IP IP 385/99

Determination of the viable aerobic microbial content of fuels and fuel components boiling below 390 °C — Filtration and culture method

This standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

Foreword

Knowledge of microbiological techniques is required for the procedures described in this standard.

1 Scope

This standard describes two procedures for the determination of the viable microbial content of fuels and fuel components boiling below 390 °C. Procedure A is suitable for enumeration of viable microbial units up to 25 000 per litre. Procedure B is suitable for enumeration of viable microbial units above 25 000 per litre.

The procedures employ two selective microbiological growth media which nominally allow separate enumeration of bacteria and fungi. But as the media are not exclusively selective, it is possible that the determination of viable microbial units from the medium which favours bacterial growth will also include some fungi and vice versa. The microbiological procedures for distinguishing bacterial growth and for distinguishing between yeasts and moulds within the fungal content are not within the scope of this standard.

NOTE 1 If the approximate contamination level is unknown it is recommended both procedures are carried out.

NOTE 2 Some of the principles described in procedure B can also be used to determine the viable microbial content of water associated with the fuel. However procedures to determine the microbial content of water are not given in this standard.

2 Normative references

The following document contain provisions which, through reference to this text, constitute provisions of this standard. At the time of publication, the edition indicated was valid. All standards are subject to revision, and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below.

Institute of Petroleum's *Guidelines for the investigation of microbial content of fuel boiling below 390 °C and associated water,* 1996.

3 Principle

After separation of any water phase, known volumes of fuel are filtered aseptically through membrane filters. Viable microorganisms collected on the filters are assayed by either placing the filters directly onto agar growth media or eluting the micro-organisms, diluting the eluent if necessary, and placing aliquots of the eluent and / or dilutions of the eluent onto agar growth media. After incubation the number of colonies are counted and from these the number of viable bacteria and fungi present in a given volume of the original fuel sample are calculated.

4 Materials and reagents

Unless otherwise specified, use only reagents of recognised analytical grade and only distilled or deionized water.

4.1 ¼ Strength Ringer's Solution

Sodium chloride	2,25 g
Potassium chloride	0,105 g
Calcium chloride $6H_2O$	0,12 g
Sodium bicarbonate	0,05 g
Water	1 l

Dissolve salts in 1 l of distilled or deionized water and sterilise by placing in an autoclave (5.19) at 121 °C ± 2 °C for 15 min. Dispense 10 ml aliquots aseptically into sterile bottles (5.17).

NOTE 3 ¼ Strength Ringer's salts are available in tablet form from various manufacturers.

4.2 Detergent solution

Polyoxyethylene(20)sorbitan monooleate (Tween 80 ®[1]) - 0,1% (*V/V*) aqueous solution sterilised by passing through a 0,2 µm membrane filter (5.21) into a sterile vessel, or placing in an autoclave (5.19) at 121 °C ± 2 °C for 15 min.

4.3 Alcohol

Ethanol, Propan-2-ol or Industrial Methylated Spirit, if necessary sterilise by passing through a 0,2 µm membrane filter (5.21) into a sterile vessel.

4.4 Malt Extract Agar (MEA)

The pH of this medium favours the growth of fungi (moulds and yeasts) over that of bacteria.

Malt Extract	30 g/l
Mycological Peptone	5 g/l
Agar	15 g/l

Suspend 50 g of the MEA in 1 l of distilled water and boil to dissolve. Adjust the pH to 5,4 ± 0,2 using either 1 mol/l hydrochloric acid (4.6) or sodium hydroxide 10% (*m/V*) (4.7). Place in an autoclave at 115 °C ± 2 °C for 10 min. Cool to approximately 50°C and pour approximately 20 ml into a number of 90 ml Petri dishes (5.7) and allow to cool.

Take one Petri dish containing the MEA at 20°C - 30°C and using a flat surface electrode and pH meter check that the pH is in the range 5,4 ± 0,2. If the reading is outside this range reject the batch and make a fresh mixture.

