Procedures & Guidelines

Quality Control Routines in a dairy Industry

1. Introduction

This document is intended to serve as guideline for the daily Quality Control routines in a dairy industry. It covers the most common quality control methods, but it does not claim any completeness due to the continuous findings and new developments in the field of scientific techniques applied to industrial Quality Control.

The area of application of the present guideline is the dairy industry. It recommends procedures for raw material as well as intermediate and end product, nevertheless the focus is mainly plain milk therefore there might be other important quality controls to perform on other dairy products not included in the present guideline.

This document is a description of methods and procedures that are commonly used nowadays in the industry; it does not pretend to fulfil the possible legal requirements about quality controls, whose responsibility stands with the single producer.

Furthermore it is important to notice some of the following methods are frequently used in certain countries and they might not have a worldwide application but rather be regionally well-know and used.

2. RAW MATERIAL QUALITY CONTROL

I Physical-Chemical Analysis

2.1. Determination of pH values

2.1.1. Objective

To evaluate the pH values in liquid foodstuff as a preliminary quality control to identify microbiological spoilage as well as chemical contamination.

2.1.2. Definitions

pH –corresponds to the inverse of the hydrogen ion concentration in moles per litre. Thus, by definition:

$$pH = \log \frac{1}{[H^+]} = - \log [H^+]$$

The pH scale is commonly used between the values 1-14 and is usually in aqueous solution. [50]

Buffer solution –is a solution which, within a certain limits, "resists" the attempt to have its pH modified. The pH value suffers little change due to the addition of acids or bases. It is basically either a weak acid with its corresponding salt or a weak base with its corresponding salt.

2.1.3. Method basis

Consists of the evaluation of the hydrogen ion concentration (pH) using a potentiometer.

2.1.4. Materials used for test

2.1.4.1. Glassware

- Beaker (50 ml)

2.1.4.2. Reagents

- Buffer solution (pH 4.0 and 7.0)
- Potassium chloride solution (saturated or specified by the manufacturer)
- Distilled water

2.1.4.3. Equipment

- pH meter (Potentiometer)

2.1.4.4. Other materials

- Paper to dry the electrode

2.1.4.5. Analysis methodology

- Calibrate the pH meter (potentiometer), first in a pH 7.0 buffer solution, then in a buffer solution pH 4.0 (wash the electrode with distilled water between solutions).
- After calibration, wash the electrode with distilled water, dry it by lightly blotting and dip it into the beaker containing the sample.
- Proceed to read the pH value
- Wash, dry and store the electrode in potassium chloride solution

* **Note**: the temperature of sample should be considered. Although there is a Temperature compensated for most of the pH meter (potentiometer), but still the best Should be considered at 20-25ºC.

2.2. Milk acidity determination

2.2.5. Objective

To evaluate the acidity of milk samples submitted to normal solution of NaOH, in order to identify the result of an intense microbiological metabolism in the sample and obtain a rough esteem of the milk quality.

2.2.6. Definitions

2.2.6.1. Indicators

These are weak acids or bases that display color changes within a narrow pH range. The color changes are due to structural modifications, including the ones related to the production of resonance forms, such as those produced with phenolphthalein. The acid form (colorless) predominates in a pH lower than 8.2 and the basic form (pink) is apparent at pH higher than 10.0. An even number of the two forms can be found at pH 9.4, called turning point.

2.2.7. Introduction

The acidity evaluation can bring up data concerning the product conservation status. The acidity evaluation methods can either validate the acidity subject to titration or supply information about the concentration of free hydrogen ions (pH).

The titration methods use indicators, which either produce or change their color at certain hydrogen ion concentrations when titrating with standard alkali. Acidity can be expressed in ml of a normal solution, percent or grams of the main acid component.

For milk, the acidity is usually expressed Soxhlet degrees, in Dornic degrees or in lactic acid percentage.

2.2.7.1. The natural milk acidity

The natural milk acidity is due to:

- The presence of dissolved acid phosphates, citrates, casein, albumin and carbon dioxide.
- The secondary reactions released by the phosphates.
- The constant amount of the casein and phosphate content present. It neither goes up nor makes milk sour.
- No free lactic acid, in the case of milk, which has just been milked.

2.2.7.2. Developed acidity

This acidity is mainly due to the lactic acid formed by the microbial degradation of lactose, and, occasionally, to the lipids which are being modified. In microbial metabolism, each lactose molecule breaks up into four lactic acid molecules in the reaction:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4\ C_3H_6O_3$$

The developed acidity caused by lactic fermentation contributes to lowering the pH to the range of 4-5. All the organic acids present in milk are in this range.

2.2.7.3. Potential acidity

It is the acidity subject to titration. Titration with a sodium hydroxide solution evaluates the hydrogen ions initially present in milk and the hydrogen ions decomposing during the titration.

Potential acidity can be expressed in several units: Soxhlet-Henkel degrees (SH), Dornic degrees (D), Thorner degrees and lactic acid percentage are the most common.

A Soxhlet-Henkel degree (SH) is obtained by titrating 100 ml of milk with a sodium hydroxide solution N/4, every milliliter corresponds to 1°SH.. The newly formed milk must have 6.4 to 7.2° SH.

The Dornic degree (D) is obtained by titrating 100 ml of milk with a sodium hydroxide solution N/9, every milliliter corresponds to 1°D.. The normal value of the potential acidity of milk is between 15-22 Dornic degrees.

The Thorner degree (Th) is obtained by titrating 100 ml of milk with a sodium hydroxide solution 0.1N, every milliliter corresponds to 1°Th.

Lactic acid percentage is obtained by dividing the Dornic degree by 100

Conversion factors Table:

	°SH	°Th	°D
°SH	1	2,5	2,25
°Th	0,4	1	0,9
°D	0,444 (= 4/9)	1,111 (= 10/9)	1

2.2.7.4. Explaining the conversion

One mole of lactic acid (90g) neutralizes one mole of sodium hydroxide (40g). A solution of one Dornic degree contains 4.44g of NaOH (40/9) in 1,000 ml of water, and a solution 0.1N of NaOH contains 4.00g of the same base.

$$CH_3CH(OH)COOH + NaOH \rightarrow CH_3CH(OH)COONa + H_2O$$

90g 40g

Thus, the relation between these two quantities leads us to conclude that

One Dornic degree is equivalent to 1.111 (= 4.44/4) ml of a sodium hydroxide solution 0.1N.

Similarly the relation between the other units can be calculated.

Practical example

Say the acidity titration of 10 ml of milk consumes 1.9 ml of a 0.1N sodium hydroxide solution.

By converting the milk acidity into the different units we obtain:

1.9 ml multiplied by a factor 10 to obtain °Th (because they refer to 100ml of milk): 19°Th

19°Th multiplied by a factor 0.9 to obtain 17.1°D and divided by 100 to obtain the lactic acid percentage i.e. 0.171%.

The value in °SH is equal to 19 ml multiplied by a factor 0.4 i.e.7.6°SH

2.2.7.5. Real acidity

The real acidity of milk depends on the concentration of hydrogen ions and is evaluated by the pH value using the pH meter.

The content of organic salts offers milk a buffer system without changing the pH values, and is responsible for protein stability.

There is no direct relationship between real acidity and potential acidity (total acidity). Fresh milk can show high potential acidity and low real acidity, or vice versa.

The lactic acid fermentation does not initially affect the potential acidity and the pH value does not change. This means that an increase of the potential acidity does not necessarily imply pH or real acidity modifications. For fresh milk, the pH range of 6.6-6.8 is considered normal.

2.2.7.6. Factors influencing an increased acidity

- Time and conservation conditions of milk.

- Milk taken from cows that suffered severe mastitis.

- Cow breed.

- Presence of colostrums (milk from the first four days after delivery).

- Influence of cow feeding

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2.2.8. Materials used for test

2.2.8.1. Glassware

- Volumetric pipettes (10 ml)

- Erlenmeyer (125 ml)

- Graduated burette (10 ml)

2.2.8.2. Reagents

- Sodium hydroxide solution 0.1N or Dornic alkaline solution (NaOH N/9)

- Alcoholic solution of phenolphthalein. 2 g of indicator in 75 ml of 95% ethanol plus 20 ml of water.

2.2.9. Method recommendations

- Use dry glassware since the presence of water during the indicator process interferes in the titration.
- It is recommended to use approximately two drops of the indicator (about 0.1 ml) to evaluate acidity. A difference of up to 3º D can be detected when larger quantities are used (ten drops).
- When powdered milk is tested, an appropriate recostitution must be done before analysis. For example:

  Whole powdered milk = 1 + 7

  Skimmed powdered milk = 1 + 10

- It is generally used 5 g of SNF (Solid Not Fat) reconstituted with water. The final number of milliliter used in the titration must be multiplied by the factor 2 (it is recognized as a standard to refer to 10 g of SNF)
- Follow the sample titration with a control; until the dye turning point is achieved (a weakly pink color will develop).

2.2.10. Analysis methodology

- Pipette 10 ml of the sample into a 125 ml Erlenmeyer flask.
- Add about two drops of the 1% phenolphthalein alcoholic solution.
- Proceed with titration using the sodium hydroxide 0.1N until a pinkish color appears.
- Read and record the result in millilitres of alkaline solution and than multiply by the appropriate factor (see table cap.2.2.3.3).

[4, 5, 50]

2.3. Alcohol test

2.3.1. Objective

Assess the stability of milk proteins by their precipitation with alcohol under different concentrations.

2.3.2. Materials used for test

2.3.2.1. Glassware

- Petri dishes
- Graduated pipettes (2 ml)

2.3.2.2. Reagents

- Neutralized ethyl alcohol solutions (pH 7.0), 70, 72, 74, 75, 76, 78 and 80° GL.

2.3.3. Analysis methodology

- With graduated pipettes (2 ml), stir onto a Petri dish 2 ml of milk and 2 ml of ethyl alcohol 74° GL; shake the dish carefully.
- If neither clots nor flakes form, carry out a new analysis by increasing the alcohol concentration. These analyses must be carried out until clots or flakes are seen.

2.3.4. Result assessment and interpretation

- The “alcohol number" is the highest concentration of alcohol mixed with the same amount of milk, which will not lead to clot formation or precipitation.
- Note that Tetra Pak recommends a product stabile to 74° GL alcohol. IDF suggests 72° GL alcohol.

[51, 52]

2.4. Alizarol test

2.4.1. Objective

Assess the quality of raw materials through the verification of the protein stability and degree milk acidity.

