AFRICAN STANDARD

DARS 1469: 2026

First Edition 2026

Anti-bacterial solid toilet soap — Specification

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This African Standard was prepared by the ARSO Technical Committee Number 35 on Surface Active Agents (ARSO/TC 35).

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Introduction

Human skin provides a favourable environment for the existence and multiplication of a variety of microbes. The conventional toilet soap washes away the germs but does not kill them. The function of an antibacterial or antiseptic toilet soap is not only to clean the skin, but also to reduce drastically the bacterial count on the skin. This prevents skin infections and perspiration odour caused by the decomposition of perspiration by bacteria.

The antibacterial toilet soap is especially effective against *staphylococcus* and similar bacteria which have the habit of residing in the under layers of skin. The antibacterials are substantive to the skin and this tackles the microbes between two washes. Antibacterial toilet soaps shall be used regularly to be effective.

In the present edition hexachloroprene has not been permitted to use as antibacterial agent. Trichlorocarcanilide (TCC) on heating decomposes to chloroanilines which can be harmful to skin and hence the limit and method for determination of chloroaniline is incorporated.

Anti-bacterial solid toilet soap — Specification

1 Scope

This Draft African standard specifies the requirements and methods of sampling and test for antibacterial solid toilet soap.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

DARS 496-2:2017, *Synthetic detergent powders — Part 2: Composition-based household hand wash*

DARS 1524-1 *Cosmetics — Safety of cosmetic products ingredients — Part 1: List of prohibited substances*

DARS 1524-2 Cosmetics — Safety of cosmetic products ingredients — Part 2: List of substances which cosmetic products must not contain except subject to the restrictions laid down

ISO 456, *Surface active agents — Analysis of soaps — Determination of free caustic alkali*

ISO 685, *Analysis of soap — Determination of alkali content and total fatty matter content*

ISO 673, *Analysis of soap — Determination of matter insoluble in ethanol*

ISO 862, *Surface active agents — Vocabulary*

ISO 8212, Soaps and detergents — Techniques of sampling during manufacture

3 Definitions

For the purpose of this standard the terms and definitions given in ISO 862 and the following shall apply.

ISO and IEC maintain terminological datatbases for use in standards at the following addresses
- IEC Electropedia: available at http:/.www.electropedia.org/
- -ISO online browsing platform: available at http://www.iso.org/obp

4 Requirements

4.1 General requirements

4.1.1 Antibacterial solid toilet soap shall be, thoroughly saponified, milled soap or homogenized soap or both, white or coloured, perfumed, and compressed in the form of firm and smooth cakes, tablets or bars and shall possess good cleaning, lathering and antibacterial properties.

4.1.2 Ingredients used in the manufacture of the product such as Perfume, colouring dyes etc shall be in accordance with DARS 1524-1 and DARS 1524-2.

4.1.3 The soap shall contain permitted antibacterial agent as specified in DARS 1524-1 and DARS 1524-2.

4.2 Specific requirements

Antibacterial solid toilet soap, shall also comply with the specific requirements specified in Table 1.

Table 1 — Specific requirement for antibacterial solid toilet soap

	Characteristic	Requirement	Method of test
1	Total fatty matter, percent by mass, min	76.0	ISO 685
2	Free caustic alkali, as sodium hydroxide (NaOH), percent by mass, max	0.1	ISO 456
3	Free carbonated alkali, as sodium carbonate (Na_2CO_3), percent by mass, max	1.0	ISO 685
4	Matter insoluble in alcohol, percent by mass, max	2.5	ISO 673
5	Antibacterial agent Triclosan (TCN) and Trichlorocarbanilide (TCC) percent by mass, max TCN percent by mass, max TCC percent by mass, max	1,0 (in combination) 0,3 (singly) 1.5 (singly)	Annex B
6	Chloroaniline content, ppm max	10	Annex C
7	Phosphate	0	DARS 496-2:2017
8	Antibacterial activity	Shall pass test	Annex A/D
9	Irritation to product	shall not be irritating to normal skin	Annex E
10	Heavy Metals Lead, mg/kg, max Arsenic, mg/kg, max Mercury, mg/kg, max	 10 2 1	ISO 21392

NOTE 1 - The total amount of heavy metals as lead, mercury and arsenic, in combination, in the finished product should not exceed 10 mg/kg.

NOTE 2 - The heavy metals including lead, mercury and arsenic may be as a result of contamination during processing and should not be deliberately added as ingredients.

NOTE 3 Trichlorocarbanilide (TCC) is not heat stable and decomposes into chloroanilines on prolonged heating above 60 °C. If TCC is used in soap, the manufacturer should take care that such soap is not subjected to temperature above 60°C during the entire manufacturing process or during storage.

5 Packing and marking

5.1 Packing

The product shall be packed in a suitable, well-closed container, to protect the integrity of the product during transportation and sale.

