Effect of Carbon Dioxide and Oxygen Enriched Atmospheres on Microbiological and Chemical Changes in Refrigerated Tuna (*Thunnus alalunga*) Steaks

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Bacterial populations and several physicochemical parameters (metmyoglobin, pH, total volatile nitrogen, nucleotide breakdown products, nonvolatile amines, lipid autoxidation, and short-chain fatty acids) and sensory attributes (odor and color) were measured in refrigerated tuna steaks stored at 2 °C in air, CO_2/air (20/80) (v/v), CO_2/air (40/60) (v/v), and CO_2/O_2 (40/60) (v/v) atmospheres. The effects of modified atmospheres on bacterial growth and physicochemical parameters are discussed. From a microbiological point of view, the shelf life of refrigerated tuna steaks was extended about 1 week when CO_2 enriched atmospheres were used. Metmyoglobin formation was minimum when the atmosphere was enriched with O_2. Therefore, the most effective atmosphere was CO_2/O_2 (40/60) (v/v), in which the shelf life of tuna may be doubled with respect to both microbial condition and color appearance.

Keywords: *Modified atmospheres; shelf life; tuna*

INTRODUCTION

Tuna is a highly valued fish caught all over the world by a variety of fishing methods. In many countries (e.g. the United States) nearly all tuna marketed is canned, although the demand for fresh and frozen steaks and fillets is growing, probably because of the interest in Japanese foods, such as sashimi, and the popularity of tuna as a grilled item in restaurants. In Spain, tuna is very popular and is consumed fresh, canned, and frozen; in 1992, 21 500 tons of fresh refrigerated tuna were sold on the Spanish market, representing 3.8% of total refrigerated fresh fish sales (MAPA, 1993).

In spite of diverse initial microbial populations, as occurs in meat, bacterial spoilage of refrigerated fish aerobically stored results from Gram-negative psychrotrophic organisms (Eklund, 1982; Van Spreekens, 1977) dominated by *Pseudomonas*, *Alteromonas*, and *Shewanella* (Hobbs, 1991; Lindsay et al., 1986). As these floras are effectively inhibited by 20% or more carbon dioxide enriched atmospheres (Clark and Lentz, 1969; Christopher et al., 1979), they have been increasingly used in recent years in the distribution of red meats, poultry, and seafood (Reddy et al., 1992; Davis, 1993). In some instances, such as red meats, the atmosphere is also enriched in oxygen (60–80%) to inhibit metmyoglobin formation (McDougall and Taylor, 1975; Ordóñez and Ledward, 1977; Asensio et al., 1988), which delays the meat surface discoloration problem (Finne, 1982). In Europe, modified atmosphere packaged (MAP) fish by end use represented 8% of the market in 1986 and 12% in 1990 (Brody, 1993) and is expected to increase further in the future.

The use of modified atmosphere to extend fish shelf life has been studied by many authors, and some excellent reviews have recently been published (Stammen et al., 1990; Skura, 1991; Reddy et al., 1992; Davis, 1993). Unlike other fish species, tuna meat has a darker color because it contains a considerable amount of myoglobin (Wheaton and Lawson, 1985). For this reason, tuna bears a greater resemblance to red meat than other fish and is probably one of the factors that must be controlled if the shelf life is to be extended by using modified atmospheres. However, in contrast with meat, tuna contains trimethylamine oxide (TMAO), although it is present in much lower concentrations than in other species (Hebard et al., 1982). The reduction of TMAO yields trimethylamine (TMA), which, together with other volatile derivatives, contributes to the off-odor of spoiled fish (Liston, 1980; Hebard et al., 1982).

This work was undertaken to clarify the above points and because the use of MAP on tuna has not yet been investigated (Reddy et al., 1992; Davis, 1993). The objective was to study the effect of three modified atmospheres on both bacterial floras and several physicochemical parameters that may limit the shelf life of refrigerated tuna.

MATERIALS AND METHODS

Preparation and Storage of Samples. Portions (about 5 kg) from the central part of the body, without gut, of three tuna (*Thunnus alalunga*) were purchased from a local fishmonger. They were transported to the laboratory in crushed ice in less than 1 h. Once at the laboratory, samples 2–3 cm thick were immediately made with a sterile knife in a walk-in cold room (2 °C). The steaks from both ends were discarded. Samples were divided into four batches to be packaged in four different atmospheres. Gas mixtures were supplied by Carburos Metálicos S.A. (Barcelona, Spain) and consisted of CO_2/air (20/80) (v/v), CO_2/air (40/60) (v/v), CO_2/O_2 (40/60) (v/v), and air that was used as control. All tuna samples were individually packaged in laminated film bags (25 × 30 cm) of low gas permeability (Cryovac BB4L; diffusion coefficient of 35 cm^3/24 h m^2 bar to O_2 and 150 cm^3/24 h m^2 bar to CO_2). MAP was made in a EUVAC 65 vacuum chamber; once the air was evacuated by vacuum pressure, plastic bags were flushed by injection with the selected gas mixture and heat sealed. All samples were stored in a walk-in cold room at 2 ± 1 °C until sampling. Two bags from each animal and atmosphere were drawn for analysis. At each sampling time, the odor of the headspace gas of the bags was recorded.

