# IV. General Test Methods

## 1. Gas Chromatography

This method is to identify and quantify the separated phases from an evaporated sample. The evaporated sample gets separated by the interaction with column filler phase in the column while is carried by a gas (carrier gas). If the column filler is solid, it is called gas-solid chromatography. If the column filler consists of inert solid coated with liquid, it is called gas-liquid chromatography.

In the former case, passing of solution is delayed by absorption or expulsion and for the latter case, it is done by distribution between gaseous mobile phase and stationary liquid. The components that affects the separation include the flow rate of carrier gas, length and inside diameter of column, particle size of solid porous support material, type of liquid used, the relative volume of liquid vs. solid porous support material, and temperature.

### A. Apparatus

Basic equipment for Gas Chromatography consists of carrier gas inlet, sample injection port, column, detector and data recording equipment.

### B. Procedure

Unless stated otherwise, the following method is to be used. After pre-setting measurement value, column detector temperature and carrier gas flow rate of the carrier gas are set up by the conditions as specified for each item. A prescribed volume of test solution or standard solution specified for each item is injected into the sample injection port using a micro-syringe for gas chromatography. The separated components are collected by a detector and a chromatogram is obtained using a recorder.

The peak location of the component on chromatogram is indicated by retention

time (time after injecting test solution up to the peak position) or retention volume (retention time x carrier gas volume). These values are characteristic of each substance under certain conditions.

These are used to identify the components in the sample

Quantitative information can be obtained from the peak area or peak height from a chromatogram. Generally, one of the following methods is used.

(1) Internal Standard Method

A set of standard solutions are prepared by incrementally adding a known amount of standard test material to a certain amount of internal standard material as specified for each item. A certain amount of each standard solution is injected. A calibration curve is prepared from the obtained chromatogram by plotting the ratios of peak areas or peak heights between standard test material and internal standard material (vertical axis) vs. the ratio between the amounts of standard test material and internal standard or the amount of standard test material (horizontal axis).

A test solution is prepared by the method specified for each item. The same amount as in standard solution is added to the test solution. Then a chromatogram is obtained under the same conditions as in standard solutions. The amount of the component is obtained from the ratio of peak area or peak height between the test component and the internal standard. The internal standard is selected so that it is stable and completely separable from the test component as well as other components in the test material.

(2) Absolute Calibration Curve Method

A set of standard test solutions are prepared with incremental concentrations. A certain amount of each solution is precisely injected. A calibration curve is prepared from the obtained chromatogram by plotting the peak areas or peak heights of the standard test component (vertical axis) vs. the amount of the standard test component. material and internal standard or the amount of standard test material (horizontal axis). A test solution is prepared by the method specified for each item. A chromatogram is obtained under the same

conditions as the calibration curve. The content of the test component is obtained from the calibration curve. In this method, all the test conditions should be kept strictly consistent.

(3) Area Percentage Method

The peak area sum of all the components is set as 100. The content ratio of each component is obtained from peak area ratio. Peak height and peak area in (1), (2), and (3) are usually measured by the following methods.

(A) Peak Height Method

A vertical line is drawn from the peak vertex to the horizontal axis. A tangent line connecting the base line of the peak is drawn. The length between the peak vertex and the intersection is measured.

(B) Peak Area Method

1) Width at half-height method : Peak width at the half maximum is multiplied by the peak height.

2) Weight Method : The peak is cut out from the chromatogram and weighed directly.

3) Automatic Integration Method : Signals from the detector is integrated using an automatic integrator.

Note : Reagents and solutions used in the test should not show any peaks that interfere with the measurement.

## 2. Residue on Ignition

Sulfuric acid is added to the sample, which is then heat treated to test the amount of residues. Unless otherwise specified, 1~2 g of sample is precisely weighed into a previously weighed platinum, quartz, or porcelain crucible. sample is wetted with sulfuric acid and slowly reduced to ash by heating. After cooling, 1 ml of sulfuric acid is added to the crucible, which is then slowly heated until sulfuric acid vapor subsides. Then the crucible is heat treated at 450~550° until

the residues turn white. The crucible is cooled in a desiccator and weighed. If there is no specification for the heat treatment time, it is heated until the weight becomes constant.

## 3. Loss on Drying and Loss on Ignition

This method is used to measure the amount of water content and other volatile material in a sample upon drying or heat treatment.

### A. Loss on Drying

If a sample is a large crystal or lump, it is ground quickly to a diameter of approximately 2 mm or less. Unless otherwise specified, 1 ~ 2 g of the ground sample is layed out flatly in a weighing bottle(which is previously dried for approximately 30 minutes and weighed) to form a layer of 5 mm or less and dried in a drier without a cap as specified for each sample. The cap is placed on the bottle, which is then weighed. If it is dried by heating, the weight is measured after cooling. If the sample melts at a temperature lower than the specified drying temperature, it is dried for 1 ~ 2 hours at a temperature which is 5～10° lower than the melting point and dried at the specified temperature.

### B. Loss on Ignition

Should follow the procedure in loss on drying. Unless otherwise specified, heat treatment temperature is carried out at 450～550° and platinum, quartz or porcelain crucible is used instead of weighing bottle.

## 4. Refractive Index

Refractive Index of a material is a light velocity ratio within a material vs. in vacuum. It is the ratio of incident angle vs. refraction angle of the sinusoidal wave

of the light. Generally, refractive index varies with wavelength and temperature. In this test, refractive index (n) is measured using D (589 nm) from sodium spectrum as a light source at a temperature t° in air. Unless otherwise specified, refractive index $n_D^{20}$ is measured using Abbe refractometer at a temperature within ±0.2°of the specified temperature.

## 5. Lead Limit Test (Dithizone Method)

Unless otherwise specified, the lead limit test by dithizone is carried out by one of the following methods. In this test, water with lowest possible lead content is used. Glass apparatus should be washed with diluted warm nitric acid (1→2) followed by water.

### A. Solution

◦**Amonium citrate solution** : 45 g of ammonium citrate (II) are dissolved in 100 ml of water, where 2~3 drops of phenol red solution are added. Ammonia water is drop-wise added to this solution until the solution becomes red. To remove lead from this solution, it is extracted with 20 ml each of dithizone solution until the color of dithizone solution becomes its characteristic green.

◦**Potassium cyanide solution** : 50 g of potassium cyanide are dissolved in 100 ml of water. This solution is processed with dithizone solution by following the same procedure as in the previous ammonium citrate solution section. The residual dithizone is extracted out from the solution with chloroform. Water is added to the resulting solution to make the total volume to 500 ml.

◦**Ammonia potassium cyanide solution** : To 20 ml of potassium cyanide solution in the previous section, 15 ml of ammonia water and water are added to make the total volume to 100 ml.

◦**hydroxylamine hydrochloride solution** : Hydroxylamine hydrochloride (20 g) is dissolved in water (total volume = approximately 65 ml), where 2~3 drops of thymolblue solution are added. Ammonia water is added to the resulting solution until the color turns yellow, where 10 ml of 4% sodium diethyldithio carbamate solution is added and set aside for 5 minutes. It is then extracted with 10~15 ml each of chloroform. At the end point, a mixture of 5 ml of the extract and 5 drops of cupric sulfate solution (1→100) does not becomes yellow color. To the aqueous phase, 2~3 drops of thymol blue solution, where dilute nitric acid is added until the solution turns red. The total volume of the solution is make to 100 ml with water.

◦**Dthizone solution for extraction** : Dithizone (30 mg) is dissolved in 1,000 ml of chloroform, where 5 ml of alcohol is added. The solution is stored in a cold place. Before use, necessary amount of the solution is mixed with approximately a half the volume of 1% nitric acid. Water layer is removed from the mixture.

◦**Dthizone standard solution** : Dithizone (10 mg) is dissolved in 1,000 ml of chloroform. It is then stored in a light shielded lead free bottle in a cold place.

◦**Lead standard solution (for dithizone)** : Lead standard solution (10 ml) is diluted to 100 ml with 1% nitric acid. This solution is prepared just before use. It contains 1 μg of Pb per 1 ml.

B. Procedure

Unless otherwise specified, a specified amount of test solution is taken into a separatory funnel, where 6 ml ammonium citrate solution (10 ml for lead limit test in iron salt), 2 ml of potassium cyanide solution, 2 ml of hydroxylamine hydrochloride solution, and 2 drops of phenol red solution are added. Ammonia water is added until the solution turns red. To this solution, 5 ml of dithizone solution for extraction is added and the solution is shaken for approximately 15 seconds. The chloroform phase is transferred into another separatory funnel. This extraction is repeated three times. The collected chloroform extracts are mixed

well by shaking for 30 seconds with 20 ml of 1% nitric acid and the chloroform layer is discarded. With small amount of chloroform, acid solution is washed several times (if necessary) to completely remove dithizone. Small drops of chloroform are removed from the surface of acid solution. To this acid solution, 5 ml of dithizone standard solution and 4 ml of potassium cyanide solution are added and mixed for 30 seconds by shaking. The violet color in the chloroform layer should not be deeper than that of the solution, which is prepared by the same procedure with 8 ml of lead standard solution (for dithizone) instead of sample.

## 6. Methoxyl Determination

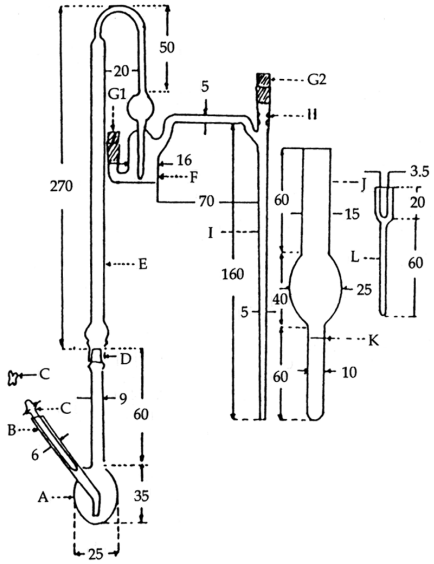
This method is to quantitatively analyze methoxyl group by the following procedure. Hydriodic acid is added to the sample, which generates methyl iodide upon heating. Methyl iodide is oxidized with bromine, where potassium iodide and diluted sulfuric acid. The resulting iodine is titrated with sodium thiosulfate solution.

$$ROCH_3 + HI = CH_3I + ROH$$
$$CH_3I + 3Br_2 + 3H_2O = CH_3Br + 5HBr + HIO_3$$
$$HIO_3 + 5HI = 3I_2 + 3H_2O$$

### A. Apparatus

The apparatus is depicted in the figure below (unit : mm).

A : flask for decomposition
B : gas inlet tube
C : ground joint
D : air cooling part




E : gas washing part

F : glass stopper

G : round face ground joint

H : gas pipe

J : absorbent tube,

K : gas outlet tube

## B. Preparation of Scrubbing Solution and Absorbing Solution

1) Washing solution : Red phosphor (1 g) is dispersed in 100 ml of water.

2) Absorbent solution : Potassium acetate (15 g) is dissolved in 150 ml mixture of glacial acetic acid and anhydrous acetic acid (9:1), 145 ml of which is mixed with 5 ml of bromine. This solution is prepared just before use.

## C. Procedure

Gas washing part E is filled to 1/2 with washing solution and absorbent tube J is filled with approximately 20 ml of absorbent solution. sample, which corresponds to approximately 6.5 mg of methoxyl group ($CH_3O$ : 31.03), is precisely weighed into a flask for decomposition A, where boiling stone and approximately 6 ml of hydroiodic acid are added. The ground joint C of A is

wetted with 1 drop of hydroiodic acid, connected to an air condenser D, which is then connected to the round face ground joint G where appropriate amount of silicone grease is applied. Nitrogen or carbon dioxide is introduced through the gas inlet B. Using an appropriate regulator, the gas flow rate is adjusted to roughly 2 bubbles per second through the gas outlet E. A is immersed in an oil bath, which is heated so that it reaches 150° in 20 ~ 30 minutes. It is then boiled for 60 minutes at that temperature. After removing the oil bath, it is cooled in air and G is removed. The content in J is transferred into a 500 ml Erlenmeyer flask (with a stopper) that is filled with 10 ml of sodium acetate solution is (1→5). J is washed several times with water into the flask. Water is added to bring the total volume to approximately 200 ml. While shaking, formic acid is drop-wise added until the red color of bromine disappears and 1 ml of formic acid is added. After adding 3 g of potassium iodide and 15 ml of dilute sulfuric acid, a cap is placed and the flask is shaken gently and set aside for 5 minutes. The separated iodine is titrated with 0.1 N sodium thiosulfate solution (indicator : 1 ml of starch solution). Separately, a blank test is carried out by following the same procedure.

0.1 N sodium thiosulfate solution 1 ml = 0.5172 mg $CH_3O$

## 7. Thin Layer Chromatography

### A. Preparation of Thin Layer Plate

A glass plate with smooth surface and uniform thinkness (50×200 mm or 200×200 mm) is fixed on a thin layer preparation plate. Glass surface is wiped clean with gauze wetted with alcohol. An appropriate amount of absorbant is well dispersed in water (approximately 1:1) by shaking for approximately 30 seconds. Using an applicator, the glass plate is uniformly coated with the dispersion (0.2 ~ 0.3 mm thickness). After setting aside for approximately 10

minutes, it is dried for 30 minutes at 105~120°(or follow other directions if specified), activated, and stored in a desiccator.

B. Procedure

Developing solution is filled up to approximately 10 mm from the bottom of the developing bath. A cap is placed on the bath so that the bath is saturated with the vapor of the developing solvent. Test solution and standard solution are spotted at 2 cm from the bottom of the thin layer plate. These spots are separated approximately 1.5 cm from each other and their diameter should not exceed 5 mm. Spots are completely dried and the plates are carefully inserted into the developing bath so that the solvent doesn't touch the spots. The bath is sealed with a cap and the solvent is developed up to 10~15cm (approximately 15~60 minutes). The plates are air-dried and oveserved under a UV light (254 nm or 360 nm) or sunlight. If necessary, a spray reagent is used and the spots are compared. Rf values are obtained by the following equation.

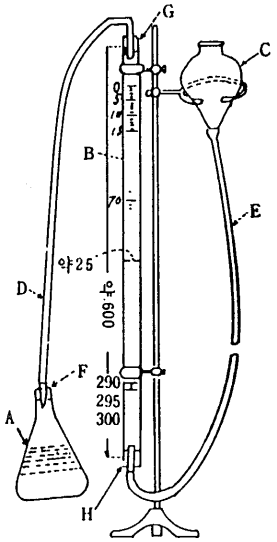
$$Rf = \frac{Distance\ from\ the\ starting\ line\ to\ the\ center\ of\ the\ spot}{Distance\ from\ the\ starting\ line\ to\ the\ solvent\ front}$$

## 8. Quantitative Test for Generated Gas

A. Apparatus

The apparatus is depicted in the figure below (unit : mm).

A : Thick Erlenmeyer flask for gas generation (approximately 250 ml volume)
B : Gas burette (300 ml volume with 0.5 ml graduation)
C : Level bottle (approximately 600 ml volume)
D and E : Rubber tube
F, G and H : Rubber stopper

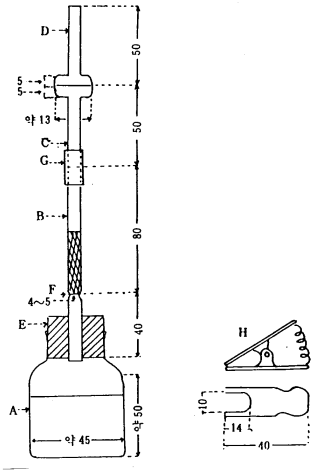



Dilute sulfuric acid is added to a level bottle C so that a weak acidity is established with methyl orange. However, when ammonia is being measured, distilled water is used instead of diluted sulfuric acid. After the temperature reaches 85° by adding 200 ml of boiling water to gas generation flask A, 2 g of sample (2 g of sample with a mixing ratio given in the directions if it is Type 2 synthetic swelling agent) is wrapped in Obrite paper, which is then again wrapped with a fliter paper. It is then placed in the flask, which is immediately connected to the gas burette with a rubber tube. The flask is gently shaken occasionally. The volume of the generating gas is measured in 3 minutes.

## 9. Arsenic Limit Test

### A. Apparatus

The apparatus is depicted in the figure below (unit : mm).




A : approximately 60 ml generator bottle with 40 ml indicating line.

B : glass tube with 6.5 mm inner diameter

C and D : a ground joint glass tube with 6.5 mm inner diameter and 18 mm outer diameter at the joint. Inner joint and the outer joint forms a concentric circle.

E : rubber stopper

F : narrow part of the glass tube B. Glass wool is inserted up to this part.

G : rubber board

H : clamp

The glass tube B is filled with glass wool up to the height of approximately 30 mm from F and soaked uniformly with a 1:1 mixture of water and lead acetate solution. Then, the solution is sucked in from the bottom of the tube. The excess liquid in the wool and the glass wall is removed.

Right before the use of apparatus, a mercuric bromide test paper is inserted in the joint of the glass tubes C and D. Both tubes are secured with a clamp.

## B. Procedure

Unless otherwise specified, a required amount of test solution is transferred into the generator bottle and, if necessary, it is neutralized with ammonia water or ammonia solution. Then, 5 ml of diluted hydrochloric acid (1→2) and 5 ml of

potassium iodide solution are added, which is set aside for 2~3 minutes. To this solution, 5 ml of stannous chloride solution is added, which is set aside for 10 minutes. Water is added to bring the total volume to 40 ml, where 2 g of granular zinc. The glass tubes, B, C, and D are inserted into the rubber stopper, which is then placed on the generator bottle. The bottle is immersed (up to approximately 3/4 of its height) in a water bath at 25° and kept for 1 hour.

C. Preparation of Color Standard

Unless otherwise stated, 1 ml of arsenic standard solution or the amount corresponding to the specifications is transferred into the generator bottle. The same procedure described above is followed.

D. Arsenic Limit

The procedures in B and C should be simultaneously followed and at least two sets of apparatus should be used. Right after the test, the mercuric bromide test paper is taken out and the colorimetry is carried out while avoiding direct sunlight. Here, the resulting color in B should not be darker than that in C. If the colors obtained from the same procedure are different, the test should be repeated.