5.2 Marking

The packages shall be securely closed and marked with the following particulars:

a) name of the product “Anti-bacterial solid toilet soap”

b) name and address of manufacture, supplier or importer and/or trade mark if any;

c) net weight;

d) batch or lot number; and

e) date of manufacture; and

f) best before date

g) State the antibacterial agent used and its level.

h) List of ingredients

7 Sampling

7.1 Preparation of test samples

For the purposes of this standard, scale of sampling and preparation of test samples shall be as prescribed as prescribed in ISO 8212.

7.2 Number of tests

7.2.1 Tests for determination of total fatty matter and free caustic alkali and matter insoluble in alcohol shall be conducted on each of the individual samples separately.

7.2.2 Tests for determination of all the remaining characteristics shall be conducted on the composite sample.

7.3 Criteria for conformity

7.3.1 For each of the characteristics which has been determined on the individual samples (see 7.2.1) the mean $(\bar{X})$ and the range *(R)* of the test results shall be calculated as follows:

Mean $(\bar{X})$ = sum of test result/number of test result

Range *(R)* = the difference between the maximum and the minimum value of test results. The lot shall be deemed as conforming to the requirements given in 7.2.1 if the expression $(\bar{X} - 0.6R)$ is greater than or equal to minimum value given in Table 1 and $(\bar{X} + 0.6R)$ is less than or equal to maximum value given in Table 1.

7.3.2 For declaring the conformity of a lot to the requirements of other characteristics determined on the composite sample, the test results for each of the characteristics shall satisfy the relevant requirement.

Annex A
(normative)

Determination of antibacterial activity

A.1 General

Two methods have been prescribed, namely, serial dilution method and substantivity test. The serial dilution test shall be the screening test and the substantivity test shall be the absolute test.

A.2 Serial dilution test

A.2.1 Outline of the method

Antibacterial activity is determined by serial dilution method by comparing the effectiveness of antibacterial chemicals present in 10 micrograms of soap per millilitre specified as the maximum inhibitory concentration.

A.2.2 Apparatus

A.2.2.1 Culture tube, rimless, 150 mm x 18 mm

A.2.2.2 Sterilized pipettes, 10 ml, 5 ml and 1 ml capacities

A.2.2.3 Loop, made of stainless steel or platinum wire

A.2.2.4 Conical flasks, 250 ml capacity.

A.2.3 Nutrient broth

A.2.3.1 Dissolve 5 g of beef extract, 5 g of sodium chloride, 10 g of peptone in one litre of distilled water by warming over a water bath. Cool and adjust the pH to 7.2 to 7.6 with sodium hydroxide solution. Distribute 9 ml each to the culture tubes. Plug the tube with non-absorbent cotton wool and sterilize in an autoclave for half an hour at 1 kg/cm2 pressure.

A.2.3.2 Take 99 ml and 90 ml of distilled water in 250-mL conical flasks. Plug them with non-absorbent cotton wool and sterilize in an autoclave.

A.2.3.3 Get a pure stain of *Staphylococcus aureus,* ATCC 6538 P. Maintain on nutrient agar medium. Transfer to a fresh slant every month and keep in the cold. Use a 24 h nutrient broth culture for the experiment.

A.2.4 Procedure

A.2.4.1 Aseptically transfer 1 g of the soap sample to the flask containing 99 ml of water. Dissolve by slight warming not exceeding 60 °C. Transfer 10 ml of this solution to another flask containing 90 ml of water. Take 1 ml of this solution and add 9 ml of nutrient broth in a culture tube. This gives a concentration of 100 μg/ml.

A.2.4.2 To three tubes containing 9 ml nutrient broth add 1 ml each of the above solution to get a concentration of 10 μg/ml soap per ml of nutrient broth in each tube. Inoculate the tubes with a loopful of the 24 h culture of *Staphylococcus aureus* and keep them in an incubator maintained at 37 °C ± 2 °C. Keep a control tube of nutrient broth containing the same concentration of soap.

A.2.4.3 If after 24 h incubation period, the liquid in all the three tubes is as clear as the control, the soap sample passes the test. Any turbidity more than the control shows the growth of bacteria.

A.3 Substantivity test

A.3.1 Basic principles

For a soap to have antibacterial activity, it shall satisfy two criteria:

a) it shall show, antibacterial activity on the skin even after the soap is rinsed away, that is, the germicide should be retained on the skin under the conditions of use; and

b) the antibacterial activity should be retained on the skin for some period so as to provide protection to the skin.

The test devised gives a measure of both these properties. The test involves application of soap solution on the forearm, rinsing it off in running water and allowing it to dry. A mixed culture of skin flora isolated from five individuals (see A.3.2.1) is applied immediately in prescribed areas and assayed by swabbing at 0 and 10 min. The percent reduction in survivors in 10 min is determined. Similarly the soap solution after rinsing is allowed to remain on the skin for 2 h. The test micro-organisms are applied to the skin at this time in prescribed areas and assayed by swabbing at 0 and 10 min. The percent reduction in survivors is determined. If the reduction in survivors at this time is greater than 45 %, the germicide is said to be substantive.