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Microbiological Analysis. Bacteria were collected at each sampling time by swabbing a 4 cm^2 area of the fillet surface three times with sterile cotton swabs. Total viable counts (TVC) were made on Plate Count agar (PCA, Oxoid) incubated at 24 °C for 36 h. Enterobacteriaceae were determined in double-layer violet–red–bile–glucose agar (VRBG, Oxoid) incubated at 32 °C for 48 h. Lactic acid bacteria (LAB) were counted in double-layer, pH 5.6, MRS agar (Oxoid) incubated at 30 °C for 3 days and *Brochothrix thermosphacta* in STAA agar (Oxoid) with streptomycin sulfate (500 mg/L) (Oxoid), cycloheximide (50 mg/L) (Oxoid), and thallous acetate (50 mg/L) (Oxoid) incubated at 20 °C for 3 days.

To characterize the dominant organisms, 20% of the colonies from PCA plates were randomly chosen (Ordóñez, 1979) and subcultured onto tryptone soya broth (TSB). Characterization of Gram-negative and Gram-positive microorganisms was carried out as described by Dainty et al. (1979). For the ascertainment of *Br. thermosphacta*, the cellular fatty acid content of isolates was determined. For that, the method of Dodd and Dainty (1992) was used. The fatty acid methyl esters were analyzed by gas chromatography with a Konik KNK 3000 HRGC chromatograph equipped with a dual flame ionization detector. The capillary column (25 m, internal diameter 0.22 mm) was packed with BP5 (0.25 μm) on fused silica. The identification of different fatty acid methyl esters was made by comparison with authentic standards (Sigma). One strain of *Br. thermosphacta* 847 (ATCC11589) and another of *Lactobacillus casei* 478 (ATCC393) provided by the Spanish Type Culture Collection were used as reference.

Chemical Analysis. The pH measurements were made with a Crison-micro pH Model 2001 pH meter on homogenates of 10 g of fish muscle and 10 mL of distilled water.

Total volatile nitrogen was determined according to the Conway microdiffusion method (Pearson, 1973).

Nonvolatile amines were extracted according to the methodology of Spinelli et al. (1974) and analyzed by HPLC in a Beckman Model 332 apparatus equipped with Model 110 A pumps, a Spectra/flo fluorometer, and a column (25 cm × 4.6 mm) filled with ODS-2 on Spherisorb. Chromatography was carried out according to the method of Ordóñez et al. (1991).

For short-chain fatty acid determination, samples of 15 g of fish were homogenized with 45 mL of distilled water using a Polytron Model PT 10-35 blender, and 0.6% hexanoic acid solution was added as internal standard. The homogenate was centrifuged (10000*g* for 10 min) and the resulting pellet reextracted with 20 mL of distilled water and recentrifuged. The two supernatants were combined, and the volume was recorded. Proteins were precipitated by adding 5 M NaOH (final pH of 12.0) and 25% $ZnSO_4$ followed by heating in a boiling water bath. To remove protein precipitate, the mixture was filtered through a Whatman No. 54 paper. The filtrate was taken to dryness by freeze-drying. Extraction and quantification of the short-chain fatty acids were carried out as reported by Ordóñez et al. (1991) using a Konik KNK 3000 HRGC chromatograph equipped with a capillary column (30 m × 0.53 mm) packed with DB-FFAP (1 μm) on fused silica. Identification of different short-chain fatty acids and the response factor calculations for the quantifications were made by comparison with standards (BDH).

For nucleotide breakdown product determination, portions of tuna muscle (10 g) were homogenized with 5 mL of 3% $HClO_4$ and centrifuged (10000*g* for 30 min). The supernatant was neutralized with 2.5 N KOH and filtered through a 0.45 μm pore filter. ATP and related compounds were analyzed by HPLC in a Pharmacia LKB apparatus equipped with Model 2248 pumps, a VWM 2141 UV detector, and a Supelcosil Model LC-18-T column (15 cm × 4.6 mm) filled with ODDMS (3 μm). Peak resolution was accomplished using a gradient elution, with the mobile phase in pump A consisting of pH 5.7, 0.1 M KH_2PO_4 and 4 mM TTBA and that in pump B of pH 6.8, 0.1 M KH_2PO_4/methanol (70/30) (v/v). Identification and quantification was made by comparison with authentic standards (Fluka). The results were expressed as *K* values, calculated according to the method of Sayto et al. (1959) by the following formula: *K* value (%) = 100 (inosine + hypoxantine)/(ATP + ADP + AMP + IMP + inosine + hypoxantine).

The percentage of metmyoglobin (MetMb) was determined spectrophotometrically by measuring the reflectance at 525 and 572 nm (Stewart et al., 1965). The 0% and 100% MetMb values were established after treatment of the tuna samples with a 20% (w/v) solution of sodium dithionite and a 1% (w/v) solution of potassium ferricyanide, respectively (Ledward, 1970). Each MetMb value was the mean of at least 12 determinations.

Lipid autoxidation was determined by the reaction of malonaldehyde with thiobarbituric acid (TBA) according to the method of Tarladgis et al. (1960).

Sensory Analysis. At each sampling time, after 1 min of equilibration at room temperature, the color of three samples (each one from a different animal) was assesed by an untrained panel of 10 members using the hedonic scale of Hansen (1980). This scale ranged from ideal (10), very good (8), regular (6), borderline (4), poor (2), to very poor (0).