E. Notice on Procedure

(1) The test solution used in this test or the solution used to make the test solution should not be involved in color reaction at all in the test.

(2) To ensure that the generated gas does not leak at all, the joints, where the mercuric bromide test paper is inserted, should be kept tight.

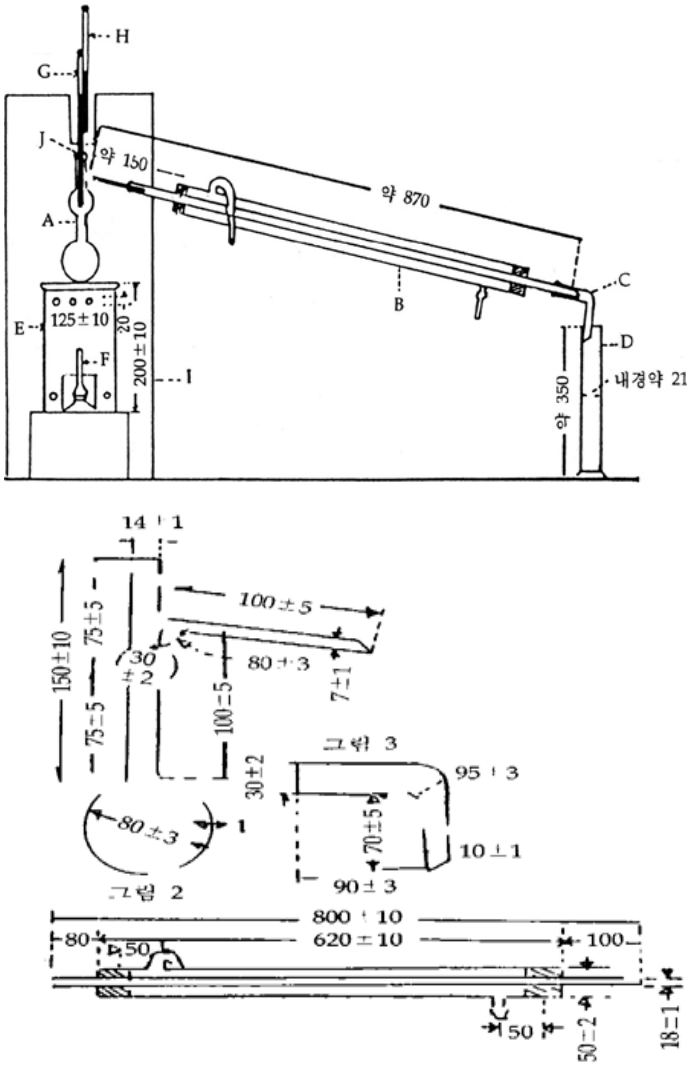
(3) The color of the mercuric bromide test paper fades by the exposure to light, heat and moisture. Therefore, colorimetry should be carried out immediately. The discoloration can be avoided temporarily by storing the paper in a desiccator.

## 10. Boiling Point and Amount of Distillate

Method 1

A. Apparatus

The apparatus is depicted in the figures (1~4) below (unit : mm).




A : Distillation Flask(It is made of hard glass with the capacity of about 300 ml. Refer to figure 2.)

B : Cooling Tube(It is made of hard glass. See the figure 4)

C : Adapter (refer to figure 3)

D : volumetric cylinder (100 ml with 1 ml graduation)

E : Support (It is a metal cylinder with several ventilation holes and it can control the flame of the burner. At the top of the support, two asbestos plates, which are approximately 6 mm thick and have a 30~40 mm circular hole at the center, are placed.)

F : Burner

G : Thermometer

H : Auxiliary Thermometer (The mercury column should be located at the center of the mercury reservoir.)

I : Wind Protector

J : Cork stopper

Glass apparatus should be completely dried. The end of the adaptor (C) should be in touch the wall of the volumetric cylinder (D). Boiling stone or capillary tube is added to the distillation flask (A). The top of the flask and the separation tube are insulated with asbestos wool.

**B. Procedure**

With a volumentric cylinder (D), 100 ml of the test solution is measured into a distillation flask (A). This volumetric cylinder does not have to be washed and it can be used as a receiving vessel. Once the apparatus is set up, water is circulated through the cooling tube, the distillation flask is heated, and the distillation is carried out for 10 minutes. Heating is adjusted so that 4~5 ml of distillate is collected per 1 minute. Unless otherwise specified, the boiling point range is determined such that the lowest is when the fifth drop of the distillate is collected and the highest is when the last drop of liquid evaporates from the bottom of the distillation flask. The correction for the exposed part of the thermometer and atmospheric pressure is done by the following equation.

Correction for the exposed part of the thermometer

$$T_1 = t + 0.00015(t - t_1)n$$

$T_1$ : corrected temperature of the exposed part of the thermometer

t : temperature of thermometer

$t_1$ : temperature of auxiliary thermometer

n : number of degrees of mercury column located at the exposed part of the thermometer

Correction for the atmospheric pressure

$$T = T_1 + 0.00012(760-P)(273+T_1)$$

T : corrected temperature

P : pressure when the test is carried out

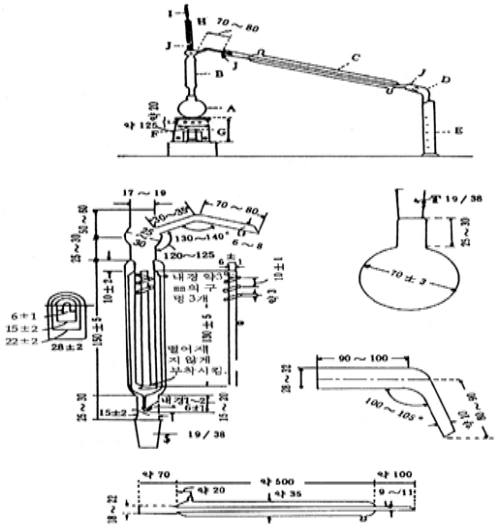
For the distillate that flows out at the temperature of 80°, the sample is cooled to below 15° before the test and 100 ml of the liquid is used as a sample for the test. A piece of paper is cut to fit into the end of the adaptor and used as the cover of the volumetric cylinder, which is immersed in a water bath at 15° or lower up to the 100 ml mark. Then the amount of distillate is recorded at the same temperature at which the sample is extracted.

### Method 2

This method is used to measure the amount of distillate in liquid at distillation temperature of 170° or lower.

### A. Apparatus

The apparatus is depicted in the figures (1 ~ 5) below (unit : mm).



A : Distillation flask (It is made of hard glass with the capacity of about 200 ml. See the figure 2.)

B : Separation tube (It is made of hard glass and about 1 mm thick. See the figure 3)

C : Cooling tube (It is made of hard glass. See the figure 4.)

D : Adapter (It is made of hard glass. See the figure 5.)

E : Volumetric cylinder (100 ml with 1 ml graduation)

F : Support (Same as Method 1)

G : Burner

H : Thermometer

I: Auxiliary thermometer (The mercury column should be located at the center of the mercury reservoir.)

J : Cork stopper

Glass apparatus should be completely dried. The end of the adaptor (D should be in touch the wall of the volumetric cylinder (E). Boiling stone or capillary tube is added to the distillation flask (A). The distillation flask and the separation tube (B) (except for the side arm) are insulated with glass wool.

### B. Procedure

Should follow the procedure in Method 1.

However, the flow rate is kept at 3~4 ml per minute.

## 11. Specific Gravity

Specific gravity is defined as the ratio of the mass of the sample to the mass of an equal volume of the standard material. In this specification, the specific gravity $(d_t^{t'})$means the means the ratio of the weight of the sample to that of an equal volume of water at t'° and t°. Unless otherwise specified, the specific gravity

(d) means the ratio of the weight of the sample ($d_{20}^{20}$) to that of an equal volume of water at 20°, and is determined by one of the following methods.

### A. Measurement by Pycnometer

A pycnometer is a container made of glass with a capacity of usually 10 to 100 ml. It has a ground, glass stopper fitted with a thermometer, and has a side tube with a mark and a ground glass cap. A pycnometer is previously washed, dried, and weighed (W). After removing the stopper and the cap, the pycnometer is completely filled with a sample, which is then kept at 1～3° lower than the specified temperature. The cap is placed while carefully preventing formation of bubbles. The temperature is gradually raised until the thermometer shows the specified temperature. The excess sample above the mark is removed through the side arm, which is then capped and wiped clean on the outside. It is then weighed ($W_1$). Again, using the same specific gravity bottle, the same procedure is repeated with distilled water. It is then weighed ($W_2$). Specific gravity (d) is obtained by the following equation.

$$d = \frac{W_1 - W}{W_2 - W}$$

### B. Measurement by Mohr-Westphal Balance

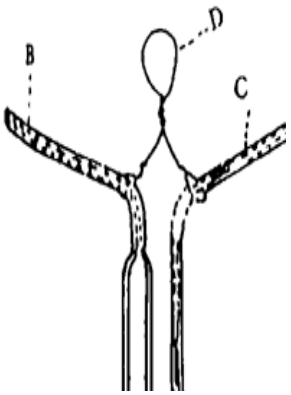
While maintaining the balance horizontal, a glass weight, where a thermometer is inserted, is hung at the right end of the scale bar. The glass weight is immersed in distilled water in a cylinder. The biggest rider is hung at the mark 10, and the balance is leveled by adjusting the screw at a specified temperature. The same procedure is repeated with the sample and the specific gravity is recorded at the position of rider when the balance is leveled. The liquid level should be adjusted so that the length of the metal needle submerged in the liquid is the same as that in distilled water case.

### C. Measurement by Hydrometer

A hydrometer with a required precision at a specified temperature is used. The hydrometer is cleaned with alcohol or ether. After well mixing the sample by

shaking, the hydrometer is floated after bubbles disappear. At a specified temperature, the specific gravity is recorded from the top of the meniscus when the hydrometer is stationary. However, if the reading directions are provided for the hydrometer, those should be followed.

D. Measurement by Sprengel-Ostwald Pycnometer




A Sprengel-Ostwald pycnometer is a vessel made of glass with a capacity of usually 1 to 10 ml. As shown in the figure 1, both the ends are thick-walled fine tubes (A), one of which has a mark (C). A platinum or an aluminium wire (D) is hung at this mark. Previously cleaned and dried pycnometer is weighed (W). Another fine tube (B) that has no marks is immersed in the sample, which is kept at a temperature 3 ~ 5° lower than the specified temperature. A rubber tube or a ground fine tube is attached at the end of the other tube (A), and the sample is gently sucked in until it comes up above the mark C, while preventing formation of bubbles. The pycnometer is immersed in a water bath, which is kept at a specified temperature for about 15 minutes. The end of the fine tube (B) is blocked with a piece of filter paper and the sample front is brought up to the mark. Then, the apparatus is removed from the water bath, wiped clean, and weighed ($W_1$). The same procedure is repeated using the same pycnometer and distilled water (instead of the sample) and it is weighed ($W_2$). Specific gravity (d) is obtained by the following equation.

$$d = \frac{W_1 - W}{W_2 - W}$$

## 12. Optical Specific Rotation

Specific rotation of an optically activated material and it solution are expressed by the equation (1) and (2), respectively. The symbol + and - denote dextrorotatory (right) and levorotatory(left), respectively. The symbol ° is attached to upper right of a number representing the degree.

$$[\alpha] = \frac{\alpha}{ld} \quad \cdots\cdots\cdots\cdots(1)$$

$$[\alpha] = \frac{100\alpha}{lc} \quad \cdots\cdots\cdots\cdots(2)$$

α : Corrected angular rotation, in degrees

l : Path length of the liquid (dm)

d : Specific gravity

c : Smount of sample (g) in 100 ml solution

Optical rotation $\alpha_x^t$ and specific rotation $[\alpha]_x^t$ are determined with the specific monochromatic light x (expressed by wavelength or the name of the light source) at a temperature t°. Unless otherwise specified, it is measured under the conditions of temperature at 20°, path length of 100 mm, and the D line (589.0 and 589.6 nm) in sodium spectrum

## 13. Water Determination (Karl Fischer Method)

The determination of water is based upon the quantitative reaction of water with iodine and sulfur dioxide under the presence of pyridine and an methyl alcohol as

shown in the reactions below.

$$H_2O + I_2 + SO_2 + 3C_5H_5N \rightarrow 2(C_5H_5N + H)I^- + C_5H_5N\ SO_3$$

$$C_5H_5N\ SO_3 + CH3OH \rightarrow (C_5H_5N + H)O^-SO_2 \cdot\ O\ \cdot CH_3$$

## A. Apparatus

It usually consists of two automatic burettes, a titration flask, and a stirrer. If necessary, an electronic device is used to determine the end point. Karl-Fischer solution is highly hygroscopic, therefore, moisture absorption from outside should be prevented. Silica gel, phosphorus pentoxide, or granular calcium chloride is used to prevent moisture absorption.

## B. Reagent and Test Solution

◦Karl Fischer Methyl Alcohol: Magnesium powder (5 g) is added to 1,000 ml of methyl alcohol, which is heated using a reflux condenser with a calcium chloride tube. If necessary, the reaction is accelerated by adding 0.1 g of mercuric chloride. When the generation of bubbles stops, methyl alcohol is distilled while avoiding introduction of moisture. The amount of moisture is kept at less than 0.5 mg per 1 ml. Moisture should be avoided for storage.

◦Karl Fischer Pyridine: Potassium hydroxide or barium oxide is added to pyridine. The container is capped with a stopper, which is then set aside several days. It is then distilled and the distillate is stored in a moisture free environment. Then distill and store by avoiding atmospheric moisture. The amount of moisture is kept at less than 1 mg per 1 ml. It is stored in a moisture free environment.

◦Karl Fisher solution

(A) Preparation : Iodine (63 g) is dissolved in 100 ml of Karl-Fischer pyridine, which is cooled in an ice bath. Dry sulfur dioxide passed through until its weight reaches 32.3 g, where Karl-Fischer methyl alcohol is added to bring the total volume to 500 ml. It is then set aside for 24 hours. Since

the solution degrades with time, it should be standardized right before use. It should be stored in a dark, moisture-free, and cool place.

(B) Standardization : 25 ml of Karl Fischer methyl alcohol are transferred into a titration flask, which is heated until the color of the solution changes from yellow to reddish brown. Exactly 50 mg of water is added to this solution, which is heated immediately. While avoiding moisture, the solution is titrated with the Karl Fischer solution to the endpoint, where the same color change occurs as above. 1 milliliter of Karl-Fischer solution corresponds to f mg of water ($H_2O$).

$$f = \frac{Weight\ of\ Water(H_2O)(mg)}{Amount\ of\ Karl\ Fischer\ Solution(ml)\ used\ for\ titration}$$

◦Standard solution of water and methyl alcohol

(A) Preparation : Karl Fischer Methyl alcohol (500 ml) is transferred into a 1,000 ml flask, where 2 ml of water and Karl Fischer methyl alcohol are added so that the total amount reaches 1,000 ml. The solution should be standardized right after the standardization of the Karl Fischer solution. It should be stored in a dark, moisture-free, and cool place with a small temperature variation.

(B) Standardization : As described in (5), 20 ml of Water-Methyl Alcohol Solution are transferred into a titration flask and titrated with the Karl Fischer solution until the color of the solution changes from yellow to reddish brown. One milliliter of Water-Methyl Alcohol Solution corresponds to f' mg of water ($H_2O$).

$$f' = \frac{F \times Titration\ amount\ of\ Karl\ Fischer\ Solution(ml)}{Amount\ of\ Water-Methyl\ Alcohol\ Solution(ml)}$$

In principle, the titration using Karl Fischer solution should be carried out at the same temperature as its standardization temperature. If the sample is not colored, the endpoint can be determined visually. In this case, the point, where the solution

becomes reddish brown changing from yellow (vice versa in case of back titration) in color, is the endpoint. If the sample is colored, the endpoint is determined electrically (Dend Stop End Point Method). In this case, two platinum electrodes are immersed in the solution to be titrated, and a constant current (5 ~ 10 μA) is applied to the solution using a variable resistor. Then Karl Fischer solution is drop-wise added. As the titration proceeds, the needle of the microammeter starts to swing vigorously and after a few seconds it comes back to its starting position. When the titration reaches the endpoint, the microampere meter swings even more vigorously (50 ~ 150 μA) for 30 seconds or longer. At this point, the titration is considered to be at the endpoint. In case of back titration, the needle of the microampere meter immediately returns back to its starting position at the end point under the presence of excess amount of Karl-Fischer solution. An apparatus with the Magic Eye can be used in replace of microampere meter. Unless otherwise directed, the titration with the Karl Fischer solution can be carried out by one of the following two methods. Normally, back titration is preferable in case of electrical method. The f of the Karl Fischer solution decreases with time. Precisely 20 ml of Water-Methyl Alcohol standard solution is titrated by following the standardization procedure of Water-Methyl Alcohol standard solution and f is obtained by the following equation.

$$f = \frac{f' \times Amount\ of\ Water-Methyl\ Alcohol\ Solution(ml)}{Titration\ Amount\ of\ Karl\ Fischer\ Solution(ml)}$$

(A) Direct Titration : Karl Fischer Methyl alcohol (25 ml) is placed in a dried titration flask, which is then titrated with the Karl Fischer solution to the endpoint. A precisely measured amount (preferably containing 10 to 50 mg of water) of sample is quickly transferred into the titration flask, which is stirred vigorously and then titrated again to the end point.

$$\text{Water content (\%)} = \frac{Amount\ of\ titrant(Karl-Fischer\ Solution)(ml)\times f}{Weight\ of\ sample(mg)} \times 100$$

(B) Back Titration : Approximately 20 ml of Karl Fischer methyl alcohol is placed in a titration flask, where an excess amount of Karl Fischer solution is added to the end point while stirring vigorously. to the endpoint. A precisely measured amount (preferably containing 10 to 50 mg of water) of sample is quickly transferred into the titration flask, which is stirred vigorously with an excess amount of Karl-Fischer solution and then titrated with water-methyl alcohol standard solution to the end point.

