Microbiological Examination Methods of Food and Water

A Laboratory Manual 2nd Edition

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CRC Press
Taylor & Francis Group
A BALKEMA BOOK

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Second Edition

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CRC Press
Taylor & Francis Group
Boca Raton London New York Leiden

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

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Originally published in Portuguese as: 'Manual de Metodos de Análise Microbiológica de Alimentos e Água ©2010, Livraria Varela, Sao Paulo, Brazil

English edition 'Microbiological Examination Methods of Food and Water: A Laboratory Manual', CRC Press/Balkema, Taylor & Francis Group, an informa business ©2013 Taylor & Francis Group, London, UK

Translation to English: Paul van Dender†

CRC Press/Balkema is an imprint of the Taylor & Francis Group, an informa business

Typeset by Apex CoVantage, LLC

Published by: CRC Press/Balkema
Schipholweg 107c, 2316 XC Leiden, The Netherlands
e-mail: Pub.NL@taylorandfrancis.com
www.crcpress.com – www.taylorandfrancis.com

Library of Congress Cataloging-in-Publication Data
Names: Silva, Neusely da., author.
Title: Microbiological examination methods of food and water : a laboratory manual / Neusely da Silva, Marta Hiromi Taniwaki, Valeria Christina Amstalden Junqueira, Neliane Ferraz de Arruda Silveira, Margarete Midori Okazaki & Renato Abeilar Romeiro Gomes.
Other titles: Manual de metodos de analise microbiologica de alimentos e agua. English
Description: Second edition. | Leiden, The Netherlands ; Boca Raton : CRC Press/Balkema, [2018] | Includes bibliographical references and index.
Identifiers: LCCN 2018034220 (print) | LCCN 2018035352 (ebook) | ISBN 9781315165011 (ebook) | ISBN 9781138091887 (hardcover : alk. paper) | ISBN 9781138057111 (pbk. : alk. paper)
Subjects: MESH: Bacteriological Techniques—methods | Food Microbiology—methods | Water Microbiology | Laboratory Manuals
Classification: LCC QR115 (ebook) | LCC QR115 (print) | NLM QW 25.5.B2 | DDC 579/.16—dc23
LC record available at https://lccn.loc.gov/2018034220

ISBN: 978-1-138-05711-1 (Pbk)
ISBN: 978-1-138-09188-7 (Hbk)
ISBN: 978-1-315-16501-1 (eBook)

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Preface

This manual was prepared with standardized methods published by renowned international organizations such as International Organization for Standardization (ISO), American Public Health Association (APHA), AOAC International, Food and Drug Administration (FDA) and United States Department of Agriculture (USDA).

The manual Includes methods for the enumeration of indicator microorganisms of general contamination (total aerobic mesophilic bacteria, lactic acid bacteria, yeasts and molds), indicators of hygiene and sanitary conditions (coliforms, *E. coli*, enterococci), spore-forming bacteria (aerobic thermophilic and mesophilic bacteria, anaerobic thermophilic and mesophilic bacteria, *Alicyclobacillus*), spoilage fungi (thermoresistant molds, osmophilic yeasts, preservative resistant yeasts) and pathogenic bacteria (*Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, *Cronobacter*, *Campylobacter*, *Yersinia enterocolitica*, *Vibrio cholerae*, *Vibrio parahaemolyticus*). The chapter covering the examination of water includes methods for the detection and determination of coliforms, *E. coli*, *Pseudomonas aeruginosa, Clostridium perfringens* and enterococci.

The major objective of the book is to provide an illustrated laboratory manual with an overview of current standard microbiological methods for the examination of food and water. The didactic setup and the visualization of procedures in step-by-step schemes allows student and practitioner to quickly perceive and execute the procedure intended. Each chapter provides numerous methods for a certain examination, and also provides simple or quick alternatives. The chapters' introductions summarize the existing knowledge regarding the target microorganism(s) and present the most useful information available in literature.

The book is intended for laboratory education of undergraduate and graduate students in food engineering and related disciplines and as an up-to-date practical companion for researchers, analysts, technicians and teachers.

In this second edition several methods have been revised according to new editions of the references used, including:

- The *Compendium of Methods for the Microbiological Examination of Foods*, 5th edition (2015), from American Public Health Association (APHA).
- The *Standard Methods for the Examination of Water & Wastewater*, 22nd edition (2012), from American Public Health Association (APHA), American Water Works Association (AWWA) & Water Environment Federation (WEF).
- The *Official Methods of Analysis of AOAC International*, 20th edition (2016), from AOAC International.
- The *Bacteriological Analytical Manual* [BAM Online] from Food and Drug Administration (last update).
- The *Microbiology Laboratory Guidebook* [MLG Online], from Food Safety and Inspection Service, United States Department of Agriculture (last update).

New methods have also been described, including:

- Aerobic mesophilic bacteria in foods – plate count methods ISO 4833-1/2:2013
- Method ISO 21528-1/2:2017 for *Enterobacteriaceae* in Foods
- Method BAM/FDA:2017 for Coliforms and *E. coli* in Food
- Method ISO 9308-1:2014 for Coliforms and *E. coli* in Water
- Method BAM/FDA:2012 for *Cronobacter* in Infant Formula
- *Clostridium perfringens* in water – membrane filtration method ISO 14189:2013
- *Clostridium perfringens* in foods – plate count method ISO 7937:2004
- Lactic acid bacteria in foods - Plate count method ISO 15214:1998

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1 Sampling, transport and storage of samples for analysis

Revision history

Item 1.2.1.c (revised) Sterilization of flasks and utensils should be done at 121±3° C for at least 15 minutes in autoclave. In sterilization oven should be made at 170±10°C for at least one hour (ISO 7218:2007/Amd.1:2013).

Item 1.2.4 (revised) Suppressed in the 22nd edition *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012) the recommendation to add EDTA to water samples with high metal content.

Item 1.3.5 (revised) The storage temperature of water samples under refrigeration recommended by the 22nd edition of the *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012) changed from 10°C to 8°C, emphasizing the recommendation that these samples should not be frozen.

1.1 Introduction

Most of the recommendations and guidelines contained in this chapter are taken from the American Public Health Association (APHA), as described in the 5th Edition of the *Compendium of Methods for Microbiological Examination of Foods* (Salfinger and Tortorello, 2015). When different from or complementary to those of the *Compendium*, they were complemented with information and recommendations from the 22nd Edition of the *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012), specific to the analysis of water, the 17th Edition of the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004), specific to the microbiological examination of dairy products and the standards of the International Organization for Standardization (ISO 6887-4:2017; ISO 7218:2007/Amd.1:2013), recommended for performing tests using ISO methods.

Some of the terms used throughout this text come from the terminology used by the International Commission on Microbiological Specification for Foods (ICMSF, 1986) for lot sampling, and their meaning should be thoroughly understood.

1.1.1 Lot

A lot is defined as an amount of food of the same composition and physical, chemical and sensory characteristics, produced and handled in one and the same production run and under exactly the same processing conditions. In practice, a lot generally is the quantity of food produced within a certain time interval during an uninterrupted period of processing of a production line.

1.1.2 Lot sample and sample unit

A lot sample is a fraction of the total amount produced, withdrawn randomly, to evaluate the conditions of the lot. In the case of foods filled into individual packages, a lot sample is composed of *n* individual packages. In the case of bulk foods, which are not filled into individual packages, a lot sample is composed of *n* aliquots of a measured volume or weight of the product. Individual packages or aliquots are called sample units and – for the purpose of assessing the lot – are examined individually. From the combined results of analysis relative to *n* sample units, it is possible to infer the characteristics

of the lot as a whole, although the result of the examination of one single sample unit may never be taken as representative of the lot.