2.4.2. Introduction

This test combines the alcohol test and the colorimetric pH determination (with the alizarin indicator) so that the casein clotting point and the pH turning point are simultaneously visualized.

Alizarin, when submitted to alcohol solution (75° GL), shows the acidity and the stability of the milk proteins.

2.4.3. Materials used for test

2.4.3.1. Glassware

- Test tube (20 or 25 ml)
- Graduated pipettes (2 ml)

2.4.3.2. Reagents

- Alizarin solution or alizarol (ready or lab-prepared): dissolve 2g of alizarin into 100 ml of neutralized ethyl alcohol 75° GL.

2.4.4. Analysis methodology

- Pipette 2 ml of milk and 2 ml of the reagent into a test tube; shake slowly by inversion.

2.4.5. Result assessment and interpretation

- Violet color: alkaline sample.
- Yellow color: acid milk.
- Brown-reddish color: normal milk.

There is no coagulation in normal milk; it only occurs when acidity is high.

Criteria for Alizarol test evaluation:

Milk	pH	%TA	Flocculation	Colour
Fresh milk	6,66 - 6,75	0,14 – 0,16	None	Light purple
Slightly Sour	6,30 – 6,50	0,17	Possible small flakes	Brownish-pink
Sour	6,00 – 6,20	0,18 – 0,19	Small flakes	Brownish-yellow
Very sour	<6,00	0,20+	Big/Large flakes	Yellow
Sweet coagulation	6,60 – 6,75	0,14 – 0,16	Big /Large flakes	Light purple
Mastitis	6,80 +	NA	Small flakes	Violet
Added alkaline	6,80 +	NA	None	Violet

[52]

2.5. Freezing point determination

2.5.1. Objective

To evaluate the freezing point of milk in order to detect adulteration by water addition to the product.

2.5.2. Introduction

The evaluation of the milk freezing point is accomplished using the Digital Cryoscope. The addition of water to milk not only reduces its quality, but also leads to spoilage or contamination that can present a health hazard. Milk has an average freezing point of -0.54°C. When milk is mixed with water, its freezing point is closer to 0°C.

2.5.3. Materials used for test

2.5.3.1. Glassware

- Cryoscopy tubes
- Graduated pipette (2 ml)

2.5.3.2. Reagents

- Calibration solution for the machine and anti-freeze solution:

Standard “A” solution: distilled water (-0.000°C freezing point)

Standard “B” solution: sodium chloride solution (-0.600°C freezing point). Put approximately 12 g of sodium chloride into an oven at 300°C for 5 hours or at 130°C for at least 24 hours. Cool down the sample in a desiccator. Weigh exactly 10.161 g and dissolve into distilled water, bringing the volume up to 1,000 ml. Let the solution stabilize for 24 hours.

A suitable cooling liquid for the cryoscope is 33% aqueous solution of propylene glycol

2.5.3.3. Equipment

- Thermistor Cryoscope

2.5.3.4. Other materials

- Absorbent paper
- Tube rack

2.5.4. Analysis methodology

Test the sample and the sodium chloride solution after they have reached the same temperature.

If the sample total acidity exceeds 20 ml of 0.1mol/l sodium hydroxide solution per 10g of non Fat solids , the final result of the test will not be representative of the original milk

Proceed according to the instructions supplied by the equipment manufacturer.

[28, 30]

2.6. Density determination

2.6.1. Objective

To analyze the milk density in order to estimate the solid content.

2.6.2. Introduction

The milk density varies between 1.028 and 1.033 g/ml at 15.5°C.

The density changes according to the milk temperature, washing and skimming.

Milk components:

- Water: d = 1.000 g/cm^3
- Fat: d = 0.930 g/cm^3
- Protein: d = 1.346 g/cm^3
- Lactose: d = 1.666 g/cm^3

2.6.3. Materials used for test

- Graduated cylinder
- Lacto-density meter

2.6.4. Analysis methodology

- Slowly pour approximately 250 ml of milk into the graduated cylinder; avoid producing foam.
- Lay down the Lacto-density meter carefully. After its stabilization, record the temperature (T) and density (Dt). The density is generally referred to at the temperature of 20°C (sometimes 27°C in tropical countries).

[1]

2.6.5. Calculation

Apply one of the following formulae:

D = Dt + (T - 20) x 0.25 (temperature of 25°C)

D = Dt + (T - 27) x 0.3 (temperature of 30°C)

Being:

D = density at 15°C

Dt = density read from the hydrometer

T = temperature of the reading

2.7. Milk determination of fat content

2.7.1. Objective

To evaluate the content of lipids or fat in milk samples in order to fulfil legal and commercial requirements.

2.7.2. Introduction

There are two methods used to evaluate the content of fat in milk:

- Gravimetric method
- Volumetric method

2.7.3. Gravimetric method

This method is the technique most commonly used to analyze food for fat content. The method is based on the continuous extraction of lipids using organic solvents, mainly ethyl ether, petroleum ether or benzene. A Soxhlet machine removes the solvent and the lipids are then weighed and assessed.

In some food, such as butter and margarine, the content of fat can be evaluated by difference. In this gravimetric method, food is placed in an appropriate flask and held over a Bunsen burner flame allowing the water present in the food to be released. First, the result for volatile substances is obtained in oven at 105°C, and then the direct extraction of fat is carried out by shaking with ethyl ether, decanting and separating the fat from the ether solution. The fat is then dried in an oven at 105°C, cooled down, and weighed. The content of fat is obtained by weight differences.

Some foods contain fat, which is intimately associated with proteins and carbohydrates, such as milky blends, soy products, wheat products (powdered and with fiber). For these foods, it is necessary to pre-treat the sample with an acid hydrolysis step, preferably using concentrated hydrochloric acid and heat. After hydrolysis, fat is submitted to drying and the quantity of fat present is gravimetrically determined by continuous extraction with petroleum ether, 40-60°C (Weibull-Stoldt method).

The direct cool extraction method is commonly used for the fat analysis in liquid foods. In this method, the sample is placed in a separation funnel and weighed. This method is useful when no heating is required for a lipid extraction. In addition, this method can be used in other evaluations, such as the peroxide content evaluation and the Kreiss reaction. [6, 14]

2.7.4. Volumetric method

The Gerber method is the most applied method for evaluating lipids in milk and other dairy products such as milk cream, cheese and yogurt.

This method is based on the release of fat by the breakdown of emulsified milk. The fat is released by cracking the protein layer (casein) surrounding fat droplets. This is accomplished through the addition of sulfuric acid (specific-density: 1.825 g/mL). Isoamyl alcohol is added to avoid the formation of lather, which eases the separation of the fat layer from other food. The separation is achieved by centrifugation in a Gerber centrifuge. The fat content is directly read in the butyrometer, either in percent grams or percent milliliters.

To automatically evaluate the fat in milk and other dairy products, machines such as Milko-tester can also be used. [3]

2.7.5. Milk determination of fat by using the Gerber method

2.7.5.1. Materials used for test

Glassware

- Volumetric pipette (11 ml)
- Graduated pipette (1 ml)

Reagents

- Sulfuric acid (d=1.812-1.820 at 20°C)
- Amyl or Isoamyl alcohol (d=0.808-0.818 at 20°C)

Equipment

- Gerber heat centrifuge or Gerber centrifuge with water bath at 65ºC (Another centrifuge can be used instead of the Gerber one)
- Gerber milk butyrometer or another butyrometer
- Graduated pipettes (1 and 10 ml)
- Thermometer (0-100ºC)

Other materials

- Butyrometer racks
- Absorbent paper
- Suction bellow
- Cloth

2.7.6. Analysis methodology

- Transfer 10 ml of sulfuric acid using a graduated pipette into a butyrometer.
- Slowly add 11 ml of the sample with a volumetric pipette. Attention must be given so that the sample is not burned when in contact with the acid.

- Add 1 ml of Isoamyl alcohol. These additions must be carried out without soaking the internal part of the butyrometer neck; if this does occur, carefully clean the area with absorbent paper).
- Cork the butyrometer, wrap it in a cloth, and stir until thoroughly dissolved.
- place the butyrometer in the centrifuge;bring the centrifuge to the operating speed required to give a relative centrifugal acceleration of 350±50g within 2 min, and then maintain this speed for 4 min.
- Remove the butyrometer from the centrifuge and if necessary, adjust the stopper to bring the fat column on the scale .place the butyrometer ,stopper downwards ,in the water bath at 65±2ºC for not less than 3 min and not more than 10 min .the water level shall be above the top of the fat column.
- Manipulate the cork by placing the light yellow, transparent layer (lipids) inside the butyrometer-graduated shaft.
- The reading of the oily layer (top of colomn minus bottom of the colomn) can be used to calculate the grams of fat per 100 g of milk or grams of fats per 100 ml of milk

2.7.6.1. Observations

This method must only be used with high quality reagents whose densities are appropriate and indicated in the above method.

Observe the order that the reagents and the sample are carefully placed inside the butyrometer. The acid is denser than milk and if the acid was added after the milk, the acid would pass through the milk and burn the milk. On the other hand, amyl alcohol is a solvent, which dissolves and carries fat; if the solvent is added before the milk, it will cause protein clotting.

Note that the butyrometers must be placed inside the centrifuge so that the weights are balanced.

If the centrifuge to be used is not heated, use water bath to warm the butyrometer after centrifuging. [6]

2.8. Determination of total dry, degreased extract

2.8.1. Objective

To evaluate the concentration of the total dry degreased extract from a milk sample.

2.8.2. Introduction

The total dry extract (or total solids) is mainly represented by the proteins, lactose, and fat present in milk. Variations can occur in its content due to factors such as animal breed, feeding, suckling period, etc.

The dry degreased extract represents the difference between the total dry extract and the fat found in milk.

2.8.3. Materials used for test

- Ackermann discs

2.8.4. Analysis methodology

- Figure out the TDE with Ackermann discs by matching the graduations of the internal circle and medial circle, which correspond to density and fat, respectively. The position of the arrow in the internal circle indicates the total dry extract (TDE).
- The TDE is determined by subtracting the amount of grams for the TDE percent from the grams for fat percent. [11]

2.9. Methyl blue reduction test (Resazurin test)

2.9.1. Introduction

The evaluation of the total amount of microorganisms present in raw milk can be done through direct or indirect methods.

The indirect processes are more commonly applied due to speed and low cost; they are, however, not as precise as the direct methods. The indirect method more frequently used to evaluate the total amount of microorganisms present in raw milk is methyl blue reduction. The bacteriological quality of milk is determined in this process. Basically, methyl blue is added to milk and the time required to discolour the mixture is verified. Other indicators, such as resazurin, are also applied.