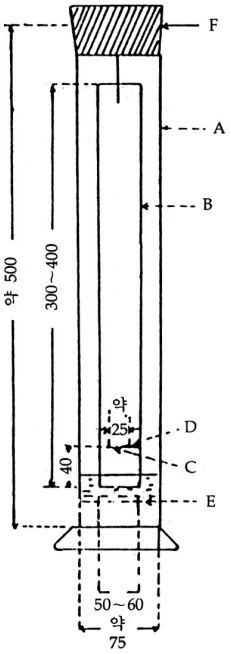
Water content (%)=

$$\frac{(Karl\ \ Fischer\ \ solution(ml)\times f)-[(water-methyl\ \ alcohol(ml))\times f']}{Weight\ \ of\ \ Sample(mg)}\times 100$$

# 14. Paper Chromatography

## Method 1

A. Apparatus




The apparatus is depicted in the figures below (unit : mm).

A : cylindrical glass vessel

B : chromatography filter

C : position of reference solution

D : position of test solution

E : developing solvent

F : rubber or glass stopper

## B. Procedure

With a pencil, a straight line is drawn at approximately 40 mm from the bottom edge of the chromatography paper (B). On this line, a specified amount of the

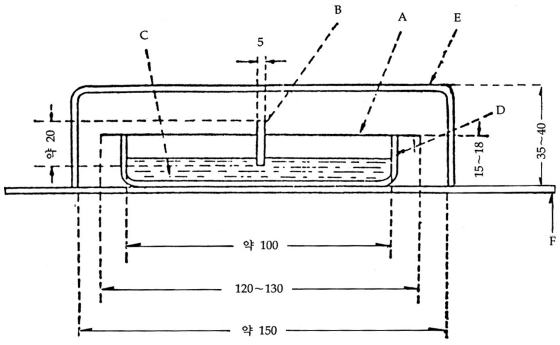
test solution and the reference solution are spotted with a micro pipette or a capillary and dried, where these solutions are prepared as specified for each item. The spots should be approximately 25 mm apart from each other. Using a thread or needle, the filter paper is hung vertically on the stopper (F) in a cylindrical glass vessel

(A) that contains a specified developing solvent (E) without touching the wall of the vessel.

The filter paper is immersed approximately up to 10 mm from bottom edge in the solvent. The vessel is sealed and set aside. When the solvent front reaches a specified distance from the spots, the paper is removed from the vessel and dried. The positions and colors of the developed spots from the test and reference solutions are observed under natural sunlight and then UV light. If necessary, it is colorized by the specified method.

## Method 2

### A. Apparatus




The apparatus is depicted in the figures below (unit : mm).

A : circular chromatography paper (diameter 120~130 mm)

B : cylindrical filter paper (Thimble Filter)

C : developing solvent

D : petridish

E : glass hermetical container

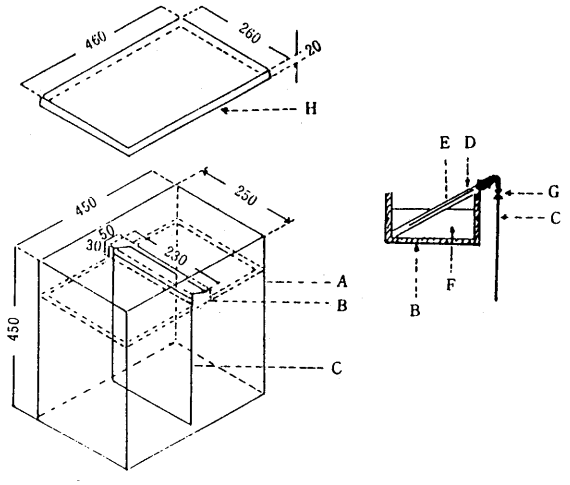
F : glass tube

### B. Procedure

At the center of a circular chromatography paper (A), a circle with 10 mm radius is drawn with a pencil. On this line, a specified amount of the test solution and the reference solution are spotted with a micro pipette or a capillary and dried, where these solutions are prepared as specified for each item. The total number of the spots should be 6 ~ 8 and they should be apart at the same interval along the circle. A hole with 5 mm diameter punched out at the center of the chromatography paper, where the cylindrical filter paper (Thimble Filter) (B) is inserted. The circular chromatography paper (A) is placed on top of a petri dish (D) with a developing solvent (C) so that the cylindrical filter paper (Thimble Filter) is submerged into the solvent up to approximately 5 mm from the bottom. It is then set aside in a hermetical container. When the solvent front reaches a specified distance, the chromatography paper is removed from the container and dried in air. The same procedure as in Method 1 is followed.

## Method 3

### A. Apparatus




The apparatus is depicted in the figures below (unit : mm).

A : a box made of hard synthetic resin

B : developing container made of hard resin (50x30x230)

C : chromatography paper

D & E : glass plate (70×220)

F : developing solvent

G : position for test solution or reference solution

H : cover

## B. Procedure

Chromatography paper is cut to 200 mm width and 400 mm length. A parallel line is drawn with a pencil at 50 mm from the short side. On this line, a specified amount of the test solution and the reference solution are spotted with a micro pipette or a capillary and dried in air. The spots should be approximately 25 mm apart from each other. This paper is sandwiched with two glass plates (D & E) so that the paper is exposed up to 40 mm from the bottom (i.e. 10 mm from the line is covered with glass plate). The glass plates are placed in a container (B) with a specified developing solvent (F), which is

then set aside in a hermetical box (A). When the solvent front reaches a specified distance, the paper is removed from the box and dried in air. The same procedure as in Method 1 is followed.

C. $R_f$

The position of the test solution or the reference solution in Method 1 or 3 is A, and the solvent front is B. The center of the developed spot is C from the test solution or the reference solution. The ratio of fronts ($R_f$) is obtained from the following equation.

$$R_f = \frac{Distance\ of\ AC}{Distance\ of\ AB}$$

Rf is a characteristic value for a material under the same conditions such as the developing temperature, the properties of chromatography paper, and the choice of developing solvent.

## 15. Softening Point Measurement

### A. Apparatus

It is depicted in figure 1.

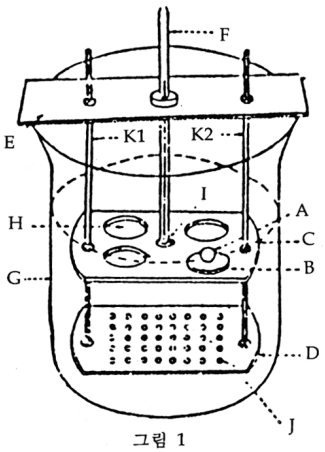


그림 1

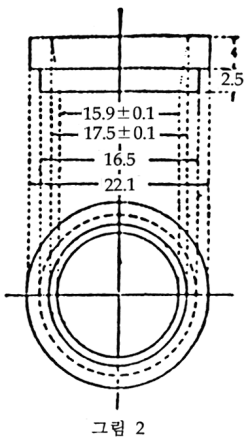


그림 2

A : iron ball (diameter 9.5 mm, weight 3.5 g)

B : a round brass plate, figure 2 (unit : mm).

C : metallic round support plate (approximately 80 mm× 60 mm× 2 mm), which has a hole (I) at the center for mercury reservoir for a thermometer and 4 fix holes (H) for a round plate around the center hole. The distance between the edge of I and the center of H is 17 mm or less.

D : bottom plate (approximately 80 mm× 60 mm× 2 mm) with 40 convection holes (J).

E : settling plate (approximately 126 mm× 28 mm× 2 mm)

F : thermometer (mercury type)

G : beaker (inner diameter 85 mm or higher, height 127 mm or higher)

H : fix hole in the circular plate (diameter 19 mm)

I : hole for mercury reservoir (diameter 2 mm)

J : convection hole (diameter approximately 4 mm)

$K_1$ & $K_2$ : supporting pole

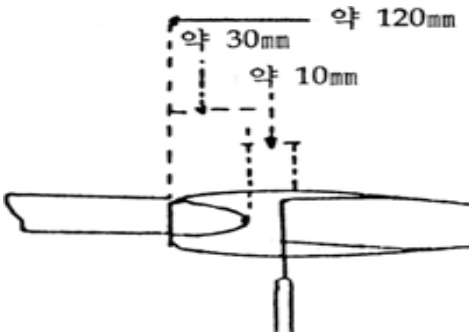
The distance from the bottom of E to the top of B is 80 mm or longer. The distance from the bottom of B to the top of D is 25.4 ± 0.2 mm. The distance from the bottom of D to the bottom of G is 20~30 mm. The center of mercury reservoir of the thermometer (F) should be at the same level of the bottom of B.

## B. Procedure

Sample is melted as quickly and at temperature as low as possible. The round plate B is placed on the flat metal plate. The melted sample is placed carefully in the round plate so that bubbles are not formed and cooled. With a slightly heated small knife, the part of the sample that is swollen over the top of the round plate is scraped off. Freshly boiled and cooled water is poored into the beaker (G) (over 90 mm in depth) and the water is kept at 15~35°. At the center of the sample surface in the round plate, the iron ball (A) is placed and the plate is inserted into the fixing hole (H). The distance between the top of

the round plate and the water surface is kept at 50 ± 2 mm. After 15~20 minutes, heating is started. The burner flame is adjusted so that it is uniformly distributed from the center to the edge of beaker bottom. In 3 minutes of heating, the rising rate is maintained at 5 ± 0.5° per minute. The softening point is where the sample softens, gets dropped from the round plate, and finally contact with the bottom plate. For each measurement, 4 round plates are used and at least 2 measurements are done. An average value is obtained.

## 16. Flame Coloration Test




Platinum wire used for this test has a diameter of approximately 0.8 mm. Its straight tip is used as it is. If the sample is solid, a small amount of hydrochloric acid is added to make it into a paste, a small amount of which is stained to approximately 5 mm from the tip of the platinum wire. While keeping the wire horizontal as shown in the figure, it is tested in a colorless flame. If the sample is liquid, the platinum wire is dipped into the sample up to 5 mm from the tip. It is then tested by following the same procedure as the solid sample. When potassium in a sodium salt is tested, the flame is observed using a cobalt glass. Frame color reaction persists approximately for 4 seconds

# 17. Test Methods for Chloride and Sulfate Slats

This method is used to test the allowed limit of chlorides or sulfates in a sample

## A. Chloride Limit Test

Unless otherwise specified, a specified amount of sample is dissolved in approximately 30 ml of water in a Nestler tube. If the solution is alkaline, it is neutralized with dilute nitric acid and then 6 ml of dilute nitric acid is added. It is then diluted with water to 50 ml. If it is specified to use a test solution, it is diluted to 50 ml with water in a Nestler tube. In another Nestler tube, a specified amount of 0.01 N hydrochloric acid is added, where 6 ml of nitric acid and water are added to bring the total volume to 50 ml. If the solution is not clear, both solutions are filtered under the same conditions. To both solutions, 1 ml each of silver nitrate solution is added and well mixed. While avoiding the direct sunlight, the mixtures are set aside for 5 minutes. Both tubes are compared in terms of turbidity with a black background.

## B. Sulfate Limit Test

Unless otherwise specified, a specified amount of sample is dissolved in approximately 30 ml of water in a Nestler tube. If the solution is alkaline, it is neutralized with dilute hydrochloric acid and then 1 ml of dilute hydrochloric acid is added. It is then diluted with water to 50 ml. If it is specified to use a test solution, it is diluted to 50 ml with water in a Nestler tube. In another Nestler tube, a specified amount of 0.01N sulfuric acid is added, where 1 ml of hydrochloric acid and water are added to bring the total volume to 50 ml. If the solution is not clear, both solutions are filtered under the same conditions. To both solutions, 2 ml each of barim chloride solution is added, well mixed, and set aside 10 minutes. Both tubes are compared in terms of turbidity with a black background.

# 18. Thermometers

Generally, Needle shape thermometer (stick shape) or mercury thermometer (stick shape) is used after correction. However, for congealing point, melting point, boiling point, and distillation range, Needle shape thermometer (stick shape) is used. Specifications for needle shape thermometer (stick shape) are as follow.

| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 |
|---|---|---|---|---|---|---|
| liquid | mercury | mercury | mercury | mercury | mercury | mercury |
| filling gas | nitrogen | nitrogen | nitrogen | nitrogen | nitrogen | nitrogen |
| temperature range | -17 ~ 50° | 40 ~ 100° | 90 ~ 150° | 140 ~ 200° | 190 ~ 250° | 240 ~ 320° |
| smallest tick | 0.2° | 0.2° | 0.2° | 0.2° | 0.2° | 0.2° |
| major tick (per) | 1° | 1° | 1° | 1° | 1° | 1° |
| tick label (per) | 2° | 2° | 2° | 2° | 2° | 2° |
| length(mm) | 280 ~ 300 | 280 ~ 300 | 280 ~ 300 | 280 ~ 300 | 280 ~ 300 | 280 ~ 300 |
| diameter of lower body (mm) | 6.0°±0.1 | 6.0°±0.1 | 6.0°±0.1 | 6.0°±0.1 | 6.0°±0.1 | 6.0°±0.1 |
| length of mercury reservoir (mm) | 12 ~ 15 | 12 ~ 15 | 12 ~ 15 | 12 ~ 15 | 12 ~ 15 | 12 ~ 15 |
| distance from the bottom of mercury reservoir to the lowest tick (mm) | 75 ~ 90 | 75 ~ 90 | 75 ~ 90 | 75 ~ 90 | 75 ~ 90 | 75 ~ 90 |
| distance from the top to the highest tick (mm) | 35 ~ 50 | 35 ~ 50 | 35 ~ 50 | 35 ~ 50 | 35 ~ 50 | 35 ~ 50 |
| distance from the bottom of mercury reservoir to submerge line (mm) | 60 | 60 | 60 | 60 | 60 | 60 |
| handle shape | ring | ring | ring | ring | ring | ring |
| allowed error | 0.2° | 0.2° | 0.2° | 0.2° | 0.2° | 0.2° |

# 19. Atomic Absorption Spectrophotometry

Metal atom is dissociated from a test solution by an appropriate method into an atomic vapor. Use ground state absorbs specific wavelengths from light, using a Spectrophotometric method, an absorbance is measured and from this absorbance a concentration of the target element is obtained. There are two methods in atomizing a metal, Flame Type and Cold Vapor Type.

## A. Apparatus

Generally, it consists of light source, atomization part, spectrometer, and photometer. Light source is used a hollow cathode lamp or a discharge lamp. There are two types for an atomization part, Flame Type (direct vaporizer) and Cold Vapor Type. Cold Vapor Type is further divided into reductive evaporation and thermal evaporation. Flame type of atomizer consists of a burner and a gas flow regulator. Reductive evaporator consists of a hermetic container and a pump. Thermal evaporator consists of a quartz dish and a heater. A spectrometer is used a diffraction grid or a prism. Photometer consists of a detector and an indicating instrument.

## B. Preparation of Test solution

Unless specified sample weight in the monograph, 5～10g of sample is accurately weighed into crucible or platinum plate, dried, carbonized, and reduced to ash at 450～550°. If it isn't reduced to ash, cool it. As ashing supplement, 2~5 ml of nitric acid(1→2) or 50% magnesium nitrate solution or aluminum nitrate · calcium nitrate solution (40 g of aluminum nitrate and 20 of calcium nitrate are dissolved in 100 ml of water) are added, wetted, dried, and continued ashing. If ashing is not enough, repeat above process one time. If necessary, 2～5 ml of nitric acid(1→2) is added and reduced to ash, lastly. After being reduced to ash, the residue is wet with water, and 2~4 ml of hydrochloric acid is added and evaporated to dryness. Specified solvents for each test method (1N hydrochloric acid for tin, 0.5 N nitric acid for other metals) are added, heated, and dissolved. Filter with filter paper if insoluble substances exist. Unless specified solvent, 0.5

N nitric acid is added to make 25ml, test solution. However, for tin, nitrate or nitric acid should not be used as ashing complement. For other metals, they are used only if they don't affect test procedure. Proceed under the same manner for blank test solution to correct test solution.

### C. Procedure

Unless otherwise specified, a test solution is prepared by a specified procedure for each item and tested by one of the following methods.

(1) Flame Type

A specified lamp is used as a light source. The lamp is switched on. The spectrometer is adjusted to the specified wavelength to be analyzed and an appropriate current setting is established. A specified mixture of combustible and support gases is ignited. Gas flow rate and pressure are adjusted. Zero point correction is carried out by sparying a solvent into the flame. A test solution prepared by a specified procedure is sprayed into the flame and its absorption is measured.

(2) Cold Vapor Type

A specified lamp is used as a light source. The lamp is switched on. The spectrometer is adjusted to the specified wavelength to be analyzed and an appropriate current setting is established. In case of a reductive evaporator, a test solution is placed in a hermetic container with an appropriate reducing agent and evaporated. In case of a thermal evaporator, a sample is evaporated by heating. Absorption by this atomic vapor is measured.

### D. Assay

Usually, one of the following methods is followed. For a quantitative analysis, interference and blank correction (background) should be considered.

(1) Calibration Curve Method

3 or more standard solutions having different concentration are prepared. A calibration curve is prepared from the absorption measurements of these solutions. A test solution having a measurable concentration is prepared and

its absorption is measured. The concentration of the target atom is obtained from the calibration curve.

(2) Standard Material Method

Standard solution is incrementally added to a set of (at least 3) test solutions having the same amount. Solvent is added to each solution so that the total volume is identical. Absorption of each solution is measured. Absorption is plotted against the standard element concentration. The concentration of the test element is obtained from the distance between the origin and the intersection between the extrapolated regression line and the horizontal axis. However, this method is only valid when the calibration curve in (1) is a straight line that passes through the origin.

(3) Internal Standard Method

Standard solution is added to a certain amount of internal standard element so that a known amount of standard test element is contained incrementally. Then, solvent is added to each solution so that the total volume is identical. Absorption of each solution is measured. Absorption ratio is plotted against the added standard element concentration. The same amount of internal standard element is added to a test solution. The ratio between absorption by the test element (obtained by the same conditions as in calibration curve) and the internal standard element is obtained. Using this ratio, the concentration of test element is obtained from the calibration curve.

Note : Reagent and Test Solution should not interfere with the measurement.

