

## ANNEX I

## Detection methods

## CHAPTER I

**REFERENCE METHOD OF DETECTION**

## Magnetic stirrer method for pooled sample digestion

1. Apparatus and reagents
  - (a) Knife or scissors and tweezers for cutting specimens
  - (b) Trays marked off into 50 squares, each of which can hold samples of approximately 2 g of meat, or other tools giving equivalent guarantees as regards the traceability of the samples
  - (c) A blender with a sharp chopping blade. Where the samples are larger than 3 g, a meat mincer with openings of 2 to 4 mm or scissors must be used. In the case of frozen meat or tongue (after removal of the superficial layer, which cannot be digested), a meat mincer is necessary and the sample size will need to be increased considerably
  - (d) Magnetic stirrers with thermostatically controlled heating plate and teflon-coated stirring rods approximately 5 cm long
  - (e) Conical glass separation funnels, capacity of at least 2 litres, preferably fitted with teflon safety plugs
  - (f) Stands, rings and clamps
  - (g) Sieves, mesh size 180 microns, external diameter 11 cm, with stainless steel mesh
  - (h) Funnels, internal diameter not less than 12 cm, to support the sieves
  - (i) Glass beakers, capacity 3 litres
  - (j) Glass measuring cylinders, capacity 50 to 100 ml, or centrifuge tubes
  - (k) A trichinoscope with a horizontal table or a stereo-microscope, with a substage transmitted light source of adjustable intensity
  - (l) A number of 9 cm diameter petri dishes (for use with a stereo-microscope), marked on their undersides into 10 × 10 mm square examination areas using a pointed instrument
  - (m) A larval counting basin (for use with a trichinoscope), made of 3 mm thick acrylic plates as follows:
    - (i) the bottom of the basin to be 180 × 40 mm, marked off into squares,
    - (ii) the sides to be 230 × 20 mm,
    - (iii) the end to be 40 × 20 mm. The bottom and the ends must be inserted between the sides, to form two small handles at the ends. The upper side of the bottom must be raised 7 to 9 mm from the base of the frame formed by the sides and the ends. The components must be stuck together with glue suitable for the material
  - (n) Aluminium foil

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*Status: Point in time view as at 05/12/2005.*

*Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 2075/2005 (repealed). (See end of Document for details)*

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- (o) 25 % hydrochloric acid
- (p) Pepsin, strength: 1: 10 000 NF (US National Formulary) corresponding to 1: 12 500 BP (British Pharmacopoea) and to 2 000 FIP (Fédération internationale de pharmacie)
- (q) Tap water heated to 46 to 48 °C
- (r) A balance accurate to at least 0,1 g
- (s) Metal trays, capacity 10 to 15 litres, to collect the remaining digestive juice
- (t) Pipettes of different sizes (1, 10 and 25 ml) and pipette holders
- (u) A thermometer accurate to 0,5 °C within the range 1 to 100 °C
- (v) Siphon for tap water.

## 2. Collecting of specimens and quantity to be digested

- (a) In the case of whole carcasses of domestic swine, a specimen weighing at least 1 g is to be taken from a pillar of the diaphragm at the transition to the sinewy part. Special trichinae forceps can be used provided an accuracy of between 1,0 and 1,15 g can be guaranteed.

In the case of breeding sows and boars, a larger sample weighing at least 2 g is to be taken from a pillar of the diaphragm at the transition to the sinewy part.

In the absence of diaphragm pillars, a specimen of twice the size 2 g (or 4 g in the case of breeding sows and boars) is to be taken from the rib part or the breastbone part of the diaphragm, or from the jaw muscle, tongue or abdominal muscles.

- (b) For cuts of meat, a sample weighing at least 5 g of striated muscle, containing little fat is to be taken, where possible from close to bones or tendons. A sample of the same size is to be collected from meat that is not intended to be cooked thoroughly or other types of post-slaughter processing.
- (c) For frozen samples, a sample weighing at least 5 g of striated muscle tissue is to be taken for analysis.

The weight of meat specimens relates to a sample of meat that is free of all fat and fascia. Special attention must be paid when collecting muscle samples from the tongue in order to avoid contamination with the superficial layer of the tongue, which is indigestible and can prevent reading of the sediment.

## 3. Procedure

### I. Complete pools (100 g of samples at a time)

- (a)  $16 \pm 0,5$  ml of hydrochloric acid is added to a 3 litre beaker containing 2,0 litre of tap water, preheated to 46 to 48 °C; a stirring rod is placed in the beaker, the beaker is placed on the preheated plate and the stirring is started.
- (b)  $10 \pm 0,2$  g of pepsin is added.
- (c) 100 g of samples collected in accordance with point 2 is chopped in the blender.
- (d) The chopped meat is transferred to the 3 litre beaker containing the water, pepsin and hydrochloric acid.