In *Salmonella* tests the criterion for foods is absence in any of the sample units examined. In such a case it is common to composite (mix together) sample units to perform one single analysis. The presence of *Salmonella* in the composite sample is unacceptable, irrespective of how many or which sample units are contaminated. Greater details will be presented in the specific chapter on *Salmonella*.

1.1.3 Lot sampling plans

Whenever the goal is to evaluate lots or batches, the taking of *n* sample units must follow a statistically adequate sampling plan. The most commonly used are the two- or three-class plans established by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002, 2011).

The two-class sampling plan classifies lots into two categories, acceptable or unacceptable, depending on the analysis results of *n* sample units. Two-class sampling plans are used more in the case of presence/absence tests, such as *Salmonella*, for example, in which absence is acceptable and the presence in any of the *n* sample units is unacceptable.

The three-class sampling plan classifies the lots into one of three categories: (1) acceptable; (2) intermediate quality but marginally acceptable; and (3) unacceptable. Three-class sampling plans are recommended for quantitative tests, for which the goal or standard is not the absence, but values that fall within a range between *m* and *M*. The parameters used in these plans for making decisions regarding the lots tested are:

n: the number of sample units that need to be randomly taken from one and the same lot and which are to be examined individually. The *n* sample units constitute the representative sample of the lot. As for non-quantitative and presence/absence tests (*Salmonella* or *Listeria monocytogenes*, for example) the sample units may be composite or pooled and subjected to a single analysis. However, when pooling or compositing samples the instructions and guidelines described in the chapters dealing with the specific tests in question should be consulted and strictly adhered to.

m: the microbiological-limiting criterion established for a given microorganism, in a given food. In a three-class sampling plan, this value separates an acceptable lot from a lot of intermediate but marginally acceptable quality.

M: a tolerable limit, above the microbiological limiting criterion *m*, and which may be reached by a certain number (*c*) of sample units, but may not be exceeded by any of these. In a two-class plan, *M* separates an acceptable lot from an unacceptable one. In a three-class plan, *M* separates a lot of intermediate but marginally acceptable quality from an unacceptable lot.

c: among the *n* sample units that constitute a representative sample of the lot, *c* is the maximum number of units that may be accepted with counts above the limiting criterion *m*, provided none of these units exceed the *M* limit. In those cases in which the microbiological criterion is absence, *c* is equal to zero, and consequently, the two-class sampling plan is to be applied.

1.1.4 Analytical unit

A sample unit generally contains a quantity of product greater than necessary for performing the analysis, for the simple reason that, when collecting a sample unit, it is important to collect quantities enough to allow for storing counter-samples and preventing accidental losses. The analytical unit is the amount of food that is actually used to perform one or more tests on the sample unit. The number of analytical units necessary for the analysis depends on the number and types of tests that will be performed on one and the same sample unit; that is, one for general quantification tests (total aerobic mesophilic counts, yeast and mold counts, total coliform/fecal coliform/*Escherichia coli* counts, *Staphylococcus aureus* counts, *Bacillus cereus* counts, *Clostridium perfringens* counts), one for each presence/absence test (*Salmonella*, *Listeria monocytogenes* and all the tests requiring enrichment in specific broth) and one for any other test that requires the sample to be subjected to a differentiated treatment (counts of spore-forming bacteria, counts of heat-resistant molds and others).

1.2 Collecting samples for analysis

Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends that, whenever possible, samples packaged in individual packages should be collected and sent to the laboratory in their original commercial packaging, sealed and intact. Each packaging unit of an individual package of the product constitutes a sample unit and

as many sample units should be collected as required by the sampling plan. If the packaging unit or individual package contains an amount of food insufficient for performing the required analyses and the keeping of counter-samples, a sufficient number of individual packages should be collected as part of one and the same sample unit. At the time of analysis, the contents of these individual packages should be placed together in one single sterile flask, which must be subsequently thoroughly mixed before withdrawing an analytical unit of the mixture. If the product does not allow for such mixing, the analyst should take, from each of the individual packages, portions of approximately equal weight, to compose or complete the analytical unit for that particular sample unit.

In the case of foods contained in vats, tanks or large containers, impossible to be transported to the laboratory, representative portions should be transferred from the bulk product to sterile collecting flasks or bags under aseptic conditions.

1.2.1 Selection and preparation of containers for the sampling of foods contained in non-individual packages

a) Use flasks or bags with leak-proof caps, made from non-toxic material approved for food contact and, preferably, autoclavable or pre-sterilized. The use of glass flasks or containers is not recommended due to the risk of breakage, contamination of the sampling environment with pieces of broken glass and loss of sample material.

b) Choose flasks of appropriate size for the amount of food to be collected. To determine the quantity of sample to be collected, consider that each sample unit should contain, at least, twice the number of analytical units that will be used in the tests and, preferably, three or four times that amount (to allow for proper separation of counter-samples and prevention of possible spills or losses). Also consider that only three-quarters of the sampling flasks capacity should be filled with the sample (to prevent overflow and to allow proper mixing of sample before withdrawing the analytical units).

c) Non-pre-sterilized flasks and utensils that will be used to collect food samples (such as spatulas, spoons, scissors, tweezers, openers, corer samplers, etc.) should, preferably, be sterilized individually in an autoclave at 121 ± 3°C for at least 15 minutes or in a sterilizing oven at 170 ± 10°C for at least 1 hour (ISO 7218:2007/Amd.1:2013). Some other methods may be used as an alternative, such as flame sterilization, immersion in ethanol and alcohol combustion, and treatment(s) using disinfectant solutions. In the latter case, only disinfectants approved for use on food-contact surfaces should be used, in strict adherence to the manufacturer's instructions and followed by 12 rinsing cycles with sterile distilled water to remove all residues. Non-sterile flasks or bags showing – after having been subjected to an internal surface washing test – counts of viable microorganisms smaller than 1 CFU/mL of their holding capacity, may be used directly without previous sterilization.

1.2.2 Procedures for the sampling of foods contained in non-individual packages

a) Before starting to collect the sample unit, the whole mass of the food should be thoroughly mixed, to ensure that the microorganisms will be evenly distributed throughout the food. Next, using appropriate utensils or instruments, withdraw the amount of product necessary to compose or complete the sample unit.

b) If it is not possible to thoroughly mix the food mass before initiating sampling, portions should be taken from different parts of the content, until obtaining the amount of product appropriate to compose or complete the sample unit. Avoid withdrawing portions of the regions close to the surface or opening of the tank or container.

b.1) To collect powder samples from different parts of tanks or large packages, corer samplers or vertical double-tube samplers, long enough to reach the center of the food mass, may be used. A different sterile sampler or sampling device should be used for each sample unit to be collected, or the instrument should be sterilized between one sampling operation and the next.

b.2) To compose or complete a sample unit with portions taken from different points of foods that consist of one large solid piece, sterile knives, tweezers and forceps should be used to cut the food into smaller pieces.

