

Viability and Antigenicity of *Anisakis simplex* after Conventional and Microwave Heating at Fixed Temperatures

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ABSTRACT

Inactivation of parasites in food by microwave treatment may vary due to differences in the characteristics of microwave ovens and food properties. Microwave treatment in standard domestic ovens results in hot and cold spots, and the microwaves do not penetrate all areas of the samples depending on the thickness, which makes it difficult to compare microwave with conventional heat treatments. The viability of *Anisakis simplex* (isolated larvae and infected fish muscle) heated in a microwave oven with precise temperature control was compared with that of larvae heated in a water bath to investigate any additional effect of the microwaves. At a given temperature, less time was required to kill the larvae by microwaves than by heated water. Microwave treatment killed *A. simplex* larvae faster than did conventional cooking when the microwaves fully penetrated the samples and resulted in fewer changes in the fish muscle. However, the heat-stable allergen Ani s 4 was detected by immunohistochemistry in the fish muscle after both heat treatments, even at 70°C, suggesting that Ani s 4 allergens were released from the larvae into the surrounding tissue and that the tissues retained their allergenicity even after the larvae were killed by both heat treatments. Thus, microwave cooking will not render fish safe for individuals already sensitized to *A. simplex* heat-resistant allergens.

Intensive studies have been carried out on methods for killing *Anisakis* (Nematoda: Anisakidae) third-stage (L3) larvae to prevent consumers from being infected after consuming lightly cooked infected fish, thus avoiding anisakiasis and allergic sensitization. Freezing, thermal processing, and high pressure can kill *Anisakis simplex* s.l., although the minimal lethal conditions differ among studies (1, 6, 9, 14, 19, 26, 28, 35, 38–40). These differences may be due to batch-to-batch variation among the nematodes, resistance to treatment, or differences in the conditions under which *A. simplex* viability was evaluated.

Microwave ovens are currently employed to cook fish and fish products. Heating by microwaves involves primarily dielectric and ionic mechanisms. Because of their polar nature, water molecules in food are often the primary component responsible for dielectric heating. Oscillatory migration of ions in food also generates heat under the influence of the oscillating electric field (20). Many studies have been conducted on the use of microwaves for the reduction of microorganisms in various foods (2, 7, 15); however, few reports include the effect of microwaves on parasites, and those reports that exist are mainly concerned

with nematodes that infect fish (5, 24, 38). It is generally accepted that thermal effects are the main cause of mortality of microorganisms and parasites in microwave-heated foods, although nonthermal effects of microwaves include selective heating of microorganisms, electroporation, cell membrane rupture, and cell lysis by electromagnetic energy coupling. However, the general consensus is that the nonthermal effects are likely to be due to the lack of precise measurements of the time-temperature history and spatial variations (20).

Anisakis infestation of fish is an important issue for the fish industry and food safety authorities (11). *Anisakis* is distributed worldwide, with higher prevalence of some species found in fishing areas. Ten *Anisakis* species have been described; these are associated with 25 marine mammals (pinnipeds and cetaceans), in which the larvae reach sexual maturity, and 73 fish and cephalopod species, which act as intermediate hosts for the L3 larvae (25). Humans are considered accidental hosts because they are not necessary for the parasite to complete its life cycle.

Anisakis infestation of fish and cephalopods is a potential human health risk because of the possibility of consumers becoming infected with live L3 larvae and because some of the secretion and/or excretion products and somatic and cuticle proteins from the parasite can cause an

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allergic response (4, 11). The majority of larvae in fish are found in the viscera, mesenteries, peritoneum, and internal organs; they are found to a lesser extent in the muscle, and then mainly in the belly flaps. However in Pacific salmon, most of the larvae are located in the hypoaxial muscle (8, 33). Fishborne parasitic anisakiasis was once limited to consumers living in countries where raw or undercooked fish is traditionally consumed. However, other factors, including growing international markets, improved transportation systems, population movements, and the spread of ethnic cooking methods in developed countries, have increased exposure to these parasites. To avoid anisakiasis, in some countries it is compulsory to consume only products in which the larvae have been killed by thermal treatments such as heating or freezing, although the recommended time-temperature conditions differ.

To date, 12 *Anisakis* allergens have been described. Of these, Ani s 4 is a heat-, acid-, and pepsin-resistant allergen that frequently causes anaphylaxis due to exposure to *A. simplex* (21, 27, 29). For this reason, Ani s 4 is often used to study the effects of various cooking and storage treatments on the allergenicity of infected fish.