A.3.2 Method

A.3.2.1 Test micro-organisms

The test organisms consist of a mixed skin flora, prepared by collecting washings from the arms and forearms of at least five individuals using 50 ml of sterile water in each case. Ten ml aliquot of each washing is individually inoculated into flasks containing 90 ml of sterilized nutrient broth. Culture is allowed to grow overnight at 30 °C and flask showing turbidity are pooled together. The mixed culture is transferred through broth and grown as above at least three times and finally maintained as Tryptone-Agar-Glucose Yeast Extract (TGYE) agar, Trypticase Soy Agar (TSA), Nutrient Agar (NA) or similar agar slants. For a test culture, an overnight slant culture is suspended into sterile saline and adjusted to a cell population of 1 x 107 cells per ml.

A.3.2.2 Test procedure

A.3.2.2.1 A number of 4 cm^2 areas (2 cm x 2 cm) are marked out on the innerside of the forearm. 0.1 ml aliquot of an 8 % soap solution with germicide is applied onto individual squares and allowed to dry for 1 min. The areas are then washed with a gentle flow of tap water for two min, dried by blowing warm air. The retentivity of the germicide on skin and its antibacterial action are then assayed by applying 0.1 ml of mixed skin flora (10^7 cells/ml) onto four such squares at 0 h. Two of the squares are swabbed immediately using standard sterile cotton swab on a stick. Swabs are placed in 5 ml saline solutions. Contents are shaken well in a vortex mixer and tenfold dilutions are prepared. Bacterial cells are assayed on TGYE agar, TSA or NA plates to determine the initial count. After 10 min, two other squares are swabbed and assayed in a similar manner.

A.3.2.2.2 In another set of tests, soap solutions are applied to the four more squares, rinsed and dried. After allowing 2 h interval, 0.1 ml of culture is applied as above to four squares. Two of the squares are swabbed and assayed at 0 h and remaining two after 10 min. Survivals at 0 h and after 2 h are determined.

A.3.2.2.3 The soap shall be considered to have passed the test if the percent kill is greater than or equal to 45 % after two hours challenge.

Annex B
(normative)

Determination of TCC and TCN in soaps by HPLC

B.1 Principle

TCC and TCN are antibacterial agents, which are separated from other components in soap by normal phase or reverse phase liquid chromatography, detected spectrophotometrically and quantified by comparison with standard TCC and TCN. The method can estimate as low as 1 ppm of the above compounds:

Procedures for both normal and reverse HPLC has been described and provide the option to use either method whichever is available to the users. Both methods are comparable.

B.2 Normal phase HPLC

B.2.1 Reagents

B.2.1.1 Iso-octane, HPLC grade.

B.2.1.2 Iso-propanol (2-propanol), HPLC grade

B.2.1.3 Hexane, HPLC grade.

B.2.1.4 Standard TCC, 99 % pure

B.2.1.5 Standard TCN, 99 % pure

B.2.2 Apparatus

B.2.2.1 High Performance Liquid Chromatograph consisting of a pump, a sample injector of fixed volume with UV detector having variable wavelengths and a recorder.

B.2.2.2 Standard volumetric flasks

B.2.2.3 Pipettes

B.2.2.4 Magnetic stirrer

B.2.2.5 Millipore filter apparatus with 0.5 µ filter

B.2.2.6 Column

B.2.2.6.1 Silica column, stainless steel 25 cm x 0.46 cm packed with Normal phase-silica 5µ (Lichrosorb Si-60)

B.2.2.6.2 Cyano column, stainless steel 25 cm x 0.40 cm packed with (Lichrospher 100) cyano 5µ.

NOTE Either, of the above columns can be used depending on the availability.

B.2.2.7 Mobile phase

B.2.2.7.1 For silica column — Transfer 20 ml of iso-propanol into a 500 ml volumetric flash and make upto mark with iso-octane and mix well. Assemble millipore filter apparatus and filter the solvent system prior to use.

B.2.2.7.2 For cyano column — Transfer 50 ml of HPLC grade iso-propanol (2-propanol) into a 500 ml volumetric flask, fill upto the mark with hexane and mix well assemble milllipore filter apparatus and filter the solvent system prior to use.

B.2.2.7.1 For silica column — Transfer 20 ml of iso-propanol into a 500-ml volumetric flask and make up to mark with iso-octane and mix well. Assemble millipore filter apparatus and filter the solvent system prior to use.

B.2.2.7.2 For cyano column — Transfer 50 ml of HPLC grade iso-propanol (2-propanol) into a 500-mL volumetric flask, fill up to the mark with hexane and mix well. Assemble millipore filter apparatus and filter the solvent system prior to use.

