

Removal of *Anisakis simplex* allergens from infected fish during the washing step of *surimi* production

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Abstract

BACKGROUND: The washing operation of fish muscle is one of the key steps in the production of *surimi*. The aim of this study was to assess in parasitised minced fish the effect of the washing steps on the allergen removal of *Anisakis simplex* and on protein yield during *surimi* processing. Experimentally infected hake (*Merluccius merluccius*) (50 *Anisakis simplex* s.s L3 larvae per 100 g of muscle) underwent three successive washing steps with water, phosphate buffer (20 mmol L⁻¹), sodium bicarbonate (60 mmol L⁻¹), or sodium hypochlorite (0.27 mmol L⁻¹) in the *surimi* processing (4 kg muscle, 1:4 w/v for each solution). Total protein concentration and *A. simplex* antigens and allergens were evaluated in each waste fraction.

RESULTS: The highest removal of *Anisakis* 4 and *A. simplex* antigens was achieved by using phosphate buffer, together with a good protein yield in the raw *surimi*. Decrease of the concentration of allergens and antigens as a function of the washing steps rendered a linear trend ($R^2 = 0.95$ and 0.98 for *Anisakis* 4 and *A. simplex* antigens, respectively).

CONCLUSION: The conditions for an optimal removal of *Anisakis* allergens can be established and calculated as a function of the washing steps. This approach opens a line to utilise parasitised fish in a safer way.

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Keywords: *A. simplex*; raw *surimi*; *Anisakis* 4; allergens; immunoblotting

INTRODUCTION

Human infection due to eating fish parasitised by live *Anisakis* sp. larvae in the third stage (L3) is considered nowadays a relevant health problem associated to fish consumption. Consumers can become infected with the live larvae when the parasitised fish is consumed raw or when treatments given to the fish as technological processes, cooking or storing practices, do not kill the larvae.^{1,2} In those cases, one of the consequences is that subjects can suffer from allergic symptoms due to sensitisation to secretion/excretion products and some somatic and cuticle proteins of *Anisakis* larvae.^{2–5} It is accepted that an initial infection of the gastric or intestinal mucosa with live L3 larvae is needed to develop allergic symptoms. Once the consumer is sensitised, symptoms of allergy can be observed after ingestion of fish containing allergenic proteins even in products that have been correctly treated to kill the larvae, since there are some allergens with a high thermal stability.^{2,3,6–11}

Chemical, biochemical and physical characteristics of *Anisakis* allergenic proteins, such as their structure, molecular weight, isoelectric point, number of SH groups, or thermal stability, among others, have been described by different authors.¹² These characteristics can influence protein stability during application of technological processes, modifying their allergenic properties. Therefore, they can be used to reduce their allergenicity.

Some of the steps of *surimi* production and gel formation can mitigate allergenicity either by removal of the allergenic proteins during the washing step or by structural modifications by heating. In the washing operation of fish muscle myofibrillar proteins are concentrated and water-soluble components leached, particularly sarcoplasmic proteins which hinder the correct formation of the network required for making gels.^{13,14} For this, water alone, or different washing solutions and pHs in the range of 6.5 to 7.5 may be used depending on the raw material.^{15,16}

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Phosphate buffer maintains the functionality of muscle protein during extraction.^{17,18} Oxidising agents such as sodium hypochlorite (NaClO) may improve the gel-forming ability in low-quality fish flesh by inducing protein oxidation and formation of disulfide bonds.¹⁹ On the other hand, sodium bicarbonate (NaHCO₃) has been reported to help in the lipid extraction, as well as in the removal of other compounds that may affect the gel formation process.^{18,20,21}

The aim of this study was to assess, in parasitised minced fish, the effect of the washing steps on the allergen removal of *Anisakis simplex* and protein yield during *surimi* processing.

MATERIALS AND METHODS

Hake

Twenty-one gutted hakes (*Merluccius merluccius*) purchased from the Fisheries Technological Centre in Celeiro Port, Lugo (CEPTEC) were sent to the laboratory at the Institute of Food Science, Technology and Nutrition (ICTAN-CSIC). The hakes were caught in July 2011 in the North East Atlantic fishing grounds (FAO Major Fishing Area 27-VIIj). The temperature of the fish on arrival to the laboratory was $\leq 1^{\circ}\text{C}$. The fish was stored with ice in cold room ($2 \pm 1^{\circ}\text{C}$) for $<24\text{ h}$.

