ANNEXURE – 1

MARKET SURVEY QUESTIONNAIRE

Name of the concern :

Address :

Please tick (✓) the appropriate

1. The fruit products available in the market.

Available fruit products	Banana	Guava	Papaya
Jam Jelly Candy RTS/Juice Sauce Squash Chutney Pickle Frozen puree Glazed fruits Canned fruits Fruit bar			

2. The major customer preferring the fruit products

Fruit products	Children	Adolescents	Adults
Jam Jelly Candy RTS/Juice Sauce Squash Chutney			

Pickle Frozen puree Glazed fruits Canned fruits Fruit bar			

3. Major Market outlet for the fruit products. Tick **(✓)** whichever is sold in your concern.

Fruit products	Tick **(✓)**
Jam Jelly Candy RTS/Juice Squash Frozen puree Fruit bar	

4. Tick (✓) your satisfactory level on the following attributes of the fruit product.

Attributes	**Satisfied**	**Dissatisfied**	**Convinced**
Price comparability Quality comparability Affordability Product labeling Product packing Innovative Safety of the product			

5. Please Tick (✓) your expectations

Expectations of the consumers	Agree	Disagree
• Like to buy a minimally processed fruit product • Interested to buy a innovative product of locally available fruits • Like to purchase a certified product only • Ready to buy a quality product at reasonable price • Feel comfortable to buy a convenience fruit product		

ANNEXURE 2

QUESTIONNAIRE TO ELICIT INFORMATION REGARDING THE PREFERENCES OF TARGET CONSUMER FOR PRODUCT FORMULATION

Name

Class

Criteria	Tick
Availability of fruit product Highly available moderate Less available Not available	
Most preferred new fruit product Fruit candy Fruit juices Fruit sauce Fruit bars	
Quality attributes preferred Taste Energy Healthy Refreshment	
Most preferred fruit product Banana Guava Papaya	
Place of purchase Departmental stores Retail shops Whole sale shop	
Factors considered while purchasing Packing Labeling Advertisement Peers opinion Price	

ANNEXURE – 3

RECIPE FOR MIXED FRUIT PICKLE

Ingredients

Raw papaya	-	150 g
Guava	-	150 g
Amla	-	100 g
Salt	-	45 g
Vinegar	-	10 ml
Oil	-	200 ml
Red chili powder	-	50 g
Mustard powder	-	1 tsp.
Asafoetida	-	a pinch
Fenugreek powder	a pinch	

Method

Blanch the fruit for 2 minutes and soak them in salt and vinegar for 3 days and dry in the sun for 2-8 hours. Heat oil and add mustard, asafetida and fenugreek powder. Fry the fruits for 45 to 20 minutes and add chili powder and try. Bottle in clean and dry bottles.

ANNEXURE – 4

SCORE CARD FOR CANDIES

Name :

Student / staff :

Product :

Date :

Appearance	Score	V_1	V_2	V_3	V_4	V_5
Very appealing	5					
Appealing	4					
Moderately appealing	3					
Fairly appealing	2					
Neither appealing nor appetizing	1					
Total						

Color	Score	V_1	V_2	V_3	V_4	V_5
Cocoa brown	5					
Dark brown	4					
Light brown	3					
Pale brown	2					
Brown	1					
Total						

Flavour	Score	V_1	V_2	V_3	V_4	V_5
Highly acceptable	5					
Acceptable	4					
Moderately acceptable	3					
Slightly acceptable	2					
Not acceptable	1					
Total						

Taste	Score	V_1	V_2	V_3	V_4	V_5
Excellent	5					
Very good	4					
Good	3					
Fair	2					
Poor	1					
Total						

Texture	Score	V_1	V_2	V_3	V_4	V_5
Hard	5					
Breakable	4					
Sticky	3					
Semisoft	2					
Soft	1					
Total						

ANNEXURE 4A

SCORE CARD FOR BANANA SAUCE

Name :
Product :
Class :
Date :

Appearance	Score	V1	V2	V3
Very appealing	5			
Appealing	4			
Moderately appealing	3			
Appetizing	2			
Neither appealing nor appetizing	1			

Color	Score	V1	V2	V3
Light yellow	5			
Pale yellow	4			
Dull yellow	3			
Light yellow	2			
Pale brown	1			