Each final value is the mean and the corresponding standard deviation calculated with the data obtained from each panelist, which data are the mean values assigned to the three different tuna steaks.

RESULTS AND DISCUSSION

Microbial Flora, Metmyoglobin Formation, and Color Assessment. Changes in different microbial groups during storage of refrigerated tuna steaks packaged in air, CO_2/air (20/80) (v/v), CO_2/air (40/60) (v/v), and CO_2/O_2 (40/60) (v/v) are shown in Figure 1. Steaks packaged in air (Figure 1A) presented a spoilage pattern similar to that observed for unpackaged refrigerated fish aerobically stored (Hobbs, 1983). After 9 days of storage, the TVC reached values slightly higher than 10^7 CFU/cm^2, at which the off-odors (putrid, stale, cabbage) were noticed, and by the 11th day the steaks were coated with slime, indicative of extensive bacterial growth (> 10^8 CFU/cm^2). Counts on VRBG achieved values close to those observed on the PCA plates, reaching levels of about 10^7 CFU/cm^2 at 9 days of storage but 1 log unit lower than that of TVC at the end of the experiment (11 days). At this time, identification of the colonies on the PCA plates revealed 5% *Br. thermosphacta* and less than 1% *Lactobacillus* spp. The remainder of the bacteria were Gram-negative oxidase positive (82%) and negative (12%). From these results, it may be deduced that the spoilage floras were dominated by Gram-negative psychrotrophs [presumably *Pseudomonas, Alteromonas,* and *Shewanella* types, according to Hobbs (1991)] and Enterobacteriaceae. Obviously, the Gram-negative flora was responsible for the spoilage. An objectionable color was also observed (Figure 2B) in samples at the same time as the off-odors.

After the paper by Shewan (1971), in which the literature of the microbiology of fish and fish products between 1930 and 1970 was reviewed, and the work of Stenström and Molin (1990), in which the microorganisms of many spoiled fish species were studied, it seems clear that *Pseudomonas*, with *Ps. fragi* as the major group, and *Shewanella putrefaciens* are the main organisms implicated in the spoilage of aerobically stored refrigerated fish. These organisms are Gram-negative, oxidase positive. Thus, it was assumed that the majority (82%) of bacteria present in the spoiled tuna packed in air filled bags belonged to the *Pseudomonas/Shewanella* group. It has also been reported (Molin et al., 1983) that Enterobacteriaceae may form part of the dominant organisms in aerobically stored refrigerated fish as these were found in the present work. However, *Br. thermosphacta* and *Lactobacillus* spp. have not been reported among the more abundant organisms (Shewan, 1971; Hobbs, 1991) in aerobically stored unpackaged

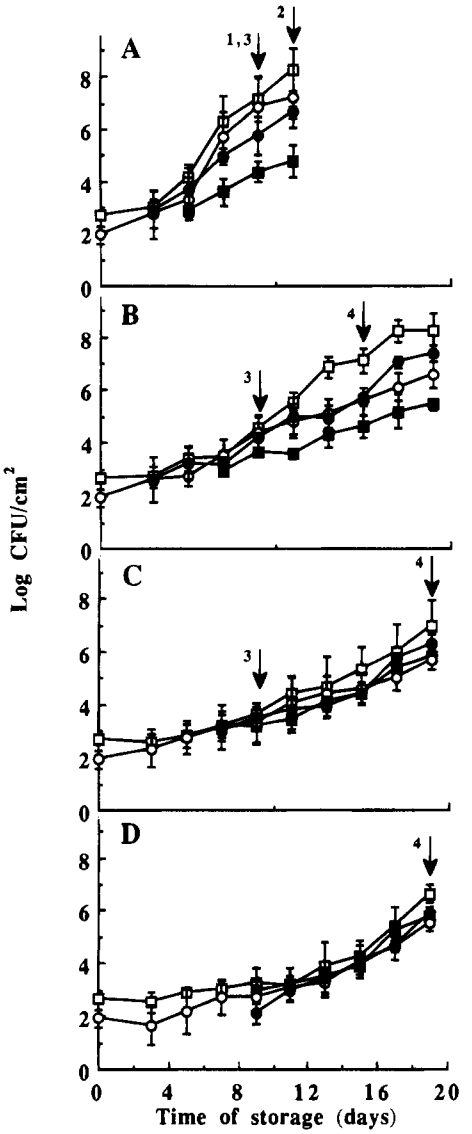

Figure 1. Changes in total viable (□), lactobacilli (■), Enterobacteriaceae (○), and *Br. thermosphacta* (●) counts on refrigerated tuna (*T. alalunga*) steaks stored in air (A), CO_2/air (20/80) (v/v) (B), CO_2/air (40/60) (v/v) (C), and CO_2/O_2 (40/60) (v/v) (D) atmospheres. 1, Off-odor (putrid, stale cabbage); 2, slime; 3, objectionable color (light green-brown); 4, off-odor (pungent, sour, strong marinade).

fish, although they have been detected when the fish is placed in an air-filled vessel (Stenström, 1985; Molin et al., 1983; Molin and Stenström, 1984). The same observation have been made for meat (Roth and Clark, 1975; Dainty et al., 1983). The incidence of *Br. thermosphacta* in these cases may be the result of the gradual accumulation of CO_2 in the bags or vessel during storage (Erichen and Molin, 1981), reaching concentrations of up to 12% after 2 weeks (Molin et al., 1983).

