91/180/EEC: Commission Decision of 14 February 1991 laying down certain methods of analysis and testing of raw milk and heat-treated milk
Official Journal L 93, 13 April 1991, pp. 1-48.

THE COMMISSION OF THE EUROPEAN COMMUNITIES,
Having regard to the Treaty establishing the European Economic Community,
Having regard to Council Directive 85/397/EEC of 5 August 1985 on health and animal-health problems affecting intra-Community trade in heat-treated milk (1), as last amended by Directive 89/662/EEC (2), and in particular Article 10 (2) thereof,
Whereas Article 10 (2) provides that the Commission shall lay down the methods of analysis and testing to be used to monitor compliance with the standards relating to raw milk and heat-treated milk;
Whereas it is necessary, for raw milk, to lay down methods in particular to determine the plate count, the cell count, the freezing point and the presence of antibiotics;
Whereas it is necessary, for pasteurized milk, to lay down methods in particular to determine the absence of pathogens, the number of coliforms, the plate count, the absence of phosphatase, the presence of peroxydase, the absence of antibiotics and the freezing point;
Whereas it is necessary, for sterilized milk and UHT (ultra high temperature) milk, to lay down methods in particular to determine the plate count and the absence of antibiotics;
Whereas for technical reasons, it is opportune as a first step to lay down reference methods of analysis and testing to ensure compliance with certain standards; whereas it is in particular necessary to continue to examine the conditions under which routine, analytical and test methods must be applied; whereas, awaiting the results of this examination, it is the responsibility of the Member States to use appropriate routine methods for ensuring compliance with the standards laid down in Directive 85/397/EEC;
Whereas the determination of reference methods of analysis and testing includes the determination of the analytical procedures to be followed and the laying down of precision criteria to ensure a uniform interpretation of results;
Whereas the measures provided for in this Decision are in accordance with the opinion of the Standing Veterinary Committee,
HAS ADOPTED THIS DECISION:

Article 1 The reference procedures for analysis and testing of raw milk shall be the following:
- determination of the freezing point,
- enumeration of micro-organisms - plate count test at 30 oC,
- enumeration of somatic cells,
- detection of antibiotics and sulphonamides.

Article 2 The reference procedures for analysis and testing of pasteurized milk shall be the following:
- determination of the freezing point,
- determination of phosphatase activity,
- determination of peroxidase activity,
- enumeration of micro-organisms - plate count test at 30 oC,
- enumeration of micro-organisms - plate count test at 21 oC,
- enumeration of coliforms - colony count at 30 oC,
- detection of antibiotics and sulphonamides,
- detection of pathogenic micro-organisms.

Article 3
The reference procedures for analysis and testing of UHT milk and sterilized milk shall be the following:
- enumeration of micro-organisms - plate count test at 30 oC,
- determination of the freezing point,
- detection of antibiotics and sulphonamides.

Article 4
The reference procedures for analysis and testing and the precision criteria to be applied must be given effect to and the collection of samples carried out in accordance with the rules set out in Annex 1.

Article 5
The reference procedures for analysis and testing referred to in Articles 1, 2 and 3 are set out in Annex 2.

Article 6
This Decision shall be reexamined before 31 December 1992 in order to take account of developments in scientific and technical knowledge. Article 7 This Decision is addressed to the Member States.
Done at Brussels, 14 February 1991.
For the Commission
Ray MAC SHARRY
Member of the Commission
(1) OJ No L 226, 24. 8. 1985, p. 13.(2)
OJ No L 395, 30. 12. 1989, p. 13.

ANNEX I

CONTENTS Page

I. GENERAL PROVISIONS
1.
Introduction
General provisions with respect to reagents, equipment, expression of results, precision and test reports are described. Competent authorities of Member States and enforcement laboratories charged with the sampling and testing of milk must respect the general provisions.
2.
Reagents
2.1.
Water
2.1.1.
Wherever mention is made of water for solution, dilution or rinsing purposes, distilled water, deionized water or demineralized water of at least equivalent purity shall be used unless otherwise specified. For microbiological purposes it shall be free from substances that might affect or influence the growth of micro-organisms under test conditions.
2.1.2.
Wherever reference is made to 'solution' or 'dilution' without further indication, 'solution in water' or 'dilution with water' is meant.
2.2.
Chemicals
All chemicals used shall be of recognized analytical reagent quality unless otherwise specified.
3.
Equipment
3.1.

Lists of equipment
The lists of equipment given in the different reference procedures contain only those items with a specialized use and items to a particular specification.
3.2.
Analytical balance
Analytical balance means a balance capable of weighing at 0,1 mg.
4.
Expression of results
4.1.
Results
Unless otherwise specified the results stated in the analytical report should be the mean arithmetic value obtained from two tests which satisfy the repeatability-criterion (5.1) stated for that method. If the repeatability-criterion is not satisfied, then the test must be repeated, or the result declared invalid.
4.2.
Calculation of percentage
Except when otherwise specified, the result shall be calculated as a percentage by mass of the sample.
5.
Precision criteria: repeatability and reproducibility
5.1.
The precision criteria given in each procedure is defined as follows:
5.1.1.
Repeatability (r) is the value below which the absolute difference between two single test results obtained with the same procedure on identical test material, under the same conditions (same operator, same apparatus, same laboratory, and a short interval of time) lies.
5.1.2.
Reproducibility (R) is the value below which the absolute difference between two single test results obtained with the same procedure on identical test material, under different conditions (different operators, different apparatus, different laboratories and/or different time) lies.
5.1.3.
Unless otherwise specified the values for the repeatibility- and reproducibility-criteria given in the relevant clauses of each procedure represent the 95 % confidence level intervals as defined by ISO 5725: second edition 1986. They are calculated from the results of recognized collaborative trials which have been used to evaluate the procedure. However, for some of the procedure, collaborative trials have not been carried out. In this case the values of the repeatibility and the reproducibility have been estimated.
5.1.4.
The collaborative trials and studies referred to in 5.1.3 should be planned and conducted in accordance with international guidelines.
6.
Test report
The test report shall specify the method of analysis used as well as the results obtained. In addition, it shall give any details of procedure used not specified in the method of analysis or which are optional, as well as any other circumstances that may have influenced the results obtained. The test report shall give all the information necessary for the complete identification of the sample.
II. SAMPLING OF RAW AND HEAT-TREATED MILK
1.
Scope and field of application
This procedure specifies the reference procedure for sampling of raw and heat-treated milk. The procedures for sampling and transport and storage of samples are applicable to raw milk from producer's deliveries and for raw and heat-treated milk in storage and transport tanks. The procedures given in 2, 4.4, 5 and 6 are applicable to the sampling of heat-treated milk for direct consumption.

2.
General
Sampling of raw and heat-treated milk in cans, tanks, etc. shall be carried out by a skilled operator who has had suitable training before undertaking the sampling of milk.
If considered appropriate the competent authorities or testing laboratory shall instruct sampling personnel in sampling techniques to ensure that the sample is representative of and in conformity with the entire batch.
If considered appropriate the competent authorities or testing laboratory shall instruct sampling personnel about marking of the sample to ensure the unambiguous identity of the sample.
3.
Sampling equipment
3.1.
General
Sampling equipment shall be made of stainless steel, or other suitable material of adequate strength and of a construction suitable for the intended purpose (mixing, sampling etc.).
Plungers and agitators for mixing liquids in containers, shall have a sufficient area to produce adequate mixing of the product, but without causing the development of a rancid flavour.
Dippers must have a solid handle of sufficient length to enable a sample at any depth of the container to be obtained. The capacity of the dipper shall be not less than 50 ml.
Sample containers and closures should be of glass, suitable metals or plastics.
The materials of which sampling equipment (including containers and closures) is constructed must not cause any change in the sample which could affect the results of the examinations.
All surfaces of sampling equipment and sample containers shall be clean and dry, smooth and free from crevices, corners shall be rounded.
3.2.
Sampling equipment for microbiological examination
Sampling equipment including containers shall, in addition to the provisions given in 3.1, be sterile.
If the same sampling equipment is used for successive sampling, it shall be cleaned and sterilized after each sampling in accordance with instructions or requirements laid down by the testing laboratory or competent authority and so that the integrity of successive samples is preserved.
4.
Sampling technique
4.1.
General
Irrespective of the tests to be performed the milk shall be thoroughly mixed prior to sampling, by either manual or mechanical means.
The sample shall be taken immediately after mixing while the milk is still agitated.
When several samples of milk in tanks are taken at the same time for different tests, the sample for microbiological examination shall be taken first.
The volume of the sample shall be to the testing requirements. The capacity of the sample containers used shall be such that they are filled almost completely by the sample, thus allowing proper mixing of the contents before testing, but avoiding churning during transport.
4.2.
Manual sampling
4.2.1.
Sampling from milk buckets and cans
To obtain agitation rapidly move a plunger up and down in the bucket or can while ensuring that the milk is properly mixed and that no cream adheres to the neck of the can. A sample representative of the whole consignment shall be obtained in accordance with 4.2.4.
4.2.2.
Sampling from refrigerated farm milk tanks or vats
Mechanically or manually agitate the milk until sufficient homogeneity is obtained.

If the volume of milk is such that the mechanical agitator cannot mix the milk, carry out manual agitation.

4.2.3.

Sampling from a weighbowl

It is essential that the milk, when tipped into a weighbowl, is adequately mixed. It may be necessary to use additional manual or mechanical agitation to ensure even distribution of fat. When the volume of the consignment to be sampled exceeds the capacity of the weighbowl, a sample representative of the whole consignment shall be obtained in accordance with 4.2.4.

4.2.4.

Sampling a divided bulk

Where the quantity of milk to be sampled is in more than one container, take a representative quantity from each container and note the quantity of milk to which each sample relates. Unless the samples from each container are to be tested individually, mix portions of these respresentative quantities in amounts which are proportional to the quantity in the container from which each sample was taken. Take sample(s) from these bulked proportionate amounts after mixing.

4.2.5.

Sampling from large vessels - storage, rail and road tanks

4.2.5.1.

Mix the milk by an appropriate procedure, before sampling.

To mix the contents of large vessels or of storage, road or rail tanks, the use of mechanical agitation is advised (4.2.5.2).

The extent of mixing shall be appropriate to the period of time over which the milk has been at rest. The efficiency of the procedure of mixing applied in any particular circumstances shall be demonstrated as being adequate for the purposes of the analysis envisaged; the criterion of mixing efficiency particularly influences the similarity between analytical results from samples taken either from different parts of the consignments, or from the outlet of the tank at intervals during discharge. A procedure of mixing milk shall be considered efficient if the difference in fat content between two samples, taken under these conditions, is less than 0,1 %.

In a large vessel with a bottom discharge outlet there may be, at the discharge point, a small quantity of milk which is not representative of the whole contents even after mixing. Therefore samples should preferably be taken through a manhole. If samples are taken from the discharge outlet, run off sufficient milk to ensure that the samples are representative of the whole.

4.2.5.2.

Mixing of the contents of large vessels or of storage, rail or road tanks can be carried out:

- by a mechanical agitator built into the tanks and driven by an electric motor,
- by a propellor or agitator driven by an electric motor and placed on the manhole with the agitator suspended in the milk,
- in the case of rail or road tankers by recirculation of the milk through the transfer hose attached to the tanker unloading pumps and inserted through the manhole,
- by clean filtered compressed air. In this case minimal air pressure and volume should be used to prevent the development of rancid flavour.

4.3.

Automatic or semi-automatic sampling

Automatic or semi-automatic sampling devices for sampling raw milk from producers' deliveries can be used in accordance with instructions given by the testing laboratory or other competent authority.

Such equipment shall, prior to being used and at regular intervals when in use, undergo appropriate tests as prescribed by the responsible authority. The suitability of the sampling procedures must be verified in order to establish:

- the minimum volume of milk collected which can be properly sampled,
- the rate of any carry-over (which is related to the minimum volume of sample),
- the ability to give a representative sample of the bulk after proper agitation.

When using automatic or semi-automatic sampling equipment, the responsible competent national authority can prescribe:
- the minimum volume of milk from which samples must be taken,
- the minimum volume of sample,
- the maximum carry-over,
- which analysis can be performed or which precautions shall be taken.

4.4.
Sampling of heat-treated milk for direct consumption in retail packings

Samples of heat-treated milk for direct consumption in retail packages are to be the complete sealed package. If possible the samples must be taken from the packaging machine or cold room in the treatment establishment as soon as possible after processing. For pasteurized milk on same day as processing.

The samples are taken from each type of heat-treated milk (pasteurized, UHT-treated and sterilized) in numbers corresponding to the examinations which will be made and in accordance with instructions laid down by the testing laboratory or other competent authority.

5.
Identifications of the sample

The sample shall be marked with an identification code so that it can be readily identified using instructions given by the testing laboratory or competent authority to thus ensure the identity of the sample (see 2).

6.
Transport and storage of samples

Instructions about the conditions of transport, storage and time between sampling and analysis of milk shall be prepared by the testing laboratory according to the type of milk and the procedure of analysis to be used. The instructions shall be laid down in accordance with the competent national authority.