NOTE 4 The addition of an antibiotic, chlortetracycline, will inhibit bacterial growth. Alternatively further lowering the pH of the medium to 3,5 - 4,0 with lactic acid (4.9) will improve inhibition of bacterial growth.

If the medium is required at pH 3,5 cool to 47 °C and acidify with 10 % lactic acid. Once acidified do not re-heat the medium.

If the pH 5,4 medium is required to be inhibited with an antibiotic, add 1 ml of a 0.1 % aqueous solution of chlortetracycline (filter sterilised) per 100 ml of MEA, mix by shaking and immediately pour into the Petri dishes.

NOTE 5 MEA is available in dehydrated form from various manufacturers. If such material is used follow the manufacturers instructions regarding sterilization. Prepoured plates, with or without added antibiotic, can be purchased.

NOTE 6 An alternative medium to MEA can be used, providing its ability to promote comparable growth of yeasts and moulds which are likely to be encountered in tested samples can be demonstrated.

NOTE 7 Alternative antibiotics may be used providing their ability to inhibit growth of bacteria but not yeasts and moulds has been validated.

4.5 Tryptone Soya Agar, TSA (Soya bean Casein Digest Agar)

This medium favours the growth of bacteria. Yeasts and moulds may also develop but usually not so luxuriantly as on the selective MEA medium.

Tryptone	15,0 g/l
Soya peptone	5,0 g/l
Sodium chloride	5,0 g/l
Agar	15,0 g/l

Suspend 40 g of the TSA in 1 l distilled water. Bring to the boil and dissolve completely. Sterilize by placing in an autoclave (5.19) at 121 °C ± 2 °C for 15 min. Pour approximately 20 ml into 90 ml Petri dishes (5.7) and allow to cool. Take one Petri dish containing the TSA at approximately 20°C - 30°C and using a flat surface electrode and pH meter check the pH. If this is found to be

1) Tween is the registered trademark of Atlas Chemical Industries Inc.

outside the required range of pH (7,3 ± 0,3) reject the batch and make a fresh mixture.

NOTE 8 TSA is available in dehydrated form from various manufacturers. If such material is used follow the manufacturers instructions regarding sterilization. Prepoured plates can be purchased.

NOTE 9 Alternative media to TSA can be used, providing their ability to promote comparable growth of bacteria which are likely to be encountered in tested samples can be demonstrated.

4.6 Hydrochloric acid, 1 mol/l.

4.7 Sodium hydroxide, 10 % (*m/V*) aqueous

4.8 Chlortetracycline (optional), 0,1 % (*m/V*) aqueous sterilized by passing through a 0,2 µm filter (5.21).

4.9 Lactic Acid (optional), 10 % (*m/V*) aqueous sterilized by passing through a 0,2 µm filter (5.21).

5 Apparatus

5.1 Measuring cylinders, glass, nominal capacity 100 ml and 1 l.

5.2 Pipettes, glass or sterile disposable plastic, nominal capacity 1 ml with 0.1 ml graduations, and nominal capacity 10 ml, or adjustable volume pipettor and sterile disposable plastic tips.

5.3. Mixed esters of cellulose membrane filters, presterilized, preferably gridded, 47 mm diameter, nominal pore size 0,45 µm.

NOTE 10 Whilst the recommended filter material is mixed esters of cellulose the selection of membrane material will depend on individual preference and fuel type.