When the steaks were packaged in atmospheres enriched with 20% of CO_2 (Figure 1B), bacterial growth was delayed. Values higher than 10^7 CFU/cm^2 were only reached after 15 days of storage, at which time a pungent, sour, and/or strong marinade odor was perceived. Counts on STAA were higher than on VRBG at the end of the experiment, but the former only reached levels of 10^7 CFU/cm^2 after 19 days of storage. Counts on MRS were the lowest throughout the experiment. Characterization of the PCA plate colonies showed a significant proportion of *Br. thermosphacta*

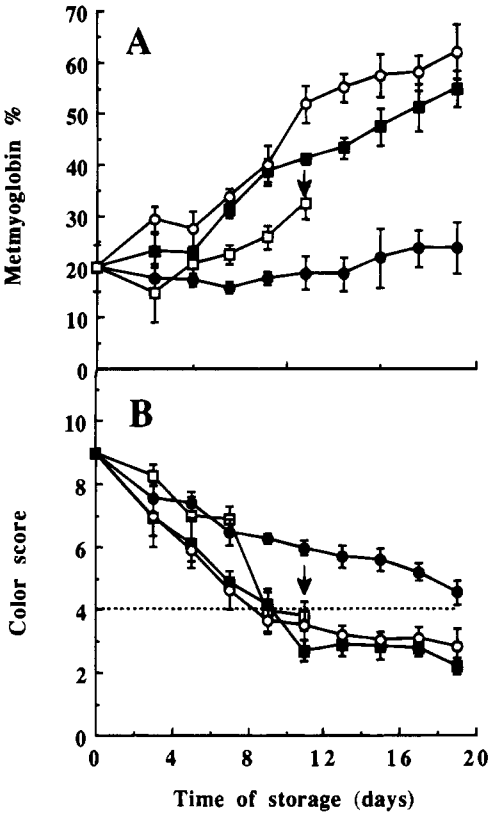

Figure 2. Changes in metmyoglobin percentage (A) and color scoring (B) on refrigerated tuna (*T. alalunga*) steaks stored in air (□), CO_2/air (20/80) (v/v) (■), CO_2/air (40/60) (v/v) (○), and CO_2/O_2 (40/60) (v/v) (●) atmospheres. The dotted line indicates the sample rejected level by the test panel. The arrows indicate when the slime was detected in samples stored in air.

and *Lactobacillus* spp., accounting for 60% and 5% of the total floras, respectively. The remaining percentage belonged to Gram-negative bacteria both oxidase positive and negative in proportions of approximately 1:2. This means that *Pseudomonas*/*Shewanella* organisms were not completely inhibited by 20% of CO_2, although it gave rise to an important change in the dominant bacteria, which were mainly constituted by *Br. thermosphacta*. From a microbiological point of view, it was possible to extend the shelf life of the refrigerated tuna up to 6 days compared to samples aerobically stored. However, the panel test evaluated the tuna color as objectionable (green-brown) at a similar time as the air-packed tuna, i.e. after about 9 days of storage (Figure 2B). In this case the color played an important role as a limiting factor of the refrigerated tuna shelf life. The MetMb concentration reached a final value of about 50% (Figure 2A).

The predominance of *Br. thermosphacta* observed in this study in CO_2/air (20/80) (v/v) atmosphere is in disagreement with the results of most authors that have investigated the microbial changes in MAP fish. From the review of Davis (1993), it may be deduced that lactobacilli have almost always been found as the dominant organisms in fish packed in CO_2 enriched atmospheres and *Br. thermosphacta* has only been occasionally detected at relatively elevated levels (Molin et al., 1983). However, it is very common to find this bacterium as the predominant one in meats stored under this kind of atmosphere together with lactobacilli and Enterobacteriaceae (Newton et al., 1977; Gill and Tan, 1979; Asensio et al., 1988). The enumeration of *Br. thermosphacta* is usually carried out by either direct

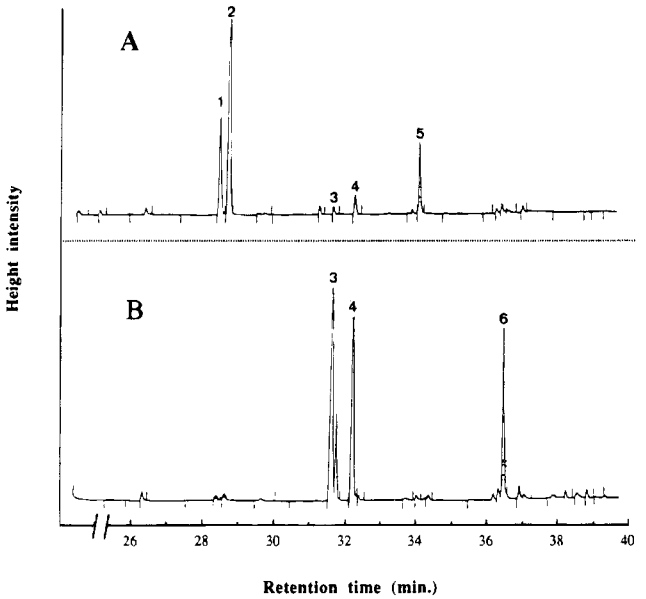

Figure 3. Chromatograms showing results of GLC analysis of *Br. thermosphacta* (A) and *L. casei* (B) cellular fatty acid composition. 1, iso C-15:0; 2, anteiso C-15:0; 3, iso C-16:0; 4, C-16:0; 5, anteiso C-17:0; 6, not identified.