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- (e) The mincing insert of the blender is immersed repeatedly in the digestion fluid in the beaker and the blender bowl is rinsed with a small quantity of digestion fluid to remove any meat still adhering.
- (f) The beaker is covered with aluminium foil.
- (g) The magnetic stirrer must be adjusted so that it maintains a constant temperature of 44 to 46 °C throughout the operation. During stirring, the digestion fluid must rotate at a sufficiently high speed to create a deep whirl without splashing.
- (h) The digestion fluid is stirred until the meat particles disappear (approximately 30 minutes). The stirrer is then switched off and the digestion fluid is poured through the sieve into the sedimentation funnel. Longer digestion times may be necessary (not exceeding 60 minutes) in the processing of certain types of meat (tongue, game meat, etc.).
- (i) The digestion process is considered satisfactory if not more than 5 % of the starting sample weight remains on the sieve.
- (j) The digestion fluid is allowed to stand in the funnel for 30 minutes.
- (k) After 30 minutes, a 40 ml sample of digestion fluid is quickly run off into the measuring cylinder or centrifuge tube.
- (l) The digestion fluids and other liquid waste are kept in a tray until reading of the results is completed.
- (m) The 40 ml sample is allowed to stand for 10 minutes. 30 ml of supernatant is then carefully withdrawn by suction to remove the upper layers and leave a volume of not more than 10 ml.
- (n) The remaining 10 ml sample of sediment is poured into a larval counting basin or petri dish.
- (o) The cylinder or centrifuge tube is rinsed with not more than 10 ml of tap water, which has to be added to the sample in the larval counting basin or petri dish. Subsequently, the sample is examined by trichinoscope or stereo-microscope at a 15 to 20 times magnification. Visualisation using other techniques is allowed, provided examination of positive control samples has been shown to give an equal or better result than traditional visualisation methods. In all cases of suspect areas or parasite-like shapes, higher magnifications of 60 to 100 times must be used.
- (p) Digests are to be examined as soon as they are ready. Under no circumstances should examination be postponed until the following day.

Where the digests are not examined within 30 minutes of preparation, they must be clarified as follows. The final sample of about 40 ml is poured into a measuring cylinder and allowed to stand for 10 minutes. 30 ml of the supernatant fluid is then removed, leaving a volume of 10 ml. This volume is made up to 40 ml with tap water. After a further settling period of 10 minutes, 30 ml of the supernatant fluid is withdrawn by suction, leaving a volume of no more than 10 ml for examination in a petri dish or larval counting basin. The measuring cylinder is washed with no more than 10 ml of tap water and these washings are added to the sample in the petri dish or the larval counting basin for examination.

If the sediment is found to be unclear on examination, the sample is poured into a measuring cylinder and made up to 40 ml with tap water and then the above procedure

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is followed. The procedure can be repeated 2 to 4 times until the fluid is clear enough for a reliable reading.

## II. Pools of less than 100 g

Where needed, up to 15 g can be added to a total pool of 100 g and examined together with these samples in accordance with 3(I). More than 15 g must be examined as a complete pool. For pools of up to 50 g, the digestion fluid and the ingredients may be reduced to 1 litre of water, 8 ml of hydrochloric acid and 5 g of pepsin.

## III. Positive or doubtful results

Where examination of a collective sample produces a positive or uncertain result, a further 20 g sample is taken from each pig in accordance with 2(a). The 20 g samples from five pigs are pooled and examined using the method described above. In this way samples from 20 groups of five pigs will be examined.

When *Trichinella* is detected in a pooled sample from five pigs, further 20 g samples are collected from the individual pigs in the group and each is examined separately using the method described above.

Parasite samples are to be kept in 90 % ethyl alcohol for conservation and identification at species level at the Community or national reference laboratory.

After parasite collection, positive fluids (digestive juice, supernatant fluid, washings, etc.) are to be decontaminated by heating to at least 60 °C.

## CHAPTER II

### EQUIVALENT METHODS

- A. Mechanically assisted pooled sample digestion method/sedimentation technique
  - 1. Apparatus and reagents
    - (a) Knife or scissors for cutting specimens
    - (b) Trays marked off with 50 squares, each of which can hold samples of approximately 2 g of meat, or other tools giving equivalent guarantees as regards the traceability of the samples
    - (c) Meat mincer or electrical blender
    - (d) A stomacher lab-blender 3 500 thermo model
    - (e) Plastic bags suitable for the stomacher lab-blender
    - (f) Conical separation funnels, capacity 2 litres, preferably fitted with teflon safety plugs
    - (g) Stands, rings and clamps
    - (h) Sieves, mesh size 180 microns, external diameter 11 cm, with stainless steel or brash mesh
    - (i) Funnels, internal diameter not less than 12 cm, to support the sieves
    - (j) 100 ml glass measuring cylinders

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- (k) A thermometer accurate to 0,5 °C within the range 1 to 100 °C
  - (l) A vibrator, e.g. an electric shaver with the head removed
  - (m) A relay which will switch on and off at one-minute intervals
  - (n) A trichinoscope with a horizontal table or a stereo-microscope, with a sub-stage transmitted light source of adjustable intensity
  - (o) A larval counting basin and a number of 9 cm diameter petri dishes as in Chapter I(1) (l) and (m)
  - (p) 17,5 % hydrochloric acid
  - (q) Pepsin, strength 1: 10 000 NF (US national formulary) corresponding to 1: 12 500 BP (British Pharmacopoeia) and to 2 000 FIP (Fédération internationale de pharmacie),
  - (r) A number of 10 litre bins to be used for decontamination of apparatus, e.g. with formol, and for digestive juice remaining where specimens test positive
  - (s) A balance accurate to 0,1 g.
2. Collecting of specimens and quantity to be digested

As stipulated in Chapter I(2).