# 20. Inductively Coupled Plasma Emission Spectroscopy

## A. Apparatus

Generally, it consists of excitation source part, sample injection port, light emission part, spectrometer, photometer, an indication and recording part. Excitation source part is composed of an electric power source, a control system, and circuit to supply and control the electric energy which excites and emits an element in a sample. This part also includes gas supply system and cooling apparatus. The sample injection port is composed of a nebulizer and a spray chamber. The light emission part is composed of a torch tube and a high-frequency induction coil. The spectroscope part is composed of a light-converging system and a spectroscope such as a diffracting grating. The photometry part is composed of a detector and a signal processing system. The indication and recording part is composed of a display and a recording device. The ICP-atomic emission spectrometry includes single-element-sequential-type- and multiple-element-sequential-type-measuring methods using a wavelength scanning spectroscope, and a simultaneously measuring method using a wavelength-fixed-type polychrometer.

## B. Preparation of Test Solution

Unless specified sample preparation in the monograph, proceed as directed under preparation of test solution(na) in Atomic Absorption Spectrophotometry.

## C. Procedure

Confirm that all live parts are normal. Switch on the excitation source part and the control system. When a vacuum-type spectroscope is used to measure the emission line in vacuum-ultraviolet region, purge sufficiently the light-path between the light emission part and the spectroscope with argon or nitrogen gas for 10 minutes. Set the flow rate for argon or nitrogen gas to the specified rate, switch on the high frequency power supply, and generate the plasma. Correct the wavelength of spectroscope with the emission spectral line of a mercury lamp. Introduce the test solution and the standard solution or control solution prepared

as specified in the individual monograph and measure the emission intensity of an appropriate emission line of the object element.

D. Assay

Usually, the determination is done using one of the following methods. In the determination, the interference and blank correction (background) should be corrected.

(1) Calibration Curve Method

Prepare standard solutions of three or more different concentrations, measure the emission intensities of these standard solutions, and prepare a calibration curve from the obtained values. Then, measure the emission intensity for the test solution with a concentration adjusted to a measurable range, and determine the amount(concentration) of the object element from the calibration curve.

(2) Standard Addition Method

To equal volumes of three or more test solutions, add to each the standard solution so that the stepwise increasing amounts of the object element are contained in the solutions, and add the solvent to make a definite volume. Measure the emission intensity for each solution, and plot the amounts(concentrations) of added standard object element on the abscissa and the emission intensities on the ordinate on the graph. Extend the calibration curve obtained by linking the plots, and determine the amount (concentration) of object element from the distance between the origin and the intersecting point of the calibration curve on the abscissa. This method is applicable only when the calibration curve drawn as directed in section (1) above is a straight line passing through the origin.

(3) Internal Standard Method

Prepare several solutions containing a constant amount of the specified internal standard element, and known graded amounts of the standard object element. For these solutions, measure the emission intensities of the standard object element and internal standard element at the analytical wavelength of

each element under the same measuring conditions, and obtain the ratios of each emission intensity of standard object element to the emission intensity of the internal standard element. Prepare a calibration curve by plotting the amounts (concentrations) of standard element on the abscissa and the ratios of emission intensity on the ordinate. Then, prepare the test solutions, adding the same amount of internal standard element as in the standard solution. Proceed under the same conditions as for preparing the calibration curve, obtain the ratio of the emission intensity of standard object element to that of internal standard element, and determine the amount (concentration) of the object element from the calibration curve.

Note : For this test, avoid the use of reagents, test solutions, and gases which interfere with the determination.

# 21. Mercury Test

Unless specified test in the individual monograph, proceed under one of the following methods.

## A. Cold Vapor Atomic Absorption Spectrophotometry

1) Apparatus

(1) Atomic Absorption Spectrometer : quartz absorption cell is attached

(2) Lamp : Hollow cathode mercury lamp

(3) Mercury vapor apparatus

2) Solution

(1) Stannous chloride solution : 10 g of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) is dissolved in 1N sulfuric acid to make 1,000m*l*.

(2) Mercury standard solution

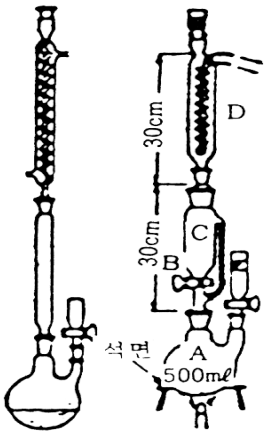
0.135g of mercury (ll) chloride is dissolved in 100 ml of 10% nitric acid and water is added to make 1,000m*l*. When using, this solution is 1.000 times diluted with 1% nitric acid, standard solution.

Mercury standard solution 1m*l* = 0.1 μg Hg

3) Preparation of Test solution

Unless specified test in the individual monograph, 5~10g of sample is transferred into a flask for decomposition. 10 ml of water and 20 ml of nitric acid are added, shaken slowly and 20 ml of sulfuric acid is slowly added. A reflex condenser is attached to the flask, which is boiled until brown smoke is not generated. When the solution doesn't become colorless~light yellow transparent solution, 5 ml of nitric acid is added after cooling, and repeat the process above. After cooling, 50 ml of water and 10 ml of 10% urea solution area added and boiled for 10 minutes. It is cooled, 1 g of potassium permanganate is added, occasionally shaken for 10 minutes, and allow to stand. Repeat this until purple-pink color remains. After boiling for 20 minutes, purple-pink color is discharged, then cool it. 1 g of potassium permanganate is

added and heated for 20 minutes again. When purple-pink color of the solution is discharged, repeat 2 times adding and heating of potassium permanganate, and cooled. Add 20 % hydroxylamine hydrochloride solution carefully until the solution becomes colorless and transparent. After cooling, the decomposed solution is transferred to another flask and inside, connecting part of a reflex condenser and a flask for decomposition are washed with water. Rinsing water is added to this, make a certain amount with water, test solution.




Example of mercury decomposition apparatus

4) Procedure

100 ml each of test solution and blank test solution whose concentration of sulfuric acid is previously adjusted to 20%(v/v) is taken to test solution bottle. After being connected to vapor apparatus, 10 ml of stannous chloride solution is added, and immediately, stopper is placed. Absorbance is measured at 253.7 nm by circulating air in absorption cell using diaphram pump. Separately, water is added to 1, 5, 10, 15, 20m*l* each of mercury standard solution to make 100 ml, respectively. Standard solution proceed in the same manner as test solution and calibration curve is prepared by measuring absorbance. Absorbance of test solution is substituted to the calibration curve and the content of mercury is

calculated.

## B. Gold amalgam Atomic Absorption Spectrophotometry

1) Apparatus

Use mercury measurement apparatus, which automatizes combustion of sample, collection by gold amalgam, and measurement by cold vapor Atomic Absorption Spectrophotometry. Mercury measurement apparatus whose a special catalyst is set on the combustion part, can be used.

2) Reagent and Solution

(1) Mercury standard stock solution : 0.135g of mercury (ll) chloride is dissolved in 0.001% L-cysteine solution to make 1,000m*l*.

Mercury standard stock solution 1m*l* = 100μg Hg

(2) Mercury standard solution : Undiluted mercury standard solution is diluted with 0.001% L-cysteine solution to make 0 ~ 200ng/m*l*.

(3) Additives : When using (a) aluminum oxide and (b) calcium hydroxide · sodium carbonate(1:1), activate for 30 minutes at 950°.

3) Procedure

Approximately 1 g of additive (a) is uniformly spread on ceramic boat and in case of solid sample, 10~300 mg of finely cut and homogenized sample is taken. In case of liquid sample, 0.1~0.5 ml of sample is completely infiltrated into additive (a). On that, about 0.5 g of additive (a) and 1 g of additive (b) are uniformly spread in turn to form the layer. In case of automatic mercury measurement apparatus whose a special catalyst is set on the combustion part, additive is not added to nickel boat and only sample is taken. Boat is transferred into combustion furnace and air or oxygen is flowed about the rate of 0.5 ~ 1*l*/min. It is heated about 900°, mercury is spilled, and collected in collection tube. Collection tube is heated about 700°, mercury vapor is sent to cold vapor Atomic Absorption Spectrophotometry apparatus and absorbance is measured, A. Separately, absorbance is measured in the same manner with additive on ceramic boat, Ab. Separately, calibration curve is prepared from absorbance, which is obtained by same

preparation using mercury standard solution. Value of A − Ab is substituted to calibration curve and the content of mercury in sample is calculated.

## 22. Assay for Alkali Salt of Organic Acid

Unless otherwise specified, sample (corresponding to approximately 0.3 g of sodium) is precisely weighed into a quartz or platinum crucible with 20~30 mm diameter. It is slowly heated initially, then continuously ramped up, and completely carbonized for approximately 2 hours. The crucible turns dark red at the heating temperature (300~400°). Care must be taken so that the burner flame should not touch the carbonized material. After cooling, carbonized material is crushed with a glass rod and transferred into a beaker along with the crucible. Approximately 50 ml of water is added to the beaker, where 50 ml of 0.5 N sulfuric acid is added. The beaker is covered with a watch glass and heated for 1 hour in a water bath. The content is filtered. If the filtrate is colored, sample is taken freshly and carbonized sufficiently. Residues on the beaker, crucible, and filter paper are washed well with warm water until the wash water does not turn a blue litmus paper red. The wash water is added to the filtrate. The excess acid is titrated with 0.5 N sodium hydroxide solution (indicator : 3 drops of methyl red solution). A 1 ml equivalent is multiplied to the amount of consumed acid to obtain the amount of salts in the sample.

This method is not to be applied for the alkali salts of organic acids that contains sulfur or halogens.

# 23. Melting Point

Melting Point means the temperature at which or within the range of which a solid completely melts and is determined by an appropriate one of the methods given

below. For convenience of measurement, solids are classified into the following two types

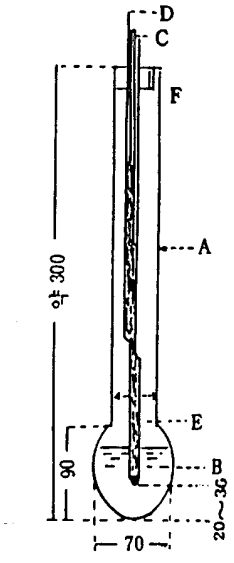
Class 1 substances : easily powdered material

Class 2 substances : fat, fatty acids, paraffin, a material that is very difficult to be powdered

## A. Procedure for Cass 1 Substances

(1) Apparatus

The apparatus is depicted in the figures below (unit : mm).




A : Round bottom flask for melting point measurement

B : Following solutions are used.

Measurement for 220° or below : Cupric sulfate

Measurement for 200 ~ 300° : Sulfuric acid and potassium sulfate (7:3 in weight) are dissolved by stirring and heating.

C : Thermometer

D : Auxiliary thermometer

E : Capillary (inner diameter approximately 1mm, length 50 ~ 70mm, one end is blocked)

F : Ventilation hole

(2) Procedure

sample is finely ground into powder and, unless otherwise specified, dried for approximately 24 hours in a desiccator (sulfuric acid). It is packed into a capillary (E) up to a thickness of 2.5 ~ 3.5 mm. If it is specified to be tested in a sealed container, the open end is sealed. The capillary is attached to the side of a thermometer so that the sample layer is located at the center of the mercury bulb. The thermometer is placed and fixed at the center of a round bottom flask (A) for melting point measurement with a cork or rubber stopper. An auxiliary thermometer (D) is placed so that the center of its mercury reservoir is located at the middle position between the solution surface and the mercury level of thermometer at the melting point (t). An appropriate thermometer is used depending on the measuring temperature range. When the apparatus is set up, the solution is heated to a temperature that is approximately 10° lower than the expected melting point. Ramping rate is set to 3° per minute up to a temperature that is approximately 5° lower than the expected melting point. Then it is set to 1° per minute. The temperature, where the contact part between inner wall of the capillary and the sample becomes damp or the sample crumbles, is recorded as the beginning of melting point. The temperature, where the sample melts completely and becomes transparent, is recorded as the end of melting point.

Correction for the exposed part of thermometer is done by the following equation.

$$T = t + 0.00015(t-t')n$$

T : corrected temperature

t : temperature reading by the thermometer

t' : temperature reading by the auxiliary thermometer

t" : If there is not marks in the thermometer at the solution surface, the temperature is read by inserting externally.

n : degrees in the exposed thermometer part (t - t")

**B. Procedure for Cass 2 Substances**

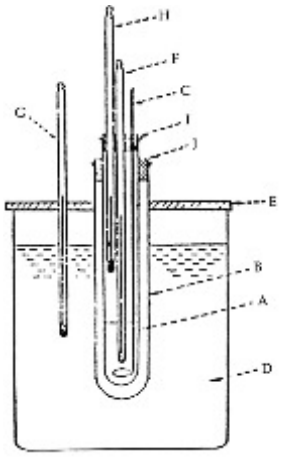
A sample is melted at a lowest possible temperature and it is sucked into a capillary up to approximately 10 mm. This capillary is cooled for approximately 24 hours at 10° or lower, or for at least 2 hours in an ice bath. The capillary is tied to the thermometer so that the sample is located at center of the mercury reservoir. It is then submerged into a beaker with water so that the top of the sample is approximately 10 mm below the water surface. While stirring continuously, the water is heated at a rate of increase of approximately 1° per 2 minutes until the temperature reaches a point approximately 5° below the expected melting point. The melting point is where the sample floats within the capillary.

## 24. Congealing Point

**A. Solid at Room Temperature**

(1) Apparatus

The apparatus is outlined in figure 1.




<figure 1>

A : Test tube (inner diameter approximately 22 mm, length approximately 160 mm)

B : Large test tube (inner diameter approximately 33 mm, length approximately 150 mm)

C : Stirring pole (diameter approximately 1 ~ 3 mm)

D : Cooling bath, water or ice is used and the temperature is kept at approximately 5° lower than the congealing point.

E : Wooden cover

F & G : Thermometer

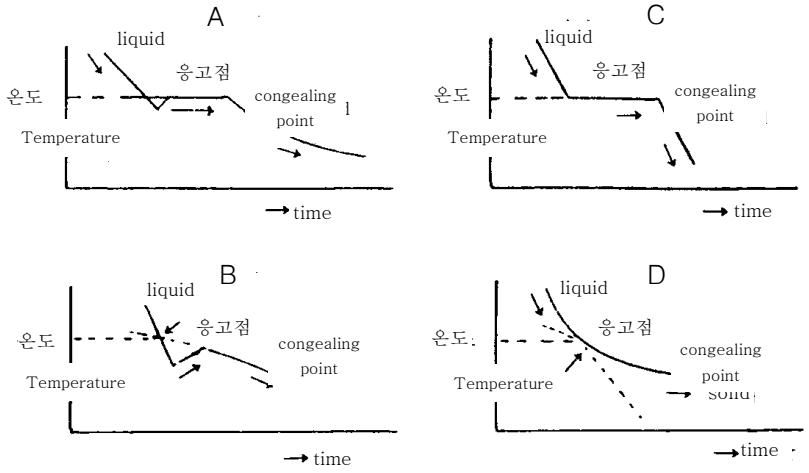
H : Auxiliary thermometer

I & J : Cork stopper

(2) Procedure

Approximately 20 g of sample is placed in a well dried test tube (A), where a thermometer (F) an auxiliary thermometer (H), and a stirring pole (C) are set up using a cork stopper. Mercury bulb of the thermometer (F) is positioned slightly lower than the center of the sample and that of the auxiliary thermometer (H) is positioned at the middle between the surface of the sample and the temperature reading at the congealing point by the thermometer (F). sample in test tube (A) is completely melted in a water or sulfuric acid bath at a temperature that is approximately 10° higher than the expected congealing

point. The melted sample is transferred into a large test tube (B), which is then submerged into a cooling bath (D).It is stirred with a stirring pole at the rate of 1 time for 2 seconds. In the beginning, the temperature falls slowly. The temperature slightly rises as crystallization starts and remains constant for a while. The temperature reading at this point is corrected for the exposed part of the thermometer by the following equation. This corrected temperature is congealing point.




$$T = t + 0.00015(t - t')n$$

T : Corrected temperature

t : Temperature reading by thermometer

t' : Temperature reading by auxiliary thermometer

n : The number of degrees in the exposed part of the thermometer

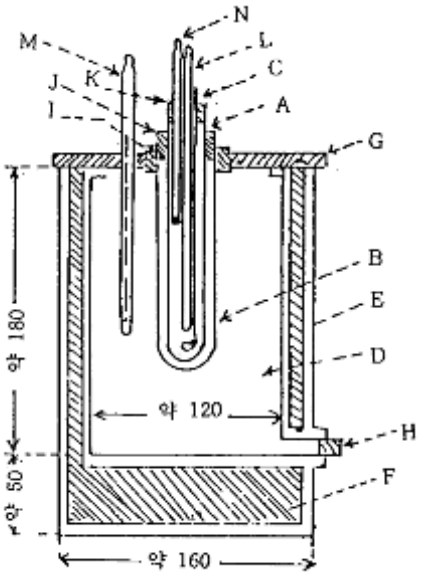
If there are significant amount of impurities in the sample, the congealing point curve shows a shape as depicted in figure B, C, or D (not figure A). In figure B and D, the intersection of extrapolated lines for solid and liquid phases is the congealing point. In figure C, the method in figure A is followed. In any cases,

the correction for the exposed part should be done

## B. Liquid at Room Temperature

(1) Apparatus

The apparatus is outlined in figure 2.




<figure 2>

A : Test tube (inner diameter approximately 22 mm, length approximately 160 mm)

B : Large test tube (inner diameter approximately 33 mm, length approximately 160 mm)

C : Stirring pole (diameter approximately 1 ~ 3 mm)

D : Cooling bath, water or ice is used and the temperature is kept at approximately 5° lower than the congealing point.