Flavour	Score	V1	V2	V3
Highly acceptable	5			
Acceptable	4			
Moderately acceptable	3			
Slightly acceptable	2			
Not acceptable	1			

Taste	Score	V1	V2	V3
Excellent	5			
Very good	4			
Good	3			
Fair	2			
Poor	1			

Consistency	Score	V1	V2	V3
Smoothly	5			
Pouring	4			
Dropping	3			
Thick	2			
Sticky	1			

ANNEXURE – 4B

SCORE CARD FOR GUAVA SAUCE

Name :
Product :
Class :
Date :

Appearance	Score	V1	V2	V3
Very appealing	5			
Appealing	4			
Moderately appealing	3			
Appetizing	2			
Neither appealing nor appetizing	1			

Color	Score	V1	V2	V3
Cream	5			
Light green	4			
Pale green	3			
Light brown	2			
Pale brown	1			

Flavour	Score	V1	V2	V3
Highly acceptable	5			
Acceptable	4			
Moderately acceptable	3			
Slightly acceptable	2			
Not acceptable	1			

Taste	Score	V1	V2	V3
Excellent	5			
Very good	4			
Good	3			
Fair	2			
Poor	1			

Consistency	Score	V1	V2	V3
Smoothly	5			
Pouring	4			
Dropping	3			
Thick	2			
Sticky	1			

ANNEXURE – 4C

SCORE CARD FOR PAPAYA SAUCE

Name :
Product :
Class :
Date :

Appearance	Score	V1	V2	V3
Very appealing	5			
Appealing	4			
Moderately appealing	3			
Appetizing	2			
Neither appealing nor appetizing	1			

Color	Score	V1	V2	V3
Orange	5			
Light orange	4			
Pale orange	3			
Dark pink	2			
Pale pink	1			

Flavour	Score	V1	V2	V3
Highly acceptable	5			
Acceptable	4			
Moderately acceptable	3			
Slightly acceptable	2			
Not acceptable	1			

Taste	Score	V1	V2	V3
Excellent	5			
Very good	4			
Good	3			
Fair	2			
Poor	1			

Consistency	Score	V1	V2	V3
Smoothly	5			
Pouring	4			
Dropping	3			
Thick	2			
Sticky	1			

ANNEXURE – 5

HEDONIC RATING SCALE

Attribute	Score	V_1	V_2	V_3	V_4	V_5
Like extremely	9					
Like very much	8					
Like moderately	7					
Like slightly	6					
Neither like nor dislike	5					
Dislike slightly	4					
Dislike moderately	3					
Dislike very much	2					
Dislike extremely	1					

ANNEXURE – 6

ESTIMATION OF ENERGY (Analytical Chemistry by Dr.Pearson)

Energy of any food products can be derived by the given calculation.

Calculation

Energy (Kcal) = (9.45 x fat) + (4.1 x carbohydrate) + (5.65 x protein)
Carbohydrate = 100 – (moisture + ash + protein + fat).

ANNEXURE – 7

ESTIMATION OF PROTEIN BY MICRO-KJELDAHL METHOD

Estimation of Total Nitrogen

Aim

To determine total nitrogen in the given sample.

Materials Required

50 per cent NaOH, 4 per cent boric acid, 0.1 NHCl, double indicator.

Procedure

0.03 gm of sample kept for digestion. After digestion the digested sample taken to Kjeldal apparatus add 15 ml 50 per cent NaOH and then process carried out in the Kjeldal apparatus finally collect the vapours contains 5 ml of 4 per cent boric acid, after collection of vapours in the container boric acid add few drops of double indicator and titrate against 0.1 NHCl.

Calculation

$$\frac{\text{T.V. x N x 0.014}}{\text{Swt (or) S. Vol.}} \text{ x } 100 = \quad \%$$

Note

Preparation of double indicator:
Protein = Total Nitrogen x 6.25

ANNEXURE – 8

ESTIMATION OF FAT BY VOLUMETRIC METHOD

Aim

To determine the fat content in the given sample.

Chemicals Required

Petroleum ether.

Procedure

Take 2 gm sample and 50 ml petroleum ether and then crush well by using mortar and pestle. Then filter in a Petri plate by using water bath, then calculate the final weight.