3. Procedure

I. Grinding

Grinding the meat samples in a meat mincer beforehand will improve the digestion quality. If an electrical blender is used, the blender must be operated three to four times for approximately one second each time.

II. Digestion procedure

This procedure may involve complete pools (100 g of samples at a time) or pools of less than 100 g.

- (a) Complete pools (100 samples at a time)
  - (i) The stomacher lab-blender 3 500 is fitted with a double plastic bag and the temperature control set at 40 to 41 °C.
  - (ii) One and a half litres of water preheated to 40 to 41 °C is poured into the inner plastic bag.
  - (iii) 25 ml of 17,5 % hydrochloric acid is added to the water in the stomacher.
  - (iv) 100 samples weighing approximately 1 g each (at 25 to 30 °C) taken from each individual sample in accordance with 2 are added.
  - (v) Lastly, 6 g pepsin is added. This order must be followed strictly to avoid decomposition of the pepsin.
  - (vi) The stomacher is then allowed to pound the content of the bag for 25 minutes.
  - (vii) The plastic bag is removed from the stomacher and the digestion fluid is filtered through the sieve into a 3 litre beaker.

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- (viii) The plastic bag is washed with approximately 100 ml of water, which is then used to rinse the sieve and lastly added to the filtrate in the beaker.
  - (ix) Up to 15 individual samples can be added to a total pool of 100 samples and examined together with these samples.
- (b) Smaller pools (less than 100 samples)
- (i) The stomacher lab-blender 3 500 is fitted with a double plastic bag and the temperature control set at 40 to 41 °C.
  - (ii) A digestion fluid is prepared by mixing about one and a half litres of water and 25 ml of 17,5 % hydrochloric acid. 6 g of pepsin is added and the whole mixed at a temperature of 40 to 41 °C. This order must be followed strictly to avoid decomposition of the pepsin.
  - (iii) Of the digestion fluid, a volume corresponding to 15 ml per gram of sample is measured (e.g. for 30 samples the volume required is  $30 \times 15 \text{ ml} = 450 \text{ ml}$ ) and transferred to the inner of the two plastic bags, together with the meat samples weighing approximately 1 g (at 25 to 30 °C) taken from each individual sample in accordance with 2.
  - (iv) Water at a temperature of approximately 41 °C is poured into the outer bag to make up a total volume in the two bags of one and a half litres. The stomacher is then allowed to pound the content of the bag for 25 minutes.
  - (v) The plastic bag is removed from the stomacher and the digestion fluid is filtered through the sieve into a 3 litre beaker.
  - (vi) The plastic bag is washed with approximately 100 ml of water (at 25 to 30 °C), which is then used to rinse the sieve and lastly added to the filtrate in the beaker.
- III. Recovery of larvae by sedimentation
- Ice (300 to 400 g of ice flakes, scaly ice or crushed ice) is added to the digestion fluid to bring its volume up to about 2 litres. The digestion fluid is then stirred until the ice has melted. In the case of smaller pools (see II(b)), the amount of ice must be reduced correspondingly.
  - The chilled digestion fluid is transferred to a 2 litre separation funnel, equipped with a vibrator in an extra clamp.
  - Sedimentation is allowed to proceed for 30 minutes, during which time the sedimentation funnel is vibrated intermittently, i.e. one minute vibration followed by a one-minute pause.
  - After 30 minutes, a 60 ml sample of the sediment is quickly run off into a 100 ml measuring cylinder (the funnel is rinsed with detergent solution after use).
  - The 60 ml sample is allowed to stand for at least 10 minutes, after which time the supernatant is withdrawn by suction to leave a volume of 15 ml, to be examined for presence of larvae.
  - For suction, a disposable syringe, equipped with a plastic tube, can be used. The length of the tube must be such that 15 ml remains in the measuring cylinder when the flanges of the syringe rest on the cylinder's rim.
  - The remaining 15 ml is poured into a larval counting basin or two petri dishes and examined using a trichinoscope or stereo-microscope.

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- The measuring cylinder is washed with 5 to 10 ml of tap water and the washings are added to the sample.
- Digests are to be examined as soon as they are ready. Under no circumstances is examination to be postponed until the following day.

Where the digests are unclear or they are not examined within 30 minutes of their preparation, they must be clarified as follows:

- the final sample of 60 ml is poured into a measuring cylinder and allowed to stand for 10 minutes; 45 ml of supernatant fluid is then removed by suction and the remaining 15 ml is made up to 45 ml with tap water,
- after a further settling period of 10 minutes, 30 ml of supernatant fluid is removed by suction and the remaining 15 ml is poured into a petri dish or larval counting basin for examination,
- the measuring cylinder is washed with 10 ml of tap water and these washings are added to the sample in the petri dish or the larval counting basin for examination.

