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VERSITA

Recovery of Anisakid larvae by means of chloro-peptic digestion and proposal of the method for the official control

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Abstract

Anisakidae larvae belonging to the genera *Anisakis* and *Pseudoterranova*, are the most responsible for zoonosis transmitted by fish products (anisakidosis). Acquired by the consumption of raw or undercooked marine fish or squid, the anisakid larvae may cause pathogenic diseases like gastric or intestinal anisakiasis and gastro-allergic disorders. In accordance with current EU legislation, the fresh fish products must be inspected visually in order to detect the possible presence of visible parasites. It is recognized that the visual method is not accurate enough to detect the larvae of parasites in food preparations containing raw or practically raw seafood and it clearly emerges that the official system of control needs to be able to utilise an most efficient analytical technique. In this work, the authors have drawn up and validated an analytical method, which involves artificial digestion and the use of a heated magnetic stirrer, based on the EU Regulation n. 2075/2005. The larvae isolated are then subjected to morphological identification at genus level by using optical microscope. The method, proved to be suitable for the detection of live and dead larvae of anisakidae in ready-to-eat foodstuffs containing raw fish or cephalopods and it is fast and accurate. The method showed high levels of sensitivity and specificity, and the suitability of its use in official food control was confirmed. Its use should be incorporated systematically into specific monitoring programs for the control of foodstuffs containing raw fish products.

Keywords

Anisakidae, digestion method, raw fish, official food control

Introduction

Anisakid nematodes are recurrently found in the abdominal cavity and muscle of numerous fish species of commercial interest: these parasites are of medical and economic importance due to public health implications and for the subsequent effects on the marketability of fish products. Larval forms of anisakid nematodes, in particular those belonging to the genera *Anisakis* and *Pseudoterranova*, are in fact the main causative agents of human anisakidosis, a fish-borne zoonosis due to the ingestion of infected raw or undercooked fish or cephalopods. In the humans the accidental intake of alive anisakid larvae may cause pathogenic diseases like gastric or intestinal anisakiasis and gastro-allergic disorders (Audicana and Kennedy 2008, Hochberg and Hamer 2010).

The European Food Safety Authority has declared that all saltwater and freshwater fish may contain larvae that are potentially dangerous to human health, if consumed raw or lightly cooked, and that no fishery can be regarded as free from Anisakidae.

In this regard, European regulations have for a long time mandated the decontamination by means of freezing of all fish products destined to be consumed raw or practically raw. Nevertheless, the fact that live larvae of parasites may be found in such products, or in gastronomic preparations that contain them, indicates that compliance with this obligation is lacking or insufficient. Not only does this constitute a potential threat to public health, it also implies an infringement of the law. In accordance with current EU legislation, fresh fish products must be inspected visually in order to detect the possible presence of visible parasites. However, no method has been prescribed for the detection of parasites in ready-to-eat foodstuffs containing raw or practically raw fish products. As the visual method is not accurate enough to detect the larvae of parasites in such products, it clearly emerges that the official system of control needs to be able to utilise an analytical tech-

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nique designed for this purpose. Such a technique should be sufficiently sensitive, reproducible, rapid, easy to carry out and able to detect the presence of even one single parasite larva in gastronomic preparations containing fish products that are raw or practically raw (salted, marinated, smoked or merely prepared); moreover, it should be able to distinguish live larvae from dead larvae without interfering in any way with the vitality of the larvae themselves.

In this work, the authors have designed an artificial enzymatic digestion procedure based on Regulation EU 2075/2005 with appropriate modifications: this method of analysis has been applied on ready-to-eat preparations containing raw or practically raw fish products obtained from potentially dangerous fish species.

Materials and Methods

A total of 250 tests were carried out, 120 of which focused on validating the method. The method utilised the technique of artificial digestion in acid solution (PH:1,4) with HCl, at 0,055M and liquid pepsin (660 U Ph Eur/ml) at concentration of 1,5% at 44–46°C, continuously stirred for 30 minutes. Any larvae present in the digested solution were able to survive artificial digestion thanks to their gastro-resistant cuticula, and were easily detectable by means of macroscopic and microscopic visual inspection of the sediment collected on Petri plates, showing a good mobility, resembling to mobility showed before digestions. The method is based on the digestion technique indicated in Reg 2075/2005 for the detection of Trichinae in meat, with appropriate modifications.