The heat resistance of helminths is low. Exposure to 56 to 60°C for several minutes usually is sufficient to eliminate infectivity (13); nevertheless, differences in viability between batches of larvae have been observed, with some larvae remaining alive after 10 min at 60°C (39). Survival of microorganisms and parasites after microwave treatment has differed among different studies for many reasons, including the lack of standardization of wattage among standard domestic microwave ovens (most of them operate in the range of 800 to 1,200 W), uneven distribution of the microwaves by the magnetrons, and differences in size, shape, composition, and amount of dipolar components in the food. Some product-specific factors, particularly moisture and salt content, have a much greater effect on microwave processing than on conventional processing because of their effect on dielectric properties. The Institute of Food Technologists produced a review on the effect of heating foods by microwaves for the U.S. Food and Drug Administration (20). All of these parameters affect the regular heating of food, sometimes leaving hot and cold spots where the temperature may be lower than that required to inactivate microorganisms or parasites (16, 18). Most microwaves in standard domestic ovens usually penetrate only to about 10 to 15 mm from the surface (17), so thicker foods are heated by conduction from the hot to the cold parts; therefore, a standing period is necessary to equilibrate the temperature inside the food. Because of these factors, the time-temperature conditions applied may not be sufficient to kill *Anisakis* larvae. The U.S. Food and Drug Administration recommends an internal temperature of 74°C in all parts of microwaved animal foods plus an additional 2 min of standing time for the covered food to achieve temperature equilibrium (36). In a previous study, we noted that most of the data relating to the ingestion of microwaved fish infected with *Anisakis* sp. were obtained from clinical reports in which the microwave heating parameters were not well described (38).

Thermal effects are considered the most important factor influencing the survival of microorganisms and parasites treated with microwaves; nevertheless, questions arise as to whether additional effects are associated with microwave treatment. Studies of the effects of conventional heating and sublethal microwave radiation on the metabolic activity of *Staphylococcus aureus* revealed that microwave treatment affected the bacteria in a way that could not be attributed solely to thermal effects (10). The inactivation of microorganisms by microwave has been explained by the increase in temperature when food is heated in the presence of water (37).

Most commercial microwave ovens do not measure the temperature inside the product during operation. In one study of the effects of microwaving on fillets of arrowtooth flounder (*Atheresthes stomias*) containing *A. simplex* larvae, the survival of *A. simplex* depended on the dimensions and weight of the fish sample. In samples rotated and stirred during treatment, a temperature of 77°C was necessary to inactivate the larvae in the infected fillets, whereas microwave treatment of isolated larvae resulted in death of the larvae at temperatures $\geq 69.8^\circ\text{C}$ (1). In another study, hake sandwiches made with two steaks (each 1 cm thick) artificially infected with *A. simplex*. The sandwiches were heated for 3 min in a standard domestic microwave oven, resulting in the death of all larvae. The final temperature measured at the thermal center immediately after microwave treatment was $\geq 66^\circ\text{C}$, and due to the thickness of the sandwich the researchers assumed that the microwaves penetrated the entire depth of the sample (35).

In addition to infection resulting in anisakiasis, *Anisakis* larvae also represent a potential risk to consumers because of the possibility of provoking an allergic response. Some *Anisakis* allergens are very heat resistant (22, 27) and have been detected even after microwave treatment had killed the larvae (38).

The aim of this study was to determine whether microwaves penetrating the entire depth of the sample have an effect in addition to heat on *A. simplex* L3 larvae and to determine the effect of microwave compared with conventional heat treatment on *A. simplex* antigens. The effects of heat on larval viability and structure and on Ani s 4 antigens in fish muscle were investigated after treatment in a microwave oven with precise microwave distribution and temperature control of the samples and in a water bath at temperatures already proven to kill the larvae.

MATERIALS AND METHODS

Materials. Live *A. simplex* s.l. L3 larvae were obtained in September and November 2007 from ovaries and viscera of heavily parasitized hake (*Merluccius merluccius*) obtained at the central fish market (MERCAMADRID) in Madrid. Approximately 100 g of larvae adhering to the ovaries and visceral tissue were sent each time to the laboratories at the Instituto del Frio and immediately stored at $5 \pm 1^\circ\text{C}$ for further use. Five hake caught in November 2007 in the Northeast Atlantic fishing grounds also were used to study the effect of microwave and conventional heating on infested muscle. These fish had an average weight of $1,145 \pm 265$ g and an average length of 50 ± 5.4 cm.

