SRI LANKA STANDARD 516 : PART 11 : 1999
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MICROBIOLOGICAL TEST METHODS

PART 11 : GENERAL GUIDANCE FOR ENUMERATION OF LIPOLYTIC ORGANISMS

SRI LANKA STANDARDS INSTITUTION

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SLS 516:Part 11:1999

Gr. 4

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SRI LANKA

Sri Lanka Standards are subject to periodical revision in order to accommodate the progress made by industry. Suggestions for improvement will be recorded and brought to the notice of the Committees to which the revisions are entrusted.

This standard does not purport to include all the necessary provisions of a contract.

SRI LANKA STANDARD
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FOREWORD

This Sri Lanka Standard was finalized by the Sectoral Committee on Agriculture and Food Products and was authorized for adoption and publication as a Sri Lanka Standard by the Council of the Sri Lanka Standards Institution on 1999-11-11.

This Standard is intended to provide general guidance for microbiological examination of food for human consumption and animal feeds. However, owing to the number and variety of these products, it may be necessary in special cases, to make certain changes to these guidelines or even to use other methods. Such changes or other methods will be indicated in the relevant Sri Lanka Standard specific to the product.

In the preparation of this standard the assistance derived from the Compendium of methods for the microbiological examination of foods (Third edition 1992) prepared by the American Public Health Association is gratefully acknowledged:

1 SCOPE

This standard gives general guidelines for enumeration of lipolytic organisms present in products intended for human consumption or feeding of animals.

2 REFERENCES

SLS 393 : Handling food samples for microbiological analysis.
SLS 516 : Microbiological Test Methods
Part 1 – General guidance for enumeration of micro-organisms colony count technique at 30^0C

AHPA compendium of methods for the microbiological examination of foods.

3 DEFINITIONS

For the purpose of this standard the following definition shall apply:

Lipolytic organisms: Any microorganism causing oxidative and or hydrolytic deterioration of lipids present in food by the activity of lipolytic enzymes.

4 SAMPLING

Sampling shall be carried out in conformity with the relevant Sri Lanka Standard for the product concerned and **SLS 393**.

5 APPARATUS AND GLASSWARE

Usual laboratory equipment and in particular the following shall be used:

5.1 Instruments for preparation of samples, sterilized prior to use by autoclaving or in an oven.

5.2 Balance accuracy 0.1 g. (Electronic)

5.3 Apparatus for homogenization

5.3.1 *Mechanical blender* operating at not less than 8000 r.p.m and not more than 45,000 r.p.m with glass or metal jars fitted with lids and resistant to the conditions of sterilization. Alternatively a stomacher with sterile bags may be used.

5.3.2 *Mixer.* capable of mixing 1ml or 2 ml of the sample or of a dilution, in a tube of adequate dimensions with 9 ml or 18 ml of diluent, to obtain a homogeneous suspension and working on the principle of eccentric rotation of the contents of the test tube (Vortex mixer).

5.4 Apparatus for dry sterilization (oven) or wet sterilization (autoclave).
Other than the equipment which is supplied sterile, particularly that made of plastic material glassware shall be sterilized either
- in an oven at 170^0C to 175^0C for not less than 1 hour or
- in an autoclave at $121\pm 1\ ^0$C for not less than 20 minutes.

5.5 Water bath. Controlled at $45 \pm 1\ ^0$C

5.6 Incubator, controlled at $30 \pm 1\ ^0$C

5.7 Colony counting equipment, Consisting of an illuminated base with a dark background and a mechanical or electronic digital counter.

5.8 Tally register

5.9 Petri dishes, glass or plastic, of diameter 90 mm to 100 mm.

5.10 Total delivery pipettes, having a nominal capacity of 1 ml and 10 ml graduated in 0.1 ml and 1 ml respectively.

5.11 pH meter, accurate to $\pm$ 0.1 pH unit at 25^0C.