counting on STAA agar or colony identification assuming any Gram-positive catalase positive rod to be *Br. thermosphacta*. Care must be taken in the determination catalase, as its production depends on growth conditions and age of culture as old cultures or those grown above 25 °C may be catalase negative and the reaction is also medium-dependent (Dodd and Dainty, 1992). In MAP proteinaceous foods (meats and fish), *Lactobacillus* is the genus to which *Br. thermosphacta* is most closely related and with which it is most likely to be confused during isolation. Therefore, in the present work, besides enumerating *Br. thermosphacta* directly on STAA agar, colonies isolated from PCA were identified according to the method developed by Dodd and Dainty (1992), based on cellular fatty acid (CFA) composition. The patterns of CFA of lactobacilli and *Br. thermosphacta* are very different, the most important feature being the high concentrations in *Br. thermosphacta* of iso C-15:0, anteiso C-15:0, and anteiso C-17:0, while these are practically absent in *Lactobacillus* spp. (Figure 3). The assignment of a given Gram-positive rod isolated from PCA to one or the other genus was therefore considered as conclusive.

In contrast with meat packed in 20% CO_2 enriched atmosphere (Asensio et al., 1988), Gram-negative, oxidase positive bacteria were not completely inhibited in tuna. This has been attributed to the sensitivity of these bacteria to pH and CO_2. The normal pH of meat is about 5.5, while that of fish is close to or higher than 6.0. *Alteromonas* (*Shewanella*) *putrefaciens* is unable to grow on meat of normal pH (Gill and Newton, 1979), but it does grow on abnormally high pH DFD meat (Hood and Mead, 1993). On the other hand, *Pseudomonas* spp. are markedly inhibited by CO_2 (Gill and Tan, 1979), but *Shewanella* spp. are much more resistant (Molin and Stenstron, 1984). Therefore, as the pH of tuna increased throughout storage reaching values higher than 6.0 (Figure 4), the Gram-negative, oxidase positive organisms detected in the CO_2/air (20/80) (v/v) atmosphere were tentatively considered as belonging to the *Shewanella* genus.

Bacterial counts of steaks mantained in both CO_2/air (40/60) (v/v) and CO_2/O_2 (40/60) (v/v) (Figure 1C,D)

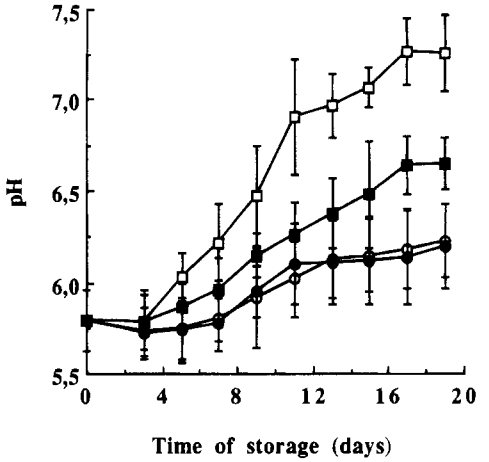

Figure 4. Changes in pH of refrigerated tuna (*T. alalunga*) steaks stored in air (□), CO_2/air (20/80) (v/v) (■), CO_2/air (40/60) (v/v) (○), and CO_2/O_2 (40/60) (v/v) (●) atmospheres.

presented the same microbiological pattern. A strong delay of bacterial growth was observed. Counts remained at a low level (less than 10^5 CFU/cm^2) during the first 11−15 days, and after 19 days, the bacterial numbers did not exceed 10^7 CFU/cm^2. Counts on PCA, MRS, VRBG, and STAA were quite similar, but the characterization of the floras growing on PCA at the end of the storage period showed that *Br. thermosphacta* was the dominant microorganism (77% and 69% of the total floras in each of the above-mentioned atmospheres, respectively). *Lactobacillus* spp. and Enterobacteriaceae were present in much smaller percentages (20−30%) at an approximate ratio of 2:1. No Gram-negative oxidase positive bacteria were detected, which indicates that *Pseudomonas/Alteromonas/Shewanella* group was efficiently inhibited by 40% CO_2. However, a great difference in the tuna color between samples stored in both atmospheres was observed (Figure 2B). The pattern of tuna color stored in CO_2/air (40/60) (v/v) was similar to that of samples maintained in CO_2/air (20/80) (v/v), while no objectionable color was assessed until 19 days of storage in samples stored in CO_2/O_2 (40/60) (v/v). Therefore, the shelf life of tuna held in the latter atmosphere may be doubled with respect to both microbiological condition and color. Figure 2A also shows metmyoglobin formation during the storage of refrigerated tuna in the four different atmospheres assayed. The results confirm those obtained in the sensorial analysis. The slowest metmyoglobin accumulation was observed when tuna was packed in CO_2/O_2 (40/60) (v/v) enriched atmosphere.

pH and Total Volatile Nitrogen. The pH increased throughout the period of tuna storage in refrigeration (Figure 4). This increase was maximum in samples stored in air with mean values ranging from 5.8 (day 0) to almost 7.3 (day 19). Samples stored in CO_2/air (40/60) (v/v) and CO_2/O_2 (40/60) (v/v) showed similar pH changes (from 5.8 to 6.2) with values lower than those found in samples stored in CO_2/air (20/80) (v/v) (from 5.8 to 6.65). These results are in agreement with the flora composition. As the Gram-negative oxidase positive organisms were inhibited by CO_2, the pH increases were slower. In aerobically stored refrigerated fish, the increase in pH is due to TMAO reduction by spoilage bacteria (Hebard et al., 1982) to TMA, which in turn is converted into other basic volatiles. The generation of these substances was also inhibited when tuna was stored in CO_2 enriched atmospheres (Figure 5), yielding a lower pH.

