INTRODUCTION

The Recommended International Code of Hygienic Practice for Egg Products was adopted by the Codex Alimentarius Commission at its 11th Session in 1976. At its 12th Session in 1978, the Commission adopted Microbiological Specifications for Pasteurized Egg Products (Appendix II of the Code). In 1985, the 16th Session adopted amendments to the Code to include melange.

This Code of Hygienic Practice is to be regarded as advisory in nature and individual governments should decide what use they wish to make of the Code. The Commission has expressed the view that codes of practice dealing with specific categories of foods might provide useful checklists of requirements for national enforcement authorities.

RECOMMENDED INTERNATIONAL CODE OF HYGIENIC PRACTICE FOR EGG PRODUCTS CAC/RCP 15-1976 (amended 1978, 1985)

1. SECTION I - SCOPE

This Code of Practice is designed to:

A. Prevent deterioration in the quality of eggs in shell intended for egg products.

B. Provide guidance on the hygienic production, storage, packaging and transport of whole egg, egg albumen, egg yolk and other products consisting wholly or mainly of one or more of the constituents of egg, intended for human consumption.

C. Provide guidance on hygienic practice relating to premises, equipment and personnel used or engaged in the production of these products.

Unless specifically stated otherwise, the word "Egg" in this Code relates to domesticated chickens (hens) eggs intended for processing as above. However, the principles of this Code may be applied equally to eggs of other domesticated birds.

2. SECTION II - DEFINITIONS

. ***Approved*** means approved by the official agency having jurisdiction.

. ***Egg*** means eggs (in shell) of domesticated chickens (hens).

. ***Egg products*** means the content of eggs, as whole egg or only the yolk or only egg albumen or a mixture of yolk and albumen in liquid, frozen or dried form, single or in combination with other foods or drinks to a minimum content of 50% egg product.

3. SECTION III - RAW MATERIAL REQUIREMENTS

3.1 Environmental Sanitation in Production Areas

3.1.1 **Hygienic disposal of human and animal wastes**. Adequate precautions should be taken to ensure that human and animal wastes are disposed of in such a manner as not to constitute a public health or hygienic hazard and extreme care should be taken to protect eggs

from contamination with these wastes, particularly those eggs that may be consumed without heat treatment.

3.1.2 **Animal, plant pest and disease control**. Treatment with chemical, biological or physical agents should be done only in accordance with the recommendations of the official agency having jurisdiction; by or under the direct supervision of personnel with a thorough understanding of the hazards involved, including the possibilities of toxic residues being retained by the product.

3.2 **Production, Storage and Collection of Eggs on the Farm**

3.2.1 **Health of farm stock**. Only eggs derived from healthy stock should be used in the production of egg products for human consumption.

3.2.2 **Equipment and product containers**. Equipment and egg containers should not constitute a hazard to health. Containers which are re-used should be of such material and construction as will facilitate thorough cleaning, and should be so cleaned and maintained as not to constitute a source of contamination to the product.

3.2.3 **Sanitary techniques**

3.2.3.1 Eggs should be collected as frequently as necessitated by the climatic conditions Collecting twice a day has been found satisfactory. The eggs should be handled as little as possible. In particular, rough handling should be avoided.

3.2.3.2 Throughout handling and storage it is essential that steps be taken to prevent:

3.2.3.2.1 Contamination of the shell with dirt, bedding materials or by animals, insects, vermin, birds, chemical or microbiological contaminants or other objectionable substances.

3.2.3.2.2 Exposure to unfavourable temperatures.

3.2.3.3 **Cleaning**. Eggs should not be cleaned on the farm. If, exceptionally, they are cleaned on the farm, this should be done only with the approval of the official agency having jurisdiction which should be satisfied as to the method of cleaning employed, including the time/temperature conditions of any washing process and the detergents/disinfectants used.

3.2.4 **Removal of obviously unfit materials**. Unfit eggs should be segregated during collection to the fullest extent practicable, and should be disposed of in such a place and such a manner as will prevent contamination of other eggs or water supplies.

3.2.5 **Storage on the farm**. Eggs should be stored in a cool room to which they should be taken immediately after collection. They should not be stacked or packed into boxes until they are cool, and the room should be kept free from strong smelling substances and odours. Eggs should be stored at such a temperature and relative humidity as will minimize deterioration having regard to local climatic conditions. Temperatures of 8^{o}-15^{o}C (46-59^{o}F) and relative humidities of 70% - 85% have been found satisfactory.

Thin-shelled or hair-cracked eggs should be carefully handled and packed in a separate container to prevent breakage before delivery to the breaking plant.

3.3 **Handling In-shell Cracked Eggs on the Farm**

3.3.1 Thin-shelled or hair cracked eggs or cracked eggs with shell membranes intact

should be carefully handled and packed in a separate container to prevent breakage before delivery to the breaking plant.

3.3.2 If there is a danger that this type of egg will break during the transport to the egg-breaking plants the following procedure should be followed.

3.3.3 Only clean hair cracked eggs (not washed) or clean cracked eggs (not washed) with shell membranes intact may be broken on the farm.

3.3.4 This procedure should be in accordance with Section 4, Sub-section 4.4.4.1

3.3.5 Egg products collected on the farm may not be strained nor be separated into egg-yolk and egg-albumen.

3.3.6 This egg product should be collected in clean and, if necessary, disinfected containers with suitable closures and should be chilled in accordance with Sub-section 4.4.4.4, Section 4. This procedure should preferably be performed in a separate room. The room used for the operation should be in accordance with the requirement set forth in Sub-section 4.1.1.

3.3.7 All measures should be taken to protect the product from contamination.

3.3.8 The egg products should be collected and transported from the farm where they are produced as soon as possible only to the egg product plant and transported at a temperature between 0-5^{o}C.

3.4 **Handling In-shell Cracked Eggs at the Packing Station**

3.4.1 The same procedures should be followed as prescribed in Sub-sections 3.3.2 to 3.3.8.

3.5 **Transportation**

3.5.1 **Facilities**. Conveyances for transporting eggs should be adequate for the purpose intended and should be of such material and construction as will permit thorough cleaning and should be so cleaned and maintained as not to constitute a source of contamination to the eggs.

3.5.2 **Handling procedures**. All handling procedures should be such as will prevent the eggs from being contaminated.

Eggs should be collected from the producers' premises and delivered to the processing plant as soon as possible, and be maintained during transport at such a temperature as will minimize deterioration having regard to local climatic conditions.

4. SECTION IV - PLANT, FACILITIES AND OPERATING REQUIREMENTS

4.1 Plant Construction and Layout

4.1.1 **Location, size and sanitary design**. The building and surrounding area should be such as can be kept reasonably free of objectionable odours, smoke, dust, or other contamination; should be of sufficient size for the purpose intended without crowding of equipment or personnel; should be of sound construction and kept in good repair; should be of such construction as to protect against the entrance and harbouring of insects or birds or vermin; and should be so designed as to permit easy and adequate cleaning.

The construction and layout of the processing premises should be such as to secure a regulated flow in the process from the arrival of the eggs at the premises to the finished product, and should provide for correct temperature conditions at all stages of the process.

4.1.2 **Sanitary facilities and controls**

4.1.2.1 **Separation of processes**. Areas where eggs and other raw materials are received or stored should be so separated from areas in which final product preparation or packaging is conducted as to preclude contamination of the finished product. Areas and compartments used for storage, manufacture or handling of edible products should be separate and distinct from those used for inedible materials. The food handling area should be completely separated from any part of the premises used as living quarters. Separate rooms should be provided for unpacking and washing of the eggs and for storing the finished product. Candling, breaking, pasteurizing and filling should be so separated as to protect against cross-contamination.

4.1.2.2 **Water supply**. An ample supply of cold water should be available and an adequate supply of hot water, where necessary. The water supply should be of potable quality. Standards of potability shall not be less than those contained in the latest edition of "International Standards for Drinking Water", World Health Organization.

4.1.2.3 **Ice**. Ice should be made from water of potable quality and should be manufactured, handled, stored and used, so as to protect it from contamination.

4.1.2.4 **Auxiliary water supply**. Where non-potable water is used - for such purposes as fire control - it must be carried in completely separate lines, identified preferably by colour and with no cross-connection or back-siphonage with the lines carrying potable water.

4.1.2.5 **Plumbing and waste disposal**. All plumbing and waste disposal lines (including sewer systems) must be large enough to carry peak loads. All lines must be watertight and have adequate traps and vents. Disposal of waste should be effected in such a manner as not to permit contamination of potable water supplies. The plumbing and the manner of waste disposal should be approved by the official agency having jurisdiction.

Drainage systems which include solid matter traps should be designed so as to allow them to be emptied. When located within or immediately outside the plant, solid matter traps should be emptied and cleaned as necessary and in accordance with the requirements of the official agency having jurisdiction.

4.1.2.6 **Lighting**. Premises should be well lit. Light bulbs and fixtures suspended over food in any step of preparation should be of the safety type or otherwise protected to prevent food contamination in the case of breakage. The illumination in any part of a workroom should be not less than 325 lux units (30 foot candles), and at points requiring close examination of the product they should be illuminated at an intensity of not less than 540 lux units (50 foot candles). Reflector filaments should be designed to allow easy dismantling, cleaning, and reassembling.

4.1.2.7 **Ventilation**. Premises should be well ventilated. Special attention should be given to the venting of areas and equipment producing excessive heat, steam, obnoxious fumes or vapours or contaminating aerosols. Good ventilation is important to prevent both condensation (which may drip into the product) and mould growth in overhead structure - which growth may fall into the food. Ventilation should be planned to allow for adequate changes of air and to ensure that the direction of air flow is never from a dirty area to a clean one.

4.1.2.8 **Toilet-rooms and facilities**. Adequate and convenient toilets should be provided and toilet areas should be equipped with self-closing doors. Toilet rooms should be well lit and

ventilated and should not open directly into a food handling area. They should be kept in a sanitary condition at all times. There should be associated hand-washing facilities within the toilet area and notices should be posted requiring personnel to wash their hands after using the toilet.

4.1.2.9 **Hand-washing facilities**. Adequate and convenient facilities for employees to wash and dry their hands should be provided wherever the process demands. They should be in full view of the processing floor. Single-use towels are recommended, where practicable, but otherwise the method of drying should be approved by the official agency having jurisdiction. The facilities should be kept in a sanitary condition at all times.

4.2 **Equipment and Utensils**.

4.2.1 **Materials**. all food contact surfaces should be smooth, free from pits, crevices and loose scale, non-toxic, unaffected by food products, capable of withstanding repeated exposure to normal cleaning, and non-absorbent unless the nature of a particular and otherwise acceptable process renders the use of a surface, such as wood, necessary.

4.2.2 **Sanitary design, construction, and installation**. Equipment and utensils should be so designed and constructed as will prevent hygienic hazards and permit easy and thorough cleaning. Stationary equipment should be installed in such a manner as will permit easy and thorough cleaning.

Wooden equipment should not be used in the breaking, pasteurizing, or filling rooms.

All pumps, pipes, vessels, and contact surfaces should be of stainless steel or other approved material.

