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16161

Distr.
RESTRICTED

IO/R.26
20 February 1987

UNITED NATIONS
INDUSTRIAL DEVELOPMENT ORGANIZATION

ENGLISH

ASSISTANCE IN THE ESTABLISHMENT OF A FOOD TESTING AND QUALITY CONTROL LABORATORY

XP/AFG/84/001/11-51

AFGHANISTAN

Technical report: Establishment of food testing and quality control laboratory in Kabul *

Prepared for the Government of the Democratic Republic of Afghanistan
by the United Nations Industrial Development Organization

Based on the work of Radovij Legetic, expert in food testing and quality control

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V.87 81922

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1. Background

Within the project XP/AFG/84/001 the establishment of an operational food testing and quality control laboratory in Kabul, D.R. of Afghanistan, has been foreseen.

Afghanistan has no organized laboratory control of food or water. Besides that there does not exist any authority to control the food on the market, prepared for export or imported. Regulations or standards in written form are not at all existing in the country.

Equipment and all accessories for the laboratory have been specified and delivered, as a help, by the Government of Yugoslavia. In addition, to establish the laboratory, the services of an expert in food testing and quality control for the duration of two months have been obtained from the United Nations Industrial Development Organization in Vienna.

2. Description of the expert's post

My task as UNIDO expert was as follows:

- To advise on the changes to be undertaken at the premises in order to prepare them for the installation of the equipment for the food testing and quality control laboratory;
- To prepare the lay-out for the equipment;
- To assist in organizing and setting up the laboratory for food testing and quality control, and advise on the selection of testing methods and procedures according to the products and materials to be tested;
- To carry out practical tests on basic methods of food analyses;
- To train counterpart personnel in modern methods of food testing, laboratory analysis and quality control and in their application to the specific products and technology processes;

- To prepare a final report, setting out the findings of the mission and recommendations to the Government of the D.R. of Afghanistan on further action which might be taken.

3. Description of the situation before I started the mission

- No organized control of food, water and other goods exists, neither in the market nor in health protection zones. This fact is creating a lot of problems for the health of the population, for market and export promotion, and led to the actual situation in the country, i.e. without any food testing and quality control system.

- The purpose of a national food control is
  - to ensure the supply of safe, nutritious and honestly presented food;
  - to protect consumers from food which is contaminated, decomposed, adulterated, injurious or packed or labelled in a false or misleading way;
  - to promote a better quality control of food by food processors and distributors and thus encourage development of the food industry on the basis of sound scientific lines;
  - to improve the export potential and enable a better control of the food imports.

If consumers have confidence in the quality and safety of food, ensured by an effective food control service, trade increases at both the local and international level due to improvements in quality and consumer acceptance. The so increased local demand encourages the food processors and the international trade brings better results in terms of foreign exchange. Such a service, if run efficiently, can lead to more varied and nutritious diets, local products can substitute costly imports and any necessarily imported goods can be closely controlled. Besides obvious economic and public health benefits the advantages in social terms can also be considerable.

The laboratory is located in part of the Institute of Parazitology, which is part of the Ministry of Public Health.

- For the chemical food testing five rooms have been provided; their dimensions are given in the attached lay-out. The micro-biological laboratory is accommodated in the same building, on the same floor.

- All rooms of the laboratory are repaired and properly prepared; however, they are short in water supply, which could not be finished until the end of my mission.

- The sewage water discharging system could not be finished by the end of my mission.

- Electricity is partly inadequate, some of the consumers request better dimensioned wires than the ones installed.

- Three people - Messrs. Asadulah Hashemi, Quader Weda and Jangisha - are members of the counterpart group. This group is set up to carry on with the laboratory work after the laboratory's completion. These three members of the counterpart staff are qualified for the work and expressed their great interest in it and enthusiasm about it. However, they would still need some additional training in a well organized laboratory, for the duration of one month.

- The equipment supplied by the Yugoslav Government was transported from Yugoslavia to here under very difficult conditions and some of the items got lost or were more or less damaged during transport.

4. <u>Situation before the completion of the mission</u>

- All items were unpacked and the situation is the following: Some of the items - especially furniture - and some of the equipment and chemicals were totally destroyed during the transport from Yugoslavia to Afghanistan. The list of broken

and missing equipment is attached to this report as Addendum 2.

- Some of the equipment (ballances, centrifuge, vacuum pump, etc.) has been repaired by myself.

- I have requested the maintenance department to improve the electric wires which had not been installed properly. This could be finished in a proper manner shortly before my departure.

- I contacted carpenters for new furniture, having designed the new items myself and I also instructed them on how to repair the destroyed furniture.

- I started, carried out and finished the training of the personnel. This part of my activities had to be reduced since the training with regard to the methods related to the broken and lost equipment could not be carried out. Also some of the instruments could not be totally or partly connected to the electric circuit net, since the wiring and the water supply were still not adequate at that time. The missing chemicals also were a problem : still the training has been carried out satisfactorily for both sides. A list of the required items and chemicals is attached as Addendum 3.

- I prepared the lay-out myself; I had to take all the measures since there were no official indications. I informed the acting officer of UNDP Kabul, Mr. Mulkh Malhotra, the authorities of the D.R. of Afghanistan, the Yugoslav authorities and UNIDO Vienna about all the problems I encountered in Kabul. This has been done during the second half of November 1986. My letter to Mr. Malhotra is attached as Addendum 5, Addendum 1 gives the methods of chemical and microbiological analysis of food and water, Addendum 2 an equipment specification of the food testing and quality control laboratory, Addendum 3 is a list of all the chemicals and instruments necessary and Addendum 4 a plan of the chemical laboratory.

5. Observations and conclusions

During the short period (two months) I carried out the main part of tasks given in the job description for post XP/AFG/84/001/11-51, which has been considered as success.

The laboratory itself will possibly be operated by this personnel as the leading team. The three men are provided to head three (the chemical, food and water analysis departments) of the laboratory, however they need approximately four laboratory technicians during the initial period.

The food testing and quality control laboratory should be responsible for the enforcement of the food legislation - through an appropriate inspection and sampling of the food production, food processing, storing and marketing facilities and both chemical and bacteriological analyses of the food samples. This should include responsibility for the hygiene in food handling, at market places where food is being sold, mobile food distribution schemes, slaughter-houses, factories, dairies, warehouses, etc.

The food control service should also play a strong advisory role when dealing with farmers, packers, processors, marketing personnel and consumers in order to ensure better compliance with the food legislation, thus assisting in the country's development.

The laboratory cannot work effectively without well-trained inspectors to carry out inspections, prepare appropriate reports, take and submit samples to the laboratory and take action on laboratory reports and others. Afghanistan does not have effective inspectors and also no proper regulation and standardization laws. In addition, the inspectors should acquire experience at other leading institutes in this field. It is not sufficient that analyses are carried out in a proper way, it is also necessary that every test result can be compared with some proposed figures or other measurable quantitative and qualitative criteria.

6. Recommendations

In order to solve these problems, it is necessary that two men from the mentioned staff are trained at a specialized university/ institute for a duration of one month each. The purpose of this additional training is the following: Ability to organize such laboratory departments and laboratories as a whole; carry out the routine analytical and special methods as well. The laboratory has to be designed and built to provide for a central food testing and quality control laboratory at the state level, i.e. in future the human and technical potential of the laboratory is to be further developed and the people to be trained have to be made familiar with this idea in the training programme.

The second activity which has to be undertaken is the establishment of a regulation and standardization scheme for quality examinations, a quality control scheme both from the chemical and microbiological point of view. And this has to take place as soon as possible.

My suggestion is that a separate project be started in the very near future.

- The duration of the mission should be one year;
- Three international experts with following qualifications ought to be engaged:

  - One food technologist with wide experience to carry out all activities of the project, able to lead the international and national staff, establish all necessary regulations and standards in the subject field;
  - One food chemist to propose the rules and methods of the various food chemical analyses;
  - One food microbiologist to prepare all necessary technical and methodical rules in the testing of food and to prepare technological starters, also to be used in various productions.

- The local staff to be selected has to be familiar with the procedures in the subject field, capable to carry out all activities,

which an implementation of these procedures will entail and properly involved in the state legislation system.

- The tasks of both the national and international personnel will be to establish Afghan food regulations and to determine all food marketing in the field of food composition and microbiology, packing, market and export presentation possibilities.

- The problems with respect to health protection, export promotion and control of imports would be difficult and practically impossible to solve without these activities and their results regarding food, water supply and tobacco products.

ADDENDUM I

METHODS OF CHEMICAL ANALYSIS OF FOOD

Acidity in Milk

Principle

The sample is for to titrate with sodium hydroxide to a phenolphtalein endpoint using milk containing rsaniline as a comparing basins.

Apparatus

1. Two loo ml evaporating basins
2. Burette, 5 ml or larger

Reagents

1. O.1. N sodium hydroxide solution
2. Rosaniline acetate solution

Dissolve o.12 g of rosaniline acetate in 96% ethanol containing o.5 of glacial acetic acid and dilute to loo ml. Store in dark.
Dilute 1 ml to 5oo ml with 96% ethanol:water, 1:1.

3. Phenolphtalein.

Dissolve 1 g phenophtalein in llo ml 96% ethanol and add o.1 N sodium hydroxide dropwise until a faint pink colour is obtained. Dilute to 2oo ml with distilled water.

Procedure

Pipette lo ml of milk into each evaporating basin. To one add 1 ml of dilute rosaniline solution and stir with a glass rod. To other add 1 ml of phenolphtalein, stirring the sample until the colour is the same as that of the rosaniline comparative standard.

Calculation

$$\% \text{ m/V lactic acid} = \frac{\text{titre}}{1000} \times 0.1 \times 90 \times \frac{100}{10 \text{ ml of milk taken}} =$$

$$\text{titre} \times 0.09$$

where % m/V = percent mass in volume

2. Acidity in Milk in dgrees Soxlet-Henkel

Apparatus

1. 50 ml automatic or ordinary burette
2. 50 ml Erlenmeyer flask

Reagents

1. 0.1 N sodium hydroxide
2. 1% phenolphtalein solution in ethanol

Procedure

In Erlenmeyer flask pipette 20 ml milk, add three drops of phenolptalein solution. Then titrate with 0.1 N sodium hydroxide, stirring the sample, until the colour, pink-red, is obtained and constant one to two minutes.

Calculation

^{o}SH = ml of 0.1 N sodium hydroxide x 2

Fat in Milk

Method of Rose - Gotlieb

Principle

The sample is treated with ammonia and ethanol, the latter to precipitate protein and the former to dissolve the precipitate, and the fat extracted with the diethyl ether and petroleum ether. The mixed ethers are evaporated and the residue weighed. The method is considered suitable for reference purposes. Strict adherenec to detail is necessary in order to obtain reliable results.

Apparatus

1. Extraction tube siphon (see diagram)
2. loo ml flat bottomed flask with G/G joint

Reagents

1. Ammonia sp. gr. o.88o (six-fifths the amount of ammonia sp. gr. o.91o should be used)
2. Petroleum ether B.R. 4o-6o°C
3. Diethyl ether, peroxide-free
4. Ethyl alcohol 96%
5. Mixed ether. Equal volumes of petroleum and diethyl ethers.

Procedure

Accurately weigh about lo mg of homogenous sample into an extraction tube. This is conveniently done on a top-pan balance accurate to a miligram by standing the tube in a plastic beaker or any other light container. For weighing on a analytical balance it may be necessary to attach a piece of wire to the neck of the tube and hitch this tc the Pan-hook of the balance.

Add 1 ml o.88o ammonia and properly mix. Add lo ml alcohol and again mix thoroughly. Add 25 ml of diethyl ether, close the tube with a wetted ground glass stopper or well fitted rolled cork, shake very gently and release the pressure without loss of ether, and repeat a couple of times until the tube can be shaken vigorously without risk of pressure build-up. Shake that for one minute. Add 25 ml of petroleum ether, rinsing the stopper and neck with some of it, wet the stopper with water again and shake vigorously for half a minute.
Weigh dry loo ml flat-bottomed ground glass flask. Leave the extraction tube to stand half an hour or more until the layers are slearly separated, insert the siphon tube so that the orifice is 2-3 mm above the aqueous layer and blow gently so that the etherial extract siphons into the weighed flask. Raise the siphon a litte but do not remove it. Rinse the tip of it with about 5 ml of mixed ethers and without shaking, siphon to the flask. Use further 5 ml of mixed ethers to rinse the cork of the siphon and the neck of the tube and again transfer without shaking the tube, then romove the siphon from the tube. Rinse the tip of the siphon and the neck of the flat-bottomed flask. The evaporation of the solvent in the flask can be started while the second extraction is in progress.

Connect the flask to a Soxlet apparatus and collect the redistilled mixed ethers (which may be used for rinsisng) or distill of the solvents in any other convenient way.

Add 15 ml of diethyl ether to the tube and shake vigorously for one minute taking the same precautions as before.
Extract a second time with a 15 ml of each solvent and rinse as before.
Evaporate all the solvent in the flask, completing the process on the water-bath and finally in the oven, drying to constant weight. Leave in the desiccator t· coll at least an hour and do not wipe the flask just before weighing.
Weigh, then add petroleum eteher to dissolve the fat and carefully decant taking care to leave any sediment in the flask. Rinse the flsak with petroleum ether until all the fat is removed.,but any sediment remaining. Dry in the oven and weigh as before. The difference in weights show out the weight of the fat extracted from the milk.
For the most accurate work ir may be checked that the residue form fourth extraction is negligible and a blank extraction may be done using lo ml of water in place of the sample.

Calculation

$$\% \text{ fat m/m} = \frac{\text{weight of fat}}{\text{weight of milk}} \times 100$$

Fat in Milk

Method by Gerber

Principle

All proteins of milk are burned down by using the concentrated sulphuric acid, (s.d. 1.915L.925 kg/l). The milk is mixed with a H_2SO_4 and amyl alcohol in special gerber tube permitting solution of the protein present and release of the fat.
The tubes are centrifuged and the fat rinsing into the calibrated part of the tube is measured as a percentage of the sample m/m. This method is suitable as a routine or screeing test.

Apparatus

1. Gerber butyrometer tubes, with lock-stopper key
2. Gerber centrifuge, 30 cm diameter
3. Milk pipette 11 ml

Reagents

1. Suphuric acid ap.gr. 1.815- 1.825
2. Amyl alcohol

Procedure

Measure 10 ml of suphuric acid into the butyrometer tube preferably by use of an automatic dispenser, without the wetting the neck of the tube. Mix the sample gently but thoroughly and fill the pipette above the graduation line. Wipe the outside of the pipette and allow the milk level to fall so that the top of the meniscus is level with the mark. Run the milk into the butyrometer tube without wetting the neck of butyrometer tube. Add 1 ml of amyl alcohol. Close with a stopper, shake until homogeneous, inverting to complete admixture of the acid and centrifuge for 4 minutes after the centrifuge has reached 1100 rpm. The tubes should be put in the centrifuge so as to ensure the radial symmetry and as evenly spread as possible, in order to protect the bearings of the centrifuge. Allow the centrifuge to come to rest remove the butyrometer tubes and place in water-bath at 65°C if centrifuge has no heater.
REad off the percentage of fat after three minutes, adjusting the height in the tube as and if necessary by movemente of the lock-stopper with the key.

Dried Milk Solubility

Principle

The powder is shaken with water and the total of the suspension determined before and after centrifuging. The amount of powder remaining in suspension after centrifuging expressed as a percentage of the total amount in suspension is taken as a measure of the sulubility.

Apparatus

1 Centrifuge with 5o ml tubes.

Procedure

Shake 4 g of powder with 32 ml of water at 5o°C for 1o seconds in a 5o ml centrifuge tube and keep the tube in water at 5o°C for 5 minutes. Centrifuge the suspenisons from half-cream and full-cream samples for 1o minutes at 2ooo rpm. Cool in a refrigerator and remove the fat layer after prising from walls of the tube with a needle. Warm to room temperature, break up the deposit with a glass rod and shake vigirously until suspension appears homogeneous. For all types of sample, pipette 2 ml weigh into a tared metal dish with lid and determine the total solid by drying on a water-bath and then in the oven 1 1/2 hours. Centrifuge for 1o minutes at 2oo rpm and determine the total solids of 2 ml supernate.

Calculation

$$\% \text{ solubility} = \frac{100 \times T_1 \times S_2}{T_2 \times S_1}$$

where: T_1 = weight of suspension taken for total determination before centrifuging

T_2 = weight of suspension taken for total solids determination after centrifuging

S_1 = weight of dired solids remaining after the evaporation of T_1

S_2 = weight of dried solids remaining after the evaporation of T_2

Interpretation

Spray dried powders are almost completely soluble, roler dried to the extent of 80% or more. The results depend on the exact conditions of test and the acidity of the powder so it is advisible to compare doubtful samples with spray dried powder known to be easonably fresh. The powders become less soluble with age, thereby affecting the quality.

Moisture in Butter

Principle

The weighed sample is dried to ccnstant weight at 100°C.

Apparatus

1. Metal dish, flat-bottomed, about 7 cm diameter, 3 cm deep, preferably with a lid
2. Oven at 100°C
3. Analytical balance reading 0.1 mg.

Procedure

Keep the sample at 32-35°C in the airtight container and shake vigorously until a homogeneous lum-free emulsion is obtained. It is convenient to put ih the dish a glass rod with a flattened end and lonf enough so that the other end can rest on the rim. Dry in the oven, cool at least half an hour in the desiccator and weigh, add 3-4 g of butter to the dish and rapidly and accurately weigh. Stir in a little alcohol to facilitate evaporation, leave the dish on a boiling water-bath, stirring occasionally, until no water is visible on the bottom of the dish. Wipe the outside of the dish and transfere to the oven. Dry to constant weight (less than 2 mg differenece successive weighings).

Calculation

$$\% \text{ moisture} = \frac{\text{weight lost in oven}}{\text{weight of sample}} \times 100$$

Reichert, Polenske, Kirschner values of Butter

Principle

Butter is distinguished from other fats by the presebce of the glycerol ester of relatively low molecule weight fatty acids, especially butyric but also caproic, capric, caprilic, lauric and myristic. These acids are wholly or partly steam-volatile and water-soluble.

The fat is saponificated with sodium hydroxide, the melt acidified and distilled under standard conditions. The distillate is filtered and the solubile acids titrated (Reichert value). The insoluble acids are dissolved in alcohol and titrated (Polenske value). The titrated soluble acids are treated with silver sulphate and the filtrate is acidified and re-distilled and distillate titrated (Kirschner value). The Reichert reflects the mount of butyrici and caproic acid present, the Kirschner butyrici alone and the

Polenske chiefly caprylic, capric and lauric with some contribution from myristic and even palmitic acids.
The procedure should be carried out without a sample in oreder to obtain a blank value which is usually about o.5 ml.