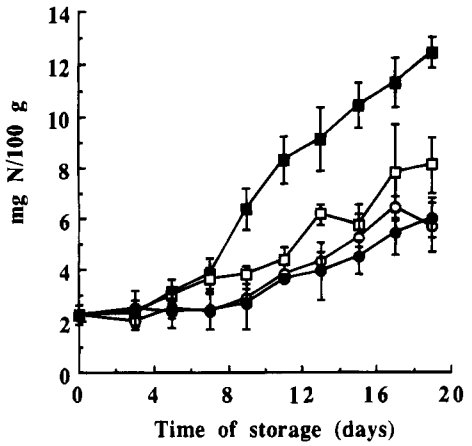

Figure 5. Changes in total volatile nitrogen on refrigerated tuna (*T. alalunga*) steaks stored in air (□), CO_2/air (20/80) (v/v) (■), CO_2/air (40/60) (v/v) (○), and CO_2/O_2 (40/60) (v/v) (●) atmospheres.

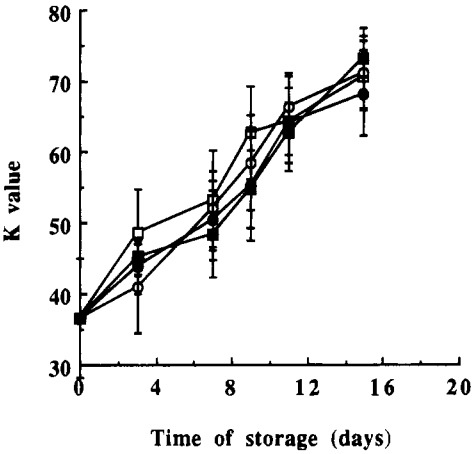

Figure 6. Changes in *K* value of refrigerated tuna (*T. alalunga*) steaks stored in air (□), CO_2/air (20/80) (v/v) (■), CO_2/air (40/60) (v/v) (○), and CO_2/O_2 (40/60) (v/v) (●) atmospheres.

Nucleotide Breakdown Products (*K* Value). Figure 6 shows the changes in *K* value at various times throughout the storage period. Results in the four atmospheres were quite similar, ranging from almost 30 to 70, and also similar to those reported for other fish stored aerobically in ice (Malle and Le Pezennec, 1992). Hence, no effect of either CO_2 or CO_2 and O_2 enriched atmospheres was observed.

Nonvolatile Amines. Table 1 shows the changes in the nonvolatile amine concentrations of refrigerated tuna steaks stored in modified atmospheres. Histamine increased constantly during storage. In the three CO_2 enriched atmospheres, the increases were delayed compared to the fish stored in air, in which the histamine content reached about 39 μg/g at the time of spoilage (11 days). The histamine levels were similar in tuna stored in the CO_2 enriched atmospheres after 19 days of storage. The accumulation of substantial amounts of histamine is very common in scombroid-type fish such as tuna and mackerel (Arnold and Brown, 1978). The amine is formed from histidine by bacteria that possess the enzyme histidine decarboxylase. Many bacteria are able to generate histamine, but only a few species have been associated with histamine production during fish spoilage (Taylor et al., 1978). These species include some Enterobacteriaceae members, such as *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei* (Behling and Taylor, 1982). Therefore, accumulation of histamine recorded in the present work in all samples was probably due to the microbial activity of Enterobacteriaceae, which is supported by the fact that the delay in histamine formation observed in CO_2 enriched atmospheres was in agreement with the inhibition of Enterobacteriaceae in samples stored in these atmospheres (Figure 1). Counts on VRBG from tuna stored in air were higher than 10^6 CFU/cm^2 at 9–11 days of storage, while this Enterobacteriaceae level was not reached in either 20% CO_2 or 40% CO_2 enriched atmospheres until 19 days of storage.

Similar arguments may be constructed for cadaverine, with Enterobacteriaceae being the main producers (Slemr, 1981; Edwards et al., 1985).

Tyramine showed a different trend. The highest levels of this amine were found in samples packed in all CO_2 enriched atmospheres. *Br. thermosphacta* was the dominant organism in these samples, but this bacterium is not an amine producer (Edwards et al., 1985). The responsible organisms may therefore well be the lactic acid bacteria since it has been reported (Edwards et al., 1987) that some strains of the genus *Lactobacillus* isolated from vacuum-packed meat and other meat products produce tyramine.