Shell eggs moving into the breaking room should be conveyed in containers constructed of stainless steel, aluminium, approved plastic material, or in single-use trays. Breaking tables should be constructed of stainless steel, aluminium or plastic material. As far as practicable, plastic materials used for these purposes should be free from cracks and scratches and should be capable of withstanding the regular cleaning and disinfection process.

Machines and containers for liquid egg should be of stainless steel or other suitable material and should be so constructed as to permit the ready elimination from the liquid egg supply of all the egg contents that are unfit for further processing.

Any device for the separation of egg yolk from egg white should be of approved sanitary design and construction.

Equipment and utensils used for inedible or contaminated materials should be so identified and should not be used for handling edible products.

4.3 **Hygienic Operating Requirements**

4.3.1 **Sanitary maintenance of plants, facilities and premises**

4.3.1.1 The building, equipment, utensils and all other physical facilities of the plant should be kept in good repair and should be kept clean and maintained in an orderly, sanitary condition. Waste materials should be frequently removed from the working area during plant operation and adequate waste receptacles should be provided. Detergents and disinfectants employed should be appropriate to the purpose and should be so used as to present no hazard to public health.

4.3.1.2 All equipment should be cleaned and disinfected at all major breaks in work periods,

whenever necessary to remove contamination, and at the end of the day's work. Disinfection should also be carried out before commencement of the day's work. Steam condensate should not be allowed to remain in any equipment. Between disinfection and work periods equipment should be handled as little as possible.

4.3.1.3 Whenever the process is stopped for approximately 30 minutes or more all hand breaking equipment and easily removable parts of breaking machines should be cleaned and disinfected. At the same time the surfaces of breaking tables should be cleaned and liberally hosed with clean hot water.

4.3.1.4 Where "in-place" cleaning is carried out and inspection at the end of the day indicates defective "in-place" cleaning, the equipment should be dismantled and cleaned.

4.3.1.5 The final stage of cleaning and disinfecting should be a thorough rinse with hot water.

4.3.2 **Disposal of waste materials**

Waste material, which includes empty shells and reject eggs, should be stored in such a manner as not to cause a nuisance from offensive odours, insects, birds or vermin. It should be removed regularly and frequently, and at least at the end of the day, from processing rooms either by means of suitable containers, conveyor belts or water troughs. In addition it should be removed from the premises daily. Immediately after emptying, receptacles and equipment used for storage and consolidation of waste material should be cleaned and disinfected, as also should the paved areas used for the storage of such waste receptacles.

4.3.3 **Vermin control**. Effective measures should be taken to protect against the entrance into the premises and the harbourage on the premises of insects, rodents, birds or other vermin.

4.3.4 **Exclusion of domestic animals**. Dogs, cats and other domestic animals should be excluded from areas where food is processed or stored.

4.3.5 **Personnel health**. Plant management should advise personnel that any person afflicted with infected wounds, sores, or any illness, notably diarrhoea, should immediately report to management. Management should take care to ensure that no person, while known to be affected with a disease capable of being transmitted through food, or known to be a carrier of such disease micro-organisms, or while afflicted with infected wounds, sores, or any illness, is permitted to work in any area of a food plant in a capacity in which there is a likelihood of such person contaminating food or food-contact surfaces with pathogenic organisms.

4.3.6 **Toxic substances**. All rodenticides, fumigants, insecticides or other toxic substances should be stored in separate locked rooms or cabinets and handled only by properly trained personnel. They should be used only by or under direct supervision of personnel with a thorough understanding of the hazards involved, including the possibility of contamination of the product.

4.3.7 **Personnel hygiene and food handling practices**

4.3.7.1 All persons working in a food plant should maintain a high degree of personal cleanliness while on duty. Clothing, including suitable head-dress, should be appropriate to the duties being performed and should be kept clean.

4.3.7.2 Hands should be washed as often as necessary to conform to hygienic operating practices.

4.3.7.3 Spitting, eating, chewing and the use of tobacco should be prohibited in food handling areas.

4.3.7.4 All necessary precautions should be taken to prevent the contamination of the food product or ingredients with any foreign substance.

4.3.7.5 Minor cuts and abrasions on the hands should be appropriately treated and covered with a suitable waterproof dressing. Adequate first-aid facilities should be provided to meet these contingencies so that there is no contamination of the food.

4.3.7.6 Gloves used in food handling should be maintained in a clean, hygienic and sound condition; gloves should be made of an impermeable material except where their usage would be inappropriate or incompatible with the work involved.

4.4 Operating Practices and Production Requirements

4.4.1 Eggs and other raw materials

Acceptance criteria. No eggs or other raw materials should be accepted by the plant if they are known to contain toxic substances. Eggs or other raw materials containing decomposed or extraneous material which will not be removed or reduced to acceptable levels by normal plant procedures of sorting or preparation, should also not be accepted.

4.4.2 **Storage and handling of shell eggs**. On receipt at the plant, eggs should be processed as soon as possible. Until they are processed, they should be stored in their cases in a cool clean room. The temperatures and relative humidities mentioned in Section 3.2.5 would be suitable. Cases should be stored in such a way as to permit cleaning underneath. Eggs should be unpacked in a room completely separated from the processing rooms. Egg outer cases should not be taken into the breaking room.

4.4.3 **Inspection and sorting**. Eggs should be candled before breaking, either at the plant or elsewhere if preferred, within a specified time approved by the official agency having jurisdiction. Dirty eggs should be cleaned before breaking out, using methods approved by the official agency having jurisdiction, including the time/temperature conditions and any detergent/sanitizer used.

Cracked eggs with shell membranes intact should be segregated in shallow containers constructed of suitable materials and should be carefully examined by experienced breakers before processing.

Cracked eggs with shell membranes broken should be dealt with as waste material, but if the breakage has occurred within the plant during candling or handling they should be segregated in a suitable receptacle used for this purpose only. Such eggs should be processed without delay.

Eggs should be candled before being passed into the breaking area. Where breaking by crushing is used, special care is necessary during candling to eliminate defective eggs.

To avoid cross-contamination, eggs other than chicken eggs should be segregated and handled and processed separately at the end of the day's processing of chicken eggs. All equipment should be cleaned and sterilized before the processing of chicken eggs is resumed.

4.4.4 **Preparation and processing**

4.4.4.1 **Breaking individually**. Eggs should be broken either by hand or machine into cups or trays and each egg should be inspected for appearance and, if possible, for odour.

Egg substance having an abnormal odour or appearance should be rejected and removed, together with any contaminated breaking equipment. Such equipment should be cleaned and disinfected before being used again. After touching rejected eggs, the breakers should immediately wash their hands with odourless soap/detergent in hot water.

Separation of egg yolk from egg white should be carried out in a hygienic manner.

Hygienic practices should be observed for the removal of shell fragments, and, where customarily removed, for blood spots and meat spots.

After breaking, a centrifuge may be used to remove the last part of the egg albumen out of the egg shells, but only eggs that have been washed with the method described in Sub-section 4.4.4.2 may be centrifuged.

4.4.4.2 **Breaking by crushing**. Breaking by crushing, when authorized by the official agency having jurisdiction, should meet the following minimum requirements:

Bulk crushing machines used for breaking out eggs for the preparation of whole egg product should be of a suitable type and be so constructed and operated as to prevent unfit eggs from entering the liquid egg product. Eggs which have been washed prior to arrival at the breaking plant should not be used. The eggs should be processed within 24 hours of candling, provided that where the eggs are held under controlled temperature conditions so as to retard spoilage and the growth of microorganisms, they may be held for a period not exceeding 72 hours without re-candling.

The eggs should be conveyed on rollers of stainless steel or other suitable material through a hot water bath maintained at a minimum temperature of 60^{o}C (140^{o}F), rinsed under hot water sprays at a minimum temperature of 80^{o}C (177^{o}F) and afterwards air dried before being ejected on to a conveyor belt, constructed of suitable material, in the crushing section.

The eggs should be crushed to remove their contents, after which all shell fragments should be removed from the conveyor belt. At the end of each day's work the machines should be cleaned, scrubbed with a suitable disinfectant and rinsed with clean hot water.

4.4.4.3 **Straining and collection**. The liquid egg should be strained either by suitable strainers, centrifuges or other suitable equipment. If strainers are used a supply of clean disinfected stainless steel, monel, or other suitable strainers should be available to enable frequent changes to be made. If necessary, a clean, disinfected stainless steel or other suitable container should be used to collect liquid egg when strainers are being changed. This unstrained liquid egg should be returned immediately for straining.

4.4.4.4 **Chilling**. Where pasteurization does not immediately follow breaking, liquid egg products should be chilled rapidly in equipment capable of reducing the product temperature to 7^{o}C (45^{o}F). If the product is to be stored before pasteurization, storage should be in suitably insulated tanks for a period preferably not exceeding 24 hours and never exceeding 48 hours. Liquid egg yolk may be held at a temperature not exceeding 10^{o}C (50^{o}F) if storage is not to exceed a period of 8 hours.

If it is intended to store liquid egg products for more than 48 hours, they should be

stored at temperatures below 0°C (32°F).

4.4.4.5 **Pasteurization**

4.4.4.5.1 Liquid egg products should preferably be pasteurized as part of a continuous process.

Egg products received from the farms or packing stations should be pasteurized in the plant.

4.4.4.5.2 All egg products should be subjected to a treatment approved by the official agency having jurisdiction as being a treatment which will destroy *salmonella*.

4.4.4.5.3 The raw liquid whole egg should be pasteurized by an approved process of heating at a temperature sufficiently high and for a time sufficiently long to ensure the destruction of *salmonella* organisms, for example at a temperature of 64°C (148°F) for at least 2½ minutes, or by other approved treatment which will give the same results.

The pasteurization of liquid albumen will, and liquid egg yolk may, require different time/temperature combinations.

4.4.4.5.4 On completion of pasteurization, all liquid products should be immediately cooled to a temperature not exceeding 7°C (45°F).

4.4.4.5.5 The plate pasteurizing apparatus should include such devices as may be necessary to ensure a constant rate of flow of liquid egg, thermostatic control of the heating of the liquid egg, and the automatic diversion of flow of any liquid egg not sufficiently heated. The batch pasteurizing apparatus should include thermostatic controls and also a stirring mechanism to mix the liquid egg to be pasteurized to ensure uniformity of temperature.

4.4.4.5.6 A continuous recording should be made of each pasteurization run, and charts showing pasteurization temperatures and times should be dated and kept available for inspection for at least one year.

4.4.4.5.7 Dried egg products processed from liquid egg which has not been pasteurized beforehand should be subjected to an approved heat treatment process, for example the hot room process, in the dried form and preferably in the container, to destroy *salmonellae*.

4.4.4.5.8 The various products should be protected from contamination at all stages after pasteurization.

4.4.4.6 **Storage**

4.4.4.6.1 Pasteurized liquid egg may be held in disinfected, insulated and covered tanks fitted with a low speed agitator and a thermometer, or in disinfected churns, provided that the temperature of the egg does not exceed 5°C (41°F) during the holding period.

4.4.4.6.2 Products which are sufficiently preserved to prevent deterioration, for example by salting or by sugaring, need not be chilled.

