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Microbiology of the food chain — Detection of *Trichinella* larvae in meat by artificial digestion method

Microbiologie de la chaîne alimentaire — Recherche des larves de Trichinella *dans la viande par une méthode de digestion artificielle*

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Foreword

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Introduction

Trichinella spp. are the causative agents of human trichinellosis, a disease which is a public health hazard and, as a result, also represents an economic problem in porcine animal production. Due to the zoonotic importance of this infection in many countries, the main efforts have focused on control and/or eradication of *Trichinella* from domestic pigs, the most important source of human infection worldwide. Digestion methods for detection of *Trichinella* larvae in muscle samples from pigs and other susceptible animal species intended for human consumption (e.g. horses, wild boars, walruses, and bears), are effective for preventing clinical trichinellosis in humans. Due to the limits of sensitivity of digestion methods, these methods might not detect infected animals with very small numbers of larvae in muscle samples, that can pose a risk for subclinical infections in humans.

Microbiology of the food chain — Detection of *Trichinella* larvae in meat by artificial digestion method

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This International Standard specifies a method of detection of *Trichinella* spp. muscle stage larvae in meat of individual animal carcasses intended for human consumption. It is applicable to the examination of meat from domestic and sylvatic animal species, which can be infected by nematodes of the genus *Trichinella*.

This method does not allow the identification of the species or genotype of detected parasites; species or genotype identification can be carried out by molecular methods.

The method described in this International Standard is intended to be used in conjunction with the guidelines in the OIE Manual of Diagnostic Tests and Vaccines and by the International Commission on Trichinellosis (ICT) for *Trichinella* testing and the inspection of carcasses intended for human consumption, unless it has been demonstrated by other means that the animal was not at risk for exposure to *Trichinella*.

The artificial digestion/magnetic stirrer method is considered to be the standard method because it has proven to give the most reliable results in validation studies.

NOTE Provided equivalence with the method described within this International Standard can be documented, alternative methods can be used for analysis.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for the application 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 7218:2007, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

International Commission on Trichinellosis (ICT), "Quality Assurance in Digestion Testing Programs for *Trichinella*", *Recommendations and Guidelines*. 2012

World Organisation for Animal Health (OIE), Chapter 2.1.16 — "Trichinellosis", *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. 7th ed. 2012

3 Terms and definitions

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

3.1
***Trichinella* muscle larvae**
ML
first larval stage (L1) of nematodes belonging to the genus *Trichinella*, which is located in striated muscles of animals worldwide and can infect humans

Note 1 to entry: These larvae are approximately 0,7 mm to 1,1 mm in length and 0,03 mm in width.

3.2
digestion assay
method to detect *Trichinella* larvae in muscle tissue by an enzymatic digestion step to release larvae from muscle tissues followed by filtration and sedimentation steps, and detection of recovered larvae by microscopy

3.3
larvae per gram
lpg
number of *Trichinella* larvae present in one gram of muscle tissue

4 Principle

4.1 General

The artificial digestion method, which is accepted as the international reference method (see OIE, Terrestrial Manual 2012, Chapter 2.1.16 — Trichinellosis, page 307), relies on enzymatic degradation of muscle fibres in a fluid composed of pepsin and hydrochloric acid followed by a series of sedimentation and washing steps; equivalent validated methods can also be used. Test performance is greatly influenced by the sample size (1 g samples will enable the reliable detection of infections of ≥3 lpg, and 3 g to 5 g samples will enable reliable detection of ≥1 lpg), by the type of the selected muscle, by the method used, and by the skills of the technician performing the test. A sensitivity of at least one to three larvae per gram shall be achieved to protect human health; consequently, an appropriate size of muscle sample should be collected from a predilection muscle of the target animal. There are no internal quality controls that can be used while performing the method. Some key elements in the principle of its use are as follows (see 4.2 to 4.9).

