



## Optimization of the pepsin digestion method for anisakids inspection in the fishing industry

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### ABSTRACT

During the last 50 years human anisakiasis has been rising while parasites have increased their prevalence at determined fisheries becoming an emergent major public health problem. Although artificial enzymatic digestion procedure by CODEX (STAN 244-2004: standard for salted Atlantic herring and salted sprat) is the recommended protocol for anisakids inspection, no international agreement has been achieved in veterinary and scientific digestion protocols to regulate this growing source of biological hazard in fish products. The aim of this work was to optimize the current artificial digestion protocol by CODEX with the purpose of offering a faster, more useful and safer procedure for factories workers, than the current one for anisakids detection. To achieve these objectives, the existing pepsin chemicals and the conditions of the digestion method were evaluated and assayed in fresh and frozen samples, both in lean and fatty fish species. Results showed that the new digestion procedure considerably reduces the assay time, and it is more handy and efficient (the quantity of the resulting residue was considerably lower after less time) than the widely used CODEX procedure. In conclusion, the new digestion method herein proposed based on liquid pepsin format is an accurate reproducible and user-friendly off-site tool, that can be useful in the implementation of screening programs for the prevention of human anisakiasis (and associated gastroallergic disorders) due to the consumption of raw or undercooked contaminated seafood products.

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## 1. Introduction

Anisakid roundworms (*Anisakis*, *Contracaecum* and *Pseudoterranova*) are recurrently found in the abdominal cavity (including gut) and flesh of a large variety of fish and cephalopod species of commercial interest, regularly consumed by humans. The third larval stage is transmitted through the consumption of raw or minimally processed

seafood, and may cause pathogenic diseases like gastric or intestinal anisakiasis (Kikuchi et al., 1990; Esteve et al., 2000; Lopez-Serrano et al., 2003; Nawa et al., 2005; Mineta et al., 2006), and gastro-allergic disorders (Alonso-Gómez et al., 2004; Plessis et al., 2004; Nieuwenhuizen et al., 2006; Audicana and Kennedy, 2008; Hochberg and Hamer, 2010). The effects of anisakids on decreasing commercial value of fish (Vidacek et al., 2009) and its impact on human health has given these parasites a public health concern, which was recently recognized by the Panel on Biological Hazards of the European Food Safety Authority (EFSA, 2010). During the last 50 years, the significance of this double effect has been growing as parasites have increased their prevalence being more relevant in North Atlantic fisheries (Smith and Wootton, 1979; McClelland et al., 1985; Adams et al., 1997;

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Abollo et al., 2001; Rello et al., 2009), and due to the lack of awareness of this potential threat among consumers. Consequently, several methods have been developed for detection, diagnosis and identification of parasites in fish, from visual inspection (Hartmann and Klaus, 1988), light microscopy (Rijpstra et al., 1988), candling (Wold et al., 2001; Butt et al., 2004), pepsin digestion (Lysne et al., 1995; Lunestad, 2003; Thien et al., 2007; Thu et al., 2007), UV illumination (Adams et al., 1999; Levsen et al., 2005; Marty, 2008), ultrasound (Hafsteinsson et al., 1989; Nilsen et al., 2008), X-rays (Nilsen et al., 2008), conductivity (Nilsen et al., 2008), electromagnetism (Haagensen et al., 1993; Choudhury and Bublit, 1994), magnetometry (Jenks et al., 1996), immunodiagnoses (Xu et al., 2010), multilocus electrophoresis (Mattiucci et al., 1997; Abollo et al., 2001), RT-PCR (Fang et al., 2011), real-time FRET (Fluorescence Resonance Energy Transfer) (Monis et al., 2005; Intapan et al., 2008), PCR (Zhu et al., 2002; Abe et al., 2005; Pontes et al., 2005), to Imaging Spectroscopy (Heia et al., 2007). Nevertheless, although all these methods have been used and are being applied by fishery operators or laboratories as integrated strategies in official and self-control tests, none of them has been accepted as the international standard accomplishing with industrial requirements. That lack of a gold standard for any of the above given methods, mainly for a fast and easy visual detection, has historically hampered the consensus of parasite detection and diagnosis protocols at the fishing industry, thus reducing consumer confidence towards seafood companies.