4.4.4.7 **Drying**

4.4.4.7.1 Glucose removal should, where applicable, be carried out prior to pasteurization by an approved method.

4.4.4.7.2 Drying should be carried out by an approved process. The drying plant used for the product should, where applicable, include a cyclone separation system in preference to the bag type separation.

4.4.4.7.3 The product should be continuously removed from the drying chamber, cooled, and packed as soon as possible into suitable containers. If the glucose has not been removed, the product should be stored at a temperature not exceeding 10°C (50°F).

4.4.5 **Packing, cooling and freezing**

4.4.5.1 Empty containers should be stored in a clean, dry place and kept free from dust, vermin, insects and any foreign matter. They should not transmit to the product objectionable substances beyond limits acceptable to the official agency having jurisdiction and should provide appropriate protection from contamination. They should be inspected immediately before use to ensure they are in a clean and satisfactory condition. Prior to filling, containers should, where necessary, be disinfected by steam, hot air, hot water, a disinfectant, or any combination of these, but the container should be well drained before filling.

Only containers ready for immediate use should be kept in the filling room.

4.4.5.2 The filling of containers should be a continuous process. The filled containers should be immediately sealed and taken either to the cooling room or to the freezing chamber without undue delay. Care should be taken during filling to avoid spillage and any excess egg should be removed.

4.4.5.3 Containers should be stacked in these rooms so as to permit free circulation of air around the containers.

4.4.5.4 The rate of freezing should be sufficient to prevent deterioration of the product and be completed within 24 hours of filling. The chilled product should be stored at a temperature not exceeding 5°C (41°F). After freezing, the product should be stored at a temperature which gives adequate protection to the product.

4.4.6 **Transportation of liquid egg products in bulk**

4.4.6.1 Tanks or containers used for transporting liquid egg products should be constructed of stainless steel or other suitable material, and be designed to facilitate cleaning and adequate drainage. They should be refrigerated or sufficiently insulated to maintain the egg product at a temperature of not more than 5°C (41°F), and preferably should not be used for any other purpose.

4.4.6.2 Pipes and connections used for the filling and discharge of the liquid egg products should be of suitable design and materials and should be cleaned and disinfected after use, and disinfected before re-use.

4.4.6.3 Liquid egg products should not be discharged from a road tanker or mobile container into a vessel containing liquid egg products from a previous delivery.

4.4.6.4 Tankers and mobile containers should be cleaned and disinfected as soon as practicable after emptying and disinfected before being re-filled. The final stage of cleaning and disinfecting should be a thorough rinse with hot water. Delivery of liquid egg products from the compartment of a tanker should be to one point only.

4.4.7 **Defrosting of frozen egg products**

4.4.7.1 When frozen egg products are being defrosted, they should be brought to their liquid state as quickly as possible without causing deterioration, but with as little increase of the temperature of the product above 0°C (32°F) as possible.

4.4.7.2 Defrosted egg products should be used immediately.

4.4.8 **Marking of containers**

All containers should be so marked as to identify the place and date of manufacture of the product.

4.5 **Hygiene Control Programme**

It is desirable that each plant in its own interest designate a single individual, whose duties are preferably divorced from production, to be held responsible for the cleanliness of the plant. The staff should be a permanent part of the organization and should be well trained in the use of special cleaning tools, in methods of disassembling equipment for cleaning, and in the significance of contamination and the hazards involved. Critical areas, equipment and materials should be designated for specific attention as part of a permanent sanitation schedule.

4.6 **Laboratory Control Procedures**

Appropriate methods of sampling and microbiological examination may be utilized to ensure the absence of *salmonellae* from the product and to test for the effectiveness of time/temperature combinations or other means of pasteurization or for the possibility of post-pasteurization contamination.

The alpha amylase test, which has been found to be valuable as an immediate indication of the attainment of a specific time/temperature relationship, may be used as an index of this attainment.

In addition to any control by the official agency having jurisdiction, it is desirable that each plant in its own interest should have access to laboratory control of the sanitary quality of the products processed. Such control should reject all food that is unfit for human consumption.

5. **SECTION V - END PRODUCT SPECIFICATIONS**

Microbiological, chemical, physical or extraneous materials specifications may be required depending upon the nature of the product. Such specifications should include sampling procedures, analytical methodology, etc., as required for the particular product (see Annex II).

Where used as an indication of specific time/temperature relationships the alpha amylase test should be negative. The product should meet microbiological criteria which will be attached at a later date.

ANNEX I

THE ALPHA AMYLASE TEST

The alpha amylase test in relation to the heat treatment of whole egg is analogous to the phosphatase test which is used for testing the efficiency of the pasteurization of milk. It depends on the fact that heat destroys the alpha amylase activity in whole egg in proportion to the degree of heat treatment given.

The temperature and holding time for the pasteurization of bulked liquid egg is not less than 64°C (148°F) for two and a half minutes, a time and temperature combination which is lethal to *salmonella* organisms.

When untreated whole egg is mixed with a starch solution the alpha amylase present degrades the starch so that the normal blue violet coloration which occurs when iodine and starch are mixed does not develop. The intensity of the blue violet colour varies inversely with the amount of alpha amylase present. The alpha amylase test is therefore a test of the degree of heat treatment given to the whole egg mixture when it is pasteurized, and provides evidence that a satisfactory time/temperature combination has, or has not, been reached.

This Annex is designed to help those who may be required to carry out the test on liquid whole egg.

THE TEST

1. **The Examination of the Sample**

The sample of liquid egg should be tested as soon as possible after receipt at the testing laboratory, but it must be allowed to come to room temperature immediately before the test.

If the sample of liquid egg has to be stored before testing it should be kept below 40°F (approximately 4.5°C) and later brought to room temperature before carrying out the test.

Any samples which show signs of decay, or evidence of deterioration, should not be tested.

A sample which contains any sugar, citric acid, or salt of citric acid, or any substance which contains sugar, citric acid or any such salt, should not be sent for testing as these substances interfere with the reaction.

2. **Precautions**

The following precautions must be taken:

(a) distilled or de-ionized water must be used in the preparation of reagents or in the dilution of reactants;
(b) contamination of liquid egg or reagents with saliva must be avoided;
(c) all glassware must be clean and dry before use;

(d) a fresh pipette must be used for each sample of liquid egg;
(e) pipettes must not be contaminated with saliva;
(f) in the event of a sample failing to pass the test, any glassware which has come

into contact with the liquid egg must be sterilized and cleaned as laid down in Section 5.

3. **Reagents**

(a) **Starch solution.** Different starches give a slight variation in performance which may affect both the shade and intensity of the colour that is produced. This variation does not in any way affect the basis of the test. The starch solution should be prepared as follows:

. Weigh an amount of analytical reagent quality soluble starch equivalent to 0.70 g of dry starch. The moisture content of the starch should be determined by drying a sample at 100°C or 212°F for 16 hours (or at 160°C or 320°F for one hour).

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. Mix this quantity of starch to a thin cream with cold water. Transfer the whole quantity of this cream to about 50 ml of boiling water; boil for one minute and cool by immersion in cold water. Add three drops of toluene and dilute with water to 100 ml in volumetric flask.

. This solution must not be used if more than a fortnight old.

(b) **Solution of Iodine**

(i) **For immediate use**

Approximately milli-normal, as specified in the "British Pharmacopoeia" 1973, Appendix II-A.[1] This solution must be freshly made before use but may be made by dilution from a stronger solution with appropriate adjustment of potassium iodide concentration.

(ii) **Stronger stock solutions**

The iodine solution can be made from 12.7 g of iodine dissolved in a solution of 25 g of potassium iodide in 30 ml of distilled water to give an approximate N/10 solution. The potassium iodide solution can be made from 335 g of potassium iodide dissolved and made up to 1 litre with distilled water. Immediately before the test 1 ml of each solution (iodine and potassium iodide) are mixed and made up to 100 ml with distilled water which give the approximate milli-normal solution for use.

(c) **Solution of trichloroacetic acid**

15 per cent weight in volume aqueous solution of trichloroacetic acid of analytical reagent quality.

4. **Apparatus**

The following may be used:

[1] Iodine 0.001 N
Iodine and potassium iodide dissolved in water to contain in 1000 ml the following quantities of I and KI: 0.1269 g I and 3.6 g KI.

(a) Graduated pipettes Grade B bulb 2 ml, 5 ml, and 10 ml, or Grade B bulb 2 ml and Grade A 10 ml straight-sided.
(b) Grade B volumetric flasks of 100 ml and 1,000 ml capacity.
(c) A 50 ml measuring cylinder.
(d) Filter funnels of 3-4 in. diameter.
(e) Whatman No. 12 fluted filter papers of 12.5 cm diameter or equivalent.
(f) Wide neck conical flasks of 100 ml capacity and/or universal containers.
(g) Test tubes approximately 7" X 1".
(h) Burettes and automatic syringes may be used for measuring iodine, trichloroacetic acid, and distilled water.
(i) A water bath capable of maintaining at a temperature of 44^{o}C ± 0.5^{o}C (111.2^{o}F ± 0.9^{o}F).

5. Cleaning and Care of Apparatus

The cleaning and care of apparatus is especially important.

(a) After use all glassware should be rinsed in water and adhering egg washed off, if necessary with N/10 sodium hydroxide. The glassware must then be washed with chromic or dilute hydrochloric acid, followed by a thorough rinsing with water and distilled water.
(b) Apparatus used for samples which have failed the test should be sterilized in a bactericidal solution of hypochlorite or carbolic acid before cleaning.
(c) New glassware should be cleaned by soaking in chromic or dilute hydrochloric acid solution and then rinsed in warm water, rinsed in distilled water, and finally dried.
(d) Glassware used for the test shall not be used for any other purpose and must be kept apart from all other apparatus in the laboratory.
(e) Traces of egg, protein or detergent may cause false failure.

6. Method

Weigh out 15.0 g of sample of liquid egg into a 100 ml conical flask or universal container, or a 7" X 1" boiling tube can be used if stoppered.

Add. 2.0 ml of the starch solution and mix thoroughly.

If the egg is at all viscous, it may be difficult to ensure that the egg and starch are properly mixed. As this is essential the egg and starch should be mixed as well as possible before, during and after incubation.

Place the mixture in the water bath for 30 minutes at 44^{o}C ± 0.5^{o}C. Remove from the water bath, shake, and with the minimum of delay add 5 ml of this mixture to 5 ml of trichloroacetic acid solution contained in a conical flask, large test tube or universal container. Shake and mix thoroughly again. Add. 15 ml water and shake and mix again.

Remove the suspended matter by filtration or centrifugation. Add 10 ml of the clear filtrate (after rejecting the first runnings), or the supernatant liquor, as the case may be, to 2 ml of the solution of iodine.

7. Interpretation

A standard Lovibond Comparator Disc 4/26 containing seven reference colour standards, and designed for use with a Special Purposes Comparator and 25 mm cells may be

used for determining the colour.

There are many intershades between blue and violet and those on the standard disc indicate the likely range.

The sample shall be deemed to have passed the alpha amylase test if the filtrate, or liquor in the solution of iodine immediately turns a blue violet colour. For this purpose colours which are more blue-violet than No. 3 on the Standard Lovibond Comparator Disc 4/26, or of a comparable spectrophotometric standard, shall be taken as satisfactory. With 1 cm cells using a wave length of 585 mμ the comparable spectrophotometric standard, compared against water, has an optical density of 0.15.