5.12 Flasks of capacity 125 ml to 300 ml or media bottles of suitable capacity.

6 CULTURE MEDIA DILUENT AND REAGENTS

6.1 Basic Materials

6.1.1 In order to improve the reproducibility of the results, it is recommended that for the preparation of culture media, dehydrated basic components or complete dehydrated media be used. The manufacturer's instructions shall be strictly followed.

6.1.2 The chemicals and materials used for preparing culture media shall be respectively of analytical reagent grade and microbiological grade.

6.1.3 The water used shall be distilled or deionized and free of substances which would be toxic or inhibitory for the growth of micro-organisms under the test conditions. It is recommended that water used shall also be microbiologicaly suitable water as specified in APHA – compendium of methods for the microbiological examination of foods.

6.1.4 If the media and diluent are not used immediately after preparation they shall be kept in a refrigerator at a temperature between 0^0C and ± 5^0C for not longer than one month in order to prevent any change in their composition & functionality.

6.2 Diluent (0.1% peptone water – Clause 6.1 of SLS 516:Part1:1991)

6.3 Media

6.3.1 Tributyrin agar – Double layer medium

6.3.1.1 Base layer medium

Composition

Tributyrin (FFA free)	50.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Preparation

Sterilize tributyrin in which the FFA have been removed,. in the autoclave for 30 minutes at 121^0C. Dissolve the agar in 1000 ml distilled water by heating and sterilize for 15 minutes at 121^0C. Cool the above sterile ingredients to 50^0C and mix in a warm sterile blender for one minute. Pour 3.0 to 4.0 ml into the bottom of sterile petridishes. Preferably plates shall be used immediately or may be stored 3 to 4 days, in the refrigerator.(plates shall be dried by an appropriate method before use).

6.3.1.2 Nutrient overlay medium

One of the following nutrient overlay media shall be used.

a) Nutrient agar

Composition

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Suspend ingredients in distilled water and melt agar by gentle boiling. Dispense into suitable flasks or bottles and sterilize at 121^0C for 15 minutes. Final reaction should be pH 7.3 ± 0.2.

b) Standard methods agar (Plate count agar)

Composition

Tryptone (Pancreatic Digest of Casein)	
or tripticase	5.0 g
yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Preparation

Dissolve ingredients in distilled water by boiling. Dispense into bottles or flasks and autoclave at 121^0C for 15 minutes. Final reaction shall be pH 7.0 ± 0.1

c) Casein soy peptone agar (Trypticase tryptic soy agar)

Compostion

Tryticase or Tryptone	15.0 g
Phytone or soytone	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Preparation

Suspend ingredients in 1000 ml of distilled water, mixing thoroughly, Heat with frequent agitation and boil for about 1 minute to dissolve completely and dispense into suitable bottles or flasks. Autoclave at 121^{0}C for 15 minutes. Final reaction should be pH 7.3 ± 0.2.

6.3.1.3 Any one of the following fats in which the free fatty acids (FFA) have been removed (See Note) shall be used as a substitute for tributyrin.

Corn oil, Soyabean oil, Lard, Tallow.

When any of these fats are used 200 ml of victoria blue B solution (1:5000) sterilized by membrane filtration (0.45 cm) shall be included into the base layer medium prepared in 800 ml of distilled water.

Note
Dissolve the fat substrate in petroleum ether (5 to 10 g/ 100 ml) and pass it through a column of activated alumina. Remove the petroleum ether from the purifed triglyceride by evaporating it on a steam table under a stream of nitrogen.