#### IV. Positive or doubtful results

Where the result is positive or uncertain, the provisions laid down in Chapter I(3)(III) shall apply.

#### B. Mechanically assisted pooled sample digestion method/‘on filter isolation’ technique

##### 1. Apparatus and reagents

As stipulated in Chapter II(A)(1).

Additional equipment:

- (a) 1 litre Gelman funnel, complete with filter holder (diameter 45 mm);
- (b) filter discs, consisting of a circular stainless steel mesh with an aperture of 35 microns (disc diameter: 45 mm), two rubber rings 1 mm thick (external diameter: 45 mm; internal diameter: 38 mm), the circular mesh being placed between the two rubber rings and bonded to them using a two-component glue suitable for the two materials;
- (c) an Erlenmeyer flask, capacity 3 litres, fitted with a side tube for suction;
- (d) a filter pump;
- (e) plastic bags, capacity at least 80 ml;
- (f) equipment for sealing the plastic bags;
- (g) rennilase, strength 1: 150 000 soxhlet units per gram.

##### 2. Collecting of specimens

As stipulated in Chapter I(2).

##### 3. Procedure

###### I. Grinding

Grinding the meat samples in a meat mincer beforehand will improve the digestion quality. If an electrical blender is used, the blender must be operated three to four times for approximately one second each time.

###### II. Digestion procedure

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This procedure may involve complete pools (100 g of samples at a time) or pools of less than 100 g.

- (a) Complete pools (100 samples at a time)

See Chapter II(A)(3)(II)(a).

- (b) Smaller pools (less than 100 samples)

See Chapter II(A)(3)(II)(b).

### III. Recovery of larvae by filtration

- (a) Ice (300 to 400 g of ice flakes, scaly ice or crushed ice) is added to the digestion fluid to bring its volume up to about 2 litres. In the case of smaller pools, the amount of ice must be reduced correspondingly.

- (b) The digestion fluid is stirred until the ice has melted. The chilled digestion fluid is then left for at least three minutes to let the larvae coil.

- (c) The Gelman funnel, fitted with a filter holder and filter disc, is mounted on an Erlenmeyer flask connected to a filter pump.

- (d) The digestion fluid is poured into the Gelman funnel and filtered. Towards the end of filtration, the digestion fluid can be helped to pass through the filter by applying suction with the filter pump. Suction must cease before the filter becomes dry, i.e. when 2 to 5 ml of fluid is left in the funnel.

- (e) Once all the digestion fluid has been filtered, the filter disc is removed and placed in an 80 ml capacity plastic bag, together with 15 to 20 ml of rennilase solution. The rennilase solution is made by adding 2 g of rennilase to 100 ml of tap water.

- (f) The plastic bag is sealed twice and placed between the inner and outer bags in the stomacher.

- (g) The stomacher is allowed to pound for three minutes, e.g. while it is working on a complete or incomplete pool.

- (h) After three minutes, the plastic bag, complete with filter disc and rennilase solution, is removed from the stomacher and opened with scissors. The liquid contents are poured into a larval counting basin or petri dish. The bag is washed out with 5 to 10 ml of water, which is then added to the larval counting basin for examination by trichinoscope or to the petri dish for examination by stereo-microscope.

- (i) Digests must be examined as soon as they are ready. Under no circumstances is examination to be postponed until the following day.

*Note:*

Filter discs must never be used when not completely clean. Unclean discs must never be allowed to dry out. Filter discs can be cleaned by leaving them in rennilase solution overnight. Before use, they must be washed in fresh rennilase solution using the stomacher.

### IV. Positive or doubtful results

Where the result is positive or uncertain, the provisions laid down in Chapter I(3)(III) shall apply.

- C. Automatic digestion method for pooled samples of up to 35 g



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1. Apparatus and reagents
  - (a) Knife or scissors for cutting specimens
  - (b) Trays marked off with 50 squares, each of which can hold samples of approximately 2 g of meat, or other tools giving equivalent guarantees as regards the traceability of the samples
  - (c) A Trichomatic 35<sup>®</sup> blender with filtration insert
  - (d) Hydrochloric acid 8,5 ± 0,5 % weight
  - (e) Transparent polycarbonate membrane filters with a diameter of 50 mm and a pore size of 14 microns
  - (f) Pepsin, strength 1: 10 000 NF (US National Formulary) corresponding to 1: 12 500 BP (British Pharmacopoeia) and to 2 000 FIP (Fédération internationale de pharmacie)
  - (g) A balance accurate to 0,1 g
  - (h) Tweezers with a flat tip
  - (i) A number of microscope slides with a side-length of at least 5 cm or a number of petri dishes at least 6 cm in diameter, marked on their undersides into 10 × 10 mm square areas using a pointed instrument
  - (j) A (stereo-)microscope with transmitted light (magnification 15 to 60 times) or a trichinoscope with a horizontal table
  - (k) A bin for collection of waste liquids
  - (l) A number of 10 litre bins to be used for decontamination of apparatus, e.g. with formol, and for digestive juice remaining where specimens test positive
  - (m) a thermometer accurate to 0,5 °C within the range 1 to 100 °C.