In the instructions the following points shall be included:
- during transport and storage precautions shall be taken to prevent exposure to contaminating odours and to direct sunlight. If the container used for samples is transparent, it shall be stored in a dark place,
- samples of raw milk taken for microbiological analysis shall be transported and stored between 0 oC and 4 oC. The time between sampling and analysis shall be as short as possible and, in no event more than 36 hours. The competent authority can accept a storage temperature between 0 oC and 6 oC if the time between sampling and analysis is not more than 24 hours,
- samples of pasteurized milk taken for microbiological analysis shall be transported and stored between 0 oC and 4 oC. The time between sampling and analysis shall be as short as possible, in no event more than 24 hours,
- milk samples other than raw milk and pasteurized milk for microbiological analysis shall be stored in the laboratory under refrigeration and the time between sampling and analysis shall be as short as possible.

Special precautions for some analyses are given under the different procedures.

ANNEX II

CONTENTS Page

I. DETERMINATION OF FREEZING POINT

1.
Scope and field of application

This procedure specifies the reference procedure for the determination of the freezing point of raw, pasteurized, UHT-treated and sterilized whole milk, partly skimmed milk and skimmed milk using an apparatus (the thermistor cryoscope) in which the thermostatically controlled bath is cooled by electrical refrigeration and a thermistor probe replaces the mercury-in-glass thermometer.

There are two types of instrument available. The first one is an instrument that seeks the maximum freezing point on the 'plateau' on the freezing curve, while the second one, for commercial reasons, is set to read at a fixed time after the onset of freezing. As freezing point curves may differ from one milk to another and between milk and the standard solutions used for calibration, this reference procedure requires the use of plateau-seeking instruments. Fixed time instruments may be used for routine screening measurements.

The freezing point can be used to estimate the proportion of extraneous water in the milk, provided that the acidity of the sample does not exceed 0,18 g of lactic acid per 100 ml (see 7.4).

2.
Definition

Freezing point of milk: The value obtained when measured according to the procedure described and expressed in degrees Celsius (oC).

3.
Principle

A test portion of milk is supercooled to the appropriate temperature, depending on the instrument and crystallization is induced by mechanical vibration which causes the temperature to rise quickly to a plateau which corresponds to the freezing of the sample.

The instrument is calibrated by adjusting it to give the correct readings for two standard solutions, using the same procedure as for samples of milk. In these conditions, the plateau gives the freezing point of the milk in degrees Celsius.

4.
Apparatus and glassware

Usual laboratory equipment and, in particular:

4.1.
Cryoscope

The cryoscope consists of a thermostatically controlled cooling bath, thermistor probe (a semi-conductor resistance thermometer) with associated circuit and a galvanometer or 'read-out', sample agitator and a device for initiating the freezing together with sample tubes.

4.1.1.
Cooling bath
Two types of cooling bath can be used.
4.1.1.1.
Immersion type
A well-insulated bath containing a suitable cooling liquid, which is stirred so that the temperature difference between any two points in the liquid does not exceed 0,2 oC. The temperature of the liquid shall not fluctuate by more than p 0,5 oC from the nominal value stated by the manufacturer.
It is important that the liquid in the cooling bath be maintained at a constant level. All of the surface of the sample tube below the volume mark shall be covered by the cooling liquid.
4.1.1.2.
Circulation type
A continuous stream of suitable cooling liquid is circulated around the sample tube. The temperature of the liquid shall not fluctuate by more than p 0,5 oC from the nominal value stated by the manufacturer.
A suitable cooling liquid is a 33 % (v/v) aqueous solution of ethane 1,2 diol (ethylene glycol).
4.1.2.
Thermistor and accompanying circuit
The thermistor shall be of the glass probe type, of diameter not greater than 1,80 p 0,2 mm and with a lead diameter not greater than 0,31 mm. The time constant of the thermistor shall be less than two seconds and the value of v (see note) shall be high. The working voltage, current and dissipation constant should be such that the thermistor temperature is not raised by more than 0,0005 oC above its surroundings at -0,512 oC. The maximum tolerance on the resistance shall be p 5 %.
When the probe is in the working position in the cryoscope, the tip of the glass bead shall lie in the axis of the sample tube and at a point 44,6 p 0,1 mm below the top of the tube (see figure on page 15). A template shall be provided to enable the user to set the probe in this position.
Note
v defines the resistance-temperature characteristics of the thermistor according to the formula:
$$- \frac{d R}{d T} \times \frac{1}{R} = \frac{v}{T^2},$$

where:
T is the temperature in Kelvin,
R
is the resistance in ohms at temperature T,
$- \frac{d R}{d T} \times \frac{1}{R}$ is the temperature coefficient.
v

is a constant that depends upon the material used to make the thermistor. In current practice a value in excess of 3 000 is recommended.

4.1.3.

Measuring and read-out device

4.1.3.1.

Principle of measurement

The instrument used shall operate on the principle of seeking the first 'plateau' in the freezing point curve. The plateau is the part of the curve in which the temperature remains constant to within

p 0,002 oC for a minimum of 20 seconds.

4.1.3.2.

Manual operation

The resistance of the thermistor shall be balanced by means of a Wheatstone bridge or similar device, using the highest quality stable resistors whose tolerance is not greater than p 10 % and whose temperature coefficient does not exceed 2 × 10-& oC.

The variable (balancing) resistor shall not depart from linearity over the whole of its range by more than 0,3 % of its maximum value.

There shall be a means of adjusting the resistors for calibration purposes.

The measuring dial shall be graduated at intervals not greater than 0,001 oC.

4.1.3.3.

Automatic operation

The read-out device shall provide a discrimination of at least 0,001 oC over the range 0 to -1 oC.

The stability of the read-out device and its associated circuit shall be such that successive indications of the same temperature do not vary by more than 0,001 oC.

The linearity of the circuit shall be such that no error greater than p 0,001 oC is introduced at any point within the range -0,400 oC to -0,600 oC when the instrument is correctly operated.

4.1.4.

Stir wire

A wire of metal inert to milk and with a diameter between 1 and 1,5 mm is used to stir the test portion.

The stir wire should be adjusted for amplitude and shall be mounted vertically with its lower end level with the tip of the thermistor probe. A tolerance of about 1,5 mm above or below this position is permitted.

The stir wire shall vibrate laterally with a sufficient amplitude stated by the manufacturer (not less than p 1,5 mm) to ensure that the temperature within the test portion remains uniform during the determination. At no time during its normal stirring operation shall the stir wire strike the thermistor probe or the wall of the tube.

4.1.5.

Device for initiation of freezing

This may be any device that when operated instantaneously initiates freezing of the sample so that the temperature of the test portion rises towards the freezing point. The stir wire may be used for this purpose; one procedure is to increase the amplitude of vibration for one to two seconds so that the stir wire strikes the wall of the sample tube.

4.1.6.

Sample tubes

The sample tubes (see figure 1) shall be made of glass and be 50,8 p 0,1 mm long, 16,0 p 0,1 mm in external diameter and 13,5 p 0,1 mm in internal diameter. The wall thickness throughout the tube shall not vary by more than 0,1 mm.

The tubes should carry a volume mark 29,8 mm below the end (21 mm above the base of the tube) to indicate a sample volume of 2,5 p 0,1 ml.

4.1.7.

Electricity supply

The supply voltage shall be stabilized, either within the apparatus or externally so that fluctuation does not exceed p 1 % of the nominal value when the mains supply fluctuates by p 6 %.

4.2.

Analytical balance.

4.3.

One-mark volumetric flasks, 1 000 ml capacity, class A.

4.4.

Drying oven, well ventilated, capable of maintaining a temperature of 130 p 1 oC

or

Electric furnace, ventilated, capable of maintaining a temperature of 300 p 25 oC

4.5.

Dessicator

5.

Reagents

5.1.

Borosilicate-glass-distilled water, boiled and cooled to 20 p 2 oC in a flask fitted with a carbon dioxide absorption tube.

5.2.

Sodium chloride, analytical reagent quality, finely ground, dried for five hours at 300 p 25 oC in a furnace or alternatively dried in an oven at 130 p 1 oC for at least 24 hours and cooled to room temperature in an efficient desiccator.

5.3.

Preparation of standard solutions

Weigh out the appropriate amount (see Table 1) of dry sodium chloride (5.2) in a weighing bottle. Dissolve in distilled water (5.1), transfer quantitatively to a 1 000 ml one-mark volumetric flask and dilute to the mark with the water at 20 p 2 oC.

Store at about 5 oC in well-stoppered polyethylene bottles of a capacity of not greater than 250 ml at not longer than two months.

Freezing point of sodium chloride solutions at 20 oC

g NaCl/l	oC
6,859	- 0,408
7,818	- 0,464
8,149	- 0,483
8,314	- 0,492
8,480	- 0,502
8,646	- 0,512
8,811	- 0,521
8,977	- 0,531
9,143	- 0,541
10,155	- 0,600

Before using a standard solution, gently invert and rotate the bottle several times to mix its contents thoroughly. At no time should a standard solution be agitated violently so as to incorporate air.
Samples of a standard solution should be withdrawn from the bottle by pouring the solution into a clean dry beaker, i.e. pipettes should never be used for this purpose.
Solutions should not be used from bottles less than one quarter full, and if not preserved with a fungicide (for example thiomersal solution, 10 g/l), should not be used if older than two months.
6.
Calibration of the thermistor cryoscope
The cryoscope must be established so that the temperature of the surrounding air does not deviate more than 1 oC from the temperature at which the calibration is carried out. The cryoscope must not be exposed to sunlight or draught and room temperature over 26-27 oC.
Ensure that the cryoscope is in good working condition in accordance with the manufacturer's instructions, and has been switched on for at least 12 hours prior to calibration. Check the position of the probe, the amplitude of vibration of the stir wire and the temperature of the cooling liquids.
Select two standard solutions (see Table 1 above) which closely bracket the expected value of the freezing point of the milk samples to be tested. The difference in freezing points between the two solutions should preferably not be less than -0,100 oC.
(In some designs of currently available cryoscope the circuit associated with the thermistor is designed to be balanced at a specific value of freezing point within the measuring range of the instrument. In these cases the use of a standard solution having this freezing point as one of the calibrating solutions facilitates the calibration procedure, and the manufacturer shall indicate this value).
Pipette 2,5 p 0,1 ml of one standard into a clean, dry sample tube and operate the cryoscope.
Note
The sample tubes used during calibration should be made from the same type of glass and washed and rinsed with demineralized water at the same time as those used during testing of the milk samples. The temperatures of the standard solutions should be similar to those of the milk samples.
Adjust the calibration controls, as indicated by the manufacturer, until the cryoscope reading is equal to the freezing point of the standard solution. Repeat the procedure with the other standard solution, and continue alternating in this way until successive readings on each solution, without further adjustment of the calibration controls, give the correct value of the freezing point of each. The cryoscope is then ready for use and will indicate directly the freezing point of the milk sample, without the application of any correction.
7.
Preparation of the test sample
7.1.
Store samples, if necessary, at a temperature between 0 and 5 oC.
7.2.
Remove any visible foreign bodies or solid butterfat from the sample, if necessary by filtering into a clean, dry vessel, and mix the sample gently. The filter, if used, shall be inert to milk and shall be effective when used at laboratory temperature.
7.3.
The milk may be tested when it is at its storage temperature (between 0 and 5 oC) or may be allowed to reach laboratory temperature immediately before commencing the test. However, it is necessary that the standard solutions and the milk samples are at the same temperature when used.
7.4.
Determine the titratable acidity of the milk as nearly as possible at the same time as the freezing point test. Samples with an acidity exceeding 0,18 g of lactic acid per 100 ml of milk cannot be examined.