The precision of this test decreases the longer the milk is stored, since the traditional mesophilic flora is replaced by Psychrotrophic flora.

2.9.2. Method

In this methyl blue test, approximately 10 ml of milk are poured into a graduated cylinder. A small amount of methyl blue (add 1 ml of a solution prepared by diluting 5 ml of a saturated solution of methyl blue in ethanol 96º GL in 95 ml of sterile water .this solution shouldn't be exposed to light and discarded after two months of preparation) is added to the milk, which makes the solution a bluish colour. This mixture is incubated in a 37°C water bath. The time required to turn the mixture to a white colour again is measured as the discolouring time. Based on the determined discolouring time, milk is commonly divided into three quality classes. **The shortest discolouring time indicates the highest level of metabolic activity and, consequently, the largest amount of micro-organism present in the milk. [52]**

Classes	Time
1	> 2 h
2	30 min to 2 h
3	30 min

II RAW MILK ALTERATION AND ADULTERATION

2.10. Qualitative test to verify the presence of amide in milk

2.10.1. Introduction

Amide is used as a thickener, and it corrects the density of water-added milk.

In tests, amide with iodine (Lugol) forms a bluish adsorption compound.

2.10.2. Materials used for tests

2.10.2.1. Glassware

- Beaker (5 ml)
- Graduated pipettes (1 and 10 ml)

2.10.2.2. Reagents

- Iodine alcohol solution 1% (Lugol).

2.10.2.3. Equipment

- Bunsen burner with wire screen

2.10.2.4. Other materials

- Cold water bath.

2.10.3. Analysis methodology

- Pipette 10 ml of the test milk into a 50 ml beaker. Warm up to boiling. Cool in ice water bath. Add 3 drops of 1% iodine alcohol solution or Lugol. Observe.

2.10.4. Result assessment and interpretation

- Positive reaction: a blue ring appears.
- Negative reaction: a brown ring appears.

*** Observations:**

- The bluish colour will disappear with heating.
- Carry out a blank test.

2.11. Qualitative test to verify saccharose in milk

2.11.1. Introduction

Saccharose can be added to milk so that water addition is disguised and density is raised.

In research, resorcin links with aldoses when acids are present giving a pinkish colour.

2.11.2. Materials used for test

2.11.2.1. Glassware

- Test tubes (25 ml, 20x200)
- Graduated pipettes, 1 and 10 ml

2.11.2.2. Reagents

- Concentrated hydrochloric acid
- Alcohol resorcin solution 20%

2.11.2.3. Equipment

- Water bath with thermostat

2.11.3. Analysis methodology

- Pipette 10 ml of suspected milk into a test tube. Add 1 ml of concentrated hydrochloric acid and 2 drops of 20% alcohol resorcin solution. Stir carefully. Warm up in boiling water bath for 5 minutes. Observe.

2.11.4. Result assessment and interpretation

- Positive reaction: pinkish red colour.
- Negative reaction: bluish yellow colour.

*** Observation:**

Do not consider the result in case the colour appears after a short span of time; this will be caused by lactose hydrolysis.

2.12. Qualitative test to check the presence of chlorides in milk

2.12.1. Introduction

This test is based on the precipitation of chlorides, in the form of silver chloride. The presence of chlorides in milk might indicate the extensive use of chemical fertilizers on the feeding for the cows (pesticides).

2.12.2. Materials used for test

2.12.2.1. Glassware

- Graduated pipettes (1.5 and 10 ml)
- Test tubes

2.12.2.2. Reagents

- Silver nitrate solution 0.1N
- Potassium chromate solution 5%

2.12.3. Analysis methodology

- Pour 5 ml of milk into a test tube.
- Add 4-5 drops of 5% potassium chromate water solution. Stir.
- Add 2.5 ml of 0.1N silver nitrate and stir.

2.12.4. Result assessment and interpretation

The yellowish colour indicates the presence of chlorides in amounts higher than the normal range. If there are chlorides in milk within the normal range, the colour can vary from dark orange to dark red.

2.13.Determination of formalin as preservative in milk

I Floroglucin method

Floroglucin reacts with formalin, producing the reddish hydroxymethylated derivative.

2.13.1. Materials used for test

2.13.1.1. Glassware

- Graduated pipettes (5 ml)
- Test tubes

2.13.1.2. Reagents

- Floroglucin solution 1%
- Sodium hydroxide (NaOH) solution 10%

2.13.2. Analysis methodology

- Transfer 10 ml of milk into a test tube.Add 1 ml of 1% Floroglucin solution and 2 ml of 10% sodium hydroxide solution.

2.13.3. Result assessment and interpretation

- When formalin is present, a salmon-pink color will appear. The reaction is fast.

II Ferric chloride method

2.13.4. Materials used for test

2.13.4.1. Glassware

- Graduated cylinder (5 ml), test tube and pipette (1 ml)

2.13.4.2. Reagents

- Sulfuric acid (1+1)
- 1% Ferric chloride ($FeCl_3$) solution

2.13.5. Analysis methodology

- Transfer 5 ml of sample and 1 ml of sulfuric acid (1+1) into a test tube.
- Add a drop of 1% ferric chloride and boil.

2.13.6. Result assessment and interpretation

When Formalin is present, a pinkish color will appear.

2.14. Determination of hydrogen peroxide as preservative in milk

I First method: Guaiacol

2.14.1. Materials used for test

2.14.1.1. Glassware

- Test tubes (20 ml)

2.14.1.2. Reagents

- 1% Guaiacol solution
- Unprocessed milk

2.14.2. Analysis methodology

- Transfer 10 ml of sample into a test tube.
- Add 2 ml of 1% Guaiacol solution and 2 ml of unprocessed milk.

2.14.3. Result assessment and interpretation

- The appearance of the salmon-pink colour shows that hydrogen peroxide is present in the sample.

II Second method: vanadium oxide

2.14.4. Materials used for test

2.14.4.1. Glassware

- Graduated cylinder (10 ml)
- Test tube

2.14.4.2. Reagent(vanadium Oxide solution)

- 1g of vanadium oxide (V_2O_5)
- 100 ml of H_2SO_4 6%

2.14.5. Analysis methodology

- Pour 10 ml of milk into a test tube.
- Add 10-20 drops of the reagent. Stir.

2.14.6. Result assessment and interpretation

A pinkish or reddish colour will appear in the presence of H_2O_2.

[46]

3.15. RAW MILK ADULTERATION (quick methods)

I Test for detection of hydrogen peroxide

Take 5 ml milk in a test tube and then add 5 drops of paraphenylene diamine and shake it well. Change of the colour of milk to blue confirms that the milk is added with hydrogen peroxide.

II Test for detection of Salt

Addition of salt in milk is mainly resorted to with the aim of increasing the corrected lactometer reading.

How to detect?

Five ml of silver nitrate (0.8%) is taken in a test tube and added with 2 to 3 drops of 1% potassium dichromate and 1 ml of milk and thoroughly mixed. If the contents of the test tube turn yellow in colour, then milk contains salt in it. If it is chocolate coloured, then the milk is free from salt.

III Test for detection of pulverized soap

Take 10 ml of milk in a test tube and dilute it with equal quantity of hot water and then add 1 – 2 drops of phenolphthalein indicator. Development of pink colour indicates that the milk is adulterated with soap.

IV Detection of detergents in milk

Take 5 ml of milk in a test tube and add 0.1 ml of bromocresol purple solution. Appearance of violet colour indicates the presence of detergent in milk. Unadulterated milk samples show a faint violet colour.

V Test for detection of Starch

Addition of starch also increases the SNF content of milk. Apart from the starch, wheat flour, arrowroot, rice flour are also added.

How to detect?

Take 3 ml milk in a test tube and boil it thoroughly. Then milk is cooled to room temperature and added with 2 to 3 drops of 1% iodine solution. Change of colour to blue indicates that the milk is adulterated with starch.

VI Test for detection of glucose

Usually poor quality glucose is added to milk to increase the lactometer reading. There are two tests available to detect the adulteration of milk with glucose.

How to proceed?

1. Phosphomolybdic or Barford Test

Take 3 ml of milk in a test tube and add 3 ml Barford's reagent and mix it thoroughly. Then keep it in a boiling water bath for 3 min and then cool it for 2 min by immersing in tap water with out disturbance. Then add 1 ml of phosphomolybdic acid and shake. If blue colour is visible, then glucose is present in the milk sample.

2. Diacetic test

Take a strip of diacetic strip and dip it in the milk for 30 sec to 1 min. If the strip changes colour, then it shows that the sample of milk contains glucose. If there is no change in the colour of the strip, then glucose is absent. In this method the presence of glucose in milk can be quantified by comparing the colour developed with the chart strip.

VII Test for detection of urea

1. Urea is generally added in the preparation of synthetic milk to raise the SNF value.

Five ml of milk is mixed well with 5 ml paradimethyl amino benzaldehyde (16%). If the solution turns yellow in colour, then the given sample of milk is added with urea.

2. Take 5 ml of milk in a test tube and add 0.2 ml of urease (20 mg / ml). Shake well at room temperature and then add 0.1 ml of bromothymol blue solution (0.5%). Appearance of blue colour after 10-15 min indicates the adulteration milk with urea.

III MICROBIOLOGICAL ANALYSES

2.16. Total Spore count and Heat resistance spore count

2.16.1. Objective

To enumerate total spores and heat resistance spores.

2.16.2. Definitions

2.16.2.1. Spores

Bacterial spores are highly resistant structures created when spore forming organisms experience environmental stress.

Spores do not multiply as such, if favourable conditions are present (for instance, the correct oxygen supply, temperature, etc.) each spore creates a vegetative cell (viable cell), which is able to multiply.

2.16.3. Introduction

The sporogenic bacteria commonly present in food belongs to the genera *Bacillus*, *Clostridium* and *Desulfotomaculun*. These, due to the heat resistance of the spores, are generally associated with the spoilage of thermally processed products that are packed into hermetic packages (commercially sterile products). The introduction of such spores in this kind of food occurs mainly because of raw material used during the formulation procedures, such as spices, sugar, flours and powdered milk,.

According to the heat resistance of the spores and the optimum growth temperature, the sporogenic bacteria are divided into two groups:

2.16.3.1. Thermophilic sporogenic bacteria

These include species whose optimum growth temperature is around 55°C and whose spores are highly heat resistant,

There are two thermophilic sporogenic types associated with the deterioration of commercially sterile products: aerobic and anaerobic thermophilic.