E : Container for cooling bath, metallic container with insulation material (F)

F : Insulation material

G : Wooden cover

H, I, J, & K : Cork stopper

L & M : Thermometer

N : Auxiliary thermometer

(2) Procedure

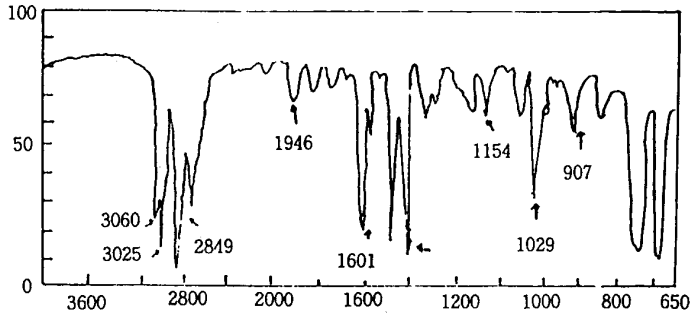
Should follow the same procedure for solid material with approximately 20 ml of sample.

## 25. Infrared Spectrophotometry

This method is used to qualitatively or quantitatively analyzing a sample based on the fact that a material shows a characteristic absorption pattern depending on its chemical structure in infrared absorption spectrum in a range of 4,000~667 $cm^{-1}$. Infrared beam is passed through a sample and an absorption is measured at each wavenumber. A spectrum is plotted as a graph with wavenumbers on the abscissa and transmittance(%) or absorption on ordinate.

### A. Apparatus and Procedure

Double beam infrared spectrophotometer is set up in a clean room, where the humidity is kept at 50% and lower and vibration is kept minimal. The ideal room temperature is 20~25°. The linearity of the absorption should be within ± 1% in a transmittance(%) range of 20~80% and the transmittance(%) is measured twice and its reproducibility should be within ± 0.5%. The reproducibility of wavenumber should be within ± 5 $cm^{-1}$ near 3,000 $cm^{-1}$ and within ± 1 $cm^{-1}$ near 1,000 $cm^{-1}$, respectively. When a polystyrene film (approximately 0.03 m in thickness) is used, it is adjusted so that the absorptions occur at the wave numbers as shown in the following figure.




## B. Preparation of sample

A sample is prepared so that the transmittance% for the strongest absorption band falls within 20~80%. Sodium chloride, potassium bromide, or potassium bromide iodine ($K_2BrI$) is used as a window plate material.

(1) Potassium Bromide Disk Method

In a quartz mortar, 1~2 mg of solid sample and 100~200 mg of potassium bromide (IR spectroscopy grade) are quickly ground and mixed into fine powder while preventing moisture absorption. The mixture is then palletized under a reduced pressure of 5 mmHg using a dry press by applying a pressure of 5~10 t/cm$^2$ against the pallet face for 5~8 minutes.

(2) Solution Method

Solid or liquid sample is dissolved in a solvent specified for each item. The solution is injected into a liquid cell. The same solvent is injected into a reference cell. The thickness of liquid cell is 0.1 mm or 0.5 mm.

(3) Paste Method

Solid sample is finely ground and mixed with fluid paraffin in a mortar. The paste is inserted between two window plates. Care must be taken so that air is not introduced into the assembly.

(4) Liquid Film Method

Liquid layer, that is formed with 1~2 drops of liquid sample between two window plates, is measured. If it is necessary to have thicker liquid layer,

Aluminum foil is inserted between two window plates to increase the path length.

(5) Thin Film Method

Sample is dissolved in a specified solvent for each item. A window plate is coated with this solution. The solvent is dried off with a heat gun. The remaining thin film of the sample is measured. If the sample is in a form of film with a thickness of 0.02 mm or less, it is measured directly.

(6) Gas Sample Measurement

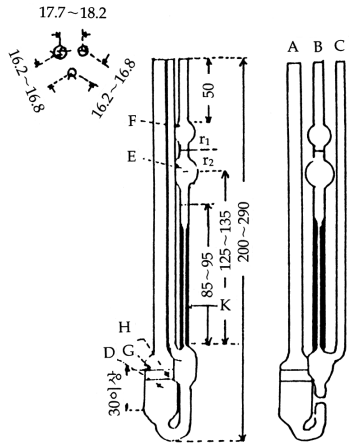
A gas cell with a path length of 5～10 cm is evacuated and filled with a sample up to a pressure specified for each item. If necessary, a gas cell with 1 m or longer can be used.

## 26. Viscosity

### 1. Viscosity measurement by capillary tube viscometer

The unit of viscosity is the Centistokes (cSt) and the viscosity is measured with the following Ubbelohde viscometer or Cannon Ubbelohde viscometer.

**A. Apparatus**




The apparatus is depicted in the figures below (unit : mm).

A, B & C : Tube part

D, E & F : Spherical part

G, H, I & J : Marking line

K : Capillary

The relation between the inside diameter of capillary and the measurable range of the viscosity is as follows.

| Inner Diameter | Viscosity Range |
|---|---|
| 0.56~0.60 | 2~10 |
| 0.75~0.79 | 6~30 |
| 0.85~0.89 | 10~50 |
| 1.07~1.13 | 20~100 |
| 1.40~1.46 | 60~300 |
| 1.66~1.67 | 100~500 |
| 1.92~1.98 | 200~1,000 |
| 2.63~2.71 | 600~3,000 |
| 3.01~3.11 | 1,000~5,000 |
| 3.58~3.66 | 2,000~10,000 |

## B. Procedure

A sample is placed in a tube A, preventing the formation of bubbles in the sample solution. When the viscometer stands vertically, the surface of the liquid sample should be placed in the middle between the marking line G and H of the spherical part D. Then the viscometer is immersed into an isothermal water bath at a specified temperature until the spherical part F of a tube B is fully submerged under water and fixed vertically. It is then set aside for 20 minutes until the temperature of the sample reaches the specified temperature. With the tube C covered with a finger, the sample is sucked in through the tube B until the sample surface reaches the center of spherical part F. Then the tube C is

opened and tube B is closed with a finger. When the sample in the lower part of the capillary drops, the tube B is opened and the time (t) taken for the meniscus to move from I to J is measured (in 0.1 seconds). For a set of measurements (2 or more), an average value is obtained and its difference form individual measurement should not be more than 0.1% at 16° or higher and not be more than 0.5% at 16° or lower. Viscosity is obtained by the following equation.

$$V = Kt \ (cSt)$$

where K is the viscometer constant, which is obtained from the same operation with distilled water or a standard solution of which viscose is already known. The temperature where K is attained may differ from the temperature where the viscosity of the sample is measured. If t is less than 200, a measurement is repeated using a viscometer with a capillary, which has a smaller inner diameter r of the capillary is smaller.

## 2. Viscosity measurement by rotational viscometer

### A. Apparatus

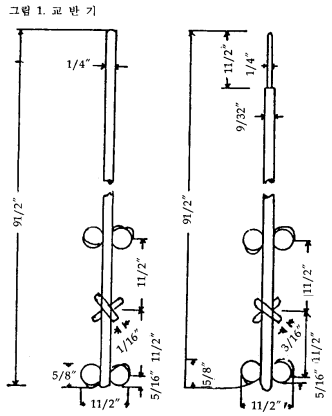
-Viscometer : Model LVP Brookfield or its equivalent (which can measure 25 ~ 10,000 cps at 25°) is used. It comes with a set of spindles to be used for different range of viscosity.

| range(cps) | spindle No. | speed (rpm) | scale | factor |
|---|---|---|---|---|
| 10 ~ 100 | 1 | 60 | 100 | 1 |
| 100 ~ 200 | 1 | 30 | 100 | 2 |
| 200 ~ 1,000 | 2 | 30 | 100 | 10 |
| 1,000 ~ 4,000 | 3 | 30 | 100 | 40 |
| 4,000 ~ 10,000 | 4 | 30 | 100 | 200 |

-Stirrer : As shown in figure 1, the stirrer is equipped with a stirring pole and a variable speed controller which can go up to 1,500 rpm.

(Note: AH Thomas Co Catalogue No. 9240-K with a stainless-propeller of 1°-1/2 inch 3 blade type may be used)

-Container for a sample : A glass container with 13.3 mm in depth, 60 mm in outer diameter and 236 ml in volume is used.




<figure 1>

B. Procedure

4 g of sample (or a specified amount for each item) is placed in the container with a known weight, where water is added to bring the total weight to 400 g. The blade of the stirring pole is positioned in the middle of the liquid, which is stirred at 800 ± 100 rpm. After 1.5 hours, the speed is adjusted appropriately so that air is not introduced and it is stirred for 30 minutes.

After removing the stirring bar, the temperature of the sample is maintained at 25°in an isothermal water bath of 25 ± 0.2°, unless otherwise specified. An appropriate spindle and speed are selected and the spindle is spun until the reading becomes constant. The viscosity is calculated by multiplying the coefficient in the table above with viscosity reading.

## 27. Heavy Metal Limit Test

This is a method to determine the allowable total limit of metallic impurities contained in a sample by colorizing of a test solution with sodium sulfate solution. The allowed limit of the metallic component is expressed in the equivalent color and

indicated in the amount of the lead in the standard reference solution (ppm of sample).

◦Lead Standard Stock Solution : After dissolving 159.8 mg of lead nitrate in 10 ml of dilute nitric acid, the solution is diluted to 1000 ml with water. For the preparation and storage of this solution, a glass container that does not contain soluble lead salts should be used

◦Lead Standard Solution : 10 ml of lead standard stock solution is diluted to 100 ml with water. This solution is prepared before use and contains 0.01 mg per 1 ml. For example, when 1 g of sample is tested using 1.5 ml of lead standard solution as a reference, the sample contains 15ppm of lead.

**Procedure**

Unless otherwise specified, a specified amount of sample is placed in a Nestler tube and dissolved in approximately 40 ml of water. The total volume is brought up to 50 ml with 2 ml of dilute acetic acid and water. Separately, an amount of lead standard solution (equivalent to the specified allowed limit) is dilute to 50 ml with 2 ml of dilute acetic acid and water in a Nesler tube. After adding 2 drops each of sodium sulfate solution to each tube, well mixing, and setting aside for 5 minutes, both tubes are observed for color comparison with a white background.

## 28. Nitrogen Determination

### A. Kjeldahl Method

(1) Apparatus

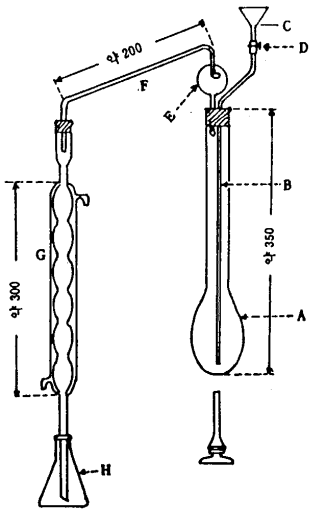
The apparatus is depicted in the figures below (unit : mm).

Ground joints may be used.

A : flask for decomposition (hard glass with 500 ~ 800 mL)

B : glass tube

C : funnel for addition of alkaline solution
D : rubber tube (there is a pinch cork at the connection between B & C)
E : Wagner tube
F : distillation tube
G : condenser
H : absorption flask (capacity : about 300 mL)




(2) Procedure

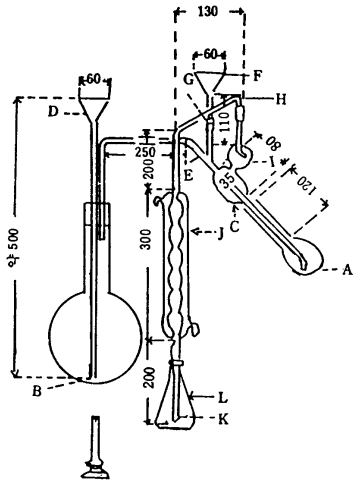
Unless otherwise specified, an amount of a sample corresponding to 20 ~ 30 mg of Nitrogen is placed in a flask for decomposition (A), add 5 g of potassium sulfate powder, 0.5 g of cupric sulfate, and 20 ml of sulfuric acid. The flask is tilted at a 45 degree angle, and heated gently until bubbles subside. It is boiled further at a higher temperature until it becomes blue transparent solution. And then boiled for 1 ~ 2 hours. After cooling, 15 ml of water is slowly added to the solution. 2 or 3 granules of boiling tips or granulated zinc are added to the solution and the apparatus is set-up as shown in the figure. 25 ml of 0.1 N sulfuric acid and 50 ml of water are added into the absorption flask (H). The end of the condenser (G) is immersed in the solution. Using a funnel (C), 85 ml of sodium hydroxide solution (2→5) is slowly added and its residue is washed down with a small amount of water. The pinch cork at D is closed and the decomposition flask is gently shaken to mix the content. It is then gently heated to boil and then boiled vigorously until 2/3 of the content is distilled out. The end of the cooling device is removed from the solution surface and its end is washed with water. Distillation is continued for a while. The excess amount of acid is titrated with 0.1 N sodium hydroxide solution (indicator : 3 drops of mixed solution of bromcresol green and methyl red). Separately, a blank test is carried out by the same procedure.

0.1 N sulfuric acid 1 ml = 1.401 mg N

## B. Semi-micro Kjeldahl Method

(1) Apparatus

It is made of hard glass as shown in the figure (units : mm). Ground joints may be used.




A : flask for decomposition (capacity : about 200 mL)

B : steam generator (capacity : about 1,000 mL)

C : Wagner tube

D : funnel for water

E : steam tube

F : funnel for alkaline solution

G : rubber tube (there is a pinch cork at the connection between E & F)

H : tube

I : small hole (inner diameter is almost same as the inner diameter of the tube)

J : condenser

K : end of cooling device (tip is angled)

L : absorption flask (capacity : about 300 mL)

If the apparatus is stored as assembled, Wagner tube (C) and its associated tubes need to be wrapped with asbestos mixed with magnesium carbonate dispersed in water. Decomposition flask (A) needs to be wrapped with cloth or asbestos sheet. Glycerin is applied to the rubber stopper between distillation device and decomposition flask. Rubber stopper and rubber hose are boiled in sodium hydroxide solution for 10 minutes and washed well with water before use. In a steam generator (B), water, 2 ~ 3 drops of sulfuric acid, and boiling stone are added. After use, the absorption flask (L) is washed thoroughly with water and sealed for storage.

(2) Procedure

An amount of sample corresponding to 2~3 mg of nitrogen is placed in the flask for decomposition (A), where 1 g of the mixture of powdered potassium sulfide and copper sulfide (10:1) is added. If the sample was attached to the neck of the flask, it is flushed it in a minimum amount of water. After slowly adding 7 ml of sulfuric acid along the inner wall of the flask, 1 ml of hydrogen peroxide is carefully added by the same method. The flask is heated on an asbestos net until the content becomes a blue transparent solution and carbonized matter on the inner wall disappears. If decomposition is insufficient, a small amount of hydrogen peroxide is added and cooled. Then it is heat treated again.

After cooling, 20 ml of water is carefully added and cooled and the flask is connected to the distillation apparatus, which is previously cleaned with steam. In the absorption flask (L), 15 ml of boric acid solution (1→25) and 3 drops of mixed solution of bromcresol green methyl red solution are added. An appropriate amount of water is added so that the tip of the cooling tube (K) is submerged into this solution. 30 ml of sodium hydroxide solution (2→5) is added through the funnel (F), which is washed with 10 ml of water. The pinch cork at G is closed and steam is passed through to start distillation. After collecting 80~100 ml of distillate, the tip of the distillation tube is removed from the solution and the tip is washed with a small amount of water. Water is added to bring the total volume to 157~180 ml, it is titrated with 0.01 N sulfuric acid. When the solution becomes almost colorless near the end point of titration, 1 drop of the mixture of bromcresol green·methyl red solution is added and further titrated. The end point is where the solution becomes slightly red in color. Separately, a blank test is carried by the same procedure.

0.01 N sulfuric acid 1 ml = 0.1401 mg N

## 29. pH Determination

pH is measured with a pH meter using a glass electrode.

pH represents an activity of hydrogen ion in a solution and is defined by the following equation. In a dilute solution, this value is very close to a natural log of a reciprocal value of hydrogen ion concentration.

$$\text{pH} = pHs + \frac{E - Es}{2.3026\,RT/F}$$

pHs : pH value of a pH standard solution

E : Voltage of a battery formed by combination of glass electrode and reference electrode in a test solution. Its constitution is expressed as follows.

Glass electrode | test solution || reference electrode

Es : Voltage of a battery formed by combination of glass electrode and reference electrode in a pH standard solution. Its constitution is expressed as follows.

Glass electrode | pH standard solution || reference electrode

R : Gas constant

T : Absolute temperature

F : Faraday constant

Values of 2.3026 RT/Fat various temperature is listed in the talbe below.

| Temperature | 2.3026 RT/F | Temperature | 2.3026 RT/F |
|---|---|---|---|
| 5° | 0.05519 | 35° | 0.06114 |
| 10° | 0.05618 | 40° | 0.06213 |
| 15° | 0.05717 | 45° | 0.06313 |
| 20° | 0.05817 | 50° | 0.06412 |
| 25° | 0.05916 | 55° | 0.06511 |
| 30° | 0.06015 | 60° | 0.06610 |

◦**Preparation of pH standard solution** : pH standard solution is used as a standard for pH. Water used in pH standard solution is prepared by the following procedure. Purified water is distilled. Distillate is boiled for at least 15 minutes to remove carbon dioxide. It is cooled with a carbon dioxide absorption tube (sodium carbonate). pH standard solution is stored in a hard glass or polyethylene bottle. Extended storage may cause change in pH. Acidic pH standard solution should be used in 3 months and alkaline pH solution should be stored with a carbon dioxide tube (sodium carbonate) and used in 1 month.

1) **Oxalate pH Standard Solution** : Potassium tetra-oxalate (pH measurement grade) is ground into powder and dried in a desiccator (silica gel), 12.71 g (0.05 gram moles) of which is dissolved in water to make 1 L.

2) **Phthalate pH Standard Solution** : Potassium hydrogen phthalate (pH measurement grade) is ground into powder and dried at 110° until the weigh becomes constant, 10.21 g (0.05 gram moles) of which is dissolved in water to make 1 L.

3) **Phosphate pH standard solution** : Monopotassium phosphate and Sodium hydrogen phosphate anhydrous (both pH measurement grade) are ground into powder and dried at 110℃ until the weigh becomes constant. 3.40 g (0.025 gram moles) of Monopotassium phosphate and 3.55 g (0.025 gram molecule) of sodium hydrogen phosphate are dissolved in water to make 1 L.