Anisakis simplex larvae

Live *Anisakis simplex* L3 larvae were obtained from heavily infected ovaries and viscera of hake caught in the North East Atlantic fishing grounds and provided by CEPTEC. Approximately 500 g of larvae with tissue attached were sent together with the hake to the laboratory and were immediately stored at $5 \pm 1^{\circ}\text{C}$. Live larvae were removed from the tissue with forceps, cleaned by successive washings with distilled water, and stored at $5 \pm 1^{\circ}\text{C}$ until use.

Sample preparation

The process was performed at pilot-plant scale. Hakes were beheaded manually, deboned in an extruder (Baader 694; Baader, Lübeck, Germany) with drum holes of 3.0 mm of diameter.

For larvae conditioning and to ensure an even distribution, lots of 11 g of clean larvae (equivalent weight of 2000 larvae) were first added to 0.10 kg of mince and then mixed with the remaining 3.9 kg. The infected mince was stored in a chill room ($5 \pm 1^{\circ}\text{C}$) for 24 h.

In order to homogenise the muscle and simulate the retention of larvae in this step, the infected muscle (4 kg per lot) was extruded again in the same conditions. Then the muscle was washed in a 25 L tank (Prototype, Pilot plant) using 16 L of washing solutions each time. Each lot underwent three successive washes [muscle:washing solution, 1:4 (w/v); 10 min] with mechanical stirring every 5 min, followed by decanting through a rotary screen (15 min) to facilitate the removal of the waste fraction (WS1) which was collected for analysis. Each lot underwent two additional washing cycles and corresponding waste fractions (WS2 and WS3) were also collected. After the last wash the muscle was pressed (Screw press Baader 523; Baader) to remove excess liquid. The temperature in all the process was $\leq 5^{\circ}\text{C}$. pH was measured during the washing steps and adjusted when necessary to 7.0–7.5 in the third washing.

Thus four lots of washed raw *surimi* (RS) were obtained after the washing and pressing steps according to the solutions used: water (H₂O), phosphate buffer pH 7.5 (20 mmol L⁻¹), sodium bicarbonate (60 mmol L⁻¹, NaHCO₃), and sodium hypochlorite (0.27 mmol L⁻¹,

NaClO). Portions of RS and waste fractions WS1, WS2, and WS3 were frozen stored at $-20 \pm 2^{\circ}\text{C}$, until analysis.

Taxonomic identification of *Anisakis* larvae

For identification studies ≥ 40 clean larvae were frozen stored ($-30 \pm 1^{\circ}\text{C}$) until used. Taxonomic identification was made following the protocol described by D'Amelio.²²

Proximate composition and pH

Moisture, ash, crude protein (LECO FP-2000 nitrogen/protein analyser; LECO Corp., St Joseph, MI, USA; using a nitrogen to protein conversion factor of 6.25) and crude fat contents of hake muscle were measured and expressed in g kg⁻¹ of muscle.^{23,24} pH was determined at room temperature using 10 g of muscle in 100 mL of distilled water. Measurements were carried out with a glass electrode (pH meter Metrohm model 827 pH lab; Metrohm AG, Herisau, Suiza). Crude protein (mg g muscle⁻¹) and pH of RS and WS1–WS3 were measured in triplicate when needed by the same methods.