For comparative test north light or fluorescence should be used.

When samples fail they should be re-tested immediately together with heated controls. When failures are confirmed samples should be examined for *salmonellae*.

ANNEX II

SECTION V - END PRODUCT SPECIFICATIONS

MICROBIOLOGICAL SPECIFICATIONS FOR PASTEURIZED EGG PRODUCTS

The present microbiological specifications for egg products contain:

(1) Number of field samples from a lot.[1]

(2) Sampling methods.

(3) Reference methods for the detection of *Salmonellae*, and for the enumeration of mesophilic aerobic bacteria and coliform bacteria.

(4) Microbiological sampling plans and limits.

1. Number of field samples from a lot

1.1 Dried whole eggs

Take 10 field samples, all of which are used for the detection of *Salmonellae*, and select at random 5 of these field samples to be examined also for mesophilic aerobic bacteria and coliform bacteria.

1.2 Frozen whole eggs

Take 10 field samples, all of which are used for the detection of *Salmonellae*, and select at random 5 of these field samples to be examined also for mesophilic aerobic bacteria and coliform bacteria.

1.3 Other egg products

Take 10 field samples, all of which are used for the detection of *Salmonellae*.

2. Sampling methods

For all egg products take field samples of at least 200 grammes[2].

2.1 Dried whole eggs[3]

[1] A lot is a quantity of food produced under identical conditions, all packages of which should bear a lot number that identifies the production during a particular time interval, and usually from a particular "line" or other critical processing unit.

[2] For further information see International Commission on Microbiological Specifications for Foods (1974) **Microorganisms in Foods II. Sampling for Microbiological analysis: principles and specific applications**. Toronto, University of Toronto Press.

[3] For further information see "**Official Methods of the Association of Official Analytical Chemists**" (12th Ed., 1975, sections 46.003 and 46.004).

Equipment. Sterile grain trier long enough to reach to the bottom of containers to be sampled. Sterile sample containers with tight closures, sterile spoon, alcohol lamp or other burner, alcohol, cotton, clean cloth or towel and water pail.

Method. For small packages, randomly take one unopened package for each of the required number of field samples required. For larger containers, such as boxes, bags, etc., remove top layer with sterile spoon or other sterile instrument, and with a sterile trier, remove at least 3 cores from the centre, midway between the centre and periphery and from the periphery respectively. Aseptically transfer the cores to a sterile sample container. Samples should be stored in a refrigerated or a cool place until analysis takes place.

2.2 **Frozen whole eggs**[1]

Equipment

(a) Electric or hand drill with a sterile 40 x 2.5 cm auger, hammer and steel strip 30 x 5 x 0.5 cm or other suitable tool for opening cans, sterile spoon, precooled sterile containers (screw-cap jars or friction-top cans), alcohol lamp or other burner, alcohol, cotton, clean cloth or towel, and water pail.

(b) It is advisable when using an electrically powered drill when sampling to fit a baffle on the drill to prevent aerial contamination of the product.

Method

(a) Drill 3 cores from top to bottom of container: first core in centre, second core midway between centre and periphery, and third core near edge of container. Transfer drillings from container with a sterile spoon to a prechilled sample container.

(b) Keep field samples refrigerated with solid CO_2 or other suitable refrigerant, if analysis is to be delayed or sampling point is at some distance from laboratory.

2.3 **Other egg products**

Proceed as for dried or frozen egg products, whichever is appropriate.

3. REFERENCE METHODS

3.1 EGG PRODUCTS - DETECTION OF *SALMONELLAE* (REFERENCE METHOD)

1. **SCOPE**

A Reference Method for the detection of *Salmonellae* (including *Arizona* but excluding *Salmonella typhi*) in egg products.

2. **FIELD OF APPLICATION**

The method can be applied to egg products covered by the Recommended International Code of Hygienic Practice for Egg Products.

[1] For further information see "**Official Methods of the Association of Official Analytical Chemists**" (12th Ed., 1975, sections 46.003 and 46.004).

3. REFERENCE

Modification of ISO/DIS 3565.[1]

4. DEFINITIONS

4.1 *Salmonellae*: Micro-organisms which form typical colonies on solid selective media and which possess the biochemical and serological characteristics described when the test is carried out according to this method.

4.2 Detection of *Salmonellae*: Determination of the presence or absence of these microorganisms, in a particular mass, when the test is carried out according to the method described.

5. PRINCIPLE

The detection of *Salmonellae* necessitates four successive stages, because they are usually present in low numbers and often in the presence of considerably larger numbers of other members of *Enterobacteriaceae*.

5.1 **Pre-enrichment**: incubating the samples in a non-selective liquid medium at 37°C.

5.2 **Enrichment**: incubated pre-enrichment media of samples from a single lot are incubated in groups of ten into single flasks of each of two liquid selective media.

5.3 **Plating out**: inoculation of the two enrichment media onto solid, selective diagnostic media which, after incubation at 37°C, are examined for the presence of colonies which by their characteristics are considered presumptive *Salmonellae*.

5.4 **Confirmation**: subculturing of colonies of presumptive *Salmonellae* and determining their appropriate biochemical and serological characteristics.

6. CULTURE MEDIA, DILUENTS AND REAGENTS

6.1 Basic materials

6.1.1 For uniformity of results, it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or a dehydrated complete medium, be used. The water used shall be distilled water or water of at least equivalent purity.

6.1.2 The manufacturers' instructions should be rigorously followed when dehydrated complete media are used.

NOTE - With regard to brilliant-green, note the specifications given in the Annex (Section 12).

[1] ISO 3565: Meat and meat products. Detection of *Salmonellae* (Reference Method), 1975.

6.2 Culture media

6.2.1 *BUFFERED PEPTONE WATER*

Composition

peptone	10.0 g
sodium chloride	5.0 g
disodium hydrogen phosphate ($Na_2 HPO_4.12H_20$)	9.0 g
potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
water	1 000 ml

Preparation

Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it is 7.0 ± 0.1 at 20°C. Transfer the medium in quantities of 225 ml into bottles of 500 ml capacity. Sterilize the medium for 20 min at 121 ± 1°C.

6.2.2 *TETRATHIONATE MEDIUM (MULLER KAUFFMANN)*

6.2.2.1 Base

Composition

meat extract	5.0 g
peptone	10.0 g
sodium chloride	3.0 g
calcium carbonate	45 g
water	1 000 ml

Preparation

Add the dehydrated base components or the dehydrated complete base to the water and boil until complete dissolution of the soluble components. Adjust the pH so that after sterilization it is 7.0 ± 0.1 at 20°C. Sterilize the base for 20 min at 121 ± 1°C.

6.2.2.2 Sodium Thiosulphate Solution

Composition

sodium thiosulphate ($Na_2 S_2 0_3. 5H_20$)	50.0 g
water to a final volume of	100 ml

Preparation

Dissolve the sodium thiosulphate in a part of the water. Dilute to the final volume. Sterilize the solution for 20 min at 121 ± 1°C.

6.2.2.3 Iodine Solution

Composition

iodine	20.0 g
potassium iodide	25.0 g
water to a final volume of	100 ml

Preparation

Dissolve the potassium iodide in a minimal volume of water and add the iodine. Shake till complete solution and dilute to the final volume. Store the solution in a tightly closed opaque container.

6.2.2.4 **Brilliant Green Solution**

Composition

brilliant green	0.5 g
water	100 ml

Preparation

Add the brilliant green to the water. Store the solution at least for one day in the dark to allow auto-sterilization to occur.

6.2.2.5 **Ox Bile Solution**

Composition

ox bile, desiccated	10.0 g
water	100 ml

Preparation

Dissolve the desiccated ox bile in the water by boiling. Sterilize the solution for 20 min at 121 ± 1°C.

6.2.2.6 **Complete Medium**

Composition

base (6.2.2.1)	900 ml
sodium thiosulphate solution (6.2.2.2)	100 ml
iodine solution (6.2.2.3)	20 ml
brilliant green solution (6.2.2.4)	2 ml
ox bile solution (6.2.2.5)	50 ml

Preparation

Add to the base, under aseptic conditions, the other ingredients in the above-mentioned order. Mix the liquids well after each addition. Transfer the complete medium in quantities of 1 000 ml aseptically into sterile bottles. Store it at 4°C in the dark until needed but use it within one week after preparation.

6.2.3 *SELENITE CYSTINE BROTH*

6.2.3.1 **Base**

Composition

tryptone	5.0 g
lactose	4.0 g
disodium hydrogen phosphate ($Na_2 HPO_4 . 12H_20$)	10.0 g
sodium acid selenite	4.0 g
water	1 000 ml

Preparation

Dissolve the ingredients with the exception of sodium acid selenite in the water by boiling for 5 min. After cooling add the sodium acid selenite. Adjust the pH to 7.0 ± 0.1 at 20°C store at 4°C.

6.2.3.2 **L-Cystine Solution**

Composition

L-cystine	0.1 g
N sodium hydroxide (NaOH)	15 ml

Preparation

Dilute to 100 ml with distilled water, do not autoclave.

6.2.3.3 **Complete Medium**

Add L-cystine solution to base at the rate of 0.1 ml per 10 ml of base. Adjust pH to 7.0 ± 0.1 at 20°C. Transfer the complete medium in quantities of 1 000 ml aseptically to sterile bottles. Use the medium on the day of preparation.

6.2.4 *BRILLIANT-GREEN/PHENOL RED AGAR (EDEL AND KAMPELMACHER)*

6.2.4.1 **Base**

Composition

meat extract	4.0 g
peptone	10.0 g
sodium chloride	3.0 g
disodium hydrogen phosphate ($Na_2 HPO_4 . 12H_20$)	0.8 g
sodium dihydrogen phosphate (NaH_2PO_4)	0.6 g
agar, readily soluble[1]	12.0 g
water	900 ml

[1] Use Oxoid No. 1 Agar or similar product.

Preparation

Dissolve the dehydrated base components or the dehydrated complete base in the water by boiling. Adjust the pH so that after sterilization it is 7.0 ± 0.1 at 20°C. Transfer the base to tubes or bottles of not more than 500 ml capacity. Sterilize the base for 15 min at 121 ± 1°C.

6.2.4.2 **Sugar/Phenol Red Solution**

Composition

lactose	10.0 g
sucrose	10.0 g
phenol red	0.09 g
water to a final volume of	100 ml

Preparation

Dissolve the ingredients in the water. Heat in a water bath for 20 min at 70°C. Cool to 55°C and use immediately.

6.2.4.3 **Brilliant Green Solution**

For composition and preparation of this solution, see 6.2.2.4.

6.2.4.4 **Complete Medium**

Composition

base (6.2.4.1)		900 ml
sugar/phenol red solution (6.2.4.2)	100 ml	
brilliant green solution (6.2.4.3)	1 ml	

Preparation

Under aseptic conditions, add the brilliant green solution to the sugar/phenol red solution cooled to approximately 55°C. Add to the base at 50 to 55°C and mix.