Specifically, acidified pepsin solution has been largely applied as a confirmatory invasive protocol to detect absence or presence of nematodes in fish products (Lunestad, 2003), and as a tool to quantify parasitic infections and to estimate the number of parasites in the fish musculature (Lysne et al., 1995; Thien et al., 2007; Thu et al., 2007). Some additional variations of the pepsin digestion method from CODEX STAN 244-2004 protocol have been developed by some authors (CX/FFP 08/29/7; Dixon, 2006) with attempts to go further, specifically in improving the method and more widely in developing faster methodologies for biological threats detection.

According to the two definitions of “optimization” provided here (“to achieve maximum efficiency in storage capacity or time or cost” and “to make as effective, perfect, or useful as possible”), the aim of this work was to improve and optimize the current artificial digestion protocol of CODEX by (1) evaluating three different brands of commercial pepsins on different fish products (e.g., lean/fatty and fresh/frozen), (2) implementing new conditions on the basis of the current digestion procedure, and (3) comparing the new practice proposed with the currently used one. As a result, a new analytical methodology is offered based on the modification of the existing artificial digestion of fish flesh provided by CODEX.

## 2. Materials and methods

### 2.1. Samples

Fresh fishes obtained at retail both of European hake (*Merluccius merluccius*) and Atlantic mackerel (*Trachurus*),

were used as representative samples of lean and fatty fish species, respectively. Half of them were processed in fresh and half were immediately frozen at  $-20^{\circ}\text{C}$  for at least 24 h, and afterwards processed. Three different commercial pepsins were preselected to be evaluated: a commonly used pepsin (pepsin 1), the recommended reagent in CODEX protocol (pepsin 2) and a novel liquid format (pepsin 3). For understanding and presenting their proteolytic activities, equivalences between different units used in commercial pepsins were taken into account (Langdon, 2009). Proteolytic activities indicated by the three manufacturers for the three pepsins were: 800–2500 Units/mg of protein, 2000 Units/g FIP (International Pharmaceutical Federation), and 660 U Ph Eur (European Pharmacopeia)/ml, respectively. Authors understand that enzymatic activities specified do not need verification because it would not be viable to develop routine protocols, since it should be necessary to perform a check of any pepsin before its use. Therefore, in order to minimize any imprecision related to the reagents, all of the pepsins used in this study were acquired, stored, prepared and treated properly under the same criteria and under identical conditions (specified by manufacturers).

### 2.2. Pepsin assays

Briefly, six aliquots of 25 g each from both fresh and frozen fish species were digested with the three different pepsins at  $37^{\circ}\text{C}$  during 30 min in an ACM-11806 magnetic stirrer with thermostated heating multiplate. The weight/volume pepsin ratio used was 1:20, understanding that ratio as 1 g of fish for 20 ml of a 0.5% pepsin solution in HCl 0.063 M pH 1.5. Undigested muscle residues of each kind of fish and pepsin were weighed and compared, without taking into account the weight due to the parasites in the positive samples.

In order to compare the two pepsins that previously had given higher percentages of digested muscle, appropriate calculations were made to determine the pepsin dose necessary in each case to prepare solutions containing the same proteolytic activity. To this end, density of liquid pepsin ( $1.215\text{ kg/m}^3$ ) and equivalence units previously mentioned were taken into account. Enzymatic activity was set at 5000 FIP Units/g, because this is the resultant value when applying the CODEX method. One more time, six samples of 25 g each of fresh hake and mackerel were digested with the two pepsins during 30 min at  $37^{\circ}\text{C}$ , using a weight/volume ratio (1:20). Undigested tissues of each kind of fish and pepsin were weighed and compared again, without taking into account the weight due to the parasites in the positive samples.

### 2.3. Electrophoretic profile

In addition to the digestions assays, electrophoretic profiles of the two previously selected pepsins were obtained in vertical SDS-PAGE discontinuous gels (10% acrylamide in the separating gel). Electrophoretic separations were carried out at 40 mA/slab, 100 V and 150 W, using Tris–Tricine buffer (Schägger and von Jagow, 1987) in a Mini Protean® System (BioRad Laboratories, Hercules, USA). Low

molecular weight-SDS Marker Kit (GE Healthcare, Buckingham, UK) was employed as reference. The gels were stained with silver, following the protocol described by Heukeshoven and Dernick (1985).