4.2 Sample size

For inspection of individual food animal carcasses for public health purposes, the sample shall be collected from predilection sites (see Annex A) and the sample size shall be risk-based, as determined by a competent authority, but not less than 1 g per carcass.

4.3 Blending/grinding of the muscle sample

Meat samples are chopped using a blender or grinder to increase the surface area for enzymatic degradation. The blending or grinding procedure shall be adjusted to maximize digestion efficiency.

NOTE Too little blending or grinding can result in poor digestion, while too much blending or grinding can damage or disrupt muscle larvae.

4.4 Preparation of the digest fluid

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

NOTE 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.

4.5 Digestion of the chopped meat

Viable *Trichinella* larvae are resistant to the pepsin-HCl digest fluid and therefore can be recovered free from muscle tissue. Dead *Trichinella* larvae can be destroyed by artificial digestion.

To facilitate an efficient and rapid digestion, a maximum ratio of 1:20, meat to digest fluid, and a temperature of 45 °C ± 2 °C, shall be maintained throughout the process. The time required for digestion shall be at least 30 min, but in the case of muscle samples which are less digestible, such as from wildlife

carcasses, or the tongue of many species (see Annex A), the digestion time should be increased but, unless otherwise validated for a particular sample matrix, shall not exceed 60 min in total.

NOTE If the time-temperature conditions are less than the required values, there might be an incomplete digestion of the muscle tissue. Conversely, elevated temperature (over 50 °C) or prolonged digestion times might result in the destruction of larvae or the inactivation of the pepsin.

4.6 Filtration of the digest fluid

Following digestion, the digest fluid shall be filtered through a sieve with specific mesh size (6.11), in order to retain undigested tissue, but allowing larvae to pass through. Sieves shall be free of debris prior to use and shall be pre-wetted to allow digest fluid to pass through quickly.

4.7 Sedimentation of the digest fluid

Sedimentation of larvae is performed in a separatory funnel (primary sedimentation) and a glass tube (secondary sedimentation). Larvae are collected in these primary and secondary sediments. Sedimentation times shall be of 30 min and 10 min for the primary and secondary sedimentation, respectively (see Annex B for sedimentation times for frozen muscle samples). If the sedimentation times are less than stipulated above, not all larvae might be settled and might not be recovered in the collected sediment.

4.8 Microscopic examination

Microscopic examination of the secondary sediment allows qualified analysts to recognize *Trichinella* larvae and distinguish from many other nematodes, organisms, or artefacts. Knowledge of the basic morphological characteristics of *Trichinella* larvae, including size (0,7 mm to 1,1 mm in length and 0,03 mm in width), shape, and colour is required to examine the sediment (see Figure C.1 and Figure C.2). The most distinguishing feature of *Trichinella* larvae, not recognized by stereomicroscopy but by compound microscopy, is the stichosome, which consists of a series of discoid cells lining the oesophagus and occupying the anterior half of the body. *Trichinella* larvae can appear coiled (when cold) or motile (when warm), or C-shaped (when dead).

To be qualified to perform routine digestion testing, the analyst shall adequately perform the artificial digestion/magnetic stirrer method using *Trichinella* spiked samples (proficiency testing panel) to consistently recover and identify larvae. Analysts should be qualified based on performance of proficiency testing in accordance with guidance from the International Commission on Trichinellosis.

4.9 Verification of findings

If positive or doubtful findings occur, confirmation and identification at the species level should be performed by a qualified reference laboratory, as defined by "ICT guidelines Recommendations for Quality Assurance in Digestion Testing Programs for *Trichinella*", Part 1 — Quality assurance in regulatory testing for *Trichinella,* i.e. "a laboratory formally recognised by a national or international authority for a required level of scientific and diagnostic expertise for a particular animal disease and/or testing methodology".