2.16.3.2. Mesophilic sporogenic bacteria

These include species whose optimum temperature is around 30-35°C and whose spores are highly heat resistant and which are also able to survive after severe thermal treatments are applied to low acidity food.

There are two types of mesophilic sporogenic bacteria associated with the deterioration of commercially sterile food: aerobic and anaerobic mesophilic.

2.16.4. Materials used for test

2.16.4.1. Glassware

- Graduated cylinder (250 ml)
- Test tubes or dilution flasks with 9 ml or 90 ml of diluent, respectively (All of these materials, on the whole, must be sterilized in an autoclave at 121°C for 15 minutes)
- Sterile screw-thread test tubes
- Sterile Petri dishes
- Sterile graduated pipettes (1 and 10 ml)
- Erlenmeyer (500 or 1,000 ml) with plate count agar (PCA). Screw-thread test tubes can be prepared, each with approximately 20 ml of the preparation (All of these materials, on the whole, must be sterilized in an autoclave at 121°C for 15 minutes)

*** Observations:** All sterile glassware must be sterilized in an oven at the minimum temperature of 170ºC for over 2 hours. Graduated glassware must be sterilized in autoclave at 121°C for 30 minutes.

2.16.4.2. Reagents

- 70% Ethyl alcohol (ethanol). Preparation: Pour approximately 200 ml of absolute ethyl alcohol onto a 250ml-graduated cylinder, dip in the alcoholmeter and gradually add distilled water until 70º GL are reached. Observation: Verify the procedures monthly. If any alteration in the solution concentration is detected, add absolute ethyl alcohol until 70° GL are reached. Store the solution in a closed container and keep it in a fresh place.
- Culture medium: plate count agar (PCA)
- Diluent: 0.1% peptonized water (1g of peptone/1,000 ml of distilled water), pH = 7. If necessary, adjust the pH either with 0.1 N hydrochloric acid (to lower the pH) or 0.1N sodium hydroxide (to raise the pH).

2.16.4.3. Equipment

- Incubators at 35 and 55 ± 1°C
- Thermostatic bath at boiling temperature
- Semi-analytical scale
- Autoclave
- Sterilization oven at 170°C
- Alcoholmeter

2.16.4.4. Other materials

- Magnifying glass or colony counter
- Pipette-rack to accomplish sterile pipettes
- Bunsen burner
- Matches

- Cotton
- Scissors
- Chronometer
- Distilled water

2.16.5. Analysis methodology

- Shake the package carefully.
- Cleanse the external area of the package with cotton soaked in 70% ethanol to remove any contaminant (cleanse the counter surface as well).
- Open the package with scissors (these must be treated through the flame of a bunsen burner).
- Aseptically pour 3-5 ml of the sample with a pipette onto a sterile screw-thread test tube.
- Place the tube in a thermostatic bath at 80°C (total spores) and at boiling temperature100°C (heat resistant spores) for 10 minutes. An additional tube (with a thermometer inside) with 10.0 ml of product must also be placed in the bath (the time counting is only started after the additional tube reaches the specified temperature).
- Carry out appropriate series dilutions (see item 2.16.5.1).
- Inoculate, out of the dish centre,1 ml of each dilution into separate, sterile and empty Petri dishes (this procedure will ease the subsequent mixing with the culture medium), opening the dishes only enough to let the pipette in, close to the Bunsen burner. To increase the counting precision, it is recommended to inoculate two or more dishes per dilution (duplicates or triplicates).
- Add approximately 15-20 ml of PCA (previously melted and cooled to at 45-48°C, harmless to skin touch) on the dishes (This is the depth plating method).
- Smoothly revolve the dishes in eight-like movements; making sure the mixture does not touch the dish lids and wait for the agar to solidify.
- Invert the dishes, incubate them at 35-37°C (total spores) for a period of 48hrs,35-37°C (thermoresistant mesophilic spores), and 55°C (thermoresistant thermophilic spores) for 5 days.

2.16.5.1. Preparation of the 10^{-1} dilutions

- Aseptically transfer 1 ml of the sample into 9 ml of the diluent (in a test tube), or 10 ml of the sample into 90 ml of the diluent (in a dilution flask), and stir.
- It is recommended to avoid dipping pipettes in a depth larger than 2.5 cm as the sample content is poured with the pipette.
- When selecting pipettes to be used for analysis, always choose pipettes, which have a capacity 10 times larger in volume than the amount to be collected. For instance, when pouring amounts of 1 ml, use at least 10 ml pipettes.

2.16.5.2. Preparation of series dilutions

- Dilution 10^{-2}: Aseptically transfer 1 ml of the dilution 10^{-1} into 9 ml of diluent, or 10 ml of the dilution 10^{-1} into 90 ml of diluent, and stir.
- The subsequent dilutions are obtained in a similar way, by transferring either 1 or 10 ml of the previous dilution into 9 or 90 ml of the diluent, respectively.
- The diluent used to prepare the dilutions 10^{-2} and above must be the same as used to prepare the first dilution 10^{-1}.
- The amount of required dilutions depends on the contamination level expected. For instance, if the expected counting is around 2,500-25.000/g per sample, the dilutions recommended for dish counting are 10^{-1}, 10^{-2} and 10^{-3}, so that dishes with 25-250 colonies are found. In case there is no way to previously evaluate the sample contamination level, a larger amount of dilutions must be prepared and inoculated (10^{-1} and 10^{-7}).
- During the transference of amounts between the dilutions, always use a different pipette for each dilution. Before removing the amount to be transferred, vigorously shake the tube or the flask. The pipette must be thoroughly filled and the amount discharged from the upper mark, even though a smaller amount than the pipette is to be released. It is recommended to avoid releasing amounts from the last or next-to-last lower marks of the pipettes. The amount must be released with the pipette tip touching the internal wall of the tube or flask, so that the liquid is poured down the wall.
- Pipettes should not be buckled. In case the tip touches any non-sterile surface, such as the external area of the pipette-rack, the tip of the other pipettes or the external tube/dilution flask walls, for instance, the pipette must be discharged and replaced by another.

2.16.6. Result assessment and interpretation

- With a magnifying glass or colony counter, count all colonies developed on the agar dish, and which present an amount between 25 and 250 colonies.
- Proceed with the calculation, multiplying the amount of colonies by the inverse of the inoculated dilution. In case more than one dish is used for dilution (duplicate or triplicate), consider as colony number the arithmetic average of the counting obtained in each of the dishes.
- Express the results in number of spores/ml or g. When presenting the results, use exponential notation and only one place decimal after the comma.
- Autoclave, at 121°C for 30 minutes, all the dishes before the material is discharged.

[51, 53]

2.17. Total counting of psychotropic aerobes

2.17.1. Objective

To count the total amount of psychrotrophic aerobes.

2.17.2. Definitions

2.17.2.1. Psychrotrophic microorganisms

These organisms develop under temperatures between 0 and 30°C, with optimum temperatures below 25°C.

2.17.3. Introduction

The counting method of microorganisms on plates is a general method used to count many different types, such as the mesophilic aerobes, thermophile aerobes, psychrotrophic aerobes, mould, yeast and others. This versatility comes from the theory that each microbial cell present in a sample will form a visible and isolated colony when the cell is fixed in an appropriate solid culture medium.

If both the medium (enrichment medium, selective medium, or differential medium) and the incubation conditions (temperature and atmosphere) are changed, it is possible to isolate particular genus or species. The correct relation is obtained between the colony number and the "colony forming units" (CFU), which is the number of clusters that are characteristic of certain microorganisms.

Dairy products are prone to contamination of psychrotrophic bacteria, which can lead to spoilage and lower quality products. The surface plating method is recommended to estimate the total amount of psychrotrophic aerobic microorganisms. This procedure avoids exposing the cell to the heat from the melted agar, since psychrotrophic microorganisms are vulnerable to high temperatures.

2.17.4. Materials used for test

2.17.4.1. Glassware

- Graduated cylinder (250 ml)
- Sterile test tubes or dilution flasks with 9 ml and 90 ml of diluent, respectively
- Sterile Petri dishes
- Sterile pipettes (1 ml)
- Erlenmeyer flasks (500 or 1,000ml) with the Plate Count Agar (PCA). Screw-cap test tubes may also be prepared with approximately 20 ml of medium.

* **Observations:** All the glassware must be sterilized in an oven at a minimum temperature of 170°C for over 2 hours. Graduated glassware must be sterilized in an autoclave at 121°C for 30 minutes.

2.17.4.2. Reagents

- 70% Ethyl alcohol (ethanol). Preparation: Pour approximately 200 ml of absolute ethyl alcohol onto a 250 ml-graduated cylinder, dip the alcoholmeter and gradually add

distilled water until 70º GL are reached. Observation: Verify the procedures monthly. If any alteration in the solution concentration is detected, add absolute ethyl alcohol until 70°GL are reached. Store the solution in a closed container and keep it in a fresh place.

- Culture medium: Plate count agar (PCA)
- Diluent: 0.1% peptonized water (1g of peptone/1,000 ml of distilled water), pH 7.0. If necessary, adjust the pH with 0.1N hydrochloric acid (to lower the pH) or with 0.1N sodium hydroxide (to raise the pH).

2.17.4.3. Equipment

- Refrigerator at 6,5 ± 0,5°C or incubators at 18 ± 1°C / 24 ± 1°C
- Bath with thermostat
- Laboratory scale
- Autoclave
- Sterilization oven at a minimum temperature of 170°C
- Alcoholmeter
- Oven at 35 ± 1°C

2.17.4.4. Other materials

- Drigalski loop
- Magnifying glass or colony counter
- Pipette-rack to accomplish sterile pipettes
- Bunsen burner
- Cotton
- Scissors
- Distilled water

2.17.5. Analysis methodology

- Shake the package carefully.
- Cleanse the external area of the package with cotton soaked in 70% ethanol to remove any contaminant (cleanse the counter surface as well).
- Open the package with cleansed and flamed scissors and carry out the appropriate series dilutions (see item 2.17.5.1.).
- For the surface plating procedure, the dishes must be previously prepared with 15-20 ml of the Plate Count Agar (PCA), melted and subsequently solidified. Before using, the medium surface must be dried (this procedure can be done in an oven at 50°C for 2 hours or 30-35°C for one night, lids closed) or in an layer-flux chamber, 0.5-1 hour, with lids partly open.
- Inoculate 0.1 ml of each dilution on the previously prepared dish surfaces, one or more dishes for each dilution. Spread the inoculate over the whole surface of the medium with a Drigalski loop until the excessive liquid is absorbed. Pipettes of 1 ml must be

used to transfer the inoculate of 0.1 ml. Do not blow the pipette and do not change the direction of the pipette tip when applying the last drop.