#### 2.4. New assay conditions

Once the best pepsin formulation was selected after the electrophoretic profile was performed, three modifications were introduced and tested during digestions in fresh and frozen samples, making the fish muscle more accessible to the enzyme action: (1) the use of the selected commercial pepsin, (2) a new weight/volume ratio for digestion solution (1:10 instead of 1:5 that CODEX protocol recommends) and (3) the homogenization and flattening of the samples before digestion in a blender for food (Smasher® AES Chemunex). For testing the reproducibility and comparing CODEX protocol with the new method after introducing new conditions (hereinafter Liquid Pepsin or LP protocol), a total of 240 digestions were carried out employing at each time 200 g of fresh and frozen hake and mackerel muscles; 120 digestions following the CODEX protocol and 120 testing the LP protocol. All assays were carried out with a pepsin concentration of 0.5% at an acidified (pH = 1.5 with HCl at 0.063 M) pepsin enzyme solution, and incubation temperature of 37 °C. After finishing every digestion, undigested muscle residues from each fish type and method were weighed, recorded and compared, without taking into account the weight due to the parasites in the positive samples.

#### 2.5. Larvae viability

In order to verify larvae viability during the definitive assays, 40 from the 240 digestions that were carried out were controlled for this aspect (20 digestions of each type of fish species; among them 10 digestions of frozen and another 10 of fresh fishes, and of those 10 digestions, 5 were carried out using each method). Anisakid-positive samples were arranged by introducing 10 larvae of *Anisakis* spp. inside anisakid-negative samples of muscle for digestion. All larvae inoculated were extracted from the muscle where they would be introduced, so larvae inoculating fresh fish samples were alive before digestions (not in the case of frozen fish digestions). Separately, 10 live and free (without muscle) anisakid larvae were digested at 37 °C in 1000 ml digestion solution following LP protocol in order to check their integrity after 210 min of digestion.

### 3. Results

#### 3.1. Samples and pepsin assays

The significance of digestions after using the three different commercial pepsins at the same concentration (0.5%) and different enzymatic activity between them is shown in Table 1. This table also illustrates digestion conditions during these assays. The two pepsin formulations that provided higher percentages of digested muscle, both for lean and for fatty fish samples, were 2 and 3.

**Table 1**  
Comparison among 3 different commercial pepsins (each one with its own enzymatic activity), in 500 ml of water and 2.5 g of pepsin (at concentration of 0.5%), in an acid solution (pH = 1.5) with HCl, at 0.063 M. Six assays were carried out using each pepsin, with muscular samples of 25 g of lean (*Merluccius merluccius*) and fatty (*Trachurus trachurus*) fresh fish.

| Pepsin name | Enzymatic activity    | Fish muscle (g) | Weight/volume ratio | Digestion time (min) | Digestion temperature (°C) | Fish species                 | Digestions (N) | Resulting muscle residue mean (g) ± SD | Digested muscle mean (%) ± SD | Commercial reference           |
|-------------|-----------------------|-----------------|---------------------|----------------------|----------------------------|------------------------------|----------------|----------------------------------------|-------------------------------|--------------------------------|
| Pepsin 1    | 800–2500 U/mg protein | 25              | 1:20                | 30                   | 37 °C                      | <i>Merluccius merluccius</i> | 6              | 1048 ± 0.18                            | 95.809 ± 0.7                  | Sigma-Aldrich P7000-100G       |
| Pepsin 2    | 2000 FIP U/g          | 25              | 1:20                | 30                   | 37 °C                      | <i>Trachurus trachurus</i>   | 6              | 4.933 ± 1.04                           | 80.270 ± 4.15                 | Merck 10 1.07190-1000G         |
|             |                       |                 |                     |                      |                            | <i>Merluccius merluccius</i> | 6              | 0.820 ± 0.2                            | 96.719 ± 0.78                 |                                |
|             |                       |                 |                     |                      |                            | <i>Trachurus trachurus</i>   | 6              | 3.180 ± 0.71                           | 87.281 ± 2.83                 |                                |
| Pepsin 3    | 660 U Ph Eur/ml       | 25              | 1:20                | 30                   | 37 °C                      | <i>Merluccius merluccius</i> | 6              | 0.085 ± 0.02                           | 99.649 ± 0.12                 | Panreac liquid 176408.1214-5lt |
|             |                       |                 |                     |                      |                            | <i>Trachurus trachurus</i>   | 6              | 1.337 ± 0.45                           | 94.652 ± 1.82                 |                                |