Calculation

$$\text{Fat content} = \frac{(\text{Plate Weight} + \text{Oil Weight}) - \text{Plate Weight} \times 100}{\text{Sample Weight}}$$

ANNEXURE – 9

DETERMINATION OF TOTAL CARBOHYDRATE BY ANTHRONE METHOD

Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The basic units of carbohydrates are the monosaccharide which cannot be split by hydrolysis into simpler sugars. The carbohydrate content can be measured by hydrolyzing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharide.

PRINCIPLE

Carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm.

Materials

- 2.5 N – HCl
- Anthrone Reagent : Dissolve 200 mg anthrone in 100 ml of ice cold 95 per cent H_2SO_4. Prepare fresh before use.
- Standard Glucose: Stock – Dissolve 100 mg in 100 ml water. Working standard – 10 ml of stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of toluene.

Procedure

1. Weight 100 mg of the sample into a boiling tube.
2. Hydrolyse by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N – HCl and cool to room temperature.
3. Neutralise it with solid sodium carbonate until the effervescence ceases.
4. Make up the volume to 100 ml and centrifuge.
5. Collect the supernatant and take 0.5 and 1 ml aliquots for analysis.

6. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard, '0' serves as blank.
7. Make up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water.
8. Then add 4 ml of anthrone reagent.
9. Heat for eight minutes in a boiling water bath.
10. Cool rapidly and read the green to dark green colour at 630 nm.
11. Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.
12. From the graph calculate the amount of carbohydrate present in the sample tube.

Calculation

Amount of carbohydrate present in 100 mg of the sample

$$= \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

ANNEXURE – 10

ESTIMATION OF TOTAL ASH

Weight accurately 5 g in silicon dish

↓

Ignite material (sample) in muffle furnace at 600°C
(Not to spill out the sample)

↓

Ashing the sample will take 2 hours

↓

Remove cool in desiccators and weigh

Calculation

$$\text{Total ash (\%)} = \frac{\text{Weight of dish with final ash - Weight of empty dish}}{\text{Weight of sample}}$$

ANNEXURE – 11

DETERMINATION OF ASH INSOLUBLE IN DIL HCL

Reagents
(a) Dilute HCl – Approx 5 N

Procedure

To the ash contained in the dish, add 25 ml of dil HCl, cover with a watch glass and heat on a water bath for 10 minutes. Allow to cool and filter the contents of the dish through Whatman filter paper No 42 or its equivalent. Wash the filter paper with water until the washings are free from acid and return it to the dish. Keep it in the electric oven for 3 hrs to dry. Ignite in a muffle furnace at 550 – 600 0 C till white or grey ash is obtained. Cool in a desiccators

Repeat the process of igniting, cooling and weighing till the difference in two consecutive weighing is less than 1 mg. Note the lowest weight.

Calculation
Ash insoluble in dil HCl = (W2 – W) x 100
On dry wt basis W1 – W
Where
W2 = weight in gm of dish with the acid insoluble ash
W =Weight in gm of empty dish
W1 =Weight in gm of the dish with the dried material.

ANNEXURE – 12

DETERMINATION OF TOTAL DIETARY FIBRE

(Based on AACC Method 32 – 05 and AOAC Method 985.29)

Definition

This method is the simplified modification of the AACC total dietary fibre (TDF) method, 32-05, and the AACC soluble/insoluble dietary fibre method (for oat products), 32-21.

1. Principle: Briefly, 1 g dried food samples (duplicate) are subjected to sequential enzymatic digestion by heat-stable (x-amylase, protease, and amyloglucosidase).

2. Soluble/insoluble dietary fibre determination: Insoluble dietary fibre (IDF) is filtered and then residue is washed with warm distilled water. Combined solution of filtrate and water washings are precipitated with 4 volumes of 95 per cent ethanol (Et OH) for soluble dietary fibre (SDF)

determination. Precipitate is then filtered and dried. Both SDF and IDF residues are corrected for protein, ash and blank for the final calculation of SDF and IDF values.

3. Total dietary fibre determination: SDF is precipitated with EtOH and residue is then filtered, dried and weighed. Total dietary fibre (TDF) value is corrected for protein and ash content.

Scope

This method determines soluble, insoluble and total dietary fibre content in processed foods and raw materials, such as cereal products, fruits and vegetables.