B.2.2.8 HPLC conditions

Detector wavelength flow rate: 280 nm

Flow rate: 0.5 ml/min

Injection volume: 20 µl

Retention time

Silica column
TCN - 7.5 min
TCC - 19.2 min

Cyano column
TCN - 4.0 min
TCC - 7.5 min

B.2.3 Procedure

B.2.3.1 Standard preparation (see note under B.3.4)

Weigh accurately 25 mg of triclosan (TCN) and 25 mg of TCC into a 100-ml volumetric flask and make up to volume with the mobile phase and mix well. Pipette 1.0 ml of this solution in a 50 ml volumetric flask and dilute with mobile phase. Final concentration of TCC and TCN is 250 µg/50 ml (5.0 ppm).

B.2.3.2 Sample preparation

Weigh accurately 1 g of homogenized sample into a 100-ml standard flask, and dilute to the mark with mobile phase. Pipette 10 ml of the supernatant liquid to a 50-ml volumetric flask, dilute with mobile phase, to the mark, and filter through 0.45 µm filter.

B.2.3.3 Chromatography

Equilibrate the column, maintained at a temperature of 30 °C, with the mobile phase with a flow rate of 0.5 ml /min for iso-octane - iso-propanol mobile phase and 1.0 ml/min for Hexane - iso-propanol mobile phase for 30 min. Set the wavelength at 280 nm. Inject 20 µl of standard solution and then sample solutions.

Measure area of the peaks of respective retention time for standard and sample.

B.2.4 Calculation

$$\text{TCN, \% by mass} = \frac{\text{Area of sample for TCN} \times \text{Concentration of standard TCN}}{\text{Area of standard TCN} \times \text{Concentration of sample}} \times 100$$

$$\text{TCC, \% by mass} = \frac{\text{Area of sample for TCC} \times \text{Concentrat ion of standard TCC}}{\text{Area of standard TCC} \times \text{Concentrat ion of sample}} \times 100$$

B.3 Reverse phase

B.3.1 Reagents

B.3.1.1 *Methanol* — HPLC grade.

B.3.1.2 *Sodium Dihydrogen Phosphate Monohydrate* — Chemical grade.

B.3.1.3 Standard TCC

B.3.1.4 Standard TCN (TCS)

B.3 Reverse phase

B.3.1 Reagents

B.3.1.1 Methanol, HPLC grade.

B.3.1.2 Sodium Dihydrogen Phosphate Monohydrate, Chemical grade.

B.3.1.3 Standard TCC

B.3.1.4 Standard TCN (TCS)

B.3.2 Apparatus

B.3.2.1 Column

Octyldimethylsilyl (C-DB)

Supercosil LC-8-DB - 15 cm x 4.6 mm, 5µ

B.3.2.2 Mobile phase

MeOH/0.01 M Phosphate buffer 62:38 v/v

0.01 M Phosphate buffer: Dissolve 1.38 g sodium dihydrogen phosphate monohydrate in 1 000 ml of distilled water. Prepare to pH 3.0 by 10 % phosphate solutions.

B.3.3 Procedure

B.3.3.1 Standard preparation (see Note under B.3.4)

B.3.3.1.1 Weigh accurately about 90 mg of TCN. Dissolve in methanol and make up to 1 000 ml volumetric flask with methanol.

B.3.3.1.2 Weigh about 110 mg of TCC, dissolve well with methanol, and make up the volume to 1 000 ml.

B.3.3.1.3 Accurately pipette 10 ml of the solution prepared in (B.3.3.1.1) into the (B.3.3.1.2) volumetric flask containing TCC. And make up to the volume with methanol. Then accurately pipette 5 ml of the solution into a 50-ml volumetric flask. Make up to the volume with methanol. Filter this standard solution through 0.45 µm filter.

B.3.3.2 Sample preparation

Weigh accurately about 1.0 g of product, dissolve in methanol and make up to 100 ml in a volumetric flask with methanol. Filter this sample solution through 0.45 μm filter.

B.3.3.3 HPLC conditions

Detector wavelength	280 nm
Column temperature	35 ºC
Flow rate	1.0 ml/min
Injection volume	10 μl

Prepare the standard solution and the sample solution at the same time. Inject the standard solution three times and calculate the average of each ingredients peak count. Inject 10 μg the sample solution and determine each ingredients percentage by the calculation shown.

B.3.4 Calculations

$$\text{TCN, \% by mass} = \frac{M_s \times A_r \times F}{A_s \times M_t \times 100}$$

$$\text{TCC, \% by mass} = \frac{M_s \times A_r \times F}{A_s \times M_t \times 100}$$

where

A_r is the peak area of the test sample,

A_s is the averaged peak area of the standard,

F is the purity of standard (percent).

M_s is the mass, in grams, of the standard, and

M_t is the mass, in grams, of the test sample,

NOTE Both TCC and TCN are photosensitive, hence standards should be freshly prepared.

Annex C
(normative)

Determination of chloroaniline

C.1 Principle

The chloroanilines are extracted from soap with dimethyl sulfoxide and diazotized with nitrous acid. The reaction products are then coupled with N-1-(naphthyl) ethylenediamine hydrochloride to produce coloured compounds which are estimated spectrophotometrically.