Proximate composition. A mince of dorsal muscle obtained from all the fish obtained was used for composition measurements. Moisture and ash (3) and crude fat (31) were determined as previously described. Crude protein (FP-2000 nitrogen-protein determinator, LECO Corp., St. Joseph, MI) was determined using a nitrogen-to-protein conversion factor of 6.25. All determinations were performed in triplicate. The results were expressed in grams per kilogram of muscle.

Sample preparation. To standardize the conditions of each heating treatment, hake muscle was infected by placing 12 L3 larvae on a 1-cm-thick steak obtained from the dorsal muscle (10 cm long, 10 cm wide) and was covered with another steak. Each sandwich was vacuum sealed in heat-resistant bags (oxygen permeability of 30 cc/m²/24 h measured at 23°C and 75% relative humidity; Wipak Gryspeert model PAE 110 K FP, ILPRA Systems España, S.L., Mataró, Spain) and stored under chilled conditions (5 ± 1°C) for 20 h to let the larvae penetrate the muscle (35). No *Anisakis* larvae were visually detected in the steaks used for infection with live larvae. Hake sandwiches without added larvae were used as controls.

Microwave treatment. A preliminary assay (September batch) was performed using tubes containing five live larvae to standardize the heating temperature. The larvae were microwave heated in 30 ml of distilled water inside capped tubes in an Ethos One Microwave Labstation (Milestone Inc., Shelton, CT) at 50 and 60°C for 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 min and at 70°C for 1, 2, 3, 4, and 5 min to compare the effects of microwave heating with those of conventional heating applied to the same batch (39). The microwave oven was equipped with a temperature control system and Easywave software, which allowed precise programming of time and temperature conditions of the samples during the microwave treatment at an established maximum power level. The oven was equipped with a rotating microwave diffuser for optimal microwave distribution in the cavity, and the samples were rotated in the oven during treatment. The oven was programmed to reach the required temperature in 1 min. The maximum power of the treatments was 800 W at 50 and 60°C and 1,000 W at 70°C. After each treatment, the liquid and larvae were immediately transferred to cuvettes for further measurements. All trials for the various time-temperature conditions were performed in triplicate.

The microwave treatment of *A. simplex* in the infected sandwiches was applied with the Ethos One Microwave Labstation. The vacuum-sealed sandwiches were placed inside the oven on a surface that rotated during treatment (60°C for 5 min and 70°C for 3 min). The conditions were selected to achieve a cooked appearance of the fish muscle. The initial time (1 min) was set in all cases after the required temperature (±0.5°C) was reached. The maximum power applied at 60 and 70°C was 800 W and 1,000 W, respectively. The characteristics of the samples and the microwave oven used were chosen to ensure that the target temperatures were reached in the entire sandwich.

Conventional heating. The conditions applied and the effect of conventional heating on the viability of isolated *A. simplex* larvae from the September batch have been published (39). In infected hake sandwiches, final temperatures of 60 and 70°C were reached by heating the sandwiches inside the vacuum-sealed bags in a water bath at 93°C. The temperatures were selected on the basis of the results obtained previously (39). The temperature was measured and recorded at 30-s intervals with thermocouples (Ebi-2T-313, Ebro, Ingolstadt, Germany) inserted in the thermal center in selected sandwiches. The time required to reach 60 and 70°C

(±2°C) was 6.5 and 11 min, respectively. As for the microwave treatment, the initial time was set once the selected temperature was reached. The sandwiches had a cooked appearance at both temperatures.

At least two sandwiches were used with each time-temperature condition in microwave or conventional heating treatments. After heat treatment, the sandwiches were opened and the larvae were immediately transferred to cuvettes. The larvae and the heated hake muscle were used for further measurements.

Mobility and appearance. Spontaneous and stimulated (touching but not damaging the larvae with a needle) movements and changes in appearance from bright translucent to opaque before and after treatments were observed in the isolated larvae and in larvae removed from the hake steaks. When movement of the larvae was not observed immediately, 3-min videos were recorded (Handycam DCR-Sr70, Sony, Tokyo, Japan) immediately after treatment and at intervals during a 2-h period. When no movement was recorded, the larva was counted as dead.