Apparatus

1. Distillation apparatus

2. Reagents

1. Sodium hydroxide solution, 50%
2. Glycerol
   (mix soda solution + glycerol in proportion 1+9)
3. Dilute sulphuric acid, 25 ml per litre, and adjust so that 40 ml exactly neutralise 2 ml of the sodium hydroxide solution.
4. Pumice powder or anti-bumping granules.
5. Phenolphtalein solution o.5% in denatured ethanol.
6. Barium hydroxide approx. o.o5 M, accurately standardized.
Shake 20 g of the octahydrate with a litre of water until the crystals dissolve and leave a couple of days for the barium sulphate to settle out. Store in a bottle with a guard-tube containing powdered soda-lime to prevent ingress of carbon dioxide. Standardize the solution against o.1 M hydrochloric acid or potassium hydrogen phtalate.
NaOH may be used instead of bariumhydroxide if the Kirschner value is not going to be determined.
7. Silver sulphate.

Procedure

Weigh 5 g (± o.o1) of the oil (obtained by melting the butter and filtering) into the distilling flask, and add 2o ml of the glycerol caustic mixture. The weighing may be done by attaching the flask to the pan hook of an analytical balance by a piece of wire and carefully adding the sample until the tare +5 g is attained. Saponify by gently heating over a small flame with constant swirling, until the liquid no longer foams and becomes clear. Allow the flask to cool to about 9o°C, add 9o ml of recently boiled disstilled water of about the same temperature and mix.

The liquid should remain clear. Add o.6 to o.7 g of the pumice and then 5o ml sulphuric acid solution (o.5 ml). Connect the flask immediately to the distillation apparatus and warm it gently until the free fatty acids form a clear surface layer.
Start heating and regulate the flame so as to collect in the measuring flask 11o ml of distillate in 19-21 minutes, taking the moment when first drop forms in the condenser at the beginning of distillation period. Regulate the water flux in the condenser so as to maintain the temperature of the water leaving the condenser at 2o± 1°C.
If the temperature of water for cooling exceeds 2o°C as in tropical and subtropical areas, and if no special arrangements can be made, the measuring flask should stay in the water-bath at 2o ±1°C about 1 hour. When exactly 11o ml of distillate has been made and collected, remove the burner immediately and substitute a small beaker for the measuring flask.

Mix the contents of the measuring flask by gently shaking and immerse the flask in water-bath at 20±1°C for 10 to 15 minutes, the 110 ml mark on the flask being 1 cm below the level of the water in the water-bath and the flask being turned from time to time. Stopper the flask and mix by inverting it 4 to 5 times without shaking. Filter the 110 ml of distillate through a dry medium speed filter paper (diameter 80-90 mm) which fits sungly into funnel. Its important to have clear filtrate.
The filter should be of such size that 15 ml poured into it will fill it completely. Pipette 100 ml of the filtrate into a conical flask of 300 ml, add 0.5 ml of phenolphtalein indicator solution and titrate with standardized aqueous alkali solution to a pink colour persistent dor 1/2 to 1 minute. Calculate the Reichert value according to the formula below. Retain the nutralized filtrate for the determination of Kirschner value.

Conduct a blank test without fat and instead of sapnifying over a naked flame, heat on a boiling water-bath for 15 min. Not more than 0.5 ml of the standardized alkali solution should be required for the titration of the blank. If the volume exceeds this, prepare fresh reagent solutions.

Determination of Insoluble Vol-tile Fatty Acid Value (Polenske value)

Rinse the filter with three successive 15 ml portions of distilled water at a temperature of 20±1°C, each having previously passed through the condenser, the small beaker and the measuring flask. Place one funnel and filter in the neck of a dry clean conical flask of 200 ml capacity. Dissolve the insoluble fatty acids by repeating the washing procedure but using 150 ml portions of ethanol (95-96% previously neutralized).
Titrate the combined ethanolic washings the standardized aqueous alkali solution using 0.5 ml phenolphtalein indicator solution, to a pink colour persistent for 1/2 to 1 minute.

Determination of Volatile Fatty Acid with Soluble Silver Salts (Kirschner Value)

Add 0.5 g of finely powdered silver sulphite to the neutralized solution from the Reichert determination. Leave the flask in the dark one hour with occasional shaking and filter the contents through a dry filter, in the dark. Transfer 100 ml of the filtrate to a dry Polenske flask, add 35 ml of cold recently-boiled distilled water, 10 ml of the dilute sulphuric acid solution and a little pumice powder or about 30 cm Al-wire, about 1 mm thick wound into a coil about 5 mm accross. Connect the flask to the standard omiting immersion in a water-bath at 20°C. Titrate 100 ml of the filtrate with 0.05 M barium hydroxide solution.

Calculation

Reichert value (soluble volatile fatty acid value):

Reichert value = 1.1 ml of 0.05 M barium hydroxide required for neutralization. The alkali will not normally be exactly 0.05 M, so the titre must be multiplied by a suitable factor after deduction of the blank titre.
Report the result rounded to the first decimal place.

Insoluble volatile Fatty acid Value (Polenske value

Polenske value = ml of 0.05 M barium hydroxide required to neutralize the alcohol soluble acids.

Volatile fatty acids with soluble silver salts (Kirschner value):

$$\text{Kirschner value} = \frac{\text{titre} \times 1.21 \times (100+C)}{10,000}$$

where C is number of ml of 0.05 M barium hydroxide required in the Reichert titration.

M e a t

Hydroxypyroline in Meat and Meat Products

Principle

Hydrolysis and a test portion in constant hydochloric acid solution containing tin (II) chloride. Neutralization, filtration and dilution. Oxidation of the hydroxyproline by chloramine-T, followed by the formation of a red compound with 4-dimethylamin benzaldehyde. Photometric measurment at a wavelength of 558 nm.

Apparatus

1. Mechanical meat mincer, laboratory size, fitted with a plate with holes not exceeding 4 mm in diameter.
2. Round or flat-bottomed hydrolisis flask capacity about 200 ml, wide necked, equipped with an air-cooled or water-cooled condenser.
3. Electric heating device.
4. Filter paper discs, diameter 12.5 cm (eg. S or S No.287)
5. pH meter
6. Aluminium or plastic foil
7. Water bath, thermostatically controlled at $60 \pm 0.5^{\circ}C$
8. Spectrophotometer, capable of being used at wavelength of 558 ± 2 nm or photoelectric colorimeter with an interference filter with absorption maximum at 558 ± 2 nm.
9. Glass cells of 1 cm optical path length.

Reagents

All reagents shall be of analytical reagent quality. The water used shall be distilled water or water of at least equivalent purity.

Tin (II) chloride, hydrochloric acid solution

Dissolve 7.5 g of tin (II) chloride dihydrate ($SnCl_2 x 2H_2O$) in water, dilute to 500 ml and add 500 ml of hydrochloric acid ($_{20}$ 1.19 g/ml).

Hydrochloric acid, approx. 6 N solution

Mix equal volumes of hydrochloric acid ($_{20}$= 1.19 g/ml) and water.

Sodium hydroxide, approx. 10 N solution

Dissolve 40 g of sodium hydroxide in water. Cool and dilute to 100 ml.

Sodium hydroxide, approx. 1 N solution

Dissolve 4 g of sodium hydroxide in water. Cool and dilute to 100 ml.

Buffer solution pH 6.0

Dissolve in water: 50 g of citric acid monohydrate ($C_6H_8O_7 x H_2O$), 12 ml of acetic acid (960 g of CH_3COOH per litre), 120 g of sodium acetate trihydrate ($CH_3COONa \times 3H_2O$), 34 g of sodium hydroxide. Dilute to 1000 ml with water. Mix this solution with 200 ml of water and 300 ml of propan -1-ol. This solution is stable for several weeks at +4°C.

Chloramine-T reagent

Dissolve 1.41 g of N-chloro-p-toluenesulphanomide, sodium salt (Chloramine-T) in 1o ml of water and successively add 1o ml of propan-1-ol and 8o ml of the buffer solution pH 6.o. Prepare this solution immediately before use.

Colour Reagent

Dissolve 1o g of 4-dimethylaminobenzaldehyde in 35 ml of perchloric acid solution (6o percent (m/m)) and then slowly add 65 ml of propan-2-1. Prepare this reagent on the day of use. Important: purification of the 4-dimethylaminobenzaldehyde is necessary. Proceed as follows:
- prepare a saturated solution of the 4-dimethylaminbezaldehyde in hot 75% (V/V) ethanol. Cool first at room temperature, and finally in a refrigerator. After about 12 hours filter on a Buchner filter funnel. Wash with a littel 7o percent (V/V) ethanol. Again dissolve in a hot 7o (V/V) ethanol. Add cold water and agitate thoroughly. Repeat this procedure until a sufficient quantity of milk-white crytals has been formed. Place in the refrigerator over night. Filter on the Buchner funnel, wash with 5o percent (V/) ethanol and vacuum dry over phosphorus (V) oxide.

Hydroxypyroline Standard Solution

Prepare a stock solution by dissolving loo mg of hydroxypyroline-alpha-carbonic acid (hydroxyproline) in water. Add 1 drop of hydrchloric acid solution and dilute to loo ml with water.
On the day of use, dilute 1 ml of the stock solution to loo ml with water in the volumetric flask. Then prepare four standard solutions by diluting 1o, 2o and 4o ml of this soultion to loo ml with water to obtain hydroxyproline concentrations of 1, 2, 3 and 4 g/ml respectively.

Sample

Proceed from a representative sample at least 200 g. See ISO 7100. Store sample in such a way that deterioration and change in composition are prevented.

Procedure

Preparation of test sample

Raw meat and raw meat products: reduce intact meat to small cubes (0.5 cm^3) by cutting it while it is cold (below $0^{\circ}C$) using sharp knife. Either place the sample in a container and seal the latter hermetically, or vacuum pack the sample in a heat-resistant plastic film. Then heat so as to maintain a temperature of at least $70^{\circ}C$ for at least 30 minutes in geometrical centre of the sample. Cool and proceed as follos:
Cooked meat and Cooked meat products: make the sample homogenous by passing it at least twice through the meat mincer and mixing. Keep the homogenized sample in a completely folled , airtight, closed container and store it in such a way that deterioration and chang in compostion are prevented. Analyse the test sample as soon as possible, but strictly within 24 h.

Test portion

Weigh to the nearest 1 mg about 4 g of the test sample into the hydrolysis flask. Ensure that none of the sample adheres to the side-wall of the flask.

Hydrolysis

Add some boiling chips and 100±1 ml of hydrochloric acid solution containing tin (II) chloride. Heat to gentle boiling using the heating device and maintain for 16 h under reflux (convenietntly overnight).

(Note: if desired by the analyst, the hydrolysis may be alternatively accomplished in two periods, each of 7 to 8 hours on consecutive days. This alternative procedure has been provided experimentally to the yield results tha are not significantly different from those obtaine with a single hydrolysis period of 16 hours.)

Filter the hot hydrolisate through paper into a 2oo ml one-mark volumetric flask. Wash the filter three times with lo ml portions of hot hydrchloric acid solution and add the washings to the hydrlisate. Make up to the mark with water.
(Note: the hydrlisate can be kept at this state for at least one week under refrigeration).

Colour Measurement

By usi g a pipette, transfer into a beaker a volume V ml of the hydrolisate so as to obtain a hydroxypyroline concentration the range 1 to 4 g/ml after diltuion to 25o ml.
(Note: in most cases, V will be the order of 5 to 25, depending on the amount of connective tissue present in the sample).
With the aid of the pH.meter, adjust pH to 8±o.2 by dropwise addition first of lo N sodium hydroxide solution. Remove the tin hydroxide precipitate on the filter at least twice with 5o ml portions of water and collecting the filtrate and washings in a 25o ml one-mark volumetric flask. Make up to the mark with water and mix.
Transfer 4 ml of this solution into a test tube and add 2 ml of chloramine-T reagent. Mix and leave at room temperature for 2o±1 minute. Add 2 ml of the colour reagent , mix thoroughly and cap the tube with aluminium or plastic foil.

Transfer the tube quickly into the water bath, controlled at 60±5°C and heat for exactly 20 minutes. Cool under running tap water for at least 3 minutes. Measure the absorbance at 558±2 nm in a glass cell (quvete) using the spectrophotometer or the photoelectric colorimeter equipped with a interference filter. Sustract the absorbance measured for the blank solution and read tha hydroyproline concentration of the diluted hydrlysate from the calibration obtained as describe above.

Blank test

Carry out in duplicate the same procedure substituting water for the diluted hydrolysate.

Calibration Curve

Carry out the same procedure again but substituting 4.00 ml of each of four diluted standard hydroxyproline solutions for the diluted hydrolysate.
Plot the measured absorbance values, corrected for the blank value, against the concentrations of the standard hydroxyproline solutions, and construct the best fitting streight line through the plotted points and the origin.

Duplicate determination

Carry out two independent determinations, starting from different test portions.

Expression of Results

Calculate hydroxyproline content, H , of the sample, as a percentage by mass, from the formula:

$$H = \frac{5h}{m \times V}$$

where:
h = the hydroxyproline concentration in micrograms per millitre, of the diluted hydrolysate
m = the mass in grams of the test portion
V = the volume in millitres of solutions taken for dilution to 250 ml.

Take as the result the aritmetic mean of two values provided that the requirement for repeatibility is satisfied. Report the result to the nearest 0.01 percent.

Repeatibility of single values

The difference between the results of values obtained simultaneously or in rapid succession on to duplicate test portions by the same analyst shall not exceed 5 percent of their arithmetic mean.

Peas - microscopical examination

Principle

Microscopical examination permits differentiation of wrinkle-seede and smooth seeded peas. The method applies both quick frozen and canned products.

Apparatus

1. Compound microscope - 100 to 250 magnification
   - phase contrast
2. Microscope slide and cover glass
3. Spatula

Reagents

1. Ethanol - 95% (V/V)
2. Glycerin

Procedure

Preparing the mount. Remove the small portion of endosperm and place on a glass slide, using a spatula, grind the material with 95% V/V ethanol. Add a drop of glycerin, place a cover glass on the material and examine under a microscope.

Identification

Starch granules of the wrinkled-seeded types (garden peas, sweet) show up a clear cut, well defined, generally spherical particles. Starch granules of small-seede types (round, early continental) show up as an amorphous mass with no well defined geometric shape.

Formal number

Principle

By the addition of formaldehyde one H is liberated per molecule of aminoacid. It is titrated with alkali. The secondary amino-group of histidine does not react; those of proline and hydroxyproline react about 75%. Tertiary nitrogen and guanidine -groups undergo no reaction.

Apparatus

1. pH-meter

Reagents

1. Sodium hydroxide, 0.25 M
2. Formaldehyde solution: pure formalin of at least 35% is brought exactly to pH 8.1 with dilute sodium hydroxide as determinated by means of pH meter.
3. Hydrogen peroxide, pure, 30%.

Procedure

25 ml fruit juice (for lemon juice 10 ml + 10 distilled water) or the corresponding amount of concentrate diluted to this volume are neutralized in a beaker with 0.25 M sodium hydroxide to pH 8.1 on the pH-meter; 10 ml of the above formaldehyde solution is then added. After ca. 1 minute the solution is titrated potentiometrically to pH 8.1 with 0.25 M sodium hydroxide.
If more than 20 ml 0.25 sodium hydroxide are taken up, the titration is to be repeated using 15 ml formaldehyde solution instead of 10 ml. When sulphur dioxide is present the sample is treated with a few drops of 30% hydrogen peroxide before neutralization.

Calculation

The amount of alkali used in the titration expressed as ml 0.1 N alkali and referred to 100 ml fruit juice or 100 g concentrate, is equal to the formol number of the sample under test.
Remark : in the literature the formol number may also be found defined as ml N alkali for each 100 ml. Sample which corresponds to values 10 times smaller than those given by the preceding method of calculation.
Results are to be expressed in whole numbers.

Chemical methods of cereal and pulse analysis

1. Gluten in wheat flour

Principle

Starch is gently washed out of the sample and the gluten remaining is dried and weighed.

Apparatus

1. Porcelain dish or mortar with spatula or pestle
2. Bolting cloth (approx. 60 GG)
3. Oven

Procedure

Weigh 25 g flour into the dish or mortar, add sufficient tapwater (about 15 ml) to form firm ball of dough and work the dough with spatula or pestle, taking cakre that no material adheres to the utensil. Allow the dough to stand in water at room temperature for one hour.

Knead the dough gently in a stream of tapwater over the bolting clotth until starch and all soluble matter are removed. This operation requires approximatively 12 minutes. To determine whether or not the gluten is free or nearly free of starch, let one or two drops of wash-water obtained by squeezing the gluten, fall into a beaker of clear water. This will become cloudy if starch is till present. Allow the gluten thus obtained to stand in water one hour, press as dry as possible between the hands, roll into a ball, place in a tared flat-bottomed dish and weigh as moist gluten. Dry at 100°C to constant weight (24 hours), cool and weight. Express the weight as a percentage. Crude gluten thus obtained is not pure protein but contains liquids, ash and some starch.

2. Acidity in Flour

Principle

The acidity in an aqueous extract prepared under standard conditions is determined by titration and calculated as lactic acid.

Apparatus

1. Waterbath at 40°C.

Reagents

1. 0.1 M $NaOH$

Procedure

Weigh 18 g of flour into a 500 ml conical flask and add 200 ml of carbon dioxide free distilled water. Stand the flask in a waterbath at 40°C for one hour so that the flask is covered to just about the level of liquid. Swirl occasinally to ensure complete mixing. After 1 hour, filter and titrate 100 ml with 0.1 M $NaOH$.

Calculation

$$\text{Acidity, \% lactic acid} = \frac{\text{ml of 0.1 M NaOH}}{1000} \times 0.1 \times 90$$

$$\times \frac{100}{9} = \frac{\text{ml of 0.1 M NaOH}}{10}$$

90 = equivalent weight of catic acid.

3. Talc on rice or barley

Principle

The talc is floated of, filtered, digested and weighed.

Reagents

1. 10% ammonia solution
2. Hydrogen peroxide, 3% (10 volume)
3. Hydrochloric:cromic acid mixture. Carefully dissolve 10 g of chromic trioxide in 100 ml of water and add to 900 ml of contracted hydrochloric acid.

Procedure

Shake 2o g of sample with the dilute hydrogen peroxide solutions. Heat to about 6o°C so that the gas formed causes the particles of talc to come away from the surface. Decant off the liquid containing the talc, wash the grains several times with water and add these washings to the decanted liquid. Heat the liquor with the hydrochloric/chromic acid mixture to oxidize suspended meal, fillter off talc, wash, ignite and weigh.
In unpolished rice the residue does not normally exceed o.o25%.

Moisture in whole loaf of bread

Principle

The loaf is weighed, cut and air dried product is weighed and ground and the moisture determined on a small portion. The total moisture is calculated as a percentage of the whole loaf as received. The method is not applicable to bread containing fruit.

Procedure

Accurately weigh loaf of bread immediately upon receipt, using scales sensitive to at least o.2 g. It is impossible to weigh accurately at this time, seal sample in air-tight container and weigh as soon as possible.
Preserve sample in such a manner that no loss of bread solids can occur whereby loss should be calculated as moisture.
Cut bread into slices 2-3 mm thick. Spread slices on paper, let dry in warm room (15-2o hours) and when apparently dry, break into fragments. If bread is not entirely crisp and brittle, let it dry longer - until it is in equilibrrium with moisture of air - so that no moisture changes occur during grinding.