5 Reagents

5.1 Tap water, heated to 47 °C ± 2 °C.

5.2 Hydrochloric acid (25 %, molar concentration: 7,8 to 7,9, or any other percentage).

5.3 Pepsin (Powder or granular: 1: 10.000 NF, 1: 12.500 BP, 2.000 FIP; liquid: 660 U/ml).

NOTE The activity of pepsin powder is expressed per gram, either in "NF" (US National Formulary), "BP" (British Pharmacopoea), or "FIP" (Fédération Internationale de Pharmacie); 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 10g of 1:10.000 NF.

5.4 Ethanol (70 % to 90 % ethyl alcohol).

5.5 Sodium hypochlorite.

6 Apparatus

Usual microbiological laboratory equipment (see 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 and removing non-digestible tissue.

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

6.4 Glass, plastic, or **steel blender**, with a sharp chopping blade (regularly inspected and/or exchanged).

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

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

6.7 Stir bar, (5 cm in length minimum).

6.8 Glass beakers, (minimum 3 l capacity).

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

6.10 Funnels (glass, **plastic,** or **steel)**, (minimum diameter of approximately 15 cm).

6.11 Sieve, made of brass or stainless steel, specific mesh size between 180 µm to 200 µm (with diameter of approximately 10 cm or larger).

6.12 Conical glass separatory funnels (minimum 2,5 l in capacity), preferably with polytetrafluoroethylene (PTFE) safety plugs (stopcocks).

6.13 Tubes or **measuring cylinders**, (50 ml or 100 ml glass).

6.14 Petri dishes (approximately 90 mm in diameter), gridded with squares of approximately 1 cm, or equivalent equipment for larval counting.

6.15 Stereo-microscope, with a sub-stage transmitted adjustable light source, or trichinoscope with a horizontal table. Image capture and storage capability (camera) are recommended, but not required to document suspect results.

6.16 Pipettes, with capacity of 1 ml, 10 ml, and 25 ml.

6.17 Small vials, for collection of recovered larvae.

Plasticware or teflonware should not be used for beakers or separatory funnels (other than stopcocks) since a rough surface and electrostatic charge can contribute to larval adherence to the inner surface of the equipment.

Equipment listed at 6.2, 6.4, and 6.11 should be regularly cleaned and free of any tissues which could contain larvae from previous analyses.

7 Sampling, labelling, and transport

Sampling, labelling, and transport are not part of the method specified in this International Standard, but are indicated in Annex A. If there are no specific International Standards dealing with sampling of the animal carcasses or parts of them, it is recommended that the interested parties come to an agreement on this subject.

In order to obtain the required sensitivity for *Trichinella* testing in animals, an appropriate size of muscle sample shall be collected from a predilection muscle (see Annex A). Muscle samples taken from the carcass for digestion testing should be at least twice the mass required for examination to allow for trimming of non-digestible tissues.

At the reception of the samples, laboratory personnel shall check the suitability of samples for testing by checking mass, composition, condition, labelling, and correspondence with transmission documents (receipt as specified in ISO 7218:2007, 8.3).

Muscle samples should be tested as soon as possible or stored at 2 °C to 8 °C to slow decomposition and avoid freezing.

8 Sample preparation

Samples used for testing shall be free from fat, tendons, and fascia. If tongue tissue is used, the tough indigestible surface layer of connective tissue shall be removed before testing. The minimum individual sample size for testing by artificial digestion should be determined by the competent authority (see "ICT guidelines Recommendations for Quality Assurance in Digestion Testing Programs for *Trichinella*", Part 5. Recommendations on essential components and minimum requirements for a *Trichinella* testing laboratory certification program — B.6 Sample collection and handling). Samples from individual animals may be pooled; the maximum muscle mass in a pool to be digested shall be 100 g, but up to 15 g of additional muscle tissue may be added when needed. For pools with a lower total muscle mass (e.g. 50 g), the digest fluid volume and ingredients may be adjusted accordingly to a minimum of 1 l.

9 Procedure

9.1 General

A scheme of the magnetic stirrer method is shown in Figure C.1.