2. Collecting of specimens

As stipulated in Chapter I(2).

3. Procedure

- I. Digestion procedure

- (a) Place the blender with the filtration insert, connect the waste tube and place the tube so it drains into the waste bin.
- (b) When the blender is switched on, heating will start.
- (c) Before this is done, the bottom valve located below the reaction chamber must be opened and closed.
- (d) Up to 35 samples weighing approximately 1 g each (at 25 to 30 °C) taken from each individual sample in accordance with point 2 are then added. Ensure that larger pieces of tendons are removed as they may clot the membrane filter.
- (e) Pour water up to the edge of a liquid chamber connected to the blender (approximately 400 ml).

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- (f) Pour about 30 ml hydrochloric acid ((8,5 %) to the edge of the smaller, connected liquid chamber.
- (g) Place a membrane filter under the coarse filter in the filter holder in the filter insert.
- (h) Lastly, add 7 g of pepsin. This order must be followed strictly to avoid decomposition of the pepsin.
- (i) Close the lids of the reaction and liquid chambers.
- (j) Select the period of digestion. A short digestion period (5 minutes) must be set for pigs at the normal slaughter age and a longer time (8 minutes) for other samples.
- (k) When the start button on the blender is turned on, the process of dispensing and digestion starts automatically, followed by filtration After 10 to 13 minutes the process is completed and stops automatically.
- (l) Open the lid of the reaction chamber after checking that the chamber is empty. If there is foam or any digestion liquid remaining in the chamber, repeat the procedure in accordance with V.

## II. Recovery of larvae

- (a) Remove the filter holder and transfer the membrane filter to a slide or Petri dish.
- (b) Examine the membrane filter using a (stereo-) microscope or a trichinoscope.

## III. Cleaning equipment

- (a) Where the result is positive, fill the blender reaction chamber with boiling water until it is two-thirds full. Ordinary tap water is poured into the connecting liquid chamber until it covers the lower sensor. Automatic cleaning then takes place. Decontaminate the filter-holder and any other equipment, e.g. using formol.
- (b) After work is completed for the day, fill the blender liquid chamber with water and put it through a standard cycle.

## IV. Use of membrane filters

Each polycarbonate membrane filter may be used no more than five times. The filter is to be turned between each use. In addition, the filter must be checked after each use for any damage which would make it unsuitable for further use.

## V. Method to be applied when digestion is incomplete and filtration cannot be carried out

Once the blender has been put through an automatic cycle in accordance with C(3)(I), open the lid of the reaction chamber and check whether there is foam or any liquid remaining in the chamber. If this is the case, proceed as follows:

- (a) close the bottom valve below the reaction chamber;
- (b) remove the filter holder and transfer the membrane filter to a slide or Petri dish;
- (c) put a new membrane filter in the filter holder and attach the filter holder;
- (d) fill the blender liquid chamber with water until the lower sensor is covered;
- (e) carry out the automatic cleaning cycle;

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- (f) after the cleaning cycle has ended, open the lid of the reaction chamber and check whether any liquid remains;
- (g) if the chamber is empty, remove the filter holder and transfer the membrane filter to a slide or Petri dish with tweezers;
- (h) examine the two membrane filters in accordance with C(3)(II). If the filters cannot be examined, repeat the entire digestion process with a longer digestion time in accordance with C(3)(I).

#### VI. Positive or doubtful results

Where the result is positive or uncertain, the provisions laid down in Chapter I(3)(III) shall apply.

### CHAPTER III

#### TRICHINOSCOPIC EXAMINATION

1. Apparatus
  - (a) An incandescent-lamp trichinoscope with 30 to 40 times and 80 to 100 times magnification or a stereomicroscope with a substage transmitted light source of adjustable intensity
  - (b) A compressorium being a pressure glass consisting of two glass plates (one of which is divided into equal fields)
  - (c) Small curved scissors
  - (d) Small forceps
  - (e) A knife for cutting specimens
  - (f) Small numbered containers for storing the specimens separately
  - (g) A dropping pipette
  - (h) A glass of acetic acid and a glass of potassium hydroxide solution for brightening any calcifications and softening dried meat.
2. Collecting of specimens

In the case of whole carcasses, several hazelnut-size samples are taken from each animal:

- (a) in domestic swine, such samples are taken from both diaphragm pillars at the transition of the sinewy part;
- (b) in wild boar samples are taken from both diaphragm pillars at the transition of the sinewy part and in addition from the jaw, the muscles of the lower leg, the intercostal muscles and the tongue muscles, giving a total of six samples from each individual animal;
- (c) if certain muscles are not available for sampling, a total of four samples are taken from the muscles that are available;
- (d) in pieces of meat, four hazelnut-size samples of striated muscle tissue containing if possible no fat, taken from different points, are taken from each piece, where possible close to bones or tendons.