C.2 Safety precautions

Dimethyl sulfoxide (DMSO) is readily absorbed into the skin. Inhalation or skin penetration shall be avoided.

DMSO should never be pipette using mouth. Always use pipette bulb. The standard chloroanilines and N-1-(naphthyl) – ethylenediamine hydrochloride shall not be allowed to come into contact with the skin. If they should, then wash the contaminated parts thoroughly with soap and water.

A supply of diluted sodium hypochlorite should be at hand at all times to deal with accidental spillages of chloraniline solution. Spillage on laboratory surface should be treated immediately with the sodium hypochlorite solution, followed by water.

C.3 Reagents

C.3.1 Dimethyl Sulphoxide(DMSO), AR grade.

C.3.2 Hydrochloric Acid – Concentrated(specific gravity — 1.18).

C.3.3 Sodium Nitrite – 0.4 percent w/v analytical grade, freshly prepared (aqueous).

C.3.4 Ammonium Sulphamate, 2 % w/v solution freshly prepared, (aqueous).

C.3.5 N-1-(naphthyl) Ethylene, 0.1 % w/v solution diamine hydrochloride freshly prepared (aqueous).

C.3.6 n-Butanol, AR grade

C.3.7 Sand, acid purified 40 — 100 micron mesh.

C.3.8 Solvent mixture

DMSO	5 volumes
n-Butanol	2 volumes
distilled water	2 volumes
hydrochloric acid	1 volume

Mix n-butanol, water and HCL, cool the mixture and add DMSO.

C.3.9 4-Chroroaniline and 3, 4-Dichloroaniline, AR grade.

C.4 Apparatus

C.4.1 Spectrophotometer, suitable for use at 554 nm

C.4.2 Cuvettes — Glass (matched pair) 10 mm

C.4.3 Water bath — Thermostatically controlled at 25 °C

C.4.4 Stop watch

C.4.5 Standard laboratory glassware

C.4.6 Filter Paper, Whatman No. 541

C.5 Procedure

C.5.1 Preparation of Calibration Curve

C.5.1.1 Dissolve 0.349 8 g of 3, 4-dichloroaniline and 0.2753 g of 4-chloroaniline in solvent mixture (see C.2.8) in a 250 ml amber volumetric flask.

Dilute to mark with solvent mixture. 1 ml = 2.5 mg mixed chloroanilines (stock solution).

C.5.1.2 Dilute this stock solution with solvent mixture as given below:

a) Take 5 ml of stock solution and dilute it to 250 ml with solvent mixture.

1 ml = 50 µg mixed chloroanilines.

b) Take 5 ml of the above solution [see C.5.1.2(a)] and further dilute to 250 ml with solvent mixture.

1 ml = 1 µg mixed chloroanilines.

Use this solution for preparation of calibration curve.

Transfer using a burette 0, 1 ml, 2 ml, 5 ml, 10 ml, 20 ml, 40 ml into 50 ml amber volumetric flasks.

C.5.1.3 From a burette, add sufficient solvent mixture to make total volume to 40-mL in each flask. The flasks are incubated in a water bath at 25 °C for 20 min: After exactly 20 min, add 2-mL of reagent (see C.3.3) into each flask and return them to the water bath for exactly 10 min (measure with a stop watch).

Then add 2-mL of reagent (see C.3.4) into each flask and return them to the water bath for exactly 10 min. Swirl the flask occasionally.

Then add 2-ml of reagent (see C.3.5) into each flask and remove them from the water bath. Dilute to volume with distilled water, mix and allow to stand for 30 min. Measure absorbance at 554 nm against the blank solution as prepared in C.5.1.4.

C.5.1.4 In preparing the blank solution, take 40 ml of solvent mixture in a 50 ml amber volumetric flask. Incubate the flask in a water bath at 25 °C for 20 min. After exactly 20 min, add 2 ml of reagent (see C.3.3) into the flask and return it to the water bath for exactly 10 min. Then add 2 ml of reagent (see C.3.4) into the flask and return it to the water bath for exactly 10 min (swirl the flask occasionally). Then add 2 ml of reagent (see C.3.5) into the flask and remove it from the water bath. Dilute to volume with distilled water, mix and allow to stand for 30 min. Use this blank solution for preparation of calibration curve only.

C.5.1.5 Prepare a graph by plotting weight (µg) of chloroanilines contained in each 50 ml flask against absorbance. The linear calibration will pass through the origin/or determine the average absorbance (*AA*) of 1 µg of mixed chloroanilines by dividing sum of absorbances of all different aliquots of the standard by sum of µg of chloroanilines in all different aliquots of standard.