Weigh, grind and weigh again to check absence of grinding losses. Mix well and determine the moisture content on a small weighed portion (about 2 grams) by drying 1 hour at 130°C as the flour samples.

Calculation

% moisture in bread = % moisture in dry ground samples

$$\frac{\text{weight of air-dried slices}}{\text{weight of fresh loaf}}$$

Starch acid hydrolisis

Principle

Starch is converted by acid hydrolisis into reducing sugars which are determined by volume using Fehling's solution.

Apparatus

1. 250 ml flask, suitable for refluxing

Reagents

1. Hydrochloric acid 1.19 density
2. Potassium hydroxide solution about 1%
3. Decolourising charcoal
4. Fehling's solution
5. Methylene blue solution (1%)

Method

Into 250 ml flask place a sample containing about one gram of starch. Add 100 ml of distilled water and two ml of hydrochloric acid. Bring to the boil and reflux for three hours.
Transfer the contents of the flask and rinse into a 200 ml graduated flask. Cool and nearly neutralize with potassium hydroxide solution. Add distilled water to 200 ml and filter through a litter decolourising charcoal.

Then pour the solution into a graduated burette and reduce 10 ml of Fehling's solution by the following method:
- Into a flat-bottomed flask of about 250 ml pour 10 ml of Fehling's solution (5 ml of solution A and 5 ml of solution B). Shake until clear and add 40 ml of distilled water and small quantity of quartz or pumice.
Place the flask on a square asbestos plate with a round hole of about 6 mm diameter in the centre, the asbestos is resting on a piece of wire gauze. Heat the flask at such a rate that the liquid begins boiling after about two minutes.
From the burette, add to the boiling liquid successive quantities of the sugar solution until the blue colour of Fehling's solution becomes hardly discernible; then add 2 or 3 drops of methilene blue solution as indicator and complete the titration by adding further quantities of the sugar solution, drop by drop, until the blue colour of the indicator disappears.
For greater accuracy repeat the titration under the same conditions but adding without a break almost all the sugar solution required to reduce the Fehling's soltuion. In this second titration, the reduction of the Fehling's solution should occur within three minutes.

Edible oils and fats

Soap test in edible oils.

Principle

Detection of alcalinity using bromphenol as indicator.

Apparatus

15o x 15 mm test tube

Reagents

1. Solution of o.1% of brom phenol blue in 96% V/V ethanol
2. Acetone, analytical grade containing 2% V/V water.

A few drops of the solution of bromphenol blue should give a yellow to yellow-green colour to the 2% water in acetone.

Procedure

Place lo ml of the acetone and 1 drop of the bromphenol blue solution ina test tube. The solution should have a yellow colour.
(If not, rinse the test tube with acetone until the blue colour disappears). Add lo g of the oil to the test tube, stopper with a clean stopper, shake and allow to settle. The presence of blue colour in the upper acetone layer indicates the presence of soap.

Exoression of results

The results is expressed as positive or negative.
The test is applicable to any edible oil.

2. METHODS OF MICROBIOLOGICAL ANALYSIS OF FOOD AND WATER●

1. ENUMERATION OF MESOPHILIC AEROBIC BACTERIA (Aerobic Plate Count)

1.1. Reference

FAO EC/Microbiol/75/Report 1/Annex

1.2. Principle

This method is based on the assumption that the microbial cells present in a sample mixed with an agar medium each form visible, separated colonies. This is obtained by mixing decimal dilutions of the food sample homogenate with the medium. After incubation of the plates at 30°C for 72 hours the number of mesophilic aerobic bacteria per g of food sample is calculated from the number of colonies obtained in selected Petri dishes at levels of dilutions giving a significant result.

It should be borne in mind that this method, as all other methods, has some limitations. Microbial cells often occur as clumps, clusters, chains, or pairs in foods, and may not be well distributed irrespective of the mixing and dilution of the sample. Consequently, each colony that appears on the agar plate may arise from a single cell or from groups of cells and hence the colony count may not reflect the actual number of the viable bacteria in the food. Moreover, some microorganisms may fail to grow and form visible colonies on the agar medium as a result of unfavourable conditions of temperature, oxygen or nutrition, or because the cells are weak.

1.3. Apparatus and Glassware

a) Petri dishes 90-100 mm, glass or plastic

b) Pipettes 1, 5 and 10 ml, graduated (total-flow)

c) Water bath, 45 ± 1°C

d) Incubator, 30 ± 1°C

e) Colony counter

1.4. Culture media and diluent

a) Buffered peptone water (BPW) P.1.9

b) Plate count agar (PCA) P.1.50

1.5. Procedure

1.5.1. Preparation of food homogenate

Weigh 25 g of the mixed sample aseptically into a sterile blender jar or into a Stomacher bag and add 225 ml of BPW. Blend the food at a speed of 15000 - 20000 r p m for not more than 2.5 min or mix in the Stomacher for 20 sec.

1.5.2. Dilution

a) Mix the food homogenate by shaking and pipette 1.0 ml into a tube containing 9 ml of the BPW, mix carefully by aspirating 10 time with a pipette.

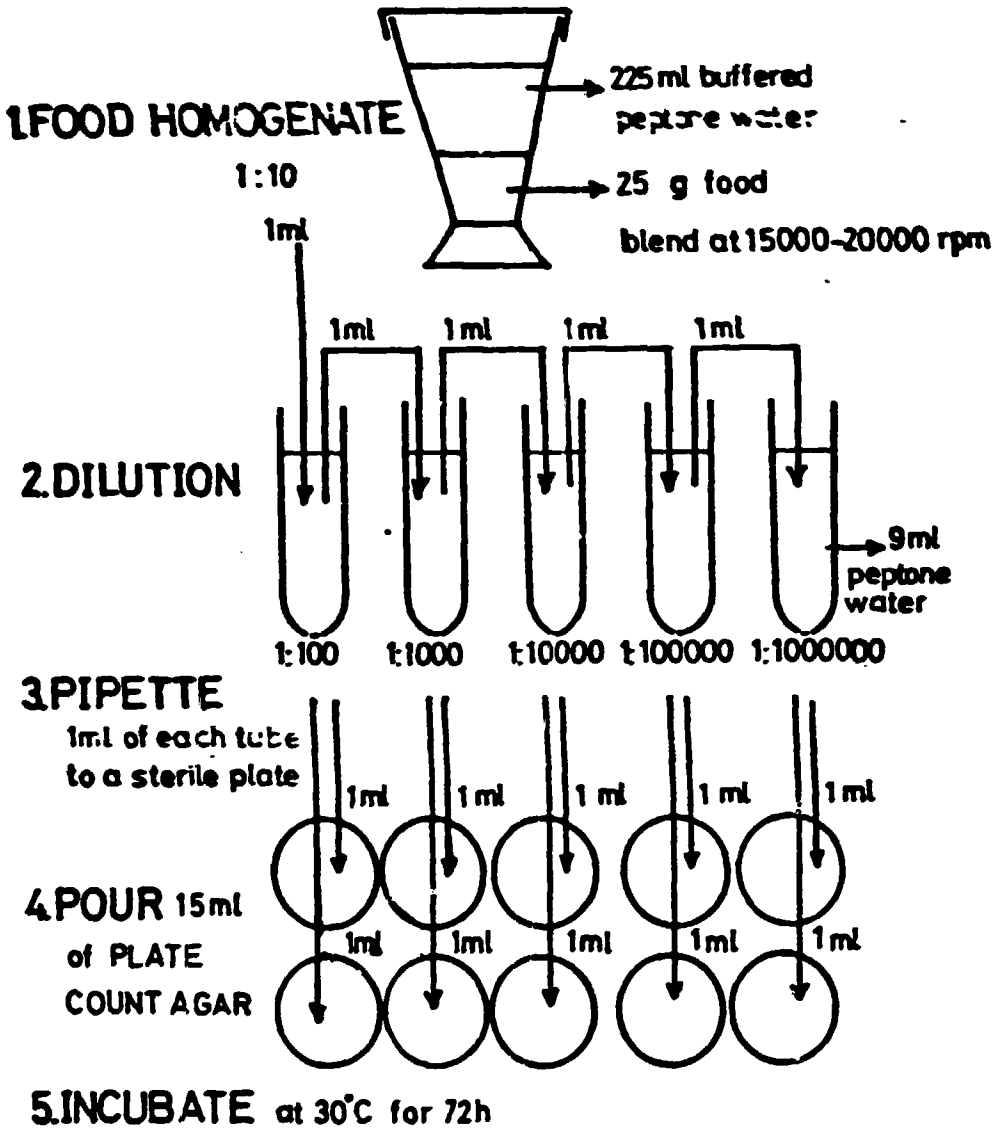
b) From the first dilution, transfer with the same pipette 1.0 to 2nd dilution tube containing 9 ml of the BPW, mix with a fresh pipette, and

c) Repeat using a 3rd, 4th tube or more until the required number of dilutions are made.

d) Shake all dilutions carefully.

● A schematic outline of the activities involved is given following each of the first 10 methods.

PL.1: AEROBIC PLATE COUNT(APC)



5.INCUBATE at 30°C for 72h

6.COUNT colonies on plates containing 30-300 colonies and multiply the number of the colonies by the dilution

REFAI

Fig. 6

1.5.3. Pour plating

a) Pipette 1.0 ml of the food homogenate and of each dilution of the homogenate into each of the appropriately marked duplicate dishes.

b) Pour into each Petri dish 15 ml of the PCA (kept at 45 ± 1°C in a water bath) within 15 min of the time of original dilution.

c) Mix the sample dilution and agar medium thoroughly and uniformly; allow to solidify.

1.5.4. Incubation

Incubate the prepared dishes, inverted, at 30 ± 1°C for 72 ± 3 h.

1.5.5. Counting the colonies

Following incubation, count all colonies on dishes containing 30 - 300 colonies and record the results per dilution counted.

1.5.6. Calculation

a) When the dishes examined contain no colonies, the result is expressed as : less than 1×10^1 bacteria per g or ml.

b) When the dishes (dilution 1 in 10) contain less than 30 colonies, the result is expressed as : less than 3×10^2 ($30 \times 10 = 3 \times 10^2$).

c) When the colonies are more than 30, count the colonies in both plates of a dilution and calculate the average, retaining only two significant digits and multiply by the inverse of the corresponding dilution to obtain the number of bacteria per g or ml.

Example : dilution 1/100 dish 1 : 175 colonies
dish 2 : 208 colonies
calculation : 175 + 208 = 383 ÷ 2 = 191 → 190 = 190 x 100
Results : 1.9×10^4 bacteria per g of food.

2. ENUMERATION OF COLIFORM BACTERIA
(Determination of the most probable number, MPN)

2.1. Reference

FAO EC/Microbiol/75/Report 1/Annex V. and Bacteriological Analytical Manual for Foods, 1976, 4th Ed. Food and Drug Administration, U.S.A.

2.2. Principle

This method is based on an MPN procedure using lauryl sulphate tryptose broth in a presumptive test, followed by confirmation of gas-positive tubes using Brilliant-Green lactose bile broth, each being incubated at 37°C for 24-48 hours. For testing for foecal coliforms EC (E. coli) broth is used, incubated at 44.5°C for 48 hours. For testing for E. coli the gas-positive tubes are streaked on L-EMB (P.1.18) medium and IMViC (D.2.5.9-e) tests are done.

2.3. Apparatus and Glassware

a) Test tubes (18 mm x 180 mm)

b) Durham tubes (10 mm x 75 mm)

c) Pipettes 1 ml (total-flow)
d) Incubators, 35 ± 1°C, 37 ± 1°C
e) Water bath, 45.5 ± 0.05°C

2.4. Culture media and reagent

a) Brilliant-Green lactose bile broth 2% (BGLB) F.1.7
b) Buffered peptone water F. 1.9
c) Indol medium and reagent F.1.25 and F.1.26
d) Koser's citrate F.1.29
e) Lauryl sulphate tryptose broth (LST) F.1.31
f) Levine's eosin methylene blue agar (L-EMB) F.1.18
g) Voges-Proskauer (VP) medium F. 1.77

2.5. Procedure

2.5.1. Preparation of food homogenate
Prepare as described under D 1.5.1

2.5.2. Dilution
Prepare as described under D.1.5.2 a) and b)

2.5.3. Inoculation
a) Inoculate each of 3 tubes of LST broth (containing inverted Durham tubes) with 1.0 ml of the food homogenate (1 in 10).

b) Carry out the same operation from the first (1 in 100) and the second (1 in 1000) dilution tubes, using a new sterile pipette for each dilution.

2.5.4. Incubation
Incubate the LST tubes at 37 ± 1°C for 24 and 48 h.

2.5.5. Reading of enrichment tubes (presumptive test)
Record tubes showing gas production after 24 h, and reincubate negative tubes for further 24 h, then record tubes showing gas production.

2.5.6. Confirmed test for coliforms
a) Transfer a loopful from each gas-positive tube of LST to a separate tube of BGLB broth.

b) Incubate the BGLB tubes at 37 ± 1°C for 48 h.

c) The formation of gas confirms the presence of coliform bacteria. Record the number of positive tubes that were confirmed as positive for coliforms.

2.5.7. Calculation (MPN)
Note the MPN appropriate to the number of positive tubes from the following table for example :
3 in 1 : 10, 1 in 1 : 100 and 0 in 1 : 1000
The table shows that MPN = 43 coliforms/g or ml.

MPN index and 95% confidence limits when 3 tubes are used

Number of Positive tubes			MPN per g or ml	95% confidence limits	
1:10	1:100	1:1000		Lower	Upper
0	0	0	<3		
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1,300
3	3	1	460	71	2,400
3	3	2	1,100	150	4,800
3	3	3	>2,400		

2.5.8. Test for faecal coliforms

a) Simultaneously with the confirmatory procedure using brilliant green lactose broth, transfer should be made from all positive presumptive tubes to EC medium.

b) The inoculated EC tubes are incubated at 45.5°C for 24 h, and gas formation is recorded. The bacterial density is estimated from the tables of MPN (see D.2.5.7).

c) For the differentiation of coliforms refer to the IMViC reactions (D.2.5.9-e).

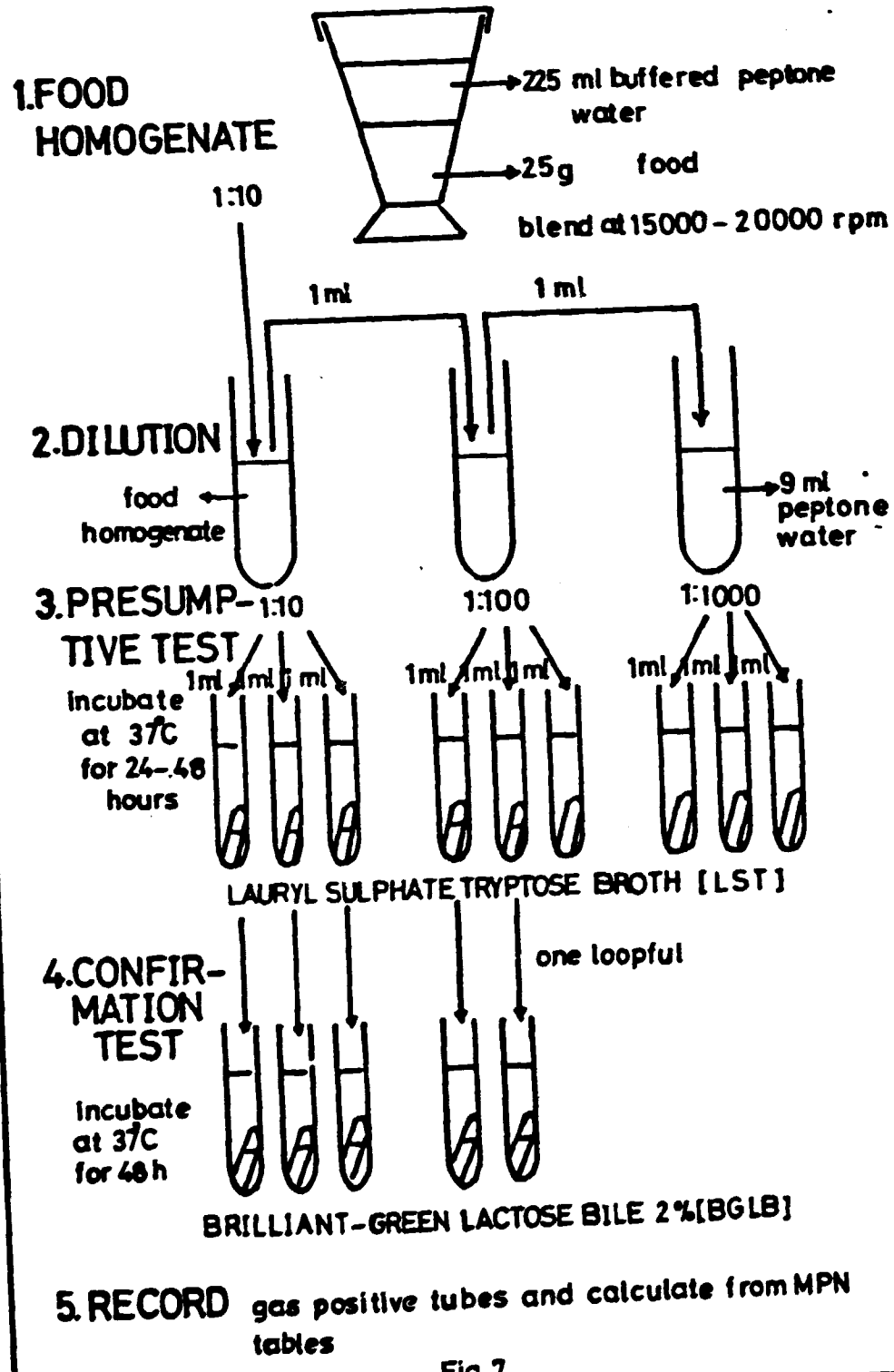
2.5.9. Test for Escherichia coli

a) Transfer a loopful from each gas-positive tube of LST to a separate tube of EC broth.

b) Incubate the EC tubes for 48 h at 44.5°C; production of gas is positive.

c) Streak one plate L-EMB agar from each positive tube in a way to obtain discrete colonies and incubate 18-24 h at 35°C.

PL.2: ENUMERATION OF COLIFORMS



Refai

Fig. 7

d) Transfer 2-3 of the suspected colonies from each L-EMB plate to PCA slants and incubate the slants for 18-24 h at 35°C. At the same time make Gram stains of each culture.

e) Perform indole, methyl red, VP and citrate tests (IMViC test). For indole and VP test see Salmonella (D.4.5.4. 1-b V and VI).

For MR test: inoculate a tube of VP medium and incubate for 48 h at 35°C; add 5 drops of methyl red indicator to each tube. MR + = red colour.

For Citrate utilization inoculate a tube of Koser's citrate medium and incubate 96 h at 35°C and examine for growth.