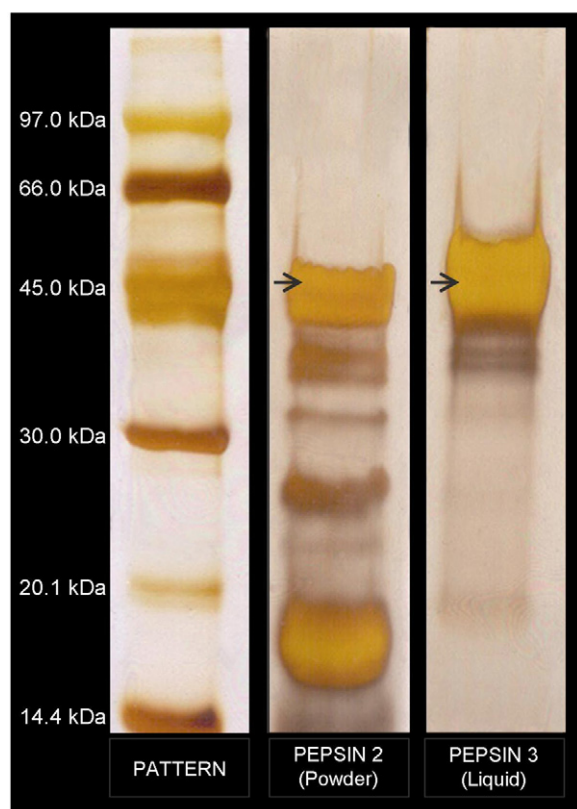


**Fig. 1.** SDS-page silver staining profile obtained from the two selected commercial pepsins assayed. Low molecular weight standard (14–97 kDa) from GE Healthcare was used as pattern. Additional bands with lower molecular weight than pepsin were obtained at one of them. Black arrow: pepsin band.

When both pepsin formulations were compared by equaling their enzymatic activities to 5000 FIP U/g, pepsin 3 showed the least fish residue in both types of fish, as Table 2 demonstrates. This table also illustrates commercial pepsins proprieties, their enzymatic activity (in FIP units), the required weight used of each one to equal enzymatic activities, and digestion conditions during these assays.

### 3.2. Electrophoretic profile

SDS-PAGE profile of pepsin extract 3 showed one band with a molecular weight corresponding to pepsin. However pepsin 2 offered a multiple band profile below to that molecular weight (Fig. 1), perhaps as autolytic consequence.

### 3.3. New assay conditions

According to obtained results at initial pepsin assays and due to its proteolytic and handling characteristics, liquid pepsin formulation (no. 3) was the selected reagent to test the new conditions (LP protocol) simultaneously to the established and current digestion protocol (CODEX). In order to obtain a maximum weight of 1 g of undigested residue in the faster of the two tested methods, for both procedures fresh samples of *M. merluccius* were digested

**Table 2**  
Comparison among 2 different commercial pepsins (at different concentration each one; enzymatic activities have been equalled at 5000U FIP), in 500 ml of acid solution (pH = 1.5) with HCl at 0.063 M. Six assays were carried out using both pepsins, with muscular samples of 25 g of lean fresh fish (*Merluccius merluccius*) and fatty (*Trachurus trachurus*) fresh fish.

| Pepsin name | Enzymatic activity | Enzymatic activity (FIP) | Pepsin dose (g) | Fish muscle (g) | Weight/volume ratio | Digestion time (min) | Digestion temperature (°C) | Fish species                 | Digestions (N) | Resulting muscle residue mean (g) ± SD | Digested muscle mean (%) ± SD |
|-------------|--------------------|--------------------------|-----------------|-----------------|---------------------|----------------------|----------------------------|------------------------------|----------------|----------------------------------------|-------------------------------|
| Pepsin 2    | 2000 FIP U/g       | 2000 FIP U/g             | 2.5             | 25              | 1:20                | 30                   | 37 °C                      | <i>Merluccius merluccius</i> | 6              | 0.881 ± 0.8                            | 96.477 ± 3.2                  |
|             |                    |                          |                 |                 |                     |                      |                            | <i>Trachurus trachurus</i>   | 6              | 1.838 ± 0.9                            | 92.647 ± 3.61                 |
| Pepsin 3    | 660 U Ph Eur/ml    | 543 FIP U/g              | 9.2             | 25              | 1:20                | 30                   | 37 °C                      | <i>Merluccius merluccius</i> | 6              | 0.785 ± 0.19                           | 96.86 ± 0.4                   |
|             |                    |                          |                 |                 |                     |                      |                            | <i>Trachurus trachurus</i>   | 6              | 0.055 ± 0.01                           | 99.78 ± 0.1                   |

