

European Pharmacopoeia, Fourth Edition (2002)**2. Methods of analysis.****2.2.3. POTENTIOMETRIC DETERMINATION OF PH**

The pH is a number, which represents conventionally the hydrogen ion concentration of an aqueous solution. For practical purposes, its definition is an experimental one. The pH of a solution to be examined is related to that of a reference solution (pH_s) by the following equation:

$$\text{pH} = \text{pH}_s - (E - E_s) / k$$

in which E is the potential, expressed in volts, of the cell containing the solution to be examined and E_s is the potential, expressed in volts, of the cell containing the solution of known pH (pH_s)

Table 2.2.3.-1. - *Values of k at different temperatures*

Temperature °C	k
15	0.0572
20	0.0582
25	0.0592
30	0.0601
35	0.0611

The potentiometric determination of pH is made by measuring the potential difference between 2 appropriate electrodes immersed in the solution to be examined: one of these electrodes is sensitive to hydrogen ions (usually a glass electrode) and the other is the reference electrode (for example, a saturated calomel electrode).

Apparatus. The measuring apparatus is a voltmeter with an input resistance at least 100 times that of the electrodes used. It is normally graduated in pH units and has a sensitivity such that discrimination of at least 0.05 pH unit or at least 0.003 V may be achieved.

Method. Unless otherwise prescribed in the monograph, all measurements are made at the same temperature (20 °C to 25 °C). Table 2.2.3.-2 shows the variation of pH with respect to temperature of a number of reference buffer solutions used for calibration. For the temperature correction when necessary, follow the manufacturer's instructions. The apparatus is calibrated with the buffer solution of potassium hydrogen phthalate (primary standard) and one other buffer solution of different pH (preferably one shown in Table 2.2.3.-2). The pH of a third buffer solution of intermediate pH read off on the scale must not differ by more than 0.05 pH unit from the value corresponding to this solution. Immerse the electrodes in the solution to be examined and take the

reading in the same conditions as for the buffer solutions.

When the apparatus is in frequent use, checks must be carried out regularly. If not, such checks should be carried out before each measurement.

All solutions to be examined and the reference buffer solutions must be prepared using *carbon dioxide-free water R*.

PREPARATION OF REFERENCE BUFFER SOLUTIONS

Potassium tetraoxalate 0.05 M. Dissolve 12.61 g of C₄H₃KO₈, 2H₂O in *carbon dioxide-free water R*, and dilute to 1000.0 ml with the same solvent.

Saturated potassium hydrogen tartrate at 25 °C. Shake an excess of C₄H₅KO₆ vigorously with *carbon dioxide-free water R* at 25 °C. Filter or decant. Prepare immediately before use.

Potassium dihydrogen citrate 0.05 M. Dissolve 11.41 g of C₆H₇KO₇ in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent. Prepare immediately before use.

Potassium hydrogen phthalate 0.05 M. Dissolve 10.13 g of C₈H₅KO₄, dried at 110 °C to 135 °C, in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Potassium dihydrogen phosphate 0.025 M + disodium hydrogen phosphate 0.025 M. Dissolve 3.39 g of KH₂PO₄ and 3.53 g of Na₂HPO₄, both dried for 2 h at 110 °C to 130 °C, in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Potassium dihydrogen phosphate 0.0087 M + disodium hydrogen phosphate 0.0303 M. Dissolve 1.18 g of KH₂PO₄ and 4.30 g of Na₂HPO₄, both dried for 2 h at 110 °C to 130 °C, in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Disodium tetraborate 0.01 M. Dissolve 3.80 g of Na₂B₄O₇, 10H₂O in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent. Store protected from carbon dioxide atmosphere.

Sodium carbonate 0.025 M + sodium hydrogen carbonate 0.025 M. Dissolve 2.64 g of Na₂CO₃ and 2.09 g of NaHCO₃ in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Table 2.2.3.-2. - *pH of reference buffer solutions at various temperatures*

Temperature °C	Potassium tetraoxalate 0.05 M	Potassium hydrogen tartrate (Sat. 25 °C)	Potassium dihydrogen citrate 0.05 M	Potassium hydrogen phthalate 0.05 M	Potassium dihydrogen phosphate 0.025 M + disodium hydrogen phosphate 0.025 M	Potassium dihydrogen phosphate 0.0087 M + disodium hydrogen phosphate 0.0303 M	Disodium tetraborate 0.01 M	Sodium carbonate 0.025 M + sodium bicarbonate 0.025 M
	C ₄ H ₃ KO ₈ , 2H ₂ O	C ₄ H ₅ KO ₆	C ₆ H ₇ KO ₇	C ₈ H ₅ KO ₄	KH ₂ PO ₄ + Na ₂ HPO ₄	KH ₂ PO ₄ + Na ₂ HPO ₄	Na ₂ B ₄ O ₇ , 10 H ₂ O	Na ₂ CO ₃ + NaHCO ₃
15	1.67		3.80	4.00	6.90	7.45	9.28	10.12
20	1.68		3.79	4.00	6.88	7.43	9.23	10.06
25	1.68	3.56	3.78	4.01	6.87	7.41	9.18	10.01
30	1.68	3.55	3.77	4.02	6.85	7.40	9.14	9.97
35	1.69	3.55	3.76	4.02	6.84	7.39	9.10	9.93
$\Delta\text{pH}^{(1)}/\Delta t$	+0.001	-0.0014	-0.0022	+0.0012	-0.0028	-0.0028	-0.0082	-0.0096

(1) pH variation per degree Celsius.

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2.2.32. LOSS ON DRYING

Loss on drying is the loss of mass expressed as per cent *m/m*.

Method. Place the prescribed quantity of the substance to be examined in a weighing bottle previously dried under the conditions prescribed for the substance to be examined. Dry the substance to constant mass or for the prescribed time by one of the following procedures.