Reagent

1. Phosphate buffer, 0.08 M, pH 6.0. Dissole 1.400 g Na phosphate anhydrate (Na_2HPO_4) (or 1.753 g dehydrate) and 9.68 g Na phosphate monobasic monohydrate (NaH_2PO_4) (or 10.94 g dehydrate) in approximately 700 ml distilled water. Dilute to 1 l with water. Check pH with pH meter.
2. Sodium hydroxide solution, 0.275 N. Dissolve 11.00 g ACS grade NaOH in approximately 700 ml distilled water, using appropriate handling precautions, in 1 l volumetric flask. Cool and dilute to volume with water.
3. Hydrochloric acid solution, 0.325 N. Dilute stock solution of known titer (i.e., 325 ml of 1.0 N HCl) to 1 l with water in volumetric flask.

Procedure

Preparation of Sample

Total dietary fibre should be determined on an as-is basis on dried, low-fat or fat-free sample. Homogenize sample and dry overnight in 70°C vacuum ovens. Cool in desiccators, reweigh and record weight loss due to drying. Dry-mill portion of dried sample to 0.3 – 0.5 mm mesh. If sample cannot be heated, freeze-dry before milling, if high fat content (> 10%) prevents proper milling, defat with petroleum ether three times with 25 ml portions (per g of sample) before milling. When analyzing mixed diets, always extract fat before determining total dietary fibre. Record weighs loss due to fat. Correct final % dietary fibre determination for both moisture and fat removed. Store dry-milled sample in capped jar in desiccators until analysis is run.

Method

Run blank through entire procedure along with samples to measure any contribution from reagents to residue.

1. Weigh duplicate 1 g samples, accurate to 0.1 mg, into 400 ml tall-form beakers. Sample weights should differ by less than 20 mg from each other. Add 50 ml phosphate buffer (pH 6.0) to each beaker and check pH with pH meter. Adjust if pH does not equal 6.0 ± 0.1.
2. Add 50 μL heat-stable α-amylase solution.
3. Cover beaker with aluminium foil and place in boiling water bath for 15 minutes. Shake gently at 5 min. intervals.
4. Cool solutions to room temperature.
5. Adjust to pH 7.5 ± 0.1 by adding 10 ml 0.275 N NaOH solution. Check pH with pH meter.
6. Add 100 μl of protease solution.
7. Cover beak with aluminium foil and incubate at 60°C with continuous agitation for 30 min.
8. Cool and add 10 ml 0.325 N HCl solution to adjust pH to 4.5 ± 0.2. Check pH with pH meter.
9. Add 200 μl amyloglucosidase, cover with aluminium foil and incubate 30 min. at 60°C with continuous agitation.
10. Add 280 ml 95% EtOH preheated to 60°C (measure volume before heating). Let precipitate form at room temperature for 60 min.
11. Weigh crucible containing Celite to nearest 0.1 mg, then wet and distribute bed of Celite in crucible by using stream of 78% EtOH from wash bottle.
12. Apply suction to draw Celite onto fritted glass as even mat. Maintain suction and quantitatively transfer precipitate from enzyme digest to crucible.
13. Wash residue successively with three 20 ml portions of 78% EOH, two 10 ml portions of 95% EtOH, and two 10 ml portions of acetone. In some cases, gums may form during filtration, trapping liquid in residue. If so, break surface film with spatula to improve filtration. Long filtration times can be avoided by careful intermittent suction throughout filtration.
14. Dry crucible containing residue overnight in 70°C vacuum oven or 105°C air oven.
15. Cool in desiccators and weigh to nearest 0.1 mg. Subtract crucible and Celite weights to determine weight of residue.
16. Analyse residue from one sample of set of duplicates for protein by AACC method 46-13, using N x 6.25 as conversion factor.
17. Incinerate second residue sample of duplicate for 5 hr. at 525°C. cool in desiccators and weigh to 0.1 mg. Subtract crucible and Celite weights to determine ash.

ANNEXURE – 13

ESTIMATION OF MOISTURE BY GRAVIMETRIC METHOD

Weigh accurately 5 gm sample in dish (evenly distributed)

↓

Place the dish in Hot air oven at 105°C

↓

Drying time : 5 hrs

↓

Remove the dish from oven and keep it in desicator for 15 mins (approx.)