4) **Borate pH standard solution** : Sodium borate is dried in a desiccator (sodium bromide soaked in water) until the weight becomes constant, 3.81 g (0.01 gram moles) of which is dissolved in water (total volume = 1 L).

5) **Carbonate pH standard solution** : 2.10 g (0.02 gram moles) of sodium hydrogen carbonate for pH measurement, which is dried in a desiccator (silica gel) until the weight becomes constant and 2.65 g (0.025 gram moles) of sodium carbonate, which is dried at 300~500° until the weight becomes constant are dissolved in water to make 1 L.

6) **Calcium hydroxide pH standard solution** : Calcium hydroxide (pH measurement grade) is ground into powder, 5 g of which is placed in a flask.

It is mixed and saturated with 1 L of water at 23 ~ 27°. Supernatant is filtered and the filtrate is used (approximately 0.02 M).

pH values of these standard solutions at various temperatures are shown in the following table. pH values not listed in the table are obtained by interpolation. **Structure of pH meter** : A pH meter is generally comprises a detecting part that has a glass electrode and a reference electrode and the indication part that indicates pH corresponding to the detected electromotive force. Indication part has a tap for the regulation of asymmetric electric potential and for the temperature compensation and a tap for the sensitivity regulation. The reproducibility of the pH meter should be within ± 0.05 when pH of pH standard solution is measured 5 times (electrode should be washed well with water after each measurement)

pH Values of pH Standard Solution

| Temperature | pH Standard Solution | | | | | |
|---|---|---|---|---|---|---|
| | oxalate | phthalate | phosphate | borate | carbonate | KOH |
| 0° | 1.67 | 4.01 | 6.98 | 9.46 | 10.32 | 13.43 |
| 5° | 1.67 | 4.01 | 6.95 | 9.39 | 10.25 | 13.21 |
| 10° | 1.67 | 4.00 | 6.92 | 9.33 | 10.18 | 13.00 |
| 15° | 1.67 | 4.00 | 6.90 | 9.27 | 10.12 | 12.81 |
| 20° | 1.68 | 4.00 | 6.88 | 9.22 | 10.07 | 12.63 |
| 25° | 1.68 | 4.01 | 6.86 | 9.18 | 10.02 | 12.45 |
| 30° | 1.69 | 4.01 | 6.85 | 9.14 | 9.97 | 12.30 |
| 35° | 1.69 | 4.02 | 6.84 | 9.10 | 9.93 | 12.14 |
| 40° | 1.70 | 4.03 | 6.84 | 9.07 | | 11.99 |
| 50° | 1.71 | 4.06 | 6.83 | 9.01 | | 11.70 |
| 60° | 1.73 | 4.10 | 6.84 | 8.96 | | 11.45 |

**Procedure** : The glass electrode should be kept in water for more than several hours before use. The power of the pH meter should be turned on for at least

5 minutes before use. The detection part is washed with water, which is then is wiped with a filter paper. When a single point correction is performed, the tap for temperature compensation should be matched with the temperature of pH standard solution. Then the detection part is immersed for longer than 2 minutes in the pH standard solution which has the nearest pH value to the pH of test solution. The tap for the regulation of asymmetric electric potential is adjusted so that the pH reading matches with the pH of the standard solution at that temperature.

When a two-point correction is performed, the tap for temperature compensation is matched with the solution temperature. It is immersed in a phosphate pH standard solution that has the nearest pH value to a test solution. The tap for the sensitivity adjustment or the tap for the temperature compensation (regardless of the temperature of the standard solution) is manipulated by the same procedure as described before. After the adjustment, the detection part is washed well with water, which is then is wiped with a filter paper and pH is read.

Note : Detailed structure and Procedure differ with pH meter.

A solution with pH 11 and alkaline metal ions has large errors, so an electrode with a small alkali-related errors should be used (necessary correction should be done). It is desirable to match the temperature of test solution with that of pH standard solution.

# 30. Identification

This is used to identify each item. Unless otherwise specified, the concentration of test solution is approximately 1%.

**(1) Sodium**

(A) When potassium pyroantimonate solution is added to a neutral ~ weakly alkaline solution (1→20) of sodium, white crystalline precipitate is formed (scratching the inner wall of the test tube with a glass rod accelerates the precipitation).

(B) When sodium is tested by the Flame Coloration Test, it shows a yellow color.

**(2) Salicylate**

(A) When 5 ~ 6 drops of dilute ferric chloride solution is added to a neutral solution of salicylate, the solution becomes purple then colorless.

(B) When dilute hydrochloric acid is added to a salicylate solution (1→20), crystalline precipitate is created. The precipitate is separated, washed with cold water, and dried. The melting point of the precipitate is 158 ~ 161°.

**(3) Benzoate**

(A) When a solution of benzoate (1→20) is acidified with dilute hydrochloric acid, crystalline precipitate is formed. The precipitate is separated, washed with cold water, and dried. The melting point of the precipitate is 122°.

(B) When ferric chloride solution is added to a neutral solution of benzoate (1→20), reddish brown precipitate is produced. When diluted hydrochloric acid is added, white precipitate is separated out.

**(4) Calcium**

(A) When calcium salt is tested by the Flame Coloration Test, it shows a red color.

(B) When ammonium oxalate solution (1→30) is added to an acid solution of calcium salt with hydrochloric acid, white precipitate is formed. The separated precipitate is insoluble in dilute acetic acid, but it is soluble in dilute hydrochloric acid.

(5) Citrate

(A) When a mixed solution of phyridine·anhydrous acetic acid (3:1) is added to 2~3 mg of citrate, the color becomes deep red.

(B) Potassium permanganate solution (1/3 volume) is added to an acidic solution of citrate (1→20) with sulfuric acid, which is heated until the color disappears. White precipitate is produced by drop-wise adding bromine solution.

(6) Nitrite

(A) When dilute sulfuric acid is added to nitrite solution (1→20), yellowish brown gas with characteristic smell is generated. If a small amount of crystalline ferrous sulfate is added the solution, it becomes dark brown in color.

(B) When 2~3 drops of potassium iodide solution is added to a solution of nitrite, where dilute hydrochloric acid is drop-wise added, the solution becomes yellowish brown. Eventually blackish purple precipitate is formed. The solution becomes deep blue when starch solution is added.

(7) Sulfite and Hydrogensulfite

(A) When iodine potassium·iodide solution is drop wise added to an acidic solution of sulfite or hydrogen sulfite in acetic acid, the color of the solution is disappears.

(B) When a same amount of dilute hydrochloric acid is added to an acidic solution of sulfite or hydrogen sulfite in acetic acid (1→20), sulfur dioxide ($SO_2$) smell is generated but the solution doesn't turn turbid (distinct from thiosulfate). When 1 drop of sodium sulfate solution is added, the solution becomes turbid with white color, which gradually becomes yellow precipitate.

(8) Aluminum

(A) When ammonium chloride solution and ammonia solution are added to a solution of aluminum salt (1→20), white gel-like precipitate is produced. The precipitate does not dissolve by adding an excess amount of ammonium solution.

(B) When sodium hydroxide solution is added a solution of aluminum salts (1→20), white gel-like of precipitate is created. The precipitate dissolves by adding an excess amount of sodium hydroxide solution.

(C) When ammonium solution is added to a solution of aluminum salts until a small amount of precipitate is produced, where 5 drops of alizarin S solution (1→1,000) are added. The color of the precipitate changes to red

**(9) Ammonium**

An excess amount of sodium hydroxide solution is added to ammonium salts. Upon heating, a gas with ammonia odor is generated. This gas turns a red litmus paper (wetted with water) blue.

**(10) Chloride**

(A) Sulfuric acid and potassium permanganate are added to a solution of chloride salt (1→20). Upon heating, gas with chlorine odor is generated. This gas turns the color of potassium iodine starch paper (wetted with water) to blue.

(B) When silver nitrate solution is added to a chloride solution, white precipitate is created. The precipitate does not dissolve by adding dilute nitric acid, but it does dissolve by adding an excess amount of ammonia solution.

**(11) Peroxide**

(A) To a 1:1 mixture of ethyl acetate and peroxide solution, 1~2 drops of potassium bichromate solution is added. When the solution is acidified with dilute sulfuric acid, the aqueous layer becomes blue. When the mixture is shaken and settled to separate, the blue color migrates to the ethyl acetate layer.

(B) When potassium permanganate solution (1→300) is added a solution of peroxide in sulfuric acid, bubbles are created and the color disappears.

**(12) Permanganate**

(A) A solution of permanganate has a reddish purple color.

(B) When an excess amount of hydrogen peroxide is added to an acidic solution of permanganate in sulfuric acid, bubbles are generated and then disappear.

(C) When an excess amount of oxalic acid solution is added to an acidic solution of permanganate in sulfuric acid, the color of the solution disappears

**(13) Potassium**

(A) When potassium salts is tested by the Flame Coloration Test, it shows a light purple color. If the flame is yellow, it shows as reddish purple color

through a cobalt glass.

(B) When sodium hydrotartarate solution is added to a neutral solution of potassium salt(1→20), white crystalline precipitate is formed. (The scratching on the inner wall of the test tube with a glass rod accelerates the precipitation.) The precipitate separated from the solution dissolves when ammonia solution, sodium hydroxide solution or sodium carbonate solution is added

**(14) Glycerophosphorate**

(A) When ammonium molybdate solution is added to the solution of glycerophosphorate, precipitate is not produced when the solution is cold. Upon boiling for an extended period of time, yellow precipitate is formed.

(B) Glycerophosphorate is mixed with a same amount of potassium hydrogen sulfate powder. When the mixture is gently heated in a direct fire, an irritating odor of acrolein is generated.

**(15) Acetate**

(A) When diluted sulfuric acid (1→2) is added to acetate, acetic acid smell is created upon heating.

(B) When sulfuric acid and a small amount of alcohol are added to acetate and heated, an odor of ethyl acetate is generated.

(C) When ferric chloride solution is added to a neutral solution of acetate (1→20), it turns reddish brown. Upon heating, it forms reddish brown precipitate. When hydrochloric acid is added, the precipitate dissolves and the solution becomes yellow in color

**(16) Bromate**

(A) When 2～3 drops of silver nitrate solution is added to an acidic solution with nitric acid of bromate (1→20), white precipitate is formed, which dissolves by heating. If a drop of sodium nitrite solution is added, light yellow precipitate is produced.

(B) When 5～6 drops of sodium nitrite solution is added to an acidic solution with nitric acid of bromate (1→20), yellow ~ reddish brown color appears. If 1

ml of chloroform is added and mixed by shaking, chloroform layer shows yellow ~ reddish brown color.

**(17) Tartarate**

(A) When silver nitrate solution is added to a neutral solution of tartarate (1→20), white precipitate is formed. If nitric acid is added to the separated precipitate, it dissolves. If ammonia solution is added to the separated precipitate and heated, it dissolves and forms a silver mirror.

(B) 2 drops of acetic acid, 1 drop of ferrous sulfate solution, and 2 ~ 3 drops of hydrogen peroxide are added to tartrate solution (1→20), where an excess amount potassium hydroxide is added. The mixture turns reddish purple ~ purple.

(C) To 5 ml of sulfuric acid, 2 ~ 3 drops of Resorcin solution (1→50) and 2 ~ 3 drops of potassium bromide are added. This solution is added to 2 ~ 3 drops of tartarate solution (1→20). It is then heated for 5 ~ 10 minutes in a water bath. The solution becomes deep blue in color. When the resulting solution is cooled and mixed with an excess amount of water, it becomes red.

**(18) Nitrate**

(A) When ferrous sulfate solution is added on top of a cooled mixture (1:1) of nitrate solution and sulfuric acid, a dark brown band is formed at the interface.

(B) When sulfuric acid and copper fragments are added to a nitrate, reddish brown gas is generated.

(C) Even when potassium permanganate solution is added to an acidic solution of nitrate in sulfuric acid, the solution does not decolorizes (distinct from nitrite).

**(19) Carbonate**

(A) When diluted hydrochloric acid is added to carbonate, bubbles are generated due to formation of gas. If the gas is passed through calcium hydroxide solution, white precipitate is formed. (same as bicarbonate)

(B) When magnesium sulfate solution is added to carbonate solution (1→20), white precipitate is produced. When dilute acetic acid is added, the precipitate dissolves.

(C) A cold solution of carbonate turns deep red by adding phenolphthalein

solution. (distinct from bicarbonate)

(20) Bicarbonate

(A) When diluted hydrochloric acid is added to bicarbonate, bubbles are generated due to formation of gas. If the gas is passed through calcium hydroxide solution, white precipitate is created. (same as carbonate)

(B) When magnesium sulfate solution is added to bicarbonate solution (1→20), white precipitate is not created at normal temperature. However, white precipitate is formed upon boiling.

(C) A cold solution of bicarbonate does not turn red by adding phenolphthalein solution. Even if it does get colored, the red color is extremely pale (distinct from carbonate).

(21) Thiocyanate

(A) When excess amount of silver nitrate is added to thiocyanate solution, white precipitate is formed. The precipitate does not dissolve by adding diluted hydrochloric acid, but dissolves by adding aqueous ammonia solution.

(B) When ferric chloride solution is added to thiocyanate solution, it turns scarlet in color and this color does not disappear by adding hydrochloric acid.

(22) Ferrous salt

(A) When potassium ferricyanide is added to a weakly acidic solution of ferrous salts, blue precipitate is formed. The precipitate does not dissolve when diluted hydrochloric acid or diluted nitric acid is added.

(B) When sodium hydroxide solution or ammonia solution is added to ferrous salt solution, gel-like white precipitate is formed (If this is well shaken, the color becomes greyish green and gradually turns reddish brown). When sodium sulfide solution is added, the color of precipitate changes to black. When diluted hydrochloric acid is added, the precipitate dissolves

(23) Thiosulfates

(A) When iodide·potassium iodide is drop-wise added to an acidic solution in acetic acid of thiosulfate, the color of the solution disappears.

(B) When a same amount of diluted hydrochloric acid is added to thiosulfate

solution, sulfur dioxide smell is generated and the solution gradually becomes turbid with white color. When this is set aside, its color is changed to yellow.

(C) When an excess amount of silver nitrate standard solution is added to thiosulfate solution, white precipitate is formed. If the precipitate is set aside, its color is changed to black.

**(24) Ferric salts**

(A) When potassium ferrocyanide is added to a weakly acidic solution of ferric salt, blue precipitate is created. The precipitate does not dissolve when diluted hydrochloric acid or diluted nitric acid is added.

(B) When Sodium hydroxide solution or ammonia solution is added to ferric salt solution, gel-like reddish brown precipitate is formed. When sodium sulfate solution is added, the color of precipitate changes to black. When diluted hydrochloric acid is added to the separated precipitate, the precipitate dissolves. The solution is turbid with white color.

(C) If ammonium thiocyanate solution is added to a neutral or weakly acidic solution of ferric salt, deep red color appears. This color persists even by adding hydrochloric acid but disappears by adding mercuric chloride.

**(25) Cupric salts**

(A) If a clean iron fragment is placed in an acidic hydrochloric acid solution of cupric salt (with hydrochloric acid), red metal is precipitated from its surface.

(B) When a small amount ammonia solution is added to cupric salt solution, light blue precipitate is formed. If ammonia standard solution is added to this solution, the precipitate dissolves and its color turns deep blue.

(C) When potassium ferrocyanide solution is added to cupric salt solution, reddish precipitate is formed. When dilute acetic acid is added to a portion of this solution, the precipitate does not dissolve. When ammonia solution is added to another portion of this solution, the precipitate dissolves and its color turns deep blue.

**(26) Lactate**

When potassium permanganate solution is added to an acidic sulfuric acid

solution (1→20) and heated, acetaldehyde smell is generated.

(27) Magnesium

When ammonium chloride solution and ammonium carbonate solution are added to magnesium salt solution, precipitate is not created. However, if sodium phosphate dibasic is added to the resulting solution, white crystalline precipitate is created. The separated precipitate is insoluble in ammonia solution.

(28) Sulfate

(A) When barium chloride solution is added to sulfate solution, white crystalline precipitate is formced. The precipitate is insoluble in hydrochloric acid or weak nitric acid.

(B) When Lead acetate solution is added to a neutral solution of sulfate, white precipitate is formed. If ammonium acetate solution is added, the precipitate dissolves.

(C) Even if the same amount of weak hydrochloric acid is added to sulfate solution, it does not become turbid (distinct from thiosulfate). Also, Sulfur dioxide odor is not generated. (distinct from sulfite).

(29) Phosphate (Orthophosphate)

(A) When Silver nitrate solution is added to a neutral solution of phosphate, yellow precipitate is formed. The precipitate dissolves when diluted nitric acid or ammonia solution is added.

(B) Ammonium molybdate solution is added to a neutral or acidic nitric acid solution of phosphate. When this solution is heated, yellow precipitate is produced. The precipitate dissolves when sodium hydroxide or ammonia solution is added.

(30) Bromide

(A) When silver nitrate standard solution is added to bromide solution, light yellow precipitate is formed. The precipitate is hardly soluble in diluted nitric acid or ammonia solution. The precipitate is separated, where ammonia water is added and mixed by shaking. Solution is separated from the precipitate. When the solution is acidified with dilute nitric acid, it becomes turbid with

white color.

(B) If chlorine standard solution is added to bromide solution, yellow~reddish brown color appears. When chloroform or carbon disulfide is added to a portion of this solution and mixed, the lower layer has yellow~reddish brown color. If phenol is added to another portion of the solution, white precipitate is formed.

**(31) Zinc**

(A) Under the presence of sodium acetate, zinc salt produces white precipitate by hydrogen sulfide. The precipitate is insoluble in acetic acid, but is soluble in diluted hydrochloric acid. The similar precipitate is created in a neutral or alkaline solution by ammonium sulfide.

(B) When Potassium ferrocyanide is added to zinc salt solution, white precipitate is produced and it is insoluble in diluted hydrochloric acid.

**(32) Iodide**

When chlorine solution is added to an aqueous solution of iodide, iodide is extricated while changing the color from yellow to red. If chloroform is added to this solution and the mixture is shaken, the chloroform layer shows purple color. If starch standard solution is added to isolated iodine, the solution turns blue. If silver nitrate solution instead of starch standard solution is added, yellow precipitate is formed, which is insoluble in nitric acid and ammonia solution.