b.3) In the case of large blocks of frozen foods, such as frozen fish blocks and frozen seafood blocks, volumes of frozen liquid egg, etc., the most adequate procedure is to use an electric drill (with a previously sterilized drill bit) in combination with a sterile funnel. Insert the drill bit in the funnel (the lower opening inside diameter of which should be only slightly greater than the diameter of the drill bit) and position the bit onto the point of the block from which a sample is to be taken. Turn on the drill and scrapings of the frozen food will move towards the surface and accumulate in the funnel, from where they can be transferred to an adequate collecting flask.

b.4) When samples are collected using faucets or tubes, the outer part of the outlet should be cleaned with ethanol 70% and sterilized by flame, if the material is fire resistant. The initial amount of product should be discarded before starting collection of the sample material. This will wash out the pipe and remove any accumulated dirt or residue particles.

b.5) For the sampling of margarine and similar products ("spreads") ISO 6887-4:2017 recommends removing the external layer (3 to 5 mm) and withdraw the sample units using a previously sterilized corer sampler. Insert the instrument diagonally, without reaching the bottom, rotate it in a complete circle and pull the sampler out, lifting out a conical portion of the product.

c) Remember that the external surface of collecting flasks and bags is not sterile. For that reason, do not hold flasks or bags directly above the mass of food, as contaminants may fall or otherwise be introduced into the product. Likewise, never insert a collecting flask directly into the product, but use an appropriate utensil instead to withdraw the sample units.

d) When withdrawing the collecting instrument filled with collected product, do not hold it above the other pre-sterilized instruments, because spatters of the food may contaminate the instruments that will be utilized later on.

e) Open the collecting flasks or bags only as far or wide enough to insert the product and close/seal immediately.

f) Do not touch the internal surface of collecting flasks or bags and their respective caps or closures.

g) Contaminated foods may contain microorganisms that are harmful to health. These samples should be collected by staff who are well trained in the handling of microorganisms and who are aware of the care required for protecting their health and safety. In case of doubt, each sample should be treated as if it were contaminated.

1.2.3 Sampling of foods involved in foodborne diseases

Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends that samples of all suspected foods should be collected and analyzed as soon as possible. However, it is of no use to collect samples that have undergone temperature abuse or that are already in a state of partial deterioration. The results of such analyses will be of no or little use to the conclusions of the investigation. If there are not any leftovers from suspected meals, one of the following alternatives may be tried: collect samples from similar meals, prepared at a later point in time but under the same conditions, collect samples of the ingredients and raw materials used in the preparation of the suspected meals and collect all containers and cooking utensils used to hold or prepare the suspected meals.

1.2.4 Sampling of water

Chapter 60 of the 5th Edition of the *Compendium* (Robin and Feng, 2015) deals with the collecting of samples of bottled water, which is considered a food by the Codex Alimentarius. These samples must be collected from their original sealed packaging. If there is any interest or need to collect smaller volumes from packaging of greater holding capacities, the entire content should be homogenized by inverting the packaging several times in quick succession. Next, the mouth or outlet should be disinfected with ethanol 70% and, under aseptic conditions, the seal broken open with a sterile or flame-sterilized knife or pair of scissors. Do not collect but dispose of the initial volume or runoff and then collect the sample in an adequate sterile flask.

To collect other kinds of water, section 9060A of the 22nd Edition of the *Standard Methods for the*

Examination of Water and Wastewater (Hunt, 2012) provides the following guidelines:

To collect samples from faucets or pipes, clean the external area of the outlet with a solution of 100 mg/L sodium hypochlorite or ethanol 70%, in addition to flame-sterilizing it if it is made of fire-resistant material. Open the faucet completely and let the water run for approximately 2 to 3 minutes to flush out any debris or impurities and clean out the piping system. Reduce the flow of water to collect a sample without spilling water droplets out of the collecting flask.

To collect water from wells or cisterns with a pump, the water should be pumped out for at least 5 to 10 minutes to allow the temperature of the water to stabilize before starting the actual sampling. In case there is no pump available, collecting flasks should be prepared by attaching a weight onto the base or bottom and then introducing the flask directly into the well. Care is needed not to contaminate the sample with material and impurities that may have accumulated onto the surface of the water.

To collect water from rivers, lakes or water reservoirs, hold the collecting flask by its base and then lower it into the water until it is totally immersed and covered by the water surface with the mouth of the bottle turned downward. Turn the mouth of the flask into the direction of the water flow with a slightly upward slope, so that the water will be retained. If there is no water flow or current, push the flask forward horizontally, in the direction opposite to that of the hand.

Samples of chlorinated water should have any residual chlorine neutralized immediately after the samples are taken, to immediately eliminate its bactericidal effect against the microbiota present. To that purpose, the collecting flasks should have 0.1 mL of a 3% sodium thiosulfate ($Na_2S_2O_3$) solution added (before sterilization) for each 100 mL of sample to be collected. This amount is sufficient to neutralize 5 mg of residual chlorine per liter of sample. In situations in which the concentration of residual chlorine exceeds 5 mg/L, utilize 0.1 mL of a 10% sodium thiosulfate solution for each 100 mL sample. This quantity is enough to neutralize 15 mg residual chlorine per liter of sample. Sterile plastic bags or flasks, which are commercially available and already contain sodium thiosulfate, may also be used. If the sample is collected and sent to the laboratory by the interested person, without previous neutralization of the chlorine, a sterile sodium thiosulfate solution should be added immediately upon arrival of the sample, under aseptic conditions.

1.3 Transportation and storage of samples until analysis

As a general rule, food samples should always be transported and stored in exactly the same way and under the same conditions the food is transported and stored until marketed. The guidelines below should be followed to ensure the integrity of the product until the time of analysis.

1.3.1 Foods with low water activity

Foods with low water activity (dehydrated, dried or concentrated), which are microbiologically stable, may be transported and stored at room temperature, although they should be protected against moisture.

1.3.2 Frozen foods

Frozen foods should be transported and kept frozen until the time of analysis. Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends storage at –20°C. ISO 7218:2007/Amd.1:2013 recommends –15°C, preferably –18°C. The transportation should be carried out in Styrofoam boxes with dry ice with certain precautions and care. The product should not come into contact with the dry ice because the absorption of CO_2 may change the pH. If the lid does not make the packaging airtight and gas proof and/or if the packaging is gas permeable and/or becomes fragile or brittle at low temperatures, a secondary packaging should be used. Generally, wrapping in thick paper or paperboard is sufficient to avoid this problem. Labels and tags used to identify the samples should be waterproof, smudge proof and fade proof to avoid the loss of important data.

1.3.3 Refrigerated foods

Refrigerated foods should be transported and kept under refrigeration from the moment they are collected until the time of analysis. Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends, as a general rule, that these samples be transported and stored at a temperature between 0°C and 4.4°C with a maximum time interval of 36 hours between sampling and analysis. If it is impossible to perform the analysis within 36 hours, the samples should be frozen or retained at

refrigeration temperatures for up to 18 hours, depending on the type of product, the reason for analysis and the type of analysis. ISO 7218:2007/Amd.1:2013 recommends that this kind of food sample be transported at a temperature between 1°C and 8°C and stored at 3 ± 2°C, with a maximum interval of 36 hours between sampling and analysis (24 hours in the case of highly perishable samples). In case it is impossible to perform the analysis within the maximum time interval stipulated, the samples should be frozen and kept under the same conditions as those described for frozen samples (–15°C, preferably –18°C), provided freezing does not interfere with recovery of the target microorganism(s) (see the exceptions below).

Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends that transportation be carried out in Styrofoam boxes containing ice. The *Compendium* further recommends the use of reusable gel ice packs, to avoid liquid from accumulating inside the boxes. If gel ice is not available, common ice may be used, provided it is pre-packed in plastic bags. Tightly closed Styrofoam boxes, with ample space inside for ice, in amounts sufficient enough to cover all sample-containing flasks, can keep the samples at appropriate refrigeration temperatures for up to 48 hours, in most situations. As a general rule, these samples should not be frozen, and for that reason, the use of dry ice inside the Styrofoam boxes is not recommended. If the transport of the samples requires a prolonged period of time, making the use of dry ice necessary, the sample packages should not come into direct contact with the dry ice packs, to avoid freezing. Labels and tags used to identify the samples should be waterproof, smudge proof and fade proof to avoid losing important data.

Exceptions: For certain microorganisms, differentiated rules apply, specified in the specific chapters. Samples of shellfish should be analyzed within 6 hours after sampling, and should not be frozen (Taylor *et al.*, 2015). Samples of refrigerated liquid egg should be analyzed, if possible, within 4 hours after sampling, and should not be frozen (Ricke *et al.*, 2015). Samples of non-heat-treated fermented or acidified products of plant origin should be stored under refrigeration for no longer than 24 hours, and should not be frozen (Pérez-Diaz *et al.*, 2015). ISO 6887-3:2017 recommends that the transport of raw fish, crustaceans, cephalopods, bivalve mollusks, gastropods, tunicates and echinoderms be done between 0°C and 10°C, without freezing. Laboratory samples shall be stored at 3 ± 2°C, and microbiological examination should be initiated within 24 hours of collection.

1.3.4 Commercially sterile foods in sealed packages

Commercially sterile foods in sealed packages under normal circumstances may be transported and stored at room temperature, and should be protected against exposure to temperatures above 40°C (ISO 7218:2007/Amd.1:2013). Samples of bottled carbonated soft drinks, sold at room temperature, may also be transported and stored under these same conditions. Blown packages should be placed inside plastic bags due to the danger of leakage of materials of high microbiological risk. Transportation and storage can be carried out under refrigeration, to prevent explosion. However, if there is any suspicion of spoilage caused by thermophilic bacteria, refrigeration is not indicated because it may destroy vegetative cells (Parkinson and Francis, 2015).

1.3.5 Water samples

For water samples Chapter 60 of the 5th Edition of the *Compendium* (Robin and Feng, 2015) recommends that bottled water in its original, sealed packaging may be transported and stored at room temperature, without the need of refrigeration. Water contained in opened packages or water samples transferred to other containers should be transported and stored under refrigeration (temperature not specified). The samples should be analyzed within an interval of preferably 8 hours, but not exceeding 24 hours.

For other types of water, part 9060B of the 22nd Edition of the *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012) provides the following guidelines:

a) **Drinking water for compliance purposes**. Preferably hold samples at temperatures below 8°C (without freezing) during transit to the laboratory. Do not exceed a 30-hour holding time from collection to analysis for coliform bacteria. Do not exceed an 8-hour holding time for heterotrophic plate counts.
b) **Nonpotable water for compliance purposes**. Hold samples at temperatures below 8°C (without freezing), during a maximum transport time of six hours.
c) **Other water types**. Hold samples at temperatures below 8°C (without freezing) during transport and until time of analysis. Holding time should not exceed 24 hours.

1.4 References

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2 Preparation of samples for analysis

Revision history

Figures 2.1 e 2.2 (revised) Figures revised according to ISO 17604:2015.

Item 2.2.2 (revised) The procedure recommended for homogenization and withdrawal of analytical units from frozen solid foods was revised according the 2nd edition of ISO 6887-1:2017 and ISO 6887-3:2017.

Item 2.3.3 (revised) The diluents recommended by different regulatory agencies for food analysis was revised according the new editions of publications and standards.

Item 2.4 (revised) The uncertainty of volume measurement allowed by ISO 6887-1:2017 is 2%. The resuscitation step recommended by ISO 6887-4:2017 before preparing the second dilution of hard and dry products and for low moisture products is about one hour at ambient temperature (18-27°C).

Annex 2.1. (revised) The procedures recommended by different regulatory agencies for homogenizing the content and withdrawal of the analytical unit of different types of foods for analysis was revised according the new editions of publications and standards.

Annex 2.2. (revised) Variations recommended by different regulatory agencies for the preparation of the first dilution of different types of foods for analysis was revised according the new editions of publications and standards.

2.1 Introduction

Most of the guidelines contained in this chapter were taken from the American Public Health Association (APHA), as described in the 5th Edition of the *Compendium of Methods for Microbiological Examination of Foods* (Salfinger and Tortorello, 2015). When different from or complementary to those of the *Compendium*, they were completed with information and recommendations from the 22nd Edition of the *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012), specific to the microbiological examination of water, the 17th Edition of the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004), specific to the examination of dairy products and several standards developed by the International Organization for Standardization (ISO 6887-1:2017; ISO 6887-2:2017; ISO 6887-3:2017; ISO 6887-4:2017; ISO 6887-5:2010; ISO 7218:2007/Amd.1:2013; ISO 17604:2015), recommended for tests performed using ISO method(s).

The preparation of samples for analysis involves three steps: (1) homogenization of the content and withdrawal of the analytical unit, (2) preparation of the first dilution of the analytical unit and (3) the preparation of serial decimal dilutions for inoculation into or onto culture media.

Before starting procedures certain precautions are recommended, to ensure that all activities be conducted under aseptic conditions:

Make sure that the work area is clean and that all doors and windows are closed to avoid air currents.

Disinfect all working surfaces with an appropriate disinfectant (ethanol 70%, 500 ppm benzalkonium chloride solution, 200 ppm sodium hypochlorite solution or any other chlorine-based compound is adequate).

Wash and disinfect your hands with a disinfectant appropriate and safe for skin contact. Verify the

necessity or not to use gloves in the chapters specifically dealing with pathogen tests.

Work inside vertical laminar flow cabinets to prevent contamination of the sample by the environment and contamination of the environment and the analyst by the sample. In case a vertical laminar flow cabinet is not available, work in an area located as close as possible to the flame of a Bunsen burner, which, when working well, will produce a steady blue flame. When handling powdered samples, it is not recommended to work very close to the flame of a Bunsen burner. ISO 7218:2007 stipulates the use of a separated area or a laminar flow cabinet.

Avoid the formation of aerosols when opening tubes, flasks or plates after agitating or releasing the content of pipettes or flame-sterilizing inoculation loops.

Never use a pipette by mouth; use mechanical pipettes instead.

After use, place the pipettes and other utensils in disposable trays and not directly onto the surface of the bench.

All instruments and utensils used to open packages and withdraw analytical units (scissors, tweezers, knives, spatulas, etc.) must be previously sterilized (in an autoclave or sterilization oven) or immersed in ethanol 70% and flame sterilized at the time of use.

Before opening the packages, disinfect the external area with ethanol 70%, maintaining contact until the alcohol has fully evaporated. In the case of flexible packages, cut open with a sterile pair of scissors. In the case of rigid packages with a screw cap, unscrew and remove the cap aseptically. In the case of cans that come with an "easy open" lid with wide opening, open the can aseptically and remove the lid. In the case of cans without an "easy open" feature, use a sterile can opener. In the case of cans, glass containers, boxes and other packaging intended to be subjected to the commercial sterility test, differentiated guidelines should be followed, as described in a specific chapter. The objective of these procedures is to ensure the integrity of the sealing system, for later analyses of the package, if necessary. Observe and note any abnormality concerning either the package itself or its content, such as blowing, leakage, off-odors and/or strange or atypical appearance, the presence of foreign objects and so on.