Emission of fluorescence. The emission of a bluish fluorescence by the larvae was examined in a dark chamber by exposure to a UV light source (366 nm) held 15 cm above the larvae. Emission of fluorescence was recorded immediately and at 1 and 2 h after each treatment. Pictures were taken under visible and UV light (Coolpix 5000 digital camera, Nikon, Tokyo, Japan). The intensity of the fluorescence was rated in arbitrary units as maximum, medium, slight, or no fluorescence.

Scanning electron microscopy. Changes in the cuticle and the shape of the larvae after microwave or conventional heating were observed by scanning electron microscopy. The samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and postfixed with OsO₄. After dehydration in a graded series of acetones, the samples were dried at the critical point (030 critical point dryer, Balzer, BAL-TEC, Schalksmühle, Germany) and coated with gold-palladium in a metallizer (SCD004, Balzer). Micrographs were taken with a scanning electron microscope (JSC 6400, Jeol, Akishima, Tokyo, Japan) at 20 kV. A significant number of microwave and conventionally heated larvae were immediately fixed as previously described (38). A large number of scanning electron micrographs were evaluated to select the ones that were most representative.

Immunohistochemistry. For immunohistochemistry, a rabbit anti-Ani s 4 antiserum generated by immunization of rabbits with 0.7 µg of recombinant Ani s 4 (Sigma-Aldrich, Haverhill, UK) was used to evaluate the presence of Ani s 4 in parasitized fish muscle. Small pieces of infected and noninfected muscle tissues were treated as previously described (29, 32, 38). The tissues were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (PBS) for 1 h at 4°C. After washing in PBS, samples were dehydrated in successive ethanols with progressive lowering of temperature to -30°C and then embedded in Lowicryl K4M resin (Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections (70 nm) of both infected and noninfected muscles were obtained with an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria) and collected on 50-mesh formvar-coated nickel grids. Tissue sections were incubated with rabbit polyclonal antisera raised against recombinant Ani s 4 diluted 1:20 with PBS containing 1% bovine serum albumen (BSA). After washing with PBS, the sections were incubated with a goat anti-rabbit immunoglobulin G (80 ng/ml) secondary antibody conjugated to 10-nm gold particles (British Biocell, Cardiff, UK) and diluted

1:100 with 1% PBS-BSA for 1 h at room temperature. After PBS and water washings, sections were stained with 2% uranyl acetate and Reynold's lead citrate and examined in a Jeol 1010 electron microscope. Controls were treated in the same way but excluding the primary antibody.

Color of fresh muscle. The color of microwave and conventionally heated hake muscle was measured in muscle homogenates in a HunterLab model D25-9 colorimeter (D45/21) (Hunter Associates Laboratory Inc., Reston, VA) using a CIELab scale of L*(lightness: 100 = white, 0 = black), a* (−a*, greenness; +a*, redness), and b* (−b*, blueness; +b*, yellowness) (42). The measurements were performed for each sandwich in triplicate.

Shear resistance. The shear resistance was evaluated by packing 25 g (± 0.5 g) of fresh and heated (microwave and conventionally heated sandwiches) hake muscle into cylindrical stainless steel containers (30 mm height by 30 mm diameter), which were hermetically sealed with screw tops and bottoms and kept at $5 \pm 1^\circ\text{C}$ until measured (≤ 18 h). The cooled samples were cut horizontally into two slices (15 mm height), and each determination was made on two slices in a TA-XT2 Texture Analyser (Stable Microsystems Ltd., Godalming, UK) fitted with a Kramer shear cell at a maximum force of 25 kN (kilonewtons) and a speed of 2 mm s^{-1} . The maximum load was measured and expressed as Newtons per gram. The measurements were made in triplicate for each sandwich.

Statistical treatment. Means and standard deviations were calculated for proximate composition of the fish muscle. Results of color and shear resistance of the muscle heated by microwave and conventional methods were subjected to a one-way analysis of variance and Tukey's honestly significant difference test using the SPSS 9.0 program (SPSS, Chicago, IL). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Proximate composition. The proximate composition of the hake muscle was $799 \pm 1.5 \text{ g kg}^{-1}$ moisture, $196 \pm 0.9 \text{ g kg}^{-1}$ crude protein, $11 \pm 3.7 \text{ g kg}^{-1}$ crude fat, and $13 \pm 0.4 \text{ g kg}^{-1}$ ash.