Spermine and spermidine were the amines detected at the highest level at the beginning of the study, and they stayed at similar concentrations during the whole storage period, with the exception of the spermidine in air-stored tuna. In these samples, an increasing trend was observed during the last half of storage, possibly due to condensation of putrescine (Karmas and Mietz, 1978). However, the presence of both polyamines at regular levels seems to be common since a similar behavior has also been observed in refrigerated (Nakamura et al., 1979) and vacuum-packed meats (Edwards et al., 1985) and in meats stored in modified atmospheres (Ordoñez et al., 1991).

A pattern similar to that of the former mentioned amines was found for putrescine. A clear increase was only observed in the air-stored tuna. This is expected in refrigerated proteinaceous food aerobically stored, i.e. in both fish (Karmas and Mietz, 1978) and meat (Nakamura et al., 1979; Edwards et al., 1983). The dominant *Pseudomonas* (Slemr, 1981; Taylor and Sumner, 1986) and many Enterobacteriaceae (Taylor and Sumner, 1986) are endowed with ornithine decarboxylase activity, but the formation of putrescine requires the presence of bacteria which use arginine for ornithine production (Dainty et al., 1986).

TBA Value. Figure 7 shows the changes in malonaldehyde content (milligrams per kilogram of wet tuna) throughout the storage period. No consistent pattern of changes in the TBA value was found; the levels were almost always lower than 3 mg of malonaldehyde/kg of wet muscle. It therefore seems that this parameter is not clearly affected by the modified atmospheres.

Short-Chain Fatty Acids. No relevant results were derived from the short-chain fatty acid analysis (data not shown). Acetic acid showed a small increase from the beginning (0.8 μg/g of wet muscle to reach final values between 4 and 6 μg/g) in all types of atmosphere used. Propionic and *n*-butyric acid stayed at similar levels (0.5–1.5 μg/g) throughout storage. These latter substances seem to be of nonmicrobial origin (Dainty and Hibbard, 1980). Isobutyric, isovaleric, and *n*-valeric acids were not detected in the first days of storage and only in small (less than 1 μg/g) or trace amounts in the latter half of the storage period. The metabolism of *Br.*

Table 1. Changes in Nonvolatile Amines (Micrograms per Gram of Wet Muscle) in Refrigerated Tuna Steaks Stored in Air, CO_2/Air (20/80) (v/v), CO_2/Air (40/60) (v/v), and CO_2/O_2 (40/60) (v/v) Atmospheres

atmosphere	days	histamine	tyramine	spermidine	spermine	tryptamine	phenylethylamine	putrescine	cadaverine
air	0	1.54	ND[a]	3.13	21.87	ND	ND	0.86	1.83
	5	6.28	1.45	4.78	18.99	ND	2.07	1.00	1.56
	9	29.65	3.09	4.03	12.28	1.75	1.98	2.30	14.60
	11	38.86	2.72	5.24	18.73	1.42	3.11	2.25	12.16
	13	49.01	4.43	5.32	14.88	1.74	2.19	3.70	14.80
	15	67.01	3.91	6.28	18.34	1.18	3.02	5.92	31.71
	17	111.54	2.14	9.05	29.38	1.00	3.63	8.62	75.23
	19	140.12	2.45	10.49	27.06	0.94	4.82	10.53	112.31
CO_2/air (20/80) (v/v)	0	1.54	ND	3.13	21.87	ND	ND	0.86	1.83
	5	1.96	ND	3.08	24.56	ND	2.19	1.12	2.19
	9	2.98	0.86	4.69	22.18	0.75	2.64	0.99	1.34
	11	5.96	1.16	4.33	21.39	0.80	3.40	1.27	1.65
	13	8.69	1.87	5.10	24.89	0.58	4.02	1.38	2.23
	15	15.36	5.64	3.71	22.87	0.76	4.58	1.24	5.45
	17	25.97	12.92	5.01	14.19	1.85	5.45	1.82	11.35
	19	33.08	14.00	4.99	23.70	1.90	5.75	2.11	12.78
CO_2/air (40/60) (v/v)	0	1.54	ND	3.13	21.87	ND	ND	0.86	1.83
	5	2.17	ND	2.28	16.77	ND	1.39	0.82	1.35
	9	5.81	1.53	3.24	18.50	0.92	3.84	0.53	2.38
	11	8.96	4.62	3.23	18.45	1.35	2.89	1.15	2.89
	13	12.47	1.98	3.64	25.35	2.22	2.90	0.93	2.76
	15	23.99	2.58	3.56	26.46	1.47	2.71	0.82	8.46
	17	31.49	8.45	2.92	17.12	1.22	10.05	1.01	10.29
	19	36.37	9.39	1.94	20.60	2.94	8.97	0.62	17.92
CO_2/O_2 (40/60) (v/v)	0	1.54	ND	3.13	21.87	ND	ND	0.86	1.83
	5	1.80	0.94	3.35	11.94	ND	2.10	0.75	0.55
	9	1.85	0.65	2.53	12.33	0.83	2.41	0.75	1.02
	11	2.43	0.90	4.20	15.46	1.46	2.84	0.86	1.63
	13	2.57	1.25	3.57	20.47	0.94	3.70	1.12	2.47
	15	9.07	1.98	3.36	14.57	1.26	4.28	1.47	8.83
	17	22.47	3.38	3.12	20.75	1.24	2.94	1.66	14.36
	19	35.35	8.48	3.28	21.40	1.77	6.46	1.35	15.98

[a] ND, not detected.