Antigen source

Antigenicity of the *A. simplex* L3 in parasitised fish flesh was studied by dot blot using rabbit anti-*A. simplex* crude extract or anti-Ani s 4 antisera. Anti-*A. simplex* crude extract and anti-Ani s 4 antisera were generated by immunisation of rabbits with 1 mg of parasite crude extract and 0.7 mg of recombinant Ani s 4 respectively, as previously described.⁹

Quantification of *Anisakis simplex* antigens by dot blot

Three microlitres of WS1, WS2, and WS3 were dotted in duplicate to a nitrocellulose membrane and dried for 10 min at 37°C . After blocking, membranes were incubated with rabbit polyclonal anti-Ani s 4 or anti-crude extract antisera (1/10 000 dilution, 1 h, room temperature) and then with alkaline-phosphatase-labelled goat anti-rabbit IgG and revealed for 30 min with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) alkaline phosphatase substrate solution. A reference curve was made in duplicate, dotting 5, 2.5, 1.25, 0.62, 0.31 and 0 μg of an *A. simplex* crude extract or recombinant Ani s 4. Membranes were scanned and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA) was used to define the boundaries of proteins dots, to assess pixel number and density, and to obtain final numerical data for quantifying. The results were expressed in micrograms of Ani s 4 or *A. simplex* antigens per gram of muscle ($\mu\text{g g}^{-1}$).

Statistical analysis

For RS, WS1, WS2, or WS3, one-way analysis of variance was performed as a function of the washing solution (water, phosphate buffer, sodium bicarbonate or sodium hypochlorite). Comparison between means was assessed by the Tukey HSD test. The level of significance was set at $P < 0.05$. Linear regression analyses were performed with total protein, Ani s 4 allergen or *Anisakis* antigens concentrations as dependent variables, and the number of washes as the independent variable. SPSS 19.0 software (Chicago, IL, USA) was used for statistical analysis.

RESULTS AND DISCUSSION

The average length and weight of fish were $64.86 \pm 2.52\text{ cm}$ and $1.70 \pm 0.15\text{ kg}$ respectively. The proximate composition of

Table 1. Crude protein (mg g muscle⁻¹) and pH values of raw *surimi* (RS) and corresponding waste fractions (WS1, WS2, WS3) after the successive washing steps from infected hake minces (50 *Anisakis simplex* s.s. larvae in 100 g muscle)

Parameter	Fraction	Washing solution			
		Water	Phosphate buffer	Sodium bicarbonate	Sodium hypochlorite
Protein	RS	121.10 ± 6.98 ^a	135.94 ± 5.59 ^b	119.23 ± 0.53 ^a	125.86 ± 4.14 ^{ab}
	WS1	15.89 ± 0.70 ^a	21.15 ± 1.16 ^b	21.33 ± 0.05 ^b	20.76 ± 0.55 ^b
	WS2	11.72 ± 1.01 ^a	10.97 ± 0.47 ^a	15.05 ± 0.20 ^b	12.61 ± 1.19 ^{ab}
	WS3	6.10 ± 1.84 ^a	7.23 ± 1.37 ^a	8.40 ± 0.07 ^b	7.32 ± 0.62 ^a
pH	RS	6.77 ± 0.02 ^a	7.38 ± 0.01 ^b	7.39 ± 0.01 ^b	7.10 ± 0.01 ^c
	WS1	7.10 ± 0.04 ^a	7.40 ± 0.04 ^b	7.60 ± 0.00 ^c	7.12 ± 0.02 ^a
	WS2	7.20 ± 0.02 ^a	*7.59 ± 0.02 ^b	*8.30 ± 0.02 ^c	7.22 ± 0.04 ^a
	WS3	7.34 ± 0.02 ^a	7.44 ± 0.04 ^b	7.40 ± 0.01 ^b	7.23 ± 0.01 ^c

*pH was adjusted to ≤7.5.

^{a,b,c}Different superscript letters in the same row indicate significant differences among washing solutions ($P < 0.05$).

the hake muscle (g kg⁻¹) was: moisture 826.78 ± 25.18; protein 171.76 ± 2.16; ash 12.43 ± 0.23; and fat 5.10 ± 0.16. The initial pH of muscle was 7.22 ± 0.02. These values were within the expected range for this species.²⁵ The yield of mince from gutted fish was 54.8%. The larvae morphologically were described as Type I and taxonomically identified as *Anisakis simplex* s.s.