6.2.4.5 **Preparation of Agar Plates**

Add to sterile large-size Petri dishes (7.2.5.1) about 40 ml of the freshly prepared complete medium (6.2.4.4) having a temperature of approximately 45°C, and allow to solidify. (When large Petri dishes are not available, transfer about 15 ml of the melted medium (6.2.4.4) to sterile small Petri dishes (7.2.5.2) and allow to solidify).

Immediately before use, dry the plates carefully, preferably with the lids off and the agar surface downwards, in an oven or incubator at 50 ± 5°C for 30 min. If prepared in advance, the undried plates shall not be kept longer than 4 h at room temperature or one day in a refrigerator.

6.2.5 ***BISMUTH SULFITE AGAR (WILSON AND BLAIR, MODIFIED)***

Composition

beef extract	5.0 g

peptone or polypeptone	10.0 g
glucose	5.0 g
disodium hydrogen phosphate ($Na_2 HPO_4 . 12H_20$)	4.0 g
ferrous sulphate ($FeSO_4 . 7H_2O$)	0.3 g
bismuth sulphite	8.0 g
brilliant green 0.025 g	
agar 20.0 g	
water	1 000 ml

Preparation

Disperse the components or the dehydrated complete medium in water and boil with frequent agitation to dissolve soluble materials. Cool to 40-45°C, do not autoclave. Final pH should be approximately 7.7.

6.2.5.1 **Preparation of Agar Plates**

Add to sterile large-size Petri dishes (7.2.5.1) about 40 ml of the freshly prepared complete medium (6.2.5) and allow to solidify. (When large Petri dishes are not available, transfer about 15 ml of the melted medium (6.2.5) to small sterile Petri dishes (7.2.5.2) and allow to solidify). Store in a refrigerator and do not use before 24 h storage or after 5 days storage.

6.2.6 ***NUTRIENT AGAR***

Composition

meat extract	3.0 g
peptone 5.0 g	
agar	12.0 g
water	1 000 ml

Preparation

Dissolve the dehydrated medium components or the dehydrated complete medium in the water by boiling. Adjust the pH so that after boiling it is 7.0 ± 0.1 at 20°C. Transfer the culture medium to tubes or bottles of not more than 500 ml capacity. Sterilize the medium for 20 min at 121 ± 1°C.

6.2.6.1 **Preparation of Agar Plates**

Transfer about 15 ml of the melted medium (6.2.6) to sterile small Petri dishes (7.2.5.2) and proceed as in 6.2.4.5.

6.2.7 *TRIPLE SUGAR/IRON AGAR (TSI AGAR)*

Composition

meat extract	3.0 g
Yeast extract	3.0 g
peptone 20.0 g	
sodium chloride	5.0 g
lactose	10.0 g
sucrose	10.0 g
glucose	1.0 g
iron (III) citrate	0.3 g
sodium thiosulphate	0.3 g
phenol red	0.024g
agar	12.0 g
water	1 000 ml

Preparation

Dissolve the dehydrated medium components or the dehydrated complete medium in the water by boiling. Adjust the pH so that after sterilization it is 7.4 ± 0.1 at 20°C. Transfer the medium in quantities of 10 ml to tubes of diameter 17 to 18 mm. Sterilize the medium for 10 min at 121 ± 1°C. Allow to set in a sloping position to give a butt of depth 2.5 cm.

6.2.8 *UREA AGAR (CHRISTENSEN)*

6.2.8.1 **Base**

Composition

peptone 1.0 g	
glucose	1.0 g
sodium chloride	5.0 g
potassium dihydrogen phosphate (KH_2PO_4)	2.0 g
phenol red	0.012g
agar	15.0 g
water	1 000 ml

Preparation

Dissolve the dehydrated base components or the dehydrated complete base in the water by boiling. Sterilize the base for 20 min at 121 ± 1°C.

6.2.8.2 **Urea Solution**

Composition

urea 400 g	
water to a final volume of	1 000 ml

Preparation

Dissolve the urea in the water. Sterilize by filtration and check sterility. (For details of the technique of sterilization by filtration, reference should be made to any appropriate textbook on microbiology).

6.2.8.3 **Complete Medium**

Composition

base (6.2.8.1)	950 ml
urea solution (6.2.8.2)	50 ml

Preparation

Under aseptic conditions, add the urea solution to the base. Adjust the pH so that it is 6.8 ± 0.1 at 20°C. Transfer the complete medium in quantities of 10 ml to sterile tubes. Allow to set in a sloping position.

6.2.9 ***SEMI-SOLID NUTRIENT AGAR***

Composition

meat extract	3.0 g	
peptone	5.0 g	
agar	4.0-8.0 g	(depending on the "gel strength" obtained)
water	1 000 ml	

Preparation

Dissolve the dehydrated base components in the water by boiling. Adjust the pH so that after sterilization it is 7.0 ± 0.1 at 20°C. Transfer the medium to bottles of not more than 500 ml capacity. Sterilize the medium for 20 min at 121 ± 1°C.

Preparation of agar plates

Add to sterile small Petri dishes (7.2.5.2) about 15 ml of the freshly prepared complete medium (6.2.9). The plates shall not be dried.

6.2.10 ***SALINE SOLUTION***

Composition

sodium chloride	8.5 g
water	1 000 ml

Preparation

Dissolve the sodium chloride in the water by boiling. Adjust the pH so that after sterilization it is 7.0 ± 0.1 at 20°C. Transfer such quantities of the solution to bottles or tubes so that they will contain 90 to 100 ml after sterilization. Sterilize the solution for 20 min at 121 ± 1°C.

6.2.11 ***LYSINE DECARBOXYLATION MEDIUM***

Composition

l-lysine monohydrochloride	5.0 g
yeast extract	3.0 g
glucose	1.0 g
bromocresol purple	0.015 g
water	1 000 ml

Preparation

Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it is 6.8 ± 0.1 at 20°C. Transfer the medium in quantities of 5 ml to narrow culture tubes approximately 8 mm in diameter and 160 mm in length for anaerobic conditions. Sterilize the medium for 10 min at 121 ± 1°C.

6.2.12 ***ß-GALACTOSIDASE REAGENT (ONPG Text)***

6.2.12.1 **Buffer Solution**

Composition

sodium dihydrogen phosphate (NaH_2PO_4)	6.9 g
sodium hydroxide, approximately 0.1 N (4 g/l) solution	3 ml
water to a final volume of	50 ml

Preparation

Dissolve the sodium dehydrogenphosphate in approximately 45 ml of water. Adjust the pH to 7.0 ± 0.1 with approximately 3 ml of the sodium hydroxide solution. Add water to a final volume of 50 ml. Store under refrigeration.

6.2.12.2 **ONPG Solution**

Composition

o-nitropheny ß-D-galactopyranoside (ONPG)	80 mg
water	15 ml

Preparation

Dissolve the ONPG in the water at 50°C. Cool the solution.

6.2.12.3 **Complete Reagent**

Composition

Buffer solution (6.2.12.1)	5 ml
ONPG solution (6.2.12.2)	15 ml

Preparation

Add the buffer solution to the ONPG solution. Store the complete reagent at 4°C but not for longer than one month.

6.2.13 ***VOGES-PROSKAUER REACTION (RAPID METHOD BY BARRY AND FEENEY)***

6.2.13.1 **VP Medium**

Composition

peptone	7.0 g
glucose	5.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5.0 g
water	1 000 ml

Preparation

Dissolve the components in the water. Adjust the pH to 6.9 and filter. Sterilize the medium for 20 min at 115°C.

6.2.13.2 **Creatine Solution**

Composition

creatine monohydrate	0.5 g
water	100 ml

Preparation

Dissolve the creatine monohydrate in the water.

6.2.13.3 **a-Naphthol Reagent**

Composition

a-naphthol	6 g
ethanol, 96% (v/v)	100 ml

Preparation

Dissolve the a-naphthol in the ethanol.

6.2.13.4 **KOH Reagent**

Composition

potassium hydroxide	40 g
water	100 ml

Preparation

Dissolve the potassium hydroxide in the water.

6.2.14 ***INDOL REACTION***

6.2.14.1 **Tryptone Medium**

Composition

Tryptone	10 g
sodium chloride	5 g
water	1 000 ml

Preparation

Dissolve the components in the water. Sterilize for 20 min at 121 ± 1°C.

6.2.14.2 **Reagent (Kovacs)**

Composition

p-dimethylaminobenzaldehyde	5 g
hydrochloric acid, ? 1.19 g/ml	25 ml
tert amyl alcohol	75 ml

Preparation

Mix the components.

6.3 **Sera**

Several anti-*Salmonella* sera may be obtained commercially, i.e. anti-sera containing one or more "O" groups (so called mono-or polyvalent anti O-sera), and anti-sera containing one or more "H" groups (so called mono-or polyvalent anti H-sera). The precise description may vary and careful reading of the labels is advised. The sera should be certified for potency and specificity by an appropriate controlling authority.

7. APPARATUS AND GLASSWARE

7.1 Apparatus

7.1.1 Mechanical blender, operating at not less than 8 000 rev/min and not more than 45 000 rev/min, with glass or metal blending jars of an appropriate capacity, fitted with lids and resistant to the conditions of sterilization.

7.1.2 Apparatus for sterilization of glassware, blender jars, culture media, etc. and equipment for filter sterilization, for example asbestos pad, membrane filter, or filter candle of a suitable porosity.

7.1.3 Drying cabinet, oven or incubator for drying the surface of agar plates preferable at 50 ± 5°C.

7.1.4 Incubator for maintaining the inoculated liquid media, plates and tubes at 37° ± 1°C.

7.1.5 Incubator or water bath for maintaining inoculated liquid media at 42 to 43°C.

7.1.6 Water baths for heating and cooling solutions and culture media to the appropriate temperatures.

7.2 **Glassware**

7.2.1 The glassware shall be resistant to repeated sterilization.

7.2.2 Culture tubes and bottles for sterilization and storage of culture media, and culture tubes 8 mm in diameter and 160 mm in length for lysine decarboxylation medium (6.2.11).

7.2.3 Measuring cylinder of 100 ml capacity, subdivided in 10 ml, for preparation of the complete media.

7.2.4 Graduated pipettes with a nominal capacity of 10 ml and 1 ml, subdivided respectively in 1.0 and 0.1 ml.

7.2.5 **PETRI DISHES**

7.2.5.1 Large-Size Dish

Dish

external diameter	140 ± 2 mm
external height	30 ± 2 mm
glass thickness	1.5 ± 0.5 mm

The rim shall be ground in a plane parallel to the base. The bottom of the dish shall be flat and parallel to the base.

Lid

external diameter	150 ± 2 mm
external height	15 ± 2 mm
glass thickness	1.5 ± 0.5 mm

7.2.5.2 Small-Size Dish

Dish

internal diameter	90 ± 2 mm
external height, minimum	18 mm

The rim shall be ground in a plane parallel to the base. The bottom of the dish shall be flat and parallel to the base.

Lid

external diameter, maximum	102 mm

7.2.5.3 Alternatively, plastic Petri dishes may be used, even if of slightly different dimensions from

the glass dishes described in 7.2.5.1 and 7.2.5.2.

7.3 **Sterilization of glassware, etc.**

Sterilize glassware, etc. by one of the following methods:

- wet sterilization at not less than 121°C for not less than 20 min;

- dry sterilization at not less than 170°C for not less than 1 h.