5.4 Filter holder assembly, single or manifold.

5.5 Filter flask, of sufficient capacity to receive all the sample being filtered and the washings.

5.6 Vacuum source, not more than 66 kPa vacuum.

5.7 Petri dishes, disposable plastic or glass, sterile, nominal diameter 90 mm.

5.8 Forceps, blunt tipped.

5.9 Incubator, capable of maintaining a temperature of 25 °C ± 2 °C or any other temperature, as appropriate, ± 2 °C.

5.10 pH meter and flat pH electrode

5.11 Scalpel or scissors

5.12 Glass beaker and cover, nominal capacity 500 ml.

5.13 Gas burner

5.14 Spreading rod, glass.

5.15 Conical flask, glass 2 l capacity.

5.16 Vortex mixer

5.17 Universal bottles, glass, screw capped, 30 ml nominal capacity.

5.18 Aluminium foil

5.19 Autoclave, capable of maintaining a temperature 115 °C ± 2°C and 121 °C ± 2°C.

5.20 Oven, capable of maintaining a temperature of 170°C ± 5°C.

5.21 Membrane filter (optional), for sterilizing liquids, nominal pore size 0,2 µm.

6 Apparatus sterilization

6.1 Glass apparatus (5.1, 5.2, and 5.14)

Cover orifices with aluminium foil or place in a sterilizing can as appropriate and place in an oven (5.20) and sterilise at 170 °C ± 2 °C for 1 h or place in an autoclave (5.19) at 121 °C ± 2 °C for 15 min. If autoclaved ensure that the glassware is dry before use. Plug mouthpieces of pipettes with non-absorbent cotton wool.

6.2 Glass bottles (5.17)

Loosen caps and place in an autoclave (5.19) at 121 °C ± 2 °C for 15 min.

6.3 Filter assembly

The flask which receives the filtered fuel and wash solutions need not be sterilized.

Do not sterilise complete assembly with membrane filter in place as this can lead to distortion or cracking of the membrane.

Either:

a) Cover orifices with aluminium foil and sterilise in an oven (5.20) at 170°C ± 2°C for 1 h or,

b) Place the apparatus in an autoclave (5.19) at 121°C ± 2°C for 15 min and dry before use.

6.4 Forceps, scalpel, scissors and glass spreading rod

Place in a covered glass beaker (5.12) containing sufficient alcohol (4.3) to cover the working ends of these instruments. Immediately prior to use remove the instrument from the alcohol and pass the working end through a burner flame. After use return the instrument to the alcohol.

CAUTION Alcohol is highly flammable. Care shall be taken to prevent the ignition of the alcohol contained in the beaker.

6.5 Plastic disposable pipette tips

Place in a suitable rack or holder, cover and place in an autoclave (5.19) at 121 °C ± 2 °C for 15 min.

7 Sampling

Guidance on how to draw and store samples for microbial testing is given in the Institute of Petroleum's *Guidelines for the investigation of microbial content of fuel boiling below 390 °C and associated water.*

8 Sample preparation

8.1 Allow sample to stand for 1 h and then examine visually.

8.2 If the sample contains free water allow it to settle and then separate the water phase and associated particulate matter by pipetting from the bottom of the sample bottle.

NOTE 11 Further analysis by microscopy and conventional microbiological culture techniques can be conducted on the water phase and associated particulate matter if required.

8.3 Shake the fuel phase of sample.

8.4 Sub-sample test portions of the fuel phase using a sterile pipette (5.2) for quantities up to 10 ml or sterile measuring cylinders (5.1) for larger quantities.

9 Procedure

9.1 Sample filtration

Place a sterile 0,45 µm pore filter (5.3) on the filter support using sterile forceps (5.8). Assemble the filter holder (5.4). Apply suction and filter the test portion through the membrane filter.

For procedure A either filter two test portions (see notes 12 and 13) through two filters or, after filtration and rinsing of a single test portion through one filter, divide the filter into two.

For procedure B filter a single test portion through one filter (see notes 12 and 13).

Record the volume of fuel filtered.

NOTE 12 It is recommended that aliquots of 50 ml are filtered; however the choice of volumes will be dictated by volume of the sample and the level of contamination expected and the filterability of the fuel. Filtration of larger sample volumes will increase test sensitivity and hence is recommended for fuels which require a high standard of microbial cleanliness such as aviation kerosene.

NOTE 13 When an adequate quantity of fuel is available, the test should be carried out at least in triplicate and if possible a greater number of replicates made.