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3. Procedure
- (a) In general a compressorium is filled with  $1,0 \pm 0,1$  g of meat, normally corresponding with 28 oat-kernel-size pieces. If necessary, two compressoria need to be filled to examine 56 oat-kernel-size pieces.
  - (b) If both diaphragm pillars are present in a domestic swine, the *Trichinella* inspector cuts 28 oat-kernel-size pieces from each of the above specimens taken from a whole carcase, making 56 pieces in all.
  - (c) If only one diaphragm pillar is present, 56 pieces are cut in different places, if possible from the transition to the sinewy part.
  - (d) The samples collected from the other four muscles of wild boar are each cut into seven oat-kernel-size pieces, giving a total of 28 additional pieces.
  - (e) The *Trichinella* inspector then compresses the 56 (or 84) pieces between the glass plates so that normal print can be clearly read through the slide preparation.
  - (f) If the flesh of the specimens to be examined is dry and old, the preparations must be softened for 10 to 20 minutes before pressing with a mixture of one part of potassium hydroxide solution to about two parts of water.
  - (g) From each of the samples taken from pieces of meat, the *Trichinella* inspector cuts 14 oat-kernel-size pieces, making 56 pieces in all.
  - (h) The microscopic examination must be carried out by scanning each preparation slowly and carefully at a magnification of 30 to 40 times.
  - (i) If the trichinoscopic examination reveals suspect areas, they must be examined at the trichinoscope's most powerful magnification (80 to 100 times).
  - (j) Where the result is uncertain, the examination is repeated on other specimens and slide preparations until the information required is obtained. The trichinoscopic examination must be carried out for at least six minutes.
  - (k) The minimum time fixed for the examination does not include the time necessary for taking samples and making the preparations.
  - (l) As a general rule, the trichinoscopic examiner must not inspect more than 840 pieces a day, corresponding with examinations of 15 domestic swine or 10 wild boar.

## ANNEX II

### Freezing treatments

#### A. Freezing method 1

- (a) Meat brought in already frozen is to be kept in this condition.
- (b) The technical equipment and energy supply of the refrigeration room must be such as to ensure that the required temperature is reached very rapidly and maintained in all parts of the room and of the meat.
- (c) Insulated packaging must be removed before freezing, except in the case of meat that is already at the required temperature throughout when it is brought into the refrigeration

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room or meat so packaged that the packaging will not prevent it from reaching the required temperature within the specified time.

- (d) Consignments in the refrigeration room must be kept separately and under lock and key.
- (e) The date and time when each consignment is brought into the refrigeration room must be recorded.
- (f) The temperature in the refrigeration room must be at least  $-25^{\circ}\text{C}$ . It must be measured using calibrated thermo-electric instruments and recorded continuously. It may not be measured directly in the cold air flow. The instruments must be kept under lock and key. The temperature charts must include the relevant data from the meat inspection register on import and the date and time of commencement and completion of freezing, and must be retained for one year after compilation.
- (g) Meat of a diameter or thickness of up to 25 cm must be frozen for at least 240 consecutive hours, and meat of a diameter or thickness of between 25 and 50 cm must be frozen for at least 480 consecutive hours. This freezing process must not be applied to meat that is thicker or of a larger diameter. The freezing time is calculated from the point when the temperature in the freezing room reaches that specified in (f).

#### B. Freezing method 2

The general provisions of (a) to (e) of method 1 are complied with, and the following time-temperature combinations applied:

- (a) meat of a diameter or thickness of up to 15 cm must be frozen for one of the following time-temperature combinations:
  - 20 days at  $-15^{\circ}\text{C}$ ,
  - 10 days at  $-23^{\circ}\text{C}$ ,
  - 6 days at  $-29^{\circ}\text{C}$ ;
- (b) meat of a diameter or thickness of between 15 cm and 50 cm must be frozen for one of the following time-temperature combinations:
  - 30 days at  $-15^{\circ}\text{C}$ ,
  - 20 days at  $-25^{\circ}\text{C}$ ,
  - 12 days at  $-29^{\circ}\text{C}$ .

The temperature in the refrigeration room must be no higher than the level of the selected inactivation temperature. It must be measured using calibrated thermoelectric instruments and recorded continuously. It must not be measured directly in the cold air flow. The instruments must be kept under lock and key. The temperature charts must include the relevant data from the meat inspection register on importation and the date and time of commencement and completion of freezing, and must be retained for one year after compilation.

Where freezing tunnels are used and the above procedures are not followed strictly, the food business operator must be able to prove to the competent authority that the alternative method is effective in killing *Trichinella* parasites in pigmeat.

#### C. Freezing method 3

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*Status: Point in time view as at 05/12/2005.*

*Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EC) No 2075/2005 (repealed). (See end of Document for details)*

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Treatment consists of commercial freeze-drying or freezing of meat for specified time-temperature combinations with temperature monitored at the centre of each cut.