- Spread the material from the higher dilution dish to the lower dilution dish, sterilizing the Drigalski loop with 70% ethyl alcohol between applications.

*** Observations:** As the inoculate amount used in the surface plating is 10 times smaller than the one used in the pour plating, the method detection limit is over 100 CFU/g for solid samples and 10 CFU/ml for liquid samples. In case the contamination level expected for the sample is below that range, a larger amount of the first dilution must be inoculated and distributed into several dishes. The commonly applied distribution is: three dishes with 0.3 ml and one dish with 0.1 ml. The required time for the liquid absorption is larger in spreading on 0.3 ml dishes and special care must be taken so that moist films do not remain in the surface.

- Wait until the dishes dry (minimum of 15 minutes), invert them and incubate them at 6,5°C for 10 days.

Observations: The reference time/temperature for the total counting psychrotrophic microorganism is 6,5°C/10 days, but there are several other incubation conditions, which can be used in certain situations:

- Surface plating: 7°C/7-8 days
- Milk analysis: 18°C/45 hours
- Milk analysis and milk cream analysis: 23-25°C /24-28 hours

2.17.5.1. Preparation of the dilution

See paragraph 2.16.5.1 and 2.16.5.2

2.17.6. Result assessment and interpretation

- Proceed for the plate reading using the same procedure as per the spore count (described in paragraph 2.16.6)
- After all the results are read, the microorganism can be identified through different methods.
- Autoclave, at 121°C for 30 minutes, all the dishes before the material is thrown away. [32, 44, 59]

3. HEAT TREATMENT PROCESS CONTROL AND HEAT TREATED PRODUCT QUALITY CONTROL

3.1. Total Aerobic Plate Count for mesophilic aerobes in raw milk & pasteurized products

3.1.1. Objective

To count the total number of mesophilic aerobes.

3.1.2. Definitions

3.1.2.1. Mesophilic microorganisms

These organisms grow at temperatures between 30 and 40°C, with optimum temperatures between 30 and 35°C.

3.1.3. Introduction

For a general introduction to plate count technique see paragraph 2.17.3

3.1.4. Materials used for test

3.1.4.1. Glassware

- Graduated cylinder (250 ml)
- Sterile test tubes or dilution flasks with 9 ml and 90 ml of sterile diluent.
- Sterile Petri dishes
- Sterile pipettes (1 ml)
- Sterile Plate Count Agar (PCA) in Erlenmeyer flasks (500 or 1,000 ml). Screw-cap test tubes may also be prepared with approximately 20 ml of the medium

*** Observations:** Media must be sterilized in the autoclave at 121ºC for 15 minutes. All the glassware must be sterilized in an oven at a minimum temperature of 170°C for over 2 hours. Graduated glassware must be sterilized in an autoclave at 121°C for 30 minutes.

3.1.4.2. Reagents

- 70% Ethyl alcohol (ethanol). Preparation: Pour approximately 200 ml of absolute ethyl alcohol onto a 250 ml graduated cylinder, dip the alcoholometer and gradually add distilled water until 70º GL are reached. Observation: Verify the procedures monthly. If any alteration in the solution concentration is detected, add absolute ethyl alcohol until 70°GL are reached. Store the solution in a closed container and keep it in fresh place.
- Culture medium: Plate Count Agar (PCA) or Standard Methods Agar (SMA)
- Diluent: 0.1% peptone water (1 g of peptone/1,000 ml of distilled water), pH 7.0. If necessary, adjust the pH with 0.1N hydrochloric acid (to lower the pH) or with 0.1N sodium hydroxide (to raise the pH).

3.1.4.3. Equipment

- Incubator at 30 ± 1°C
- Water bath with thermostat
- Laboratory scale
- Autoclave
- Sterilization oven at a minimum temperature of 170°C
- Alcohol-meter

3.1.4.4. Other materials

- Magnifying glass or colony counter
- Pipette rack to accomplish sterile pipettes
- Bunsen burner
- Cotton
- Scissors
- Distilled water

3.1.5. Analysis methodology

- Shake the package carefully.
- Cleanse the external area of the package with cotton soaked in 70% ethanol to remove any contaminant (cleanse the counter surface as well).
- Open the package with cleansed and sterilized scissors and carry out the appropriate series of dilutions (see item 3.1.5.1.).
- Pipette1 ml of each dilution into separate, labelled empty Petri dishes (this procedure will ease the subsequent mixing with the culture medium), opening the dishes only enough to let the pipette in, close to the Bunsen burner. To increase the counting precision, it is recommended to inoculate two or more dishes per dilution (duplicates or triplicates).
- Add to the dishes approximately 15-20 ml of the PCA (previously melted and cooled down to 45 - 50°C, which is cool enough to touch). This is the pour plating method.
- Smoothly and slowly move the dishes in figure eight-like movements; make sure the mixture does not touch the dish lids and wait for the agar to solidify.
- Invert the dishes; incubate them for 48 hours at 30 ± 1°C for the total aerobic plate count.

3.1.5.1. Preparation of the dilution 10^{-1}

See paragraph 2.16.5.1 and 2.16.5.2

3.1.6. Result assessment and interpretation

- Proceed for the plate reading using the same procedure as per the spore count (described in paragraph 2.16.6)
- After all the results are read, the microorganism can be identified through different methods.
- Autoclave, at 121°C for 30 minutes, all the dishes before the material is thrown away.

[54, 33]

3.2. Total coliform specification – Evaluation of Pasteurization efficiency

3.2.1. Introduction

Coliforms are bacteria that are gram negative, non-sporogenic, facultatively aerobic or anaerobic rods capable of fermenting lactose with gas production in 24 to 48 hours at 35°C.

The presence of Coliforms in processed food is considered a useful indication of post-process contamination due to insufficient hygiene and sanitation procedures.

3.2.2. Materials used

3.2.2.1. Glassware

- Test tubes
- Pipettes (1 and 10 ml)
- Durham tubes (no gases/bubbles after preparation and sterilization)

3.2.2.2. Reagents

Presumptive test:

- Lauryl sulfate triptose solution (LST) – 10 ml per tube
- Distilled water

Confirmed test for coliforms:

- Green Brilliant Bile solution (BGLB) – 6-8 ml per tube
- Distilled water

3.2.2.3. Equipment

- Incubator at 35 ± 2°C

3.2.3. Analysis methodology

- Carry out the series dilution of the product in 0.1% peptone water (10^{-1}, 10^{-2} and 10^{-3} dilutions).

*** Observation:** The recommended diluent to analyze cheese, low solubility powdered milk, yogurt and other fermented milk is 2% sodium citrate solution. It is recommended to use sterile distilled water to analyze high solubility powdered milk, such as condensed milk (concentrated or evaporated).

- Transfer 1 ml of the 10^{-1} dilution into each tube of a three-tube series with 10 ml of LST.
- The same procedure must be carried out with the other dilutions, summing up to 9 tubes.

*** Observation:** the dilutions used vary according to the bacterial load estimated from the product to be analyzed.

- Incubate the tubes at 35°C for 24 hours and/or 48 hours. Observe the bacterial growth with gas formation in LST after a 24-hour incubation. If the result is negative, continue incubating for an additional 24 hours and carry out a new reading.
- Inoculate a loop from the positive LST tubes into VB.
- Incubate at 35°C for 24 hours or 48 hours.
- Verify the presence of gas.

3.2.4. Result assessment and interpretation

- The reading of the LST solution must be interpreted using the table for the most probable number as a presumptive result for coliforms.
- Observe if there is growth with gas formation in the green brilliant solution (GBLB) after a 24-hour incubation. If the result is negative for growth and gas formation, continue to incubate and proceed with a new reading after 48 hours.
- Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes (i.e. those that also produce gas when inoculated in GBLB) and the attached table [22, 41, 42, 48]

TABLE FOR THE MOST PROBABLE NUMBER – Most probable numbers (MPN) and confidence intervals at 95% of probability for several combinations of positive tubes in a three-tube series. Inoculated sample amount: 10.0, 1.0 and 0.1 g or ml.

(Bacteriological Analytical Manual, 6th ed., United Stated: Food and Drug Administration, 1984)

Three-tube series			
Combinations of positive tubes	**Confidence interval (95%)**		
	MPN/g	**Minimum**	**Maximum**
0-0-0	< 0,03	<0,05	<0,09
0-0-1	0,03	<0,05	0,09
0-1-0	0,03	<0,05	0,13
0-2-0	-	-	-
1-0-0	0,04	<0,005	0,20
1-0-1	0,07	0,01	0,21
1-1-0	0,07	0,01	0,23
1-1-1	0,11	0,03	0,36
1-2-0	0,11	0,03	0,36
2-0-0	0,09	0,01	0,36
2-0-1	0,14	0,03	0,37
2-1-0	0,15	0,03	0,44
2-1-1	0,2	0,07	0,89
2-2-0	0,21	0,04	0,47
2-2-1	0,28	0,10	1,50
2-3-0	-	-	-
3-0-0	0,23	0,04	1,20
3-0-1	0,39	0,07	1,30
3-0-2	0,64	0,15	3,80
3-1-0	0,43	0,07	2,10
3-1-1	0,75	0,14	2,30
3-1-2	1,20	0,30	3,80
3-2-0	0,93	0,15	3,80
3-2-1	1,5	0,30	4,40
3-2-2	2,1	0,35	4,70
3-3-0	2,4	0,36	13,0
3-3-1	4,6	0,71	24,0
3-3-2	11,0	1,50	48,0
3-3-3	>24	>1,50	>48,0

3.3. Milk boiling test

3.3.1. Introduction

If a milk sample fails in the test, the milk must contain many acid or rennet producing microorganisms or the milk has an abnormal high percentage of proteins like colostral milk. Such milk cannot stand the heat treatment in milk processing and must therefore be rejected. [52]

3.3.2. Materials used for test

3.3.2.1. Glassware

- Test tubes

3.3.2.2. Other materials

- Bunsen burner
- Wood forceps

3.3.3. Analysis methodology

- Boil a little milk in a test tube, constantly stirring it.
- Observe whether milk clots.

3.3.4. Result assessment and interpretation

- In normal milk there is no formation of clots.

3.4. Sensory analysis – Triangle test

3.4.1. Introduction

The sensorial analysis verifies pleasant and unpleasant changes on food and drink. This effect is measured and analysed by statistically analysing results from trained taster groups.