↓

Remove from desicator and weigh it

Calculation

$$\text{Moisture (\%)} = \frac{\text{Weight of dish with sample before drying - Weight of the dish with sample after drying}}{\text{Weight of sample taken}}$$

ANNEXURE – 14

DETERMINATION OF VITAMIN A BY SPECTROPHOTOMETRIC METHOD

Reagents

1. Aldehyde-free alcohol : Reflux 95% C_2H_5OH with KOH and A1 metal for 6-8 hours and then redistill.
2. Repurified ethyl ether : Reflux ether over $SnCl_2$ for 4-6 hours and then distill.
3. Repurified petroleum ether : Skellysolve B (SSB)
   a. Shake SSB with concentrated sulfuric acid unit acid no longer turns dark.
   b. Wash SSB with water until acid-free.
   c. Dry SSB over $CaCl_2$ and then distill.
4. Activated glycerol dichlorohydrin :
5. Ammonia: Reagent grade.
6. Chloroform: Reagent grade.
7. Vitamin A acetate: 1 g, capsule 10,000 U.S.P. units of vitamin A.

Extraction Procedure

1. Add 100 ml of sample to a 500 ml separatory funnel.
2. Add 15 ml of concentrated NH_4OH. Shake.

3. Add 100 ml of redistilled C_2H_5OH. Shake
4. Add 100 ml of peroxide-free ether. Shake vigorously.
5. Add 25 ml. of redistilled SSB. Shake vigorously.
6. Allow to stand for 20 minutes.
7. Draw off lower layer into separator funnel and re-extract with 25 ml. of ether and 10 ml of SSB.
8. Let combined extract stand for 30 minutes.
9. Filter combined extract through glass wool into a 500 ml, round-bottom flask, and rinse funnel and glass wool three times with 5-10 ml. of ether each time.
10. Evaporate ether in water bath at 60 – 70°C, under vacuum by connecting flask to aspirator.
11. Evaporate all ether and pour fat into 100 ml. beaker with about six glass beads.
12. Solidify in iced bath and pour out remaining water and alcohol.
13. Melt fat and place it in vacuum oven at 45 – 50°C under 5-10 in. vacuum for about 1 hour and then about 3-4 hours at 20-25 in. vacuum.
14. Pipette fat into a 10 ml. graduated, glass-stoppered cylinder without touching sides of cylinder.
15. Add equal amount of chloroform and mix well.

Development of Colour

1. Pipette 1 ml. of fat-chloroform solution and 4 ml of activated glycerol dichlorohydrin into a 10 ml. graduated, glass stoppered cylinder.
2. Mix contents by inverting the cylinder 10 times and place it in a water bath at 25°C.
3. Red the colour at 500 mμ in a spectrophotometer, using 1 ml. of chloroform and 4 ml. of activated glycerol dichlorohydrin as the blank.

Calculation
Construction of Standard Graph

1. Dissolve a known amount of vitamin A acetate in chloroform and dilute to known volume of 1-16 γ of vitamin A per milliliter.
2. Develop color in standard solutions and plot concentration versus log per cent transmission and determine the equation.
3. Check every lot of glycerol dichlorohydrin against a known standard of vitamin A before using.
4. Substitute the per cent transmission obtained in the equation N = a – log % / b, where N is the concentration and a and b are constants calculated by the least square method (6).
5. Multiply the final value by proper dilution factor.

Correction for Carotene Interference

1. Read standard carotene solutions (0 – 3.5 γ per milliliter of $CHCl_3$) at 420 mμ, using chloroform as a blank.
2. Read the corresponding value of that carotene concentration which will give a color with glycerol dichlorohydrin at 550 mμ.
3. Construct a standard graph for carotene at 420 mμ and 550 mμ with glycerol dichlorohydrin and determine the equations as for vitamin A.
4. Determine the per cent transmission which would be given by the amount of carotene present in the sample and obtain the equivalent per cent transmission on the 550 mμ graph. Calculate the equivalent amount of vitamin A and subtract the value from the total vitamin A.

ANNEXURE – 15

DETERMINATION OF VIT C [ASCORBIC ACID}
Volumetric Method

Ascorbic acid otherwise known as vitamin C is an antiscorbutic. It is present is gooseberry, bitter gourd etc. in high amounts. Generally it is present in all fresh vegetables and fruits. It is a water soluble and heat-labile vitamin. The method described below is easy, rapid and a large number of samples can be analysed in a short time.

