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Microbiology of the food chain — Methods for the detection of Anisakidae L3 larvae in fish and fishery products —

Part 2: Artificial digestion method

Microbiologie de la chaîne alimentaire — Méthodes de recherche des larves L3 d'Anisakidae dans les poissons et produits de la pêche —

Partie 2: Méthode de digestion artificielle

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

A list of all parts in the ISO 23036 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Nematodes of the Anisakidae family have a complex life cycle involving a high number of hosts. Adult stages of Anisakidae reside in the stomach of marine mammals, where they are embedded in the mucosa. Unembryonated eggs produced by adult females are released with the faeces of marine mammals and become embryonated in seawater, where first-stage larvae (L1) develop in the eggs. The larvae moult to become free-swimming second-stage larvae (L2) and, if ingested by crustaceans, mature into third-stage larvae (L3). This stage is infective to fish and squid, and larvae are transferred between fishes through predation, maintaining the L3 stage. Some larvae migrate from the abdominal cavity into muscle tissues. Humans are incidental hosts and can be infected after ingesting raw or undercooked infected fish or cephalopods containing viable L3.

Nematodes of the family Anisakidae are the causative agents of human anisakidosis, a disease that is not only a public health hazard affecting humans, but also represents an economic problem in fishery and food safety (the term “anisakiasis”, designating the disease caused by members of the genus *Anisakis*, is also sometimes used). Worldwide, marine and wild anadromous fishes are intermediate hosts of Anisakidae, whereas marine mammals are the definitive hosts.

Visual inspection procedures for the detection of Anisakidae larvae in fish are employed to minimize the risk that contaminated fish will reach the consumer,[1]][2] thus preventing human anisakidosis.

The UV-press and the artificial digestion of the fish muscular tissue are the methods specifically designed to detect nematode larvae in fish and to evaluate the infestation level of a batch, and have been validated and tested in multicentre collaborative studies[3] (see Clause 10).

Microbiology of the food chain — Methods for the detection of Anisakidae L3 larvae in fish and fishery products —

Part 2: Artificial digestion method

1 Scope

This document specifies a method for the detection of Anisakidae L3 larvae commonly found in marine and anadromous fishes. The method is applicable to fresh fish and/or frozen fish, as well as lightly processed fish products, such as marinated, salted or smoked. It is also suitable for visceral organs as a confirmatory method for a visual inspection scheme.

The artificial digestion method[4][5][6] is applicable to quantifying parasitic infections by estimating the number of parasites in the fish musculature and, when applied to fresh fish or lightly processed fish products (never frozen before processing), determining the viability of Anisakidae L3, which can be present.

This method does not apply to determining the species or genotype of detected parasites. Final identification is made by morphological and/or molecular methods.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
Anisakidae L3 larvae
third-stage larvae (L3) belonging to the Anisakidae family, in particular to *Anisakis*, *Contracaecum* and *Pseudoterranova* genera

Note 1 to entry: For practical purposes, the genus *Hysterothylacium* belonging to Raphidascarididae family and already classified as Anisakidae, can be also included.

3.2
artificial digestion method
method to detect Anisakidae larvae in fish muscle tissue by an enzymatic (pepsin-HCl) digestion step to release larvae from muscle tissues followed by a filtration step and detection of larvae by microscopy

Note 1 to entry: If applied to fresh fish, it allows checking of the viability of larvae.

4 Principle

4.1 General

The artificial digestion method relies on the enzymatic degradation of muscle fibres in a fluid composed of pepsin and hydrochloric acid followed by filtration and washing steps.

The procedure allows a differentiation between dead and viable anisakid larvae if the temperature of the digestion solution does not exceed 37 °C (with the exception of *Hysterothylacium* sp. larvae, which are killed at 37 °C), and assuming the fish was never frozen.

There are no internal quality controls that can be used while performing the method.

NOTE Alternative methods can be used for analysis, provided their equivalence with the methods described in this document are demonstrated.

4.2 Sample size

For inspection of fishery products for public health purposes, the sample size shall be risk-based, as determined by the competent authority.

4.3 Sample preparation

To increase the surface area for enzymatic degradation, samples are gently eased apart taking care not to disrupt larvae by checking for them. Alternatively, a Smasher/Stomacher®[1)], that facilitates the digestion but does not damage the nematode larvae, can be used.

A blending or grinding procedure should be avoided as this can damage or disrupt larvae.

4.4 Digestion of the sample

Viable Anisakidae larvae are resistant to the pepsin-HCl digest fluid and therefore can be recovered free from muscle tissues.

To facilitate an efficient and rapid digestion, a maximum ratio of 1:20, meat to digest fluid, and a temperature of 37 °C ± 2 °C shall be maintained throughout the process. The time required for digestion shall be 15 min to 30 min. For muscle samples that are less digestible, the digestion time should be increased but, unless otherwise validated for a particular sample matrix, the time shall not exceed 45 min.

4.5 Filtration of the digest fluid

Following digestion, the digest fluid shall be filtered through a sieve with specific mesh (6.9). Retained larvae shall be rinsed with tap water.

