage, counts remained about 2 log cycles lower than in batch C in the same no vacuum-packaging conditions. When the smoked sardine slices were coated with the oregano-enriched film (batch OHP), counts were considerably lower than in any of the other batches up until the end of storage. Paarup et al. (2002) observed that, the higher the pressure, the longer was the shelf-life of fresh squid muscle, and similar findings have been described for fresh salmon, hake, octopus, and prawns (Amanatidou et al., 2000; Hurtado et al., 2000, Hurtado, Montero, & Borderías, 2001; López-Caballero et al., 2000). There has been less work on the storage behaviour of high pressure-treated cold-smoked fish. Lakshmanan and Dalgaard (2004) reported that high pressure did not prolong the shelf-life of pressurized cold-smoked salmon but did record a bactericidal effect, in that counts were 1–3 log cycles lower during storage. Gómez-Estaca et al. (2006) observed the same findings for pressurized (300 MPa/15 min/20 °C) cold-smoked dolphinfish.

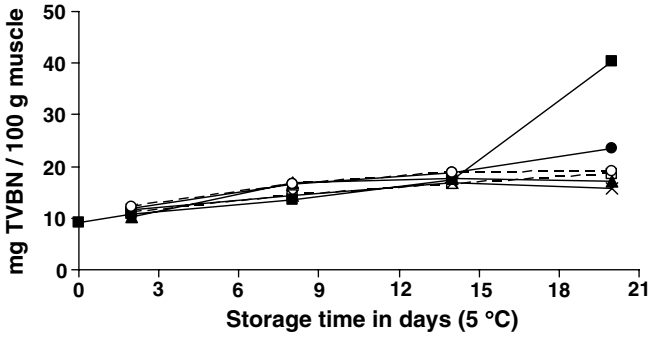
Sulphide-reducing bacteria (Fig. 5) were detectable from the onset of storage and followed a pattern similar to that for the total microorganisms. In batch C, counts rose gradually up to the end of storage, reaching about 10^6 CFU/g. The oregano and rosemary extracts had no effect, but the chitosan lowered levels considerably, to 10^3 CFU/g or lower in all cases. Pressurization kept counts below the detection threshold over the entire storage period, except on day 20 in the uncoated batch, in which levels reached 10^5 CFU/g. In agreement with findings reported for other smoked species (Lakshmanan & Dalgaard, 2004; Montero et al., 2007), luminescent bacteria were not recorded in any of the sample batches and *Enterobacteriaceae* were of minor importance on spoilage or were not detected.

The total volatile basic nitrogen (TVBN) determination (Fig. 6) did not reveal any significant differences between the batches, and the level of 25 mg/100 g was exceeded only in batch C at the end of storage. The European Union (European Commission, 1995) has set an upper limit of between 25 and 35 mg TVBN per 100 g of fish muscle, depending on the species. In any case, to date, no limits have been set for processed products, such as smoked fish.



Batch	Storage time in days					
	0	2	8	14	16	20
C	a	bx	cx	-	dx	ex
O	-	ax	bx	bx	cx	cy
R	-	ax	bx	-	cy	cy
Q	-	ax	by	cy	az	az
CHP	-	ay	az	ay	-	by
OHP	-	ay	aw	ay	-	aw

Fig. 5. Sulphide-reducing microorganisms (15 °C) in the different batches (as in Fig. 1) during chilled storage (5 °C). Results have been expressed as log CFU per g of muscle. Arrows indicate levels below the detection threshold. Stars indicate that one of the replicates was below the detection threshold (the value for the other replicate has been plotted). Different letters (a, b, c) represent significant differences between sampling dates for a given batch. Different letters (x, y, z) represent significant differences between batches on the different sampling dates.



Batch	Storage time in days				
	0	2	8	14	20
C	a	ax	ax	ax	bx
O	-	ax	byz	bx	cy
R	-	ax	bz	bx	by
Q	-	ax	xyab	bx	by
CHP	-	ax	xyb	cx	dy
OHP	-	ax	byz	cx	cy

Fig. 6. Total volatile basic nitrogen (TVBN) in the different batches (as in Fig. 1) during chilled storage (5 °C). Results have been expressed as mg of TVBN per 100 g of muscle. Different letters (a, b, c) represent significant differences between sampling dates for a given batch. Different letters (x, y, z) represent significant differences between batches on the different sampling dates.

4. Conclusions

The stability of cold-smoked sardine muscle was improved by coating the muscle with functional gelatin-based edible films. Films enriched with an oregano or a rosemary extract were able to slow lipid oxidation, but they failed to slow microbial growth. Gelatin–chitosan films were most effective at reducing microbial growth. High-pressure processing improved the microbial quality of the fish during storage but, by the end of storage, microbial counts were the same as in the untreated control batch, and hence a combination of high pressure and coating with a functional film would seem to hold out the most promise. In the experimental combinations of high pressure and coating with film enriched with an oregano extract, micro-organism counts and oxidation indices were kept well below those in the other sample batches tested for at least two weeks of chilled storage under no vacuum-packaging conditions.

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