Mobility and emission of fluorescence: isolated larvae. The percentages of isolated larvae showing spontaneous mobility and emission of fluorescence after microwave treatment in distilled water are presented in Table 1. At 50°C , the percentage of larvae that were alive after microwave treatment generally decreased over time. No movement was observed after treatments at 60 and 70°C at any time point (all larvae recorded as dead). All the larvae had a cooked, opaque appearance after 7 and 2 min at 60 and 70°C , respectively.

Emission of a bluish fluorescence by dead *Anisakis* larvae when examined with UV light at 366 nm is often used to distinguish between live and dead individuals. No fluorescence was observed among the isolated larvae after microwave treatment at 50°C , when $\geq 80\%$ of the larvae were dead (no spontaneous or stimulated mobility observed after 7 min of microwave treatment) (Table 1). At 60 and 70°C (all larvae dead under the studied conditions), fluorescent emissions were observed in only 50% of the

TABLE 1. Percentage of isolated *Anisakis simplex* L3 larvae showing movement and fluorescence after microwave treatment under various time-temperature conditions

Temp ($^\circ\text{C}$)	Time (min) ^a	% of larvae showing movement ^b	% of larvae showing fluorescence ^c		
			Slight	Medium	Maximum
50	1	70	0	0	0
	2	80	0	0	0
	3	50	0	0	0
	4	20	0	0	0
	5	70	0	0	0
	6	60	0	0	0
	7	20	0	0	0
	8	20	0	0	0
	9	20	0	0	0
	10	10	0	0	0
60	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
	6	0	0	0	0
	7	0	0	0	0
	8	0	0	0	0
	9	0	20	30	0
	10	0	0	40	40
70	1	0	0	0	0
	2	0	10	0	0
	3	0	30	50	0
	4	0	0	30	70
	5	0	0	0	100

^a Time was recorded after the selected temperature was reached.

^b Larvae were recorded as dead when no movement was observed over a 2-h period.

^c Intensity of fluorescence was rated as maximum, medium, or slight.

larvae after 9 min at 60°C and in only 10% of the larvae after 2 min at 70°C , but percentages and fluorescence intensity generally increased over time. Emission of fluorescence by the same batch of larvae heated at 60 and 70°C in a water bath was observed after shorter times (39).

Mobility and emission of fluorescence: larvae in infected sandwiches. No mobility was observed in larvae removed from the infected sandwiches heated at 60 and 70°C by microwave or conventional heating in a water bath (Table 2). The percentage of larvae emitting fluorescence after the sandwiches were heated at 60°C was similar for both types of heat treatments; at 70°C , all larvae treated by microwave heating emitted fluorescence but only 25% of the larvae heated in the water bath emitted fluorescence. Nevertheless, the intensity of emission in the microwave-treated larvae was lower than that in the conventionally heated larvae. All larvae had a cooked, opaque appearance after treatment of the sandwiches at both temperatures. The inconsistent emission of fluorescence by dead larvae killed by conventional or microwave heat treatments confirms our previous findings that this technique is not appropriate for discrimination between live and dead larvae (30, 35, 39, 40).

TABLE 2. Percentage of *Anisakis simplex* L3 larvae showing movement and fluorescence after heating the infected sandwiches by conventional means in a water bath (H) and by microwave treatment (MW) under various time-temperature conditions

Temp (°C)	Time (min) ^a	Treatment	% of larvae showing movement ^b	% of larvae showing fluorescence ^c		
				Slight	Medium	Maximum
60	5	H	0	8	8	0
		MW	0	0	8	8
70	3	H	0	0	17	8
		MW	0	100	0	0

^a Time was recorded after the selected temperature was reached.

^b Larvae were recorded as dead when no movement was observed over a 2-h period.

^c Intensity of fluorescence was rated as maximum, medium, or slight.

Larval movement, emission of fluorescence under UV light, and changes in color when stained with different dyes have been used to determine larval death because some authors consider parasite mobility alone as an inadequate means of distinguishing live from dead larvae (23). However, by monitoring mobility alone over a 2 h period we were able to clearly distinguish between live and dead *Anisakis* L3 larvae. Emission of fluorescence in live larvae can be associated with stress produced by some treatments, and a lack of fluorescence in dead larvae can be associated with the batch of larvae and the type and intensity of the treatment (30, 35, 39, 40). Various dyes have been used to discriminate between live and dead larvae (23). However, if the cuticle of the larvae has been altered by a previous treatment, the conditions applied in the standard larvae staining methodology (acid-pepsin at 37°C for 24 h) may cause ruptures in the cuticle, reducing its resistance to pepsin, and some stained larvae fragments may become visible in the medium (39).