**(33) Succinic acid salt**

Adjust pH of succinic acid salt solution (1→20) to 6-7. To 5ml of the solution, add 1ml of ferric chloride TS. A yellow -red precipitate is formed.

## 31. Readily carbonizable substances test

This is to test for the allowed limit of impurities in substances, which are easily colored sulfuric acid, when a sample is dissolved in sulfuric acid. Unless otherwise specified, a specified amount of powdered sample is dissolved (small amount at a time) in 5 ml of 94.5%~95.5% of sulfuric acid by mixing with a glass rod in a test tube made of clear hard glass. The solution is set aside for 15 minutes.

Separately, color standard solution is placed in a test tube. Both tubes are observed for comparison with a white background. Observation is made from the side and the top. If it is specified that the sample needs to be dissolved by heating, colorimeter test is carried out after heating as specified for the item.

## 32. Ash and Acid-Insoluble Ash Limit Test

### A. Ash

Unless otherwise specified, 3g of sample is placed in a crucible, which is previously dried and weighed, and reduced to ash at 550° until readily carbonizable substances disappears. It is then cooled in a desiccator and weighed. If carbonization is incomplete, it is wetted with 1~2 drops water in a cooled crucible. It is dried in a water bath and then reduced to ash again.

### B. Acid-Insoluble Ash

To the ash obtained from A, 25 ml of dilute hydrochloric acid is added and the mixture is boiled for 5 minutes. It is then filtered through a quantitative filter paper. The residue is thoroughly washed with boiling water. The filter paper is dried and burned to obtain the amount of ash. The amount of acid-insoluble ash is obtained by subtracting the filter paper ash from the total ash.

## 33. Spectrophotometry

This method is to measure the degree of absorption of light in a narrow characteristic wavelength range. Absorption spectrum which material solution shows in visible and ultraviolet region depends on the chemical structure of each material. Therefore, by detecting absorption in various wavelengths, a material can be identified. Usually, absorption of a solution at a certain concentration at max

wavelength (λmax) and min wavelength (λmin) is measured, which is then used to Identification, Purity, and Assay test.

When a monochromatic light passes through a solution of a certain material, the ratio of the transmitted light intensity (I) to the incident light intensity (Io) is called the transmittance (T). Absorbance (A) is the common logarithm of the reciprocal of transmittance.

$$T = \frac{I}{I_0} \qquad A = \log\frac{I_0}{I} = -\log T$$

Absorbance (A) is proportional to the concentration (c) of the solution and the pathlength (l) the layer of of the solution.

$$A = kcl$$

The absorbance with 1cm (l) and 1% (c) is specific optical density (E), and the absorbance with 1cm (l) and 1M (c) is molecular extinction coefficient (E). Molecular extinction coefficient at the maximum absorption wavelength is $E_{max}$. Absorption measurement is carried out with a solution using a specified solvent. It is desireable to have a concentration of a solution so that the absorption within 0.2~0.7. If the absorption is too high, the solution is diluted to an appropriate concentration.

$$E^{1\%}_{1cm} = \frac{a}{c(\%)\times l} \qquad E = \frac{a}{c(mol)\times l}$$

l : path length (cm)

a : absorption from the measurement

c(%) : concentration of test solution (w/v%)

c(mol) : concentration of test solution (mol)

## A. Apparatus and Preparation

A photoelectric spectrophotometer or photoelectric colorimeter is used as a measuring apparatus. Photoelectric spectrophotometer consists of a

monochrometer and a photoelectric photometer. A tungsten lamp and a hydrogen discharge lamp are used as light source to measure absorption in visible and UV range, respectively. A photoelectric colorimeter consists of optical filter and photoelectric photometer. A tungsten lamp is used as a light source to measure absorption in visible range. As a cuvette, quartz is used for UV absorption and glass is used for visible absorption.

First, using a specified filter for each method or a filter that has measuring wavelength as a central wavelength, ut us adjusted so that a reference solution that lies in a light path gives a zero absorption at a wavelength of spectrophotometer that matches the measuing wavelength. Then a test solution is placed in the light path and an absorption is measured. If possible, a filter with a central transmission wavelength that closely matches with the maximum absorption band of the solution. It is also recommended that wavelength band of the filter transmission is narrower than the absorption band.

In absorption measurement of each additive, "a blank test is carried out to correct" means that a sample is not used as a reference. It rather means that a solution treated by the same procedure as above is used. "A blank test is carried out using a solvent as a reference" means the same solvent to dissolve the sample is used as a reference solvent.

### B. Determination Procedure

The equation expressing absorbance (A) is Beer-Lambert's law. This applies to a certain range of concentration of a sample. When absorbance measurement is used as a Assay, this measurable range of concentration should be known. When a standard material is not specified, a pure material of the sample should be used. A set of solutions with various concentrations are prepared and absorbance for each solution is measured. A curve of absorbance vs. concentration is prepared. The linear region of the curve obeys the Beer-Lambert Law and is used as a calibration curve for quantitative analysis.

### C. Calibration of Wavelength and Absorbance Scales

Wavelength values are usually examined using quartz mercury lamp or glass

mercury lamp at 239.95, 253.65, 302.16, 313.16, 334.15, 365.48, 404.66, 435.83, 546.10 nm and hydrogen discharge lamp at 486.13, 656.28 nm. Absorbance values are examined with a 0.006 w/v% solution of potassium bichromate (standard reagent) in 0.01N sulfuric acid. $E^{1\%}_{1cm}$ of this solution at 235 (min), 257 (max), 313 (min), and 350 nm (max) are 125.2, 145.6, 48.9, and 107.0, respectively.

## 34. Coloring Matter Tests

### A. Water Insoluble substances

2 g of sample is well mixed in 200 ml of boiling water by shaking and filtered through a crucible type glass filter (1G4) that is previously weighed. Insoluble substances are washed with boiling water until the wash water becomes colorless and dried along with the filter for 3 hours at 135°. After cooling in a desiccator, the filter with insoluble substances is weighed.

### B. Chloride and Sulfate

Precisely 0.1 g of sample is weighed and dissolved in water to make 100 ml, Use this solution as the Test Solution. Separately, 0.165 g of sodium chloride, which is dried at 500 ~ 600° for 1 hr, is dissolved in water to make 1,000 ml, Standard Stock Solution of chloride ion. Also, precisely 0.148 g of sodium sulfate, which is dried at 100° for 2 hrs, is dissolved in water to make 1,000 ml, Standard Stock Solution of sulfate ion.

Standard Solutions are prepared by diluting 0.2 ml, 1 ml, 10 ml and 50 ml each of the above Standard Stock Solutions to 100 ml with water. With 20 µl each of Test and Standard Solutions, ion chromatography is carried out under the following operation conditions. A calibration curves are prepared from the peak areas of chloride ions and sulfate ions in each Standard Solution. The content of each ion is obtained from the calibration curve using a peak area of Test Solution. Then the concentrations of sodium chloride and sodium sulfate are obtained by

multiplying 1.65 and 1.48 to the amount of chloride ion and sulfate ion. Finally, the contents of sodium chloride and sodium sulfate in the sample are calculated.

Operation Conditions

-Detector : Electrical Conductivity Meter

-Packing material : Porous anion exchange resin

-Column : Stainless steel or plastic tube with inner diameter 2～4 mm, length 20～25 cm

-Eluant : 1.8 mM sodium carbonate solution
1.7 mM sodium carbonate solution

-Flow rate : 1.0～1.5 mL/minute

C. Arsenic

0.5 g of Arsenic is placed in a platinum, quartz, or porcelain crucible. 20 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450～550°. If carbonaceous substance persists, it is wetted with a small amount of nitric acid, which is further heat treated at 450～550°. After cooling, 6 ml of hydrochloric acid is added to the residue and approximately 10 ml of water is added if necessary, which is then heated in a water bath. After cooling, the solution is brought up to 25 ml with water (Test Solution). When test for arsenic is carried out with this test solution, it should not be more than 4ppm. The color reference is prepared by following the same procedure with 2ml of arsenic standard solution.

D. Heavy Metals

2.5 g of sample is reduced to ash by the same procedure in Residues on Ignition. To the resulting ash, 3 ml of hydrochloric acid and then 7 ml of water are added and mixed. It is then filtered through a quantitative filter paper (Type 5, C). The residues are washed with 5 ml of dilute hydrochloric acid and 5 ml of water, which is added to the filtrate, Solution A. The residues on the filter paper are dried along with the filter paper at 105°, which is then reduced to ash in a platinum crucible by heating at approximately 450°. 1～2 g of anhydrous sodium carbonate is added to the crucible, which is then covered and heated to

melt the carbonate. After cooling, 10 ml of water is added, which is acidified by drop-wise adding 3 ~ 6 ml of hydrochloric acid. It is transferred into a beaker with a small amount of water, and then water to make 50 ml. Use this solution as the test solution. Separately, a blank test solution is prepared by following the same procedure without the sample.

(1) Zinc : 2.5 ml of test solution is diluted to 50 ml with 10 ml of diluted hydrochloric acid(1→4) and water, solution B. Separately, 2.5 ml of zinc standard solution, 10 ml of diluted hydrochloric acid(1→4) and water are added to 2.5 ml of blank test solution, which is then diluted to 50 ml, reference solution. For the solution B and the reference solution, proceed as directed under Atomic Absorption Spectrophotometry under the following operating condition. The absorbance of solution B should not be higher than that of reference solution (not more than 200 ppm).

Operation Conditions

Combustible gas : Acetylene

Combustible supporting gas : Air

Lamp : Zinc hollow cathode lamp

Wavelength : 213.9 nm

(2) Chromium : Unless otherwise specified, 10 ml of test solution is diluted to 50 ml with 10 ml of diluted hydrochloric acid(1→4) and water, solution C. Separately, 10 ml of chromium standard solution, 10 ml of diluted hydrochloric acid(1→4) and water are added to 10 ml of blank test solution, which is then diluted to 50 ml, reference solution. For the solution C and reference solution, proceed as directed under Atomic Absorption Spectrophotometry under the following operating conditions. The absorbance of the solution C should not be higher than that of reference solution (not more than 50 ppm).

Operation Condition

Combustible gas : Acetylene

Combustible supporting gas : Air

Lamp : Chromium hollow cathode lamp

Wavelength : 357.9nm

(3) Iron : 2 ml of test solution is diluted to 50 ml with 10 ml of diluted hydrochloric acid(1→4) and water, solution D. Separately, 5 ml of iron standard solution, 10 ml of diluted hydrochloric acid(1→4) and water are added to 2 ml of blank test solution, which is then diluted to 50 ml, reference solution. For the solution C and reference solution, proceed as directed under Atomic Absorption Spectrophotometry under the following operating condition. The absorbance of solution D should not be higher than that of reference solution (not more than 500 ppm).

Operation Condition

Combustible gas : Acetylene

Combustible supporting gas : Air

Lamp : Iron hollow cathode lamp

Wavelength : 248.3nm

(4) Manganese : Unless otherwise specified, 4 ml of test solution is diluted to 50 ml with 10 ml of diluted hydrochloric acid(1→4) and water, solution E. Separately, 1 ml of manganese standard solution, 10 ml of diluted hydrochloric acid(1→4) and water are added to 4 ml of blank test solution, which is then diluted to 50 ml, reference solution. For the solution E and reference solution, proceed as directed under Atomic Absorption Spectrophotometry under the following operating condition, the absorbance of solution E should not be higher than that of reference solution (not more than 50 ppm).

Operation Condition

Combustible gas : Acetylene

Combustible supporting gas : Air

Lamp : Manganese hollow cathode manganese lamp

Wavelength : 279.5nm

(5) Other Heavy Metals : Solution A is diluted to 50 ml with water, 20 ml of the solution is transferred into a Nestler tube. After adding 1 drop of phenolphthalein solution, ammonia solution is added until the solution turns

red and 2 ml of acetic acid (1→4) is added. The resulting solution is filtered, if necessary. The filter paper is washed with water and wash water is added to the filtrate. The filtrate is diluted to 50 ml with water, Solution H. Separately, 2.0 ml of lead standard solution and 1 drop of phenolphthalein solution are added to 20 ml of blank test solution, which is treated by the same procedure as the test solution H, Use this solution as the solution I. 2 drops each of sodium sulfate solution are added to Solutions H and I. They are mixed by shaking and set aside for 5 minutes. The color of H should not be deeper than that of I, (Not more than 20ppm).

**E. Other Coloring Matters**

Ammonium acetate solution is added to 5.0 ml, 2.0 ml and 1.0 ml each of standard stock solution. Each solution is diluted to exactly 100 ml with water, standard solution. Liquid chromatography is carried out with 20 µl each of test and standard solutions under the following operation conditions. Peak area of Subsidiary Colors in test solution is measured. From the calibration curve, the amount of each pigment is obtained. The sum of each pigment is calculated.

Operation Conditions

-Detector: Visible Light Spectrophotometer

-Column: Chemically bonded C18 column with 5 µm (inner diameter 4～6 mm, length 15～30 cm) or its equivalent

-Flow Rate: 1 ml/minute

-Wavelength: 515 nm

-Carrier Phase : A: ammonium acetate solution (7.7→1,000)

B: acetonitrile : methanol (70:30)

Solution A : Solution B (100:0) → Solution A : Solution B (30:70) 25 minutes

**F. Unreacted raw materials and products of side reactions**

Ammonium acetate solution is added to 5.0 ml, 2.0 ml and 1.0 ml each of standard stock solution. Each solution is diluted to 100 ml with water, standard solution. Liquid chromatography is carried out with 20 µl each of test and standard solutions under the following operation conditions. Peak area of

Unreacted raw materials and products of side reactions in test solution is measured. From the calibration curve, the amount is obtained.

Operation Conditions

-Detector : Visible Light Spectrophotometer

-Column : Chemically bonded C18 column with 5 μm (inner diameter 4～6 mm, length 15～30cm) or its equivalent

-Flow Rate : 1 ml/minute

-Wavelength : 290 nm

-Carrier Phase : A : ammonium acetate solution (7.7→1,000)

B : acetonitrile : methanol (70:30)

Solution A : Solution B(100:0) → Solution A : Solution B (30:70) 50 minutes

G. Unsulfonated Primary Aromatic Amines

(1) As Aniline

Accurately 2 g of sample is weighed into a separatory funnel containing 100 ml of water and dissolved by adding 50 ml of water, where 5 ml of sodium hydroxide solution (4→100) and 50 ml of ethyl acetate are added, shaken well, and extracted. Ethyl acetate layer is separated out. Water layer is further extracted 50 ml of ethyl acetate and the acetate layer is added to the previous extract. It is washed with sodium hydroxide solution (4→1,000) until the color disappears. The extract is again extracted three times with 10 ml of dilute hydrochloric acid (3→10). Hydrochloric acid phase are combined and diluted to 100 ml with water, Solution A. After cooling 10 ml of Solution A for 10 minutes in a test tube in an ice bath, 1 ml of potassium bromate solution (1→2) and 0.05 ml of sodium nitrite solution (1→30) are added, which is then set aside for 10 minutes in ice.

This mixed solution is transferred into a 25 ml volumetric flask with 1ml of 0.05 mo1/l of 3-hydroxy-2,7-naphthalein sulfonate disodium solution and 10 ml of sodium carbonate solution (1→10), which is filled with water to 25 ml. It is then set aside for 15 minutes, Test Solution.

Separately, 10 mg of aniline is dissolved in 30 ml of diluted hydrochloric acid

(3→10), which is diluted to 100 ml with water. 2.0 ml of this solution is further diluted to 100 ml with dilute hydrochloric acid (1→10). This solution is treated by the same procedure as Solution A and its absorption is measured. In case of Test Solution, 10 ml of Solution A is added to 25 ml of volumetric flask, where 1 ml of 0.05 mo1/l of 3-hydroxy-2,7-naphthalein sulfonate disodium solution and 10 ml of sodium carbonate solution (1→30). Then water is added to bring the total volume to 25 ml, Reference Solution. Absorption at 510 nm for each solution is measured. Absorbance of the test solution should be less than that of the reference solution.

H. Assay

(1) Titanium Trichloride Method

(A) A specified amount of test solution is placed in a 500 ml Erlenmeyer flask, where 15 g of sodium citrate and water are added to bring the total volume to approximately 200 ml. While bubbling carbon dioxide through and boiling the solution vigorously, it is titrated with 0.1 N titanium trichloride. The end point is where the characteristic color of the sample disappears

(B) The same procedure in (A) is followed with 15 g of sodium hydrogen tartarate instead of sodium citrate.

(C) The same procedure in (A) is followed with 15g of sodium hydrogen tartarate instead of sodium citrate. However, as an indicator, 10 ml of food colorant green No.2 solution (1→1,000) is used. Separately, a blank test is carried out.

(2) Weight Method

A specified amount of test solution is placed in a 500 ml beaker, which is boiled and then cooled. To this solution, 25 ml of dilute hydrochloric acid (1→50) is added, which is boiled again. The inner wall of the beaker is washed with approximately 5 ml of water and the beaker is covered with a watch glass. It is heated for 5 hours in a water bath and cooled in air. The precipitate is filtered through a glass filter (1G4) with a known weight. It is washed 3 times with 10 ml each of dilute hydrochloric acid (1→200) and then twice with 10 ml each of water. The precipitate is dried along with the glass

filter for 3 hours at 135°, cooled in a desiccator, and weighed.

## 35. Coloring Matter Aluminum Lake Test

### A. Hydrochloric Acid- and Ammonia-Insoluble substances

20 ml of water is added to 2 g of sample, where 20 ml of hydrochloric acid is added and mixed well. After adding and mixing with 300 ml of boiling water, it is covered with a watch glass, heated for 30 minutes in a water bath, and cooled. The supernatant is filtered through a glass filter (1G4) with a known weight. The insoluble substances are transferred to the glass filter with approximately 30 ml of water. It is then washed twice with 5 ml each of water. It is again washed with 1% ammonia solution until the wash liquid becomes almost colorless. It is then washed with 10 ml of 1% hydrochloric acid. It is further washed with water until the wash water does not react with silver nitrate solution. It is dried along with the glass filter for 3 hours at 135°, cooled in a desiccator, and weighed.