during 20 min, and frozen ones for 15 min. The reason why 1 g was the determinant weight in order to establish the digestion time with each pepsin and method is because 1 g was the maximum accorded amount of undigested muscle for getting an easy and rapid finding of parasites. Although Atlantic mackerel digestions showed more difficulties during the assays (probably due to muscle characteristics and fat contain), the same criterion of 1 g was followed at the two methods, thus providing more digestion time (45 min) to fresh and frozen samples belonging to that species. Results in Table 3 show differences in relation to the amounts of undigested muscle residues from lean and fatty fishes and between procedures. This table also contains digestion protocols conditions, type of fishes and percentages of digested muscle (%).

New conditions introduced and assayed (liquid pepsin, weight/volume ratio of 1:10 and flattening of the samples before digestion) gave higher percentages of digested muscle (a lower quantity of resulting residue) after less time, both for lean and fatty fish species, than the CODEX protocol.

### 3.4. Larvae viability

Concerning larvae viability tests, after both CODEX and LP digestion protocols for both types of fishes and for both forms of preservation, all larvae introduced were recovered in perfect conditions; live larvae were recovered still alive and showing a good mobility, resembling to mobility showed before digestions (Fig. 2). Moreover, the 10 live and free larvae which were submitted to 210 min of digestion following the LP protocol, were recovered without mobility but completely entire.

## 4. Discussion

Due to the low confidence of other traditional parasite detection methods like the widely used visual inspection of abdominal cavity (Llarena-Reino et al., 2012), the norm by CODEX (CODEX STAN 244-2004) is considered the current recommended procedure for anisakids detection and counting in certain fish species and commercial displays. However, due to the lack of an officially legislated reference standard, not for CODEX protocol neither for any of the traditionally used formulas, there is no consensus in *modus operandi* to accomplish with artificial digestions for anisakids detection. An example of a similar approach in terms of performance and objectives, which has been sharply and effectively legislated, is the detection method for trichinellosis. Traditionally different detection protocols and variations had been used for meat inspections and for studies concerning *Trichinella* (Forbes and Gajadhar, 1999; Leclair et al., 2003; Gajadhar et al., 1996, 2009). Since January 2006, a Commission Regulation of the European Community of 5 December 2005 (EC No. 2075/2005) has laid down specific rules on official controls for *Trichinella* in pig meat. This detailed law required laboratories to carry out the magnetic stirrer protocol for pooled-sample digestion in fresh pig meat. Afterward, some authors concluded that pepsin powder formulations potentially caused severe allergic reactions to sensitive people (Marqués et al., 2006)

**Table 3**  
Resulting muscle residues (g) and digested muscle (%) means, comparing the Liquid pepsin (LP) protocol (new digestion assay using the selected liquid pepsin – pepsin 3 –, at concentration of 0.5% in 2000 ml of water), to CODEX STAN 244-2004 protocol (using the recommended powdered pepsin – pepsin 2 –, at concentration of 0.5% in 1000 ml of water). Both digestions were carried out in an acid solution (pH = 1.5) with HCl, at 0.063 M. A total of 240 assays with samples of 200 g of fish were carried out; 120 for each method (30 assays were performed for fresh, and 30 for frozen lean fish belonging to *Merluccius merluccius*, and the same number for fatty fish (*Trachurus trachurus*)).