C.6 Determination of chloroanilines

C.6.1 Weigh to the nearest mg 3.0 - 15 g of finely grated soap add 10.0 g - 15.0 g of acid purified sand. Transfer quantitatively the sample and the sand into a mortar and grind the mixture thoroughly

with a pestle to give a homogenous mass. Transfer the mass to a previously weighed 250 ml flat bottom flask quantitatively and reweigh. Add DMSO (100 ml), stopper firmly and attach the flask to an automatic shaker. Shake for 1 h. Filter the DMSO extract through Whatman No. 541 into a 250 ml amber volumetric flask. Wash the flask and filter paper with small aliquots of DMSO. Allow the filtrate to drain completely, dilute to volume with DMSO and mix. Transfer 20 ml DMSO extract into a 50 ml amber volumetric flask. Add 20 ml of solvent mixture. The flask is incubated in a water bath at 25 °C for 20 min. After exactly 20 min, add 2 ml of reagent (see C.3.3) into the flask and return it to the water bath for exactly 10 min (measure with a stop watch). Then add 2 ml of reagent (see C.3.4) into the flask and return it to the water bath for exactly 10 min (swirl the flask occasionally). Then add 2 ml of reagent (see C.3.5) into the flask and remove it from the water bath. Dilute to volume with distilled water, mix and allow to stand for 30 min. Read the absorbance at 554 nm against blank (prepared as below).

C.6.2 Prepare the blank solution by mixing 20 ml of DMSO extract of sample and 20 ml of solvent mixture in a 50 ml amber volumetric flask. Incubate the flask in a water bath at 25 °C for 20 min.

After exactly 20 min, add 2 ml of distilled water into the flask and return it to the water bath for exactly 10 min. Then add 2 ml of reagent (see C.3.4) into the flask and return it to the water bath for exactly 10 min (swirl the flask occasionally). Then add 2 ml of reagent (see C.3.5) into the flask and remove it from the water bath. Dilute to volume with distilled water, mix and allow to stand for 30 min. Use this solution as a blank for reading sample only.

C.6.3 Deduce the amount of chloroanilines (μg) from the calibration graph curve.

NOTE The determination should be completed in one day.

C.7 Calculations

Determine the amount of mixed chloroanilines in the aliquot of test solution from the calibration graph.

$$\text{Chloroaniline content (in ppm)} = \frac{250(M + M_1)M_3}{20M_2M}$$

where

M is the mass, in grams, of soap

M_1 is the mass, in grams, of sand

M_2 is the mass, in grams, of soap and sand transferred to the flask

M_3 is the mass, in micrograms, (μg) of mixed chloroanilines found from calibration graph/or it can be calculated as given below:

$$M_3 = \frac{\text{Mass of the sample}}{\text{Average absorbance of 1}\mu\text{g mixed chloroaniline (AA)}}$$

where

$$M_3 = \frac{\text{Sum of the OD of the standards}}{\text{Sum of concentration of standard chloroanilines in }\mu\text{g}}$$

$$\text{Weight of soap actually used, in g} = \frac{M_2M}{(M + M_1)}$$

Annex D
(normative)

Determination of microbial inhibition of cosmetic soap bars and liquid hand and body washes

D.1 Scope

This annex specifies a method for testing and comparing the microbial inhibition properties of cosmetic soap bars and liquid hand and body washes.

D.2 Application

This method applies to the verification of efficacy claimed by antibacterial soaps in the inhibition of microorganisms.

D.3 Principle

When soap bars and liquid hand and body washes that have antimicrobial properties are inoculated into medium No.1 (see D.4.3.1.1), specific active ingredients diffuse into the surrounding agar thereby creating a zone of inhibition around the product, which is measured as an indication of the microbial inhibition properties of the product.

NOTE The organisms referenced in this standard are the indicator organisms used when testing for hygiene purposes.

D.4 Test method

D.4.1 General

Sampling and testing shall be carried out by personnel familiar with microbiological procedures.

D.4.2 Accuracy

Except where otherwise specified, all the following tolerances:

temperatures	± 2 °C;
masses	± 1.0 %;
volumes	± 1.0 %; and
pH values	± 0.1 pH unit.

D.4.3 Culture medium, reagents, reference cultures and controls

D.4.3.1 Culture medium

NOTE 1 The culture medium listed in D.4.3.1.1 is commercially available in dehydrated form and is made up in accordance with the manufacturer's instructions.

NOTE 2 Glass distilled water or deionised water should be used

D.4.3.1.1 Medium No. 1

Ingredients

agar	15.0 g
beef extract	15.0 g
glucose	1.0 g
pancreatic digest of casein	4.0 g
peptone	6.0 g
Yeast extract	3.0 g
Water	1 000 mL

D.4.3.1.2 Preparation

D.4.3.1.2.1 Dissolve the ingredients in approximately 900 mL of water and mix.

D.4.3.1.2.2 Make up to 1 L.

D.4.3.1.2.3 Adjust the pH so that after sterilization the pH value is 6.6 ± 0.05.

D.4.3.1.2.4 Dispense 20 mL ± 0.2 mL volumes into suitable containers and sterilize in an autoclave for 15 min ± 0.5 min at 121 °C.