- "in a desiccator": the drying is carried out over *diphosphorus pentoxide R* at atmospheric pressure and at room temperature;
- "*in vacuo*": the drying is carried out over *diphosphorus pentoxide R*, at a pressure of 1.5 kPa to 2.5 kPa at room temperature;
- "*in vacuo* within a specified temperature range": the drying is carried out over *diphosphorus pentoxide R*, at a pressure of 1.5 kPa to 2.5 kPa within the temperature range prescribed in the monograph;
- "in an oven within a specified temperature range": the drying is carried out in an oven within the temperature range prescribed in the monograph;
- "under high vacuum": the drying is carried out over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa, at the temperature prescribed in the monograph.

If other conditions are prescribed, the procedure to be used is described in full in the monograph.

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2.4.9. IRON

Dissolve the prescribed quantity of the substance to be examined in *water R* and dilute to 10 ml with the same solvent or use 10 ml of the prescribed solution. Add 2 ml a 200 g/l solution of *citric acid R* and 0.1 ml of *thioglycic acid R*. Mix, make alkaline with *ammonia R* and dilute to 20 ml with *water R*. Prepare a standard in the same manner using 10 ml of *iron standard solution (1 ppm Fe) R*.

After 5 min, any pink colour in the test solution is not more intense than that in the standard.

2.4.14. SULPHATED ASH

Ignite a platinum, porcelain or quartz crucible at 600 +/- 50 °C for 30 min, allow to cool in a desiccator over silica gel and weigh. Place the prescribed amount of the substance to be examined in the crucible and weigh. Moisten the substance to be examined with a small amount of *sulphuric acid R* (usually 1 ml) and heat gently at as low a temperature as practicable until the sample is thoroughly charred.

After cooling, moisten the residue with a small amount of *sulphuric acid R*, heat gently until white fumes are no longer evolved and ignite at 600 +/- 50 °C until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Allow the crucible to cool in a desiccator over silica gel, weigh it again and calculate the mass of the residue.

If the mass of the residue so obtained exceeds the prescribed limit, continue the ignition, as previously, to constant mass, unless otherwise prescribed.

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2.5.9. DETERMINATION OF NITROGEN BY SULPHURIC ACID DIGESTION

SEMI-MICRO METHOD

Place a quantity of the substance to be examined (m g) containing about 2 mg of nitrogen in a combustion flask, add 4 g of a powdered mixture of 100 g of *dipotassium sulphate* R, 5 g of *copper sulphate* R and 2.5 g of *selenium* R, and three glass beads. Wash any adhering particles from the neck into the flask with 5 ml of *sulphuric acid* R, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulphuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulphuric acid in the neck of the flask; precautions should be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min, unless otherwise prescribed. Cool, dissolve the solid material by cautiously adding to the mixture 25 ml of *Water* R, cool again and place in a steam-distillation apparatus. Add 30 ml of *strong sodium hydroxide solution* R and distil immediately by passing steam through the mixture. Collect about 40 ml of distillate in 20.0 ml of 0.01 M *hydrochloric acid* and enough *water* R to cover the tip of the condenser. Towards the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with 0.01 M *sodium hydroxide*, using *methyl red mixed solution* R as indicator (n_1 ml of 0.01 M *sodium hydroxide*).

Repeat the test using about 50 mg of *glucose* R in place of the substance to be examined (n_2 ml of 0.01 M *sodium hydroxide*).

Content of nitrogen = $0.01401 (n_2 - n_1) / m$ per cent

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2.5.29. SULPHUR DIOXIDE

Introduce 150 ml of *water* R into the flask (A) (see Figure 2.5.29.-1) and pass *carbon dioxide* R through the whole system for 15 min at a rate of 100 ml/min. Place 10 ml of *dilute hydrogen peroxide solution* R neutralised with a 1 g/l solution of *bromophenol blue* R in *alcohol (20 per cent V/V)* R in the test-tube (D). Without interrupting the stream of carbon dioxide, remove the funnel (8) and introduce through the opening into the flask (A) 25.0 g of the substance to be examined with the aid of 100 ml of *water* R. Add through the funnel 80 ml of *dilute hydrochloric acid* R and boil for 1 h. Open the tap of the funnel and stop the flow of carbon dioxide and also the heating and the cooling water. Transfer the contents of the test-tube with the aid of a little *water* R to a 200 ml wide-necked, conical flask. Heat on a water-bath for 15 min and allow to cool. Add 0.1 ml of a 1 g/l solution of *bromophenol blue* R in *alcohol (20 per cent V/V)* R and titrate with 0.1 M *sodium hydroxide* until the colour changes from yellow to violet-blue.

Calculate the content of sulphur dioxide in parts per million from the expression: $128a$