2.2 Homogenization of samples and withdrawal of the analytical unit

The analytical unit is the amount of material withdrawn from a sample to be subjected to one or more tests. The number of analytical units that should be withdrawn and the amount of material of each analytical unit depends on the number and types of tests that will be performed on the same sample. In general, the following items are necessary:

a) **Analytical units for presence/absence tests with enrichment in specific broth.** One analytical unit is required for each test (*Salmonella*, *Listeria* and others). The quantity of material of each of these analytical units is defined in the chapters specifically dedicated to these tests.
b) **Analytical units for tests requiring differentiated treatment of the sample.** One analytical unit is required for each test (commercial sterility, bacterial spore counts, thermoresistant mold counts and others). The quantity of material of each of these analytical units is also defined in the chapters specifically dedicated to these tests.
c) **Analytical units for general quantification tests.** General quantification tests usually comprise total aerobic mesophilic or psychrotrophic counts and counts of yeasts and molds, lactic acid bacteria, enterococci, *Enterobacteriaceae*, coliform and/or *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens* and *Pseudomonas* spp. These tests are performed with the same analytical unit, which, most commonly, consists of 25 g or 25 mL of the sample. According to ISO 6887-1:2017 the minimum of 10 g is specified for enumeration tests in many specific standards. For qualitative tests the size is normally 25 g (or 25 mL), although alternative quantities can be used. Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends that the minimum amount or volume of the analytical unit be at least 50 g for solid foods and 10 mL, 11 mL or 50 mL for liquid products. However, in the specific chapters, the recommended amount for most cases is 25 g or less. For more information on these exceptions see Annex 2.2.

Before withdrawing the analytical unit(s), the content of the sample should be well homogenized to ensure that the portion to be removed will be representative for the material as a whole. The procedures to achieve good homogenization are different for liquid products, solid products and products with a predominantly surface contamination, as will be further specified in the following sections.

2.2.1 Procedure for homogenization and withdrawal of analytical units from liquid products

If the liquid product (viscosity not greater than that of milk) is filled in containers with enough inner space to allow for agitation, invert the packaging 25 times. If the container is filled to more than two-thirds of its inner space, invert the package 25 times in a 30-cm arc within 7 seconds. If there is not enough free space for agitation, then use a second, sterile container and transfer the sample from one container to the other, for three consecutive times. If foam is formed, let it subside by standing until totally dispersed. As for gasified samples (carbonated soft drinks and similar products), transfer the content to a sterile container with a wide mouth and, with the cap slightly open, agitate using a shaker until the gas is completely expelled (this step is unnecessary if the analytical unit is transferred directly to the filtration flask, in the tests using the membrane filtration method).

Withdraw the analytical unit with a pipette, inserting the tip of the pipette to a depth not greater than 2.5 cm below the surface of the liquid. The measurement should be volumetric and the time interval between the homogenization of the sample and the withdrawal of the analytical unit should not exceed 3 minutes. The *Compendium* (Taylor *et al.*, 2015) does not set a limit for the uncertainty of the measurement of the volume, which, according to ISO 6887-1:2017, should not be greater than 5%.

2.2.2 Procedure for homogenization and withdrawal of analytical units from solid or concentrated liquid products

In the case of solid or concentrated liquid products, follow the guidelines contained in Annex 2.1, which defines the procedures most appropriate for homogenizing and withdrawing the analytical unit of different types of foods. The *Compendium* (Taylor *et al.*, 2015) recommends that the uncertainty of mass or weight measurement be not greater than 0.1 g. ISO 6887-1:2017 recommends this measurement uncertainty not to exceed 5%.

If the sample is frozen, the *Compendium* (Taylor *et al.*, 2015) recommends thawing in the original packaging under refrigeration temperatures (≤4.4°C) for no longer than 18 hours. Alternatively, higher temperatures may be used, but not higher than 40°C, and for no longer than 15 minutes. In this case, frequent agitation of the sample is required to facilitate thawing. The use of a controlled temperature water bath and agitation is recommended. ISO 6887-1:2017 recommends thawing under refrigeration (5 ± 3°C for no longer than 24 hours), in the original packaging. Alternatively, higher temperatures may be used (18°C to 27°C for no longer than 3 hours), but, unlike the *Compendium*, ISO 6887-1:2017 does not recommend defrosting in water bath or under running cold water, as this can result in contamination of the sample if the packaging is not completely watertight. In the case of large blocks of frozen foods, which cannot be thawed under the conditions described above, the procedure recommended by ISO 6887-1:2017 can be used: with an electric drill (fitted with a previously sterilized drill bit) or a hand drill, make holes in several points of the piece. Set the speed of the drill to not more than 900 rpm to avoid fusion or dispersion of the shavings. Using a sterile spatula, collect the shavings in a sterile container or plastic bag, from which the required number of analytical units can be taken. The entire sampling operation shall not cause a significant increase in the temperature of the sample. For frozen fish, crustaceans, mollusks, tunicates and echinoderms, ISO 6887-3:2017 recommends either taking the test portion using a drill or defrost at ambient temperature (18°C to 27°C) for approximately 60 minutes but no more than 3 hours.

If the sample is heterogeneous, consisting of different layers, each of which is of a distinct and clearly different composition (filled cakes, pies, desserts and other ready-to-eat foods), the analytical unit should be put together using portions of the different layers, taking into account the actual proportion of each layer in the product. Alternatively, homogenize the entire content of the sample and withdraw the analytical unit from the macerate (ISO 6887-4:2017).

If the amount of sample sent for analysis is smaller than the analytical unit(s) required, the *Compendium* (Taylor *et al.*, 2015) recommends subjecting half of the available amount of sample material for analysis and reserving the other half as a counter-sample. If homogenization is done using a blender, the quantity of sample plus diluent (first dilution 10^{-1}) in the jar of the blender should be sufficient to cover the cutting blades of the apparatus. For meat products, ISO 6887-2:2017 recommends using all of the material for the tests.

2.2.3 Procedure for withdrawing the analytical unit using the surface swabbing technique

The surface swabbing technique applies to foods of which most microbial contamination is predominantly present or concentrated on the surface, such as bovine, swine, poultry and fish carcasses. It also applies to the analysis of the surfaces of pieces of equipment, tables, utensils and packaging.

Rubbing can be done with sterile swabs or, if the area to be sampled is large, with sterile sponges. This material can be purchased in individual, sterile packages. The sponges may be replaced by sterile cotton pads, prepared in the laboratory. The swabs may also be prepared in the laboratory, with wooden shafts of approximately 15 cm in length by 3 mm in diameter and the absorbent part in cotton measuring approximately 2 cm in length by 5 mm in diameter.

2.2.3.1 Swab sampling

Prepare tubes or flasks with 10 mL of an appropriate diluent. The *Compendium* (Taylor *et al.*, 2015) recommends 0.1% peptone water (PW) or Butterfield's phosphate buffer, and ISO 6887-1:2017 recommends saline peptone water (SPW) or buffered peptone water (BPW). Remove the swab from its sterile package, holding it by the shaft at the edge opposite to the cotton tip. Moisten the cotton in the diluent, pressing it against the walls of the flask to remove any excess liquid.