Table 1 shows the crude protein content and pH values of raw *surimi* and the waste fractions obtained with the different solutions. The protein content in RS ranged between 119 and 136 mg g muscle⁻¹, rendering a yield of 69–79% with respect to total muscle protein (172 mg g⁻¹), within the typical values for these washing processes.²⁶ The amount of protein obtained in the WS1 extracted with water was significantly lower than with the other solutions where no differences were found. In the second and third washes, the protein content in the waste fraction extracted with bicarbonate solution was significantly higher than in the other samples.

pH values varied depending on the solution used during the washing steps (Table 1). The bicarbonate-treated waste fraction, which had the highest protein content, corresponded to an increased pH value during the first two washes (7.6 and 8.3, respectively). However, in the process for making *surimi* the recommended final pH is in the range of 6.5–7.5,^{15,16} as higher pHs may decrease the gel forming ability of the *surimi* to obtain final gel products. Therefore in the last wash the pH of phosphate buffer and bicarbonate lots were adjusted to this range.

Table 2. Linear regression equation parameters (y = protein concentration; x = number of washes) for the proteins extracted with different washing solutions from infected hake mince (50 *Anisakis simplex* s.s. larvae in 100 g muscle),

Washing solution	a	m	R^2
Water	21.032	4.897	0.993
Phosphate buffer	28.370	7.459	0.924
Sodium bicarbonate	27.856	6.466	0.999
Sodium hypochlorite	27.005	6.722	0.985

a is a constant, m is the slope of the linear regression and R^2 is the coefficient of determination.

The extraction of proteins with the different solutions followed a linear trend as a function of the successive washes, with coefficients of correlation ranging from 0.999 to 0.924. Table 2 shows the equation parameters for each of the solutions. SDS-PAGE of the waste fractions stained with Coomassie brilliant blue, and performed in an independent experiment, showed that the most abundant proteins fell, for all washing solutions in the 52–25 kDa range, and no signs of myosin heavy chain loss were observed in any of these extracts, irrespective of the solutions (Fig. 1).

Figure 2 shows the quantification of Ani s 4 and *A. simplex* antigens by dot blot analysis in WS1, WS2, and WS3. Ani s 4 is described as a secreted-excreted allergen associated with episodes of anaphylaxis.^{6,27} Due to its low molecular weight (~9 kDa), stability to high temperatures, acid, pepsin and pressures,^{6–10,28–31} it has been selected to study the effect of treatments given to fish muscle in the recognition of allergens in processes where washing steps are applied as *surimi-kamaboko* gels process. The highest overall extraction of Ani s 4 was observed with water and phosphate buffer as compared to hypochlorite and bicarbonate (Fig. 2a). As regards *A. simplex* antigens, the highest amount was extracted with the phosphate buffer (Fig. 2b).

In order to establish a possible pattern of allergen and antigen loss as a function of the different extracting solutions, the results were plotted against the number of successive washes. In general, and as found for total protein extraction, a linear trend was observed for both Ani s 4 and *A. simplex* antigens (Fig. 3). The equation parameters for the concentration of Ani s 4 and *A. simplex* antigens as a function of the number of washes performed were calculated and the highest coefficient of determination was obtained for the phosphate buffer-extracted allergens/antigens ($R^2 = 0.95$ and 0.98 for Ani s 4 and *A. simplex* antigens, respectively).

These results indicate that a number of cycles needed to wash out the allergens or antigens may be inferred for each of the solutions. Calculations using the equation parameters suggest that four to five washings with phosphate buffer could be sufficient to wash out the Ani s 4 allergen or *A. simplex* antigens, whereas five to six washings would be needed for water and hypochlorite. The low coefficient of determination for bicarbonate ($R^2 = 0.16$ and 0.31 for Ani s 4 and *A. simplex* antigens, respectively) does not allow to make any predictions. In all cases the numbers of washings are beyond the experimental range and therefore these estimations should be checked in each situation of interest.