- (a) The general provisions of (a) to (e) of Method 1 are to be complied with for the following time-temperature combinations:
- 106 hours at  $-18^{\circ}\text{C}$ ,
  - 82 hours at  $-21^{\circ}\text{C}$ ,
  - 63 hours at  $-23,5^{\circ}\text{C}$ ,
  - 48 hours at  $-26^{\circ}\text{C}$ ,
  - 35 hours at  $-29^{\circ}\text{C}$ ,
  - 22 hours at  $-32^{\circ}\text{C}$ ,
  - 8 hours at  $-35^{\circ}\text{C}$ ,
  - 1/2 hour at  $-37^{\circ}\text{C}$ .
- (b) The temperature is to be measured using calibrated thermoelectric instruments and recorded continuously. The thermometer probe is inserted in the centre of a cut of meat no smaller in size than the thickest piece of meat to be frozen. This cut must be placed at the least favourable position in the refrigeration room, not close to the cooling equipment or directly in the cold air flow. The instruments must be kept under lock and key. The temperature charts must include the data numbers from the meat inspection register on import and the date and time of commencement and completion of freezing, and must be retained for one year after compilation.

### ANNEX III

#### Examination of animals other than swine

Horse meat, wild game meat and other meat that could contain *Trichinella* parasites must be examined in accordance with one of the digestion methods specified in Chapter I or II of Annex I, with the following changes:

- (a) specimens weighing at least 10 g are taken from the lingual or jaw muscle of horses and from the foreleg, tongue or diaphragm of wild boar;
- (b) in the case of horse, where those muscles are lacking, a larger-sized specimen is to be taken from a pillar of the diaphragm at the transition to the sinewy part. The muscle must be clean of connective tissue and fat;
- (c) at least 5 g of sample is digested following the reference method of detection in Chapter I of Annex I or an equivalent method in Chapter II. For each digest, the total weight of muscle examined must not exceed 100 g in the case of the method in Chapter I and methods A and B in Chapter II and 35 g in the case of method C in Chapter II;
- (d) where the result is positive, a further 50 g specimen is taken for a subsequent independent examination;
- (e) without prejudice to the rules on conservation of animal species, all meat of game animals other than wild boar, such as bears, carnivorous mammals (including marine

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mammals) and reptiles, are to be tested by sampling 10 g of muscle at the predilection sites or larger amounts if those sites are not available. Predilection sites are:

- (i) in bear: diaphragm, masseter muscle and tongue;
  - (ii) in walrus: tongue;
  - (iii) in crocodiles: masseter, pterygoid and intercostal muscles;
  - (iv) in birds: muscles of the head (e.g. masseter and neck muscles).
- (f) The digestion time must suffice to ensure adequate digestion of the tissue of these animals but must not exceed 60 minutes.

## ANNEX IV

Detailed conditions for *Trichinella*-free holdings and regions with a negligible *Trichinella* risk

For the purpose of this Annex,

‘controlled housing conditions in integrated production systems’ means a type of animal husbandry where swine are kept at all times under conditions controlled by the food business operator with regard to feeding and housing.

## CHAPTER I

### OBLIGATIONS ON FOOD BUSINESS OPERATORS

- A. The following requirements must be met by food business operators to obtain official recognition of holdings as free from *Trichinella*:
- (a) the operator must have taken all practical precautions with regard to building construction and maintenance in order to prevent rodents, any other kind of mammals and large carnivorous birds from having access to buildings where animals are kept;
  - (b) the operator must apply a pest-control programme, in particular for rodents, effectively to prevent infestation of pigs. The operator must keep records of the programme to the satisfaction of the competent authority;
  - (c) the operator must ensure that all feed has been obtained from a facility that produces feed in accordance with the principles described in Regulation (EC) No 183/2005 of the European Parliament of 12 January 2005 and of the Council laying down requirements for feed hygiene<sup>(1)</sup>;
  - (d) the operator must store feed intended for *Trichinella* susceptible species in closed silos or other containers that are impenetrable to rodents. All other feed supplies must be heat-treated or produced and stored to the satisfaction of the competent authority;
  - (e) the operator must ensure that dead animals are collected for disposal by sanitary means within 24 hours of death. However, dead piglets may be collected and stored on the holding in a properly closed container pending disposal;
  - (f) if a rubbish dump is located in the neighbourhood of the holding, the operator must inform the competent authority. Subsequently, the competent authority must assess

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- the risks involved and decide whether the holding is to be recognised as free from *Trichinella*;
- (g) the operator must ensure that piglets coming onto the holding from outside and pigs purchased are born and bred under controlled housing conditions in integrated production systems;
- (h) the operator must ensure that pigs are identified so each animal can be traced back to the holding;
- (i) the operator may introduce new animals, onto the holding only if they:
- (i) come from in holdings officially recognised as free from *Trichinella*; or
  - (ii) are accompanied by a certificate authenticated by the competent authority in the exporting country stating that the animal comes from a holding recognised as free from *Trichinella*; or
  - (iii) are kept in isolation until the results of a serological test approved by the Community reference laboratory prove to be negative. Serological sampling must commence only after the animals have been on the holding for four weeks;
- (j) the operator shall ensure that no pigs intended for slaughter have had outdoor access during the entire production period;
- (k) outdoor access during the first few weeks of life before weaning shall be permitted if all the following conditions are met:
- (i) no *Trichinella* infestations have been diagnosed in domestic animals in the country in the past 10 years;
  - (ii) an annual surveillance programme exists for wildlife susceptible to *Trichinella*. The programme shall be risk-based and shall be conducted in an area epidemiologically related to the geographical location of the *Trichinella*-free farms. The programme shall test the relevant indicator species on the basis of previous findings. The results shall show a prevalence of *Trichinella* in indicator animals below 0,5 %;
  - (iii) when outdoors, the animals shall be in properly fenced areas;
  - (iv) the monitoring program referred to in Article 11 shall be in place and monitoring shall be more frequent on the holdings involved;
  - (v) all sows and boars kept for breeding purposes on the holding shall be systematically sampled at slaughter for examination using the reference method of detection described in Chapter I of Annex I or one of the equivalent methods described in Chapter II of Annex I, and
  - (vi) steps shall be taken to prevent access by large carnivorous and omnivorous birds (e.g. crows, birds of prey).
- B. Food business operators of holdings recognised as free from *Trichinella* shall inform the competent authority where any of the requirements in point A is no longer fulfilled or where any other change has occurred that might affect the *Trichinella*-free status of the holding.



