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

Part 1: UV-press method

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

Partie 1: Méthode presse/UV

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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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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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 fish muscle tissues 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 9).

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

Part 1: UV-press 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 cold smoked.

This method is applicable to quantifying parasitic infections by estimating the number of parasites in the fish musculature.

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
UV-press method
method to detect Anisakidae larvae in fish muscle tissue by UV examination after pressing and freezing

Note 1 to entry: Under UV light, L3 appear as brightly fluorescent spots of different colours, partially depending on the anisakid species.

4 Principle

The UV-press method[4] relies on the peculiar feature of frozen Anisakidae larvae, which show fluorescence under UV light, due to the presence of the pigment ("lipochrome") Lipofuscin. Nematode detection is based on screening under UV-light of flattened and frozen fillets, of either fresh or thawed fish. The method may also be used for parasite inspection of larger fish such as farmed Atlantic salmon (*Salmo salar*), cod (*Gadus* spp.) or halibut (*Hippoglossus hippoglossus*). In these cases, each fillet or fish side has to be cut into smaller pieces, which are then processed and examined separately. Samples are placed in clear plastic bags, pressed to a 1 mm to 2 mm thin layer and then frozen. After the freezing and subsequent thawing of the fillets, a visual inspection is carried out by examining each bag containing a fillet under a 366 nm UV-light source. Any anisakid nematode larva will appear as a brightly fluorescent spot, so that it can be easily recorded and the approximate site of infection can be determined.

Empirical evidence suggests that Anisakidae larvae killed by cooking, salting and pickling show brilliant fluorescence without prior freezing, therefore the method can be used to detect nematodes in such products.

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.

5 Equipment and consumables

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

5.1 Press system, an automatic or manually operated hydraulic pressing device with a working pressure range from 7 bar to 8 bar.

5.2 Transparent plastic bags of the appropriate thickness and size (e.g. 300 mm × 700 mm × 0,075 mm LDPE). The size of the plastic bag depends on the size of both sample and pressing device (e.g. for herring fillets bags of 700 mm length and 300 mm width can be used).

5.3 UV-light source with a wavelength of 366 nm.

5.4 –20 °C freezer.

6 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.

7 Procedure

WARNING — General safety measures should be addressed when working with UV (e.g. avoid prolonged skin contact). An eye protection or a UV protecting glass sheet installed in the viewing chamber, appropriate for the type of UV lamp, should be used. Specific attention should be observed when handling pressing devices in order to avoid any harm to the personnel performing the test.

7.1 Weighing the sample

Each sample shall be weighed and its weight shall be recorded.

7.2 Preparation of the sample

Fresh or frozen fish shall be eviscerated and filleted, and each flesh side/fillet put in separate (not necessary for small fish) transparent plastic bags. Proceed in the same way if the sample to be tested is lightly processed fish.

The skin of fillets from fishes with a thick skin (e.g. red fish or salmon) should be removed, since it could prevent UV visual inspection.

There is no minimum individual sample size limit and the maximum limit depends on the press device. For large fish, such as Atlantic salmon, cod or halibut, each fillet or fish side can be cut into smaller pieces, which are then processed and examined separately.

7.3 Pressing

Put each bag containing fillets in an automatic or manually operated hydraulic pressing device at 7 bar to 8 bar, and press for a holding time of at least 5 s until the pressed samples reach 1 mm to 2 mm thickness.

A stainless steel frame can be used to ensure regular pressing. The indicated pressing conditions are recommended but not mandatory.

7.4 Freezing

After pressing, the bags containing fillets are kept in a conventional freezer at –20 °C until the fillets are frozen stiff throughout (usually 24 h).

If working with initially frozen fish, additional freezing of the pressed samples is not necessary the inspection step may follow immediately after pressing.

7.5 Thawing

Before testing, samples should be completely thawed at 4 °C or higher, not exceeding room temperature.

The time between thawing and inspection shall not exceed 12 h at room temperature, otherwise Anisakidae larvae can lose their fluorescence.

Thawing can be omitted if the results are urgently needed, since fluorescence is also visible in frozen samples.

7.6 Visual inspection

Put the bags containing the thawed fish fillet under a 366 nm UV light source placed in a dark room. The set-up of the UV-light source should be equipped with both up- and down-light. A set-up comprising six fluorescent light tubes (e.g. Philips TL-D 18 W BLB[1)]), with two and four tubes yielding down- and up-light, respectively, is recommended. Other UV light sources can also be used (e.g. LEDs).

Any anisakid larvae present in a given sample will appear as brightly fluorescent spots, partially depending on the actual anisakid species (see Annex B).

Checking on both sides of the bag, the approximate infection site of the larvae can be recorded, i.e. whether they are in the dorsal (fillets) or ventral (belly flaps) portion of the fish flesh.

NOTE Validated automatic devices can be useful to capture fluorescence images to be analysed for reporting (documentation, results) and to comply with safety measures.

1) The fluorescent light tube PHILIPS TL-D 18 W BLB is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

8 Expression of the results

Results shall be expressed as “present” or “absent” and the number of Anisakidae larvae in “x” grams of sample, per fillet section (Figure B.1).

NOTE If requested or appropriate, the localization of larvae detected can be reported.

If doubtful findings occur, confirmation and identification at the species level by molecular methods should be performed by a qualified reference laboratory.

9 Performance characteristics of the method

The values of these characteristics have been determined by a ring trial on the method organized within the framework of a European project[3].

The accuracy, defined as the closeness of a measurement to the true value, is 100 % (95 % CI: 88,4 % to 100 %).

The sensitivity, defined as the percentage of samples correctly identified as positive, is 100 % (95 % CI: 86,3 % to 100 %).

The specificity, the percentage of samples correctly identified as negative, is 100 % (95 % CI: 47,8 % to 100 %).

The limit of detection of the method is 1 larva in 100 g sample.

10 Test report

A laboratory worksheet (see Annex C) should be used by analysts to record data for test reports and is, therefore, a critical document for quality audits and trace-back investigations. Key components of the laboratory worksheet include traceability information of the sample, the International Standard used (including its year of publication), documentation that the method has been performed correctly by qualified personnel, equipment used (freezer, press and UV- transilluminator), documentation of problems and irregularities, a written record of results (including a reference to the clause which explains how the results were calculated), any deviations from the procedure, any unusual features observed and the date of the test. Laboratory worksheets should be stored according to the requirements of the competent authority.

11 Quality assurance

Each laboratory shall have a system of adequate documentation that demonstrates that anisakid testing was correctly performed according to appropriate quality assurance standards. There are no internal quality controls that can be used while performing the method.