a = number of millilitres of a 0.1 M *sodium hydroxide*

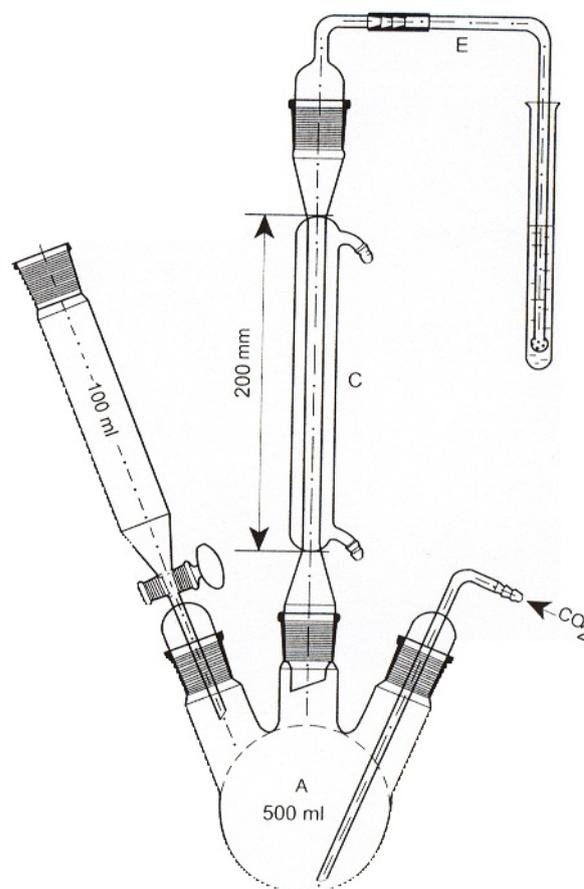


Figure 2.5.29.-1.- Apparatus for the determination of sulphur dioxide

2.5.30. OXIDISING SUBSTANCES

Transfer 4.0 g to a glass-stoppered, 125 ml conical flask and add 50.0 ml of *water* R. Insert the stopper and swirl for 5 min. Transfer to a glass-stoppered 50 ml centrifuge tube and centrifuge. Transfer 30.0 ml of the clear supernatant liquid to a glass-stoppered 125 ml conical flask. Add 1 ml of *glacial acetic acid* R and 0.5 g to 1.0 g of *potassium iodide* R. Insert the stopper, swirl, and allow to stand for 25 min to 30 min in the dark. Add 1 ml of *starch solution* R and titrate with 0.002 M *sodium thiosulphate* until the starch-iodine colour disappears. Carry out a blank determination. Not more than 1.4 ml of 0.002 M *sodium thiosulphate* is required (0.002 per cent, calculated as H_2O_2).

1 ml of 0.002 M *sodium thiosulphate* is equivalent to 34 μ g of oxidising substances, calculated as hydrogen peroxide.

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2.6.12. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS (TOTAL VIABLE AEROBIC COUNT)

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi, which may grow under aerobic conditions.

The tests are designed primarily to determine whether or not a substance that is the subject of a monograph in the Pharmacopoeia complies with the microbiological requirements specified in the monograph in question. When used for such purposes follow the instructions given below, including the number of samples to be taken and interpret the results as stated below. The tests may also be used for the test for *Efficacy of antimicrobial preservation* (5.1.3) as described in the Pharmacopoeia. They may furthermore be used for monitoring raw material quality and may be used in association with guidelines on *Microbiological quality of pharmaceutical preparations* (5.1.4). When used for such purposes, for example by a manufacturer for raw materials and/or finished product monitoring or for process validation, the conduct of the tests including the number of samples to be taken and the interpretation of the results are matters for agreement between the manufacturer and the competent authority.

Carry out the determination under conditions designed to avoid accidental contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any microorganisms, which are revealed in the test. If the product to be examined has antimicrobial activity this must be adequately neutralised. If inactivators are used for this purpose their efficacy and non-toxicity versus microorganisms are demonstrated.

Determine the total viable aerobic count by the membrane filtration method, or the plate-count method as prescribed in the monograph.

The Most Probable Number (MPN) method is reserved for bacterial counts when no other method is available. The choice of a method may be based on factors such as the nature of the product and the expected number of microorganisms. Any method, which is chosen, must be properly validated.

When used in conjunction with chapter 5.1.3 or 5.1.4, the pour-plate method, the surface-spread method and the membrane filtration method may be used.

PREPARATION OF THE SAMPLE

Sampling plan. Sampling of the product must follow a well-defined sampling plan. The sampling plan will be dependent on factors such as batch size, health hazard associated with unacceptably highly contaminated products, the characteristics of the product and the expected level of contamination. Unless otherwise prescribed, use sample(s) of 10 g or 10 ml of the substance or preparation to be examined taken with the precautions referred to above. Select the sample(s) at

random from the bulk material or from the available containers of the preparation. If necessary, to obtain the required quantity, mix the contents of a sufficient number of containers to provide each sample, depending on the nature of the substance or preparation to be examined.

An example of a sampling plan applicable to products where homogeneity with respect to the distribution of microorganisms may be a problem, is the three-class sampling plan. In this case five samples from each batch are drawn and investigated separately. The three recognised classes are:

- (i) acceptable samples, i.e. samples containing less than m CFU (colony-forming units) per gram or millilitre, where m is the limit specified in the relevant monograph;
- (ii) marginal samples, i.e. with more than m CFU, but less than $10m$ CFU per gram or millilitre;
- (iii) defective samples, i.e. containing more than $10m$ CFU per gram or millilitre.

Water-soluble products. Dissolve or dilute 10 g or 10 ml of the product to be examined in buffered sodium chloride-peptone solution pH 7.0 or in another suitable liquid. In general a one in ten dilution is prepared. However, the characteristics of the product, or the required sensitivity may necessitate the use of other ratios. If the product is known to have antimicrobial activity, an inactivating agent may be added to the diluent. If necessary adjust the pH to about pH 7 and prepare further serial tenfold dilutions using the same diluent.

Non-fatty products insoluble in water. Suspend 10 g or 10 ml of the product to be examined in buffered sodium chloride-peptone solution pH 7.0 or in another suitable liquid. In general a one in ten suspension is prepared, but the characteristics of some products may necessitate the use of larger volumes. A suitable surface-active agent such as 1 g/l of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If the product is known to have antimicrobial activity, an inactivating agent may be added to the diluent. If necessary adjust the pH to about pH 7 and prepare further serial tenfold dilutions using the same diluent.

Fatty products. Homogenise 10 g or 10 ml of the product to be examined with not more than half its weight of sterile polysorbate 80 or another suitable sterile surface-active agent, heated if necessary to not more than 40 °C, in exceptional cases to not more than 45 °C. Mix carefully and if necessary maintain the temperature in a water-bath or in an incubator. Add sufficient pre-warmed buffered sodium chloride-peptone solution pH 7.0 to make a one in ten dilution of the original product. Mix carefully whilst maintaining the temperature for the shortest time necessary for the formation of an emulsion and in any case for not more than 30 min. Further serial tenfold dilutions may be prepared using buffered sodium chloride-peptone solution pH 7.0 containing a suitable

concentration of sterile polysorbate 80 or another sterile surface-active agent.