8. **SAMPLING**

8.1 Proceed from 200 g field samples (see paras 1 and 2 at beginning of Annex II).

8.2 The frozen field samples shall be kept frozen until analysis.

9. **PROCEDURE**

9.1 **Pre-treatment of the sample**

Field samples of dried eggs shall be well mixed by shaking before the sample units are withdrawn. Field samples of frozen eggs should be thawed by placing them in cold running water only long enough to completely thaw them. The thawed field sample shall be well mixed by shaking, before removal of the sample units.

9.2 **Sample unit**

Weigh 25 g of the mixed field sample (9.1) into a sterile blender jar (7.1.1).

9.3 **Blending**

9.3.1 Add 225 ml of the buffered peptone water (6.2.1) to the jar.

9.3.2 Operate the blender according to its speed, for sufficient time to give a total number of 15 000 to 20 000 revolutions. Thus, even with the slowest blender, this time will not exceed 2.5 min.

9.4 **Pre-enrichment**

9.4.1 Transfer the contents of the blender jar aseptically to a sterile 500 ml bottle.

9.4.2 Incubate the bottle at 37 ± 1°C for not less than 16 h and not more than 20 h.

9.5 **Enrichment**

9.5.1 After the incubation period, transfer 10 ml from each of 10 bottles (9.4.2) to 1000 ml of tetrathionate medium (6.2.2), and 10 ml from each of the same 10 bottles to 1000 ml of selenite medium (6.2.3). Both enrichment broths should be warmed to 42-43°C prior to inoculation.

9.5.2 Incubate the inoculated tetrathionate and selenite media up to 2 days at 42 to 43°C. The temperature shall not exceed 43°C.

9.6 **Plating out**

9.6.1 After an incubation period of 18 to 24 h, streak from each bottle (9.5.2), using a loop with

a diameter of 2.5 to 3 mm, onto the surface of brilliant-green/phenol red agar plates (6.2.4) and to bismuth sulphite agar (6.2.5), so that well-isolated colonies are obtained. (When large Petri dishes are not available, two small Petri dishes may be streaked one after the other, using the same loop).

9.6.2 Incubate the plates with the bottom of the Petri dishes uppermost in an incubator at 37 ± 1°C.

9.6.3 After an incubation period of 2 days (see 9.5.2), repeat the plating out of the two enrichment media and place the plates in an incubator at 37 ± 1°C.

9.6.4 Examine the plates after an incubation of 20 to 24 h for the presence of typical colonies of *Salmonella*.

9.6.5 If growth is slight and no typical colonies of *Salmonella* are present, reincubate at 37 ± 1°C for a further 20 to 24 h.

Re-examine the plates for the presence of typical colonies of *Salmonella*.

NOTE - Subject any typical or suspect colony to a confirmation (9.7) because the recognition of colonies of *Salmonella* is to a large extent a matter of experience and their appearance may vary somewhat, not only from species to species of *Salmonella*, but also from batch to batch of medium. In this respect agglutination of colonies with an omnivalent *Salmonella*e antiserum may help to recognize suspected colonies.

9.7 Confirmation of presumptive *Salmonella* colonies

9.7.1 SELECTION OF COLONIES FOR CONFIRMATION

9.7.1.1 From each plate of each selective medium (see 9.6.1) select five typical or suspect colonies for confirmation.

9.7.1.2 If on one plate there are less than five typical or suspect colonies, take for confirmation all the typical or suspect colonies.

9.7.1.3 Streak the selected colonies onto the surface of nutrient agar plates (6.2.6), in a manner which will allow well-isolated colonies to develop.

9.7.1.4 Incubate the inoculated plates at 37 ± 1°C for 20 to 24 h.

9.7.1.5 Select isolated colonies for biochemical and serological confirmation.

9.7.2 BIOCHEMICAL CONFIRMATION

9.7.2.1 Inoculation and Incubation of Media

Inoculate the following media with the selected colonies (9.7.1.5) by means of an inoculating wire.

9.7.2.1.1 TSI agar (6.2.7)

Streak the agar slope surface and stab the butt. Incubate for 1 or 2 days at 37 ± 1°C. Interpret the changes in the medium as follows:

Butt

Yellow	.....	glucose converted
red or unchanged	.....	no conversion of glucose
black	.....	formation of hydrogen sulphide
bubbles or cracks	.....	gas formation from glucose

Slant surface

yellow	.....	lactose and/or sucrose converted
red or unchanged	.....	neither lactose nor sucrose converted.

9.7.2.1.2 Urea agar (6.2.8)

Streak the agar slope surface. Incubate for 1 or 2 days at 37 ± 1°C. Splitting of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later on to deep cerise.

9.7.2.1.3 Lysine decarboxilation medium (6.2.11)

Inoculate just below the surface of the liquid medium. Incubate for 1 day at 37 ± 1°C. A purple colour after growth has occurred indicates a positive reaction. A yellow colour indicates a negative reaction.

9.7.2.1.4 ß-galactosidase reagent (6.2.12)

Suspend a loopful of the suspected colony in 0.25 ml of the saline solution (6.2.10) in a tube. Add 1 drop of toluene. Put the tube in a water bath at 37 ± 1°C for several minutes. Then add 0.25 ml of the ß-galactosidase reagent and mix. Replace the tube in the water bath at 37 ± 1°C for 24 h (see Note). A yellow colour indicates a positive reaction.

NOTE - The reaction is often apparent after 20 min.

9.7.2.1.5 Voges-Proskauer reaction (6.2.13)

Inoculate two tubes by suspending a loopful of the suspected colony in 0.2 ml of the medium (6.2.13.1) in each tube. Incubate one tube at room temperature and the other at 37 ± 1°C for 48 h. After suspension, add to each tube 2 drops of the creatine solution (6.2.13.2), 3 drops of the ethanolic naphthol solution (6.2.13.3) and then 2 drops of the KOH reagent (6.2.13.4); shake after the addition of each reagent. A pink to bright red colour within 15 min indicates a positive reaction.

9.7.2.1.6 Indol reaction (6.2.14)

Inoculate a tube containing 5 ml of the medium (6.2.14.1) with the suspected colony. Incubate for 24 h at 37 ± 1°C. After incubation, add 1 ml of the indol reagent (6.2.14.2). The forming of a red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

9.7.2.2 Interpretation of the Results

Salmonellae show the following reactions[1]

[1] Edwards and Ewing, 1972.

Salmonella serotypes confirming[1]

TSI glucose (acid formation) (9.7.2.1.1)	+	100 %
TSI glucose (gas formation) (9.7.2.1.1)	+	91.9 %
TSI lactose (9.7.2.1.1)	-[2]	99.2 %
TSI sucrose (9.7.2.1.1)	-	99.5 %
TSI hydrogen sulphide (9.7.2.1.1)	+	91.6 %
Urea splitting (9.7.2.1.2)	-	100 %
Lysine decarboxylation (9.7.2.1.3)	+	94.6 %
ß-galactosidase reaction (9.7.2.1.4)	-[2]	98.5 %
Voges-Proskauer reaction (9.7.2.1.5)	-	100 %
Indol reaction (9.7.2.1.6)	-	98.9 %

9.7.3 **SEROLOGICAL CONFIRMATION**

Examine pure (9.7.1.5) non-auto-agglutinable (9.7.3.1) colonies for the presence of *Salmonella* 0 or H antigens by slide agglutination with sera according to the following procedure.

9.7.3.1 Elimination of Auto-Agglutinable Strains

Put on a carefully cleaned slide 1 drop of saline solution (6.2.10). Disperse in this drop an amount of the culture under test to obtain a homogeneous and turbid suspension. Rock the slide gently for 30 to 60 s. Observe the reactions against a dark background, preferably with the aid of a magnifying glass. The strains are considered auto-agglutinable if the bacteria have clotted to more or less distinct units. The serological confirmation of these auto-agglutinable strains by the procedures 9.7.3.2 and 9.7.3.3 is impossible.

9.7.3.2 Examination of 0-Antigens

Use pure (9.7.1.5) non-auto-agglutinable (9.7.3.1) colonies. Proceed according to 9.7.3.1, using anti-0 serum (6.3) instead of saline solution. The mono- or polyvalent sera shall be used one after another.

9.7.3.3 Examination of the H-Antigens

Inoculate the semi-solid nutrient agar (6.2.9) with a pure non-auto-agglutinable (9.7.3.1) colony. Incubate the medium for 18 to 24 h at 37 ± 1°C. Use this culture for the examination of the H-antigens according to the procedure in 9.7.3.1 but using a drop of anti- H serum (6.3) instead of saline solution.

9.7.4 **INTERPRETATION**

9.7.4.1 Strains which show typical biochemical reactions (9.7.2) and give positive serological reactions according to 9.7.3.2 or 9.7.3.3 are considered to be *Salmonellae*.

9.7.4.2 Strains which show typical biochemical reactions (9.7.2) but do not give positive serological reactions according to 9.7.3.2 or 9.7.3.3, strains which do not show typical

[1] These percentages are still under review. They only indicate that not all strains of *Salmonellae* show the reactions as marked by + or -. These percentages may vary from food product to food product.

[2] The *Salmonella* subgenus III (Arizona) may give a positive lactose and ß-galactosidase reaction; the *Salmonella* subgenus II may give a negative lactose, but a positive ß-galactosidase reaction.

biochemical reactions (9.7.2), but give positive serological reactions according to 9.7.3.2 or 9.7.3.3, and auto-agglutinable (9.7.3.1) strains which show typical biochemical reactions (9.7.2), could be *Salmonellae*.

9.7.4.3 Strains which do not show typical biochemical reactions (9.7.2) and which do not give positive serological reactions according to 9.7.3.2 or 9.7.3.3 are not considered to be *Salmonellae*.

9.7.5 DEFINITIVE CONFIRMATION

Strains which are considered to be *Salmonellae* (9.7.4.1) or which may be *Salmonellae* according to 9.7.4.2, shall be sent to a recognized *Salmonella* Reference Centre for definitive typing. This dispatch shall be accompanied by all possible information concerning the strain(s).

10. EXPRESSION OF RESULTS

If *Salmonellae* after plating out (9.6) are detected in neither of the enrichment media, report: "No *Salmonellae* isolated from the 10 (or 30) sample units of product examined".

If *Salmonellae* after plating out (9.6) are detected in one or both of the enrichment media, report: "*Salmonellae* isolated from the 10 (or 30) sample units of product examined", and whether serotyping has been used: "The identified *Salmonellae* belong to the following types: ...".

11. TEST REPORT

11.1 Indicate the method of test by quoting this Reference Method.

11.2 Give the exact name of the Centre which helped to identify the strains.

12. ANNEX: SPECIFICATION FOR BRILLIANT GREEN

12.1 BACTERIOLOGICAL PERFORMANCE

Suppression of spreading of proteus on brilliant green/phenol red agar (6.2.4), while the growth of *salmonellae* is not inhibited.

12.2 METHOD OF TEST

12.2.1 Medium

Prepare brilliant green/phenol red agar according to 6.2.4 with various concentrations of brilliant green, viz. 4.5 mg/1 to 6 mg/1.