7.5.
UHT-treated and sterilized milk shall stand at least 20 minutes in an open container before examination.
8.
Procedure
8.1.
Preliminary checks
Check that the level and the temperature of the cooling liquid is in accordance with the manufacturer's instructions and that, if appropriate, the thermistor probe is in an empty sample tube in the sample well. Switch on the cryoscope and ensure that cooling liquid is being properly stirred or circulated, as appropriate. When the cryoscope has been switched on for at least 12 hours, check the temperature of the cooling liquid and the position and amplitude of vibration of the stir wire.
8.2.
Routine calibration check
Before each test run, measure the freezing point of a standard sodium chloride solution (e.g. a solution with freezing point -0,512 oC) until two consecutive determinations do not differ by more than 0,001 oC. If the mean of these values differs from the freezing point of the standard solution by more than 0,002 oC recalibrate the cryoscope as described in 6.
If the cryoscope is in continuous use, carry out the routine calibration check If the cryoscope is in continuous use, carry out the routine calibration check at least once an hour. The instructions of the manusfacturer have to be taken into account.
8.3.
Determination of the freezing point of the milk
Gently invert and rotate the milk sample bottle several times to mix its contents. At no time should a sample be agitated so violently as to incorporate air.
Pipette 2,5 p 0,1 ml of the milk into a clean, dry, sample tube and remove any excess with a pipette. Ensure that the probe and stir wire are clean and dry, if necessary, wiping carefully with a soft, clean, lintless tissue from below upwards.
Insert the sample tube into the calibrated cryoscope according to the manufacturer's instructions. The milk will be cooled and freezing initiated at a temperature specified by the manufacturer within 0,1 oC.
(On some automatic instruments this temperature may be observed on the digital read-out; on manual instruments the necessary precision is achieved by ensuring that freezing starts when the galvanometer pointer or hair-line coincides with the appropriate mark).
If, for any reason, freezing is initiated before or after the specified temperature range abandon the test and repeat with another test portion of the milk. If the repeat sample also freezes before the specified temperature a further portion of the sample should be warmed to 45 oC and held for five minutes to allow melting of crystalline fat.
Then cool again to the testing temperature and test immediately. The temperature of the milk after initiation of freezing will rise rapidly to a value which will remain virtually constant for some time before falling again. The freezing point corresponds to the highest temperature reached during this period and this value shall be recorded.
Note
The time during which the temperature remains constant and the time interval between initiation of freezing and the attainment of the highest temperature will differ from sample to sample and will be considerably shorter for water and standard sodium chloride solutions than for milk. It is essential that it is the highest temperature that is recorded.
When the measurement has been satisfactorily completed, remove the tube, rinse with water and then dry the thermistor probe and stir with a soft, clean, lintless tissue from below upwards and make a duplicate determination on another portion of the milk sample.
If the difference between the freezing points obtained is greater than the value of repeatability (0,004 oC), carry out a duplicate determination on another portion of the sample. Provided the two determinations agree to within 0,004 oC, the values should be recorded and used to calculate the final result.

8.4.
Cooling of probe
After using the instrument, place an empty sample tube in the sample well and lower the operating head in order to keep the probe cool. (In certain designs of cryoscope this may not be possible; in such cases, it is essential to ensure that the probe is adequately cooled before taking measurements, for example, by making several dummy determinations until consistent readings are obtained).
9.
Expression of results
9.1.
Calculation
If, following the routine calibration check, the calibration is confirmed, calculate the mean of the acceptable duplicate freezing point values obtained rounded to the third decimal place.
If the sum of two acceptable duplicate values is an odd number, the mean should be rounded to the nearest even value as shown in the following example:

Freezing point (oC)

Duplicate values
Mean
- 0,544 - 0,545
- 0,545 - 0,546
- 0,544
- 0,546

9.2.
Precision
9.2.1.
Repeatability (r): 0,004 oC.
9.2.2.
Reproducibility (R): 0,006 oC. II. DETERMINATION OF PHOSPHATASE ACTIVITY
1.
Scope and field of application
This procedure specifies the reference procedure for the determination of the phosphatase activity in pasteurized milk.
2.
Definition
2.1.
The phosphatase activity is a measure of the quantity of active alkaline phosphatase present in the product, expressed as the quantity of phenol, in micrograms, liberated under the conditions mentioned in the procedure by 1 ml of the pasteurized milk.
2.2.
Any milk whose phosphatase activity is below 4 mg/ml is considered to be phosphatase negative.
3.
Principle
The phosphatase activity is evaluated from the amount of phenol liberated from the disodium phenyl-phosphate added to the sample. The phenol liberated reacts with dibromoquinonechlorimide producing dibromoindophenol (bluish in colour) which is measured colorimetrically at 610 nm. A comparison is made with a sample where the phosphatase enzyme has been destroyed.
4.
Reagents
4.1.
Barium borate-hydroxide buffer

4.1.1.
Dissolve 50,0 g of barium hydroxide [Ba(OH)2.8H2O] in water and make up to 1 000 ml.
4.1.2.
Dissolve 22,0 g of boric acid [H3BO3] in water and make up to 1 000 ml.
4.1.3.
Warm 500 ml of each solution to 50 oC, mix the solutions, stir, cool rapidly to about 20 oC, adjust the pH, if necessary, to 10,6 p 0,1 by addition of solution 4.1.1 or 4.1.2. Filter. Store the solution in a tightly stoppered container.
4.1.4.
Dilute the solution before use with an equal volume of water.
4.2.
Colour development buffer
Dissolve 6,0 g of sodium metaborate (NaBO2) or 12,6 g of (NaBO2.4H2O), and 20,0 g of sodium chloride (NaCl) in water and make up to 1 000 ml.
4.3.
Colour dilution buffer
Dilute 10 ml of the colour development buffer (4.2) to 100 ml with water.
4.4.
Buffer substrate
Dissolve 0,1 g of disodium phenylphosphate dehydrate phenol free in 100 ml of buffer (4.1.3) or dissolve 0,5 g of disodium phenylphosphate in 4,5 ml of the colour development buffer (4.2), add two drops of the BQC solution (4.6) and let stand at room temperature for 30 minutes. Extract the colour so formed with 2,5 ml of butan-1-ol and let stand until the butan-1-ol is separated. Remove the butan-1-ol and discard. Repeat this extraction if necessary.
The solution may be stored in a refrigerator for a few days; develop the colour and re-extract before use. Prepare the buffer substrate immediately before use by diluting 1 ml of this solution to 100 ml with the barium borate-hydroxide buffer (4.1.3).
4.5.
Zinc-copper precipitant
Dissolve 3,0 g of zinc sulphate (ZnSO4, 7H2O) and 0,6 g of copper (II) sulphate (CuSO4, 5H2O) in water and make to 100 ml.
4.6.
2.6-Dibromoquinonechlorimide solution (BQC-solution)
Dissolve 40 p 1 mg of 2,6-dibromoquinonechlorimide (BQC) (C6H2Br2CINO6) in 10 ml of 96 % (v/v) ethanol.
Store in a dark-coloured bottle in a refrigerator. Discard if it is discoloured or more than one month old.
4.7.
Copper (II) sulphate solution
Dissolve 0,05 g of copper (II) sulphate (CuSO4.5H2O) in water and make to 100 ml.
4.8.
Phenol standard solutions
4.8.1.
Weigh 200 p 2 mg of pure anhydrous phenol, transfer to a 100 ml volumetric flask, add water, mix and make up to the mark. This stock solution remains stable for several months in a refrigerator.
4.8.2.
Dilute 10 ml of the stock solution to 100 ml with water and mix. 1 ml contains 200 mg of phenol.
5.
Apparatus and glassware
Notes:
(a) All glassware, stoppers and sampling tools must be carefully cleaned. It is recommended to rinse them with freshly boiled distilled water or to steam them.

(b) Certain types of plastic stoppers may cause phenolic contamination and their use shall not be permitted.
Usual laboratory equipment and, in particular:
5.1.
Analytical balance
5.2.
Water bath, capable of being maintained at 37 p 1 oC.
5.3.
Spectrophotometer suitable for readings at a wavelength of 610 nm.
5.4.
Test tubes, 16 or 18 mm × 150 mm, preferably graduated at 5 and 10 ml.
5.5.
Pipettes
5.6.
Glass funnels of convenient size, for example 5 cm diameter.
5.7.
Folded filters at least 9 cm in diameter for medium filtration speed.
5.8.
Volumetric flasks for the preparation of standard solutions.
6.
Procedure
Notes:
(a) Avoid the influence of direct sunlight during the determination.
(b) Contamination with traces of saliva or perspiration can give false positive results and must be avoided. In this respect, special attention shall be given to the pipetting procedure.
6.1.
Preparation of the test sample
6.1.1.
Carry out the analysis directly after sampling. Otherwise, keep the sample in a refrigerator, but not for more than two days.
6.2.
Test portion
Pipette into each of two test tubes (5.4) 1 ml of the test sample, using one tube as a control or blank.
6.3.
Determination
6.3.1.
Heat the blank for two minutes in boiling water; cover the test tube and the beaker of boiling water with aluminium foil to ensure that the entire tube will be heated. Cool rapidly to room temperature.
6.3.2.
Treat the blank and the test sample in a similar manner for the rest of the procedure. Add 10 ml of the buffer substrate (4.4) and mix.
6.3.3.
Immediately incubate the samples in the water bath (5.2) for 60 minutes, mixing the contents occasionally (at least four times).
6.3.4.
Heat in boiling water for two minutes in the same manner as under 6.3.1. Cool rapidly to room temperature.
6.3.5.
Add 1 ml of the zinc-copper precipitant (4.5) to each tube and mix thoroughly.
6.3.6.
Filter through dry filter paper, discard the first 2 ml, refilter if necessary until the filtrate is completely clear, and collect 5 ml in a test tube.
6.3.7.

Add 5 ml of the colour development buffer (4.2).
6.3.8.
Add 0,1 ml of the BQC solution (4.6), mix and allow the colour to develop for 30 minutes at room temperature.
6.3.9.
Measure the absorbance against the control or blank in the spectrophotometer (5.3) at a wavelength of 610 nm.
6.3.10
Repeat the determination with an appropriate dilution of the sample if the absorbance as measured under 6.3.9 exceeds the absorbance of the standard containing 20 mg of phenol per tube as measured under 6.4.4. Prepare this dilution by mixing one volume of the test sample with an appropriate volume of a part of the same test sample heated carefully to boiling in order to inactivate the phosphatase.
6.4.
Preparation of the calibration curve
6.4.1.
Prepare a suitable range of diluted standards, starting from the standard phenol (4.8.2), containing 0 (control or blank), 2, 5, 10 and 20 mg of phenol per millilitre and pipette respectively 1 ml of water and 1 ml of the four phenol standard solutions into each of five test tubes.
6.4.2.
Add to each test tube 1 ml of the copper (II) sulphate solution (4.7), 5 ml of the colour dilution buffer (4.3), 3 ml of water and 0,1 ml of the BQC solution (4.6); mix.
6.4.3.
Allow the colour to develop for 30 minutes at room temperature.
6.4.4.
Measure the absorbance against the control or blank in the spectrophotometer at a wavelength of 610 nm (5.3).
6.4.5.
From the values of absorbance (6.4.4) obtained for each quantity of phenol added (6.4.1), calculate the regression line by the procedure of least squares.
7.
Expression of results
7.1.
Calculation and formula
7.1.1.
Calculate from the absorbance reading (6.3.9) the quantity of phenol using the regression line obtained (6.4.5).
7.1.2.
Calculate the phosphatase activity, expressed as micrograms of phenol per millilitre of the pasteurized milk from the following formula:
Phosphatase activity = 2,4 × A × D
where
A
is the quantity of phenol in micrograms obtained under 7.1.1;
D
is the dilution factor of the dilution according to 6.3.10 (in the case of no dilution, D = 1);
The factor 2,4 is the dilution factor (5/12 of 1 ml test sample) - See 6.2 in connection with 6.3.2, 6.3.5 and 6.3.6.
7.2.
Precision
7.2.1.
Repeatability (r): 2 mg phenol/ml.
7.2.2.

Reproducibility (R): 3 (preliminary) mg phenol/ml.
7.2.3.
If a dilution is applied according to 6.3.10 the limit mentioned in 7.2.1 and 7.2.2 is referred to the results obtained on the diluted sample.
III. DETERMINATION OF PEROXIDASE ACTIVITY
1.
Scope and field of application
This procedure specifies the reference procedure for the determination of the presence or absence of peroxidase enzyme in milk as a control of pasteurization.
2.
Definition
Peroxidase-positive reaction:
If the milk is properly pasteurized a blue colour will occur within 30 seconds after mixing.
Peroxidase-negative reaction:
No colour will occur within 30 seconds after mixing.
3.
Principle
The peroxidase enzyme decomposes hydrogen peroxide. The atomic oxygen liberated oxidizes the colourless 1,4-phenylenediamine into the purple indophenol (Storch test). The colour intensity is proportional to the enzyme's concentration.
4.
Reagents
4.1.
Solution of 1,4-phenylenediamine
Dissolve 2 g 1,4-phenylenediamine ($C_6H_8N_2$) in warm (50 oC) water and make to a volume of 100 ml. Keep the solution in a dark brown bottle with a glass stopper and store in a cool and dark place. After preparation, a solution of 1,4-phenylenediamine forms a sediment in one or two days: it must then be discarded.
4.2.
Solution of hydrogen peroxide
Dilute 9 ml of hydrogen peroxide 30 % in water and make to a volume of 100 ml. To stabilize, add 1 ml concentrated sulphuric acid per litre of solution.
The hydrogen peroxide solution is stable for one month, if kept in a cool dark place and in a bottle with glass stopper preventing any contact with organic compounds.
5.
Procedure
5.1.
Introduce 5 ml of the milk sample into a clean test tube with suitable closure.
5.2.
Add 5 ml of the 1,4-phenylenediamine solution (4.1).
5.3.
Add 2 drops of hydrogen peroxide solution (4.2).
5.4.
Watch the colour production within 30 seconds after mixing. If the blue colour occurs later than
30 seconds after the addition of the reagents the reaction is unspecific.
IV. ENUMERATION OF MICRO-ORGANISMS - PLATE COUNT TEST AT 30 oC
1.
Scope and field of application
This procedure specifies the reference procedure for the enumeration of micro-organisms by means of a colony count technique at 30 oC. The procedure is applicable to raw milk and pasteurized milk and to UHT-treated and sterilized milk preincubated at 30 oC for 15 days.
2.
Definition

The designation 'micro-organisms' means: organisms forming countable colonies when incubated aerobically under the conditions described.