Transdermal patches. Remove the protective cover sheets ("release liner") of ten patches of the transdermal preparation by using sterile forceps and place them, the adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with sterile gauze (or woven-filter type monofilament polymer grid), if necessary, and transfer the ten patches to a minimum volume of 500 ml of buffered sodium chloride-peptone solution pH 7.0 containing suitable inactivators such as polysorbate 80 and/or lecithin. Shake vigorously the preparation for at least 30 min (preparation A). Prepare another ten patches in the same way, place them in a minimum volume of 500 ml of broth medium D and shake vigorously for at least 30 min (preparation B).

EXAMINATION OF THE SAMPLE

Membrane filtration. Use membrane filters having a nominal pore size not greater than 0.45 μm and whose effectiveness to retain bacteria has been established. The type of filter material is chosen in such a way that the bacteria retaining efficiency is not affected by the components of the sample to be investigated. Cellulose nitrate filters, for example, may be used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. The filtration apparatus is designed to allow the transfer of the filter to the culture medium.

Transfer a suitable amount of the sample prepared as described in the section Preparation of the sample (preferably representing 1 g of the product, or less if large numbers of colony-forming units are expected) to each of two membrane filters and filter immediately. Wash each filter with three quantities, each of about 100 ml of a suitable liquid such as buffered sodium chloride-peptone solution pH 7.0. To this solution, surface-active agents such as polysorbate 80, or inactivators of antimicrobial agents may be added. If validated, less than three washes may be applied. Transfer one of the membrane filters, intended primarily for the enumeration of bacteria, to the surface of a suitable agar medium, such as medium B and the other, intended primarily for the enumeration of fungi, to the surface of a suitable agar medium, such as medium C. Incubate the plate of agar medium B at 30 °C to 35 °C, and the plate of agar medium C at 20 °C to 25 °C for five days, unless a reliable count is obtained in a shorter time. Select plates with the highest number less than 100 colonies and calculate the number of colony-forming units per gram or millilitre of product.

When examining transdermal patches, filter 50 ml of preparation A separately through each of two sterile filter membranes. Place one membrane to agar medium B for total aerobic microbial count, the other membrane to agar medium C for the count of fungi

PLATE-COUNT METHODS

a. Pour-plate method. Using Petri dishes 9 cm in diameter, add to each dish 1 ml of the sample prepared as described in the section Preparation of the sample and 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of bacteria (such as medium B), or 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of fungi (such as medium C) at not more than 45 °C. If larger Petri dishes are used the amount of agar is increased accordingly. Prepare for each medium at least two Petri dishes for each level of dilution. Incubate the plates at 30 °C to 35 °C (20 °C to 25 °C for fungi) for five days, unless a reliable count is obtained in a shorter time. Select the plates corresponding to one dilution and showing the highest number of colonies less than 300 (100 colonies for fungi). Take the arithmetic average of the counts and calculate the number of colony-forming units per gram or millilitre.

b. Surface-spread method. Using Petri dishes 9 cm in diameter, add 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of bacteria (such as medium B) or a liquefied agar medium suitable for the cultivation of fungi (such as medium C) at about 45 °C to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a LAF bench or in an incubator. Spread a measured volume of not less than 0.1 ml of the sample prepared as described in the section Preparation of the sample over the surface of the medium. Use at least two Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of colony-forming units proceed as described for the pour-plate method.

MOST-PROBABLE-NUMBER METHOD

The precision and accuracy of the most-probable-number method (MPN) is less than that of the membrane filtration method or the plate-count methods. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of bacteria in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least three subsequent tenfold dilutions of the product as described in the section Preparation of the sample. From each level of dilution three aliquots of 1 g or 1 ml are used to inoculate three tubes with 9 ml to 10 ml of a suitable liquid medium (such as broth medium A). If necessary a surface-active agent such as polysorbate 80, or an inactivator of antimicrobial agents may be added to the medium. Thus, if three levels of dilution are prepared nine tubes are inoculated. Incubate all tubes for five days at 30 °C to 35 °C. Record for each level of dilution the number of tubes showing microbial growth. If the reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or on a suitable I agar medium (such as agar medium B), for 18 h to 24 h at the same temperature and use these results. Determine the most probable number of bacteria

per gram or millilitre of the product to be examined from Table 2.6.12.-1.

Table 2.6.12.-1. - *Most-probable-number values of bacteria*

Three tubes at each level of dilution								
Number of positive tubes			MPN per gram	Category		95 per cent confidence limits		
0.1g	0.01g	0.001g		1	2			
0	0	0	<3			-	-	
0	1	0	3		x	<1	17	
1	0	0	3	x		1	21	
1	0	1	7		x	2	27	
1	0	0	7	x		2	28	
1	2	0	11		x	4	35	
2	0	0	9	x		2	38	
2	0	1	14		x	5	48	
2	1	0	15	x		5	50	
2	1	1	20		x	8	61	
2	2	0	21	x		8	63	
3	0	0	23	x		7	129	
3	0	1	38	x		10	180	
3	1	0	43	x		20	210	
3	1	1	75	x		20	280	
3	2	0	93	x		30	390	
3	2	1	150	x		50	510	
3	2	2	210		x	80	640	
3	3	0	240	x		100	1400	
3	3	1	460	x		200	2400	
3	3	2	1100	x		300	4800	
3	3	3	>1100			-	-	
Category 1: Normal results. obtained in 95 per cent of the cases.								
Category 2: Less likely results. obtained in only 4 per cent of cases. These are not to be used for important decisions. Results that are even less likely than those of category 2 are not mentioned and are always unacceptable.								

EFFECTIVENESS OF CULTURE MEDIA AND VALIDITY OF THE COUNTING METHOD

Grow the bacterial test strains separately in containers containing a suitable liquid medium (such as broth medium A) at 30 °C to 35 °C for 18 h to 24 h. Grow the fungal test strains separately on a suitable agar medium (such as medium C without antibiotics) at 20 °C to 25 °C for 48 h for *Candida albicans* and at 20 °C to 25 °C for 7 days for *Aspergillus niger*.