D.4.3.2 Horse serum

Sterile inactivated horse serum that is free from preservatives.

D.4.3.3 Reference cultures

D.4.3.3.1 Test organisms

Use the following test organisms:

Staphylococcus aureus	Sta 10 ATCC 6538;
Escherichia coli	Esc 20 ATCC 8739; and
Pseudomonas aeruginosa	Pse 16 ATCC 15442.

NOTE Other reference organisms may also be used in addition to the ones specified in D.4.3.3.1.

D.4.3.3.2 Preparation of test organism suspensions

From a newly opened freeze-dried culture or recently received agar culture, subculture the test organisms into bottles of 10 mL nutrient medium (D.4.3.3.2.1)

D.4.3.3.2.1 Nutrient medium

D.4.3.3.2.1.1 Ingredients

peptone	5.0 g
sodium chloride	5.0 g
yeast extract	2.0 g
beef extract	1.0 g
water	1 000 mL

D.4.3.3.2.1.2 Preparation

Dissolve the ingredients in the water and adjust the pH value to 7.1. Dispense in 10 mL volumes into suitable bottles and sterilize by autoclaving at 121 ±2 °C for 15 minutes.

D.4.3.3.3 Incubate the bottles at 37 °C for 24 h. Subculture onto nutrient agar slopes (see D.4.3.3.3.1). Incubate the slopes at 37 °C for 24 h.

D.4.3.3.3.1 Nutrient agar

D.4.3.3.3.1.1 Ingredients

Agar	15.0 g
peptone	5.0 g
sodium chloride	5.0 g
yeast extract	2.0 g
beef extract	1.0 g
water	1 000 mL

D.4.3.3.3.1.2 Preparation

Dissolve the ingredients in the water and adjust the pH value to 7.1. Dispense 10 mL and 15 mL volumes into suitable bottles and sterilize by autoclaving at 121 ±2 °C for 15 minutes. Allow only the 10 mL volumes to solidify in a sloped position.

D.4.3.3.4 From each of these slope cultures, prepare four subcultures (stock cultures) of each test organism onto 10 mL nutrient agar slopes (see D.4.3.3.3.1). Incubate the stock cultures at 37 °C for 24 h and then store in a refrigerator maintained at 4 °C, except for *Pseudomonas aeruginosa* which is stored at ambient temperature.

D.4.3.3.5 Use the stock cultures to prepare further subcultures for the test, but do not make more than six serial subcultures from each stock culture. After the sixth serial subculture, resort to n new freeze-dried culture.

D.4.3.3.6 Preparation of cultures for test suspensions

D.4.3.3.6.1 For each of the test organisms, inoculate a nutrient agar slope (see D.4.3.3.3.1) from a stock culture (see D.4.3.3.4) and incubate at 37 °C for 24 h.

D.4.3.3.6.2 For the test, use a 24h culture that has been subcultured for two successive days. After six subcultures, restart the process using a fresh stock culture (see D.4.3.3.4).

NOTE The physiological condition of the test organisms is important and might influence inter-laboratory and intra-laboratory variations in test results.

D.4.3.3.6.3 After incubation, wash the bacterial growth from the slope using 10 mL sterile water and, if necessary, scrape the agar surface. Carefully decant the suspension into a sterile Erlenmeyer flask and shake vigorously to suspend all growth in the water. Standardize the suspension, by using a spectrophotometer in conjunction with a standard curve, a haemocytometer, Petroff-Hausser counting chamber or any other suitable means, so that it contains 10^5 cfu/mL ± 10^4 cfu/mL. Use the suspension within 3 h of preparation.

D.4.3.4 Control

D.4.3.4.1 Positive control

Natural honey with no additives, e.g. colourants

D.4.3.4.2 Negative control

Sterile deionised water.

D.4.4 Procedure

D.4.4.1 Melt the contents of a bottle of the medium No. 1 (see D.4.3.1.1) and cool to 45 ± 2 °C. Add 1 mL of the sterile inactivated horse serum (see D.4.3.2) and 1 mL of S. aureus test organism suspension prepared in accordance with D.4.3.3.2. Mix well and avoid the formation of air bubbles, then pour the mixture into a Petri dish of 90 mm diameter and allow to solidify.

D.4.4.2 Using as sterile cutter that produces cylindrical wells (holes) of 8 mm diameter, make five evenly spaced straight cylindrical wells in the solidified medium. Remove and discard the plugs of medium. Seal the bottom of each well using one or two drops of the molten medium in D.4.3.1.1 and allow to solidify.

D.4.4.3 Introduce the test sample into the wells in such a way that each well is completely filled with the sample, taking care not to form bubbles. When the test sample is a soap bar, grind a sufficient amount to a powder, using a sterile pestle and mortar, and introduce the powder into the wells. Ensure that the surface of the agar remains free from the sample.

D.4.4.4 Incubate the petri dish at 37 ± 1 °C for 18 h to 24 h.