Equipment and materials used:

- Knife or scissors and pincers for sample preparation;
- Homogenizers (stomacher);
- Magnetic stirrer equipped with a thermostatically controlled heating plate and Teflon-coated mixing rods of approximately 5 cm in length;
- Conical glass separation funnels with a minimum capacity of 2 L, preferably equipped with Teflon safety stoppers;
- Supports, rings and clamps;
- Funnels: internal diameter no less than 12 cm;
- 3-litre glass beaker;
- Graduated glass cylinders with a capacity between 50 and 100 ml or centrifuge test-tubes;
- Stereo-microscope with light sources of adjustable intensity both above and below*;*
- Petri plates of 9 cm diameter (for use with the stereo-microscope), preferably marked out on the bottom into 10 × 10 mm subdivisions by means of a sharp marker;
- 25% hydrochloric acid;
- Liquid pepsin stabilised 660 U Ph Eur (European Pharmacopoeia)/ml;
- Tap water heated to 46–48°C;
- Weighing-scale calibrated to at least 0.1g;
- Metal basins with a capacity of 10–15 litres for the collection of remaining digestive liquid;
- Pipettes of various sizes (10 and 25 ml) and supports;
- Thermometer calibrated to 0.5°C for temperatures between 1 and 100°C;
- Pipettor or vacuum pump;
- Reference materials provided by the CReNA (Centro di Referenza Nazionale per le anisakiasis, IZS, Sicily) i.e. larvae of *Anisakis* spp. conserved in 70% alcohol, obtained from infested fish products.
- Lactophenol of Amman solution and Lactophenol Blue or 87% glycerol.
- 70% ethanol;
- Physiological solution;

Sample preparation

Small fresh fish (anchovies, sardines, etc.) were cut up with scissors into pieces no larger than 1 cm², without removing the spine. The viscera and the heads were cut up together with the rest of the sample. The same procedure was applied to larger fish (*Clupea harengus*, *Scomber scombrus*, *Lepidopus caudatus*, etc), though the spine, head and all fins were always removed. Processed fish which had undergone salting, marinating, filleting and/or only with the addition of seasoning and flavouring was prepared by cutting up the sample with scissors into pieces no larger than 1 cm². In the case of processed products steeped in oil, the oil was carefully removed with the aid of absorbent paper. Cephalopods were treated in the same way, following removal of the bony parts and teeth, when present.

Digestion procedure

The procedure was carried out on 100–130 g of product for each single digestion, as follows:

a) 16 ± 0.5 ml of hydrochloric acid was poured into a 3-L beaker containing 2.0 L of tap water heated to a temperature of 46°C; a stirring rod was inserted into the beaker, the beaker was placed on a preheated plate and stirring was started.
b) 30 ± 0.5 ml of liquid pepsin was added to the solution ;
c) amounts of sample weighing 130 g were minced inside a homogenizers bag;
d) about 50 mL of the solution containing water, pepsin and hydrochloric acid in was added to the matrix prepared in the homogenizers bag;
e) the bag containing both the matrix and the solution underwent treatment in the homogenizers for about 1 minute, until a slush of liquid consistency was obtained. The slush was then transferred to the beaker together with the digestion liquid; some of the digestion juice was used to wash out the bag several times after transfer of its contents to the beaker and the bag was inspected thoroughly, as the larvae tended to adhere to it;

f) the beaker was covered with a sheet of aluminium foil and placed on the magnetic stirrer, which was set to a constant temperature of 44–46°C throughout the procedure;
h) the digestion juice was stirred for about 30 minutes and then poured directly into the sedimentation funnel. Some types of matrix (cephalopods) required longer digestion times (no more than 60 minutes), while others (salted anchovies and sardines) needed only 20 minutes of digestion. Similarly, a shorter digestion time proved to be sufficient when the sample weighed less than 70 g;
i) the digestion juice was left in the sedimentation funnel for 15 minutes;
j) after 15 minutes, a 40 ml sample of the digestion juice was rapidly poured into the graduated cylinder or into a 40 ml test-tube;
l) the 40 ml sample was left to settle for 5 minutes. Subsequently, 30 ml of supernatant liquid was carefully aspirated, in order to draw off the upper layers leave a maximum volume of no more than 10 ml. Whenever the sediment was not deemed sufficiently transparent at the moment of examination, the sample was poured into a graduated cylinder and brought up to a volume of 40 ml by adding tap water and the same process was repeated. This procedure was repeated 2–4 times until the liquid was sufficiently transparent to allow reliable examination;
m) the remaining 10 ml sample of sediment was poured into a basin in order to detect and count any larvae on the Petri plate;
o) the graduated cylinder or test-tube was rinsed with no more than 10 ml of tap water, which was added to the sample in the Petri plate. The test-tube or graduated cylinder was carefully inspected, as larvae often adhered to it. The sample was then examined under the stereo-microscope at a magnification of between ×5 and ×10. Larger and very mobile larvae were already visible to the naked eye, and more easily so if a light source from below was used.