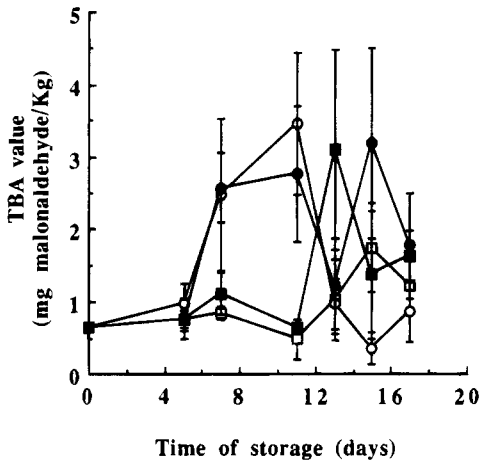

Figure 7. Changes in the TBA value of refrigerated tuna (*T. alalunga*) steaks stored in air (□), CO_2/air (20/80) (v/v) (■), CO_2/air (40/60) (v/v) (○), and CO_2/O_2 (40/60) (v/v) (●) atmospheres.

thermosphacta may be either aerobic or anaerobic, but it has been suggested (Ordoñez et al., 1991) that *Br. thermosphacta* presents an aerobic metabolism in meat stored in both CO_2 and CO_2/O_2 enriched atmospheres. Under aerobic conditions, this bacterium produces acetoin and acetic, isobutyric, and isovaleric acids (Dainty and Hibbard, 1980). Thus, these short-chain fatty acids might be expected. However, their concentrations were much lower than those found in meat stored under similar atmospheres (Ordoñez et al., 1991), which may be attributed to the different pH and chemical composition of the tuna because several environmental factors (oxygen, carbohydrate availability, pH, etc.) greatly influence the aerobic metabolism of *Br. thermosphacta* (Dainty and Hibbard, 1980).

From a public health point of view, MAP of tuna may pose a potential danger. The major concern with relation to the safety of MAP of tuna is either the histamine and tyramine production or the potential for growth and toxin production by *Clostridium botulinum*.

The opinions on the toxicity threshold values of tyramine and histamine vary widely, from 10 to 80 mg of tyramine and from 70 to 1000 mg of histamine (Renner, 1987). The highest amount of tyramine (14 μg/g) was found in tuna stored in CO_2/air (20/80) atmosphere. Similar levels of histamine were reached in tuna packaged in all modified atmospheres assayed and also similar to the values achieved in air at the time of spoilage (29–35 μg/g). According to these values (Table 1), it would be necessary to consume a very great quantity of tuna (close to 1000 g) to reach the highest toxicity level reported by Renner (1987). Furthermore, concentrations higher than those found in tuna (tyramine 140–910 μg/g and histamine 30–110 μg/g) have been reported in many cheeses (Renner, 1987) which are usually consumed. Therefore, it seems that the risk for consuming tuna packaged in modified atmosphere is not higher than that for tuna aerobically stored and other foods.

In relation to *Cl. botulinum* it has been observed that *Cl. botulinum* type E and the nonproteolytic strains of types B and F may grow and produce toxin at 3.3 °C (Varnam and Evans, 1991). Several authors (García et al., 1987; Post et al., 1985) have detected botulinal toxin in some fishes packaged in several modified atmospheres at temperatures below 5 °C, but the fish always presented evident spoilage signs. However, at tempera-

tures above 5 °C, may be detected toxin before spoilage (Post et al., 1985). Then, it seems to be that no problem related with the botulinal toxin will occur if the temperature is mantained below 5 °C. Furthermore, the inclusion of oxygen has been recommended (Hotchkiss, 1988) to reduce the risk of botulinal toxin production for MAP of finfish with low myoglobin concentrations, in which should not be necessary the atmosphere enrichment with this gas. Finally, it has been reported (Smith et al., 1990) that there is little conclusive evidence that gas packaging represents a significantly greater hazard than packaging in air.

Conclusion. Three different modified atmospheres have been assayed to extend the shelf life of refrigerated tuna steaks. Aerobically stored samples (control) were spoiled (off-odors and slime) after 9–11 days of storage, reaching bacterial counts of 10^8 CFU/cm^2. However, when the steaks were packed in atmospheres enriched in both 20% and 40% CO_2, bacterial growth was inhibited, achieving values of about 10^7 CFU/cm^2 after 15 and 19 days of storage, respectively. In these atmospheres, *Br. thermosphacta* was the dominant organism. Total volatile nitrogen and pH increased with Gram-negative bacteria counts. No effect of CO_2 enriched atmospheres on the *K* value was observed. Histamine, cadaverine, and putrescine levels increased along with Enterobacteriaceae. The highest tyramine levels were found in samples packed in CO_2 enriched atmospheres, which has been related to the tyrosine decarboxylase activity of lactobacilli. Spermidine and spermine maintained regular levels during storage, except spermidine in air-stored tuna. No consistent pattern in lipid autoxidation was found. The study of short-chain fatty acids showed no relevant results. Color played an important role as a limiting factor of the shelf life of refrigerated tuna stored in CO_2 enriched atmospheres. The most effective atmosphere was CO_2/O_2 (40/60) (v/v). It is concluded that the shelf life of tuna packed in this atmosphere may be doubled with respect to both microbial condition and color.

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