DRAFT INTERNATIONAL STANDARD

ISO/DIS 18744

ISO/TC **34**/SC **9**

Secretariat: **AFNOR**

Voting begins on:
2013-11-07

Voting terminates on:
2014-04-07

Microbiology of the food chain — Detection and enumeration of Cryptosporidium and Giardia in fresh leafy green vegetables and berry fruits

Microbiologie de la châine alimentaire — Recherche et dénombrement des Cryptosporidium et Giardia dans les légumes verts frais à feuilles et les fruits à baies

ICS: 07.100.30

ISO/CEN PARALLEL PROCESSING

This draft has been developed within the International Organization for Standardization (ISO), and processed under the **ISO lead** mode of collaboration as defined in the Vienna Agreement.

This draft is hereby submitted to the ISO member bodies and to the CEN member bodies for a parallel five month enquiry.

Should this draft be accepted, a final draft, established on the basis of comments received, will be submitted to a parallel two-month approval vote in ISO and formal vote in CEN.

To expedite distribution, this document is circulated as received from the committee secretariat. ISO Central Secretariat work of editing and text composition will be undertaken at publication stage.

Reference number
ISO/DIS 18744:2013(E)

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 18744 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).

Introduction

Cryptosporidium spp. and *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) are protozoan parasites that can cause enteric illness in humans. Both organisms are characterized by a robust transmission stage, *Cryptosporidium* oocyst and *Giardia* cyst, which can survive in moist environments (dehydration is lethal) for prolonged periods. As these transmission stages, hereafter referred to as (oo)cysts, are highly resistant to chemical and physical pressures, chlorine at the concentrations used in the treatment of drinking waters,disinfectants used for produce. , the absence of vegetative bacteria on fresh produce as indicators of faecal contamination does not necessarily indicate the absence of (oo)cysts. No practical method exists to culture these agents for the purpose of detection, and therefore direct removal from the food sample must be performed, followed by visualisation of the (oo)cysts by microscopy. The methods described in this document are for determining whether *Cryptosporidium* and/or *Giardia* (oo)cysts are present on the surfaces of fresh produce, and for their enumeration. This standard is based on the only published methods which have been tested in a multicenter collaborative trial. Alternative methods can be used following a demonstration of their equivalence with this standard following the protocol described in ISO 16140.

Microbiology of the food chain — Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits

1 Scope

This International Standard specifies a method that is applicable for the detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts on or in food products that are described herein as fresh leafy green vegetables and berry fruits. With suitable controls, it may also be applicable for the examination of other fresh produce.

The microscopy descriptions are for *Cryptosporidium* spp. oocysts and *Giardia duodenalis* cysts of size ranges which include those species or assemblages known to be pathogenic to humans.

This method is not applicable for the determination of the species or genotypes/assemblages of *Cryptosporidium* oocysts and *Giardia* cysts. The method will detect all species and genotypes / assemblages that are known to be pathogenic for humans and also others that are not. For further identification, molecular typing assays are required, but these may not be applicable if process positive controls have been spiked into the samples.

This method does not allow the determination of viability or infectivity of any *Cryptosporidium* oocysts and *Giardia* cysts which may be present.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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.*

ISO 16140, *Microbiology of food and animal feeding stuffs -- Protocol for the validation of alternative methods.*

3 Terms and definitions

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

3.1
***Cryptosporidium* oocysts**
transmission stages of *Cryptosporidium* spp.; their detection is based on reaction with specific anti-*Cryptosporidium* antibodies and typical morphological characteristics as described in Section 11 of this International Standard

3.2
***Giardia* cysts**
transmission stages of *Giardia* spp.; their detection is based on reaction with specific anti-*Giardia* antibodies and typical morphological characteristics as described in 11 of this International Standard

3.3
fresh leafy green vegetables
plant leaves eaten as a vegetable, which have not been subjected to any process, except cutting and washing

3.4
fresh berry fruits
derived from a variety of plants having small fruit characterized by a high surface : weight ratio, and which have not been subjected to any process except washing

3.5
sample process control
(Oo)cysts labelled with specific fluorogenic reporters that may be added in defined numbers to the sample prior to processing to verify that the method after the elution step is operating efficiently