D.4.4.5 At the end of this period (see D.4.4.4), remove the Petri dish from the incubator. Measure the diameter of the zone of inhibition diagonally across the well to the nearest millimeter

D.4.4.6 Take the average of the five diameters and record this to the nearest millimeters**.**

D.4.4.7 Repeat the procedure described in D.4.4.1 to D.4.4.6 (inclusive) using the *E. coli* and *P. aeruginosa test* suspensions successively.

D.4.4.8 Repeat the procedure described in D.4.4.1 to D.4.4.6 (inclusive) for the positive control and the negative control

D.5 Interpretation of results

D.5.1 For the product to pass the test, the average diameter of the zone of inhibition for each of the test organisms shall be at least 10 mm.

D.5.2 If the zones are not clearly defined for one of the reference organism (e.g. hazy, incomplete zone, or distorted shape), the test shall be repeated for the specified reference organism.

Annex E
(normative)

Testing Procedure for Assessment of Skin Irritation Potential in Human Volunteers

D.1 Objective

To assess the skin tolerance and irritation potential of cosmetic ingredients under controlled conditions using a stepwise human volunteer testing approach.

D.2 Pre-Study Requirements

Before initiation of the study:

i. Conduct a full safety assessment by a qualified safety assessor.
ii. Review chemical structure, toxicological data, and related substances.
iii. Confirm that no significant health risk to volunteers is foreseeable.
iv. Ensure compliance with ethical requirements and obtain approval where applicable.
v. Define study objectives, methodology, evaluation criteria, and adverse event management procedures.
vi. Obtain written informed consent from all participants.

D.3 Selection of Volunteers

D.3.1 Inclusion Criteria

i. Healthy informed volunteers meeting study requirements.
ii. Appropriate age, gender, and skin condition.
iii. Agreement to comply with study instructions.

D.3.2 Exclusion Criteria

i. Pregnancy or nursing.
ii. Skin damage, tattoos, scars, or active skin disease at test sites.
iii. Medication or medical history affecting skin response.
iv. Participation in conflicting or recent studies.

D.3.3 Withdrawal Criteria

Participants shall be withdrawn if:

i. Study instructions are not followed;
ii. Illness or adverse reactions occur;
iii. The participant chooses to withdraw.

D.4 Test Materials

i. Prepare test materials at concentrations appropriate for the study objective and expected exposure conditions.
ii. Use suitable vehicles that do not independently cause irritation.
iii. Include reference/control materials where appropriate.

D.5 Test Methods

Testing shall follow a stepwise progression from minimal to increased exposure until the study objective is achieved.

D.5.1 Examples of Test Types

D.5.1.1 Single Application Open Test
Apply material to exposed skin under non-occlusive conditions.

D.5.1.2 Repeat Application Open Test
Repeated applications to evaluate cumulative irritation effects.

D.5.1.3 Closed Patch Test (Occlusive or Semi-Occlusive)
Apply material using patches for defined exposure periods (e.g. 4 h, 24 h, or 48 h).

D.5.1.4 Repeat Application Patch Test
Repeated occlusive applications to assess cumulative irritation potential.

D.5.2 Test Procedure

D.5.2.1 Application

i. Assess baseline skin condition before first application.
ii. Randomize test sites where appropriate.
iii. Apply measured amounts sufficient to cover the test area without overflow.
iv. Maintain consistent patch placement during repeated studies.

D.5.2.2 Exposure Conditions

i. Define exposure duration, concentration, and application type in the protocol.
ii. Increase exposure progressively only if previous stages show acceptable tolerance.

D.5.2.3 Removal of Test Material

Remove materials gently without rubbing to avoid cross-contamination or mechanical irritation.

D.6 Evaluation of Skin Responses

D.6.1 Visual Assessment

i. Assess treatment sites at predefined intervals using a standardized scoring scale.
ii. Evaluate erythema, oedema, dryness, and related skin reactions.
iii. Use the same trained assessor and consistent lighting conditions throughout the study.

D.6.2 Instrumental Assessment

Where applicable, objective measurements may include:

i. Transepidermal Water Loss (TEWL);
ii. Redness intensity measurements;
iii. Laser Doppler flowmetry.

Measurements shall be conducted under controlled environmental conditions with calibrated instruments.

D.7 Data Analysis and Interpretation

i. Perform statistical analyses as specified in the study protocol.
ii. Compare responses of test materials with reference or control materials.
iii. Interpret results based on irritation severity, exposure conditions, and cumulative effects.

D.8 Adverse Event Management

i. Monitor participants continuously for adverse reactions.
ii. Discontinue treatment at any site reaching the predefined irritation threshold.
iii. Record and manage all adverse events according to established procedures.

D.9 Study Report

The final report shall include:

i. Study objective and design;
ii. Participant selection criteria;
iii. Test materials and procedures;
iv. Assessment methods;
v. Results and statistical analysis;
vi. Adverse events and withdrawals;
vii. Conclusions;
viii. Study protocol and informed consent documentation.

Bibliography

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