Staphylococcus aureus such as ATCC 6538 (NCIMB 9518, CIP 4.83)

Escherichia coli such as ATCC 8739 (NCIMB 8545, CIP 53.126)

Bacillus subtilis such as ATCC 6633 (NCIMB 8054, CIP 52.62)

Candida albicans such as ATCC 10231 (NCPF 3179, IP 48.72)

Aspergillus niger such as ATCC 16404 (IMI 149007, IP 1431.83)

Use buffered sodium chloride-peptone solution pH 7.0 to make reference suspensions containing about 100 colony-forming units per millilitre. Use the suspension of each of the micro-organisms separately as a control of the counting methods, in the presence and absence of the product to be examined. When testing the membrane filtration method or the plate-count method, a count of any of the test organisms differing by not more than a factor of five from the calculated value from the inoculum is to be obtained. When testing the most-probable-number method the calculated value from the inoculum is to be within the 95 per cent confidence limits of the results obtained. To test the sterility of the medium and of the diluent and the aseptic performance of the test, carry out the method using sterile sodium chloride-peptone solution pH 7.0 as the test preparation. There must be no growth of micro-organisms.

INTERPRETATION OF THE RESULTS

The bacterial count will be considered to be equal to the average number of colony-forming units found on agar medium B. The fungal count will be considered to be equal to the average number of colony-forming units on agar medium C. The total viable aerobic count is the sum of the bacterial count and the fungal count as described above. If there is evidence that the same types of micro-organisms grow on both media this may be corrected. If the count is carried out by the most-probable-number method the calculated value is the bacterial count.

When a limit is prescribed in a monograph it is interpreted as follows:

10² micro-organisms: maximum acceptable limit: 5 x 10²,

10³ micro-organisms: maximum acceptable limit: 5 x 10³, and so forth.

If a sampling plan such as the three-class sampling plan for example, is used, proceed as follows:

Calculate the total viable aerobic count separately for each of the five samples. The substance or preparation passes the test if the following conditions are fulfilled:

(i) none of the individual total viable aerobic counts exceeds the prescribed limit by a factor of ten or more (i.e. no "unacceptable samples"),

(ii) and not more than two of the individual total viable aerobic counts are between the prescribed limit and ten times this limit (i.e. no more than two "marginal samples").

The solutions and culture mediums recommended are described in the general chapter 2.6.13.

2.6.13. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS (TEST FOR SPECIFIED MICRO-ORGANISMS)

In this general method the use of certain selective media is proposed. A feature common to all selective media is that sub-lethally injured organisms are not detected. As sub-lethally injured organisms are relevant for the quality of the product a resuscitation must be included in examination procedures that rely on selective media.

If the product to be examined has antimicrobial activity this must be adequately neutralised.

Enterobacteria and certain other gram-negative bacteria

Although the test has been designed to detect bacteria belonging to the family of Enterobacteriaceae, it is recognised that other types of organisms (*e.g. Aeromonas, Pseudomonas*) may be recovered.

Detection of bacteria. Prepare the product to be examined as described in the general method 2.6.12, but using broth medium D in place of buffered sodium chloride-peptone solution pH 7.0, homogenise and incubate at 35 °C to 37 °C for a time sufficient to revive the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 h but not more than 5 h). Shake the container, transfer the quantity of the contents (homogenate A) corresponding to 1 g or 1 ml of the product to 100 ml of enrichment medium E and incubate at 35 °C to 37 °C for 18 h to 48 h. Subculture on plates of agar medium F. Incubate at 35 °C to 37 °C for 18 h to 24 h. The product passes the test if there is no growth of colonies of gram-negative bacteria on any plate.

Quantitative evaluation. Inoculate suitable quantities of enrichment broth medium E with homogenate A and/or dilutions of it containing respectively 0.1 g, 0.01 g and 0.001 g (or 0.1 ml, 0.01 ml and 0.001 ml) of the product to be examined. Incubate at 35 °C to 37 °C for 24 h to 48 h. Subculture each of the cultures on a plate of agar medium F to obtain selective isolation. Incubate at 35 °C to 37 °C for 18 h to 24 h. Growth of well-developed colonies, generally red or reddish, of gram-negative bacteria constitutes a positive result. Note the smallest quantity of the product which gives a positive result and the largest quantity that gives a negative result. Determine from Table 2.6.13.-1 the probable number of bacteria.

Table 2.6.13.-1

Results for each quantity of product			Probable number, bacteria per gram of product
0.1 g or 0.1 ml	0.01 g or 0.01 ml	0.001 g or 0.001 ml	
+	+	+	more than 10 ³
+	+	-	less than 10 ³ and more than 10 ²
+	-	-	less than 10 ² and more than 10
-	-	-	less than 10

When testing transdermal patches, filter 50 ml of preparation B as described in the general method 2.6.12 through a sterile filter membrane, place the membrane in 100 ml enrichment broth medium E and incubate at 35 °C to 37 °C for 18 h to 24 h. After incubation spread on agar medium F for the detection of Enterobacteria and other gram-negative micro-organisms.

Escherichia coli

Prepare the product to be examined as described in the general method 2.6.12 and use 10 ml or the quantity corresponding to 1 g or 1 ml to inoculate 100 ml of broth medium A, homogenise and incubate at 35 °C to 37 °C for 18 h to 48 h. Shake the container, transfer 1 ml to 100 ml of broth medium G and incubate at 43 °C to 45 °C for 18 to 24 h. Subculture on plates of agar medium H at 35 °C to 37 °C for 18 h to 72 h. Growth of red, non-mucoid colonies of gram-negative rods indicates the possible presence of *E. coli*. This is confirmed by suitable biochemical tests, such as indole production. The product passes the test if such colonies are not seen or if the confirmatory biochemical tests are negative.

Salmonella

Prepare the product to be examined as described in the general method 2.6.12, but using broth medium A in place of buffered sodium chloride-peptone solution pH 7.0, homogenise and incubate at 35 °C to 37 °C for 18 h to 24 h. Transfer 1 ml of the enrichment culture to 10 ml of broth medium I and incubate at 41 °C to 43 °C for 18 h to 24 h. Subculture on at least two different agar media chosen from agar medium J, agar medium K and agar medium L. Incubate at 35 °C to 37 °C for 18 h to 72 h. The probable presence of salmonellae is indicated by the growth of cultures having following appearance:

agar medium J: well-developed, colourless colonies,

agar medium K: well-developed, red colonies, with or without black centres,

agar medium L: small, transparent, colourless or pink, opaque-white colonies, often surrounded by a pink or red zone.