The sensorial tests are aimed at informing food manufacturers about consumer preferences and the likeability of the product. A sensory panel will help the food manufacturer

1. Identify differences and preferences among samples.
2. Select the best process to improve product quality level.
3. Help the development of new products.
4. Evaluate a product quality alteration.

There are several evaluation methods, which are for either description of the product, likeability of the product, or differentiating the product from another.

Sensorial methods:

* Paired comparison test
* Triangular test
* Duo-trio test
* Arrangement test
* Multiple comparison tests

For example, the triangular test is used to verify whether there is a significant difference between chocolate milk samples.

3.4.2. Triangular test

3.4.2.1. Objective

To verify whether there is a significant difference between two samples subjected to different treatments. For example, to verify whether changes in ingredients, processing, packaging or storing produced sensorial modifications on the final product.

3.4.2.2. Method basis

Each taster gets three coded samples and is informed that two are the same thing and one is different. The taster is asked to taste the samples, from right to left or vice-versa, and to identify the different sample.

3.4.2.3. Taster group

Twenty to forty people are generally employed for triangular tests, though only twelve can be used when differences are rather big.

Tasters do not need to be trained, but it is recommended that they are oriented before the test is carried out. This orientation will help the person to become familiar with the test procedure and the product to be tested.

3.4.2.4. Results analysis

To analyse the results, it is necessary to:

- Write down the total number of answers.
- Count the number of correct answers.
- Verify whether the number of correct answers is bigger than the one on the table for triangular test result analysis. If so, it may be possible to conclude that there is a significant difference between two samples at the test significance level.

3.4.2.5. Observations

The samples must be served in all possible combinations.

AAB	BBA
ABA	BAB
BAA	ABB

If the taster is unable to detect differences, he is still required to choose at random (this random choice is considered in the statistical analysis of the results). The probability to choose the correct answer in the triangular test is 1/3.

- This test only verifies whether there is any difference between samples. It does not assess in what samples are different, and it does not assess how or how much they differ.

3.4.2.6. Example

A chocolate manufacturer has tested new package types for their products. So, chocolate drinks, which were produced in the same lot, were packed in two different packing types and were stored under the same environmental conditions for three months. After this three-month storing period, a triangular test was carried out to verify whether there was any difference between the chocolate drinks packed in different ways.

* Results

- Total number of tests: 30

- Number of correct answers: 22

According to the table for triangular tests, for a total of 30 tests the minimum number of correct answers which leads to a significant difference between tested samples is 15 for $p<0.05$, 17 for $p<0.01$ and 19 for $p<0.001$. As 22 correct answers were obtained, it is correct to conclude that there was a significant difference ($p<0.001$) between the chocolate drinks packed in different packages.

If there were 14 correct answers, there would be no difference detected.

If there were 16 correct answers, there would be a significant difference between samples at a 5% significance level.

[55]

Triangular test application form

APPLICATION FORM

Name: _____ Date: _____

Please, taste these codified samples of ……., from right to left.
Two samples are the same kind and one is different. Circle the different sample.

328 167 894

Comments:

_____
_____
_____
_____

Triangular test table

Total number of correct answers*	Significance level (%)			
	10	**5**	**1**	**0.1**
3	3	3	-	-
4	4	4	-	-
5	4	4	5	-
6	5	5	6	-
7	5	5	6	7
8	5	6	7	8
9	6	6	7	8
10	6	7	8	9
11	7	7	8	10
12	7	8	9	10
13	8	8	9	11
14	8	9	10	11
15	8	9	10	12
16	9	9	11	12
17	9	10	11	13
18	10	10	12	13
19	10	11	12	14
20	10	11	13	14
21	11	12	13	15
22	11	12	14	15
23	12	12	14	16
24	12	13	15	16
25	12	13	15	17
26	13	14	15	17
27	13	14	16	18
28	14	15	16	18
29	14	15	17	19
30	14	15	17	19
31	15	16	18	20
32	15	16	18	20
33	15	17	18	21
34	16	17	19	21
35	16	17	19	22
36	17	18	20	22
42	19	20	22	25
48	21	22	25	27
54	23	25	27	30
60	26	27	30	33
66	28	29	32	35
72	30	32	34	38
78	32	34	37	40
84	35	36	39	43
90	37	38	42	45
96	39	41	44	48

* Source: Meilgaard, M.; Civille, G.V.; Carr, B.T. (1987).

3.5. Microbiological evaluation of UHT dairy products

3.5.1. Objective

To evaluate the presence of viable microorganism in UHT treated product by using the streak loop technique on Petri dishes.

3.5.2. Introduction

The streak loop technique is a well-known microbiological Quality Control method. It differs from a typical count of bacteria because it does not provide a quantitative figure of the microbiological quality of the product but it gives a "yes or no" answer, that is what really matters in case of commercially sterile products.

3.5.3. Materials used for test

3.5.3.1. Glassware

- Sterile, agar filled Petri dishes. All culture media must be sterilized in an autoclave at 121°C for 15 minutes.

*** Observations:** All glassware must be sterilized in oven at a minimum temperature of 170°C for over 2 hours. Graduated glassware must be sterilized in an autoclave at 121°C for 30 minutes.

3.5.3.2. Reagents

- 70% Ethyl alcohol (ethanol). Preparation: Pour approximately 200 ml of absolute ethyl alcohol onto a 250ml-graduated cylinder, dip the alcoholometer and gradually add distilled water until 70º GL are reached. Observation: Verify the procedures monthly. If any alteration in the solution concentration is detected, add absolute ethyl alcohol until 70° GL are reached. Store the solution in a closed container and keep it in fresh place.
- Culture media: Use PCA (Plate Count Agar).

3.5.3.3. Equipment

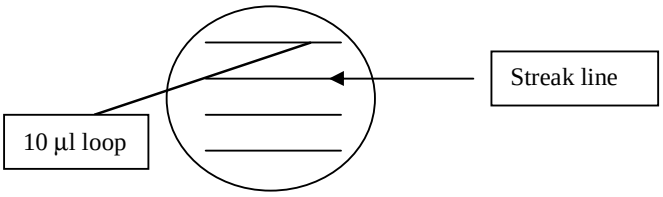
- Autoclave
- Laboratory scale
- Water bath with thermostat
- Incubator at 30 ± 1°C
- Alcohol meter

3.5.3.4. Other materials

- Bunsen burner
- Cotton
- Scissors
- Platinum loop
- Distilled water

3.5.4. Analysis methodology

- The samples must be pre-incubated for 7 days at 30°C
- Clean up the package surface with cotton and alcohol
- Cut open the packages with a scissor burnt at the Bunsen flame
- Burn the platinum loop until it gets incandescent.
- Wait few seconds, and then collect a small portion from the first sample.
- Make a straight line (see picture below) in the dish with the specific culture medium.
- Burn the platinum loop until it gets incandescent.
- Wait until the loop cools before taking the second sample.
- Repeat the procedure above for each sample (it is recommended not to exceed four streaks per plate)



- For the purpose of higher accuracy the test can be performed in double.
- Invert plates and incubate at 30°C for 72 hours
- After the incubation period is finished, remove the dishes and proceed with the evaluation of microbiological growth. A sample has to be regarded as positive if the relative streak shows some growth, however it is commonly considered an environmental contamination anytime less than 10 cfu per streak are detected.

[33, 42, 56]

3.6. Quadrant streak technique to isolate microorganism

3.6.1. Introduction

The streaking technique in Petri dishes is used to identify microbial groups, such as the mesophilic aerobes, thermophilic aerobes, psychrotrophic aerobes, mould, yeast and other types.

In order to obtain well-isolated discrete colonies, the quadrant streak technique should be used. This allows sequential dilution of the original microbial material (broth culture or colonies on a plate) over the entire surface of a fresh plate. As the original sample is diluted by streaking it over successive quadrants, the number of organisms decreases. Usually by the third or fourth quadrant only a few organisms are transferred on the inoculation loop and these produce a few isolated colonies.

3.6.2. Materials used for test

3.6.2.1. Glassware

- Graduated cylinder (250 ml)
- Sterile, agar filled Petri dishes (specific for each microorganism type). All culture media must be sterilized in an autoclave at 121°C for 15 minutes.
- Beaker (50 ml)

*** Observations:** All glassware must be sterilized in oven at a minimum temperature of 170°C for over 2 hours. Graduated glassware must be sterilized in an autoclave at 121°C for 30 minutes.

3.6.2.2. Reagents

- 70% Ethyl alcohol (ethanol). Preparation: Pour approximately 200 ml of absolute ethyl alcohol onto a 250ml-graduated cylinder, dip the alcoholometer and gradually add distilled water until 70º GL are reached. Observation: Verify the procedures monthly. If any alteration in the solution concentration is detected, add absolute ethyl alcohol until 70° GL are reached. Store the solution in a closed container and keep it in fresh place.
- Culture media: Use BHI (Brain Heart Infusion) medium for uncontaminated UHT milk. Use PCA (Plate Count Agar) for contaminated products.

3.6.2.3. Equipment

- Autoclave
- Laboratory scale
- Water bath with thermostat
- Ovens at 30 ± 1°C
- Alcohol meter

3.6.2.4. Other materials

- Bunsen burner
- Platinum loop
- Distilled water

3.6.3. Analysis methodology

– Dip the platinum loop into ethyl alcohol and burn it until it gets incandescent.

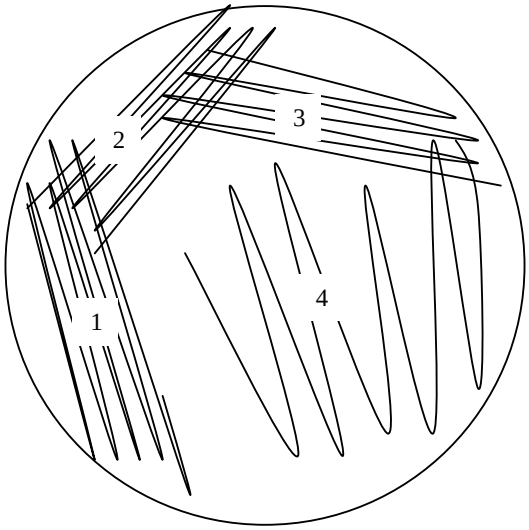
– Allow the loop to cool down and remove a small amount of bacterial growth (either a loopful form a broth culture or a single colony from a plate) with the sterile inoculating loop.

– Immediately streak the inoculating loop very gently over a quarter of the plate using a back and forth motion (see area 1 in the figure below).