12.2.2 Procedure

Inoculate a set of plates with different brilliant green concentrations with a pure culture of a swarming proteus and another set with a pure culture of *Salmonella*, and incubate these plates at 37 ± 1°C for no longer than 24 h. A satisfactory concentration of the stain should allow growth of *Salmonella* with typical pink colonies, 1 to 2 mm in diameter, and limited growth of proteus, i.e. no spreading. The concentration of brilliant green which shows this pattern should be used for the preparation of the brilliant green solution (6.2.2.4).

3.2 EGG PRODUCTS - ENUMERATION OF MESOPHILIC AEROBIC BACTERIA (REFERENCE METHOD)

1. SCOPE

A Reference Method for enumeration of mesophilic aerobic bacteria in egg products.

2. FIELD OF APPLICATION

The method can be applied to dried or frozen whole egg products covered by the Recommended International Code of Hygienic Practice for Egg Products.

3. REFERENCE

Modification of ISO/TC 34/SC 9 method for enumeration of mesophilic aerobic bacteria.

4. DEFINITION

By "**mesophilic aerobic bacteria**" are meant micro-organisms growing aerobically at 30°C under the conditions described in the present method.

5. PRINCIPLE

5.1 Inoculation in Petri dishes of melted defined culture medium with the food homogenate (1 in 10) and decimal dilutions.

5.2 Incubation of this medium aerobically at 30°C for 72 h.

5.3 Calculation of the number of mesophilic aerobic bacteria per gramme of sample unit from the number of colonies obtained in selected Petri dishes at levels of dilution giving a significant result.

6. CULTURE MEDIA, DILUENTS AND REAGENTS

6.1 Basic materials

6.1.1 For uniformity of results, it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or a dehydrated complete medium, be used. The water used shall be distilled water or water of at least equivalent purity.

6.1.2 The manufacturers' instructions should be rigorously followed when dehydrated complete media are used.

6.1.3 If the media are not used on the day of preparation, keep them in darkness at +5°C for not more than one month, taking precautions to prevent evaporation.

6.2 Culture media

6.2.1 ***BUFFERED PEPTONE WATER***

Composition

peptone	10.0 g
sodium chloride	5.0 g
disodium hydrogen phosphate (Na_2HPO_4. $12H_20$)	9.0 g
potassium dihydrogen phosphate (K_2HPO_4)	1.5 g
water	1 000 ml

Preparation

Dissolve the components in water by boiling.
Adjust the pH so that after autoclaving it is 7.0 ± 0.1 at 20°C.
Transfer to tubes or dilution bottles in quantities of 9 ml.
Sterilize for 20 min at 121 ± 1°C.

6.2.2 ***PLATE COUNT AGAR***

Composition

dehydrated yeast extract	2.5 g
pancreatic digest of casein	5.0 g
glucose	1.0 g
powdered or flaked agar-agar	12 to 18 g depending on gelatinizing properties of the product
water	1 000 ml

Preparation

Dissolve, in boiling water, the components or the dehydrated complete medium. If necessary, adjust the pH so that after sterilization, it is 7.0 ± 0.2 at 20°C (Measurement performed at 45°C with temperature correction).

Distribute the medium in tubes (e.g. 18 mm x 180 mm), with 15 ml per tube, or in bottles not exceeding 500 ml, filling only about half of the volume of the bottle.

Sterilize in an autoclave at 121 ± 1°C. for 20 min.

Before beginning the analysis, to avoid delay in pouring the agar, melt the medium completely in a bath of boiling water and cool to 45-48°C, preferably in a water bath.

6.2.3 ***NON-NUTRITIVE AGAR, CALLED "WHITE AGAR"***

Composition

powdered or flaked agar-agar	12 to 18 g depending on the gelatinizing properties of the product.
water	1 000 ml

Preparation

Dissolve the agar-agar in boiling water.

If necessary, adjust the pH so that after sterilization it is 7.0 ± 0.2 at 20°C (Measurement performed at 45°C with temperature correction).

Distribute the agar in tubes (e.g. 18 mm x 180 mm), 4 ml per tube, or in 150 ml bottles, 100 ml per bottle.

Sterilize in an autoclave at 121 ± 1°C for 20 min.

Before beginning the analysis, to avoid delay in pouring the agar, melt the medium completely in a bath of boiling water, and cool to 45-48°C, preferably in a water bath.

7. APPARATUS AND GLASSWARE

Standard laboratory equipment, and especially:

7.1 Apparatus for sterilizing glassware, culture media, etc.

7.2 Incubator regulated to 30 ± 1°C.

7.3 Glass Petri dishes or plastic dishes, diameter 90 to 100 mm.

7.4 Culture tubes or bottles for sterilization and storage of culture media.

7.5 Total-flow pipettes, nominal capacity 1 ml and graduated in 0.1 ml.

Sterilization of the glassware

Sterilize the glassware by one of the following methods:

(a) dry sterilization at not less than 170°C for not less than 1 h.

(b) wet sterilization at not less than 120°C for not less than 20 min.

8. SAMPLING

8.1 Proceed from the 200 g field samples (see paras 1 and 2 at beginning of Annex II).

8.2 The frozen field samples must be kept frozen until analysis.

9. PROCEDURE

9.1 Preparation of the sample unit, of the Food Homogenate (1 in 10) and of the decimal dilutions

9.1.1 For the pre-treatment of the field sample, the sample unit and blending to obtain the food homogenate (1 in 10), refer to *Salmonella* reference method (Sections 9.1, 9.2 and 9.3 of para 3.1).

9.1.2 **DILUTION**

9.1.2.1 Mix the contents of the jar by shaking, and pipette (with 7.5) 1 ml into a tube containing 9 ml of dilution fluid (6.2.1).

9.1.2.2 Mix the liquids carefully by aspirating 10 times with a pipette.

9.1.2.3 Transfer with the same pipette 1.0 ml to another dilution tube containing 9 ml of dilution fluid, and mix with a fresh pipette.

9.1.2.4 Repeat steps 9.1.2.2 and 9.1.2.3 until the required number of dilutions are made. Each successive dilution will decrease the concentration 10-fold.

9.2 **Pour Plating**

9.2.1 Take two sterile Petri dishes (7.3). Transfer into each of these dishes, with a sterile pipette (7.5), 1 ml of the food homogenate (1 in 10).

9.2.2 Take two other sterile Petri dishes. With a new sterile pipette, transfer to each of these dishes 1 ml of the contents of the first dilution tube.

9.2.3 Carry out the same operation from the last dilution tube. Pour into each Petri dish 15 ml of medium (6.2.2). The time elapsing between commencing to prepare the dilutions and pouring the agar into the dishes must not exceed 15 min. Carefully mix the inoculum with the medium and allow the latter to solidify by placing the Petri dishes on a cool horizontal surface. Where it is suspected that the product to be analyzed contains bacteria whose colonies are likely to spread over the surface of the media, pour onto the surface of the inoculated agar, after the latter has solidified, about 4 ml of medium (6.2.3) to provide a layer of approximately 2 mm in thickness. Allow the medium to solidify.

9.3 **Incubation of the dishes**

Invert the prepared dishes and place them in the incubator at 30 ± 1^{o}C (7.2).

9.4 **Counting the Colonies**

Examine the dishes after the prescribed incubation period. If this is not possible, they may be held at 4^{o}C for a maximum of 24 h. Count the colonies in each dish suitable for use in the calculation of the number of bacteria per gramme of product, in principle those containing between 30 and 300 colonies (unless exception, see section 9).

10. **EXPRESSION OF RESULTS**

10.1 **Method of calculation**[1]

Give the result as the number of mesophilic aerobic bacteria per gramme of dried or frozen whole egg product. Express it by a number in the range 1.0 to 9.9 multiplied by 10^{x}, x being the appropriate power of 10.

When counting, several situations may be encountered:

[1] New text from ISO document when available.

10.1.1 ***PRODUCTS CONTAINING RELATIVELY FEW MICROORGANISMS (TABLE 1)***

10.1.1.1 The dishes examined contain no colonies:

Give the result in the form: less than 1 x 10^1 bacteria per gramme of product, 10^1 being the inverse of the dilution of the food homogenate (ex. 1).

10.1.1.2 The dishes corresponding to the food homogenate (1 in 10) contain less than 30 colonies:

Give the result in the form: less than 3 x 10^2 bacteria per gramme of product (example 2).

10.1.2 ***OTHER PRODUCTS (TABLE II)***

10.1.2.1 General case: at least one dish exists which contains between 30 and 300 colonies (examples 3, 4 and 5).

Retain all dishes corresponding to the dilution or to the two successive dilutions in which this dish or these dishes are located.

For each dilution, calculate the average number of colonies. Retain only two significant digits. Thus, for a three-digit number, round off to the nearest zero. If the third digit is 5, round off to the lower zero.

Multiply the value obtained by the inverse of the corresponding dilution to obtain the number of bacteria per gramme of product.

In a case in which there are two values for the number of bacteria per gramme of product (as when two dilutions have been retained) average these two values if the ratio of the higher value to the lower value is less than 2. If not, retain the lower value.

10.1.2.2[1] Special cases: there are no dishes containing between 30 and 300 colonies.

If the numbers of colonies differ slightly from these limits at the level of two successive dilutions (example 6), proceed as for 10.1.2.1 (case for two retained dilutions).

If the dishes corresponding to one dilution contain spreading colonies, and if the number of colonies of the next dilution is lower than 30 (example No. 7), proceed with this dilution as for 10.1.2.1.

11. TEST REPORT

11.1 Indicate the method of test by quoting this Reference Method.

11.2 The test report shall give the information needed for complete identification of the sample.

[1] The text of this Section may be revised in the future.

TABLE I[1]

Examples	No. of colonies from 1 gramme of food homogenate (1 in 10)	Results (in no. of bacteria per g of product)	Explanation of Calculations
No. 1	0	fewer than 1 x 10 bacteria	$1 \times 10^1 = 1 \times 10$
No. 2	18 17	fewer than 3×10^2 bacteria	$30 \times 10^1 = 3 \times 10^2$

[1] **This Table is still under review.**

TABLE II[1]

Examples	Number of Colonies		Ratio	Results (in number of bacteria per gramme of product)	Explanation of Calculations
	dilution at $\frac{1}{100}$	dilution at $\frac{1}{1000}$			
No. 3	175 208	16 17	-	1.9×10^4	$175 + 208 = 383 : 2 = 191 \rightarrow 190 \rightarrow 190x10^2 = 1.9x10^4$
No. 4	322 278	23 29	- -	3×10^4	$322 + 278 = 600 : 2 = 300 \rightarrow 300x10^2 = 3x10^4$
No. 5	296 378	40 24	<2	3.3×10^4	$296 + 373 = 674 : 2 = 337 \rightarrow 340 \rightarrow 340x10^2 = 3.4x10^4$ $40 + 24 = 64 : 2 = 32 \rightarrow 32x10^3 = 3.2x10^4$ $\frac{3.4x10^4}{3.2x10^4} < 2$ $\rightarrow 10^4 x \frac{(3.4 + 3.2)}{2} = 3.3x10^4$
No. 6	327 330	18 25	<2	2.7×10^4	$327 + 330 = 657 : 2 = 328 \rightarrow 330 \rightarrow 330x\ 10^2 = 3.3x\ 10^4$ $18 + 25 = 43 : 2 = 21.5 \rightarrow 21x10^3 = 2.1x10^4$ $\frac{3.3x10^4}{2.1x10^4} < 2$ $\rightarrow 10^4 x \frac{(3.3 + 2.1)}{2} = 2.7x10^4$
No 7	spreaders spreaders	18 24			$18 + 24 = 42 : 2 = 21 \rightarrow 21x10^3 = 2.1x10^4$

[1] **This Table is still under review.**

3.3 EGG PRODUCTS - ENUMERATION OF COLIFORM BACTERIA: DETERMINATION OF THE MOST PROBABLE NUMBER (MPN) (*REFERENCE METHOD*)

1. SCOPE

A Reference Method for the detection of coliform bacteria in egg products.