The digestion juices were examined as soon as they were ready; in no case did examination have to be postponed to the following day. In the case of samples up to 50 g, the digestion juices and ingredients were halved (1 L of water, 8 ml of hydrochloric acid and 5 g of pepsin), while digestion times were left unchanged.

When present in the sediment, the Anisakid larvae may lie straight or curled into a spiral. They may also appear mobile or immobile. Any parasites detected in the positive samples were drawn off by means of a pipette and kept in 70% ethyl alcohol for species identification according to the procedure reported below.

Visual morphological identification of the larvae of Anisakidae.

The larvae L3 of the parasites belonging to the genus *Anisakis* have the appearance of a whitish worm, from 14 to 30 mm in length, with a circular section, and are clearly visible to the naked eye.

Identification at the level of the genus was carried out by observing the morphological features under the optical microscope. Previously fixed in 70% ethanol alcohol, the larvae were placed on a slide and a drop of lactophenol (clarifying agent) was added. When necessary, in order to improve contrast, a drop of lactophenol blue or 87% glycerol was added; this provided good results.

Under the optical microscope, we evaluated the appearance of the anterior and posterior ends and of the anterior portion of the digestive apparatus of the larvae, in accordance with the parameters reported in the bibliography (Berland 1991, Huang 1988, Smith and Wotten, 1984a, 1984b, 1984c).

To identify the larvae as belonging to the genus *Anisakis* the following morphological features were sought (Figure 1, A-B-I):
– the oesophagus comprises a muscular portion followed by a glandular or ventricular portion (longer in *Anisakis* Type I), which continues into the intestine; the oesophageal appendix and blind intestine are absent;
– the presence of a tooth at the tip of the anterior end;
– the presence (*Anisakis* Type I) or absence (*Anisakis* Type II) of a small spine or mucron at the tip of the tail.

To identify the larvae as belonging to the genera *Hysterothylacium spp.*, *Contracaecum sp* and *Pseudoterranova sp.*, the following morphological characteristics were considered (Figure 1, C-M):
– the oesophagus comprises a muscular portion followed by a glandular or ventricular portion, which does not extend directly into the intestine; in the genera *Hysterothylacium spp.* and *Contracaecum sp.*, an oesophageal appendix and blind intestine are present, while in the genus *Pseudoterranova sp.*, only the blind intestine is present.

In order to render the method applicable to official control, the results, in the event of positivity, was indicated whether the larva found in the sediment was alive or dead at the moment of visual observation. Mobile larvae were deemed to be alive, while stiff, immobile larvae were classified as dead. Therefore during the set-up phase of the method, tests were also conducted on naturally contaminated field samples.

For the validation of the analytical method, the tests were conducted on 99 samples artificially contaminated. The matrices tested were raw fish products ready to eat and more specifically fish salted and fish marinated with oil and spices (*Engraulis encrasicolus*), fish smoked (*Makaira indica*, *Clupea harengus*), fresh fish (*Scomber scombrus*, *Lepidopus caudatus*) in raw food preparation and cephalopods (*Totarodes sagittatus*). The samples were tested by the method of visual inspection to determine their negativity and subsequently were artificially contaminated with one live larva and one dead larva of *Anisakis* spp. for verification of the accuracy (sensitivity and specificity). The results of the validation tests are shown