| Digestion method                     | Pepsin name | Enzymatic activity | Fish muscle (g) | Stomacher time (min) | Digestion temperature (°C) | Weight/volume ratio | Fish species                 | Digestions (N) | Fish sample type | Digestion time (min) | Resulting muscle residue mean (g) ± SD | Digested muscle mean (%) ± SD |
|--------------------------------------|-------------|--------------------|-----------------|----------------------|----------------------------|---------------------|------------------------------|----------------|------------------|----------------------|----------------------------------------|-------------------------------|
| Codex protocol (CODEX STAN 244-2004) | Pepsin 2    | 2000 FPU/g         | 200             | –                    | 37 °C                      | 1:5                 | <i>Merluccius merluccius</i> | 30             | Fresh            | 20                   | 125.2 ± 14.91                          | 37.38 ± 7.45                  |
|                                      |             |                    |                 |                      |                            |                     |                              | 30             | Frozen           | 15                   | 126.7 ± 11.22                          | 36.63 ± 5.61                  |
|                                      |             |                    |                 |                      |                            |                     |                              | 30             | Fresh            | 45                   | 32.9 ± 5.33                            | 83.52 ± 2.67                  |
|                                      |             |                    |                 |                      |                            |                     | <i>Trachurus trachurus</i>   | 30             | Frozen           | 45                   | 36.48 ± 4.61                           | 81.76 ± 2.3                   |
|                                      |             |                    |                 |                      |                            |                     |                              | 30             | Fresh            | 20                   | 0.653 ± 0.328                          | 99.67 ± 0.16                  |
|                                      |             |                    |                 |                      |                            |                     |                              | 30             | Frozen           | 15                   | 0.475 ± 0.184                          | 99.76 ± 0.09                  |
| LP (liquid pepsin) Protocol          | Pepsin 3    | 660 U Ph Eur/ml    | 200             | 4                    | 37 °C                      | 1:10                | <i>Merluccius merluccius</i> | 30             | Fresh            | 15                   | 0.902 ± 0.24                           | 99.55 ± 0.12                  |
|                                      |             |                    |                 |                      |                            |                     |                              | 30             | Frozen           | 45                   | 0.795 ± 0.18                           | 99.60 ± 0.09                  |
|                                      |             |                    |                 |                      |                            |                     |                              | 30             | Frozen           | 45                   | 0.795 ± 0.18                           | 99.60 ± 0.09                  |