Principle

Ascorbic acid reduces the 2, 6-dichlorophenol indophenols dye to a colourless leuco-base. The ascorbic acid gets oxidized to dehydroascorbic acid. Though the dye is a blue coloured compound, the end point is the appearance of pink colour. The dye is pink coloured in acid medium. Oxalic acid is used as the titrating medium.

Materials

- Oxalic acid 4%
- Dye solution: Weigh 42 mg sodium bicarbonate into a small volume of distilled water. Dissolve 52 mg, 2, 6-dichloro phenol indophenols in it and make up to 200 ml with distilled water.
- Stock Standard Solution : Dissolve 100 mg ascorbic acid in 100 ml of 4 per cent oxalic acid solution in a standard flask (1 mg . ml).
- Working Standard: Dilute 10 ml of the stock solution to 100 ml with 4 per cent oxalic acid. The concentration of working standard is 100 μg / ml.

Procedure

1. Pipette out 5 ml of the working standard solution into a 100 ml conical flask.
2. Add 10 ml of 4 per cent oxalic acid and titrate against the dye (V_1 ml). End point is the appearance of pink colour which persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid.
3. Extract the sample (0.5 – 5 g depending on the sample) in 4 per cent oxalic acid and make up to a known volume (100 ml) and centrifuge.
4. Pipette out 5 ml of this supernatant, add 10 ml of 4 per cent oxalic acid and titrate against the dye (V_2 ml).

Calculation

Amount of ascorbic acid mg / 100 g sample

$$= \frac{0.5\,\text{mg}}{V_1\,\text{ml}} \times \frac{V_2}{5\,\text{ml}} \times \frac{100\,\text{ml}}{\text{Weight of the sample}} \times 100$$

ANNEXURE – 16

DETERMINATION OF SODIUM

1. AIM:

To determine the Sodium content in the given sample.

2. CHEMICALS REQUIRED:

Ammonium acetate, 25 ppm Nacl solution, 50 ppm Nacl solution.

3. PROCEDURE:

5 gm of the sample is dissolved in 25 ml of ammonium acetate solution. This solution is filtered. The Sodium content is calculated using flame photometer.

4. CALCULATION:

Sodium content = Sodium value X Factor X 100

$$\frac{\qquad\qquad\qquad\qquad\qquad\qquad}{\text{Sample weight}}$$

ANNEXURE – 17

DETERMINATION OF POTASSIUM

1. AIM:

To determine the potassium content in the given sample.

2. CHEMICALS REQUIRED:

Ammonium acetate, 25 ppm kcl solution, 50 ppm kcl solution.

3. PROCEDURE:

5 gm of the sample is dissolved in 25 ml of ammonium acetate solution. This solution is filtered. The potassium content is calculated using flame photometer.

4. CALCULATION:

$$\text{Potassium content} = \frac{\text{Potassium value X Factor X 100}}{\text{Sample weight}}$$

ANNEXURE – 18

DETERMINATION OF THE AEROBIC COLONY COUNT IN FOODS BY HPB METHOD

Materials and Special Equipment

1. Plate count agar (PC)
2. Peptone water diluents (0.1%) (PW)
3. 2% sodium citrate (tempered to 45°C) (for cheese samples only)
4. Sodium 2, 3, 5 triphenyltetrazolium chloride (0.1%) (optional)
5. 1N HCl and 1N NaOH
6. pH meter or paper capable of distinguishing to 0.3 to 0.5 pH units within a range of 5.0 to 8.0
7. Stomacher, blender or equivalent.
8. Incubator capable of maintaining the growth temperature required for the specific type of aerobic bacterial being enumerated (i.e., for psychrophillic bacteria: 15 - 20°C, for mesophilic bacteria : 30 - 35°C and for thermophilic bacteria : 55°C) and 45° water bath.
9. Colony counting device (optional).

Procedure

Determine which type of aerobic bacteria is being enumerated. Analyze each sample unit individually. The test shall be carried out in accordance with the following instructions.

Handling of Sample Units

- During storage and transport, the following shall apply with the exception of shelf-stable products, keep the sample units refrigerated (0 - 5°C). Sample units of frozen products shall be kept frozen.
- Thaw frozen samples in a refrigerator or under time and temperature conditions which prevent microbial growth or death.
- Analyze sample units as soon as possible after receipt in the laboratory.