Using a sterile frame of 50 cm^2 in size, delimit the area to be sampled, holding the frame firmly against the surface. Rub the swab with pressure, moving from left to right and then from bottom to top. Rotate the cotton swab tip continuously as you wipe, so that the entire surface of the cotton comes into contact with the sample. Upon completion of the rubbing or wiping, transfer the swab to the tube or flask containing the diluent, breaking off the hand-manipulated part of the wooden shaft against the inside of the flask tube, before immersing the remainder of the swab in the diluent.

Repeat this procedure one more time, covering the same sample surface area, using a dry swab this time. Place and keep the second swab in the same flask or tube containing diluent.

The liquid collected by the swabs can be used in general quantification tests or in presence/absence tests. In the second case, follow the guidelines and instructions in each of the specific chapters. This procedure samples a total surface area of 50 cm^2 and each milliliter of diluent, upon removal of the swabs, corresponds to 5 cm^2 of the sampled surface. Both the sampled surface area as the volume of diluent may vary, in accordance with the needs or the characteristics of the sample.

For the swabbing of half bovine or swine carcasses using the same procedure, ISO 17604:2015 recommends sampling the points indicated in Figures 2.1 and 2.2. Use one swab for each point and, between one point and the next, immerse the frame in ethanol 70% and flame sterilize. The swabs may be placed and kept in one and the same

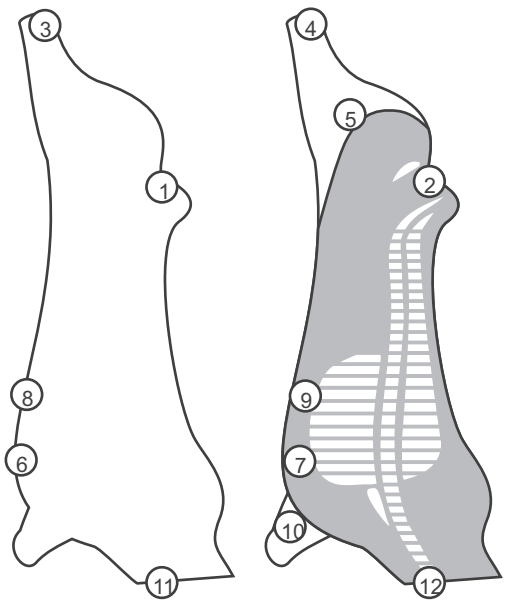

Figure 2.1 Points recommended by ISO 17604:2015 for swab sampling of bovine carcasses.

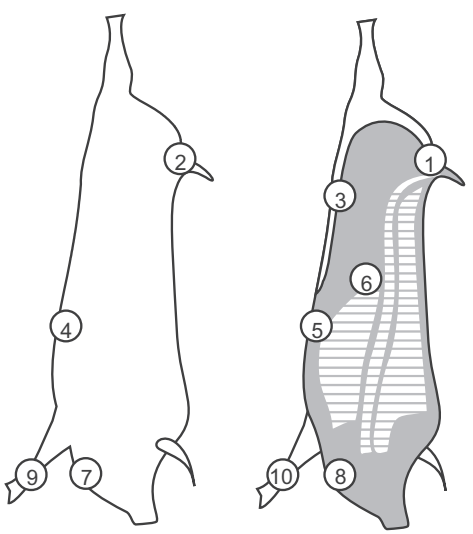

Figure 2.2 Points recommended ISO 17604:2015 for swab sampling of swine carcasses.

flask containing a total volume of diluent corresponding to a multiple of 10 mL diluent for each pair of swabs.

2.2.3.2 Sponge swab sampling

Prepare tubes or flasks with 25 mL of one of the diluents recommended for swabs. Open the plastic bag containing the sterile sponge (or cotton pad) and add an amount of diluent sufficient to moisten the sponge, without leaving behind any visible excess fluid. Hold the bag by its outside surface and massage the sponge to moisten it evenly. Thoroughly wash your hands before putting on a pair of sterile gloves and remove the sponge from the bag.

Using a sterile frame measuring 10 × 10 cm, delimit the area to be sampled by holding the frame firmly against the surface. Rub the sponge under pressure, moving it 10 times from left to right and 10 times from bottom to top. Upon completing this procedure, place the sponge back again into the bag and add the remainder of the diluent, until completing 25 mL.

The liquid collected by the sponges can be used in general quantification tests or in presence/absence tests. In the second case, follow the guidelines and instructions in each of the specific chapters. This procedure samples a total surface area of 100 cm^2, and each milliliter of diluent, after the sponge is removed, corresponds to 4 cm^2 of the sample surface. Both the sampled surface area as the volume of diluent may vary, in accordance with the needs or the characteristics of the sample.

2.2.4 Procedure for withdrawing the analytical unit using the surface washing technique

The surface washing technique is used for taking food samples of which most microbial contamination is predominantly present or concentrated on the surface, such as whole poultry carcasses, poultry cuts, fish, egg shells, grains, seeds, nuts and peanuts, which may be immersed in an adequate diluent contained in a sterile bag. The method is also used for the analysis of packages that can be closed and agitated with the diluent inside, for washing the package and collecting the sample to be examined.

2.2.4.1 Procedure for washing poultry carcasses

The following procedure is from MLG/FSIS (2017) to be used for the simultaneous examination of *Salmonella* and other microorganisms. It is also recommended by ISO 17604:2015.

Aseptically drain excess fluid from the carcass and transfer the carcass to a sterile plastic bag. Pour 400 mL of buffered peptone water (BPW) into the cavity of the carcass contained in the bag. Rinse the bird inside and out with a rocking motion for 1 minute (ca. 35 rpm). This is done by grasping the broiler carcass in the bag with one hand and the closed top of the bag with the other. Rock the carcass with a reciprocal motion in about an 18- to 24-inch arc, ensuring that all surfaces (interior and exterior of the carcass) are rinsed. Transfer the sample rinse fluid to a sterile container. Use 30 ± 0.6 mL of the sample rinse fluid obtained above for *Salmonella* analysis. Add 30 ± 0.6 mL of sterile BPW, and mix well. For analyses other than *Salmonella*, the dilutions can be made directly from the BPW rinse. Alternatively, the carcass may be rinsed in Butterfield's phosphate buffer instead of BPW. In this case, for *Salmonella* analysis add 30 ± 0.6 mL of double concentration BPW to 30 ± 0.6 mL of carcass-rinse fluid and mix well.

In this procedure each milliliter of washing liquid corresponds to the weight of the carcass divided by 400. For example, if the carcass weighs 1600 g, each milliliter of the washing liquid corresponds to 4 g of the sample.

2.2.4.2 Procedure for washing other foods

Transfer the sample to a sterile bag and weigh. Using the same diluents recommended for swabs, add to the bag the amount of diluent required for an initial 1:1 dilution (1 mL of diluent per gram of sample). Closing the mouth or opening of the bag with one hand, agitate the sample and massage the pieces inside the bag with the other hand from the outside, taking the necessary care and precautions to avoid that pointed or other protuberant parts come to pierce or puncture the package. In the case of grains, seeds, nuts and similar products, the sample may also be placed in a flask containing the diluent and agitated for 10 minutes in a laboratory shaker.

The liquid produced by this washing procedure may be used for general quantification tests or for presence/absence tests. In the second case, follow the guidelines and instructions in each of the specific chapters. In this procedure each milliliter of the washing liquid corresponds to 1 g of sample.

The volume of diluent may vary, in accordance with the needs or the characteristics of the sample.