9.1.1 Filter detergent wash

Maintaining suction, wash the membrane filter free of fuel with a 10 ml aliquot of sterile detergent solution (4.2).

9.1.2 Filter rinse

Whilst maintaining suction, wash the membrane filter free of detergent solution with three successive 10 ml portions of sterile 3 Strength Ringer's solution (4.1).

9.1.3 Remove the suction, dismantle the filtration apparatus carefully and using sterile forceps, remove the filter. Either divide the filter into two or use the whole filter and proceed in accordance with 9.2 or 9.3.

9.2 Procedure A — Placing filters directly on agar growth media

If prepoured plates are to be used examine for the presence of microbial colonies before use. Reject any which show evidence of microbial growth. Also examine plates for free moisture. If free moisture is present dry the plates before use by either leaving them unstacked on the laboratory bench for 1 h or place the plates unstacked in an incubator at 37 °C ± 2 °C until dry.

Transfer either the two membrane filters, or the two halves of the single filter, exposed surface up, onto the surface of the MEA (4.4) and TSA media (4.5) in the Petri dishes. Ensure good contact between membrane filter and medium.

9.3 Procedure B — Elution of micro-organisms

9.3.1 Elution of microorganisms from membrane filter

If prepoured plates are to be used examine for the presence of microbial colonies before use. Reject any which show evidence of microbial growth. Also examine plates for free moisture. If free moisture is present dry the plates before use by either leaving them unstacked on the laboratory bench for 1 h or place the plates unstacked in an incubator at 37 °C ± 2 °C until dry.

Using sterile forceps transfer the membrane filter to a sterile Petri dish. Cut the membrane filter into strips using a sterile scalpel or scissors and use the sterile forceps to transfer the strips to 10 ml of sterile 3 Strength Ringer's solution (4.1) in a sterile Universal bottle (5.17).

Mix the filter strips in the eluent using a vortex mixer (5.16) for 30 s to elute microorganisms from their surface.

9.3.2 Transfer of eluted microorganisms to culture media

If prepoured plates are to be used examine for the presence of microbial colonies before use. Reject any which show evidence of microbial growth. Also examine plates for free moisture. If free moisture is present dry the plates before use by either leaving them unstacked on the laboratory bench for 1 h or place the plates unstacked in an incubator (5.9) at 37 °C ± 2 °C until dry.

If required make ten-fold serial dilutions in sterile 3 Strength Ringer's solution of the eluent.

Using a pipette (5.2) place 0,1 ml of the mixed eluent, and serial dilutions if prepared, onto Petri dishes containing the MEA (4.4) medium and the TSA (4.5) medium and using a freshly flamed glass spreading rod (5.14) spread the eluent and each serial dilution onto the MEA and TSA media.

NOTE 14 Replicating the procedure will improve the precision.

9.4 Incubation of Agar Media

9.4.1 Place the dishes in an incubator (5.9) controlled at 25 °C ± 2 °C for 5 days. Invert Petri dishes containing TSA.

NOTE 15 It is recommended that the dishes are examined for growth after 3 days and again after 5 days. This should ensure that the slow developing colonies are not missed and that small colonies are not missed through overgrowth.

NOTE 16 The incubation temperature should reflect the temperature at which microbial proliferation may occur in the sampled fuel system. 25 °C is suitable for most ambient systems but an appropriately higher incubation temperature can be used when the temperature of the system sampled exceeds 30 °C.

9.4.2 After the allotted incubation period examine the dishes and record the number of colony forming units on the TSA and MEA media. Do not agitate the plates or remove the lids whilst examining. If procedure A has been followed and the colonies can not be differentiated either repeat the test using procedure B or take a smaller sample.

NOTE 16 If practicable, colonies on each medium should be identified by colour, morphology and microscopic examination; colony counts of bacteria,

yeasts and moulds can then be recorded separately. Because some yeasts grow well on the TSA medium (or alternative bacterial media) as well as on the MEA medium it is advisable to identify colony types on TSA by microscopy to determine whether they are yeasts or bacteria. If yeasts do grow on TSA, yeast colonies should be counted on MEA and TSA and the highest colony counts used to calculate numbers per litre as described in 10.1 or 10.2 below.