Preparation of Media

- Prepare plate count agar and dispense in appropriate quantities. Sterilize.
- Temper prepared melted agar in a water bath to 45°C ensuring that the water level is 1 cm above the level of the medium in the bottles.
- Clean surface of working area with a suitable disinfectant.
- Clearly mark the duplicate Petri plates.

Preparation of dilutions

- Prepare sterile 0.1% peptone water diluents.
- To ensure a truly representative analytical unit, agitate liquid or free flowing materials until the contents are homogeneous. If the sample unit is a solid, obtain the analytical unit by taking a portion from several locations within the sample unit.
- Prepare a 1 : 10 dilution o the food by aseptically blending 25 g or ml (the analytical unit) into 225 ml of the required diluents, as indicated in Table 1. If a sample size other than 25 g or ml, is used, maintain the 1 : 10 sample to dilution ratio, such as 11 (10) g or ml into 99 (90) ml.
- If a homogeneous suspension is to be obtained by blending, the blending time should not exceed 2.5 min in order to prevent over-heating. With foods that tend to foam, use blender at low speed, and remove an aliquot from below the liquid / foam interface. If a homogeneous suspension is to be obtained by shaking, shake the dilution bottles 25 times through a 30 cm arc in approximately 7 sec.
- In some instances it may be advantageous to prepare the initial dilution on a percent basis to obtain a more accurate test material weight than is attained by the dilution ratio method. i.e., a 10 solution (suspension) is represented by 10 g (ml) per 100 g (ml) of solution (suspension), whereas a 1 : 10 dilution is based on 10 g (ml) of product (solute) plus 90 g (ml) of diluents (solvent).
- Check the pH of the food suspension. If the pH is outside the range of 5.5 – 7.6, adjust the pH to 7.0 with sterile NaOH or HCl.

- Prepare succeeding decimal dilutions as required, using a separate sterile pipette for making each transfer.
- Shake all dilutions immediately prior to making transfers to ensure uniform distribution of the microorganisms present.

Plating

- Agitate each dilution bottle to resuspend material that may have settled out during preparation.
- Pipette 1 ml or 0.1 ml of the required dilutions to appropriately marked duplicate petri plates.
- In the case of products that tend to adhere to the bottom of the plants, add the inoculums to 1.0 ml of sterile diluents previously placed in the Petri plate.
- Pour 12 – 15 ml of tempered agar into each plate and mix by rotating and tilting. Allow to solidify. Plates should be poured not more than 15 min after preparation of dilutions.

Incubation

Incubate plates in the inverted position for 48 h ± 4 h. Incubation temperature is dependent on the growth temperature requirements of the target organisms (for psychrophilic bacteria : 15 – 20°C, or mesophilic bacteria : 30 – 35°C, and for themophilic bacteria : 55°C). The plates used to enumerate psychrophilic and thermophilic bacteria may be incubated up to 5 days. Other combinations of time and temperature may be used, if the lab has verified their suitability. Avoid crowding or excessive stacking of plates to permit rapid equilibration of plates with incubator temperature.

Counting Colonies

- Count colonies promptly after the incubation period.
- If possible, select plates with 20 – 200 colonies (including pinpoint colonies). If counts do not fall within this range select plates that fall nearest to the 20 – 200 range.
- If plates contain colonies which spread, select a representative portion of the plates free from spreaders, if possible, and count the colonies in this area. The total count of the entire plate is estimated by multiplying the count for the representative area counted by the reciprocal of the fraction of the plate counted. E.g. 30 colonies counted on 1/ of area of the plate, count for the whole plate: 30 x 4 = 120 colonies.

Differentiation of Colonies from Interfering Particles

- Alternatively, after incubation flood plates with 2 ml of 0.1% 2, 3, 5, triphenyltetrazolium chloride. Gently rock plates from side to side to cover the entire area with solution. Pour off excessive solution and allow the plates to remain at room temperature for 3 hrs. in an inverted position. The bacteria reduce the indicator to a formazan which colours

the colonies red and aids in distinguishing the food particles. Colonies cannot be picked for isolation after this method has been used.