3.

Principle

A defined volume of the milk sample is mixed with the culture medium in Petri dishes and incubated at 30 oC for 72 hours. The colonies are counted and the number of micro-organisms per 1 ml of raw or pasteurized milk or per 0,1 ml of preincubated UHT-treated or sterilized milk is calculated.

4.

Apparatus and glassware

The usual laboratory equipment and, in particular:

4.1.

Apparatus

4.1.1.

Hot air oven, capable of operating at 170 to 175 oC.

4.1.2.

Autoclave, capable of operating at 121 p 1 oC.

4.1.3.

Incubator, capable of maintaining a temperature of 30 p 1 oC at all points within it.

4.1.4.

pH-meter, with a temperature compensation, accurate to p 0,1 pH unit.

4.1.5.

Water bath, capable of operating at 45 p 1 oC.

4.1.6.

Lens, magnification 2-4x.

4.1.7.

Lens, magnification 8-10x.

4.1.8.

Tally counter.

4.1.9.

Mixer capable of mixing 1 ml of the milk sample or decimal dilution with 9 ml diluent and working on the principle of eccentric rotation of the contents of the test tube.

4.2.

Glassware

4.2.1.

Test tubes with suitable closures and of sufficient capacity to contain, with adequate head-space for mixing, 10 ml of the primary dilution or further decimal dilutions.

4.2.2.

Flasks of capacity 150 to 250 ml or tubes, about 20 ml capacity, to hold the culture medium.

4.2.3.

Pipettes (plugged with cotton wool) of glass or sterile synthetic material with unbroken tip of nominal capacity 1 ml and having an outlet of diameter 1,75 to 3 mm.

4.2.4.

Petri dishes, of clear uncoloured glass or sterile synthetic material, the bottom dish having an internal diameter of about 90-100 mm. The internal depth should be 10 mm minimum. The bottom shall have no irregularities which interfere with counting colonies.

4.2.5.

Sterilization of glassware:

Glassware shall be sterilized by one of the following procedures:

(a) by being maintained at 170 to 175 oC for not less than one hour in a hot air oven (4.1.1);

(b) by being maintained at 121 p 1 oC for not less than 20 minutes in an autoclave (4.1.2).

In the autoclave care should be taken to ensure adequate penetration of steam - e.g. if the equipment is sterilized in containers, these should not be tightly closed, flasks should have loose lids.

Glassware sterilized in the autoclave should be dried by venting the steam.

Pipettes must be sterilized in a hot air oven (4.1.1).
5.
Culture medium - milk plate count agar
5.1.
Composition:
Yeast extract
2,5 g
Tryptone
5,0 g
Glucose D (+) or Dextrose
1,0 g
Skimmed milk powder
1,0 g
Agar
10 to 15 g, depending on the gelling properties of the agar used.
Water
1 000 ml
Skimmed milk powder shall be free from inhibitory substances. This should be verified by comparative tests using a skimmed milk powder known to be free from inhibitory substances.
Preparation:
Suspend and dissolve the components in the following order: yeast extract, tryptone, glucose and finally, skimmed milk powder, in the water. Heating the suspension will assist this procedure. Add the agar and heat to boiling, stirring continuously until the agar is completely dissolved, or steam for about 30 minutes.
Filter through filter paper, if necessary.
Check the pH with a pH meter (4.1.4) and if necessary adjust the pH so that, after sterilization it is 6,9 p 0,1 at 25 oC using a solution (at least 0,1 mol/l) of sodium hydroxide or hydrochloric acid.
5.2.
Distribution, sterilization and storage of culture medium
Dispense the medium (5.1) in quantities of 100 to 150 ml into flasks or 12 to 15 ml into tubes (4.2.2). Stopper the flasks and tubes.
Sterilize in the autoclave (4.1.2) at 121 p 1 oC for 15 minutes.
Check the pH of the medium.
If the medium is not to be used immediately, store it in the dark at a temperature between 1 and 5 oC for no longer than one month after preparation.
5.3.
Commercial dehydrated culture medium
The culture medium (5.1) can be prepared from commercial dehydrated medium. Follow the manufacturer's instructions but add the skimmed milk powder before dissolving if it is not a component.
Adjust the pH to 6,9 p 0,1 at 25 oC as described in 5.1 and dispense, sterilize and store the medium as described in 5.2.
6.
Diluents
6.1.
Peptone/saline solution
Composition:
Peptone
1,0 g
Sodium chloride (NaCl)
8,5 g
Water
1 000 ml
Preparation:

Dissolve the components in water, heating if necessary.
Check the pH with a pH meter (4.1.4) and if necessary adjust the pH so that, after sterilization, it is 7,0 p 0,1 at 25 oC using a solution (at least 0,1 mol/l) of sodium hydroxide or hydrochloric acid.
6.2.
Distribution, sterilization and storage of diluent
Dispense the diluent (6.1) into test-tubes (4.2.1) in quantities such that after sterilization each tube contains 9,0 p 0,2 ml of diluent. Stopper the tubes.
Sterilize in the autoclave (4.1.2) at 121 p 1 oC for 15 minutes.
Check the pH of the diluent.
In the diluent is not to be used immediately, store it in the dark at a temperature between 1 and 5 oC for no longer than one month after preparation.
6.3.
Commercial dehydrated diluents
The diluent (6.1) can be prepared from commercial dehydrated tablets or powders. Follow the manufacturer's instructions. Adjust the pH as described in 6.1 and dispense, sterilize and store the diluents as described in 6.2.
7.
Procedure
7.1.
Melting of the medium
Before beginning the microbiological examination, melt the required amount of medium quickly and temper the medium to 45 p 1 oC in a water bath (4.1.5).
7.2.
Preparation of the milk sample
Mix the milk sample thoroughly, so that the micro-organisms are distributed as evenly as possible, by rapidly inverting the milk sample container 25 times. Foaming should be avoided or foam allowed to disperse. The interval between mixing and removing the test portion should not exceed three minutes.
7.3.
Preparation of the primary dilution (10-1) (raw and pasteurized milk)
Transfer with a sterile pipette (4.2.3) 1 ml of the sample (7.2) of raw milk or pasteurized milk into 9 ml of diluent (6.1) avoiding contact between the pipette and the diluent. The temperature of the diluent shall be approximately the same as that of the milk sample. Mix this primary dilution carefully in the mixer (4.1.9) for 5-10 seconds.
A primary dilution of 10-; is thus obtained.
7.4.
Preparation of further decimal dilutions (raw and pasteurized milk).
Transfer with a sterile pipette (4.2.3) 1 ml of the primary dilution (7.3) into 9 ml of diluent (6.1), following the instructions given in 7.3.
The 10-$ dilution is thus obtained.
Repeat these operations to obtain further decimal dilutions until the appropriate number of micro-organisms is expected to be obtained (8.1.1).
7.5.
Inoculation of the Petri dishes
7.5.1.
Raw milk: Transfer with a sterile pipette (4.2.3) 1 ml of the sample and/or the appropriate decimal dilution into a dish (4.2.4). At least two dilutions must be examined. Prepare one dish from each dilution chosen as appropriate (8.1.1).
7.5.2.
Pasteurized milk: Transfer with a sterile pipette (4.2.3) 1 ml of the sample and/or the appropriate decimal dilution into a dish (4.2.4). At least two dilutions must be examined. Prepare two dishes from each dilution chosen as appropriate (8.1.1).
7.5.3.

UHT-treated and sterilized milk (examined after incubation for 15 days at 30 oC - Directive Annex A, Chapter VII, point 5):
Transfer with a sterile pipette (4.2.3) 0,1 ml of the milk sample (7.2) into a dish (4.2.4).
Prepare two dishes.
7.6.
Pouring
Pour about 15 to 18 ml of the medium (7.1) into each inoculated dish.
Mix immediately after pouring by rotating the Petri dish sufficiently to obtain evenly dispersed colonies after incubation.
The time between the end of the preparation of the milk sample and mixing, according to the kind of milk, test portion or dilution with medium shall not exceed 15 minutes.
Allow to solidify on a clean, cool horizontal surface.
7.7.
Incubation of Petri dishes
Transfer the dishes to the incubator (4.1.3). Incubate the dishes inverted. Stack not more than six high. Stacks of dishes should be separated from one another and from the walls and top of the incubator.
Incubate at 30 p 1 oC for 72 p 2 hours.
7.8.
Counting of colonies
Count the colonies in the Petri dishes containing not more than 300 colonies.
Examine the dishes in subdued light. To facilitate counting, a suitable lens (4.1.6) and/or a tally counter (4.1.8) may be used. Avoid mistaking particles of precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using a lens of higher magnification (4.1.7) where required, to distinguish colonies from foreign matter.
Spreading colonies are considered as single colonies. If less than one quarter of the dish is overgrown by spreading colonies, count the colonies on the unaffected part of the dish and calculate the corresponding number for the entire dish. If more than one quarter of the dish is overgrown by spreading colonies, discard the dish.
8.
Calculation and expression of results
8.1.
Raw and pasteurized milk
8.1.1.
Use counts from all dishes containing between 10 and 300 colonies (see 8.1.3 and 8.1.4).
8.1.2.
The number of micro-organisms per 1 ml of raw or pasteurized milk is given by the formula:
$$\frac{S\ C}{(n1 + 0{,}1\ n2)\ d}$$

where:
S C
is the sum of colonies counted as in 8.1.1,
(n1 + 0,1 n2) d
is equal to the volume of sample plated in which:
n1
is the number of dishes counted in first dilution,
n2
is the number of dishes counted in second dilution,
d
is the dilution factor from which the first counts were obtained.
The count is given to two significant figures. When the digit to be rounded off is five, round off so that the figure immediately to the left is even.
Example (pasteurized milk):

Dilution 10-2: 278 and 290 colonies
Dilution 10-3: 33 and 28 colonies
Number/ml = 278 + 290 + 33 + 28
Number/ml
=
278 + 290 +33 + 28
(2 + 0,1 × 2) 10-2
Number/ml = 0,022
=
629
0,022
=
28 590
=
29 000
=
2,9 × 10%.
8.1.3.
If there are only counts less than 10, report the number of micro-organisms per millilitre as 'less than 10 x d per ml', 'd' being the reciprocal of the lowest dilution factor.
8.1.4.
If there are only counts exceeding 300 colonies but the counting is possible, calculate an estimated count and multiply by the reciprocal of the dilution factor. Report the result as the 'Estimated number of micro-organisms per ml'.
8.2.
UHT and sterilized milk
Plate counts of more than 10 colonies at 0,1 ml shall be considered to no longer satisfy the requirements in Directive 85/397/EEC.
9.
Precision
Results from internationally accepted collaborative trials are not yet available.
V. ENUMERATION OF MICRO-ORGANISMS - PLATE COUNT TEST AT 21 oC
1.
Scope and field of application
This procedure specifies the reference procedure for enumeration of micro-organisms by means of a colony count technique at 21 oC on pasteurized milk after incubation of the milk at 6 oC for five days to determine the degree of contamination of the pasteurized milk with psychotrophic micro-organisms capable of multiplying in milk at 6 oC.
2.
Definition
The designation 'micro-organisms' means: organisms forming countable colonies when incubated aerobically under the conditions described.
3.
Principle
The pasteurized milk is incubated at 6 oC for five days. A defined volume of the milk sample is mixed with the culture medium in Petri dishes and incubated at 21 oC for 25 hours. The colonies are counted and the number of micro-organisms per 1 ml of pasteurized milk is calculated.
4.
Apparatus and glassware
The usual laboratory equipment and, in particular:
4.1.
Apparatus
4.1.1.
Hot air oven, capable of operating of 170 to 175 oC.