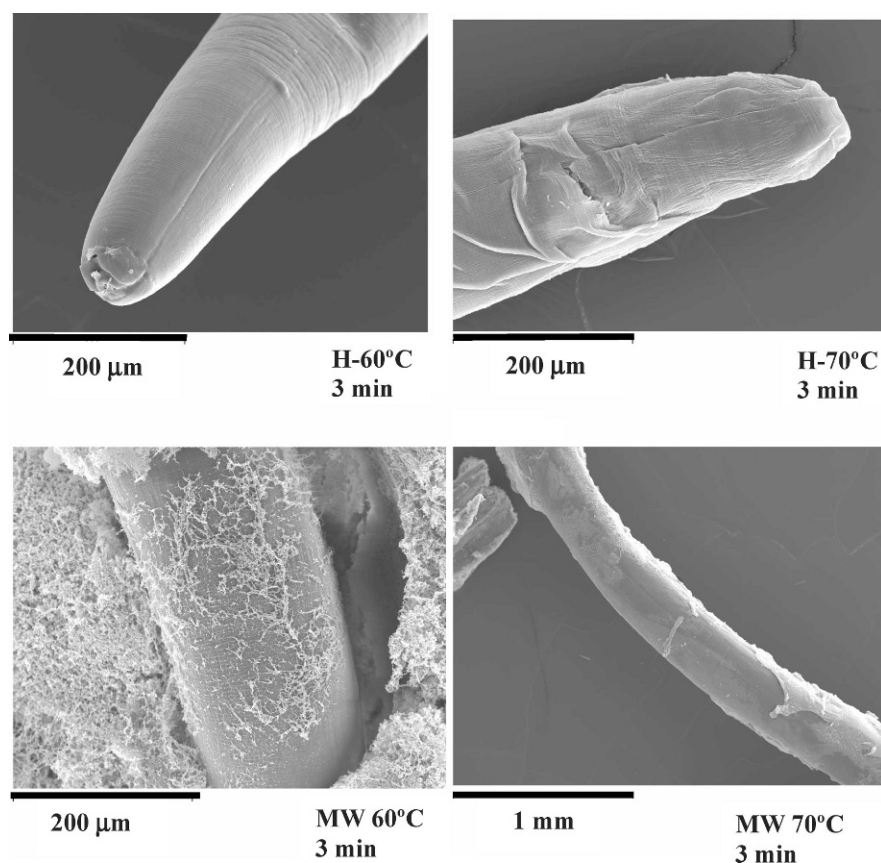
In the isolated larvae, the time taken to reach the required temperature inside the experimental tubes was 1 min in the microwave heated groups and 3 min in the conventionally heated groups (39). Nevertheless, the effect of heating on loss of mobility was observed earlier when the larvae were heated by microwave than by conventional heating in a water bath. In the sandwiches, the effect of temperature was more difficult to compare. In the microwave treated samples the time required to heat the thermal center at both selected temperatures was set at 1 min, and from the thickness of the samples it was assumed that the microwaves penetrated the entire sandwich; however, in the water bath samples, the transmission of heat was from the surface to the interior of the sandwich, and the time required to reach 60 or 70°C at the thermal center was 6.5 and 11 min, respectively. The temperature necessary to inactivate either the isolated larvae or the larvae infecting the muscle was lower than that reported by other authors (1), probably because the microwave oven used in the present study measured the working conditions more accurately. As previously indicated, the general consensus is that the nonthermal effects of microwaves such as selective heating, electroporation, cell membrane rupture, and cell lysis are likely to be due to the lack of precise measurements of the time-temperature conditions and spatial variations (20). However, in present study the size of the samples was fixed

in both heat treatments and the time-temperature conditions in the microwaved samples were applied in an oven designed to accurately attain the predetermined conditions during sample treatment, so that both heat treatments could be compared. The results for the isolated larvae suggest that although heat is the main factor responsible for the mortality of larvae after microwave treatment, nonthermal factors have additional effects.