9.2 Blending/grinding

For blending/grinding, a small amount (50 ml to 100 ml per 100 g meat) of digest fluid or tap water (45 °C ± 2 °C) shall be added to the meat in the blender/grinder to facilitate homogenization. Blending/grinding should be continued until the meat is chopped or minced thoroughly (usually three bursts of 5 s to 10 s each) but shall not last so long as to harm the larvae.

9.3 Preparation of the digest fluid

The pepsin used for the preparation of digest fluid shall have the appropriate activity required for digestion. (see 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 25 % hydrochloric acid (see Clause 5) to a glass beaker containing 2 l of tap water preheated to 45 °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: 1. water, 2. hydrochloric acid, and 3. pepsin. This will prevent degradation of pepsin which might result from direct exposure to concentrated hydrochloric acid.

NOTE 2 Stock solutions of hydrochloric acid are available in formulations other than 25 % and are to be 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 10g of 1:10.000 NF.

9.4 Digestion of the chopped meat in the glass beaker

a) After blending/grinding, the meat is transferred to a 3 l glass beaker which contains the digest fluid (see 9.3). To avoid loss of larvae due to adhering muscle tissue, the blending/grinding equipment should be thoroughly rinsed with the digest fluid in the beaker and the blender bowl should also be thoroughly rinsed with a small quantity of digest fluid which is then poured back into the glass beaker.

b) Cover the glass beaker with aluminium foil to decrease evaporation and keep a constant temperature, 45 °C ± 2 °C.

c) Regularly monitor the temperature of the digest fluid using a thermometer.

d) Ensure that during stirring, the digest fluid rotates at a sufficiently high speed to create a deep vortex without splashing.

e) Digest the sample for 30 min; if necessary, the digestion time can be increased but shall not exceed 60 min in total.

9.5 Filtration of the digest fluid

a) Ensure that the stopcock of the separatory funnel is fully closed. Pour the digest fluid carefully (to avoid overflow and spillage) into the separatory funnel through a sieve with specific mesh size (6.11).

b) Rinse the glass beaker and the sieve with an additional volume of tap water (minimum of 100 ml) to avoid larval loss due to adhering muscle tissue or larvae sticking on the surfaces of the glassware and the sieve.

c) The digestion process is considered satisfactory if residual debris remaining on the sieve consists primarily of indigestible non-muscle tissue (typically consisting of fascia and connective tissue) of no greater than 5 % of the original sample mass.

d) If undigested muscle tissue or non-muscle tissue remains on the sieve in excess of that described above, the digestion procedure shall be repeated. In the case of excessive undigested muscle tissue, the remaining fraction shall be digested and examined in addition to the initial digest, or new samples shall be collected and the entire method repeated (i.e. start procedure again from 9.2).

NOTE Since the sieve is used to separate muscle debris from *Trichinella* larvae after digestion, not to measure, the mesh size does not need to be periodically calibrated.

9.6 Sedimentation of the digest fluid in the separatory funnel

Allow the digest fluid to sediment for 30 min.

NOTE Although not necessary, gentle tapping of the funnel wall (e.g. every 10 min) can facilitate the larvae settling to the bottom of the funnel.

9.7 Collection of the primary and secondary sediment

a) Dispense approximately 40 ml of the digest fluid (primary sediment) into a glass tube.

The stopcock of the separatory funnel shall be fully and quickly opened to ensure that no larvae are trapped on the edge of the opening or fail to be flushed out due to low flow velocity.

NOTE 1 If the volume of the primary sediment is too small, larvae might remain in the digest fluid in the separatory funnel and might be lost. Conversely, there might be excessive debris affecting clarity if the volume of the primary sediment is too high.

b) Allow the 40 ml of primary sediment to stand for 10 min.

NOTE 2 If the sedimentation time is too short, larvae might not have enough time to sediment and might be lost.

c) Carefully withdraw 30 ml of supernatant by suction from the top of the fluid, leaving a volume of not more than 10 ml (secondary sediment).