2.2.4.3 Procedure for washing packages

This procedure is recommended for packages with a leak-proof cap or closure system. In the case of packages that do not have any cap/closure system or caps that are not leak proof, use the swabbing method.

Using the same diluents as those recommended for swabs, add to the package an amount of diluent sufficient to wash the entire internal surface by agitation (one-fifth of the package's holding capacity, for example). Close the package tightly and, with the hands agitate and swirl the package vigorously to remove the microorganisms adhered to the inner surface. Try to reach all the points of the inner surface, so as to guarantee complete removal of the contaminants present.

The liquid obtained by this washing procedure may be used for general quantification tests or for the presence/absence tests. In the second case, follow the guidelines and instructions in each specific chapter. In this procedure each milliliter of the washing liquid corresponds to the holding capacity of the package divided by the volume of the diluent. For example, if the holding capacity of the package is 500 mL and the volume of diluent is equal to 100 mL, each milliliter of the washing liquid corresponds to 5 cm^3.

2.2.5 Keeping of counter-samples

After withdrawing the analytical unit(s), store the remaining material under the same conditions utilized prior to analysis (ISO 7218:2007). Perishable samples need to be frozen, but it is important to know that thawing of counter-samples for the purpose of repeating microbiological test(s) is not an acceptable practice, due to the possible death of part of the microbial populations that were originally present. In the case of frozen products, this problem can be resolved by thawing for analysis only the portion required for the test(s). The remaining quantity, which was not thawed, may be kept frozen to be used as a counter-sample for later repetitions of the test(s), if necessary. In the case of refrigerated products, there is no acceptable way to keep counter-samples without freezing. In case test(s) need to be repeated, the result(s) should be interpreted taking into account the fact that population(s) of the target microorganism(s) may have been reduced due to freezing.

In the case of samples the analytical unit of which has been collected by surface swab or sponge rubbing technique or the surface washing technique, the part of the diluent retaining the contaminants and not used for subsequent microbiological testing should be frozen to serve as a counter-sample. Also in this case, it should be taken into consideration that the population(s) of the target microorganism(s) may have been reduced due to freezing.

The minimum time for keeping counter-samples is the time required for obtaining the results of the tests, but should be set at the discretion of the laboratory. The samples may be disposed of by throwing them in a dumpster, but samples deteriorated or suspected of containing microorganisms that are harmful to health should be decontaminated in an autoclave (121°C for 30 minutes) prior to final disposal (ISO 7218:2007).

2.3 Preparation of the first dilution of the analytical unit

To proceed with the analysis, the analytical unit must be diluted and homogenized with an adequate diluent, to allow inoculation into or onto culture media. The recommended diluents and initial dilution ratios vary with the type of sample and the type of test that will be performed, as described below.

2.3.1 Diluents for presence/absence tests

These tests are performed with dilution and homogenization directly in enrichment broth, specified in the corresponding chapters.

2.3.2 Diluents for tests requiring differentiated handling of the sample

Also for these tests the specific chapters should be consulted.

2.3.3 Diluents for general quantification tests

For these tests the recommendations showed at Table 2.1 apply.

Table 2.1 Diluents recommended by different regulatory agencies for food and water analysis.

Regulatory agency publication	Matrix to which it applies	Diluent recommended
APHA, *Compendium of Methods for Microbiological Examination of Foods* (Taylor *et al.*, 2015)	Foods	0.1% peptone water (PW) or Butterfield's phosphate buffer
APHA, *Standard Methods for the Examination of Water & Wastewater* (Hunt, 2012)	Water	0.1% peptone water (PW) or magnesium chloride phosphate buffer
APHA, *Standard Methods for the Examination of Dairy Products* (Davis and Hickey, 2004)	Milk and dairy products	Butterfield's phosphate buffer or magnesium chloride phosphate buffer
ISO 6887-1:2017	Foods	Saline peptone water (SPW) or buffered peptone water (BPW)
	Foods pH below 4.5	Double-strength buffered peptone water [BPW][2]
ISO 6887-2:2017	Meat and meat products	Saline peptone water (SPW) or buffered peptone water (BPW)
ISO 6887-3:2017	Fish and fishery products	Saline peptone water (SPW) or buffered peptone water (BPW)
ISO 6887-4:2017	Miscellaneous products	Saline peptone water (SPW) or buffered peptone water (BPW)
	Foods pH below 4.5	Double-strength buffered peptone water [BPW][2]
ISO 6887-5:2010	Milk and dairy products	0.1% peptone water (PW) or buffered peptone water (BPW) or saline peptone water (SPW) or Ringer's solution quarter-strength or phosphate buffer acc. ISO 6887-5
ISO 6887-6:2013	Samples from the primary production stage (from farm to slaughterhouse)	Saline peptone water (SPW) or buffered peptone water (BPW)

The *Compendium* (Taylor *et al.*, 2015) recommends, for general use in the examination of foods, 0.1% peptone water (PW) or Butterfield's phosphate buffer.

Section 4.030 of the *Standard Methods for the Examination of Dairy Products* (Davis and Hickey, 2004) recommends, for general use in the examination of dairy products, Butterfield's phosphate buffer (called phosphate dilution water) or magnesium chloride phosphate buffer (called phosphate and magnesium chloride dilution water).

The *Standard Methods for the Examination of Water & Wastewater* (Hunt, 2012) recommends, for general use in the examination of water samples, 0.1% peptone water (PW) or magnesium chloride phosphate buffer (called buffered water).

ISO 6887-1:2017 recommends saline peptone water (SPW) or buffered peptone water (BPW) for general use in the examination of foods. For foods with pH below 4.5 recommends BPW double strength.

ISO 6887-2:2017 and ISO 6887-3:2017 also recommend saline peptone water (SPW) or buffered peptone water (BPW) for general use in the examination of meat and meat products, and fish and fishery products.

ISO 6887-4:2017 recommends saline peptone water (SPW) or buffered peptone water (BPW) for general use in the examination of miscellaneous products and BPW double strength for foods with pH below 4.5.

ISO 6887-5:2010 recommends, for general use in the examination of milk and dairy products, 0.1% peptone water (PW), buffered peptone water (BPW), saline peptone water (SPW), Ringer's solution quarter-strength or phosphate buffered solution according ISO 6887-5.

There are special cases for which a different diluent is recommended. For more details on these exceptions see Annex 2.2.

2.3.4 How to prepare an initial 1:10 (10^{-1}) dilution

The initial dilution recommended for most samples is 1:10 (10^{-1}), obtained by adding m grams or milliliters of the sample to 9 × m milliliters of diluent. For example, for 25 g of sample, add 9 × 25 mL of diluent (225 mL). There are situations in which the diluent and the initial dilution are different. For more details on these exceptions see Annex 2.2.

2.3.5 How to prepare an initial dilution different from 1:10

In some special situations the first dilution is different from 1:10. To determine the volume of diluent necessary to obtain a predetermined 1:*k* dilution of the sample, use the $v = [(k.m) - m]$ ratio. For example, to obtain a 1:50 dilution of an analytical unit of 10 g, add [(50 × 10) – 10] mL of diluent (490 mL). To obtain the same dilution for an analytical unit of 20 g, add [(50 × 20) – 20] mL of the diluent (980 mL).