Scanning electron microscopy. The scanning electron micrographs of the isolated larvae heated with microwaves indicate slight dehydration of the larval body, but no major differences were associated with higher temperatures or longer exposure times (micrographs not shown). The micrographs of larvae inside the sandwiches heated in a water bath (Fig. 1) show clear changes only when the larvae were heated at 70°C. The appearance of larvae heated at 60°C did not differ from that of unheated larvae (35). When the larvae in the sandwiches were heated with microwaves, there were no appreciable changes in the shape of the larvae at either temperature, although in both cases the cuticle was covered with a layer resembling aggregated protein, apparently from the surrounding muscle.

Immunohistochemistry. The immunohistochemistry micrographs reveal changes in the muscle structure due to heating (Fig. 2). The presence of the heat-resistant allergen Ani s 4 in the fish muscle heated by conventional means and microwaves at 70°C is shown at higher magnification (Fig. 2A) in areas where no larvae were observed in the muscle. This finding suggests that Ani s 4 allergens were released from the larvae into the surrounding tissue and remained active after both heat treatments had killed the larvae, which also has been reported in fresh muscle (32). Ani s 4 and *A. simplex* crude extracts have been recognized by human sera (30). The detection of Ani s 4 by immunohistochemistry supports previous findings in which Ani s 4 and other *Anisakis* allergens were detected by immunoblotting in extracts of fresh and frozen fish muscle heated at higher temperatures in a water bath and a standard microwave domestic oven, even after pepsin treatment (38). Immunogold labeling for the detection of Ani s 4 in fish muscle is a useful method for detection of the allergen in situ, mainly when the muscle structure is altered, as occurs during long-term freezing, cooking, etc. Most of the

FIGURE 1. Scanning electron micrographs of *Anisakis simplex* L3 larvae obtained from infected hake muscle sandwiches heated by conventional heating (H) or microwave treatment (MW) to internal temperatures of 60 and 70°C.



traditional immunological methods used for screening allergens in food depend on extraction of the allergenic proteins from the food matrix; therefore, if the muscle structure is altered and myofibrillar proteins aggregate, low ionic strength soluble proteins such as *Anisakis* 4 may become trapped in the fish matrix, thus becoming unextractable under standard conditions (34).

Effect of microwave and conventional heating on hake muscle. In some studies, microwave treatments have induced more intense oxidation of fatty acids when compared with conventional heat treatments (12). Oxidation of fatty acids and formation of free radicals can modify the color and texture of fish muscle. In a previous study, some changes in color were observed when gilthead seabream

(*Sparus aurata*) muscle artificially infected with *A. simplex* larvae and stored chilled for 7 days was compared with samples that were not infected with the larvae (41).

Changes in muscle color are shown in Table 3. Heating significantly increased the lightness (L^*) of the muscle compared with the fresh sample. The increase was significantly higher in the conventionally heated muscle than in the microwave heated samples at both temperatures. Significant differences in a^* ($-a^*$, greenness; $+a^*$, redness) were found between the fresh, conventionally heated, and microwaved samples at both temperatures, with most of the heated lots changing to negative a^* values (greenness). Significant differences in a^* values between control (no larvae added) and infected samples were found in the microwaved samples at both temperatures. Differ-

FIGURE 2. Immunohistochemistry micrographs of hake muscle heated to 70°C by conventional heating (H) or microwave treatment (MW). Inset (A): Immunolocalization of the *A. simplex* allergen *Anisakis* 4 in the fish muscle. The 10-nm gold particles conjugated with the secondary antibody appear in the micrograph as dark circles (arrows).

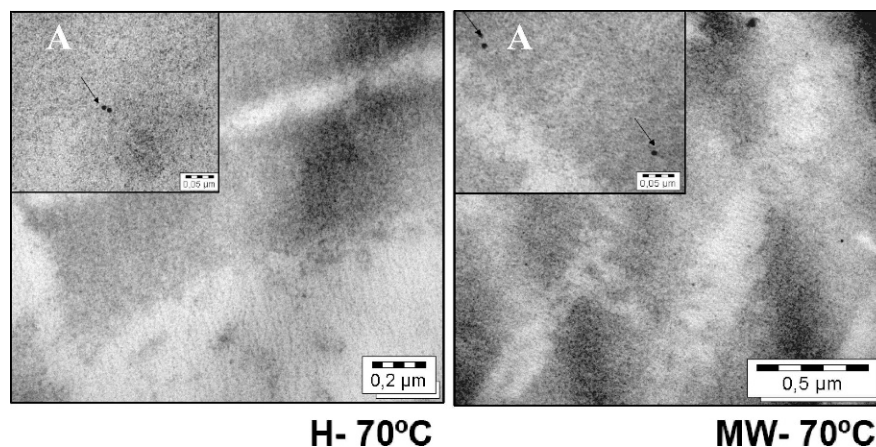


TABLE 3. Color of fresh and treated hake muscle after heating at 60 and 70°C in a conventional water bath (H) and a microwave oven (MW)^a