4.6 Verification of findings

If positive or doubtful findings occur, confirmation and identification at the species level should be performed by a qualified reference laboratory, by means of morphological and/or molecular methods.

1) Smasher and Stomacher® are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

5 Reagents

5.1 Tap water.

5.2 Hydrochloric acid, 25 %.

5.3 Pepsin, powder or granular: 1:10 000 NF, 1:12 500 BP, 2 000 FIP; liquid: 660 U/ml.

Pepsin is commercially available in powder, granular and liquid forms. The activity of the pepsin shall be certified. The pepsin shall be stored according to the manufacturer's recommendation.

The use of a liquid pepsin formulation can be advantageous as it could reduce the risk of occupational hazard, such as allergic reaction in laboratory staff.

NOTE The activity of pepsin powder is expressed per gram, either in "NF" (US National Formulary), "BP" (British Pharmacopoeia) or "FIP" (Fédération Internationale de Pharmacie). The activity of liquid pepsin is expressed in European Pharmacopoeia units per millilitre with a minimum of 660 U/ml. Other pepsin activities can be used, provided the final activity in the digest fluid is equivalent to the activity of 10 g of 1:10 000 NF.

5.4 90 % ethanol.

5.5 Acidic solution, 1 % acetic acid.

6 Equipment and consumables

Usual microbiological laboratory equipment in accordance with ISO 7218 and, in particular, the following.

6.1 Labelled collection trays or plastic bags, for samples.

6.2 Knives, scissors and forceps, for cutting samples.

6.3 Calibrated scale, for weighing samples and/or pepsin, accuracy ±0,1 g.

6.4 Magnetic stirrer, with an adjustable heating plate or magnetic stirrer put in an incubator.

6.5 Polytetrafluoroethylene (PTFE)-coated triangular stir bar, 5 cm in length, minimum.

6.6 Thermometer, accurate to at least ±0,5 °C, minimum range of temperatures from 20 °C to 70 °C.

6.7 Glass beakers, appropriate capacity.

6.8 Aluminium foil or **lids**, to cover the top of the glass beaker.

6.9 Sieve, made of brass or stainless steel, mesh size around 180 microns to 500 microns, with diameter of approximately 10 cm or larger.

6.10 Petri dishes, approximately 90 mm in diameter, for larval counting.

6.11 Small vials, for the collection of recovered larvae.

6.12 Stereo-microscope, with a sub-stage transmitted adjustable light source.

7 Sampling

Sampling is not part of the method specified in this document, but it is indicated in Annex A. If there are no specific International Standards dealing with sampling of fish, it is recommended that the interested parties come to an agreement on this subject.

8 Procedure

WARNING — Laboratory staff performing the procedure shall wear disposable gloves, a mask and a laboratory coat. When handling pepsin, contact with the powder and inhalation of dusts (mask) should be avoided. The use of a liquid pepsin formulation can be advantageous as it could reduce the risk of an allergic reaction in laboratory staff.

8.1 Preparation of the sample

Samples used for testing can be fresh, frozen or lightly processed fish, and internal organs (e.g. liver, ovary). Their condition shall be reported before testing.

Each sample shall be weighed and its weight shall be reported. The minimum individual sample size for testing by digestion shall be at least 25 g and no more than 200 g.

Whole fish shall be manually eviscerated, skinned and filleted, and the viscera and each flesh side/fillet can be put in separate transparent plastic bags to be analysed independently, depending on fillet size.

Manually ease apart the sample or, alternatively, use a Smasher/Stomacher[2].

If any larvae are found before digestion, they should be properly preserved for further observation, as described in 8.5.

For lightly processed fish containing, for example, oil and spices, the sample shall be rinsed with tap water before digestion, to remove such ingredients as much as possible.

8.2 Preparation of the digest fluid

The pepsin used for the preparation of digest fluid shall have the appropriate activity required for digestion (5.3). Digest fluid shall be made fresh for each analysis. The steps for preparing digest fluid are as follows:

a) add 16 ml of hydrochloric acid (see Clause 5) to a glass beaker containing 2 l of tap water preheated to 35 °C ± 2 °C;

b) place a stirring rod in the beaker, place the beaker on a preheated magnetic stirrer or on a magnetic stirrer in the incubator, and commence the stirring;

c) add 10 g of powder or granular pepsin (1:10 000 NF) or 30 ml of liquid pepsin (660 U/ml).

NOTE 1 The most critical step is the obligatory sequence of mixing of the digest fluid: a) water, b) hydrochloric acid, and c) pepsin. This will prevent degradation of pepsin that could result from direct exposure to concentrated hydrochloric acid.

NOTE 2 Stock solutions of hydrochloric acid are available in formulations other than 25 % and are adjusted accordingly. For example, if a 37 % stock solution of hydrochloric acid (molar concentration: 12,1) is used, 11 ml is added to 2 l of preheated tap water.

NOTE 3 Other activities of pepsin can be used, provided the final activity in the digest fluid is equivalent to the activity of 10 g of 1:10 000 NF (5.3).

2) Smasher and Stomacher® are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.