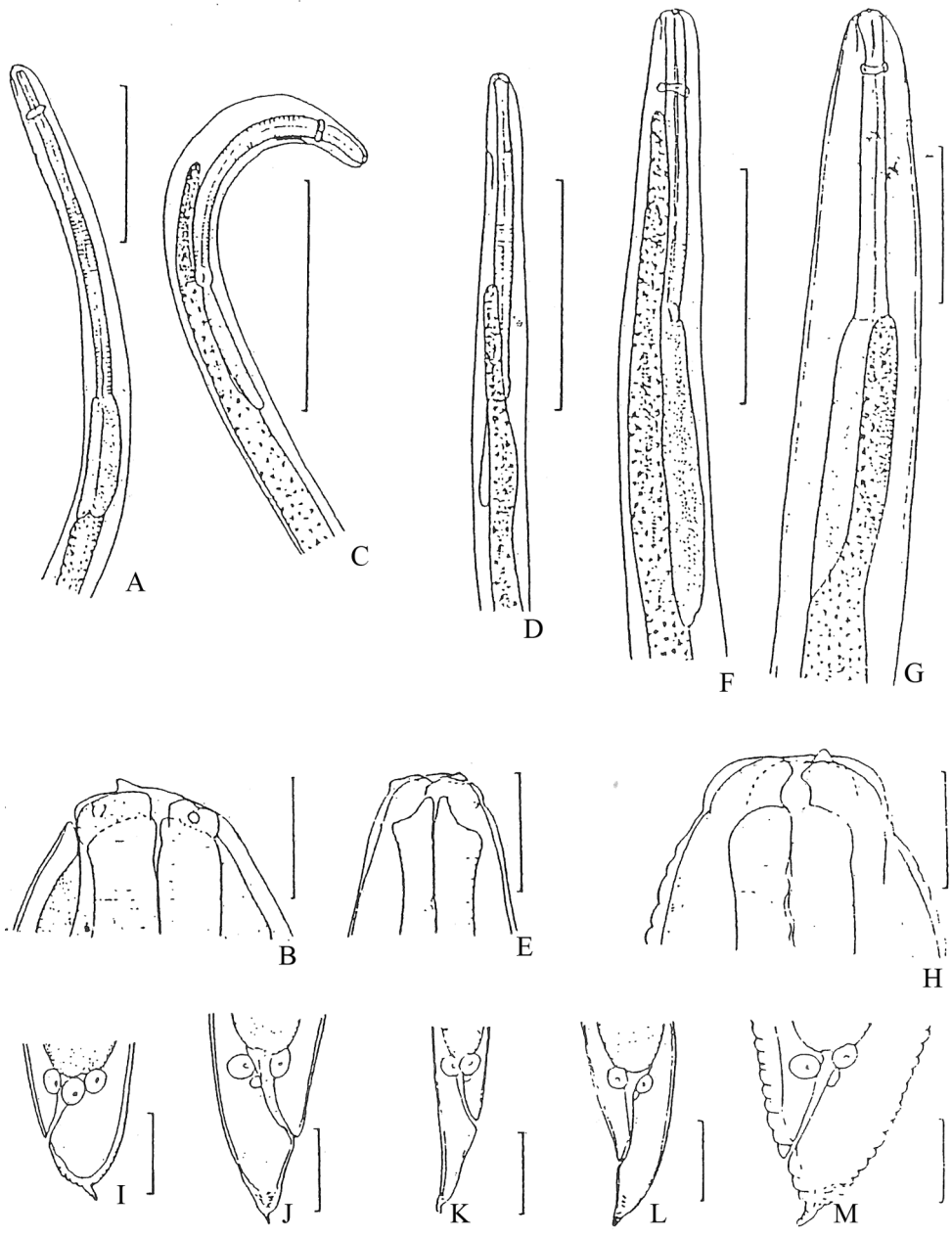

Fig.1. – Anterior end: **A–B –** *Anisakis simplex*; **C–D–E –** *Hysterothylacium spp.*; **F –** *Contracaecum sp.*; **G–H –** *Pseudoterranova decipiens*. Posterior end: **I –** *Anisakis simplex*; **J–K –** *Hysterothylacium spp.*; **L –** *Contracaecum sp.*; **M –** *Pseudoterranova decipiens* (W. Huang, 1988)

in Table II . To calculate the recovery efficiency were processed data obtained from the laboratory on samples of the proficiency tests organized by CR.e.Na (Italian National Reference Centre for Anisakiasis, at the IZS, Sicily), in the years 2012 and 2013. Proficiency Test consisted of sending 5 unknown samples of raw fish, some of which had been contaminated with a variable number of dead larvae. These samples were analyzed both by the method of visual inspection that with the digestive method . The recovery efficiency was evaluated on the total number of larvae detected as a result of the examinations compared to larvae artificially added by the organizer of interlaboratory circuit . The results are summarized in Table I.