3.6
positive process control
sample to which (oo)cysts have been added in defined numbers prior to extraction to verify that the method after the elution step is operating efficiently

3.7
negative process control
equivalent quantity of material to the sample, which is considered to be free of (oo)cysts and processed in the same manner as the tested sample

4 Principle

The principle of the method is based on removal of the oo(cysts) from the sample by elution procedures, followed by concentration in the eluate by centrifugation and isolation by immunomagnetic separation (IMS). Detection of the oo(cysts) is performed by microscopy after labelling with monoclonal antibodies (mAbs) conjugated to a fluorochrome.

5 Reagents

5.1 Reagents required for eluting oo(cysts) from leafy green vegetables and berry fruits

5.1.1 Glycine buffer, **pH 5.5** for leafy green vegetables, (Annex A.2.1)

5.1.2 Glycine buffer, **pH 3.5** for berry fruits (Annex A.2.2)

5.2 Reagents used in concentrating, fixing, staining, detection, and quality control

5.2.1 Methanol, analytical grade

5.2.2 Paramagnetic beads, for the isolation of *Cryptosporidium* oocysts and *Giardia* cysts

5.2.3 Hydrochloric acid, 0.1 M (Annex A.3.1)

5.2.4 Sodium hydroxide, 1 M (Annex A.3.2)

5.2.5 Fluorescently-labelled monoclonal antibodies (mAbs) against *Cryptosporidium* oocysts and *Giardia* cysts

5.2.6 Immunofluorescence mounting medium (Annex A.3.3)

5.2.7 4',6'-diamidino-2-phenylindole dihydrochloride dihydrate (DAPI), freeze-dried reagent

5.2.8 DAPI stock solution (Annex A.3.4)

5.2.9 DAPI working solution (Annex A.3.5)

5.2.10 Phosphate buffered saline (PBS) (Annex A.1.1)

5.2.11 Non-fluorescing immersion oil

5.2.12 Stock suspensions of *Cryptosporidium* oocysts and *Giardia* cysts (Annex B)

5.2.13 Suspensions of pre-labelled and enumerated non-viable *Cryptosporidium* oocysts and *Giardia* cysts

The fluorochrome label shall be different to that which is used for the detection of the target organism.

5.2.14 Parasite storage medium (Annex A.3.6; A.3.7)

5.2.15 Deionized water

5.2.16 Fingernail varnish (for sealing coverslips onto slides as necessary)

6 Apparatus

This method requires common microbiological laboratory equipment (see ISO 7218) and in particular the following:

6.1 Tweezers (for handling fresh produce as necessary)

6.2 Welled slides, with hydrophobic coating, wells capable of accommodating 50 µl volume, and coverslips of appropriate size

6.3 A paddle (peristaltic) blender and compatible filtered bags

6.4 Swing out centrifuge (to accommodate at least 4 x 50 ml conical centrifuge tubes per run)

6.5 Glass Leighton tubes

6.6 Rotating mixer compatible with the Leighton tubes

6.7 Magnetic clip stand compatible with the Leighton tubes

6.8 Magnetic clip stand for microcentrifuge tubes

6.9 Slide warmer tray, 37 °C to 42 °C incubator, or equivalent slide-drying apparatus

6.10 Humidity chamber (lidded box containing damp absorbent material and that can be kept darkened and held at the temperature appropriate for the immunofluorescent reagent).

6.11 Aspiration device - vacuum source equipped with liquid trap and pipette, or equivalent.

6.12 Epifluorescence microscope with 20×, 40x objectives and 100x objective immersion lens and a calibrated eyepiece graticule (reticule). Shall include FITC filter set (blue, 480 nm excitation, 520 nm emission filter) and DAPI filter set (UV, 375 nm excitation, > 420 nm emission). If sample process controls are used, an additional filter set suitable for the fluorochrome will be required. Differential interference apparatus (DIC) optics are advantageous. A photographic recording system attached to the microscope may be pertinent for recording positive or presumptive events.

6.13 FITC control slide for evaluation of fluorescence intensity and verification of proper performance of the optical system of the fluorescence microscope.