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## CHAPTER II

### OBLIGATIONS ON THE COMPETENT AUTHORITIES

- A. The competent authorities in Member States where *Trichinella* has been detected in domestic swine in the last 10 years may recognise a holding as free from *Trichinella* provided that:
- (a) at least two control visits are made in the 12 months preceding recognition of the holding to verify compliance with the requirements of Chapter I(A) of Annex IV; and
  - (b) all pigs sent for slaughter during the 24 months preceding recognition or a longer time period if the competent authority decides it to be necessary have been tested to ensure to the satisfaction of the competent authority that a sufficient number of animals from the holding have been tested using one of the parasite detection methods described in Chapters I and II of Annex I; and
  - (c) the results of the tests have been negative; and
  - (d) a risk-based wildlife monitoring programme has been put in place in those areas where wildlife and holdings applying for *Trichinella*-free status coexist; the monitoring programme optimises parasite detection by applying the most suitable indicator animal and detection technique, by sampling as wide a number of animals and taking as large a meat sample as is feasible; parasites detected in wildlife are identified at species level in a Community or national reference laboratory; the Community reference laboratory can assist by preparing a standardised protocol for a wildlife monitoring programme. Historical data may be used for the fulfilment of the requirements listed in this part.
- B. The competent authorities in Member States where *Trichinella* has not been detected in domestic swine in the last 10 years may recognise a holding as free from *Trichinella* provided that,
- the requirement in part A(d) above has been fulfilled.
- C. The competent authority may decide to recognise a category of holdings as free from *Trichinella* where all of the following conditions are met:
- (a) all the requirements set out in Chapter I(A) of Annex IV are met, with the exception of point (k), which does not apply; and
  - (b) no autochthonous *Trichinella* infestations in domestic animals have been detected in the country in the past 10 years, during which time continuous testing has been conducted on slaughtered swine population such as to provide at least 95 % confidence that where the prevalence of *Trichinella* exceeds 0,0001 %, any infestations will be detected; and
  - (c) a clear description must be available of the category of holdings, the type of farming and the type of animals involved; and
  - (d) a risk-based monitoring programme for wildlife has been established in accordance with Chapter II(A)(d) of Annex IV.
- D. In addition to the requirements laid out in Annex IV to Directive 2003/99/EC, the initial report and the subsequent annual reports to the Commission shall contain the following information:

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- (a) the number of cases (imported and autochthonous) of *Trichinella* in humans, including epidemiological data;
- (b) the results of testing for *Trichinella* in domestic swine not raised under controlled housing conditions in integrated production systems; the results must include the age and sex of affected animals, the type of management system, the type of diagnostic method used, the degree of infestation (if known), and any relevant additional information;
- (c) the results of testing for *Trichinella* in breeding sows and boars; the results must include the information mentioned under (b);
- (d) the results of testing for *Trichinella* in carcasses of wild boar, horses, game and any indicator animals;
- (e) the results of serological tests as referred to in Article 11 once a suitable test has been validated by the Community reference laboratory;
- (f) other cases where *Trichinella* is suspected, either imported or autochthonous, and all relevant laboratory results;
- (g) details of all positive results and the *Trichinella* species verification by the Community or national reference laboratory;
- (h) the data are to be submitted in the format and according to the timetable determined by EFSA for the reporting of zoonoses.
- (i) for reports concerning *Trichinella*-free holdings or category of holdings: information on the number of *Trichinella*-free holdings and summary results of inspections of *Trichinella*-free holdings, including information on farmer compliance;
- (j) for reports concerning a region with negligible risk, information is to be submitted on:
  - (i) the monitoring programme implemented according to Article 11, or equivalent information;
  - (ii) the risk-based wildlife monitoring programmes implemented according to part A(d) above, or equivalent information.

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**Changes to legislation:** There are currently no known outstanding effects for the  
Commission Regulation (EC) No 2075/2005 (repealed). (See end of Document for details)

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(1) OJ L 35, 8.2.2005, p. 1.

**Status:**

Point in time view as at 05/12/2005.

**Changes to legislation:**

There are currently no known outstanding effects for the Commission Regulation (EC) No 2075/2005 (repealed).