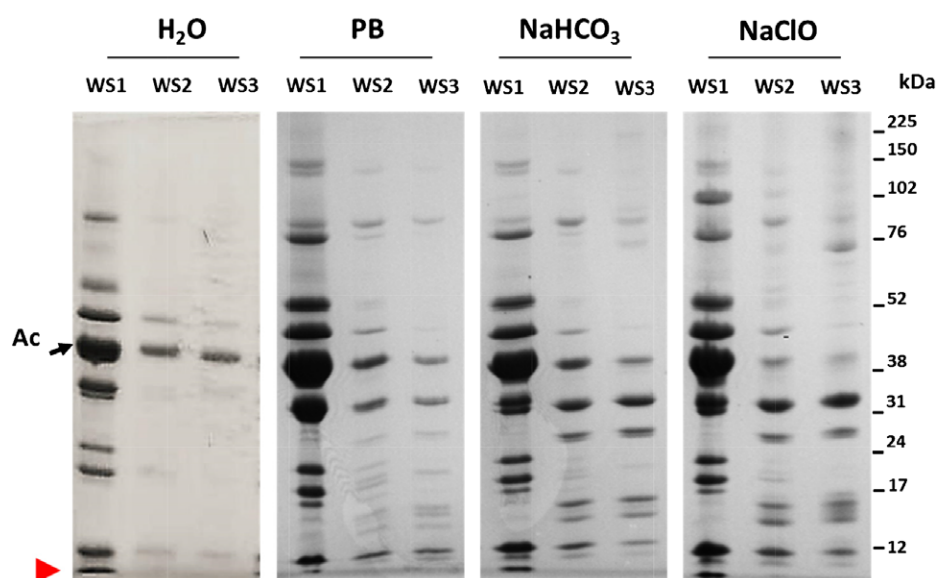


Figure 1. Electrophoresis pattern of waste fractions obtained during the successive washes (1:4, w:v, 10 min; $\leq 5^\circ\text{C}$) of infected hake mince (50 *Anisakis* spp. larvae in 100 g muscle) with different solutions. WS1, WS2, and WS3: first, second and third waste fractions respectively. H_2O , water; PB, phosphate buffer; NaHCO_3 , sodium bicarbonate; NaClO , sodium hypochlorite. Ac, actin; $\blacktriangle$, band corresponding to Ani s 4 molecular weight (MW).

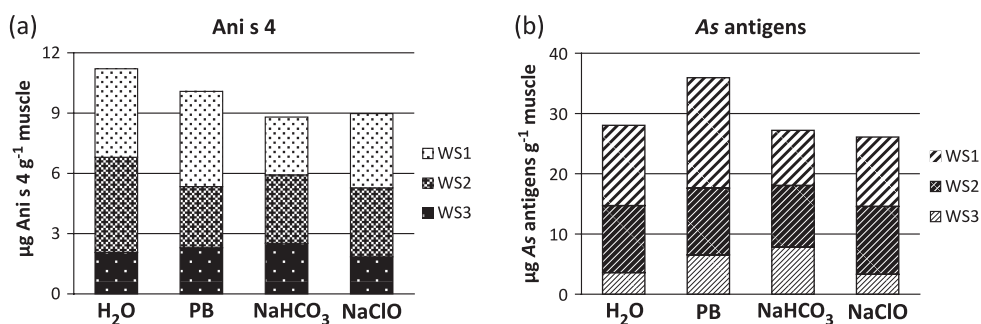


Figure 2. (a) Ani s 4 and (b) *A. simplex* antigens ($\mu\text{g g}^{-1}$) extracted with different washing solutions from infected hake mince (50 *Anisakis simplex* s.s. larvae in 100 g muscle). WS1, WS2, and WS3: first, second and third waste fractions respectively. H_2O , water; PB, phosphate buffer; NaHCO_3 , sodium bicarbonate; NaClO , sodium hypochlorite.

Figure 4 shows the ratio between Ani s 4 and the total soluble protein content for each waste fraction as a function of the washing step. It can be observed that in the third wash the extracts become enriched in Ani s 4, although the amount of both protein and allergen is actually very low.

The results show that since washing fish muscle can substantially reduce the allergen concentration, strategies can be designed for a given washing solution used in the *surimi* processing. Also, different expected infection levels, pH values, ratio of muscle to washing solution, number of washings, or washing times, will affect the extraction rate so that this strategy should be translated to each particular case. Washing of muscle could also be applied as a means of decreasing the allergen concentration for other elaboration procedures in different types of products.