Transfer separately a few of the suspect colonies to agar medium M in tubes, using surface and deep inoculation.

presence of salmonellae is provisionally confirmed if in the deep inoculation but not in the surface culture there is a change of colour from red to yellow and usually a formation of gas, with or without production of hydrogen sulphide in the agar. Precise confirmation may be carried out by, appropriate biochemical and serological tests. The product passes the test if colonies of the type described do not appear or if the confirmatory biochemical and serological tests is negative.

Pseudomonas aeruginosa

Prepare the product to be examined as described in the general method 2.6.12 and use 10 ml or the quantity corresponding to 1 g or 1 ml to inoculate 100 ml of broth medium A, homogenise and incubate at 35 °C to 37 °C for 18 h to 48 h. Subculture on a plate of agar medium N and incubate at 35 °C to 37 °C for 18 h to 72 h. If no growth of micro-organisms is detected, the product passes the test. If growth of gram-negative rods occurs, transfer some material of morphologically different, isolated colonies to broth medium A and incubate at 41°C to 43 °C for 18 h to 24 h. The product passes the test if no growth occurs at 41°C to 43 °C

When testing transdermal patches, filter 50 ml of preparation A as described in the general method 2.6.12 through a sterile filter membrane and place in 100 ml of broth medium A and incubate at 35 °C to 37 °C for 18 h to 48 h. After incubation spread on agar medium N.

Staphylococcus aureus

Prepare the product to be examined as described in the general method 2.6.12 and use 10 ml or the quantity corresponding to 1 g or 1 ml to inoculate 100 ml of broth medium A, homogenise and incubate at 35 °C to 37 °C for 18 h to 48 h. Subculture on a plate of agar medium O and incubate at 35 °C to 37 °C for 18 h to 72 h. Black colonies of gram-positive cocci, surrounded by a clear zone indicate the presence of *S. aureus*. Confirmation may be effected by suitable biochemical tests such as the coagulase test and the deoxyribonuclease test. The product passes the test if colonies of the type described do not appear on agar medium O or if the confirmatory biochemical tests are negative.

When testing transdermal patches, filter 50 ml of preparation A as described in the general method 2.6.12 through a sterile filter membrane and place in 100 ml of broth medium A and incubate at 35 °C to 37 °C for 18 h to 48 h. After incubation spread on agar medium O.

Nutritive and selective properties of the media and validity of the test

The tests described hereafter must be performed at least on each lot of dehydrated media.

Proceed as follows. Grow the following test strains separately, in tubes containing suitable media such as those indicated, at 30 °C to 35 °C for 18 h to 24 h:

<i>Staphylococcus aureus</i>	such as ATCC 6538 (NCIMB 9518, CIP 4.83): broth medium A,
<i>Pseudomonas aeruginosa</i>	such as ATCC 9027 (NCIMB 8626, CIP 82.118): broth medium A
<i>Escherichia coli</i>	such as ATCC 8739 (NCIMB 8545, CIP 53.126): broth medium A,
<i>Salmonella typhimurium</i>	no strain number is recommended (a salmonella not pathogenic for man, such as <i>Salmonella abony</i> (NCTC 6017, CIP 80.39), may also be used): broth medium A

Dilute portions of each of the cultures using buffered sodium chloride-peptone solution pH 7.0 to make test suspensions containing about 1000 viable micro-organisms per millilitre. Mix equal volumes of each suspension and use 0.4 ml (approximately 100 micro-organisms of each strain) as an inoculum in tests for *S. aureus*, *P. aeruginosa*, *E. coli* and *Salmonellae* in the presence and in the absence of the product to be examined. A positive result for the respective micro-organisms must be obtained.

Clostridia

The tests described below are intended for distinct purposes. The first method is intended for products where exclusion of pathogenic clostridia is essential and it is necessary to test for their absence. The products generally have a low total count. The second method is a semi-quantitative test for *Clostridium perfringens* and is intended for products where the level of this species is a criterion of quality.

I 1. Test for *Clostridia*

Prepare the product to be examined as described in the general method 2.6.12. Take two equal portions corresponding to 1 g or 1 ml of the product to be examined. Heat one portion to 80 °C for 10 min and cool rapidly. Do not heat the other portion. Transfer 10 ml of each of the homogenised portions to two containers (38 mm x 200 mm) or other suitable containers containing 100 ml of medium P. Incubate under anaerobic conditions at 35 °C to 37 °C for 48 h. After incubation, make subcultures from each tube on medium Q to which gentamicin has been added and incubate under anaerobic conditions at 35 °C to 37 °C for 48 h. If no growth of micro-organisms is detected, the product passes the test.

Where growth occurs, subculture each distinct colony form on culture medium Q, without gentamicin, and incubate in both aerobic and anaerobic conditions. The occurrence of only anaerobic growth of gram-positive bacilli (with or without endospores) giving a negative catalase reaction indicates the presence of *Clostridium spp.* Compare, if necessary, colony morphology on the two plates and apply the catalase test to eliminate aerobic and facultatively anaerobic *Bacillus spp.* which give positive catalase reaction. This test may be applied to discrete colonies on agar, or indirectly following transfer to a glass slide, by application of a drop of *dilute hydrogen peroxide solution R*. The formation of gas bubbles indicates a positive catalase reaction.

2. *Count of Clostridium perfringens*

Prepare the product to be examined as described in the general method 2.6.12, and prepare 1:100 and 1:1000 dilutions in buffered sodium chloride-peptone solution pH 7.0. Determine the most probable number of bacteria as described under total viable aerobic count 2.6.12, using culture medium R in tubes or other suitable containers with a small Durham tube. Mix with minimum shaking and incubate at 45.5 °C to 46.5 °C for 24 h to 48 h. The containers showing a blackening due to iron sulphide and abundant formation of gas in the Durham tube (at least 1/10 of the volume) indicate the presence of *Cl. perfringens*. Estimate the most probable number of *Cl. perfringens* by means of Table 2.6.12.-1.