4.1.2.
Autoclave, capable of operating at 121 p 1 oC.
4.1.3.
Incubators, capable of maintaining a temperature of
(a) 6 p 0,2 oC
(b) 21 p 1 oC
at all points within them.
4.1.4.
pH meter, with temperature compensation, acurate to p 0,1 pH unit.
4.1.5.
Water bath, capable of operating at 45 p 1 oC.
4.1.6.
Lens, magnification 2-4x.
4.1.7.
Lens, magnification 8-10x.
4.1.8.
Tally counter.
4.1.9.
Mixer, capable of mixing 1 ml of the milk sample or decimal dilution with 9 ml of diluent and working on the principle of eccentric rotation of the contents of the test tube.
4.2.
Glassware
4.2.1.
Test tubes, with suitable closures and of sufficient capacity to contain, with adequate head-space for mixing, 10 ml of the primary dilution or further decimal dilutions.
4.2.2.
Flasks, of capacity 150 to 250 ml or tubes, about 20 ml capacity, to hold the culture medium.
4.2.3.
Pipettes, (plugged with cotton wool) of glass or sterile synthetic material with unbroken tip of nominal capacity 1 ml and having an outlet of diameter 1,75 to 3 mm.
4.2.4.
Petri dishes, of clear uncoloured glass or sterile synthetic material, the bottom dish having an internal diameter of about 90-100 mm. The internal depth should be 10 mm minimum. The bottom shall have no irregularities which interfere with counting colonies.
4.2.5.
Sterilization of glassware
Glassware shall be sterilized by one of the following procedures:
(a) by being maintained at 170 to 175 oC for not less than one hour in an hot air oven (4.1.1).
(b) by being maintained at 121 p 1 oC for not less than 20 minutes in an autoclave (4.1.2).
In the autoclave care should be taken to ensure adequate penetration of steam, e.g. if the equipment is sterilized in containers, these should not be tightly closed, flasks should have loose lids.
Glassware sterilized in the autoclave should be dried by venting the steam.
Pipettes must be sterilized in a hot air oven (4.1.1).
5.
Culture medium - milk plate count agar
5.1.
Composition:
Yeast extract
2,5 g
Tryptone
5,0 g
Glucose D(+) or dextrose
1,0 g
Skimmed milk powder

1,0 g
Agar
10 to 15 g, depending on the gelling properties of the agar used
Water
1 000 ml
Skimmed milk powder shall be free from inhibitory substances. This should be verified by comparative tests using a skimmed milk powder known to be free from inhibitory substances.
Preparation:
Suspend and dissolve the components in the following order: yeast extract, tryptone, glucose and finally, skimmed milk powder, in the water. Heating the suspension will assist in this procedure. Add the agar and heat to boiling, stirring continuously until the agar is completely dissolved, or steam for about 30 minutes.
Filter through filter paper, if necessary.
Check the pH with a pH meter (4.1.4) and if necessary adjust the pH so that, after sterilization, it is 6,9 p 0,1 at 25 oC using a solution (at least 0,1 mol/l) of sodium hydroxide or hydrochloric acid.
5.2.
Distribution, sterilization and storage of culture medium
Dispense the medium (5.1) in quantities of 100 to 150 ml into flasks or 12 to 15 ml into tubes (4.2.2). Stopper the flasks and tubes.
Sterilize in the autoclave (4.1.2) at 121 p 1 oC for 15 minutes.
Check the pH of the medium.
If the medium is not to be used immediately, store it in the dark at a temperature between 1 and 5 oC for no longer than one month after preparation.
5.3.
Commercial dehydrated culture medium
The culture medium (5.1) can be prepared from commercial dehydrated medium. Follow the manufacturer's instructions but add the skimmed milk powder before dissolving if it is not a component.
Adjust the pH to 6,9 p 0,1 at 25 oC as described in 5.1 and dispense, sterilize and store the medium as described in 5.2.
6.
Diluents
6.1.
Peptone/saline solution:
Composition:
Peptone
1,0 g
Sodium chloride (NaCl)
8,5 g
Water
1 000 ml
Preparation:
Dissolve the components in water, heating if necessary.
Check the pH with a pH meter (4.1.4) and if necessary adjust the pH so that, after sterilization, it is 7,0 p 0,1 at 25 oC using a solution (at least 0,1 mol/l) of sodium hydroxide or hydrochloric acid.
6.2.
Distribution, sterilization and storage of diluent
Dispense the diluent (6.1) into test tubes (4.2.1) in quantities such that after sterilization each tube contains 9,0 p 0,2 ml of diluent. Stopper the tubes.
Sterilize in the autoclave (4.1.2) at 121 p 1 oC for 15 minutes. Check the pH of the diluent.
If the diluent is not to be used immediately, store it in the dark at a temperature between 1 and 5 oC for no longer than one month after preparation.
6.3.

Commercial dehydrated diluents
The diluent (6.1) can be prepared from commercial dehydrated tablets or powders. Follow the manufacturer's instructions. Adjust the pH as described in 6.1 and dispense, sterilize and store the diluents as described in 6.2.
7.
Procedure
7.1.
Melting of the medium
Before beginning the microbiological examination, melt the required amount of medium quickly and temper the medium to 45 p 1 oC in a waterbath (4.1.5).
7.2.
Preparation of the milk sample
7.2.1.
Incubate an unopened package of pasteurized milk, or if this is impossible a representative sample not less than 100 ml for 120 p 2 hours at 6 p 0,5 oC in an incubator (4.1.3(a)).
7.2.2.
After incubation mix the milk sample thoroughly, so that the micro-organisms are distributed as evenly as possible, by rapidly inverting the milk sample container 25 times. Foaming should be avoided or foam allowed to disperse. The interval between mixing and removing the test portion should not exceed three minutes.
7.3.
Preparation of the primary dilution (10-1)
Transfer with a sterile pipette (4.2.3) 1 ml of the sample (7.2.2) into 9 ml of diluent (6.1) avoiding contact between the pipette and the diluent. The temperature of the diluent shall be approximately the same as that of the milk sample. Mix this primary dilution carefully using a mixer (4.1.9) for 5 to 10 seconds.
A primary dilution of 10-; is thus obtained.
7.4.
Preparation of further decimal dilutions
Transfer with a sterile pipette (4.2.3) 1 ml of the primary dilution (7.3) into 9 ml of diluent (6.1), following the instructions given in 7.3.
The 10-$ dilution is thus obtained.
Repeat these operations to obtain further decimal dilutions until the appropriate number of micro-organisms is expected to be obtained (8.1).
7.5.
Inoculation of the Petri dishes
Transfer with a sterile pipette (4.2.3) 1 ml of the sample and/or the appropriate decimal dilution into a dish (4.2.4). At least two dilutions must be examined. Prepare two dishes from each dilution chosen as appropriate (8.1).
7.6.
Pouring
Pour about 15 to 18 ml of the medium (7.1) into each inoculated dish.
Mix immediately after pouring by rotating the Petri dish sufficiently to obtain evenly dispersed colonies after incubation.
The time between the end of the preparation of the milk sample and mixing dilution with medium shall not exceed 15 minutes.
Allow to solidify on a clean, cool horizontal surface.
7.7.
Incubation of Petri dishes
Transfer the dishes to the incubator (4.1.3(b)). Incubate the dishes inverted. Stack not more than six high. Stacks of dishes should be separated from one another and from the walls and top of the incubator.
Incubate at 21 p 1 oC for 25 hours.
7.8.
Counting of colonies

Count the colonies in the Petri dishes containing not more than 300 colonies.
Examine the dishes in subdued light. To facilitate counting, a suitable lens (4.1.6) and/or a tally counter (4.1.8) may be used. Avoid mistaking particles of precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using a lens of higher magnification (4.1.7) where required, to distinguish colonies from foreign matter.
Spreading colonies are considered as single colonies. If less than one quarter of the dish is overgrown by spreading colonies, count the colonies on the unaffected part of the dish and calculate the corresponding number for the entire dish. If more than one quarter of the dish is overgrown by spreading colonies, discard the dish.
8.
Calculation and expression of results
8.1.
Use counts from all dishes containing between 10 and 300 colonies (see 8.3 and 8.4).
8.2.
The number of micro-organisms per 1 ml of pasteurized milk is given by the formula:
(n1 + 0,1 n2) d
S C
(n1 + 0,1 n2) d

where:
S C
is the sum of colonies counted as in 8.1,
(n1 + 0,1 n2) d
is equal to the volume of sample plated in which:
n1
is the number of dishes counted in first dilution,
n2
is the number of dishes counted in second dilution,
d
is the dilution factor from which the first counts were obtained.
The count is given to two significant figures. When the digit to be rounded off is five, round off so that the figure immediately to the left is even.

Example:
Dilution 10-2: 278 and 290 colonies
Dilution 10-3: 33 and 28 colonies
Number/ml = 278 + 290 + 33 + 28
Number/ml
=
278 + 290 +33 + 28
(2 + 0,1 × 2) 10-2
Number/ml = 0,022
=
629
0,022
=
28 590
=
29 000
=
2,9 × 10%.

8.3.
If there are only counts less than 10, report the number of micro-organisms per millilitre as 'less than 10 × d per ml'; 'd' being the reciprocal of the lowest dilution factor.

8.4.
If there are only counts exceeding 300 colonies but the counting is possible calculate an estimated count and multiply by the reciprocal of the dilution factor. Report the result as the 'Estimated number of micro-organisms per ml'.
9.
Precision
Results from internationally accepted collaborative trials are not available.
VI. ENUMERATION OF COLIFORMS - COLONY COUNT AT 30 oC
1.
Scope and field of application
This procedure specifies the reference procedure for the enumeration of coliforms in pasteurized milk by means of a colony count technique at 30 oC.
2.
Definition
The designation 'coliforms' means bacteria which at 30 oC form characteristic colonies or non-characteristic colonies which ferment lactose with the production of gas under the conditions described.
3.
Principle
A defined volume of the milk sample is mixed with the culture medium in Petri dishes and incubated at 30 oC for 24 hours. Characteristic colonies are counted and if necessary the identity of the non-characteristic colonies is confirmed by testing for the ability to ferment lactose. The number of coliforms per 1 ml of pasteurized milk is then calculated.
4.
Apparatus and glassware
The usual laboratory equipment and, in particular:
4.1.
Apparatus
4.1.1.
Hot air oven, capable of operating at 170 to 175 oC.
4.1.2.
Autoclave, capable of operating at 121 p 1 oC.
4.1.3.
Incubator, capable of maintaining a temperature of 30 p 1 oC at all points within it.
4.1.4.
pH meter, with temperature compensation, accurate to p 0,1 pH unit.
4.1.5.
Water bath, capable of operating at 45 p 1 oC.
4.1.6.
Wire needle made of platinum-iridium or nickel-chromium.
4.2.
Glassware
4.2.1.
Test tubes with suitable closures and of capacity 20 ml, to hold the confirmatory medium (5.2) and Durham tubes of appropriate dimensions for use with the test tubes.
4.2.2.
Flasks of capacity 150 to 250 ml to hold the solid selective medium (5.1).
4.2.3.
Pipettes (plugged with cotton wool) of glass or sterile synthetic material with unbroken tip of nominal capacity 1-10 ml and having an outlet of diameter 1,75 to 3 mm.
4.2.4.
Petri dishes, of clear uncoloured glass or sterile synthetic material, the bottom dish having an internal diameter of about 90-100 mm. The internal depth should be 10 mm minimum. The bottom shall have no irregularities which interfere with counting colonies.
4.2.5.

Sterilization of glassware:
Glassware shall be sterilized by one of the following procedures:
(a) by being maintained at 170 to 175 oC for not less than one hour in a hot air oven (4.1.1).
(b) by being maintained at 121 p 1 oC for not less than 20 minutes in an autoclave (4.1.2).
In the autoclave care should be taken to ensure adequate penetration of steam - e.g. if the equipment is sterilized in containers, these should not be tightly closed, flasks or bottles should have loose lids.
Glassware sterilized in the autoclave should be dried by venting the steam.
Pipettes must be sterilized in a hot air oven (4.1.1)
5.
Culture Media
5.1.
Violet red bile lactose agar (VRBL agar). Solid selective medium.
Composition:
Peptone
7 g
Yeast extract
3 g
Lactose (C12H22O11.H2O)
10 g
Sodium chloride (NaCl)
5 g
Bile salts
1,5 g
Neutral red
0,03 g
Crystal violet
0,002 g
Agar
10 to 15 g (depending on the gelling properties of the agar used)
Water
1 000 ml
Preparation:
Suspend and dissolve the components in the water and leave to stand for several minutes, then mix vigorously.
Check the pH with a pH meter (4.1.4) and if necessary adjust the pH so that, after boiling, it is 7,4 p 0,1 at 25 oC using a solution (at least 0,1 mol/l) of sodium hydroxide or hydrochloric acid.
Bring rapidly to the boil, swirling from time to time and immediately dispense in quantities of 100 to 150 ml into sterile flasks (4.2.2). Temper the medium in a water-bath (4.1.5) at 45 p 1 oC.
The sterility of the medium should be checked at the time of use (see 6.4).
Use the medium within three hours of its preparation.
5.2.
Brilliant green lactose bile broth. Confirmatory medium.
Composition:
Peptone
10 g
Lactose (C12H22O11.H2O)
10 g
Dehydrated ox bile
20 g
Brilliant green
0,0133 g
Water

1 000 ml
Preparation:
Dissolve the components in the water by boiling.
Check the pH with a pH meter (4.1.4) and if necessary adjust the pH so that, after sterilization, it is 7,2 p 0,1 at 25 oC using a solution (at least 0,1 mol/l) of sodium hydroxide or hydrochloric acid.
Dispense the medium, in quantities of 10 ml, into test tubes (4.2.1) containing Durham tubes.
Stopper the tubes.
Sterilize in the autoclave (4.1.2) at 121 p 1 oC for 15 minutes.
The Durham tubes shall not contain air bubbles after sterilization.
Check the pH of the medium.
If the medium is not to be used immediately, store it in the dark at a temperature between 0 and 5 oC for no longer than one month after preparation.
5.3.
Commercial dehydrated culture media
The culture media (5.1, 5.2) can be prepared from commercial dehydrated media. Follow the manufacturer's instructions. Adjust the pH and dispense, boil or sterilize and store the media as described in 5.1 and 5.2.
6.
Procedure
6.1.
The medium
Use the medium (VRBL agar) as described in 5.1.
6.2.
Preparation of the milk sample
Mix the milk sample thoroughly, so that the micro-organisms are distributed as evenly as possible, by rapidly inverting the milk sample container 25 times. Foaming should be avoided or foam allowed to disperse. The interval between mixing and removing the test portion should not exceed three minutes.
6.3.
Inoculation of the Petri dishes
3 ml of the milk sample (6.2) are inoculated by transferring with a sterile pipette (4.2.3) 1 ml of the milk sample into each of three dishes (4.2.4).
6.4.
Pouring
Pour VRBL agar (6.1) into each inoculated dish in quantities of about 12 ml.
Mix immediately after pouring by rotating the Petri dish sufficiently to obtain evenly dispersed colonies after incubation.
The time between the end of the preparation of the milk sample and mixing test portion with medium shall not exceed 15 minutes.
Prepare for sterility purposes as a control an uninoculated dish, with 12 ml of the VRBL agar used for the inoculated dishes.
Allow to solidify on a clean cool horizontal surface until the medium has set.
After complete solidification pour at least 4 ml VRBL agar (6.1) on the surface of the inoculated medium.
Allow to solidfy.
6.5.
Incubation of Petri dishes
Transfer the dishes to the incubator (4.1.3). Incubate the dishes inverted. Stack not more than six high. Stacks of dishes should be separated from one another and from the walls and top of the incubator.
Incubate at 30 p 1 oC for 24 p 2 hours.
6.6.
Counting of colonies
6.6.1.