Classification of Coliforms by IMViC test

Indole	MR	VP	Citrate	Type
+	+	-	-	Typical E. coli
-	+	-	-	Atypical E. coli
+	+	-	+	Typical intermediate
-	+	-	+	Atypical intermediate
-	-	+	+	Typical E. aerogenes
+	-	+	+	Atypical E. aerogenes

Compute MPN of E. coli per g or ml considering as E. coli the Gram - negative, non-spore forming rods producing gas in lactose and producing ++ — or -+— IMViC pattern.

3. ENUMERATION OF FAECAL STREPTOCOCCI

3.1. Reference

Thatcher, F.S. and Clark, D.S. ed. (1968) : Microorganisms in foods : Their significance and methods of enumeration. Toronto, University of Toronto Press.

3.2. Principle

This method is based on presumptive enumeration of faecal streptococci (Lancefield group D streptococci) using Packer's Crystal-Violet Azide Blood Agar and the pour plate technique, followed by confirmation and identification of the suspected colonies.

3.3. Apparatus and Glassware

a) Petri dishes
b) Pipettes
c) Water baths, 45°C, 60°C
d) Incubator, 35 - 37°C
e) Colony counter

3.4. Culture media and reagents

a) Buffered peptone water P.1.9
b) Packer's crystal-violet azide blood agar P.1.47
c) Phenol-red sorbitol broth P.1.49
d) Thallous acetate tetrazolium glucose agar P.1.63
e) Tryptose broth, pH 9.6, and pH 7.2 P.1.68
f) Tryptose agar P.1.69
g) Tryptose bile broth 40% P.1.70
h) Tryptose salt broth P.1.72
i) Tryptose tellurite agar P.1.73
j) Tryptose TTC agar P.1.74

3.5. Procedure

3.5.1. Preparation of food homogenate
Prepare as described under D.1.5.1

3.5.2. Dilution
Prepare as described under D.1.5.2

3.5.3. Inoculation
Pipette 1.0 ml of the food homogenate and of each dilution of the homogenate to each of the appropriately marked duplicate dishes. Promptly add to each dish 15 ml of the Packer's crystal-violet azide blood agar, melted and tempered to 45°C. Mix and leave to solidify.

3.5.4. Incubation
Incubate the plates inverted at 35 - 37°C for 72 h.

3.5.5. Counting the colonies
Count all the small violet-coloured colonies on plates containing 30-300 colonies and compute the number of presumptive faecal streptococci per g of food specimens.

3.5.6. Confirmation of faecal streptococci

a) Subculture 3-5 of the violet-coloured colonies on separate dried plates of thallous acetate tetrazolium glucose agar.

b) Incubate plates inverted at 35-37°C for 48 h. Colonies with red centres are probably Streptococcus faecalis; white colonies are likely to be Streptococcus faecium; intensely red colonies are probably Streptococcus lactis.

c) Transfer two white and two red-centred colonies to separate slants of tryptose agar, incubate at 35-37°C for 24 h.

d) Prepare smear and stain with Gram.

e) From the slants (24 h old) inoculate the following :

i) slopes of tryptose agar; incubate at 45°C for 48 h (growth indicates a positive test).

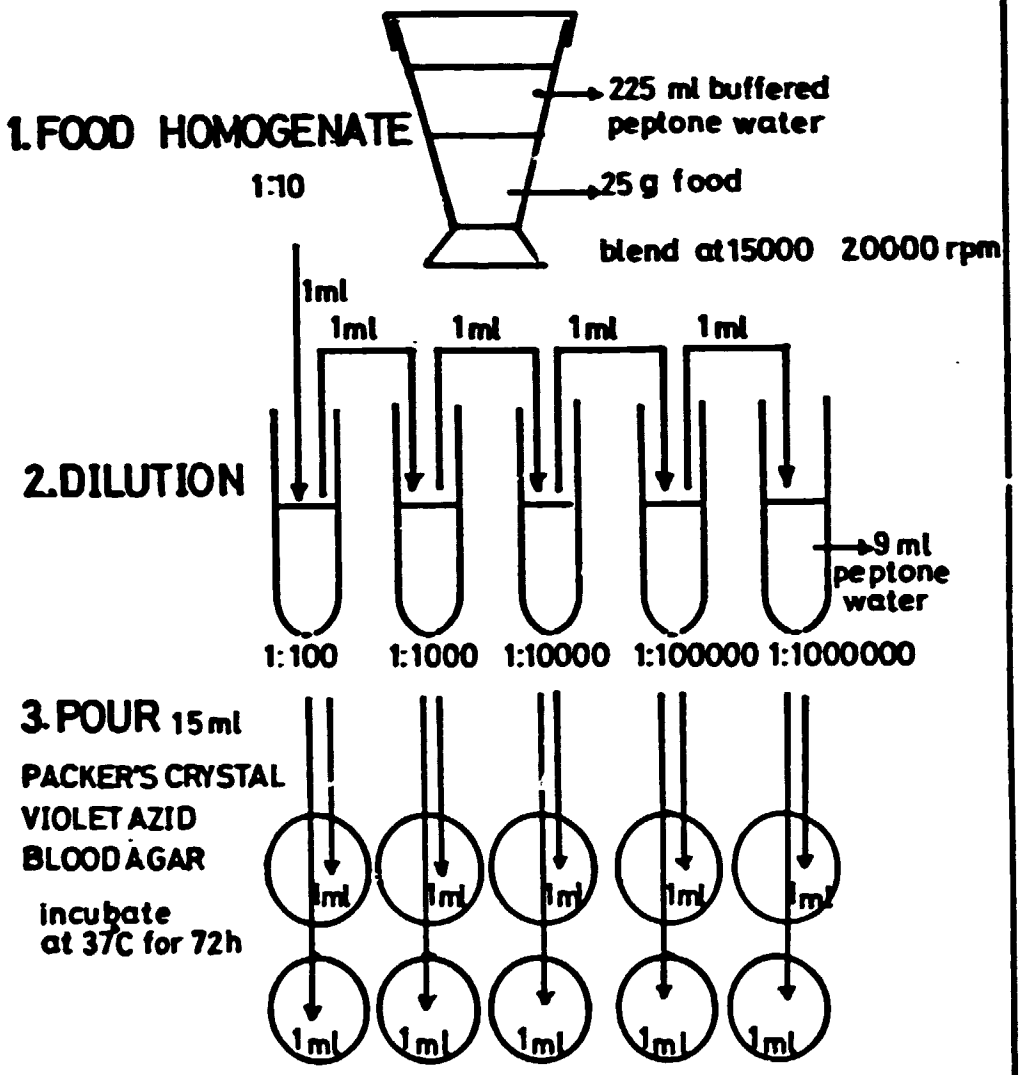
ii) tubes of tryptose bile broth 40%; incubate at 35-37°C for 48 h (Turbidity represents a positive test).

iii) Slants of trytose agar; incubate at 35-37°C for 48 h, pipette hydrogen peroxide solution on the growth. The evolution of bubbles indicates a positive catalase test. Note that, cultures on bile broth 40% and those grown at 45°C are negative for catalase.

3.5.7. Identification of species

a) Inoculate two tubes of the following media with 24-hour-old cultures of the streptococci and incubate at 35-37°C for 48 h.

PL.3: ENUMERATION OF FAEC. STREPT.



4. COUNT all small colonies with violet colour

5. CONFIRMATION

a. grow at 45°C
b. " " pH 9.6
c. " in the presence of 40% bile
d. " in 6.5% NaCl containing medium
e. resist heating at 60°C for 30 min
f. " tellurite, sorbitol, TTC

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Fig. 8

i) tryptose broth, pH 9.6
ii) tryptose salt broth
iii) tryptose tellurite agar slants
iv) tryptose TTC agar slants
v) phenol-red sorbitol broth

Growth in the first 4 media represents positive reaction, yellow colour in the last medium indicates fermentation and positive reaction.

b) Inoculate two tubes of tryptose broth, pH 7.2, preheated to 60°C and hold them at 60°C for 30 min in a water bath. Cool tubes and incubate them for 48 h at 35-37°C. Growth is a positive test.

c) Classify according to the following table :

Identification of Lancefield group D Streptococci

Test	Strept. faecalis	Strept. faecium	Strept. durans	Strept. bovis	Strept. equinus
45°C	+	+	+	+	+
40% bile	+	+	+	+	+
Catalase	-	-	-	-	-
pH 9.6	+	+	+	-	-
6.5% NaCl	+	+	+	-	-
60°C for 30 min	+	+	+	-	-
0.05% tellurite	+	-	-	-	-
Sorbitol	+	±	-	-	-
TTC agar	+	-	±	±	-

4. DETECTION OF SALMONELLA

4.1. Reference

FAO EC/Microbiol/75/Report I/Annex V

4.2. Principle

Salmonella when present are usually found in low numbers in foods and often in the presence of considerably larger numbers of other members of Enterobacteriaceae. In foods which have been heated, refrigerated, frozen or dried, viable Salmonella bacteria may be present.

This method is based therefore on giving the chance for the few numbers of normal or of stressed Salmonella bacteria to grow first in a non-selective liquid medium at 37°C (pre-enrichment). Such a medium and a temperature will allow other bacteria to grow as well, therefore this step is followed by subculturing the pre-enrichment medium into a liquid selective medium and incubating it at 42 to 43°C. The latter is inoculated into a solid selective and differential medium, and after incubation at 37°C the plates are examined for the presence of colonies which by their characteristics are considered presumptive Salmonella. These colonies are then examined for the biochemical and serological characteristics of Salmonella species.

4.3. Apparatus and Glassware

a) Test tubes (18 x 180 mm) and bottles (500 and 1000 ml capacity)
b) Test tubes (6 mm x 160 mm) for lysine decarboxylation medium
c) Measuring cylinder (100 ml)
d) Pipettes, 1 ml and 10 ml
e) Petri dishes, small size (90 - 100 mm in diam) and large size(140 - 150 mm in diam)
f) Incubators, 37°C and 42-43°C
g) Drying cabinet, 50 ± 5°C
h) Water bath 50°C

4.4. Culture media and reagents

a) Bismuth sulphite agar F.1.4
b) Brilliant-green/phenol red agar F.1.8
c) Buffered peptone water F.1.9
d) β-galactosidase reagent F.1.22
e) Indole medium and reagent F.1.25, F.1.26
f) Lysine decarboxylation medium (LDC) F.1.33
g) Nutrient-agar F.1.44
h) Saline solution F.1.54
i) Selenite cystine broth F.1.56
j) Semi-solid nutrient agar F.1.57
k) Tetrathionate medium F.1.62
l) Triple sugar/iron agar (TSI agar) F.1.65
m) Urea agar F.1.75
n) VP medium F.1.77

4.5. Procedure

4.5.1. Preparation of food homogenate

Prepare as described under D.1.5.1

4.5.2. Pre-enrichment

a) Transfer the food homogenate (25 g sample blended with 225 ml BPW) aseptically to a sterile 500 ml bottle.

b) Incubate at 37 ± 1°C for 16 - 20 h.

4.5.3. Enrichment

a) Transfer 10 ml of each pre-enrichment bottle to 100 ml tetrathionate medium and another 10 ml to 100 ml of selenite medium, previously warmed to 42-43°C.

b) Incubate at 42-43°C for 48 h.

4.5.4. Plating out

a) After 18-24 h, streak from each enrichment flask one large petri dish or 2 small ones of each of Brilliant green/phenol red agar and bismuth sulphite agar.

b) Incubate at 37 ± 1°C for 20-24 h.

c) After another 24 h repeat a) and b).

d) Examine the plates after 24 and 48 h for typical colonies of Salmonella.

4.5.5. Confirmation

4.5.5.1. Biochemical confirmation

a) Select 5 typical or suspected colonies from each and streak them on nutrient agar plates. Incubate for 20-24 h at 37°C.

b) From isolated colonies on nutrient agar plates inoculate the following media :

i) TSI agar : Streak the agar slope surface and stab the butt. Incubate at 37°C for 24-48 h. Interpret the changes in the medium as follows

Butt	
Yellow	glucose fermented
Red or unchanged	glucose not fermented
Black	hydrogen sulphide formed
Bubbles or cracks	gas formed from glucose
Slant surface	
Yellow	lactose and/or sucrose fermented
Red or unchanged	lactose and/or sucrose not fermented

ii) Urea agar : Streak the agar slop surface. Incubate at 37°C for 24-48 h. Rose-pink colour indicates positive reaction.

iii) Lysine decarboxylation medium : Inoculate just below the surface of the liquid medium. Incubate at 37°C for 24 h. A purple colour after growth indicates a positive reaction.

iv) β-galactosidase reagent : Suspend a loopful of the colony in 0.25 ml of the saline solution in a tube. Add 1 drop of toluene. Put the tube in a water bath at 37°C for several min. Add 0.25 ml of β-galactosidase reagent and mix. Put the tube again in the water bath at 37°C for 24 h. A yellow colour indicates a positive reaction.

v) VP medium : Suspend a loopful of the colony in each of two tubes containing 0.2 ml of the medium. Incubate one tube at room temperature and the other at 37°C for 45 h. Add to each tube 2 drops of the creatine solution, 3 drops of the ethanolic naphthol solution and 2 drops of the KOH reagent. Shake after the addition of each reagent and read the reaction within 15 min. A pink to bright red colour indicates a positive reaction.

vi) Indole medium : Inoculate a tube with the colony and incubate at 37°C for 24 h. Add 1 ml of the indole reagent. The forming of a red ring indicates a positive reaction.

c) Typical Salmonella Colonies

i) Brilliant-green agar

Colonies are colourless, pink to fuchsia, translucent to opaque with surrounding medium pink to red. Some Salmonella appear as transparent green colonies if surrounded by organisms fermenting lactose or sucrose, since these carbohydrate-fermenting organisms produce colonies and zones that are yellow green or green; less than 1% of the Salmonella are atypical in that they ferment lactose and appear as yellow-green or green colonies.

ii) Bismuth sulphite agar

Colonies are brown, black, sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time. Some strains produce green colonies with little or no darkening of the surrounding medium.

PL.4: DETECTION OF SALMONELLA

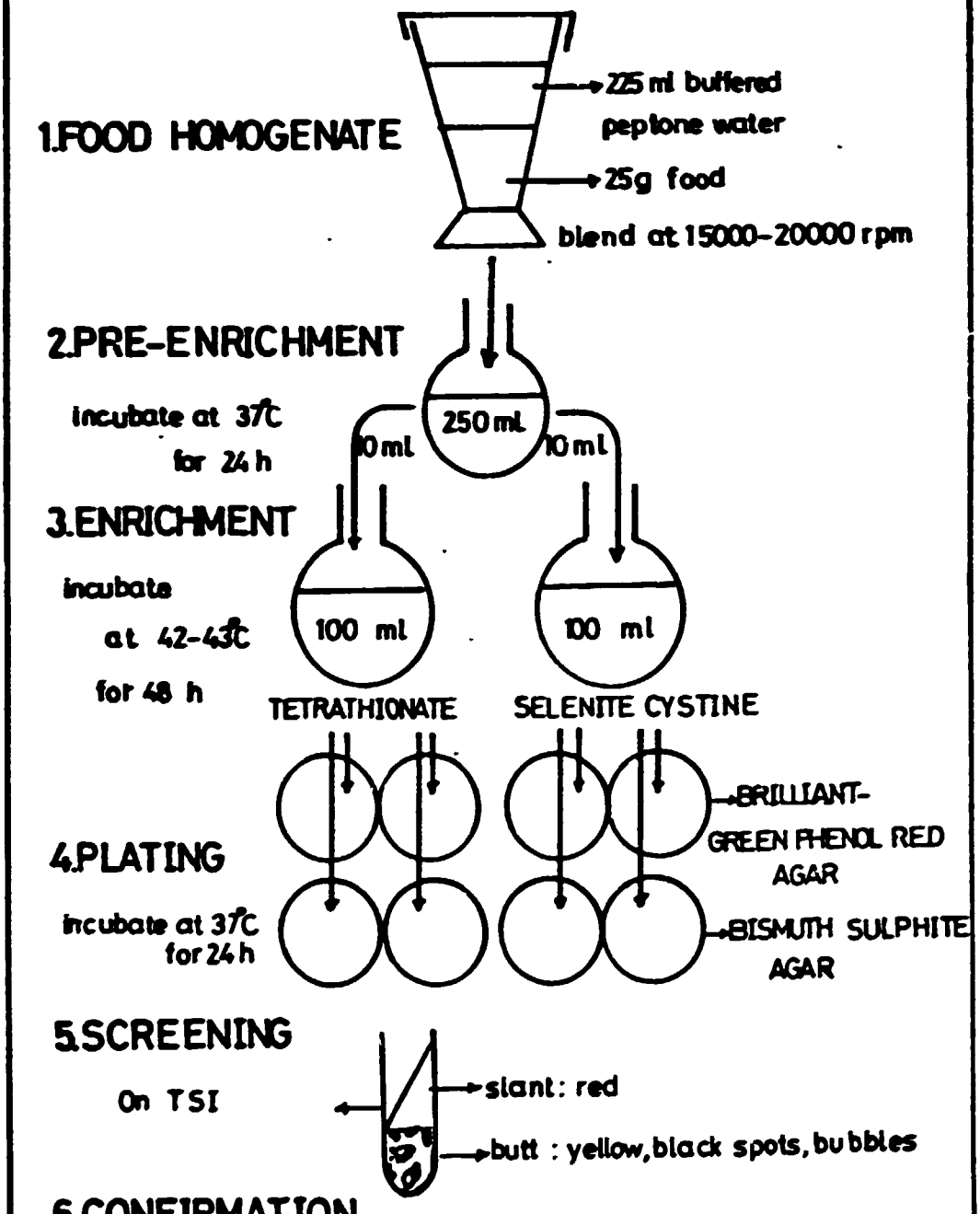

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Fig. 9

d) Biochemical reactions of Salmonella

i)	TSI agar	
	butt : yellow	+ (100%)
	black	+ (91.6%)
	bubbles or cracks	+ (91.9%)
	slant : red or unchanged	- (99.2%)
ii)	Urea agar no change of colour	- (100%)
iii)	Lysine decarboxylase purple colour	+ (94.6%)
iv)	β-galactosidase reaction no change of colour	- (98.5%)
v)	VP reaction no change of colour	- (100%)
vi)	Indole test; a yellow brown test	- (98.9%)

4.5.5.2. Serological confirmation

Examine pure non-auto-agglutinable colonies for the presence of O and H antigens by slide agglutination with poly- and monovalent sera. For the determination of H antigens, inoculate a plate of semi-solid nutrient agar and examine with H sera after incubation at 37 ± 1°C for 18-24 h.

5. ENUMERATION OF SHIGELLA

5.1. Reference

Thatcher, F.S. and Clark, D.S. ed. (1968) Microorganisms in foods. Their significance and methods of enumeration. Toronto, University of Toronto Press; and Compendium of Methods for the Microbiological Examination of Foods, 1976. APHA.