Controls

Use the following strains:

For method 1: *Clostridium sporogenes*, e.g. ATCC 19404 (NCTC 532) or CIP 79.3,

For method 2: *Clostridium perfringens*, e.g. ATCC 13124 (NCIMB 6125, NCTC 8237, CIP 103409).

If necessary combine with *Cl. sporogenes* to check selectivity and anaerobic conditions.

The following section is published for information.

RECOMMENDED SOLUTION AND CULTURE MEDIA

The following solution and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopoeia. Other media may be used if they have similar nutritive and selective properties for the micro-organisms to be tested for.

Buffered sodium chloride-peptone solution pH 7.0

Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dihydrate	7.2 g, equivalent to 0.067 M phosphate
Sodium chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified water	1000 ml

To this solution surface-active agents or inactivators of antimicrobial agents may be added, such as:

Polysorbate	80 g/l to 10 g/l
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Sterilise by heating in an autoclave at 121°C for 15 min.

Broth medium A (Casein soya bean digest broth)

Pancreatic digest of casein 17.0 g
 Papaic digest of soya bean 3.0 g
 Sodium chloride 5.0 g

Dipotassium hydrogen phosphate 2.5 g
 Glucose monohydrate 2.5 g
 Purified water 1000 ml

Adjust the pH so that after sterilisation it is 7.3 +/- 0.2. Sterilise by heating in an autoclave at 121°C for 15 min.

Agar medium B (Casein soya bean digest agar)

Pancreatic digest of casein 15.0 g
 Papaic digest of soya bean 5.0 g
 Sodium chloride 5.0 g Agar 15.0 g
 Purified water 1000 ml

Adjust the pH so that after sterilisation it is 7.3 +/- 0.2. Sterilise by heating in an autoclave at 121°C for 15 min

Agar medium C (Sabouraud-glucose agar with antibiotics)

Peptones (meat and casein) 10.0 g
 Glucose monohydrate 40.0 g
 Agar 15.0 g
 Purified water 1000 ml

Adjust the pH so that after sterilisation it is 5.6 +/- 0.2. Sterilise by heating in an autoclave at 121°C for 15 min. Immediately: before use, add 0.10 g of benzylpenicillin sodium and 0.10 g of tetracycline per litre of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per litre of medium before sterilisation.

Broth medium D (Lactose monohydrate broth)

Beef extract 3.0 g I
 Pancreatic digest of gelatin 5.0 g
 Lactose monohydrate 5.0 g
 Purified water 1000 ml

Adjust the pH so that after sterilisation it is 6.9 +/- 0.2. Sterilise by heating in an autoclave at 121°C for 15 min and cool immediately.

Enrichment broth medium E (Enterobacteria enrichment .. broth-Mossel)

Pancreatic digest of gelatin 10.0 g
 Glucose monohydrate 5.0 g
 Dehydrated ox bile 20.0 g
 Potassium dihydrogen phosphate 3 g
 Disodium hydrogen phosphate dihydrate 8 g
 Brilliant green 15 mg
 Purified water 1000 ml

Adjust the pH so that after heating it is 7.2 +/- 0.2. Heat a 100 °C for 30 min and cool immediately.

Agar medium F (Crystal violet, neutral red, bile agar with glucose)

Yeast extract 3 g
 Pancreatic digest of gelatin 7 g
 Bile salts 1 g
 Lactose monohydrate 10 g
 Sodium chloride 5 g
 Glucose monohydrate 10 g
 Agar 15 g
 Neutral red 30 mg
 Crystal violet 2 mg
 Purified water 1000 ml

Adjust the pH so that after heating it is 7.4 +/- 0.2. Heat to boiling; do not heat in an autoclave.

Broth medium G (MacConkey broth)
Pancreatic digest of gelatin 20.0 g
Lactose monohydrate 10.0 g
Dehydrated ox bile 5.0 g
Bromocresol purple 10 mg
Purified water 1000 ml

Adjust the pH so that after sterilisation it is 7.3 +/- 0.2. Sterilise by heating in an autoclave at 121°C for 15 min.

Agar medium H (MacConkey agar)
Pancreatic digest of gelatin 17.0 g
Peptones (meat and casein) 3.0 g
Lactose monohydrate 10.0 g
Sodium chloride 5.0 g
Bile salts 1.5 g
Agar 13.5 g
Neutral red 30.0 mg
Crystal violet 1 mg
Purified water 1000 ml

Adjust the pH so that after sterilisation it is 7.1 +/- 0.2. Boil for 1 min with constant shaking then sterilise by heating in an autoclave at 121°C for 15 min.

Broth medium I (Tetrathionate bile brilliant green broth)
Peptone 8.6
Ox bile, dried 8.0 g
Sodium chloride 6.4 g
Calcium carbonate 20.0 g
Potassium tetrathionate 20.0 g
Brilliant green 70 mg
Purified water 1000 ml

Adjust the pH so that after heating it is 7.0 +/- 0.2. Heat just to boiling. Do not re-heat

Agar medium J (Deoxycholate citrate agar)
Beef extract 10.0 g
Meat peptone 10.0 g
Lactose monohydrate 10.0 g
Sodium citrate 20.0 g
Ferric citrate 1.0 g
Sodium deoxycholate 5.0 g
Agar 13.5 g
Neutral red 20 mg
Purified water 1000 ml

Adjust the pH so that after heating it is 7.3 +/- 0.2. Heat gently to boiling and boil for 1 min, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

Agar medium K (Xylose, lysine, deoxycholate agar)
Xylose 3.5 g
L-Lysine 5.0 g
Lactose monohydrate 7.5 g
Sucrose 7.5 g
Sodium chloride 5.0 g
Yeast extract 3.0 g
Phenol red 80 mg

Agar 13.5 g
Sodium deoxycholate 2.5 g
Sodium thiosulphate 6.8 g
Ferric ammonium citrate 0.8 g
Purified water 1000 ml

Adjust the pH so that after heating it is 7.4 +/- 0.2. Heat just to boiling, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

Agar medium L (Brilliant green, phenol red, lactose monohydrate, sucrose agar)
Peptones (meat and casein) 10.0 g
Yeast extract 3.0 g
Sodium chloride 5.0 g
Lactose monohydrate 10.0 g
Sucrose 10.0 g
Agar 20.0 g
Phenol red 80 mg
Brilliant green 12.5 mg
Purified water 1000 ml

Heat to boiling for 1 min. Adjust the pH so that after sterilisation it is 6.9 +/- 0.2. Immediately before use, sterilise by heating in an autoclave at 121°C for 15 min, cool to 50 °C and pour into Petri dishes.