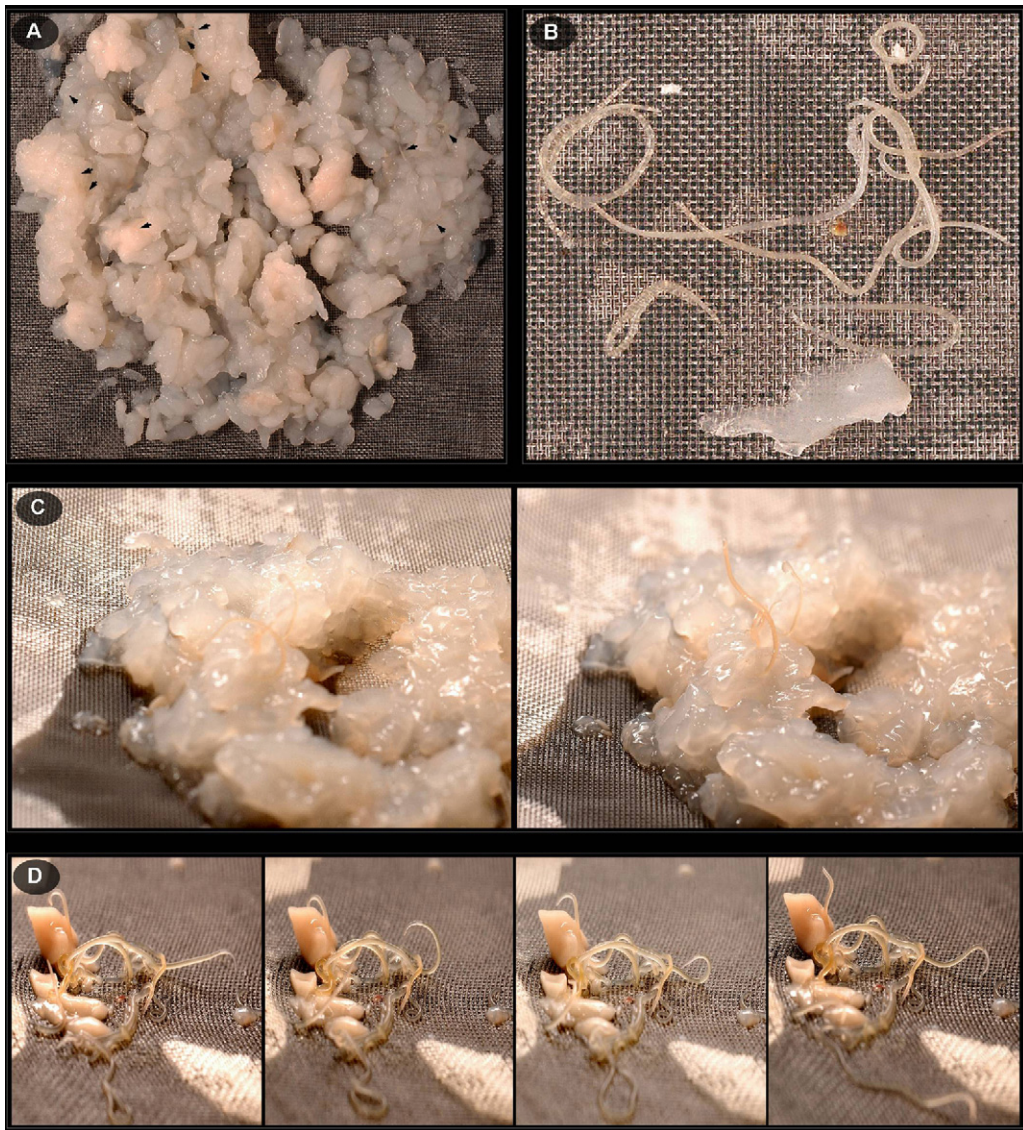


**Fig. 2.** Resulting digestions after examining and controlling the viability of the larvae. (A) Ten anisakid larvae after CODEX digestion protocol of frozen *Merluccius merluccius*. Black arrowhead: anisakid larval. (B) Ten anisakid larvae after LP (Liquid Pepsin) digestion protocol of frozen *Merluccius merluccius*. (C) Sequence of two pictures showing live anisakid larvae moving after CODEX digestion protocol of fresh *Merluccius merluccius*. (D) Sequence of four pictures showing live anisakid larvae moving after LP digestion protocol of fresh *Merluccius merluccius*.

and workers (Maddox-Hyttel et al., 2007) who handled the chemical, thus constituting a health risk. Simultaneously, the Commission Regulation of the European Community of 24th October 2007 (EC No. 1245/2007) modified Annex I of the regulation (EC) No. 2075/2005, allowing the use of liquid pepsin formulations to detect *Trichinella* in meat. Similarly, during the present study the artificial digestion protocol from CODEX has been revised in depth, detecting some limitations and disadvantages in powder pepsin forms and in the conditions. During the first assay carried out with the three pepsin formulations (digestions at the same concentration of 0.5%; Table 1), pepsin 1 offered the lowest proteolytic effectiveness. Due to this, it was removed from the study. After selected and assayed pepsin formulas 2 and 3 for the second test (digestion solutions

with the same proteolytic activity; Table 2), pepsin 3 gave better results; higher percentage of digested muscle than pepsin 2. Therefore, liquid pepsin form (pepsin 3) was more effective, and it also offered an easier handling at work procedures than pepsin formulation 2. Moreover and as mentioned above, liquid enzyme formula avoids possible allergic reactions that pepsin in powder form may cause. Additionally, the study of the purity by means of the SDS-PAGE silver staining profile was determinant to qualify pepsin formulation 3 as the cleanest, purest, fastest and the most versatile and efficient of both. This was the reason why this liquid enzyme form was selected as the most interesting pepsin to be assayed applying the settings and new conditions suggested in this study, in a comparative test between both pepsin formulations and both

procedures (CODEX and LP). That comparison revealed that LP protocol is more sensitive, efficient and accurate. It offers innovative characteristics like being more handy and easier to use than CODEX, even for unskilled personnel such as fish markets and factories workers. Therefore, since there is a non standardized safer optional method for *Trichinella* detection, it seems reasonable to consider a similar non standardized safer alternative method for anisakids detection as well, due to the important, increasing and urgent requirement of its use.

Besides increasing safety, comfort and usability, this novel procedure reduces costs and test times. This fact leads to a huge reduction of the expenses and time dedicated to quality and public health controls at industries, without variation on results reliability. These kinds of improvements are extremely significant, also for research centers to make faster progresses in specific aspects of the parasites and the public health prevention programs.

### Conflict of interest statement

The authors declare no conflict of interest.

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