Temp, time ^b	Treatment	L*	a*	b*
Fresh muscle	No larvae added	52.00 ± 1.61 A	0.70 ± 0.80 A	-1.70 ± 0.90 A
60°C, 5 min	H, no larvae added	75.70 ± 1.61 B	-1.80 ± 0.20 B	3.50 ± 1.00 B
	H, larvae added	74.71 ± 0.70 B	-1.68 ± 0.30 B	2.95 ± 0.60 B
	MW, no larvae added	62.70 ± 5.20 C	0.30 ± 0.40 C	2.50 ± 2.10 B
	MW, larvae added	71.13 ± 2.00 D	-1.15 ± 0.60 B	4.16 ± 0.70 B
70°C, 3 min	H, no larvae added	79.02 ± 0.29 E	-1.87 ± 0.21 B	4.00 ± 0.50 B
	H, larvae added	76.21 ± 1.77 B	-0.74 ± 0.25 B	5.80 ± 0.71 C
	MW, no larvae added	72.10 ± 0.43 D	-0.81 ± 0.51 B	5.07 ± 1.14 BC
	MW, larvae added	70.10 ± 1.64 C	0.05 ± 0.13 A	6.25 ± 0.43 C

^a L*, lightness (100 = white; 0 = black); -a*, greenness; +a*, redness; -b*, blueness; +b*, yellowness. Within a column, means with different letters are significantly different ($P < 0.05$).

^b Time was recorded after the selected temperature was reached.

ences in a* between fresh control muscle and fresh muscle infected with *Anisakis* larvae have been reported (41). The color of the muscle changed from negative b* values (blueness) measured in the fresh muscle to positive b* values (yellowness) in both heated samples, generally increasing at higher temperatures. Nevertheless, we have not found a clear pattern of behavior in the color values measured in samples heated with both treatments.

Conventional heating in a water bath at 70°C resulted in increased shear resistance of hake muscle compared with fresh muscle and with muscle heated conventionally at 60°C or with microwaves at 60 or 70°C (Table 4). The increase in shear resistance is related to an increase in toughness, which is associated with a decrease in sensory quality in hake.

Microwave heating has advantages over conventional cooking because *A. simplex* larvae are killed faster when the microwaves penetrate the entire sample. The results in isolated larvae support the hypothesis that heat is the main but only cause of death of larvae during microwave treatment; variations in the time-temperature conditions were not a factor because these parameters were set accurately. Fewer textural changes were observed at 70°C in the fish muscle heated in the microwave oven compared with that heated in the water bath, indicating that the muscle is less altered by microwaves under conditions most likely to kill the larvae. In addition to hot and cold spots in

samples due to the uneven distribution of microwaves in standard domestic microwave ovens, beyond a given sample depth larval death is due solely to the heat effect. In these cases, the time-temperature conditions reached at all points in the product may not be sufficient to kill the larvae. Although the larvae are dead, heat-resistant allergenic proteins such as Ani s 4 may remain in the muscle after either form of heat treatment, indicating that although microwave treatment may prevent anisakiasis and allergic sensitization in consumers, microwave cooking may not render fish safe for individuals already sensitized to *A. simplex* allergens.

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TABLE 4. Shear resistance of fresh and heated hake muscle after conventional heating (H) and microwave heating (MW) at 60 and 70°C

Temp	Treatment	Shear resistance (N g ⁻¹) ^a
Fresh muscle	No larvae added	1.25 ± 0.11 A
60°C	H, no larvae added	1.30 ± 0.03 A
	H, larvae added	1.10 ± 0.09 A
	MW, no larvae added	1.12 ± 0.04 A
	MW, larvae added	1.40 ± 0.46 A
70°C	H, no larvae added	2.70 ± 0.05 B
	H, larvae added	2.40 ± 0.13 C
	MW, no larvae added	1.20 ± 0.04 A
	MW, larvae added	1.40 ± 0.18 A

^a Means with different letters are significantly different ($P < 0.05$).

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