5.2. Principle

This method is based on the use of XLD medium (P.1.78). It contains xylose as a differentiating agent and since most Shigella do not ferment xylose, they appear as alkaline (red) colonies on the plates (presumptive Shigella). For confirmation all biochemical tests used for Salmonella (D.4.5.4.1) are to be done and those giving typical characteristics (D.5.5.6.1) are examined serologically. For further details see Edwards, P.R., and W.H. Ewing 1972. Identification of the Enterobacteriaceae. 3rd Ed. Burgess Publ. Co., Minneapolis, Minn.

5.3. Apparatus and Glassware

a) Petri dishes
b) Pipettes
c) Water bath, 45°C
d) Incubator, 37°C
e) Drying cabinet
f) Glass spreaders

5.4. Culture media and reagents

a) Buffered peptone water P.1.9
b) Carbohydrate media P.1.10
c) Indole medium P.1.25, P.1.26
d) Citrate medium P.1.29
e) Motility test medium P.1.40
f) Potassium cyanide (KCN) medium P.1.52
g) TSI P.1.65
h) Urea agar P.1.75
i) VP medium P.1.77
j) Xylose lysine decarboxylase agar (XLD) P.1.78
K) Shigella antisera

5.5. Procedure

5.5.1. Preparation of food homogenate
Prepare as described under D.1.5.1

5.5.2. Dilution
Prepare as described under D.1.5.2

5.5.3. Inoculation
Pipette 0.25 ml of food homogenate and dilutions of homogenate onto the surfaces of dried plates of XLD agar and spread with a sterile glass rod.

5.5.4. Incubation
Incubate at 37°C for 24 h.

5.5.5. Counting the colonies (presumptive Shigella)
Select all plates with 30-300 colonies and count colonies that appear uniformly red. These are presumptive Shigella.

5.5.6. Confirmation

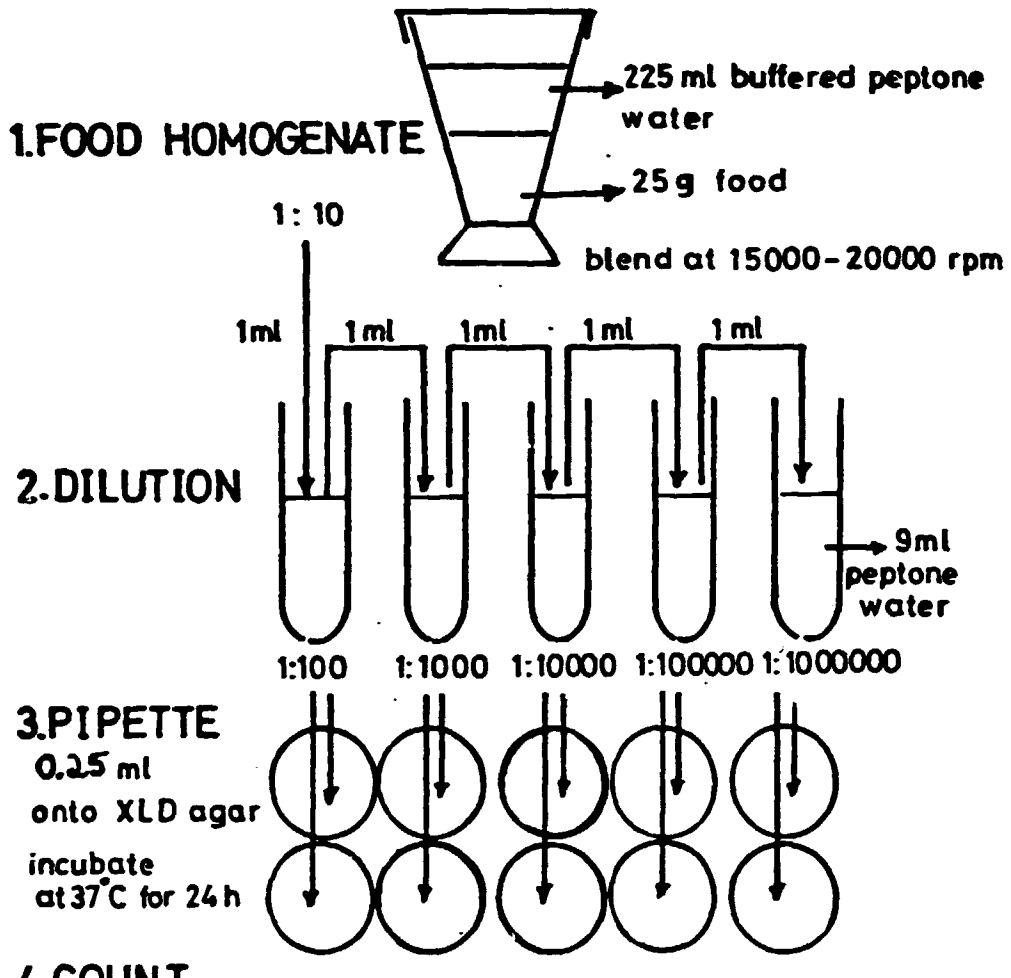
5.5.6.1. Biochemical confirmation
Proceed as in Salmonella with regard to the following tests :

a) TSI : red slant, yellow butt, no gas, no H_2S
b) Urease : negative (no red colour)
c) Motility : non-motile
d) KCN : no growth
e) Indole : positive or negative
f) VP : negative
g) Carbohydrates : no gas
h) Citrate medium: no growth

5.5.6.2. Serological identification
Examine pure non-auto-agglutinable colonies from a nutrient agar or TSI agar slant for agglutination with poly- and monovalent Shigella sera.

Cultures that appear to be Shigella on the base of biochemical reactions, but which agglutinate poorly or not at all, should be heat treated by boiling a suspension of the organisms for 15 - 30 min. After such treatment, the suspension is cooled and retested for agglutination.

PL.5: ENUMERATION OF SHIGELLA



4. COUNT colonies that appear uniformly red

5. CONFIRMATION:

a. TSI : red slant, yellow butt, no gas, no H_2S
b. UREASE : negative
c. KCN : no growth
d. CITRATE : no growth
e. CARBOHYDRATE: no gas
f. INDOL: positive or negative
g. VP : negative
h. MOTILITY : non-motile
i. SEROLOGICAL IDENTIFICATION

Refai

Fig.10

6. DETECTION OF ENTEROPATHOGENIC ESCHERICHIA COLI (EPEC)

6.1. Reference

Compendium of Methods for the Microbiological Examination of Foods, 1976, APHA.

6.2. Principle

This method is based on pre-enrichment in nutrient and MacConkey broth, enrichment in LST and EE broth and streaking on L-EMB agar. The suspected colonies are then examined for biochemical and serological characteristics of EPEC.

6.3. Apparatus and glassware

a) Water baths 44°C and 41.5°C
b) Incubator, 35°C
c) Blender
d) Test tubes, pipettes, Petri dishes

6.4. Culture media and reagents

a) Carbohydrate fermentation media P.1.10
b) Levine's eosin methylene blue agar P.1.18
c) Enteric enrichment (EE) broth P.1.19
d) Indole media and reagent P.1.25, P.1.26
e) Lauryl sulphate tryptose broth (LST) P.1.31
f) MacConkey agar P.1.34
g) MacConkey broth P.1.35
h) Nitrate broth P.1.43
i) Nutrient broth P.1.45
j) KCN medium P.1.52
k) TSI agar P.1.65
l) Urea broth P.1.75 without agar
m) VP medium P.1.77
n) *E. coli* antisera

6.5. Procedure

6.5.1. Sample preparation

Weigh two 25 g portions aseptically into 225 ml MacConkey broth and 225 ml nutrient broth in sterile blender jars and homogenize 30 sec (1:10).

6.5.2. Direct streak

Streak nutrient broth homogenate on L-EMB and MacConkey agars. Incubate at 35°C for 24 h.

6.5.3. Enrichment

Incubate MacConkey broth at 35°C for 20 h. Transfer one loop to 30 ml LST broth. Incubate at 44°C for 20 h. Incubate nutrient broth at 35°C for 6 h. Transfer one loop to 30 ml EE broth. Incubate at 41.5°C for 18 h.

6.5.4. Preliminary serological examination

Neutralize LST and EE enrichment broths with 10% $NaHCO_3$. Place one drop of each broth on a clean slide and add one drop of polyvalent OB sera and one drop of 0.5% saline. Mix drops and examine for agglutination.

6.5.5. Biochemical confirmation

Streak positive LST enrichment broth on L-EMB agar and EE enrichment broth on MacConkey and L-EMB agars. Incubate at 35°C for 24 h.

PL.6: DETECTION OF ESCH. COLI

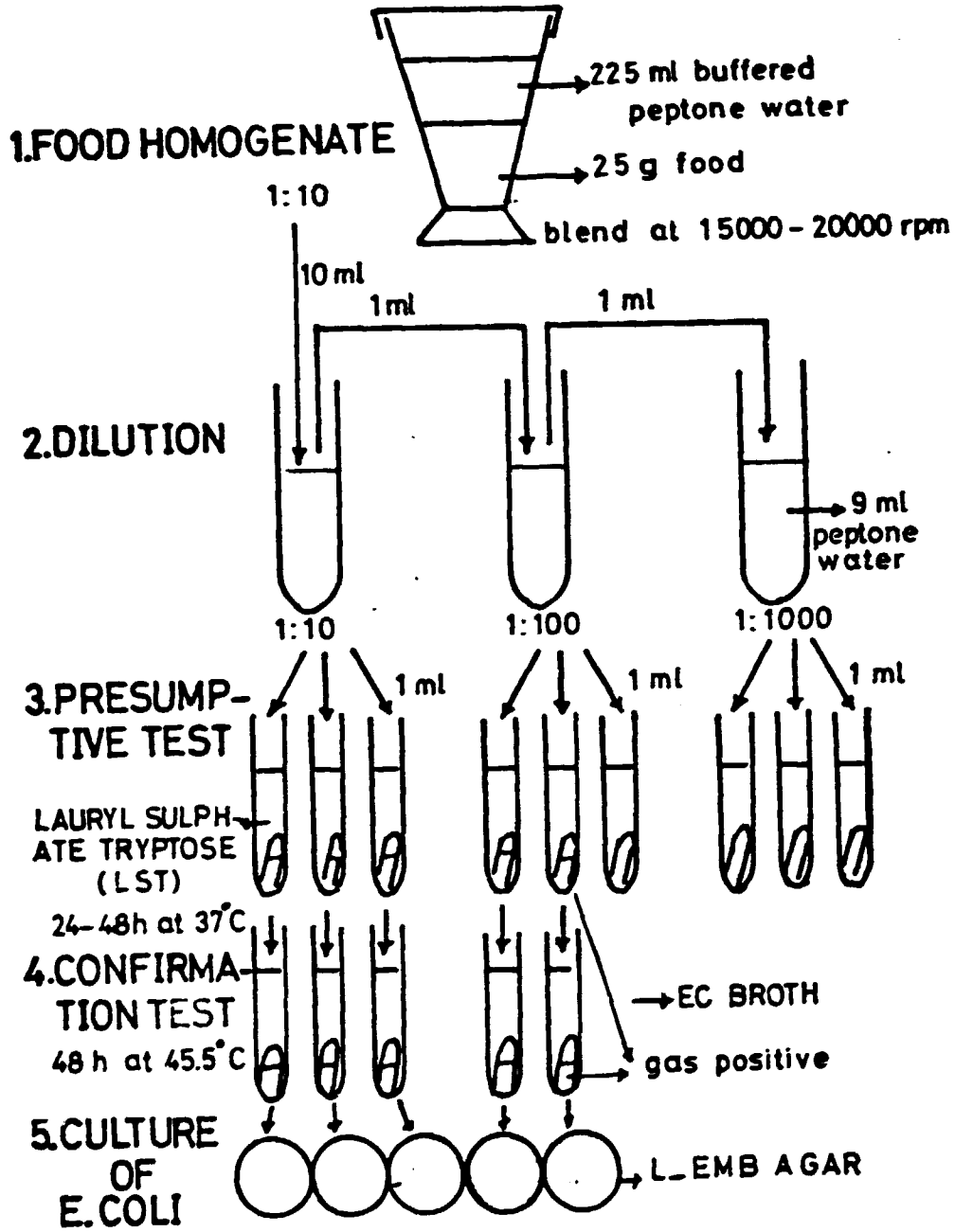


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Fig.11

6.5.5.1. Select characteristic colonies and inoculate TSI, VP, indole, urease, KCN, citrate and adonitol media. In addition, slants of plate count agar are inoculated with the same colonies to be used for serological examination.

6.5.5.2. Biochemical characteristics of E. coli

TSI	+ (acid) + (H_2S)
V.P.	-
Indole	+
Urease	-
KCN	-
Adonitol	-
Cytochrome oxidase	-

6.5.5.3. Serological identification of Enteropathogenic E. coli

a) Suspend growth from PCA in saline and further examine only cultures giving a homogenous suspension.

b) Test the suspension in polyvalent OK sera using drops of suspension, anti-serum and saline.

c) If negative in polyvalent sera, heat the suspension at 100°C for 15 min and re-examine in polyvalent sera.

d) If positive, examine in monovalent OK sera of positive polyvalent groups.

e) Confirm the identification by tube agglutination using serially diluted serum.

7. ENUMERATION OF STAPHYLOCOCCUS AUREUS

7.1. Reference

FAO EC/Microbiol/77/Report 2/Annex III

7.2. Principle

This method is based on the spreading of 0.25 ml of the food homogenate and of the subsequent decimal dilutions on the surface of Baird-Parker Agar. This medium contains various inhibitory substances which do not interfere with the growth of S. aureus. The ability of S. aureus to reduce the potassium tellurite and to hydrolyze the egg yolk present in the medium result in the appearance of black colonies surrounded by a clear zone characteristic for S. aureus. The confirmatory procedure used in this method to establish the identity of S. aureus is the coagulase test. Coagulase is a substance produced by S. aureus which clots the plasma of humans and animals.

There are many factors affecting the usefulness and reliability of S. aureus detection and enumeration procedures. Among the important factors are the physiologic state of the organism, competitive position of S. aureus in the sample menstrum and limitations of the medium used for isolation. It is to be noted that the physiology of S. aureus is diverse; for example, not all strains have the capacity to hydrolyze egg yolk or produce coagulase. Considerable divergence also has been demonstrated in the response of various strains to the toxic chemicals used in the isolation media.

7.3. Apparatus and Glassware

a) Petri dishes 10 cm
b) Pipettes 1 ml
c) Water bath 45°C
d) Incubator 37°C
e) Colony counter
f) A drying cabinet

7.4. Culture media and reagents

a) Baird-Parker agar P.1.3
b) Brain heart infusion broth P.1.6
c) Buffered peptone water P.1.9
d) Rabbit plasma (dehydrated, containing 0.1% EDTA)

7.5. Procedure

7.5.1. Preparation of the food homogenate

Prepare as described under D.1.5.1

7.5.2. Dilution

Prepare as described under D.1.5.2

7.5.3. Inoculation

Pipette 0.25 ml of homogenate and dilutions of homogenate onto the surface of previously dried Baird-Parker agar plates and spread with a sterile bent glass rod. Duplicate plates should be prepared from each dilution.

7.5.4. Incubation

Incubate plates inverted at 37°C for 24 and 48 h.

7.5.5. Counting of the colonies (presumptive S. aureus)

a) After 24 h select plates with 30-300 separate colonies which are black and shiny, with narrow white margins and surrounded by clear zones extending into the opaque medium. These are presumptive colonies of Staphylococcus aureus.

b) Mark the position of these colonies and re-incubate the plates for a further 24 hours.

c) Count all colonies with the above appearance that developed during the extended period of incubation and submit these, or a significant number of them to the coagulase test.

d) Colonies of a few strains of S. aureus may be surrounded by an opaque zone at 24 hours, and a larger number of strains may show this appearance after 48 h. On the other hand, coagulase negative staphylococci may show clearing after 48 h. Therefore, coagulase test should be made on suspect colonies.

e) Total the colonies which produced clear zones after 24 h of incubation and those appearing after 48 h and were proved to be coagulase positive.

7.5.6. Confirmation

7.5.6.1. Testing for coagulase production

a) Transfer suspect S. aureus colonies into test tubes containing 5 ml of brain heart infusion broth and incubate 20-24 h at 35-37°C.

b) Add 0.1 ml of resulting growth to 0.3 ml of rehydrated rabbit plasma in small tubes and incubate at 35-37°C.

PL.7: ENUMERATION OF STAPH. AUREUS

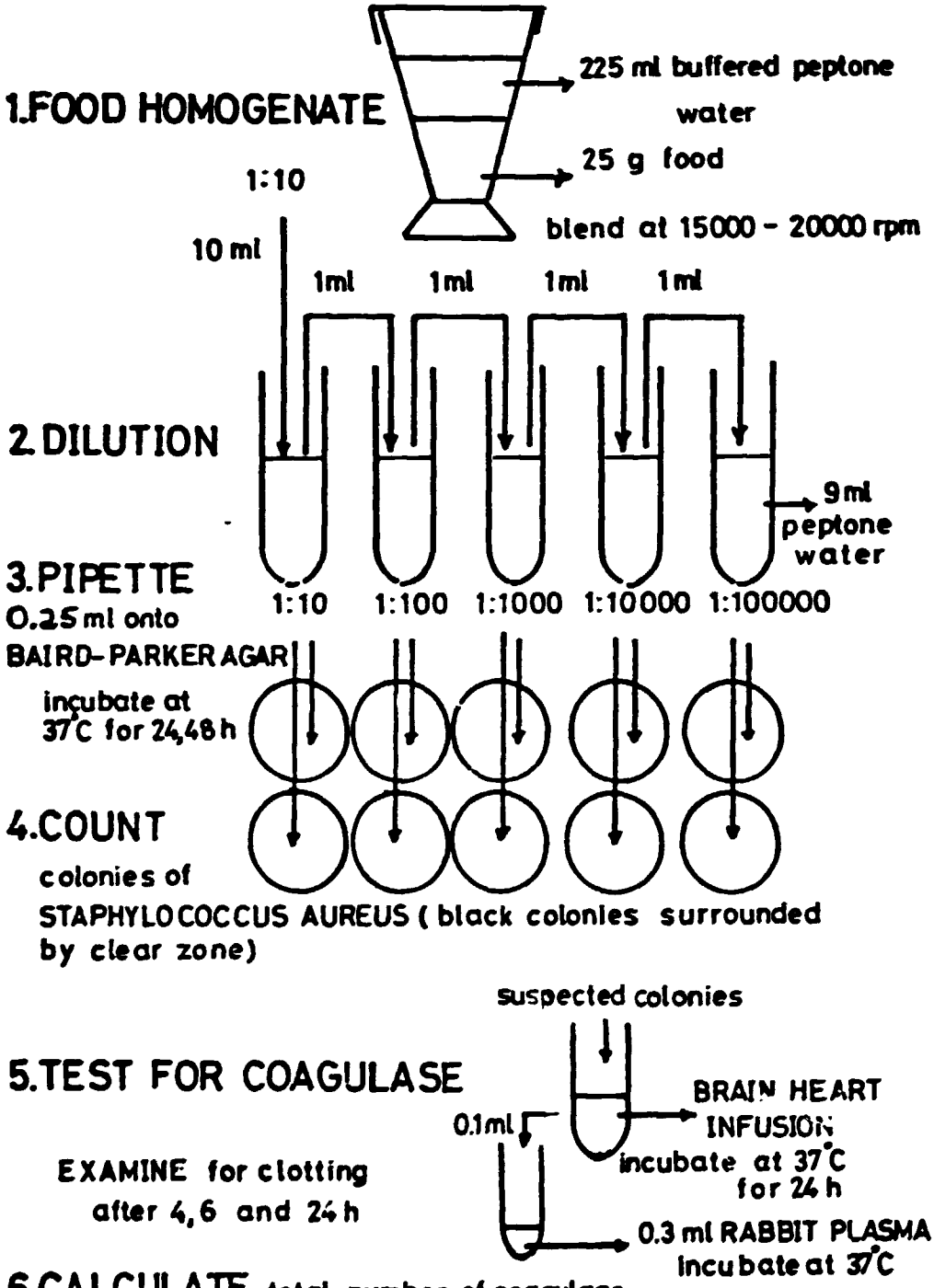

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Fig.12

c) Examine tube for clotting after 6 h. The formation of a distinct clot is evidence of coagulase activity (3+), a 4+ reaction is obtained when the entire content of the tube coagulates and is not displaced when the tube is inverted. A 3+ or 4+ reaction is considered as positive identification of Staphylococcus aureus.