NOTE 3 If the analyst considers that the digest fluid is not clear enough to be examined, a washing step can be performed (add 30 ml of tap water, allow the sediment to stand for 10 min, and repeat 9.7 c).

d) Pour the secondary sediment into a gridded Petri dish or larval counting basin. To remove larvae which can adhere to the inner surface of the glass, the tube should be rinsed with 10 ml of water which is then added to the Petri dish.

e) The final 20 ml of digest fluid in the Petri dish shall be allowed to stand for at least 1 min for any larvae to settle before microscopic examination.

9.8 Microscopic examination

a) Examine the digest fluid in the Petri dish for transparency with a stereomicroscope or trichinoscope at a 10× to 20× magnification.

b) Transparency of the digest fluid shall be checked by ability to focus on the grid lines of the Petri dish or alternatively to read a printed page under the Petri dish. If the secondary sediment is not transparent enough to enable easy identification of any larvae, it shall be clarified as follows:

1) transfer the secondary sediment and tap water rinse of the Petri dish or larval counting basin into a clean glass tube and add additional tap water to a total volume of approximately 40 ml;

2) allow to sediment for 10 min;

3) carefully withdraw the supernatant [see 9.7 c)], leaving a volume of 10 ml.

4) pour this sediment and a 10 ml tap water rinse of the tube into the original gridded Petri dish .

c) Examine the digest fluid in the Petri dish grid by grid with a stereomicroscope or trichinoscope at a 10 X to 20 X magnification for at least 10 min (this is considered the minimum time needed for a thorough examination by a skilled operator). Examination shall be done systematically, taking care to avoid movement of fluid in the Petri dish.

d) Examination should be performed immediately after digestion; if not possible, the Petri dish shall be stored refrigerated and the digest fluid shall be examined the same day of digestion.

e) Any suspect *Trichinella* or other organism findings shall be further examined by microscopy at 60 X to 100 X magnification (see Figure C.2).

f) If *Trichinella* positive findings occur, the larvae shall be transferred, as soon as possible, into a small vial (1 ml to 2 ml) filled with 70 % to 90 % ethyl alcohol (final concentration) for preservation.

g) It is recommended that confirmatory microscopic identification and molecular identification (e.g. by PCR) at the species/genotype level on all larvae found be performed by a qualified laboratory.

If required by the competent authority, suspect or positive *Trichinella* pooled samples should be traced back from the pool to the carcass of origin through digestion of progressively smaller numbers of pooled samples of increased sample size from the implicated carcasses. Pooled samples with negative results should be ruled out and those yielding positive results should continue to be sampled and tested until digestion of tissues from individual carcass(es) demonstrates the source of the positive result.

10 Documentation

Each laboratory shall have a system of adequate documentation which demonstrates that *Trichinella* testing was correctly performed according to appropriate quality assurance standards. A laboratory worksheet (see Annex D) should be used by analysts to record data for test reports and is, therefore, a critical document for quality audits and traceback investigations. Key components of the laboratory worksheet include sample tracking information, documentation that the method has been performed correctly by qualified personnel, documentation of problems and irregularities, and a written record of results. Laboratory worksheets should be stored according to the requirements of the competent authority.

11 Expression of the results

Results shall be expressed as “presence” or “absence” of *Trichinella* larvae in “x” grams of sample.

12 Safety measures

Fluids (digestive fluids, supernatant fluids, rinses, etc.) as well as glassware and other equipment, which could be contaminated with *Trichinella* larvae, shall be decontaminated by heating to at least 70 °C, or by chemical alternative methods (e.g., sodium hypochlorite at a final concentration of 0,01 % of active chlorite, at 3 h minimum) before discarding or washing.

Annex A
(normative)

Sample collection

A.1 General

In order to obtain the desired sensitivity for *Trichinella* testing in domestic or wild animals, an appropriate size of muscle sample shall be collected from a predilection muscle of the target animal species. See Table A.1 for a list of the predilection muscles by animal species. The sample size to be tested shall be determined by the competent authority based on the risk of *Trichinella* infection in animals under investigation, scientific knowledge of test sensitivity, and the purpose of testing.