6.3.2 Tributyrin Agar – Single Layer Medium

Composition

Nutrient agar, standard methods agar }	(amount recommended by manufacturer
or tryptic soy agar }	to prepare 1.0 L of medium)
Tributyrin (FFA free)	- 50.0 g
Distilled water	- 1000.0 ml

Preparation

a) Sterilize tributyrin in the autoclave at 121^{0}C for 30 minutes. Any one of fats specified in 6.3.1.3 shall be used as a substitute for tributyrin together with victoria blue B solution prepared and sterilized as specified in 6.3.1.3.

b) Dissolve the recommended amount of selected dehydrated nutrient medium (Nutrient agar, Standard methods agar or trypticsoy agar) or ingredients (sse 6.3.1.2) in 1000.0 ml of distilled water by heating. (If any other fat is used as a substitute for tributyrin 800.0 ml of distilled water shall be used). Sterilize at 121^{0}C for 15 minutes. The final pH should be as stipulated by the manufacturer.

c) Cool the above sterile ingredients to 50^{0}C, and mix together in a warm sterile blender for 1 min, before use.

7. PROCEDURE

7.1 Preparation of sample

The analytical sample shall be prepared as specified in the relevant product standard.
Make at least five standard ten-fold serial dilutions of the sample as described in clause 7 of SLS 516 Part 1:1991 using 0.1 percent peptone diluent (see 6.2).

7.2 Double layer Method

Inoculate 0.5 ml quantities of suitable dilutions of the sample prepared as described in clause 7.1 into duplicate plates of previously dried Tributyrin Agar base layer medium. (see 6.3.1.1). Spread the inoculum on the surface of the agar by uing a separate sterile spreader for each set of plates. Allow the inoculum to absorb into the medium. Pour 10 or 12 ml of Nutrient overlay medium (See 6.3.1.2) over each plate and allow to set.

7.3 Single Layer Method

Inoculate 1 ml quantities of suitable dilution of the sample prepared as described in Clause 7.1 into duplicate pertridishes, Pour 12 or 15 ml of molten tributyrin agar single layer medium (See 6.3.2) containing the fat maintained at 45 ^{0}C, into each petridish. Mix well and allow to set.

7.4 Incubation

Incubate plates at 30^{0}C ± 1^{0}C for 48 ± 2h upto 72 ± 3 h when tributyrin is the fat substrate and 4 to 7 days for other fats.

8 COUNTIMG AND REPORTING COLONIES

After the period of incubation count the colonies surrounded by lipolytic zone and report as lipolytic micro organisms per millilitre or per gram of the product (In accordance with Clause 11 of **SLS 516 Part 1 : 1991)**

Note
On tributyrin agar without Victoria Blue B lipolytic microorganisms are surrounded by a transparent zone, on an opaque background. Colonies on media containing the dye are surrounded by a dark blue zone on an opaque light blue background.

The dye acts as an acid indicator, and when fats are hydrolysed, non soluble long chain fatty acids give an intense dark blue colour. Short chain soluble fatty acids diffuse through the agar and give a weak blue colour. Indistinct zones sometimes occur because of weak lipolysis or acid production that is due to utilization of sugar.

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The Sri Lanka Standards Institution is the owner of the registered certification mark shown below. Beneath the mark, the number of the Sri Lanka Standard relevant to the product is indicated. This mark may be used only by those who have obtained permits under the SLS certification marks scheme. The presence of this mark on or in relation to a product conveys the assurance that they have been produced to comply with the requirements of the relevant Sri Lanka Standard under a well designed system of quality control inspection and testing operated by the manufacturer and supervised by the SLSI *which includes surveillance inspection of the factory, testing of both factory and market samples.*

Further particulars of the terms and conditions of the permit may be obtained from the Sri *Lanka Standards Institution, 17, Victoria Place, Elvitigala Mawatha, Colombo 08.*

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SRI LANKA STANDARDS INSTITUTION

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The principal objects of the Institution as set out in the Act are to prepare standards and promote their adoption, to provide facilities for examination and testing of products, to operate a Certification Marks Scheme, to certify the quality of products meant for local consumption or exports and to promote standardization and quality control by educational, consultancy and research activity.

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All members of the Technical and Sectoral Committees render their services in an honorary capacity. In this process the Institution endeavours to ensure adequate representation of all view points.

In the International field the Institution represents Sri Lanka in the International Organization for Standardization (ISO), and participates in such fields of standardization as are of special interest to Sri Lanka.

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