Recording Results

- Calculate the average count (arithmetic mean) of the duplicate plates.
- When reporting results (Table II) round-off the counts to two significant figures and record only the first two left hand digits: (e.g., record 2,850 as 2,900).
- If the lowest dilution plated shows no colonies, the recorded value will be the lowest average obtainable with given volume plated onto a given set of replicate plates proceeded by a "less than" (<) sign, e.g., for one milliliter and a set of duplicate plates (1 ml/plate) the value is < 0.5. The lowest possible average with one colony on one of the two duplicate plates is : (1 + 0) / 2 = 0.5. This value is for a 10° dilution (Dilution Factor = 1). For other dilutions, the numerical value of 0.5 must be multiplied by the reciprocal of the dilution: i.e., the Dilution Factor, e.g. $1 / 10^{-1} = 10$. To compute the Aerobic Colony Count (ACC), use the formula: N = A x D, where N is the number of colonies per g (ml) of product, A is the average count per plate, and D is the respective dilution factor.

ANNEXURE – 19

DETERMINATION OF ANTI OXIDANT ACTIVITY BY ORAC ASSAY METHOD

The ORAC assay is based upon the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds such as [2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH).In this manner, the ORAC assay uses a biological relevant radical source and it combines both inhibition time and degree of inhibition into one quantity. Recent modifications to this assay include the use of fluorescein as the probe, the adaptation to a high-throughput format, and the ability to measure the lipophilic, hydrophilic, and total antioxidant capacity of a substance. These modifications, along with no washing steps, have greatly simplified the ORAC assay; thereby making it ideally suited to measure the antioxidant capacity of a substance.

Assay Principle

Over time Reactive Oxygen Species (ROS), generated from the thermal decomposition of AAPH, will quench the signal from the fluorescent probe fluorescein. The subsequent addition of an antioxidant produces a more stable fluorescence signal, with signal stability depending on the antioxidant's capacity .The data points are summarized over the time by the evaluation software. This is then compared to the standard, Trolox®, and is expressed as micromoles of Trolox® equivalents (TE) per gram or per milliliter of sample (µmole of TE/g or µmole of TE/mL)

Materials and Methods

- Food samples
- Costar® 96 well black opaque plate, Corning Costar Corporation, Cambridge, MA, cat. no. 3792
- Fluorescein Sodium, 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox®), L (+)-ascorbic acid, Epicatechin gallate, [2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)] were obtained from Sigma-Aldrich
- Plate sealer, BMG LABTECH, Aylesbury, UK, Cat. No. 77400-05
- Thermostar, BMG LABTECH, Offenburg, Germany
- FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany

Test Protocol

Different dilutions of Trolox® (200 µM – 12.5 µM) and sample compounds (ascorbic acid and epicatechin gallate, two known antioxidants) were prepared in phosphate buffer (pH 7.4 10 mM). All solutions were and should be prepared fresh daily.

In every working well the following was pipetted in triplicate:

- Fluorescein, 150 µL of a 10 nM solution
- For standard, 25 µL of Trolox® dilution
- For sample, 25 µL of sample dilution
- For blank, 25 µL of phosphate buffer

The micro plates were sealed followed by incubation for 30 min at 37°C in a Thermostar micro plate incubator without shaking. Alternatively, the FLUOstar OPTIMA itself can perform the incubation step.

After incubation, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 90 sec to determine the background signal. After 3 cycles, 25 µL (240 mM) of AAPH was injected with the help of onboard injectors. Alternatively AAPH can also be added manually with a multi-channel-pipette. This has to be done as quickly as possible since the ROS-generator displays immediate activity after addition. The test was resumed and fluorescent measurements were taken up to 90 minutes.

Instrument Settings Overview

Mode:	**Fluorescence intensity, plate mode**
Optic :	Top optic, combination head
Filter:	Exc. 485 nm Em. 520 nm
No. of cycles:	60
Measurement start time:	>0.0 sec
No. of flashes:	10
Cycle time:	90 sec
Gain:	Adjusted for each plate

Recording Results

To obtain the values for Trolox® equivalents (TE) of antioxidants with known concentration over the desired concentration range one can divide the slopes of the regression curves:

$$\text{TE over considered concentration range} = \frac{\text{slope regression curve (sample)}}{\text{slope regression curve (Trolox}^{®}\text{)}}$$