2. FIELD OF APPLICATION

The method can be applied to dried or frozen whole egg products covered by the Recommended International Code of Hygienic Practice for Egg Products.

3. REFERENCE

Thatcher, F.S. and Clark, D.S., Ed. (1968): Microorganisms in foods. Their significance and Methods of Enumeration. Toronto, University of Toronto Press.

4. DEFINITION

Coliforms bacteria: microorganisms that form gas in the two media described below when the test is carried out according to the method.

5. PRINCIPLE

5.1 Enrichment

Inoculation in tubes of an enrichment medium with the food homogenate (1 in 10) and decimal dilutions.

Incubation of this medium at 37°C for 48 h.

5.2 Confirmation

From tubes with gas formation, inoculation in a confirmatory medium in tubes.

Incubation of these confirmatory tubes at 37°C for 48 h and calculation on basis of a table the most probable number of coliform bacteria per gramme of the egg product.

6. CULTURE MEDIA, DILUENTS AND REAGENTS

6.1 Basic materials

For uniformity of results, it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or a dehydrated complete medium, be used. The water used shall be distilled water or water of at least equivalent purity.

The manufacturers' instructions should be rigorously followed when dehydrated complete media are used.

If the media are not used on the day of preparation, keep them in darkness at +5°C for not more than one month, taking precautions to prevent evaporation.

6.2 Culture media

6.2.1 ***BUFFERED PEPTONE WATER***

COMPOSITION

peptone	10.0 g
sodium chloride	5.0 g
disodium hydrogen phosphate (Na_2HPO_4. $12H_2O$)	9.0 g
potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
water	1 000 ml

Preparation

Dissolve the components in water by boiling. Adjust the pH so that after autoclaving it is 7.0 ± 0.1 at 20°C. Transfer to tubes or dilution bottles in quantities of 9 ml. Sterilize for 20 min at 121 ± 1°C.

6.2.2 ***LAURYL SULPHATE TRYPTOSE BROTH***

Composition

tryptose, tryptone, or trypticase	20 g
lactose	5 g
potassium monohydrogen phosphate (K_2HPO_4)	2.75 g
potassium dihydrogen phosphate (KH_2PO_4)	2.75 g
sodium chloride	5 g
sodium lauryl sulphate	0.1 g
water	1 000 ml

Preparation

Dissolve ingredients in water and dispense in 10 ml volumes in tubes (e.g. 18 mm x 180 mm) (7.3) containing inverted Durham fermentation vials (10 mm x 75 mm) (7.4). Sterilize in an autoclave at 121°C for 10 minutes. Final pH should be approximately 6.8.

6.2.3 ***BRILLIANT GREEN LACTOSE BILE BROTH 2%***

NOTE: For preparation of ox bile solution and brilliant green solution, see *Salmonella* Reference Method.

Composition

peptone	10 g
lactose	10 g
ox bile	20 g
brilliant green	0.0133 g
water	1 000 ml

Preparation

Dissolve the peptone and lactose in 500 ml of water and add the ox bile dissolved in 200 ml of water. Bring the volume to approximately 975 ml with water and adjust the pH to 7.4.

Add 13.3 ml of a 1% aqueous solution of brilliant green bring the total volume to 1 litre, stir, and filter through cotton if necessary.

Disperse in 10 ml volumes into tubes (e.g., 18 mm x 189 mm) (7.3) containing inverted Durham fermentation vials (10 mm x 75 mm) (7.4). Sterilize in an autoclave at 121°C for 10 min.

7. APPARATUS AND GLASSWARE

Standard laboratory equipment, and especially:

7.1 Apparatus for sterilizing glassware, culture media, etc.

7.2 Incubator regulated to 37 ± 1°C.

7.3 Tubes for sterilization and storage of culture media.

7.4 Durham tubes (to be inserted in 7.3).

7.5 Total-flow pipettes, nominal capacity 1 ml and graduated in 0.1 ml.

Sterilization of the glassware

Sterilize the glassware by one of the following methods:

(a) dry sterilization at not less than 170°C for not less than 1 hour;
(b) wet sterilization at not less than 121°C for not less than 20 min.

8. SAMPLING

8.1 Proceed from the 200 g field samples (see paras 1 and 2 at beginning of Annex II).

8.2 The frozen field samples shall be kept frozen until analysis.

9. PROCEDURE

9.1 Preparation of the sample unit, of the food homogenate (1 in 10), and of the decimal dilutions.

9.1.1 For the pre-treatment of the sample, the sample unit and blending to obtain food homogenate (1 in 10), refer to *Salmonella* method (Sections 9.1, 9.2 and 9.3 of para 3.1).

9.1.2 DILUTION

9.1.2.1 Mix the contents of the jar by shaking, and pipette (with 7.5) 1 ml into a tube containing 9 ml of dilution fluid (6.2.1).

9.1.2.2 Mix the liquids carefully by aspirating 10 times with a pipette.

9.1.2.3 Transfer with the same pipette 1.0 ml to another dilution tube containing 9 ml of dilution fluid, and mix with a fresh pipette.

9.2 **Inoculation of enrichment medium**

9.2.1 Take three tubes of lauryl sulphate tryptose broth (6.2.2). Transfer into each of these tubes with a sterile pipette (7.5) 1 ml of the food homogenate (1 in 10).

9.2.2 Take three other tubes of lauryl sulphate tryptose broth (6.2.2). With a new sterile pipette, transfer to each of these tubes 1 ml of the contents of the first dilution tube.

9.2.3 Carry out the same operation from the last dilution tube.

9.3 **Incubation of the tubes**

Incubate tubes at 37 ± 1°C for 24 and 48 hours.

9.4 **Reading of enrichment tubes**

After 24 hours, record tubes showing gas production, and proceed to steep 9.5 for these tubes. Reincubate negative tubes and read these after 48 hours. Record tubes showing gas production, and proceed to step 9.5.

9.5 **Confirmation of coliforms**

Confirm that the tubes of lauryl sulphate tryptose broth selected in step 9.4 are positive for coliform bacteria by transferring a loopful of each to separate tubes of brilliant-green lactose bile broth 2% (6.2.3).

9.6 **Incubation of confirmatory tubes**

Incubate confirmatory tubes 48 hours at 37 ± 1°C and note gas production.

9.7 **Reading of confirmatory tubes**

The formation of gas confirms the presence of coliform bacteria.

9.8 **Recording the number of positive confirmatory tubes**

Record the number of enrichment tubes (9.4) in each dilution that were confirmed as positive for coliform bacteria.

If, for example, the number of positive tubes in the three dilutions were 3, 1, and 0, respectively, the results are recorded as 1:10 dilution = 3, 1:100 dilution = 1, and
1:1000 dilution = 0.

10. **EXPRESSION OF RESULTS**

10.1 **Method of calculation**

To obtain the most probable number (MPN) of coliform bacteria, proceed as follows:

10.1.1 Refer to the MPN table (Table 1) and note the MPN appropriate to the number of positive tubes. For example, in the illustration given in step 9.8 above, the values for each dilution are 3, 1 and 0 respectively. The table shows that these results indicate au MPN of 40 per gramme of the egg product.

11. TEST REPORT

11.1 Indicate the method of test by quoting this Reference Method.

11.2 The test report shall give the information needed for complete identification of the sample.

TABLE 1[1]

MOST PROBABLE NUMBER (MPN) OF COLIFORM BACTERIA IN EGG PRODUCTS PER GRAMME

Result	MPN	Confidence Limits 99%		Confidence Limits 95%	
0 1 0	3	<1	23	<1	17
1 0 0	4	<1	28	1	21
1 0 1	7	1	35	2	27
1 1 0	7	1	36	2	28
1 2 0	11	2	44	4	35
2 0 0	9	1	50	2	38
2 0 1	14	3	62	5	48
2 1 0	15	3	65	5	50
2 1 1	20	5	77	8	61
2 2 0	21	5	80	8	63
3 0 0	23	4	177	7	129
3 0 1	40	10	230	10	180
3 1 0	40	10	290	20	210
3 1 1	70	20	370	20	280
3 2 0	90	20	520	30	390
3 2 1	150	30	660	50	510
3 2 2	210	50	820	80	640
3 3 0	200	<100	1900	100	1400
3 3 1	500	100	3200	200	2400
3 3 2	1100	200	6400	300	4800

The above table contains only the most likely results which would be obtained in 95% of the cases with series of 5 tests. If one of the results does not figure in the table, it is too unlikely to be acceptable and the series of 5 tests shall be repeated.

4. SAMPLING PLANS AND MICROBIOLOGICAL LIMITS

4.1 Dried and frozen whole egg

***Salmonellae*:**

[1] The MPN table reproduced here is calculated according to: J.C. de Man (1975) The Probability of Most Probable Numbers. **European J. Appl. Microbiol., 1, 67-78.**

(a) *Salmonella* organisms should not be recovered from any of ten sample units examined when the test is carried out according to the method described (n = 10, c = 0, m = 0).[1]

(b) In products intended for special dietary purposes, *salmonella* organisms should not be recovered from any of thirty sample units examined (n = 30, c = 0, m = 0).[1]

Mesophilic aerobic bacteria:

Mesophilic aerobic bacteria should not be recovered from any of five sample units examined when the test is carried out according to the method described in a number exceeding one million per gramme, nor in a number exceeding 50,000 per gramme from three or more of the five sample units examined. (n = 5, c = 2, m = 5 x 10^4, M = 10^6).[1]

Coliform bacteria:

Coliform bacteria should not be recovered from any of five sample units examined, when the test is carried out according to the method described, in a number exceeding 1,000 per gramme, nor in a number exceeding ten per gramme from three or more of the five sample units examined. (n = 5, c = 2, m = 10, M = 10^3).[1]

4.2 Other egg products

Salmonellae:

(a) *Salmonella* organisms should not be recovered from any of ten sample units examined when the test is carried out according to the method described (n = 10, c = 0, m = 0).[1]

(b) In products intended for special dietary purposes, *salmonella* organisms should not be recovered from any of thirty sample units examined (n = 30, c = 0, m = 0).[1]

[1] n = The number of sample units to be examined.
m = The value at or below which no concern is recognized.
M = The value beyond which the lot is rejected.
c = The maximum number of sample units with values between m and M for the lot to be acceptable.
These criteria are employed in describing 3-class plans. In a 2-class plan M is not applicable.