Agar medium M (Triple sugar, iron agar)
Beef extract 3.0 g
Yeast extract 3.0 g
Peptones (casein and beef) 20.0 g
Sodium chloride 5.0 g
Lactose monohydrate 10.0 g
Sucrose 10.0 g
Glucose monohydrate 1.0 g
Ferric ammonium citrate 0.3 g
Sodium thiosulphate 0.3 g
Phenol red 25 mg
Agar 12.0 g
Purified water 1000 ml

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.4 +/- 0.2. Fill into tubes to one-third of their height, sterilise by heating in an autoclave at 121°C for 15 min and allow to cool in a position that gives a deep portion and a sloping surface.

Agar medium N (Cetrimide agar)
Pancreatic digest of gelatin 20.0 g
Magnesium chloride 1.4 g
Dipotassium sulphate 10.0 g
Cetrimide 0.3 g
Agar 13.6 g
Purified water 1000 ml
Glycerol 10.0 ml

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.2 +/- 0.2. Sterilise by heating in an autoclave at 121°C for 15 min.

Agar medium O (Baird-Parker agar)
Pancreatic digest of casein 10.0 g
Beef extract 5.0 g
Yeast extract 1.0 g

Lithium chloride 5.0 g
 Agar 20.0 g Glycine 12.0 g
 Sodium pyruvate 10.0 g
 Purified water 950 ml

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 6.8 +/- 0.2. Sterilise by heating in an autoclave at 121°C for 15 min, cool to 45 °C to 50 °C and add 10 ml a sterile 10 g/l solution of potassium tellurite and 50 ml of egg-yolk emulsion.

Medium P (Reinforced medium for clostridia)

Beef extract 10.0 g
 Peptone 10.0 g
 Yeast extract 3.0 g
 Soluble starch 1.0 g
 Glucose monohydrate 5.0 g
 Cysteine hydrochloride 0.5 g
 Sodium chloride 5.0 g
 Sodium acetate 3.0 g
 Agar 0.5 g
 Purified water 1000 ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is about 6.8. Sterilise by heating in an autoclave at 121°C for 15 min.

Medium Q (Columbia agar)

Pancreatic digest of casein 10.0 g
 Meat peptic digest 5.0 g
 Heart pancreatic digest 3.0 g
 Yeast extract 5.0 g
 Maize starch 1.0 g
 Sodium chloride 5.0 g
 Agar, according to gelling power 10.0 to 15.0 g
 Purified water 1000 ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 7.3 +/- 0.2. Sterilise by heating in an autoclave at 121°C for 15 min. Allow to cool to 45 °C to 50 °C; add, where necessary, gentamicin sulphate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

Medium R (Lactose monohydrate sulphite medium)

Pancreatic digest of casein 5.0 g
 Yeast extract 2.5 g
 Sodium chloride 2.5 g
 Lactose monohydrate 10.0 g
 Cysteine hydrochloride 0.3 g
 Purified water 1000 ml

Dissolve, adjust to pH 7.1 +/-0.1 and fill to 8 ml in 16 mm by 160 mm tubes containing a small Durham tube. Sterilise by heating in the autoclave at 121°C for 15 min and store at 4 °C.

Before use, heat the medium for 5 min in a water-bath and cool. Add to each tube 0.5 ml of a 12 g/l solution of sodium metabisulphite R and 0.5 ml of a 10 g/l solution of ferric ammonium citrate, both solutions being freshly

prepared and filtered through membranes (pore size: 0.45 µm).

NEUTRALISING AGENTS

Neutralising agents may be used to neutralise the activity of antimicrobial agents. They may be added to buffered sodium chloride-peptone solution pH 7.0, preferably before sterilisation. If utilised their efficacy and non-toxicity towards micro-organisms are demonstrated. A typical neutralising fluid has the following composition:

Polysorbate 80 30 g
 Lecithin (egg) 3 g
 Histidine hydrochloride 1 g
 Peptone (meat or casein) 1 g
 Sodium chloride 4.3 g
 Potassium dihydrogen phosphate 3.6 g
 Disodium hydrogen phosphate dihydrate 7.2 g
 Purified water 1000 ml

Sterilise by heating in an autoclave at 121 °C for 15 min.

If the solution has insufficient neutralising capacity the concentration of polysorbate 80 or lecithin may be increased. Alternatively, the neutralisers mentioned in Table 2.6.13.-2 may be added.

Table 2.6.13.-2. - *Inactivators for antimicrobial agents to be added to buffered sodium chloride-peptone solution pH 7.0*

Type of antimicrobial agent	Inactivator	Concentration	Comment
Phenolics	Sodium laurilsulfate Polysorbate 80 and lecithin Egg yolk	4 g/l 30 g/l and 3 g/l 5 ml/l - 50 ml/l	Add after sterilisation of buffered sodium chloride-peptone solution pH 7.0.
Organo mercurals	Sodium thioglycolate	0.5 g/l - 5 g/l	
Halogens	Sodium thiosulphate	5 g/l	
Quaternary ammonium compounds	Egg yolk	5 ml/l - 50 ml/l	Add after sterilisation of buffered sodium chloride-peptone solution pH 7.0.