The washing procedure presented here suggests that by setting these conditions it is possible to use this type of *surimi* to make gel products with a low associated risk for antigen exposure. The current trend is to perform less washing cycles with lower water to mince ratios, achieving good results in terms of *surimi* quality.³² However, *surimi* production using less water may not be as efficient in terms of allergen removal.

This work has been performed under controlled conditions with heavily infected fish. European Union regulations^{33–35} state that fish and fishery products that have a high rate of infection cannot be placed on the market for human consumption. This procedure would potentially allow the use of heavily infected fish or fish parts (e.g. belly flaps) which otherwise would have to be discarded.

The procedure does not exclude that some of the allergens, which interact with some muscle components and are not accessible to washing, are retained in the raw *surimi*. Also, for strategic or economic reasons, it may not be acceptable to perform more washing cycles. It would then be necessary to check to what extent this residual allergenicity might be further reduced by the application of other technological treatments (i.e. thermal treatment as applied in *kamaboko* type gels).

CONCLUSIONS

Washing of muscle can substantially reduce the allergenic capacity of heavily infected fish in a way that can be calculated as a function of the washing steps, so that the conditions for an optimal removal can be designed case by case. This approach opens a line to utilise parasitised fish in a safer way.

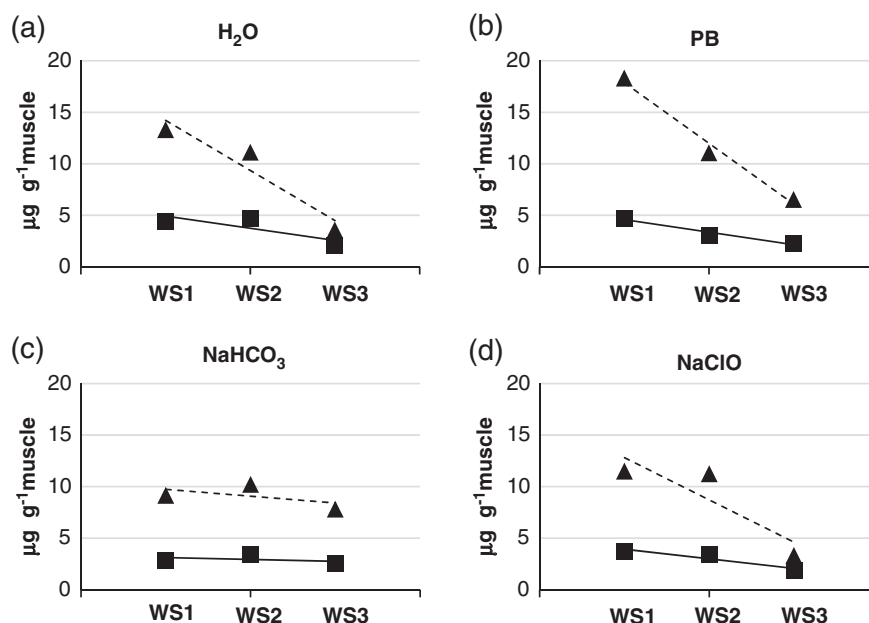


Figure 3. *Anisakis* (■) and *A. simplex* antigens (▲) during the successive washes with (a) water (H₂O), (b) phosphate buffer (PB), (c) sodium bicarbonate (NaHCO₃), and (d) sodium hypochlorite (NaClO). WS1, WS2, and WS3, first, second, and third waste fractions, respectively.

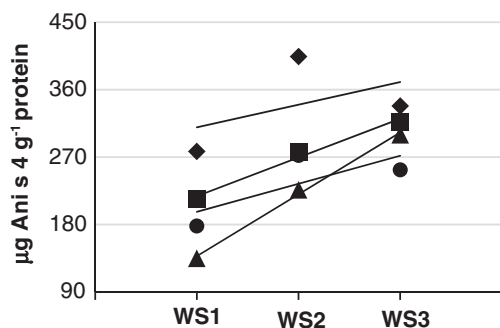


Figure 4. Ratio between *Anisakis* and protein content during the successive washes with (◆) water, (■) phosphate buffer, (▲) sodium bicarbonate, and (●) sodium hypochlorite. WS1, WS2, and WS3, first, second, and third wash waste fractions, respectively.

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