Count the colonies in Petri dishes containing not more than 150 colonies. Count the dark red coloured colonies having a diameter of at least 0,5 mm with or without surrounding precipitate, characteristic for coliforms.
6.6.2.
If all or some colonies have a non-characteristic aspect (e.g. differ in colour, in size or in the formation of precipitate from typical colonies) carry out a confirmatory test (6.7).
6.7.
Confirmatory test
According to the indications given in 6.6.2 carry out a confirmatory test on a suitable number (e.g. three to five) of non-characteristic colonies by inoculation into tubes of brilliant green lactose bile broth (5.2) using a wire needle (4.1.6). Incubate the tubes at 30 ± 1 oC for 24 ± 2 hours.
Consider colonies which produce gas in the Durham tube as confirmed coliforms.
7.
Calculation and expression of results
7.1.
Use counts (see 7.4) from dishes containing not more than 150 colonies.
7.2.
If a confirmatory test is applied, calculate the number of colonies of coliforms from the percentage of confirmed coliform colonies.
7.3.
The number of coliforms per 1 ml of pasteurized milk is given by the formula:
S C ,
S C
n

where:
S C is the total number of colonies of coliforms (7.1 in connection with 7.2) found by the examination of the milk sample (3 ml),
n

is the number of millilitres of the sample examined (6.3) (3 ml).
The count is given to two significant figures when there are more than 100 colonies. When the digit to be rounded off is five, round off so that the figure immediately to the left is even.

If there are only counts exceeding 150 colonies, report result as the 'Estimated number of coliforms per 1 ml'.
8.
Precision
Results from internationally accepted collaborative trials are not available.
VII. ENUMERATION OF SOMATIC CELLS
This procedure specifies two procedures as references procedures for the enumeration of somatic cells:
A. The microscopic method
B. The fluoro-opto-electronic method

A. Microscopic method
1.
Scope and field of application
This procedure specifies the reference procedure for the enumeration of somatic cells in raw milk.
This procedure specifies the procedure for the enumeration of the number of cells in a milk sample to calibrate and check the accuracy of the fluoro-opto-electronic procedure (see B.1).
2.
Definition

For this procedure somatic cells are those cells, e.g. leucocytes and epithelial cells whose nuclei can be stained distinctly using methylene blue.

3.

Principle

0,01 ml of milk is spread over 1 cm$ of a slide. The film is dried and stained. Counting is carried out using a microscope. The number of somatic cells counted in a defined area is multiplied by the working factor to obtain the number of cells/ml.

4.

Reagents

Analytical grade chemicals must be used.

Dye solution:

Composition:

Methylene blue

0,6 g

Ethanol - 99 %

54,6 ml

1,1,1-trichloroethane or tetrachloroethane

40,6 ml

Glacial acetic acid

6,6 ml

Warning

Tetrachloroethane is poisonous. If used the preparation and application must be carried out in a fume cupboard.

Preparation:

Mix the ethanol and 1,1,1-trichloroethane or tetrachloroethane in a bottle and heat in a water bath to 60 to 70 oC. Add the methylene blue, mix carefully, cool in a refrigerator to 4 oC during 12 to 24 hours and add the glacial acetic acid. Filter, using a filter with pore size of 10 to 12 microns or less, and store the dye solution in an air-tight bottle. If particles or sediment are formed, filter again before use.

5.

Apparatus and glassware

5.1.

Microscope, with magnification of × 500 to × 1 000.

5.2.

Microsyringe, 0,01 ml with accuracy of p 2 % or better.

5.3.

Slide, with an area 20 mm × 5 mm marked for the film, or a standard slide and a template of 20 mm × 5 mm for the film.

5.4.

Levelled hot plate, (30 to 50 oC) for drying the slides.

5.5.

Fan (hairdryer), for drying the film.

5.6.

Water bath, capable of operating at 30 to 40 oC for heating the milk sample.

5.7.

Stage micrometer slide, ruled in 0,01 mm divisions.

6.

Procedure

6.1.

Milk sample

The milk sample must be tested within six hours of sampling. During storage the temperature of the sample must not exceed 6 oC. Freezing must be avoided.

6.2.

Preparation of the sample in the laboratory

Heat the sample in a water bath (5.6) to 30 to 40 oC. Then mix carefully. Cool to the temperature at which the microsyringe (5.2) has been calibrated, e.g. 20 oC.

6.3.

Pre-treatment of the slides

Clean the slides (5.3), for example with ethanol, dry with dust-free paper, flame and cool. Store in a box to avoid dust.

6.4.

Preparation of the film

Remove 0,01 ml of milk from the sample prepared as above using a microsyringe (5.2). Carefully clean the outside of the syringe in contact with milk. Place the syringe on the slide (5.3), first drawing the outline of the shape (20 mm × 5 mm). Then fill in the area as evenly as possible. Dry the film on a levelled hot plate (5.4) until completely dry.

At least two films from each milk sample must be prepared and examined.

6.5.

Staining the films

Dip in dye solution (4) for 10 minutes. Dry, completing with fan (5.5) if required. Dip the films in tap water until all the surplus dye is washed away. Then dry again and store protecting against dust.

6.6.

Calibration of microscopic field

Depending on the magnification chosen (× 500 to × 1 000) determine with the stage micrometer slide (5.7) the diameter of the microscopic field.

7.

Counting and calculation

7.1.

Counting of cells

Use a microscope (5.1). Instead of counting cells, only cell nuclei are counted. These are clearly recognizable and for the count at least half the nucleus should be visible in the microscopic field. Count strips or fields across the middle third of the film, avoid counting strips or fields selected exclusively from the peripheral areas of the film. Careful preparation of the films, and hence reliability of the results, must be checked at least once a month by counting different parts of the film. The counting may also be performed by counting of microscopic fields spread in a system such that all parts of the film are equally represented.

7.2.

Minimum number of cells to be counted

Since microscopic counting of somatic cells may also be used for standardization of automatic and mechanized counting procedures, the coefficient of variation of counts on identical samples must not be higher than that of electronic instruments. The coefficient of variation on a milk sample containing 400 000 to 600 000 cells/ml should not exceed 5 %. The number of somatic cells to be counted in each sample must, according to the characteristics of the Poisson distribution, be at least 400 in order to meet this repeatability.

The Poisson distribution presupposes

M = V = s$,

where

M is the mean value.

V

is the variance

and

s

is the standard deviation.

The coefficient of variation is:

CV = s × 100 % or CV = 100 % or CV = 100 %

CV =

s × 100 %

M

or CV =
100 %
s
or CV =
100 %
wM-
M (mean) indicating the number of particles (cells) which have been counted (i. e. 400 for CV = 5 %).
7.3.
Calculation of the working factor
Using 0,01 ml of milk the working factor is calculated according to 7.3.1. or 7.3.2.
7.3.1
Counting strips across the film
The length of strips to be counted is 5 mm each. The breadth of a strip corresponds to the diameter of the microscopic field as determined by the stage micrometer slide (5.7).
Working factor = 20× 100
Working factor =
20 × 100
d × b
where:
d = is the diameter of the microscopic field in mm as determined by the stage micrometer slide (5.7).
b
= is the number of strips counted completely.
7.3.2.
Counting microscopic fields in the middle third of the film or with grid:
Working factor = 20 × 5 × 100 = 12 732
Working factor =
20 × 5 × 100
P × d² × s
4
=
12 732
d² × s
where:
d = is the diameter of the microscopic field in mm as determined by the stage micrometer slide (5.7).
s
= is the number of fields counted.
7.4.
Calculation of the cell content
The number of somatic cells counted (7.1 and 7.2) is multiplied by the Working Factor (7.3), to obtain the cells per ml of milk.
7.5.
Precision
Coefficient of variation (see 7.2) must not exceed 5 %.
Results from internationally accepted collaborative trials are not available.

B. Fluoro-opto-electronic method
1.
Scope and field of application
This procedure specifies the reference procedure, which after proper calibration (se A.1), can be used for counting of somatic cells in raw milk - with or without chemical preservation.
2.
Definition

For this procedure somatic cells are particles which have a minimum intensity of fluorescence due to the staining of the DNA in the nucleus of somatic cells.

3.

Principle

A part of the sample (e.g. 0,2 ml) is thoroughly mixed with buffer solution and fluorescent solution. Part of this mixture is then transferred in the form of a thin film to a rotating disc which serves as an object plane for the microscope.

Each cell produces an electrical pulse which is amplified and recorded. The number of somatic cells is printed out in thousands per ml.

4.

Reagents

Analytical grade chemicals must be used unless otherwise stated. Water should be either distilled or deionized or of equivalent purity.

4.1.

Buffer solution

Composition:

Potassium hydrogen phtalate
51,0 g
Potassium hydroxide
13,75 g
Polyethyleneglycol-mono-p(1,1,3,3-tetramethylbutyl)-phenyl-ether
(e.g. Triton X-100), 1 % by volume
10 ml
pH 5,7 to 5,9. Make up with water to 10 000 ml.

Preparation:

The individual components are mixed. Airtight storage should not exceed seven days.

4.2.

Fluorescent solution (stock solution)

Composition:

Ethidium bromide
1,0 g
Make up with water to 1 000 ml.

Preparation:

Ethidium bromide is dissolved in water. Storage in a light-proof and airtight bottle should not exceed two months.

4.3.

Fluorescent solution (working solution)

20 ml of the stock solution (4.2) are mixed with the buffer solution (4.1) to give 1 000 ml. The working solution should not be used for longer than seven days.

4.4.

Cleaning solution

Composition:

Buffer solution (4.1)
10 ml
Polyethyleneglycol-mono-p-(1,1,3,3-tetramethylbutyl)-phenyl-ether
(e.g. Triton x-100), 1 % by volume
10 ml
Ammonia, 25 % by volume
25 ml
Make up with water to 10 000 ml.

Preparation:

The individual components are mixed. Storage should not exceed 30 days.

5.

Apparatus and glassware

5.1.

Counting instrument, working according to the fluorescence optical principle.
Note
Prior to use the instrument must be calibrated. The relation between the volume of the particles to be counted and the threshold level above which the counts are made is thus determined. Calibration of the apparatus is done in accordance with the manufacturer's instructions using samples whose cell content has been determined by the microscopic procedure (A).
5.2.
Water bath, with circulation, capable of operating at 40 p 1 oC.
5.3.
Test tube, with appropriate seal, approximately 15 ml.
6.
Milk sample
6.1.
The sample must be stored at low temperature in a test tube (5.3). If the sample is not chemically preserved, it should not be counted within the first 24 hours after milking because the counts will be too low. Storage temperature must not exceed 6 oC.
6.2.
Preservation
Chemical preservation must be done within 24 hours. Preservation must be carried out as soon as possible after sampling.
6.2.1.
Chemical preservation of the sample can be achieved by addition of one of the following preservatives:
- orthoboric acid:
the final concentration of orthoboric acid in the sample must not exceed 0,6 g/100 ml. Such a preserved sample may be stored for up to a further 24 hours at 6 to 12 oC,
- potassium dichromate:
the final concentration of potassium dichromate must not exceed 0,2 g/100 ml. Such preserved samples may be stored for up to a further 72 hours at 6 to 12 oC,
- sodium azide:
the samples may be preserved with sodium azide to a final concentration of 0,024 g/100 ml, provided the sample is cooled to 6 to 12 oC immediately after sampling, and counted within 48 hours after sampling,
- bronopol:
the sample may be preserved with bronopol to a final concentration of 0,05 g/100 ml, provided the sample is cooled to 6 to 12 oC immediately after sampling, and counted within 72 hours after sampling.
6.2.2.
A sample already preserved with orthoboric acid may be further preserved for up to 48 hours using potassium dichromate.
Note
Local conditions regarding the discharge of effluents must be observed for samples preserved with potassium dichromate.
7.
Procedure
7.1.
Pretreatment of the sample
The milk to be examined shall be stored after milking for at least 24 hours at approximately 2 to 6 oC. Counting of samples on the day of milking is not advisable without pretreatment because the results may be too low. If counting of such a sample is necessary, this shall be pretreated for at least three hours with potassium dichromate (see 6.2.1).
7.2.
Preparation

The pretreated sample (see 7.1), or the untreated sample which is at least one day old, is heated in a water bath (5.2) to approximately 40 oC. The sample is then stored at room temperature until the counts are done.