– Flame the loop again and allow it to cool. Going back to the edge of area 1 that was just streaked, extend the streak into the second quarter of the plate (area 2) .

– Flame the loop again and allow it to cool. Going back to the area that was just streaked (area 2), extend the streak into the third quarter of the plate (area 3).

– Flame the loop again and allow it to cool. Going back to the area that was just streaked (area 3), extend the streak into the fourth quarter of the plate (area 4).



Inoculation of a streak plate:

1. Area of initial inoculation and first streak yields heavy growth.

2. Area of second streaks from area 1 yields less dense growth.

3. Area of third streaks from area 2 yields weak growth.

4. Area of fourth streaks from area 3 yields single colonies.

Please Note:

- Use the entire surface of the plate, not just the middle of the plate.
- Streak only one loopful of a broth culture.
- Remove only one colony or a barely visible amount of cells from a plate.

- Invert plates and incubate the under the specific conditions for the various different microorganism types.
  - Uncontaminated UHT milk: 30 ± 1°C
  - Contaminated products: dishes in duplicates for incubation at 30 ± 1°C
- After the incubation period is finished, remove the dishes and proceed with the identification of the microorganism types through Gram staining, spore staining with malachite green and phase contrast microscopy.

[33, 42, 57]

3.7. Determination of the homogenization index

I – Traditional NIZO method

3.7.1. Materials used for test

3.7.1.1. Glassware

-Special homogenisation pipette (NIZO)

-Beaker

3.7.1.2. Equipment

Gerber centrifuge (1,100 ± 100 rpm), which can be warmed up to 40°C

***Observation:** in case there is no heat centrifuge, warm the sample to 45°C.

3.7.1.3. Other materials

- Rubber cork

3.7.2. Analysis methodology

- Aspirate the 45ºC-warmed sample to the upper mark on the NIZO pipette.
- Close the pipette tip with a rubber cork, place it in the heat centrifuge.
- Separate the sample for 30 minutes.
- After the separation is done, close the upper tip of the pipette with a finger and remove the cork.
- Let milk drop slowly through the pipette tip into a beaker. When the liquid reaches the lower mark of the pipette (20-ml), stop emptying it.
- Examine the fat content from the collected product, according to the Gerber method.
- The value obtained must be compared to the fat content obtained from the examination of the original milk sample.

If the fat content of the original sample is Fc, the fat content of the separated sample is Fs and the homogenisation efficiency is H, the relation among these three quantities is:

$$H\% = \frac{100 \times Fs}{Fc}$$

If Fs = 3.0% and Fc = 3.2%, H% will be:

$$\frac{100 \times 3.0}{3.2} = 91\%$$

II – Alternative method

3.7.3. Materials used for test

3.7.3.1. Glassware

- Graduated cylinder (250 ml)
- Volumetric pipette (25 ml)

3.7.3.2. Equipment

- Refrigerator (temperature about 7°C)

3.7.4. Analysis methodology

- Place 250 ml of the product into a graduated cylinder, soon after it is sterilized.
- Store refrigerated (+/- 7°C) for 48 hours.
- Separate 25 ml from the upper part (A) and 225 ml from the lower part of the sample (B).
- Evaluate the content of fat matter or fat content through the Gerber method (see "Methods to evaluate fat in milk") in both samples.

3.7.5. Result assessment and interpretation

The homogenisation index (H) is obtained according to the following formula:

$$H = [1 - (A - B) / A] \times 100$$

The homogenisation index must be at least 90% (the ideal index is above 95%).

4. Control of aseptic critical parameters and Hygiene Controls

4.1. Determination of Peroxide residue in water

4.1.6. Analytical approach:

4.1.6.1. Materials

- 1 burette (25 ml)
- 1 volumetric pipette (1,000 ml)
- 1 graduated pipette (10 ml)
- 1 Erlenmeyer flask (250 ml)

4.1.6.2. Reagents

- 0.01N Potassium permanganate
- 2N Sulfuric acid

4.1.6.3. Procedures

- Measure exactly 100 ml of the water sample from the package with the volumetric pipette and transfer this amount into the 250 ml-erlenmeyer.
- Add 1 ml of the 2N sulfuric acid solution.
- Pour the 0.01N potassium permanganate solution with the burette until the liquid becomes pinkish and remains for over a minute.
- Make a blank test (use tap water).

4.1.6.4. Calculation

ppm of peroxide = (Vb – Va) x 1.7

Being:

Va = volume of permanganate spent in the blank test.

Vb = volume of permanganate spent during the sample titration.

4.1.6.5. Reaction

$2\ KMnO_4 + 5\ H_2O_2 + 4\ H_2SO_4 = 2\ KHSO_4 + 2\ MnSO_4 + 5\ O_2 + 8\ H_2O$

4.1.7. Another approach:

The evaluation of the peroxide residue in Tetra Brik packages can be done by the peroxide evaluation kit from Merck (Tetra Pak number: 90298-30), Merckoquant (1.10011.0001) or the Chemetrics Testkit (Tetra Pak number: 90298-31). The purpose of the test kits are a) to enable fast and convenient determination of remaining quantity of hydrogen peroxide in the product and b) to enable checking the quantity of hydrogen peroxide reaching the packages when the tube heating function is switched off on the open aseptic machine system (TBA/3 and TCA).

Both kits may be used to measure the hydrogen peroxide content in water solutions whilst only 90298-30 may be used for measuring in organic solutions (milk).

Test kit 90298-30, which consists of 100 micro quantum rods, gives a rough value. Colour change in the rods indicates contents of hydrogen peroxide.

Test kit 90298-31, which consists of a refill pack containing 30 ampoules (90298-32), comparators and test body, gives an accurate value. In addition this method also enables detection of minor residual quantities. The ampoules, which contain a reagent, automatically suck up the water and are coloured differently according to contents of hydrogen peroxide.

Check immediately after the production of packages with distilled or de-ionized water

[58]

4.2. Determination of hydrogen peroxide concentration

4.2.1. Materials used for test

- Hydrometer with thermometer
- Graduated cylinder (250 ml)

4.2.2. Analysis methodology

- Remove approximately 250 ml of peroxide from the machine and pour it into a graduated cylinder.
- Place the hydrometer in the cylinder to verify whether there is enough liquid to make the hydrometer float. If bubbles are found stuck on the hydrometer, spin swiftly until the bubbles disappear.
- Read the temperature and the density value at the liquid level.

***Observation: The peroxide must be determined by measuring both the density and the temperature (usually at 20°C).**

4.2.3. Result assessment and interpretation

The peroxide concentration is obtained with a ruler and a specific monogram **(use only the original monogram by Tetra Pak).** Connect the values of the temperature and density to obtain a percentage of peroxide on a weight to weight basis.

4.2.4. Concentrations of peroxide required during production with TBA filling machines

The optimal peroxide concentration' is 35%.

The peroxide in the TBA/3 must be changed daily. Plus must 3 ml of PSM for each litre of peroxide be added before production starts. Never reutilize this mixture in subsequent productions. Peroxide concentrations between 30% and 50% are acceptable for production on filling machine with a peroxide bath system. It is recommended that all peroxide is changed after 120 hours or 7 production days (whichever happens first).

4.2.5. Verification of peroxide consumption in TBA/3 filling machines

- Remove the hydrogen peroxide reservoir from the machine and replace it by a cylinder. (preferably plastic) with a known amount (1 to 2 litres) of hydrogen peroxide into a cylinder (preferably plastic).
- Measure the peroxide consumption at each production hour by measuring the amount remaining.

*** Observation:** It is recommended that the peroxide is prepared before production hours.

4.3. CIP efficiency Swab and Bioluminescence methods

I – Traditional swab method

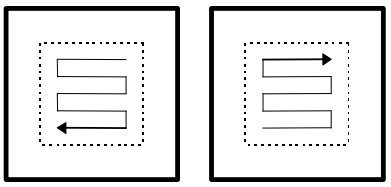
Carry out a swab on surfaces to be analysed, then soak the swab in 0.1% peptone water and incubate for 24 hours at 37°C. After incubation, proceed with spreading on PCA and incubate again for 24 hours at 37°C.

A level below 80 cfu/ cm^2 is generally regarded as an acceptable result.

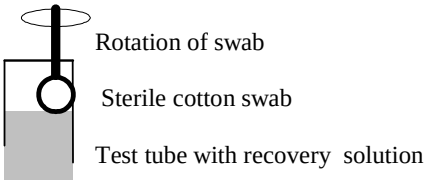
II – Another method for swab test

1. Soak a sterile cotton swab into the test tube with recovery solution.

2. Swab the area with the wet swab. (Left/right and back). Rotate the swab during the swabbing. Please note that if the area to be swabbed is wet use a dry swab.



3. Dip the swab into the same test-tube with swab-up solution and then “wring out” the absorbed liquid.



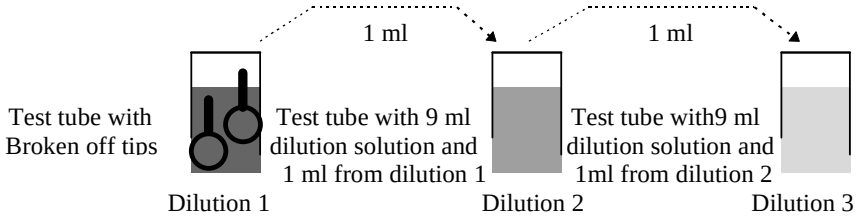
4. Break off the swabs' tip in the test tube with swab-up solution

Broken off tip
Test tube with recovery solution

5. Place (break off) the cotton swab into the test tube in such a manner that it can be closed.

Broken off tips
Test tube with recovery solution

6. Shake the test tube (with the swab/s) in a mixer. The swabs should be spinning. Make sure to use the same mixing procedures (e.g. time and speed) with each test tube.
7. Pour the content of the test tube into a sterile petri dish or dilute to the appropriate concentration procedure as follows:
8. Using a sterile pipette, take 1 ml swab-up solution from the "swab" test tube (dilution 1) and transfer to a test tube with 9 ml dilution solution (dilution 2). Thus making a tenfold dilution, which makes it easy to calculations the result.