Recommendations for optimum colony numbers and colony count confidence limits are given in annex A.

10 Calculation

10.1 Procedure A

Calculate the number of colony forming units per litre, *N*, in the sample from the colony counts on the TSA plates, and the fungi per litre in the sample from the colony counts on the MEA plate (see note 16) using the following equation:

$$N = \frac{CC \times 1000}{V}$$

where:

CC is the colony count (see 9.4.2);

V is the volume of the fuel filtered, in millilitres.

If for each medium duplicate aliquots were filtered, average the results of the duplicate estimations. If only halves of membrane filters were used for Procedure A multiply the colony count by two.

10.2 Procedure B

Calculate the number of colony forming units per litre, *N*, in the sample from the colony count on the TSA plate, and the fungi from the colony count on the MEA plate (see note 16) using the following equation:

$$N = \frac{CC \times 10^5 \times DF}{V}$$

where:

CC is the colony count, average of replicate plates (see 9.4.2);

DF is the dilution factor of the eluent (if no dilution of the eluent is made then DF = 1);

V is the volume of the fuel filtered, in millilitres.

If for each medium duplicate aliquots were filtered, average the results of the duplicate estimations.

11 Expression of results

Report the number of colony forming units as counts per litre.

12 Test report

The test report shall contain at least the following information:

a) a reference to this standard;

b) the result of the test (see clause 11);

c) sufficient detail to identify the fuel tested;

d) any deviation, by agreement or otherwise, from the procedure specified;

e) any unusual observations before, during or after testing;

f) the date of the test.

Annex A
(informative)

Optimum Colony Counts and Colony Count Confidence Limits

A.1 General

The accuracy of culture methods for the enumeration of microbes can be poor and is affected by both determinable and indeterminable factors. A principle indeterminable factor is the heterogeneity of microbial distribution in the material being sampled. The principle determinable inaccuracy is dependent on the number of colonies on a plate. This inaccuracy decreases as the number of colonies on the plate increases, up to a limit when overcrowding effects inhibit growth and/or the user can no longer discern separate colonies. Another factor is the inability of some organisms to grow on the enumeration media. Whilst techniques are employed to keep the determinable inaccuracies to a minimum, the precision that can be expected for the analysis of dissolved chemical species is not possible for this test.

A.2 Optimum colony counts

It is recommended that plates or filters containing less than 20 colonies or more than 300 colonies should not be counted. However this upper limit for colony counts is dependent on the ability of the user to discern individual colonies. Provided that a sufficiently large volume is filtered and that both Procedures A and B are used, it will usually be the case that at least one assay plate will have a colony count within the recommended range.

Where microbial contamination is low the use of plates containing less than 20 colonies may be unavoidable. In such cases it should be appreciated that accuracy and precision will be low.

A.3 Colony count confidence limits

The precision of the test is dependent on the number colonies that form on the agar plate and may be indicated by quoting 95% confidence limits. These limits define the range within which, with a 95% probability, the true colony count lies. The confidence limits for counts of colonies obtained when a single sample is placed on an agar plate or, passed through a membrane filter are given in Table A.1 below. This assumes that the distribution of organisms within the fuel sample or the aqueous extract is random and conforms to a Poisson series.

Table A.1 — 95% confidence limits

Number of colonies counted	95% confidence limit
200	172 - 228
100	80 - 120
80	62 - 98
50	36 - 64
30	19 - 41
20	11 - 29
16	8 - 24
10	4 - 16
6	1 - 11

Increased precision may be achieved by preparing three or more replicate plates from the fuel and calculating the mean colony count (see notes 13 and 14). The 95 % confidence limits for the mean of the replicates may be determined by standard statistical techniques.