2.3.6 Procedure for the preparation of the first dilution of liquid samples

In the case of liquid foods, transfer the analytical unit directly to tubes or flasks containing the amount of diluent necessary for a 1:10 dilution. Homogenize the sample with the diluent by agitation, inverting the container or package 25 times. To allow for perfect homogenization, use tubes or flasks with screw caps. They should be of a size sufficiently great to ensure that no more than two-thirds of their holding capacity is taken up by the analytical unit plus the diluent. There are special cases that require a different initial dilution. For further details on these exceptions see Annex 2.2.

2.3.7 Procedure for the preparation of the first dilution of solid or concentrated liquid samples

In the case of solid or concentrated liquid foods, transfer the analytical unit to a sterile homogenization flask or bag. Add to the sample the amount of diluent necessary to obtain a 1:10 dilution. Homogenize the analytical unit with the diluent, which can be achieved by manual agitation, shaking the flask in an inverted position 25 times through a 30-cm arc within 7 seconds (concentrated liquids, soluble powders), agitation in a peristaltic homogenizer (better known as stomacher) for 1 to 2 minutes (soft foods, pasty foods, ground or minced foods, poorly soluble powders) or in a blender (hard foods). In the case of homogenization using a blender, the *Compendium* (Taylor *et al.*, 2015) recommends using high speed during the first few seconds and low speed (8,000 rpm) for the remaining time, which should not exceed 2 minutes. If a more prolonged homogenization is necessary, it is important to prevent excessive heating of the material. For that purpose, the *Compendium* (Taylor *et al.*, 2015) recommends cooling the diluent in an ice bath before use, while ISO 6887-4:2017 recommends not homogenizing for periods longer than 2.5 minutes. There are special cases that require a different initial dilution. For further details on these exceptions see Annex 2.2.

2.3.8 Procedure for the preparation of the first dilution of samples obtained by surface swabbing or surface washing

The diluent retaining the contamination collected with swabs, sponges or surface washing is, in itself, already the first dilution of the sample. The subsequent treatment of serial decimal dilution is performed using this suspension as point of departure. Since the initial dilution is not the standard 1:10 dilution, this difference must be taken into account when doing the final calculations of the results, as described in Chapters 3 and 4.

2.4 Serial decimal dilution of the sample

The preparation and inoculation of serial dilutions of the sample are required for quantitative tests, to reduce the number of microorganisms per unit of volume and to make it possible to count them. This series of dilutions is generally decimal or 10-fold for ease of calculation of final results.

The number of dilutions necessary depends on the expected level of contamination and should be such as to allow for, in plate counts, obtaining plates with numbers of colonies varying between 25–30 and 250–300 (see Chapter 3) or between 15 and 150 in yeast and mold counts. In counts by the most probable number (MPN) method, the number of dilutions must allow for obtaining positive tubes at the lowest dilutions and negative tubes at the highest dilutions (see Chapter 4).

According to the general procedure described by the *Compendium* (Petran *et al.*, 2015), the second dilution is to be initiated immediately upon completion of the first dilution. The duration of the complete procedure, from the preparation of the first dilution until inoculation of all culture media, should not exceed 20 minutes (except when described in case-specific chapters).

According to the general procedure described by ISO 6887-1:2017, the duration of the complete procedure should not exceed 45 minutes and the time interval between the end of the preparation of the first dilution and the beginning of the second and subsequent dilutions should not exceed 30 minutes (except when specified in specific procedures).

For hard and dry products and for low-moisture products, ISO 6887-4:2017 recommends a resuscitation step before preparing the second dilution. In general, leave the sample to rest at laboratory temperature (18°C to 27°C) for about 1 hour (20 to 30 minutes in case of flours, cereal grains and by-products and animal feeds).

In all cases in which volumes are transferred, the uncertainty of the measurement must not exceed 2% (ISO 6887-1:2017).

How to prepare the second dilution (10^{-2}): Transfer aseptically 1 mL of the first dilution (10^{-1}) to 9 mL of diluent. The diluents are the same as those recommended for the first dilution. In the second dilution there are no special cases in which a different diluent is required from the one used to prepare the first dilution.

Do not dip the tip of the pipette to a depth of more than 1 cm when pipetting the volume from the first to the second dilution (ISO 6887-1:2017). If the first dilution does not contain suspended particles, the material may be agitated before transferring the volume from the first to the second dilution. If there are suspended particles, ISO 6887-1:2017 recommends not to agitate and wait until the suspended particles settle to the bottom before transferring the volume. In the case of viscous samples, which adhere to the internal wall of the pipette, ISO 6887-5:2010 recommends dispensing the volume and subsequently washing the pipette with diluent (by aspirating several times) to ensure that all the material be transferred to the second dilution.

How to prepare subsequent dilutions: Transfer 1 mL of the previous dilution to 9 mL of diluent. Before withdrawing the volume to be transferred, agitate the tube vigorously, inverting it 25 times in a 30-cm arc (within 7 seconds) or using a laboratory vortex mixer (15 seconds).

2.5 References

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Annex 2.1 Procedures for homogenizing the content and withdrawal of the analytical unit of different types of foods

a) Powdered products: Homogenize the sample by vigorously agitating and inverting the package with your hands until well mixed or stir the content with a sterile spatula or glass rod. If there is not enough free space inside the package to allow for appropriate homogenization, transfer the whole content to a larger flask and proceed in exactly the same way (ISO 6887-5:2010). Withdraw the analytical unit with a sterile spatula.

b) Pasty or ground products: Stir the content with a sterile spatula or glass rod until well homogenized. Withdraw the analytical unit with a sterile spatula (Taylor *et al*., 2015).

c) Yogurts with fruit pieces: For yogurt containing fruit pieces, the *Standard Methods for the Examination of Dairy Products* (Duncan *et al*., 2004) recommends homogenizing the entire content of the sample unit in a blender for 1 minute before withdrawing the analytical unit.

d) Cheeses: The *Standard Methods for the Examination of Dairy Products* (Duncan *et al*., 2004) recommends macerating the whole content of the sample unit (with a sterile spatula) and withdrawing the analytical unit from the mixture.

e) Very hard food products: ISO 6887-1:2017 and ISO 6887-4 2017 recommends grinding the sample until a homogeneous mixture is obtained. To avoid excessive heat in this process, do not homogenize for more than 1 minute at a time.

It is also possible to place samples inside a sterile plastic bag and beat the material with a sterile hammer, crumbling it into small bits and pieces. Mix well the fragmented sample, withdraw the analytical unit with a sterile spatula and, after addition of the diluent, keep the sample homogenized for 1 hour at 18°C to 27°C for the recovery of stressed cells (ISO 6887-4:2017).

ISO 6887-1:2017 and ISO 6887-5:2010 recommend when using a stomacher to place the sample and diluent in two or more sterile bags to prevent puncturing and possible sample spillage. When using rotary homogenizer do not homogenize for more than 2.5 minutes at a time.

f) Pieces of solid foods: Chapter 2 of the *Compendium* (Taylor *et al*., 2015) recommends using a suitable instrument (knife, sterile scissors) to break or cut smaller pieces from various points in the piece until the required amount is obtained.

g) Eggs in the shell: For analysis of the internal content, Chapter 46 of the *Compendium* (Ricke *et al*., 2015) recommends to remove any adherent material from the shell surface with a brush, immerse the eggs in ethanol 70% for 10 seconds and flame sterilize, or, alternatively, to immerse in 3:1 alcoholic solution of iodine for 10 seconds and allow to dry. Using sterile gloves open the eggs aseptically and place the internal content inside a sterile flask or bag, separating the yolk from the egg white if the analysis requires. Mix well and withdraw the analytical unit from the mixture.