7.5.7. Calculation of the colony count

The number of Staphylococcus aureus should be calculated from the percentage of confirmed colonies in relation to the total number of suspected colonies (D.7.5.5.e); this is then multiplied by 4 (0.25 ml spread) and by the dilution factor.

8. ENUMERATION OF VIBRIO PARAHAEMOLYTICUS

8.1. Reference

Bacteriological Analytical Manual for Foods. 1976. 4th ed., Food and Drug Administration, U.S.A.

8.2. Principle

This method is based on the incorporation of 3% NaCl in all media used for isolation and identification of V. parahaemolyticus.

8.3. Apparatus and Glassware

a) Test tubes
b) Measuring cylinders
c) Pipettes
d) Petri dishes
e) Incubator
f) Water bath

8.4. Culture media and reagents

a) Alkaline peptone water P.1.1
b) Carbohydrate media P.1.11
c) Arginine hydrolase broth P.1.14
d) Lysine decarboxylase broth P.1.14
e) Glucose salt teepol broth (GSTB) P.1.23
f) Hugh-Liefsen glucose broth (HLGB) P.1.24
g) Indole medium and reagent P.1.25, P.1.26
h) Motility test medium P.1.40
i) Nutrient gelatine P.1.46
j) Salt-trypticase broth (STB) P.1.55
k) Sodium chloride, 3% P.1.58
l) Thiosulfate-citrate-bile salt-sucrose agar (TCBS) P.1.64
m) TSI agar P.1.65
n) Trypticase soy agar (TSA), with 3% NaCl P.1.66
o) Trypticase soy broth (TSB), with 3% NaCl P.1.67
p) VP medium P.1.77
q) Paraffin oil
r) Gram stain P.2.10

8.5. Procedure

8.5.1. Preparation of food homogenate
Prepare as described under D.1.5.1

8.5.2. Dilution
Prepare as described under D.1.5.2

8.5.3. Inoculation
Inoculate three 10 ml portions of 1:10 dilutions into 10 ml each of double strength GSTB, and then inoculate three 1 ml portions of 1:10, 1:100, 1:1000 and 1:10000 dilutions into single strength GSTB.

8.5.4. Incubation
Incubate broth tubes overnight at 35°C.

8.5.5. Confirmation

8.5.5.1. a) After incubation, streak a loopful of the culture from the three highest dilutions of GSTB showing growth onto TCBS agar plates.

b) Incubate the plates for 18 h at 35°C.

c) The colonies of V. parahaemolyticus on TCBS appear round, 2-3 mm in diameter with green or blue centres. V. alginolyticus colonies are larger and yellow. Coliforms, Proteus and enterococci colonies are small and translucent.

8.5.5.2. Biochemical identification

a) TSI : Streak the slant and stab the butt and incubate overnight at 35°C. V. parahaemolyticus produces alkaline slant and acid butt, no gas and no H_2S (typical Shigella-like reaction).

b) Motility medium : Inoculate 4 tubes by stabbing. Diffuse circular growth occurs after 24 h incubation at 35°C.

c) Make a Gram stain from growth on TSA slant.

d) Halophilic nature : Inoculate 4 tubes of STB containing 0, 6, 8 and 10% NaCl, incubate; V. parahaemolyticus will grow well in 6 and 8% NaCl but not in 0 and 10% concentrations.

e) MR-VP test : refer to D.2.5.9-e, and 4.5.4.1-b

f) Indole test : Refer to D.4.5.4.1-b.

g) Carbohydrate fermentation : Inoculate one tube each of glucose, lactose, sucrose, maltose, mannitol, etc. from TSA slant. After incubation check for acid production.

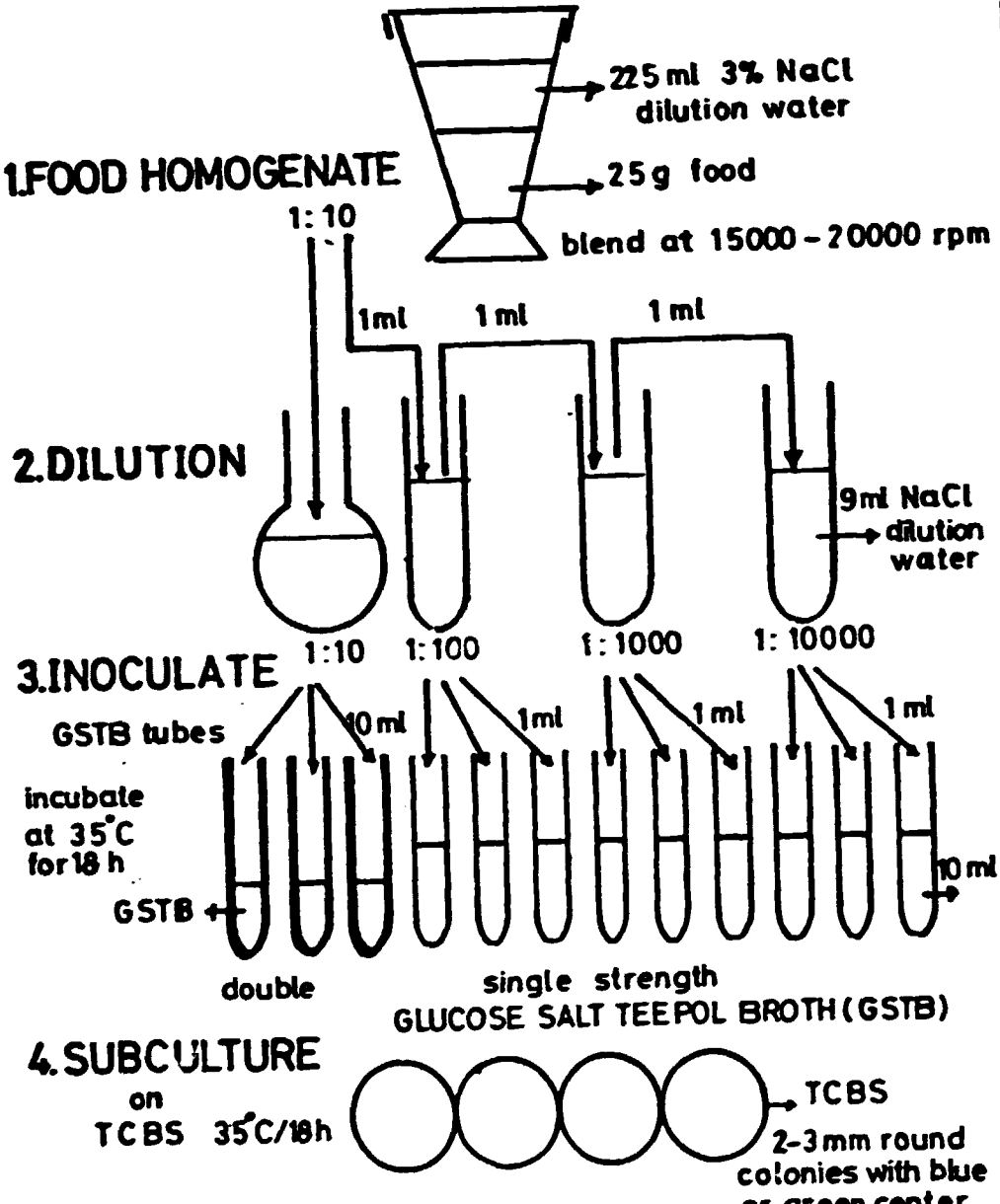
h) Glucose fermentation : Stab 2 tubes of HLGB medium, overlay one tube with sterile paraffin oil and incubate for 2 days at 35°C. Yellow coloration of both tubes indicates fermentation, in the tube without oil only indicates oxidation. V. parahaemolyticus is a glucose fermenter producing no gas.

i) Cytochrome oxidase test : Allow 2-3 drops of alphanaphthol solution to flow over a fresh slant of V. parahaemolyticus or over a colony on a plate, then follow this by an equal amount of phenylenediamine solution. The development of dark blue colour within 2 min is positive.

j) LDC : Refer to D.4.5.4.1-b

k) Growth at 42°C : Incubate an inoculated TSB at 42°C in a water bath for 24 h.

PL.8: DETECTION OF V.PARAHAEM.



Refai

Fig.13

8.5.5.3. The characteristic features of V. parahaemolyticus are :

a) Gram-negative curved rod
b) Cytochrome oxidase positive
c) Glucose oxidation/fermentation (O/F) positive, no gas
d) Colony on TCBS typical blue-green in colour
e) TSI, alkaline slant, acid butt, no gas, no H_2S
f) Positive growth at 42°C
g) Positive growth in 8% but not in 10% NaCl
h) Positive LDC
i) Negative VP
j) Negative sucrose

8.5.6. Calculation

When the blue green colonies on TCBS are finally identified biochemically as Vibrio parahaemolyticus, refer to the original positive dilutions on GSTB and apply the 3 tube MPN table (D.2.5.7) for final enumeration of the organism.

9. ENUMERATION OF BACILLUS CEREUS

9.1. Reference

Bacteriological Analytical Manual for Foods, 1976, 4th ed. Food and Drug Administration, U.S.A.

9.2. Principle

This method is based on surface plating technique using a medium containing egg yolk on which the colonies of B. cereus are recognized by being surrounded by zones of turbidity.

9.3. Apparatus and Glassware

a) Petri dishes
b) Pipettes, 1 ml
c) Incubators, 20°C, 30°C, 35°C

9.4. Culture media and reagents

a) Buffered peptone water P.1.9
b) KG agar, P.1.28
c) Nitrate broth B.1.43
d) Nutrient agar B.1.44
e) Nutrient gelatin B.1.46
f) VP medium B.1.77
g) Gram stains P.2.10

9.5. Procedure

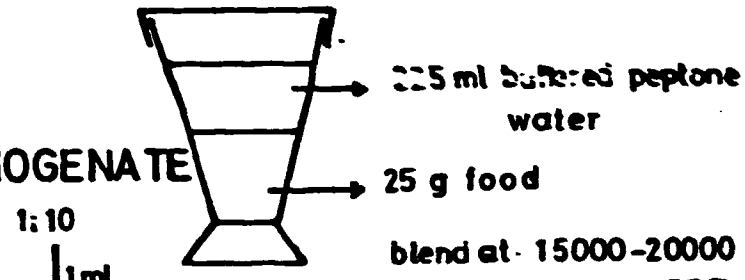
9.5.1. Preparation of food homogenate

Prepare as described under D.1.5.1

9.5.2. Dilution

Prepare as described under D.1.5.2

PL.9: ENUMERATION OF B. CEREUS

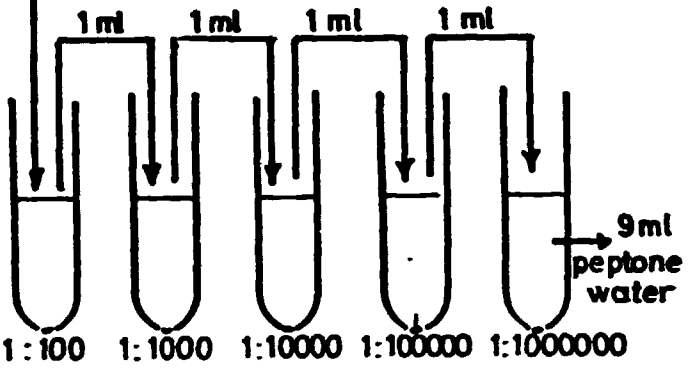


1. FOOD HOMOGENATE

1:10

1 ml

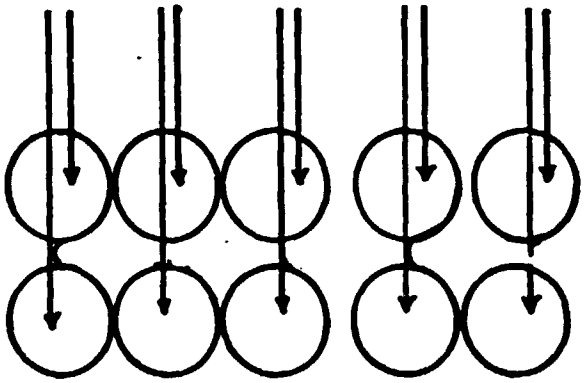
2. DILUTION



3. PLATING 0.25 ml ON

KG AGAR

Incubate at 30°C for 24h



colonies of Bacillus cereus are surrounded by a halo of dense ppt.

4. SMEAR & STAIN WITH GRAM (Gram +)

5. BIOCHEMICAL TESTS

a. nitrate red +	e. acid from mannitol +
b. starch hyd. ±	f. litmus milk pept., coag.
c. gelatine liq. +	g. motility ±
d. VP +	

Refal

Fig.14

9.5.3. Inoculation

Pipette 0.25 ml of homogenate and dilutions of the homogenate on the surface of previously dried KG agar plates and spread with a sterile bent glass rod.

9.5.4. Incubation

Incubate the plates at 30°C for 20-24 h

9.5.5. Counting of the colonies (presumptive B. cereus)

Count the colonies surrounded by a halo of dense precipitate (lecithinase activity) and calculate the total number per gram of specimen by multiplying by 4 and by the dilution factor.

9.5.6. Confirmation

a) From typical colonies make smear and stain with Gram and examine microscopically.

b) At the same time transfer some of the typical colonies to nutrient agar slants, incubate at 30°C for 24 h and from the growth inoculate the following :

i) Gelatine tube : examine for liquefaction after 24 h incubation at 20°C

ii) Nitrate broth tube : after 24 h incubation at 35°C add 2 drops of alpha-naphthol reagent. An orange colour indicates that nitrate has been reduced to nitrite.

iii) VP medium : refer to D.4.5.4.1-b

c) Characteristics of Bacillus cereus

Gelatine liquefaction	+
Nitrate reduction	+
Egg yolk reaction	+
VP reaction	-
Gram stain	+

9.5.7. Calculation

When the zone-forming colonies are confirmed microscopically and biochemically, their count gives the confirmed B. cereus.

10. ENUMERATION OF CLOSTRIDIUM PERFRINGENS

10.1. Reference

Official Methods of Analysis, Association of Official Analytical Chemists, 12th Ed. 1975.

10.2. Principle

This method is based on counting Clostridium perfringens using the pour plate technique and a selective medium containing sulphite polymyxin sulphadiazine (SPS). The sulphite is reduced by Clostridium perfringens to sulphide which reacts with the iron found in the medium to form a black iron precipitate

that gives the Clostridium colonies a black appearance. These colonies are then confirmed by additional tests. The antibiotics are inhibitory to saprophytic anaerobes and facultative anaerobes.

10.3. Apparatus and Glassware

a) Petri dishes and test tubes
b) Pipettes
c) Anaerobic jars
d) Incubator, 35°C
e) Water bath, 45°C
f) Colony counter

10.4. Culture media and reagents

a) Cooked meat enrichment medium P.1.12
b) Fluid thioglycollate medium P.1.21
c) Motility nitrate medium P.1.39
d) Peptone water diluent (0.1%) P.1.48
e) Sporulation broth P.1.59
f) Sulphite polymyxin sulphadiazin agar (SPS) P.1.61
g) Gram stain P.2.10

10.5. Procedure

10.5.1. Preparation of food homogenate

Prepare as described under D.1.5.1

10.5.2. Dilution

Prepare as described under D.1.5.2

10.5.3. Inoculation

a) Pipette 1.0 ml of the food homogenate and of each dilution of the homogenate to each of appropriately marked duplicate Petri dishes.

b) Pour 15-20 ml of SPS agar into each dish, rotate and tilt to mix the inoculum and agar and allow to solidify.

10.5.4. Incubation

a) Invert the plates and place in anaerobic jar.
b) Incubate the jar at 35-37°C for 24 h.

10.5.5. Counting of colonies (presumptive Cl. perfringens)

Select plates showing about 30-300 black colonies, count the colonies and calculate number of clostridia per g of food.

10.5.6. Confirmation of clostridium perfringens

a) Select 10 typical colonies from the SPS plates and inoculate each colony into a tube of freshly deaerated and cooled fluid thioglycollate broth.

b) Incubate at 35°C for 18-24 h

c) Examine each culture by Gram stain and check for purity of the culture. Short, thick Gram-positive rods with blunt edges .

d) If cultures are pure, inoculate separate tubes of motility-nitrate medium, sporulation broth, and cooked meat medium. Incubate 24 h at 35°C.

e) Examine tubes of motility-nitrate medium for motility, type of growth along the stab, (Cl. perfringens is non-motile) and nitrate reduction by adding 0.5-1.0 ml of α-naphthylamine solution and the same amount of sulphanilic acid (Cl. perfringens reduces nitrates, i.e. a pink or orange colour develops within 15 min).

PL10: ENUMERATION OF CL.PERFRING.