7.3.

Cell counting

The count shall be carried out using the counting instrument (5.1) within 15 minutes of the end of heating (see 7.2). Immediately before counting, the sample shall be thoroughly mixed in order to obtain as homogeneous a distribution of the somatic cells as possible.

Further dilution and preparation of the sample shall take place automatically in the instrument.

8.

Precision

Figures for repeatability (r) and reproducibility (R) from international collaborative trials are not available. In the future, precision data will be stated.

The data available on national level allow the following estimations:

(1) Cell count level between 400 000 and 500 000/ml

- standard deviation for repeatability:

sr = 20 000 cells/ml

(equivalent to a coefficient of variation or 5-4 %

- standard deviation for reproducibility:

sR = 40 000 cells/ml

(equivalent to a coefficient of variation of 10-8 %)

9.

Control of accuracy

Control of accuracy is performed using samples with known cell contents determined by microscopic cells counts at a national reference laboratory.

VIII. DETECTION OF ANTIBIOTICS AND SULPHONAMIDES

SCOPE AND FIELD OF APPLICATION

This procedure specifies the reference procedure for the detection of antibiotics and sulphonamides in raw milk and in heat-treated milk.

The reference procedure includes:

A. Qualitative method

This procedure is the initial procedure by which milk samples containing antibiotics including sulphonamides are selected. The procedure described is one of a number of similar procedures, which in principle all use Bacillus stearothermophilus var. calidolactis, ATTC 10149 as the test organism. The procedure has been chosen as representative for those tests.

B. Procedure for confirmation and identification of penicillin

This procedure must be used to confirm the results of the qualitative procedure, to identify penicillin and to determine the concentration of penicillin.

A. Qualitative procedure

1.

Scope and field of application

The procedure specifies the qualitative detection of antibiotics and sulphonamides in raw and heat-treated milk in excess of the limits laid down in the table:

Detectable concentrations of various antibiotics and sulphonamides ([1])

Test sensitivity
All negative
All positive
Benzylpenicillin
0,002

0,006
Ampicillin
0,002
0,005
Cloxacillin
0,015
0,035
Nafcillin
0,006
0,011
Tetracycline
0,10
0,40
Oxytetracycline
0,20
0,45
Chlortetracycline
0,15
0,50
Chloramphenicol
7,
15,
Dihydrostreptomycin
4,
13,
Neomycine
1,
22,
Kanamycin
9,
28,
Bacitracin
0,06
0,14
Erythromycin
1,
2,25
Rifamycin
0,01
0,14
Diaphenylsulfone
0,01
0,1
Sulphamethazine (Sulphadimidine)
0,5
1,
([1]) Benzylpenicillin and bacitracin expressed as IU/ml, all other antibiotics as mg/ml.

2.
Definition
The milk contains antibiotics or sulfonamides when the colour of the medium is not changed (see 7.1).
3.
Principle

A milk sample is added, together with nutrients to an agar gel containing pH indicator and spores of Bacillus stearothermophilus var. calidolactis ATCC 10149 (see 5.4.1), which has a good overall sensitivity and is particularly sensitive to inhibition by penicillin. Incubation resulting in the normal growth and acid production of the organism causes the pH indicator colour to change from purple to yellow. The presence in the milk of substances which are inhibitory to the growth of the organism causes the pH indicator colour to remain purple.

4.

Apparatus and glassware

The usual laboratory equipment and, in particular:

4.1.

Apparatus

4.1.1.

Incubator, capable of maintaining a temperature of 64 p 1 oC

4.1.2.

Water bath, capable of operating at 64 p 1 oC

4.1.3.

Rack, for tubes or ampoules

4.1.4.

Pipette, single service disposable tips suitable for sampling and dispensing 0,1 ml

4.1.5.

Forceps or tweezers

4.1.6.

Hot air oven, capable of operating at 170 to 175 oC

4.1.7.

Autoclave, capable of operating at 121 p 1 oC

4.1.8.

pH-meter.

4.2.

Glassware

4.2.1.

Sample bottles with suitable closures.

Note

Some rubber stoppers may deposit inhibitory substances on the neck of the bottle.

4.2.2.

Petri dishes, of clear, uncoloured glass or sterile synthetic material with flat bottoms of uniform thickness, minimum internal diameter about 140 mm.

4.2.3.

Bottles, of capacity 250 ml.

4.2.4.

Pipettes, (plugged with cotton wool) of glass or sterile synthetic material of nominal capacity 1 ml and 10 ml

4.2.5.

Glass spatulas

4.2.6

Tubes or ampoules, internal diameter about 8 mm, with caps or stoppers.

4.2.7.

Sterilization of glassware

Glassware should be sterilized by one of the following procedures:

(a) by being maintained at 170 to 175 oC for not less than one hour in an hot air oven (4.1.6);

(b) by being maintained at 121 p 1 oC for not less than 20 minutes in an autoclave (4.1.7).

In the autoclave care should be taken to ensure adequate penetration of steam, e.g. if the equipment is sterilized in containers, these should not be tightly closed, flasks or bottles should have loose lids.

Glassware sterilized in the autoclave should be dried by venting the steam.

Pipettes must be sterilized in a hot air oven.

5.
Media, solutions, test organism
The ingredients of the media must be suitable for bacteriological purposes. The water used must be glass-distilled or demineralized of at least equal purity. It must not contain substances inhibitory to the test organism.
5.1.
Media
5.1.1.
Nutrient agar
Composition
Yeast extract
2 g
Peptone
5 g
Meat extract
1 g
Sodium chloride
5 g
Agar
10-15 g
Water
1 000 ml
Preparation
Dissolve the components in water. Bring to boil, swirling time to time. Adjust pH so that after sterilization it is 7,4 p 0,1 at 25 oC.
Dispense 10 ml quantities into test tubes to make agar slants or 100 ml quantities into bottles. Sterilize at 121 p 1 oC for 15 minutes.
5.1.2.
Agar medium
Composition
Sodium chloride
2 g
Agar
15 g
Water
1 000 ml
Trimethoprim or Tetroxoprim solution (see 5.1.3) (1)
10 ml
Preparation
Dissolve the components except trimethoprim or tetroxoprim in water. Bring to boil, swirling from time to time. Add trimethoprim or tetroxoprim and sterilize at 121 p 1 oC for 15 minutes; adjust the pH so that after sterilization it is 7,0 p 0,1 at 25 oC.
5.1.3.
Trimethoprim or Tetroxoprim solution
Composition
Trimethoprim
5 mg
or Tetroxoprim
30 mg
Ethanol 96 %
5 ml/30 ml
Water to
1 000 ml
Preparation
Dissolve the trimethoprim or tetroxoprim in ethanol (5 or 30 ml) and dilute with water.

5.1.4.
Nutrient
Composition
Yeast extract
0,75 mg
Glucose
5,0 mg
Soluble starch
8,0 mg
Bromocresol purple
0,025 g
Water to
50 ml
Preparation
Dissolve the nutrients and indicator in the water, if necessary with heating, sterilize by filtration. The nutrient is commercially available as tablets.
5.2.
Standard penicillin solutions
5.2.1.
Prepare solution of penicillin of 60 mg/ml = (100 IU/ml) by dissolving crystalline sodium or potassium benzyl penicillin in sterile distilled water in a suitable stoppered sterile bottle.
5.2.2.
Prepare a working solution of penicillin by making up 1,25 ml of the penicillin solution (5.2.1)
to 1 000 ml with sterile distilled water. This working solution contains 0,075 mg
(= 0,125 IU/ml).
5.2.3.
Prepare 75 ml of a standard penicillin solution containing 0,004 mg/ml (= 0,0067 IU/ml) by adding 71 ml inhibitor-free milk (5.3) to 4 ml of working penicillin solution (5.2.2) and mixing.

5.2.4.
The penicillin solutions mentioned in 5.2.1 to 5.2.3 are to be prepared on the day the test is carried out.
5.3.
Inhibitor-free milk
Prepare as a control inhibitor-free milk by reconstituting skimmed milk powder (10 % m/v) previously tested and found to be free from inhibitory substances, in sterile distilled water. Alternatively, a sufficient quantity of fresh bulk milk tested and found to be free from inhibitory substances may be dispensed in bottles, heated for one hour at 100 oC and thereafter stored in a refrigerator at 0 to 6 oC for a maximum period of one week.
5.4.
Test organism
5.4.1.
Bacillus stearothermophilus var. calidolactus strain ATCC 10149 is used as the test organism. The strain is identical with C 953.
5.4.2.
Prepare a stock culture for maintenance of the test culture. The test culture is kept on a slant of nutrient agar (5.1.1). The agar slant is surface-streak inoculated, using a loop of the test culture, and is incubated aerobically for 48 hours at 63 p 1 oC. After incubation, the tube is sealed, using a sterile rubber stopper. The stock culture thus obtained can be kept for several months in a refrigerator at 0 to 5 oC.
5.5.

Test culture (spore suspension)
5.5.1.
20 ml of the nutrient agar (5.1.1) is transferred aseptically to a sterile Petri dish (4.2.2) and cooled to room temperature.
5.5.2.
With a sterile pipette (4.2.4) transfer 5 ml sterile distilled water to a tube with stock culture (5.4.2) and wash the spores off the agar slant using a sterile loop. This spore suspension shall be kept at 0 to 5 oC and shall be used within 36 hours.
5.5.3.
With a sterile pipette (4.2.4) transfer 0,5 ml of the spore suspension (5.5.2) to a culture plate (5.5.1) and spread the inoculum thoroughly over the whole surface with a bent glass rod. Incubate at 63 p 1 oC (4.1.1) for 16 to 18 hours.
When using a stock culture (5.4.2), or a culture which is more than 36 hours old, the subculturing procedure should be carried out at least twice with no more than an interval of 36 hours between subculturing.
5.5.4.
Transfer using a sterile pipette (4.2.4) 10 ml of distilled water to the culture plate (5.5.3) and remove spores from the surface into suspension using a glass rod.
Transfer the spore suspension to a bottle (4.2.3) containing 250 ml sterile distilled water. Close the bottle and shake thoroughly. Cultures which are not to be subcultured immediately should be stored in a refrigerator at 0 to 6 oC.
5.5.5.
The spore suspension shall have a viable colony count between 5 and 10 million per ml on plate count agar medium incubated at 63 p 1 oC for 16 to 18 hours. The spore suspension must be uniformly turbid and if it contains flocks or sediment it should be discarded and a new suspension should be prepared from the stock culture (5.4.2).
5.6.
Preparation of test tubes/ampoules
5.6.1.
Melt the agar medium (5.1.2) and cool to 55 oC.
5.6.2.
Add one part of fresh spore suspension (5.5.4) to five parts of the agar medium (5.6.1) in a tube or bottle, mix thoroughly.
5.6.3.
Transfer 0,3 ml of the inoculated media (5.6.2) calculated to give a layer 5 mm thick to a sterile tube or ampoule (4.2.6) and close with a stopper or a cap or by melting the tip. Let the test tubes/ampoules cool in an upright position, allow the medium to solidify and then let stand for at least 12 hours.
5.6.4.
The test tubes/ampoules may be used on the same day, but they may be kept several months provided they are cooled immediately after preparation and kept at 0 to 6 oC.
6.
Procedure
6.1.
Samples should be tested as soon as possible and preferably within 24 hours of sampling, maintaining the samples between 0 and 5 oC in the meantime. If it is not possible to test the samples within 24 hours, they should be maintained in deep-freeze (- 30 to - 15 oC) to minimize inactivation of penicillin.
6.2.
Identify each tube/ampoule (5.6) legibly and indelibly. Remove the cap or stopper. Place the required number for the samples and controls (5.2 and 5.3) to be examined in a suitable rack (4.1.3).
6.3.
Add 50 microliter of the nutrient mentioned in 5.1.4 to each tube/ampoule.
6.4.