Table A.1 — Predilection muscles for selected animal species, which are recommended for digestion testing for *Trichinella*

Animal species	Predilection muscles
Domestic pig (*Sus scrofa domesticus*)	Diaphragm, masseter
Horse (*Equus ferus caballus*)	Masseter, diaphragm, tongue
Wild boar (*Sus scrofa)*	Diaphragm, foreleg, tongue
Dog (*Canis lupus familiaris*)	Diaphragm, masseter, tongue
Bear (*Ursus spp.*)	Diaphragm, masseter, tongue
Walrus (*Odobenus spp.)*	Tongue
Seal (family *Phocidae)*	Diaphragm, intercostals, tongue
Crocodile (*Crocodylus niloticus)*	Intercostal, masseter
Fox (*Vulpes* spp.)	Diaphragm, foreleg, tongue
Raccoon dog (*Nyctereutes procyonoides)*	Diaphragm, foreleg, tongue

For pigs, muscle samples shall be taken from the diaphragm pillars (crus) or from the masseter. In the absence of predilection site muscles, a greater amount of muscle sample shall be taken from other striated muscles near bones or tendons.

Samples shall consist of striated muscle tissue only; avoid taking connective tissue or fat, which is not suitable for digestion testing. Samples shall be taken using a knife or other cutting instrument.

Sample mass shall be twice the mass required to perform the test, to allow for trimming of non-digestible tissues to obtain the required amount of striated muscle for the test.

A.2 Sample identification

(Pooled) muscle samples shall be labelled upon collection to trace to individual animal carcasses and their parts (e.g. for horse samples, labelling shall permit the correspondence between sample, horse head and carcass).

A.3 Documentation

Prepare, at least for each animal batch, a document reporting all relevant information about the batch and the correspondence between samples and carcasses.

A.4 Transport

Transfer samples to the laboratory as soon as possible at a temperature which prevents decomposition of samples, but avoiding freezing (transport is specified in ISO 7218:2007, 8.2).

Annex B
(normative)

Frozen samples

B.1 General

Muscle samples from animals (e.g. wildlife monitoring), which cannot be examined directly after collection or cannot be stored at refrigerated temperatures for several days, shall be stored under freezing conditions (–20 °C) until testing. Although freezing should be the last choice for *Trichinella* testing, carcasses sometimes need to be frozen before examination (e.g. foxes in an *Echinococcus multilocularis* endemic area). Freezing will kill most species and genotypes of *Trichinella,* but some species and genotypes have partial or complete resistance to freezing (e.g. *T. nativa*). Results from experimental studies show that dead larvae have a prolonged sedimentation time due to a change in their density and shape (from coiled to C-shaped larvae). Therefore, sedimentation time should be prolonged for frozen muscle samples, keeping in mind that a longer sedimentation time might result in a higher amount of debris in the sediment.

The artificial digestion method may be applied to frozen samples with modification at the following points (see B.2 to B.4).

B.2 Sampling

The mass of frozen samples tested by digestion should be increased to compensate for the reduction in test sensitivity, since freezing can affect digestion results; species that are not freeze-resistant will be killed and therefore might be destroyed during the digestion process.

B.3 Sedimentation

If muscle samples have been frozen prior to digestion, larvae of freeze-susceptible *Trichinella* species are likely to be dead. As dead larvae uncoil upon release from the muscle tissue, their sedimentation speed decreases. Therefore, sedimentation time (see 9.6) for frozen muscle samples where dead larvae are expected shall be prolonged for up to 60 min.

B.4 Microscopic examination

Dead larvae can be uncoiled and transparent; therefore, their detection by microscopy is difficult and special attention shall be paid in the examination of the digest fluid in the Petri dish.

DNA degradation occurs quickly in dead larvae, potentially precluding the molecular identification of larvae at the species or genotype level.