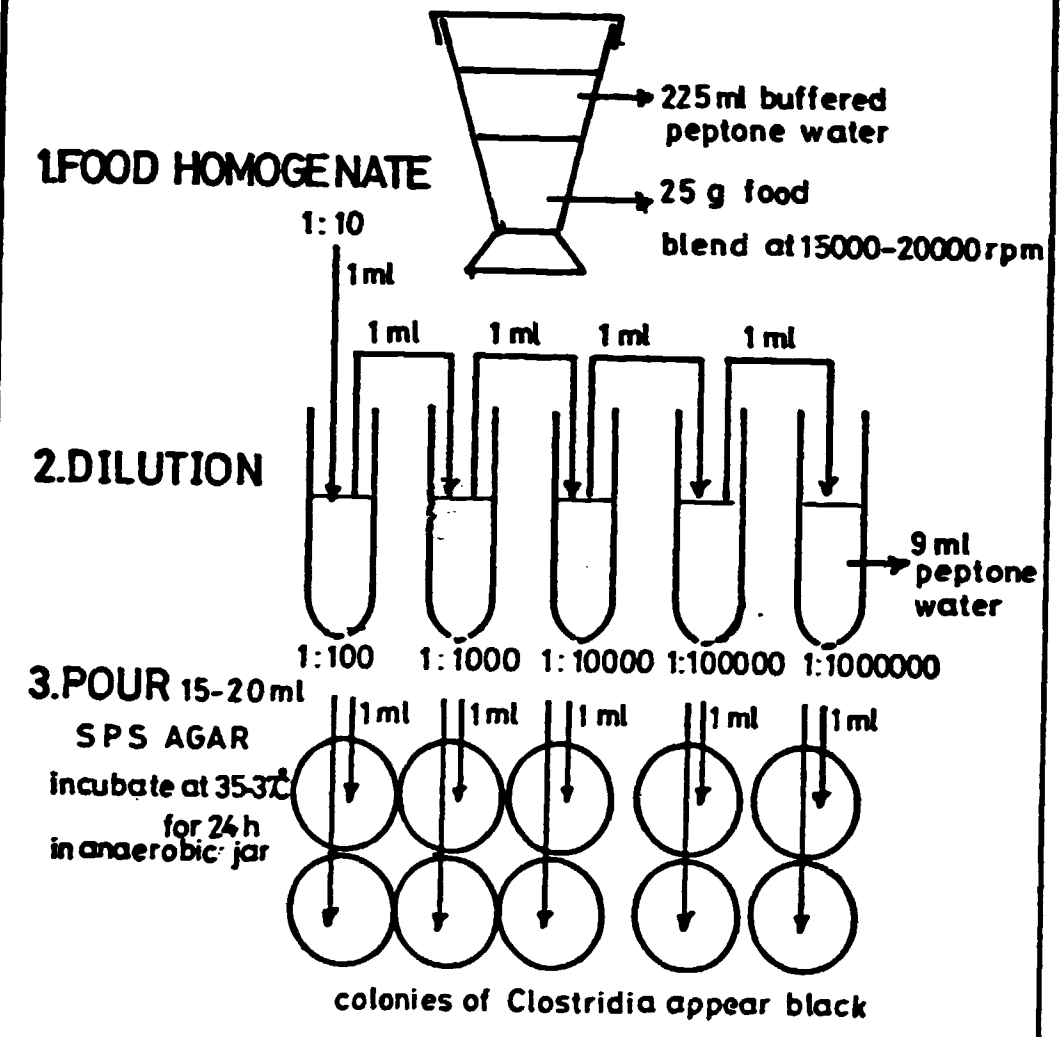


Fig.15

f) Examine sporulation broth for spores. Make a smear from sediment, air-dry, and heat-fix. Stain 10 min with malachite green. Wash with water, stain with aqueous safranin for 15 sec, rinse, blot, dry and examine microscopically. Spores will be stained green, vegetative cells red.

10.5.7. Calculation

Calculate the number of Cl. perfringens in the sample on a basis of the percentage of the colonies tested which are confirmed as Cl. perfringens.

Example :

Count of black colonies of 10^{-4} dil = 85
8 out of 10 colonies tested were confirmed as Cl. perfringen.
No. of Cl.perfringens per gram of food = $\frac{85 \times 0.8 \times 10,000}{6.8 \times 10^{5}} = 680,000$

11. DETECTION OF VIABLE CLOSTRIDIUM BOTULINUM AND BOTULINUS TOXIN

11.1. Reference

Thatcher, F.S. and Clark, D.S. ed. (1968). Microorganisms in Foods. Their Significance and Methods of enumeration. Toronto, University of Toronto Press.

11.2. Principle

This method is based on the detection of typical Gram-positive bacilli with subterminal oval spores grown on cooked meat medium and producing turbidity, gas and digestion of the meat particles.
Since the organisms very closely resemble other common nontoxinogenic clostridia, immunological detection of the specific toxin remains an essential procedure. The detection of the toxin in the culture filtrate or in the food sample is based on the protection of mice by type-specific antitoxin when injected intraperitoneally by the supernatant of the culture or the food extract.

11.3. Apparatus and Glassware

a) Pipettes
b) Hypodermic needles for injection of mice
c) Suitable centrifuge tubes
d) Mechanical food blender capable of homogenizing small food samples.
e) Centrifuge
f) Incubator, 30°C
g) Water bath, 50°C, 80°C

11.4. Culture media and reagents

a) Cooked meat medium P.1.13
b) Sterile saline solution P.1.54
c) Poly- and monovalent antitoxins

11.5. Procedure

11.5.1. Detection of viable Cl. botulinum

a) Inoculation

Place about 5 g of homogenized food sample into each of three tubes of freshly boiled and cooled cooked meat medium. Heat one of the tubes to 60°C for 15 min, and another to 80°C for 30 min in water bottles. Leave the third tube unheated.

b) Incubation

Incubate all tubes at 30°C for 5-15 days and examine for turbidity, gas production and digestion of meat particles.

c) Examination

i) After 5 days examine cultures for turbidity, gas production, digestion of meat particles, and odour. Also examine microscopically a smear stained by Gram stain. Observe morphology of organisms and note existence of typical clostridial cells, occurrence and relative extent of sporulation, and location of spores within cells.

ii) If there is no growth after 5 days, incubate and examine again after 10 days.

11.5.2. Detection of botulinus toxin in food

a) Homogenize the food sample with an equal weight of sterile saline, using a mechanical blender.

b) Centrifuge the homogenate at high speed for 1 h, preferably in a refrigerator or in a cold room.

c) Boil 2 ml of the supernatent for 10 min. to destroy toxin, if present.

d) Inject pair of mice each with 0.5 ml of the boiled supernatent. These are the control test for a heat-labile toxin, and should not die because of botulinus toxin, if present before boiling.

e) Dilute the unheated supernatent to 1:2, 1:10 and 1:100 with sterile saline.

f) Inject separate pairs of mice with 0.5 ml of the undiluted and diluted supernatent.

g) Observe mice for 72 h; typical symptoms of botulism usually begin within 24 h with ruffling of fur, followed by laboured breathing, weakness of limbs and finally totel paralysis and death.

11.5.3. Confirmation and typing of the toxin

a) Dilute monovalent antitoxins to types A, B, E and F with sterile saline to a concentration of 1 International Unit / 0.5 ml.

b) Prepare dilutions of the toxic supernatant to cover range of 10, 100 and 1000 min.lethal dose.

c) Inject several groups of mice intraperitoneally, each mouse with 0.5 ml of diluted antitoxins.

d) Challenge the mice after 30-60 min with the various dilutions of the toxic supernatant. Also inject pair of unprotected mice with each toxic dilution as control.

e) Observe mice for 72 h for symptoms of botulism.

f) The death of all groups except one means that this group is protected by specific antitoxins. This confirms the presence of botulinus toxin and indicates its type.

11.5.4. Detection of botulinus toxin in culture filtrate

Follow the same steps under 11.5.2 (starting with c) and 11.5.3

12. ENUMERATION OF YEASTS AND MOULDS

12.1. Reference

Compendium of Methods for the Microbiological Examination of Foods, 1976, APHA.

12.2 Principle

This method is the same as that used for enumeration of mesophilic aerobic bacteria but using a medium suitable for the growth of yeasts and moulds such as potato dextrose agar, mycophil agar or malt agar. From the colony smears are made and examined to make sure that the organism is a yeast or mould.

12.3. Apparatus and Glassware

a) Petri dishes
b) Pipettes
c) Water bath
d) Incubator
e) Colony counter.

12.4. Culture media and diluent

a) Buffered peptone water P.1.9
b) Mycophil or malt agar with antibiotics P.1.42, P.1.36

12.5. Procedure

12.5.1. Preparation of food homogenate

Prepare as described under D.1.5.1

12.5.2. Dilution

Prepare as described under D.1.5.2

12.5.3. Pour plating

a) Pipette 1.0 ml of each dilution into each of appropriately marked duplicate Petri dishes.

b) Pour into each Petri dish 15-20 ml of mycophil or malt agar tempered to 45°C. Mix thoroughly and allow to solidify.

12.5.4. Incubation and reporting

a) Invert plates and incubate at 20-25°C for 5 days. If excessive growth develops, count colonies first after 3 days and then again after 5 days.

b) Report as yeast and mould count per g or ml.

13. DIRECT MICROSCOPIC ENUMERATION OF MICROORGANISMS IN FOODS

13.1. Reference

Compendium of Methods for the Microbiological Examination of Foods, 1976, APHA.

13.2. Principle

This method determines the count of both dead and viable organisms in the sample. Unusually high microscopic counts are indicative of poor sanitation.

13.3. Equipment and reagents

a) Microscopic slides plain or with a delineated circular 1 cm^2 area.
b) Syringes calibrated to deliver 0.01 ml or a pipette similarly calibrated or a platinum loop with 4 mm internal diameter.
c) Drying cabinet, 40-45°C.
d) Compound microscope with stage micrometer.
e) Stains, including Gram stain.

13.4. Film preparation

a) Mix the sample well by shaking or blending.
b) Transfer 0.01 ml by syringe, pipette or loop to a clean slide and spread over 1 cm^2 area with a bent point needle.
c) Dry the prepared films without delay on a level surface at 40-45°C.
d) Films of food high in fat should be defatted by rinsing the dried slide with xylol and washing off with methanol before staining.
e) Stain with Gram stain or any other stain as required.

13.5. Microscopic examination

Films are examined at first with the high dry objective then with oil immersion. An estimate is made of the clumps of microorganisms present in 1 ml of the test portion. Clumps are counted separately if any cell or group of cells of the same counted morphological type is separated by a distance equal to or greater than twice the smallest diameter of the two cells nearest each other. Count cells of different morphology, or which are stained differently, as separate units regardless of their proximity to other cells. To examine a representative portion of the film, select a starting field midway on any side and 2 or 3 fields in from the edge. Count separate fields in a series across the film. Then start midway at the top or bottom of the film and count a series of separate fields in a line perpendicular to the first series.

13.6. Calculation

In computing the count for the film method, the average number of microorganisms per field is multiplied by the microscopic factor (MF) and by the reciprocal of the dilution used.
Average number per field x MF x reciprocal of dilution = Direct microscopic (DMC) per g or ml.
N.B. MF = 100/A where A is the microscopic field area.

14. BACTERIOLOGICAL EXAMINATION OF WATER FOR SANITARY QUALITY

14.1. Reference

Standard Methods for the Analysis of Water and Waste Water, 1971, 14th Ed. APHA.

14.2. Enumeration of Aerobic Mesophilic Bacteria (Standard plate count)

14.2.1. Apparatus and Glassware

a) Petri dishes 90-100 mm
b) Pipettes 1, 5 and 10 ml (total flow)
c) Water bath, 45°C
d) Incubator 35°C or 20°C
e) Colony counter

14.2.2. Culture media

a) Phosphate buffer solution of 10% peptone water
b) Plate count agar F.1.50

14.2.3. Procedure

a) Preparation and dilution

The sample bottle should be shaken vigorously 25 times. Serial decimal dilutions are made using phosphate buffer solution or 10% peptone water, as described under D.1.5.1

b) Plating

1.0 ml or 0.1 ml of the sample as well as of the dilutions are placed in separate Petri dish and 15.0 ml of liquefied agar medium at a temperature of 43 to 45°C are to be added to each dish. The agar and the sample should be thoroughly mixed by tilting and rotating the dish and left to solidify.

c) Incubation

Incubate the plates inverted at 35°C for 24 h or at 20°C for 48 h.

d) Counting

Only plates showing 30 to 300 colonies should be considered in determining the standard plate count. Counts may be designated as "standard plate count at 35°C" or "standard plate count at 20°C".

14.3. Enumeration of Coliforms

14.3.1. Multiple-tube fermentation technique

14.3.1.1. Apparatus and Glassware

a) Pipettes and graduated cylinders
b) Dilution bottles or tubes
c) Petri dishes 100 mm Ø and 60 mm Ø for the membrane filter
d) Fermentation tubes and vials
e) Incubators, 35°C, 44.5°C
f) pH meter

14.3.1.2. Culture media and reagents

a) Brilliant green lactose bile broth F.1.7
b) Buffered peptone water F.1.9
c) EC medium F.1.15
d) Endo agar F.1.17
e) Eosin methylene blue agar F.1.18
f) Lactose broth F.1.30
g) Lauryl tryptose broth F.1.31
h) Nutrient broth F.1.45

14.3.1.3. Procedure

a) Presumptive test

In case of non-chlorinated water inoculate 5 tubes of the presumptive medium (lactose or lauryl tryptose broth) of 10 ml quantities (double strength) each with 10 ml quantities of water, five tubes of the medium (single strength) of 5 ml quantities each with 1 ml water and another set of 5 tubes of 5 ml quantities each with 0.1 ml of the water.

In case of chlorinated or filtered water it is unnecessary to examine 0.1 ml volume. Instead we add 50 ml of water to a bottle containing 50 ml of the medium (double strength).

Incubate the inoculated tubes and bottles at 35°C for 24 and 48 h and record the presence or absence of gas formation.

b) Confirmed test

Gently shake or rotate tubes showing gas and transfer one to three loopfuls of the medium to a fermentation tube containing BGLB broth.

c) Completed test

Streak one or more Endo or L-EMB plates from each tube of BGLB showing gas and incubate at 35°C for 24 h. Find one or more typical well-isolated coliform colonies and transfer one each to a lactose broth or a lauryl tryptose broth and to a nutrient agar slant.
Record the formation of gas in the fermentation tube and make a Gram-stained preparation from the agar slant cultures.

The formation of gas in the secondary lactose broth tube and the demonstration of Gram-negative non-spore forming rod-shaped bacteria may be considered a satisfactory completed test, demonstrating the presence of a member of the coliform group in the volume of sample examined. Calculate the number from the tables of MPN (see D.14.3.1.5).

N.B. The presumptive test without confirmation may be applied to examine any sample of waste, sewage, or water known to be heavily polluted.

The confirmed test should be applied in the examination of routine samples of drinking water, of water in process of treatment and of bathing water.

The completed test should be applied only to a proportion of sample so as to establish beyond reasonable doubt the value of the confirmed test in determining the sanitary quality of such water supplies.

14.3.1.4. Faecal Coliform (MPN)

a) Simultaneously with the confirmatory procedure using BGLB broth, transfer should be made from all positive presumptive tubes to EC medium.

b) The inoculated EC tubes are incubated at 44.5°C for 24 h, and gas formation is recorded. The bacterial density is estimated from the tables of MPN (seen D.14.3.1.5)

c) For the differentiation of coliform refer to the IMViC reactions (see D.2.5.9.b).

14.3.1.5. MPN index for various combinations of positive and negative results when one 50 ml portion, five 10 ml portions and five 1 ml portions (left table) or when five 10 ml portions, five 1 ml portions and five 0.1 ml portions (right table) are used

Number of positive tubes 50	10	1 ml	MPN per ml	Number of positive tubes 50	10	1 ml	MPN per ml
0	0	0	1	1	4	0	13
0	0	1	1	1	4	1	17
0	0	2	2	1	4	2	22
0	1	0	1	1	4	3	28
0	1	1	2	1	4	4	35
0	1	2	3	1	4	5	43
0	2	0	2	1	5	0	24
0	2	1	3	1	5	1	35
0	2	2	4	1	5	2	54
0	3	0	3	1	5	3	92
0	3	1	5	1	5	4	160
0	4	0	5	1	5	5	240
1	0	0	1				
1	0	1	3				
1	0	2	4				
1	0	3	6				
1	1	0	3				
1	1	1	5				
1	1	2	7				
1	1	3	9				
1	2	0	5				
1	2	1	7				
1	2	2	10				
1	2	3	12				
1	3	0	8				
1	3	1	11				
1	3	2	14				
1	3	3	18				
1	3	4	21				

Number of positive tubes 10	1	0.1 ml	MPN per ml	Number of positive tubes 10	1	0.1 ml	MPN per ml
0	0	0	< 2	4	2	1	26
0	0	1	2	4	3	0	27
0	1	0	2	4	3	1	33
0	2	0	4	4	4	0	34
1	0	0	2	5	0	0	23
1	0	1	4	5	0	1	31
1	1	0	4	5	0	2	43
1	1	1	6	5	1	0	33
1	2	0	6	5	1	1	46
2	0	0	5	5	1	2	63
2	0	1	7	5	2	0	49
2	1	0	7	5	2	1	70
2	1	1	9	5	2	2	94
2	2	0	9	5	3	0	79
2	3	0	12	5	3	1	110
3	0	0	8	5	3	2	140
3	0	1	11	5	3	3	160
3	1	0	11	5	4	0	130
3	1	1	14	5	4	1	170
3	2	0	14	5	4	2	220
3	2	1	17	5	4	3	280
3	3	0	17	5	4	4	350
4	0	0	13	5	5	0	240
4	0	1	17	5	5	1	350
4	1	0	17	5	5	2	540
4	1	1	21	5	5	3	920
4	1	2	26	5	5	4	1600
4	2	0	22	5	5	5	2400

14.3.2. Enumeration of coliform (Membrane-Filter "LP" procedure)

14.3.2.1. Apparatus and Glassware

a) Pipette and graduated cylinders
b) Petri dishes, ø 60 mm
c) Filtration units
d) Filter membranes
e) Absorbent pads for nutrients
f) Incubator, 35°C
g) A binocular dissecting microscope
h) Water bath, 45°C

14.3.2.2. Culture media

a) Endo agar P.1.17
b) M-FC medium P.1.37

14.3.2.3. Procedure

a) Sample size

Size of the sample will be governed by the expected bacterial density. 100 to 500 ml or more can be filtered.

b) Filtration

Using sterile forceps, place a sterile filter membrane over the porous plate of the filtration unit, grid side up. Carefully place the matched funnel unit over the receptacle and lock it in place. Filtration is then accomplished by passing the sample through the filter under partial vacuum. The filter may be rinsed by the filtration of three 20-30 ml portion of sterile buffered water between samples. Unlock and remove the funnel, immediately remove the filter membrane with sterile forceps and place it on the agar with a rolling motion to avoid the entrapment of air. Filtration units should be sterilized at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between samples of filtration, and in this case new filtration requires sterilization of all membrane filter holders.

c) Incubation

The plate is incubated inverted at 35°C for 24 h.

d) Counting

The typical coliform colony has a pink to dark red colour with a metallic surface sheen. The count is best made with a low-power (10-15 magnifications) binocular wide-field dissecting microscope. Count from filters containing 20-80 coliform colonies.

e) Calculation of coliform density

The calculation, coliform density is reported in terms of total coliforms per 100 ml as follows :

$$\text{Total coliform colonies/100 ml} = \frac{\text{coliform colonies counted} \times 100}{\text{ml sample filtered}}$$

14.3.2.4. Faecal coliform (MF Procedure)

a) Preparation of culture dish

Place a sterile absorbent pad in each dish and pipette approximately 2 ml of M-FC medium to saturate the pad. Carefully remove any surplus liquid from the dish. The filter is then placed on the medium-impregnated pad.

b) Incubation

The prepared culture are placed in waterproof plastic bags for protection during submersion in the water bath for 24 h incubation at 45°C.

c) Counting

Colonies produced by faecal coliform bacteria are blue in colour. The non-faecal coliform colonies are grey to cream-coloured.

d) Calculation

As in D.14.3.2.3-e.