Mix the milk sample thoroughly and transfer with the syringe (4.1.4) 0,1 ml into the corresponding labelled tube/ampoule. Use a clean disposable tip for each sample to be transferred.
6.5.
Repeat the operation described in 6.4 in duplicate using the standard penicillin solution containing 0,004 mg/ml (= 0,0067 IU/ml) penicillin instead of the milk sample (5.2.3).
6.6.
Repeat the operation in 6.4 in duplicate using the control of inhibitor-free milk (5.3) instead of the milk sample.
6.7.
Close the tubes/ampoules and place the rack containing the tubes/ampoules in a water bath at 63 p 1 oC (4.1.2) for at least 2; /2 to 2=/4 hours.
6.8.
Remove the rack containing the tubes/ampoules from the water bath.
6.9.
Observe the color of the test medium (see 7).
7.
Interpretation of results
7.1.
A purple coloration of the test medium in any of the milk sample or control tubes/ampoules indicates the presence of antibiotics or sulphonamides at or about the 'all positive' level given in the table on page 39 in the sample. The coloration of the tubes/ampoules with standard penicillin solution (6.5) shall remain purple to prove the test medium to be sufficiently sensitive.
7.2.
A purple coloration only of a part of the test medium or an irregular coloration in any of the milk sample tubes/ampoules indicates inhibitory substances between the levels given in the table on page 39 in the sample.
7.3.
A yellow coloration of the test medium in any of the milk sample or control tubes/ampoules indicates the absence of substances inhibitory to the test organism.
7.4.
If there is a purple coloration in all tested tubes/ampoules, including the negative control, the tubes/ampoules do not contain viable spores and the samples should be retested with freshly prepared test materials.
8.
Confirmation of results
8.1.
Confirm all samples with reactions as described in 7.1 and 7.2 in accordance with 'Method B'. If a storage of milk samples before confirmation is necessary, they must be deep frozen to prevent degradation of the antibiotics.

B. Procedure for confirmation of penicillins and determination of concentration
1.
Scope and field of application
The procedure specifies the confirmatory test for penicillins or antibiotics besides penicillins and procedure for determination of the penicillin concentration in milk samples with positive (A.7.1) or dubious reaction (A.7.2).
Sensitivity of various antibiotics by the procedure
see A.1.
2.
Definition
2.1.
The milk sample contains antibiotics including sulphonamide when the sample by the procedure described gives a clear zone of at least 2 mm of inhibition around the disk.

2.2.
If a sample which contains antibiotics including sulphonamide (2.1) and to which penicillinase (betalactamase) has been added gives no clear zone or a clear zone of a smaller diameter than without penicillinase the inhibitory substance is either penicillin or both penicillin and another antibiotic including sulphonamides.
2.3.
If the zone is not inactivated by penicillinase (2.2) the inhibitory substance in the milk sample is not penicillin, but can be another residue (see Directive 85/397/EEC, Annex A, Chapter VI, A.1.f. and 2 b).
Some of the semi-synthetic penicillins, e.g. sodium cloxacillin, are not or only partly inactivated by penicillinase or completely resistant and are therefore not identified as penicillin (see 7.3).
3.
Principle
A disk of absorbent paper impregnated with the milk to be examined is placed on the surface of an agar medium inoculated with Bacillus stearothermophilus, var. calidolactis. Incubation resulting in normal growth of the organism causes the agar to become cloudy. The presence in the milk of substances which are inhibitory to the growth of the organisms is indicated by a clear zone around the disk. The size of the clear zone depends, among other things, on the concentration and type of inhibitory substance in the milk.
4.
Apparatus, glassware and equipment
4.1.
Apparatus
4.1.1.
See A.4.1.
4.1.2.
Water bath, capable of operating at 80 o p 1 oC.
4.2.
Glassware
See A.4.2.
4.3.
Paper disks, inhibitor free, diameter 9 to 13 mm, capable of taking approximately 130 mg of milk (preferably stored in a dessicator).
5.
Media, standard solutions, penicillinase solution, reagents, test organism etc.
The ingredients of the media must be suitable for bacteriological purposes. The water used must be glass-distilled or demineralized of at least equal purity. It must not contain substances inhibitory to the test organism.
5.1.
Media
5.1.1.
Nutrient agar (A.5.1.1)
5.1.2.
Test medium for the detection of inhibitory substances
Composition
Yeast extract
2,5 g
Tryptone
5 g
Glucose
1 g
Trimethoprim or Tetroxoprim solution (A.5.1.3)
10 ml
Agar

10-15 g (depending on gelling quality)
Water
1 000 ml
Preparation
The solid components are completely dissolved in water by heating and stirring before the trimethoprim or tetroxoprim solution is added. After adding trimethoprim or tetroxoprim solution the pH must be adjusted so that after sterilization it will be 8,0 + 0,1 at 25 oC. The medium is sterilized for 15 minutes at 121 p 1 oC.
5.2.
Standard penicillin solutions in milk
see A.5.2.
For the quantification of inhibitory substances (8) make standard penicillin solutions in inhibitor-free milk (A.5.3) with the following concentrations:
(a) 0,004 mg/ml (0,0067 IU/ml)
(b)
0,006 mg/ml (0,01 IU/ml)
(c)
0,03 mg/ml (0,05 IU/ml)
(d)
0,06 mg/ml (0,1 IU/ml)
5.3.
Penicillinase solution
5.3.1.
Dissolve sufficient penicillinase (betalactamase) in sterile distilled water to give a concentration of 1 000 U/ml. This solution, preferably divided into small portions, can be stored at 0 to 5 oC for up to four weeks.
Note
There is no uniform international standard for penicillinase. For the purpose of this procedure, it is assumed that 10 units of penicillinase will be sufficient to inactivate 0,6 mg (= 1 IU) of penicillin. For supplies of penicillinase of unknown strength it will be necessary to check whether this assumption is valid. Otherwise it is necessary to change the concentration of the penicillinase solution correspondingly.
5.3.2.
Instead of penicillinase solution, commercially available disks prepared with penicillinase can be used if, after a control procedure they are found to contain an appropriate amount of penicillinase.
5.4.
Test organism
See A.5.4.
5.5.
Test culture (spore suspension)
See A.5.5.
5.6.
Preparation of the test plates
5.6.1.
Melt the test medium for the detection of inhibitory substances (5.1.2) and coolto 55 oC.
5.6.2.
Add in a bottle one part of fresh spore suspension (5.5) to as many parts of the test medium for detection of inhibitory substances (5.1.2) as gives an appropriate density of colonies in the inoculated test medium and mix thoroughly.
5.6.3.
Transfer to a sterile Petri dish (A.4.2.2), previously heated to 55 oC, the inoculated test medium (5.6.2) to give a layer 0,6 to 0,8 mm thick. For a Petri dish of 140 mm internal diameter about 15 ml test medium is necessary in order to obtain a thickness of 0,8 mm.
5.6.4.

Transfer the Petri dishes to a cold horizontal surface previously checked with a spirit level, remove the lids and allow the agar medium to solidify. When the medium has solidified, the lids are replaced on the dishes which are then inverted to minimize condensation on the surface of the agar medium.

5.6.5.

The test plates thus prepared are used preferably on the same day, but they may be kept up to two weeks provided they are kept in a sealed polyethylene bag at 5 oC immediately after preparation.

5.6.6.

In order to identify the samples mark the bottom of the test plates.

6.

Procedure

6.1.

Preparation of sample

6.1.1.

Samples giving positive or dubious results at 'Method A' (A.7.1 and A.7.2) must be retested, identified and quantified as penicillin.

6.1.2.

Initially these milk samples are heated at 80 p 1 oC for 10 minutes, to avoid influence from thermolabile non-specific inhibitors.

6.1.3.

After thoroughly mixing, approximately 10 ml of the heated test milk is transferred into a suitable sterile wide-mouthed bottle. About 0,4 ml of the penicillinase solution (5.3) is added to the milk and mixed thoroughly.

6.2.

Detection of inhibitors

6.2.1.

Dip a paper disk (4.3) into the milk sample (6.1.2) using a clean dry pair of forceps. Remove any excess milk by touching the disk against the side of the sample bottle. Place the disc flat on the surface of the test plate (5.6) and press down gently with the forceps.

6.2.2.

The disks prepared with the different milk samples must be at least 20 mm from each other and at least 10 mm from the edge.

6.2.3.

In order to check sensitivity, disks (4.3) dipped in the standard penicillin solution (5.2) must be placed randomly between the milk sample disks at a rate of at least 2 % of the number of milk sample disks and at least five standard disks should be used during each test.

6.2.4.

When all the disks have been placed on the agar medium in random fashion and have been identified, invert the plates and incubate at 63 p 1 oC for 2; /2 to five hours.

6.2.5.

After incubation the plates are examined in front of a suitable light source for clear zones of inhibition around the paper disks. Clear zones are measured.

6.2.6.

The zones around the disks containing the penicillin standard solution (6.2.3) must be at least 2 mm.

6.2.7.

Clear zones around the disks containing the milk sample of at least the same size or greater than that in 6.2.6 indicates substances inhibiting the test organism.

6.3.

Identification and quantification of the inhibitory substances

6.3.1.

Procedure 6.2.1 is performed in duplicate on the heated milk sample (6.1.2) and on the sample treated with penicillinase (6.1.3). Instead of adding penicillinase to 10 ml of the milk sample a prepared penicillinase disk (5.3.2) can be dipped into this sample and placed on the test plate.

6.3.2.
Procedure 6.2.1 is performed in duplicate for each of the standard penicillin solutions mentioned in 5.2.(a)-(d).
6.3.3.
The average diameters of the clear zones of inhibition for the milk sample and for the penicillinase control as well as for the standard penicillin solutions should be determined.
7.
Interpretation of results (see 2)
7.1.
If there is no clear zone around the disk containing the penicillinase control, but there is a clear zone around the disk containing the milk sample, equal to or greater than the zone around the disk containing the standard penicillin solution (5.2(a)), the inhibitory substance in the milk sample corresponds to a sodium-(potassium)-benzyl-penicillin concentration of at least 0,004 mg/ml.
7.2.
If the average diameter of the clear zone around the disk containing the penicillinase is equal to the average diameter of the clear zone around the disk containing the milk sample the milk contains inhibitory substances which are impossible to inactivate with the penicillinase concentrations used in this procedure.
7.3.
If the average diameter of the clear zone around the disk containing the penicillinase is smaller than the average diameter of the clear zone around the disk containing the milk sample heated in accordance with 6.1.2, the milk sample contains penicillin together with other antibiotics including sulphonamides besides penicillin or semi-synthetic penicillin, which cannot be identified by the penicillinase concentration used in this procedure. Synthetic penicillins, such as sodium cloxacillin, may not be inactivated by penicillinase under the conditions of the test and may therefore be classified as inhibitors other than penicillin.
Note
Inhibitory substances other than penicillin may if required be identified using suitable procedures.
8.
Determination of penicillin content
8.1.
The determination of the penicillin content can be performed either after drawing of a standard curve or by calculation from the zone sizes obtained with the standard penicillin solutions in milk (5.2.(a)-(d)).
8.2.
Drawing of a standard curve
As there is a linear correlation between the log10 of the penicillin concentration and the diameter of the inhibitory zones, the standard curve may be drawn on semilogarithmic paper with the penicillin concentrations as the logarithmic ordinate and the inhibitory zones as the abscissa. The inhibitory zones are calculated as the average of duplicate tests. The diameters of the inhibitory zones are plotted against the standard penicillin concentrations, and the standard curve is drawn.
8.3.
Calculation
The penicillin concentrations in the milk sample can be calculated from their zone diameters using the equation or the standard curve. For accurate assay the radius of the inhibition zones should be at least two times and not bigger than five times the radius of the disks.
9.
Expression of results
9.1.
The results are expressed as content of penicillin equal to or above 0,004 mg/ml (or by indicating the determined concentration) or as content of inhibitors other than penicillin.
9.2.

Repeatability (r) and Reproducibility (R)
Figures are not available and not meaningful because a standard is included for comparison.
IX. DETECTION OF PATHOGENIC MICRO-ORGANISMS
1.
Scope and field of application
In accordance with Directive 85/397/EEC requirement Annex A, Chapter VII (2), this procedure gives the instructions which must be followed to check pasteurized milk for pathogenic micro-organisms.
2.
Definition
Examination must be made for bacteria species which are the most frequently involved in food-borne diseases.
Pasteurization is a treatment which safeguards against the presence of non-thermoresistant pathogens in milk. If the standards laid down in Annex A, Chapter VII (2) of the Directive for colony count at 30 oC and 21 oC, coliforms and phosphatase are met, a specific test for pathogens is only necessary where there is suspicion that the milk is connected with a food poisoning outbreak.
3.
Procedure
Procedures and frequencies for examination must be laid down by the national authority, in a way which makes it possible to issue Health certificates concerning heat-treated milk for intra-Community trade. For detection of pathogenic micro-organisms apply the criteria and procedures which are internationally accepted if they are available.
4.
Result report
For each pathogenic micro-organism investigated the result shall be expressed in the following way:
Number per ml of milk or 'presence' or 'absence' in the volume of pasteurized milk required by the procedure used. The report shall describe clearly the procedure used. (1) Patent legislation on the use of culture media containing anti-folate substances have to be observed.