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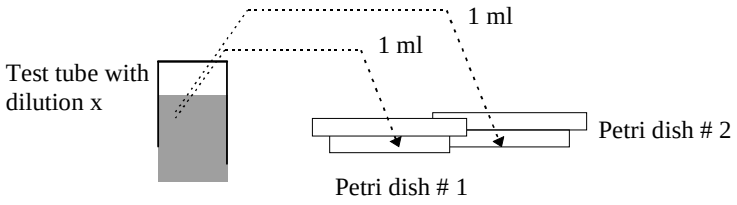
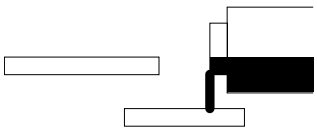
9. Shake the test tube in the mixer and plate 1 ml of the solution in a sterile Petri dish (recommended is to make two plating per dilution). Repeat the dilution as many times as necessary. (The ideal dilution should result in 30-300 cfu per 9 cm (diameter) plate

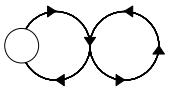


10.Then pour the agar in the petri dishes and mix carefully.



Petri dish

Petri plate



Example of mixing pattern
three "8" in each direction

11.Incubate the plates at suitable temperature and time

12.After incubations count the cfu on the plates. (Preferably on the plates with 30-300 cfu.)

III – Fast bioluminescence methods

4.3.1. Method basis

ATP is present in all living cells and brings light emission when combined with luciferase (enzyme found in bioluminescent organisms, such as fireflies). This light can be measured and determine the ATP quantity coming from the microbiological contamination, food remnants and so on. The ATP quantity shows the hygiene level.

4.3.2. Analysis procedure

1. Spread the kit swab on the area to be tested.
2. Transfer the sample into the reaction device.
3. Read the results with the specific reading machines.

4.4. Calculating the cleaning solution concentration

4.4.1. Concentration of the caustic soda solution (NaOH):

a. HCl (hydrochloric acid) evaluation

Reagent: HCl 1N

Indicator: Phenolphthalein

Titration:

• Pipette 10 ml of caustic soda solution (NaOH) [used for cleaning purposes] into an Erlenmeyer flask.

• Add about 3-4 drops of phenolphthalein

• Proceed with the 1N HCl solution titration using a burette

• Record the amount of 1N HCl (V_{HCL}) used during the dye turning point

Calculation: **NaOH Conc. = V_{HCL} x 0.4**

b. H_2SO_4 1N evaluation

Reagent: H_2SO_4 1N

Indicator: Phenolphthalein

Titration:

• Pipette 10 ml of caustic soda solution (NaOH) [used for cleaning purposes] into an Erlenmeyer flask.

• Add about 3-4 drops of phenolphthalein

• Proceed with the 1N H_2SO_4 solution titration using a burette

• Record the amount of 1N H_2SO_4 ($V_{H_2SO_4}$) used during the dye turning point

Calculation: **NaOH concentration = $V_{H_2SO_4}$ x 0.4**

***Concentrations obtained are expressed as % (vol)**

4.4.2. Concentration of nitric acid solution (HNO_3):

Reagent: 1N NaOH

Indicator: Methyl Red

Titration:

•, Pipette 10 ml of nitric acid solution (HNO_3) [used for cleaning purposes] into an Erlenmeyer

• Add about 3-4 drops of methyl red

• Proceed with the 1N NaOH titration using a burette

• Record the amount of 1N NaOH (V_{NaOH}) used during the dye turning point

Calculation: **HNO_3 Conc. = V_{NaOH} x 0.63**

4.4.3. Indicator formulation

4.4.3.1. Phenolphthalein

One gram of phenolphthalein [C_6 $H_4COO.C(C_6H_4OH)_2$] is dissolved into 60 ml of absolute ethyl alcohol; the mixture is diluted with distilled water up to 100 ml.

pH	Colour
8.3	No colour (colourless)
9.8	Red

4.4.3.2. Methyl Red

0.2 gram of methyl red [$HOOC.C_6$ $H_4H:N.C_6H_4N(CH3)_2$] is dissolved into 60 ml of absolute ethyl alcohol; the mixture is diluted with distilled water up to 100 ml.

pH	Colour
4.2	Red
6.3	Yellow

5. Environmental and Personnel Hygiene

5.1. Microbiological air load - Precipitation method

This method can make use of Petri films (3M) or 9 cm diameter Petri dishes.

5.1.1. Test Material Petri Film (3M)

5.1.1.1. Glassware

Sterile pipette (1 ml)

Dilution flask to sterilize distilled water

*** Observations:** All the sterile glassware must be sterilized in an oven at a minimum temperature of 170°C for over 2 hours. Graduated glassware must be sterilized in an autoclave at 121°C for 30 minutes.

5.1.1.2. Reagents

- Sterile distilled water

5.1.1.3. Equipment

- Incubating oven at 35 ± 1ºC

5.1.1.4. Other materials

- Petri film (3M)
- Plastic disks, specific for analysis with Petri films

5.1.2. Analysis methodology

5.1.2.1. Preparation of the Petri film

- Aseptically pour 1 ml of sterile distilled water with a pipette onto the film.
- Close and press down the dish with the plastic disk, specific for analysis with Petri films.
- Wait for the medium to solidify (about 20-30 minutes)
- Open the film, fix it with adhesive tape on the predetermined spot and leave it open for 15 minutes.
- Close and incubate the film (upright) in a culture oven at 35 ± 1ºC for 48 ± 2 hours. (A minimum manipulation is required as sample is collected so that any contamination, which could interfere with the final result, is avoided).

*** Observation:** Keep an unexposed film as control.

5.1.3. Result assessment and interpretation

- Remove the film from the culture oven after the incubation period.
- Proceed to count colonies on the film.
- Multiply the result by 2.5 (to obtain the result in 100 cm^2)
- The maximum acceptable limit is 40 CFU/100cm^2.

5.1.4. Test Material Petri dishes

5.1.4.1. Glassware

- 9-cm diameter sterile Petri dish with the culture medium agar plate count (PCA), sterile and solidified.

5.1.4.2. Reagents

- Culture medium: agar plate count (PCA)

5.1.4.3. Equipment

- Incubation oven at 35 ± 1ºC

5.1.5. Analysis methodology

- Open the Petri dishes at the predetermined locations and leave them open for 15 minutes.
- Close and incubate the dishes (at inverted position) in culture oven at 35 ± 1ºC for 48 ± 2 hours.

*** Observation:** Keep an unexposed dish as a control.

5.1.6. Result assessment and interpretation

- Remove the dish from the culture oven after the incubation period.
- Proceed with the counting the colonies on the dish.
- Multiply the result by 1.57 (to obtain the result in 100 cm^2)
- The maximum acceptable limit is 40 CFU/100cm^2.

5.2. Microbiological air load - portable air sampling devices

Please note:
The method is described for an SAS air sampler. For air samplers from other suppliers e.g. Millipore other analysis methodology may apply, even so that the principle method is the same.

5.2.1. Analysis methodology

- Remove both the protection and air filter lids from the machine
- Place the Rodac plate (55 mm diameter) with PCA
- Replace the air filter lid, and select the volume of air to be sampled according to the suspected level of the ambient load to be analyzed.
- After sampling, incubate the plate at 35±1ºC for 48±1 hours for bacterial counting.

5.2.2. Result assessment and interpretation

The result is obtained after correlating data using the enclosed table and placing the data in the following formula.

$$y = \frac{Pr}{V}$$

where:

V = the sampled volume in liters

r = colony forming unit (55 mm diameter)

Pr = effective number of colonies (probability) obtained from the enclosed statistical table using the value of "r"

y= colony forming unit by literof air

** The final result is expressed in CFU/ ft^3 or CFU/ m^3, multiplying the value of CFU/ liters by 28.32 or 1,000 respectively.

Correlation table of colony counting using RODAC standard plates (55mm)

r	Pr	r	Pr	r	Pr	r	Pr	r	Pr	r	Pr	r	Pr
1	1	32	34	62	73	92	119	122	178	158	278	194	471
2	2	33	36	63	74	93	121	123	180	159	282	195	480
3	3	34	37	64	76	94	122	124	182	160	286	196	489
4	4	35	38	65	77	95	124	125	185	161	289	197	499
5	5	36	39	66	78	96	126	126	187	162	293	198	508
6	6	37	40	67	80	97	128	127	189	163	297	199	519
7	7	38	42	68	81	98	130	128	192	164	301	200	530
8	8	39	43	69	83	99	131	129	194	165	305	201	542
9	9	40	44	70	84	100	133	130	196	166	309	202	554
10	10	41	45	71	86	101	135	131	199	167	313	203	567
11	11	42	46	72	87	102	137	132	201	168	317	204	580
12	12	43	48	73	88	103	139	133	204	169	322	205	595
13	13	44	49	74	90	104	141	134	206	170	326	206	611
14	14	45	50	75	92	105	142	135	209	171	331	207	627
15	15	46	51	76	93	106	144	136	212	172	335	208	646
16	17	47	53	77	95	107	146	137	214	173	340	209	666
17	18	48	54	78	96	108	148	138	217	174	344	210	687
18	19	49	55	79	98	109	150	139	220	175	349	211	712
19	20	50	57	80	99	110	152	140	222	176	354	212	739
20	21	51	58	81	101	111	154	141	225	177	359	213	770
21	22	52	59	82	102	112	156	142	228	178	365	214	807
22	23	53	60	83	104	113	158	143	231	179	370	215	851
23	24	54	62	84	106	114	160	144	234	180	375	216	905
24	25	55	63	85	107	115	162	145	237	181	381	217	978
25	26	56	64	86	109	116	165	146	240	182	387	218	1088
26	28	57	66	87	110	117	167	147	243	183	393	219	1307
27	29	58	67	88	112	118	169	148	246	184	399		
28	30	59	69	89	114	119	171	149	249	185	405		
29	31	60	70	90	116	120	173	150	252	186	412		
30	32	61	71	91	117	121	175	151	255	187	418		
								152	258	188	425		
* r = counted colony forming unit								153	261	189	432		
*Pr = probability								154	265	190	439		
								155	268	191	447		
								156	271	192	455		
								157	275	193	463		

5.3. Hand hygiene assessment

5.3.1. Operator's hand microbiological assessment

The operator shall be analysed regularly in terms of bacterial growth by making his hands touch PCA agar with subsequent incubation at 37°C for 24 hours.

5.3.2. Microbiologic assessment of hands before and after washing and decontamination

5.3.2.1. Analysis methodology

- Collect hand samples by making the fore-finger touch the Petri dish with PCA agar, in three different situations:
- Without washing hands;
- Washing with detergent and drying with white paper;
- Decontaminating with iodine alcohol solution (ethanol 70ºGL with 0.25% iodine).

5.3.2.2. Result assessment and interpretation

- Check the reduction rate of the bacterial growth in the PCA dish obtained from the sample hand, before washing and after washing and decontamination.

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