14.4. Test for the faecal streptococcal group

14.4.1. Multiple-tube technique

a) Apparatus and reagents

See D.14.3.1.1. and 14.3.1.2

b) Presumptive test

The method is the same as in coliform test but using a series of tubes of azide dextrose broth F.1.2.
The inoculated tubes are incubated at 35°C for 24 h. Each tube is examined for the presence of turbidity. If no turbidity shows, re-incubate for another 24 h.

c) Confirmed test

From all azide dextrose broth tubes showing turbidity after 24 or 48 h transfer 3 loopfuls to tubes containing 10 ml ethyl violet azide broth, and incubate at 35°C for 24 h.
The presence of faecal streptococci is indicated by the formation of a purple button at the bottom of the tube, or occasionally by a dense turbidity.

d) The MPN is recorded as in the case of coliforms from the MPN tables (See D.14.3.1.5).

14.4.2. Streptococcal plate count

The same as in the case of standard plate count, but here M-Enterococcus or KF Streptococcus agar is used. The plates are incubated at 35°C for 48 h ; surface and subsurface colonies produced by faecal streptococci are dark red to pink in colour with entire edges.

14.4.3. Membrane filter Technique

14.4.3.1. Culture Media

a) Brain heart infusion agar F.1.5 and broth F.1.6
b) KF Streptococcus agar F.1.27
c) M-Enterococcus agar F.1.38

14.4.3.2. Procedure

a) Sample size

A sample of 100, 10, 1 or 0.1 ml may be necessary, depending on the amount of pollution in the water sample.

b) Filtration

Follow D.14.3.2.3-b

c) Culture dish preparation

Pour by pipette 4.5 ml liquified medium into the dishes and flame the surface to eliminate bubbles.

d) Incubation

Transfer the filter membrane directly to the agar medium, invert the plates and incubate at 35°C for 48 h.

e) Counting

Colonies produced by faecal streptococci are dark red to pink in colour.

f) Calculation

Calculate from filter membranes containing 20-100 faecal streptococcus colonies. See D.14.3.2.3-e

14.4.3.3. Confirmation test

a) Find selected typical colonies from membrane filters and inoculate onto a brain heart infusion agar slant. Incubate at 35°C for 24-48 h.

b) Transfer a loopful of growth from the brain heart infusion agar slant to a clean slide. Add few drops of 3% hydrogen peroxide to the smear. The absence of bubbles constitutes a negative catalase test indicating a probable Streptococcus culture.

c) Transfer a loopful of growth from the brain-heart infusion agar into brain-heart infusion broth and incubate at 45°C for 48 h.

Also transfer a loopful of growth into bile broth [illegible]ium (10% oxgall solution) and incubate at 35°C for 3 days. Growth in the abo[illegible]edia constitutes a positive test for faecal streptococci.

15. REFERENCES

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EQUIPMENT SPECIFICATION-FOOD TESTING AND QUALITY CONTROL LABORATORY IN AFGHANISTAAN

1.	Destrcallating app el heated/20/1/h	1
2.	Balance analotical range upto 160g	1
3.	Balance multi purpose upto 100g	1
4.	Soxlet apparatus heater	missed
5.	Hot plate, elecricaly heated	2
6.	Glowing furance, electricaly heated	2
7.	Muffle furance solar	1
8.	Vacum pump N.10.6 $10m^3/h$	1
9.	Mohr-westphal balance	1
10.	Speatrophotometer	1
11.	PH-meter commpl.	1
12.	Water bath - 6 poss.	1
13.	Sand bath autom. coutr.	missed
14.	Hot-plate for kjeldhl micro 6 poss	1
15.	Centrifupe, laboratory for 10 and 100 ml tubes etc., with all items	1
16.	Colorimeter with all items	1
17.	Stirer. laboratory type a revolution comtr	missed
18.	Oil bath automaticaly controled	missed
19.	Magnetic & Hear with hot plate	2
20.	Cork borers/set	complete 1
21.	Signal clock	missed
22.	Gas burner	2
23.	Alchol lamp 100 ml	2
24.	Apparatus for paper chromatography	missed
25.	Thermometer set	1
	- 0 ÷ 50	
	- 0 ÷ 200	
	- 0 ÷ 300	
	- 0 ÷ 450	

26. Vacum pump, water-jet	1
27. Vacum pump, IV 2G 2g 2x3/h	missed
28. Refregerator	1 damaged
29. Deep freezer refrigerator	1 damaged

LABORATORY FURNITURE

30. Laboratory Table 390 x 80 x 85 cm	2 broken
31. Laboratory Table 390 x 75 x 85 cm	2 - " -
(Ceramic plate, resistant to acids and sols.)	2 - " -
32. Laboratory table for apperatus 135 x 64 x 76	3 - " -
33. Table for balance 100 x 61 x 78	2 - " -
34. Chemical closet 120 x 49 x 190	1 - " -
35. Classware closet 120 x 49 x 190	2 - " -
36. Fume cupboard (Drgester)-conetions for water, gas, illumination, light fan, 154 x 80 x 250 cm	2 - " -
37. Digestor fan, plastic 100 x 500	2 - " -
38. Laboratory chair	4 - " -
39. Writing desk	1 - " -

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40. Micro kicldahl - Apparatus Parnas-Wagner	2 missed
41. Extaction app., Soxlet compl. Flask 100 and 250	6 missed
42. Kieldahl flask 500 ml	10
43. Desrcator 250 mm	2 1 missed
300 mm	2 missed
44. Bottle with tubulature at the bottom with stop cock tubulature NB sorket 10 L	2 missed
45. Buretté, automatic (Pellet), standard gradnation (Schellenbach)	missed
50 ml	[illegible]
20 ml	missed
46. Weighing vessel with grooming cap	
40 x 60 mm	

47. Laboratory beaker, low form		
1000 ml	5	
600 ml	20	
400 ml	20	1 missed
250 ml	20	6 missed
150 ml	20	3 missed
100 ml	20	5 missed
50 ml	10	
25 ml	10	
48. Crucible, porceloin. 42x36mm	30	2 missed
49. Flask, erlenmeyer, wide mouth		
250 ml	20	
300 ml	20	10 missed
500 ml	10	
50. Measuring cylindar		
25 ml	10	
50 ml	10	
100 ml	10	9 missed
250 ml	5	
500 ml	5	
1000 ml	2	
2000 ml	2 missed	
51. Volumetric flasks with NS stopper		
25 ml	20	1 missed
50 ml	20	
100 ml	20	
200 ml	20	
250 ml	20	
500 ml	5	1 missed
1000 ml	2	1 missed
2000 ml	2	

52. Filter funel with a short tube			
	DIA 150 mm	10	
	" 80 mm	10	
	" 45	5	
53. One mark pipette (bulb pipette)			
	1 ml	10	4 missed
	5 ml	10	1 missed
	10 ml	10	6 missed
	20 ml	10	2 missed
	25 ml	10	10 broken
	50 ml	5	5 broken
	100 ml	2	1 broken
54. Graduated prpette			
	1 ml	10	
	2 ml	10	
	5 ml	10	
	10 ml	10	10 missed
	20 ml	10	
	25 ml	10	
55. Asdestos wire Gouse 13x13		10	
56. Condenser, Liebig, lenght of jacket 400.00mm		5	
Condenser, Ahein, lenght of jacket 400.00 mm		5	
57. Washing bottle			
	500 ml	5	2 missed
	1000 ml	5	1 missed
58. Tripod stand, bar lenght 100cm		10	
59. Support prpete		2	
60. Support. funnel		2	
Support test tube (wooden)		4	
61. Clamp holder (brass)			
	Double for-bourette	10	
	Fork like	10	
	Angular	10	

62. Tweezers, 23 cm	4
Double spoon, metal	
18	4
14	4
64. Support Rings	
45 mm	3
85 mm	3
65. Air injector, filter pump	2
66. Droping bottle	
30 ml	5
100 ml	5
67. Reogent bottle	
250 ml	10
500 ml	10
1000 ml	10
brush: test tube	10
burete	10
bottle	10
68. Cork, Rubber, assorted	3 KG
69. Hose, plastic different	20
70. Porcelain morier andpestle	4
71. Dish, petry 100x15 mm	50
72. Funnel, Buchner (100 ml)	5
73. Funnel, separatory	
100 ml	5 missed
250 ml	5 missed
500 ml	5 missed
74. Spatula	10
75. Rod, glass different dia	2 kg
76. glass tubular conector	
T-type	10
Y-type	10

77. Beaker cover, wathc glass form		
50 mm	10	
100 mm	10	
150 mm	10	
78. Centrifupe tube		
15 ml	12	10 missed
100 ml	12	all broken
79. Micro burete		
1 ml	10 missed	
2 ml	10 missed	
5 ml	10 missed	
80. Reflux coundenser	2	
81. Sintered glass filtration crucible proosity		
G.2	OK	
G.3	OK	
G.4 for vacum	OK	
Filtration with internal diameter of 35 mm	15	
82. Glass stopped comical flasus		
100 ml	5	
200 mi	5	
250 ML	5	
300 ml	5	
83. Destilation flask, round bottomed:		
250 ml	5 missed	
500 ml	5 missed	
1000 ml	2	1 missed
2000 ml	2	1 missed
84. Specific gravity bottle of pycnometure Ace to Rischaner		
25 ml	10	1 missed
50 ml	10 OK	
85. Funnel for pycnometure	10	all missed

CHEMICALS

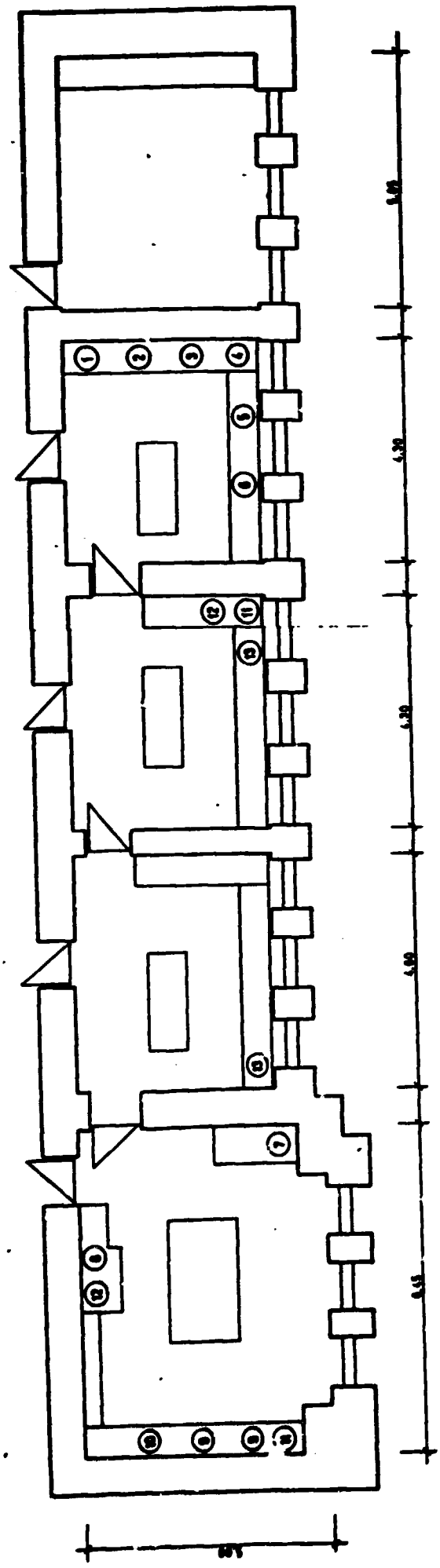
86. Petrolium ether p.a.	10 x 1	7 btl. missed
87. Diethyl ether p.a.	10 x 1	4 btl. missed
88. Chloroform ether p.a.	10 x 1	4 btl. missed
89. Suphuric acid p.a.	20 x 1	
90. Hydrochloric acid p.a.	20 x 1	1 missed
91. Indicators		
Methyl Red	1 x 20 g	
Phenolphtalein	1 x 100 g	
92. Ethyl alcholhol 96% PA	20 x 1	missed
93. Cupric sulphate	2 x 1 kg	
94. Sodium Hydroxide	5 x 1 kg	
95. Potassium sulphate	4 x 500 g	
96. Sodiumthio sulp	1 x 500 g	
97. Nitric acid	20 x 1	10 missed
98. Potassuim permanganate	1 x 250 g	missed
99. Amonium hydroxide	20 x 1L	2 missed
100.Acetic acid, gladule	10 x 1 L	missed
101.Methanol p.a.	10 x 1L	missed
102.Oxalic acid	8 x 200 g	OK
103.Silica gel	20 x 1 kg	missed
104.Fehling sol. A. p.a.	200 x 100 gel	OK
105.Fehling sol B. p.a.	200 x 100 ml	OK
106.Potassium Dichrunate	2 x 500 g	OK
107.Ammonium chloride	2 x 500 g	OK
108.Perchloric acid	5 x 1L	missed

CHEMICALS AND INSTRUMENTS NECESSARY

EDTA di-sodium salt	5 KG
EDTA di-potassium salt	5 KG
Ammonium Hydronide	5 KG
Ammonium chloride	5 KG
Magnesium chloride	5 KG
Eriocheom Black-T	0,5 KG
Soduim chloride	5 KG
Sodium acetate	5 KG
Potasuim nitrate	2 KG
Acetic Acid	15 L
Barium chloride	5 KG
Sodium Sulphate	5 KG
Sodium salicilate	2 KG
Soduim sulphate 4NH	5 KG
Sodium hydroxide	20 KG
Sulphuric acid conc 1.840	20 L
Magnesium sulphate	5 KG
Potassuim hydroxide	10 KG
Sodium Iodide	2 KG
Sodium Azide	2 KG
Sodium sulphate	KG
Sodium sulphite	KG
Cobalt chloride	2 KG
Potassium chloride	5 KG
Hydronil-Amid-hydro-chloride	2 KG
Ammonium acetate	5 KG
Ferrus amonium sulphate	2 KG
Maroxid	0,5 KG
Mercury sulphate	0,5 KG
Mercury Iodide	0,5 KG
Mercury chloride	0,5 KG

Silver Nitrate	2 KG
Phosphoric acid	5 L
Potassium sulphocyanide	5 KG
Potassium cyanide	2 KG
Dithizone diphenilethiocaebuzol	5 KG
Potassuim Permanganate	5 KG
Potassium dichromate	5 KG
Potassium hydrocarouote	5 KG
Potassium Iodide	5 KG
Ethyl alchohol	50 L
Methanol	20 L
Amhyl alc	50 L
Petrol ethar	20 L
Dyethyl ethar	50 L
Acethon	20 L
Chlorophorm	50 L
Phenol cryst	5 KG
Gerber centrifupe la 24 places	1 KOM
Butirometars formilk GERBER	50 KOM
- " - buter - " -	6 KOM
- " - chese - " -	10 KOM
Inkubator 0 + 150^{0} C	1 KOM
Calculator	2 KOM
Electroconductivity meter	1 KOM
Terbedetimeter	1 KOM
PH meter	1 KOM
Automatic byretes 50 cm^{3}, 10cm^{3} .... 50 cm^{3}	24 KM
Rephroctometer	one
Polary meter	one
Free ammonic bidestilatr ep.	one

Strainer different sizes	few
Filter paper different types	few
Indikator paper different	
Buffer tablet for water hardnes	few
Chromotograf and dryer and 10 pack paper	one
Hot plates 6x for Soxlet extractron	one
Keramic Dish 500 ml	5
Phenolphtalein	1 KG
Metal Red	1 KG
Metal Orange	1 KG
Metal blue	1 KG
Standard colors for different foods	complete
Oxalic acid standard sol.	10 L
Iodine sol.	10 L
Iodine cryst	5 KG
Rubber bulbs different sizes	50 pcs
Tin knife	10 pcs
Kiezelqur dest. apt.	2 pcs



MINISTRY OF PUBLIC HEALTH
KABUL - AFGHANISTAN
INSTITUTE FOR PARASITOLOGY

CHEMICAL LABORATORY

ROOMS & EQUIPEMENT DISPOSITION

1. ANALITICAL BALANCE
2. SPECTROPHOTOMETER
3. COLORIMETER
4. CENTRIFUGE
5. BALANCE TECH.
6. MOHR. WESTFAL BALANCE
7. SOXLET
8. DESTILER
9. HOT PLATES
10. MUFFLE FURANCE
11. HOT PLATE HEATER
12. GLOWING FURANCE
13. ELECTRICAL HEATER

1:50

PREPARED BY: RADIVOJ LEGETIĆ
UNIDO CONSULT.

ADDENDUM 5

To: Mr. M. Malhotra
UNDP, Kabul

Dear Sir,

Considering my being here, in Kabul from 3rd November this place I had a contact with a people from Ministry of Public Health trying to solve the existing problims in accordance with a Project AFG/81/001. My task is to enable this laboratory, to start it and introducing the personel in using and running this equipment as well.

This equipment consisting from various instruments, furnitures, glasswares and chemicals have been damaged more or less during its transportation along route Yugoslavia-Kabul. The complete list of all goods is given in addendum 1, with signed item what have been broken, damaged or missed. Yugoslav autorities have to try to conntact the insurance AGENCY where it was issured.

All items were dispacked in transportation from Herat to Kabul and packed again into non adequate package. This fact obtained so many troubles.

Some of instruments have been repacked and they are not mentioned as damaged.

Preparation of the necessary rooms have been done on a quite proper way. Exepc some problems with electricity supplying what is promised to be solved by Ministry of Public Health.

All furniture has been broken during transportation, only few of items could be used.

In that aim the Ministry of Public Health arranged carpenters. In that way this problem, the problem of furniture is going to be solved.

We started a program of training of personel what as first consist to introduce them in matter of existing instruments and new methods of chemical examinations.

The people in laboratory are expressing a great interest, cooperability and enthusiasm to help and to make as much progress as possible.

The instruments and chemicals what are necessary to be supplied are in list add. 2. And this list have to be submited to Yugoslav autorities to commplete the necessry irems of this laboratory.

What I suggest to do is:
First of all:

This letter with all additional items to be submitted to

- UNIDO - WIENA

- Federal Committee for Energy and Industry S. F. R. Yugoslavia

- Joint UNDO- Yugoslavia Centre
  21.000 Novisad, POB. 331
  Yugoslavia ; and

- Afghanistani authorities in this matter have to be informed.

This letter have to be completed with a:

a. list of broken, missed and damaged items;
b. list of requested chemicals and instruments for completing laboratory.

All this have been done considering the steps what introduce us into the existing project, specially telex to UNDP from Belgrade 8/02 what copy is added to this letter.

Expecing your cooperation.

Yours sincerely,

Radivoi Legetic