Results

In all the samples analysed, the larvae artificially inserted into the various matrices were recovered following execution of

Table I. Results obtained as a result of participation in the Proficiency tests C.Re.N.A. 2012 and 2013

Method	Sensibility	Specificity	Recovery
Digestive method	100%	100%	91.67%
Ispective method	100%	100%	75%

Table II. Specifications of the method used in the different matrices

Fish species	Fish sample type	Stomacher time (seconds)	Digestion temperature	Weight/ volume ratio	N° campioni	Digestion time (minutes)
Engraulis encrasicolus	Marinated/salted	30	45+1°C	1:20	20	30
Makaira indica, *Clupea harengus*	Smoked	30	45+1°C	1:20	13	30
Scomber scombrus, *Lepidopus caudatus*	Fresh product	30	45+1°C	1:20	33	30
Totarodes saggittatus	Fresh product	60	45+1°C	1:20	33	60
				Total	**99**	

the analytical method. The technique therefore proved to be highly efficacious. Moreover, in no case did the technique kill any of the worms that had been inserted live into the matrix. In the case of cephalopod samples, it proved necessary to prolong digestion times to about 45–60 minutes; however, in this case too, the larvae recovered were still alive and vital.

The method displayed values of specificity and sensitivity of 100%. The recovery, assessed on the results of proficiency tests 2012 e 2013 was 91.6%. In 2011, the method passed the accreditation test of the official national body, ACCREDIA, and was officially included among the analyses conducted by our laboratory for the Italian National Health Service.

Discussion

In the last years, several methods have been developed for detection of parasites in fishery products including visual inspection, candling, pressing, digestion and recently Polymerase Chain Reaction (PCR) but at the moment, none of this methods has been accepted as the international standard accomplishing with industrial requirements. In the European Union the official method for larvae detection is visual inspection and candling of fish fillets indicated in Regulation EC 2074/2005 but it is known that this methods not detect all the larvae present in the flesh of fish (Levsen *et al.* 2005) or in seafood preparations unlike other more accurate and efficient methods as, in particularly, artificial digestion. Several digestion methods have been proposed in the literature (Jackson *et al.* 1981) and recent studies have shown that pepsin and HCl conentration may be different according to the material to be digested (Llarena-Reino *et. al.* 2013). However the method object of the present study employs a single concentration of pepsin and hydrochloric acid for all matrices. This in order to make easier the use of the method in a laboratory of official control of foodstuffs. The concentration of pepsin and hydrochloric acid recommended, in fact, was optimal for use on different matrices, applying small variations of the times of digestion.

In this work, the method described proved to be particularly suitable for use in the sphere of the official examination of ready-to-eat foodstuffs containing raw or practically raw fish products and it is fast and accurate. In table 2 summarizes the specifications of the method used.

Indeed, the high sensitivity and specificity of the method, in addition to its ability not to interfere with the vitality of larvae, make it particularly suitable for revealing non-compliance with the obligation to decontaminate raw materials on the part of food manufacturers. Furthermore, the technique can easily be implemented within relatively short times in any laboratory that conducts analyses on foodstuffs. During the study phase of the method, several experimental tests were carried out on field samples; these demonstrated the suitability of the method for the detection of other genera of Aniskidae, including the genus *Contracaecum* sp., which is smaller and more delicate than the others. In this latter case, however, it was observed that the analytical method noticeably interfered with the vitality of this parasite, probably owing to the digestion temperature. This surely constitutes a limit; indeed, although this parasite is not of interest to public health, as it is not recognised as a zoonotic agent, the finding of live larvae of this genus in a ready-to-eat product indicates that the product has not been properly decontaminated. In this regard, experimental trials conducted in our laboratory have indicated that the digestion temperature can be considerably reduced without impairing the efficacy of the method. However, further studies will be needed before this modification can be implemented.

Another limit that needs to be mentioned is that the digestion technique is able to detect dead Aniskidae larvae that have already started to undergo processes of decomposition within the matrix to be analysed. Indeed, in this case, the processes of degradation following the decomposition of the parasite also involve the external gastro-resistant cuticula. This reduces the resistance of the larva to artificial digestion. In such conditions, the parasite risks being completely destroyed by the action of the chloro-peptic mixture, thus giving rise to false-negative results. This explains the value of the recovery to the

Proficiency test, caused by the use of dead larvae in a state of degradation, which could not resist the digestion.

The application of the chloro-peptic digestion method in the field of official inspections undoubtedly fills a gap in the management of the risk of anisakiasis. The method showed high levels of sensitivity and specificity, and the suitability of its use in official food control was confirmed. Its use, together with the inspection of food manufacturing facilities, should be incorporated systematically into specific monitoring programs for the control of foodstuffs containing raw fish products.

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