### B. Arsenic

0.5 g of Arsenic is placed in a platinum, quartz, or porcelain crucible. 20 ml of magnesium nitrate in ethyl alcohol (1→10) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450～550°. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450～550°. After cooling, 6 ml of hydrochloric acid is added to the residue and approximately 10 ml of water is added if necessary, which is then heated in a water bath. After cooling, the solution is brought up to 25 ml with water (Test Solution). When test for arsenic is carried out with this test solution, it should not be more than 4ppm. The color reference is prepared by following the same procedure with 2 ml of arsenic standard solution.

### C. Heavy Metals

2.5 g of sample is reduced to ash by the same procedure in Residues on Ignition. To the resulting ash, 5 ml of hydrochloric acid and 1 ml of nitric acid are added and lumps are crushed. It is evaporated to dryness in a water bath. Again, 5 ml of hydrochloric acid is added to crush the lumps. It is again evaporated to dryness in a water bath. The residues are dissolved by adding 10 ml of dilute hydrochloric acid and heating. After cooling, it is filtered through a quantitative filter paper (Type 5, C). The residues are washed with 30 ml of dilute hydrochloric acid, which is added to the filtrate. The filtrate is evaporated to dryness in a water bath. The residues are dissolved in 10 ml of dilute hydrochloric acid by heating. After cooling, it is filtered. The container and the filter paper are washed with a small amount of water. Wash water is added to the filtrate, where pH is adjusted to approximately 4 using ammonium acetate solution (1→10). It is then diluted to 100 ml with water. Separately, a blank test solution is prepared by following the same procedure without the sample.

(1) Zinc : To 10 ml of test solution, 10 ml of diluted hydrochloric acid(1→4) and water are added to bring the total volume to 50 ml, solution A. Separately, 2.5 ml of zinc standard solution, 10 ml of diluted hydrochloric acid(1→4) and water are added to 10 ml of blank test solution so that the total volume is 50 ml, reference solution. For the solution A and the reference solution, proceed as directed under Atomic Absorption Spectrophotometry under the following operating condition, the absorbance of solution A should not be higher than that of reference solution (not more than 50 ppm).

Operation Condition

Combustible gas : Acetylene

Combustible supporting gas : Air

Lamp : Zinc hollow cathode lamp

Wavelength : 213.9nm

(2) Iron : 4 ml of test solution is diluted to 50 ml with 10 ml of diluted hydrochloric acid(1→4) and water, solution B. Separately, 5 ml of iron standard

solution, 10 ml of diluted hydrochloric acid(1→4) and water are added to 4 ml of blank test solution, which is then diluted to 50 ml, reference solution. For the solution A and the reference solution, proceed as directed under Atomic Absorption Spectrophotometry under the following operation condition, the absorbance of solution B should not be higher than that of reference solution (not more than 250 ppm).

Operation Condition

Combustible gas : Acetylene

Combustible supporting gas : Air

Lamp : Iron hollow cathode lamp

Wavelength : 248.3nm

(3) Other Heavy Metals : 40 ml of test solution is diluted to 50 ml with water, Solution E. Separately, 40 ml of blank test solution, 2 ml of lead standard solution, and water are mixed to have 50 ml of Solution F. When 2 drops each of sodium sulfide solution is added to each Solution E & F, which is then mixed by shaking and set aside for 5 minutes, the color of Solution E should not be deeper than that of Solution F (Not more than 20ppm).

D. Barium

1 g of sample is placed in a platinum crucible, which is reduced to ash by following the procedure in Residues on Ignition. Ash is well mixed with 5g of anhydrous sodium carbonate. The crucible is covered and heated to melt the content. After heating for additional 10 minutes, it is cooled and 20 ml of water is added. The contents are dissolved by heating in a water bath.

After cooling, the solution is filtered and the residues are washed with water until the wash water does not show the reaction of sulfate salts. The residues along with the filter paper are transferred into a beaker, where 30 ml of dilute hydrochloric acid. It is well mixed and boiled. After cooling, it is filtered and the residues are washed with 10 ml of water and the wash water is added to the filtrate. The filtrate is evaporated to dryness in a water bath. The residues are dissolved by adding 5 ml of water, which is then filtered, if necessary. 0.25 ml

of dilute hydrochloric acid is added and mixed well. Water is added to the filtrate to bring the total volume to 25 ml, test solution. Separately, 0.5 ml of dilute hydrochloric acid and water are added to 0.5 ml of barium standard solution, which is then diluted to 25 ml, reference solution. Proceed test for the test solution and the reference solution as directed under Inductively Coupled Plasma Atomic Emission Spectrometry. The emission intensity of test solution should not be higher than that of reference solution (not more than 500 ppm).

**E. Assay**

(A) A specified amount of sample is placed in a 500 ml wide-mouth Erlenmeyer flask, where 20 ml of dilute sulfuric acid and then 50 ml of boiling water are added. The sample is dissolved by heating. To this solution, 150 ml of boiling water and then 15g of sodium citrate are added. While bubbling carbon dioxide through and vigorously boiling the solution, it is titrated with 0.1 N titanium trichloride solution. The end point is when the characteristic color of the sample is discharged.

(B) The same procedure in (A) is followed with 15g of sodium hydrogen tartarate instead of sodium citrate.

(C) The same procedure in (A) is followed with 15g of sodium hydrogen tartarate instead of sodium citrate. However, as an indicator, 10 ml of food colorant green No.2 solution (1→1,000) is used. Separately, a blank test is carried out.

## 36. Flavoring Substances Test

**A. Halogenated Compounds**

(1) Copper Screen Method : Copper wire, which is used at the end of copper screen of 15 mm width, 5 cm length, approximately 1 mm mesh size, is used. This copper screen is burned in a colorless flame of a burner until green color in the flame disappears and cooled in air. This is repeated several times so

that a film of oxide is formed. After cooling, the screen is coated with 3 drops of sample and burned. This is repeated 3 times. When this screen is burned in a colorless outer flame, that is adjusted to approximately 4 cm in height, green colored flame should not appear

(2) Ignition Method : A quantitative filter paper is cut into a siae of 5 cm width and 6 cm length, which is dipped into a sample and placed on a watch glass. The watch glass is placed on a tripod and the filter paper is ignited. Watch glass is immediately covered with a 1l beaker, which is wetted with water. After ignition is finished, the inner wall of the beaker is washed 10 ml of water. 1 drop of nitric acid and then 1 drop of silver nitrate solution are added to the wash water. The resulting turbidity should not be higher than that of a reference solution that is prepared by following the same procedure without a sample.

### B. Acid Value

Unless otherwise specified, approximately 10 g of sample is precisely weighed and dissolved (by heating, if necessary) in approximately 50 ml of alcohol (neutralized with 0.1 N potassium hydroxide using phenolphthalein solution) or 1:1 mixture of alcohol and ether. It is titrated with 0.1 N sodium hydroxide solution using a micro-burette until a red color persists for 30 seconds (indicator : 1 ml phenolphthalein solution). If the precipitate of sample is formed, additional solvent is added to dissolve it.

$$\text{Acid value} = \frac{Amount\ of\ 0.1N\ potassium\ hydroxide\ solution\,(ml)\times 5.611}{Weight\ of\ sample\,(g)}$$

### C. Ester Value and Ester Content

Unless otherwise specified, a specified amount of sample is precisely weighed into a 150 ml flask, where 10 ml of alcohol and 3 drops of phenolphthalein solution are added. It is neutralized with 0.1 N potassium hydroxide solution. 25 ml of 0.5 N alcoholic solution of potassium hydroxide is added. A reflux condenser is attached to the flask, which is gently boiled for 1 hour in a water

bath. After cooling, 1 ml of phenolphthalein solution is added to the solution and excess alkali is titrated with 0.5 N hydrochloric acid. Separately, a blank test is carried out.

$$\text{Ester Value} = \frac{(a-b)\times 28.05}{Weight\ of\ sample(g)}$$

The equationfor ester content below applies only to the monobasic acid esters.

$$\text{Ester content (\%)} = \frac{molecular\ weight\ of\ ester\times(a-b)\times 0.5}{Weight\ of\ sample(g)\times 1{,}000}\times 100$$

$$= \frac{ester\ value\times molecular\ weight\ of\ ester}{561.1}$$

a : consumed amount of 0.5N hydrochloric acid in blank test (ml)

b : consumed amount of 0.5N hydrochloric acid for test solution (ml)

**D. Saponification Value**

Unless otherwise specified, a specified amount of sample is precisely weighed into a 150 ml flask, where 25 ml of 0.5 N alcoholic solution of potassium hydroxide. A reflux condenser is attached to the flask, which is gently boiled for 1 hour in a water bath. After cooling, 1 ml of phenolphthalein solution is added to the solution and excess alkali is titrated with 0.5 N hydrochloric acid. Separately, a blank test is carried out.

$$\text{Saponification Value} = \frac{(a-b)\times 28.05}{Weight\ of\ sample(g)}$$

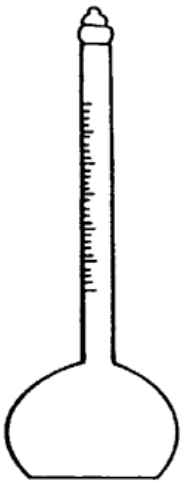
a : consumed amount of 0.5 N hydrochloric acid in blank test (ml)

b : consumed amount of 0.5 N hydrochloric acid for test solution (ml)

**E. Phenol Content**

Unless otherwise specified, the content of phenol is measured as the content of

matters in a sample that are soluble in alkali hydroxides and by the following method.



10 ml of sample is placed in 150 ml of Cassia flask, where 75 ml of potassium hydroxide solution is added in 3 portions. It is mixed well by shaking for 5 minutes. It is set aside for 30 minutes, where 1N potassium hydroxide solution is slowly added until the insoluble oil rises up to the graduated marks of the flask. After setting aside for 1 hour, the amount is measured.

Phenol Content (%) = 10 × [10  amount of insoluble oil (ml)]

### F. Alcohol Content

Alcohol content is the content of isolated alcohols that are present in a sample.

#### Procedure

Unless otherwise specified, the following method is followed.

#### Method 1

In a 100 ml flask with an air condenser, 10 ml of sample, 10 ml of anhydrous acetic acid, and 1 g of anhydrous sodium acetate (freshly melt by heating) are added and gently boiled for 1 hour in a water bath. After setting aside for 15 minutes, 50 ml of water is added, which is heated for 15 minutes in a water

bath. After cooling, the contents is transferred into a separatory funnel and the aqueous phase is separated out. Oil phase is washed with sodium carbonate solution until the wash solution becomes basic. It is then washed with sodium chloride solution until the wash solution becomes neutral. This oil phase is transferred into a dried container, where approximately 2 g of anhydrous sodium sulfate is added and mixed well by shaking. It is set aside for 30 minutes and then filtered. A specified amount of acetylated oil is precisely weighed and tested by the following ester value measurement method.

This ester value is also called acetyl value and calculated by the following equation.

$$\text{Acetyl Value} = \frac{(a-b)\times 28.05}{Weight\ of\ acetylated\ oil\,(g)}$$

(1) A sample without ester

$$\text{Alcolhol content (\%)} = \frac{Molecular\ weight\ of\ alcohol\times(a-b)\times 0.5}{[amount\ of\ acetylated\ oil\,(g)-0.02102\,(a-b)]\times 1{,}000}\times 100$$

$$= \frac{acetyl\ \ value\times molecular\ \ weight\ \ of\ \ alcohol}{561.1-(0.4204\times acetyl\ \ value\,)}$$

a : consumed amount of 0.5 N hydrochloric acid in blank test (ml)

b : consumed amount of 0.5 N hydrochloric acid for test solution (ml)

### Method 2

A specified amount of sample is precisely weighed into a 200 ml flask with a stopper, where 5 ml of anhydrous acetic acid·pyridine solution is added. The connection part is wetted with 2~3 drops of pyridine and the stopper is loosened. It is then heated for 1 hour in a water bath. After cooling, the inner wall of flask and the stopper are washed with 10 ml of water into the flask.

After a stopper is placed, it is mixed by shaking and cooled to normal temperature. The connection part and the inner wall are washed with 5 ml of neutralized alcohol into the flask. It is then titrated with 0.5N alcoholic solution of potassium hydroxide (indicator : 2~3 drops of cresol red·thymol blue solution). Separately, a blank test is carried out by the same procedure.

$$\text{Alcohol content (\%)} = \frac{Molecular\ weight\ of\ alcohol \times (a-b) \times 0.5}{Weight\ of\ sample(g) \times 1{,}000} \times 100$$

a : consumed amount of 0.5 N alcoholic solution of KOH for test solution (ml)

b : consumed amount of 0.5 N alcoholic solution of KOH in a blank test (ml)

**G. Aldehydes and Ketones content**

(1) Sodium Hydrogen Sulfite Method

Unless otherwise specified, 10 ml of sample is placed in a 150 ml Cassia flask, where 75 ml of sodium hydrogen sulfite solution is added and mixed. It is heated while shaking occasionally in a boiling water bath until the lump disappears completely. 25 ml of sodium hydrogen sulfite solution is added to the solution, shaken and mixed, which is then set aside for 10 minutes in a boiling water bath. Sodium hydrogen sulfite solution is slowly added until the insoluble oil rises up to the graduated marks of the flask. After setting aside for 1 hour, the amount is measured.

Content of aldehydes and ketones (%) = 10 × [10  amount of insoluble oil (ml)]

(2) Sodium Sulfite Method

Unless otherwise specified, 75 ml of freshly prepared 30% sodium sulfite solution and 2 drops of phenolphthalein solution are added to a 150 ml Cassia flask and isolated alkali is neutralized with acetic acid. 10 ml of sample is added to the flask, which is well shaken in a boiling water bath. Isolated alkali is occasionally neutralized with acetic acid. If the solution does not show red~pale red color by adding 3 drops of phenolphthalein solution, the flask is set aside for 15 minutes in a boiling water bath. 30% of sodium sulfite solution

(neutralized with acetic acid using phenolphthalein solution as an indicator) is slowly added until the insoluble oil rises up to the graduated marks of the flask. After setting aside for 1 hour, the amount is measured.

Content of aldehydes and ketones (%) = 10 × [10  amount of insoluble oil (ml)]

(3) Hydroxylamine Method

**Method 1**

A specified amount of sample is precisely weighed and well mixed by shaking with 50 ml of 0.5 N hydroxylamine hydrochloride solution, which is set aside or gently boiled in a water bath using a reflux condenser for a specified period of time. It is then cooled to room temperature. Isolated acid is titrated with 0.5N alcoholic solution of potassium hydroxide. Separately, a blank test is carried out by the same procedure

Content of aldehydes and ketones (%)

$$= \frac{molecular\ \ weight\ \ of\ \ aldehydes\ \ and\ \ ketones \times (a-b) \times 0.5}{Weight\ \ of\ \ sample(g) \times 1{,}000} \times 100$$

a : consumed amount of 0.5 N alcoholic solution of KOH for test solution (ml)

b : consumed amount of 0.5 N alcoholic solution of KOH in a blank test (ml)

**Method 2**

A specified amount of sample is precisely weighed and mixed well by shaking in 75 ml of hydroxylamine solution, which is set aside or gently boiled in a water bath using a reflux condenser for a specified period of time. It is then cooled to room temperature. The excess amount of hydroxylamine is titrated with 0.5 N hydrochloric acid. The end point is where the color of solution changes from violet to greenish yellow. Separately, a blank test is carried out by the same procedure.

Content of aldehydes and ketones (%)

$$= \frac{molecular\ weight\ of\ aldehydes\ and\ ketones \times (a-b) \times 0.5}{Weight\ of\ sample(g) \times 1{,}000} \times 100$$

a : consumed amount of 0.5N hydrochloric acid in blank test (ml)

b : consumed amount of 0.5N hydrochloric acid for test solution (ml)

## 37. Oils Test

Oils test is measured for acid value, saponification value and ester value about fatty acid, aliphatic alcohols, and ester of fatty acid except for flavoring.

**A. Acid value** : Unless otherwise directed, a specified amount of sample is accurately weighed and dissolved(by heating, if necessary) in approximately 50ml of mixture of ethyl alcohol and ether(1:1), previously neutralized to 0.1N alcoholic potassium hydroxide(indicator : phenolphtalein TS). After cooling, add a few drops of phenolphthalein TS and titrate, while shaking, with 0.1N alcoholic potassium hydroxide TS to the first pink color that persists for at least 30seconds.

Acid Value = Amount of 0.1N potassium hydroxide(ml) x 5.611/Weight of sample (g)

**B. Saponification Value**

Unless otherwise directed, a specified amount of sample is accurately weighed and dissolved in 40ml of ethyl alcohol(by heating, if necessary), and added 20ml of alcoholic potassium hydroxide TS. A reflex condenser is connected to the flask, which is gently boiled with shaking for 30 min in a water bath. After cooling, wash the condenser with a few ml of water, add a few drops

of phenolphthalein TS and titrate the excess potassium hydroxide with 0.5N hydrochloric acid. Perform a blank determination using the same amount of alcoholic potassium hydroxide TS.

Saponification value = (a-b) x 28.05/ weight of sample (g)

a: Consumed amount of 0.5N hydrochloric acid in blank test (ml)

b: Consumed amount of 0.5N hydrochloric acid in sample solution (ml)

C. Ester value

Unless otherwise specified, Ester value is calculated by the formula after measuring saponification value and acid value.

Ester value= saponification value - acid value

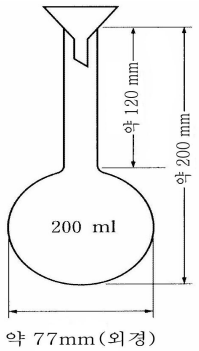
D. Hydroxyl Value

Unless otherwise specified, approximately 1g of the sample is accurately weighed and transfer it into a round flask indicated in the picture below. 5ml of pyridine acetic anhydride reagent is accurately taken and placed in the flask. Small funnel is placed on the entrance of the flask, and the bottom of the flask is immersed about 1cm in the oil bath of 95 ~ 100°. Next, shake the flask, and heat for 10minutes. After cooling, funnel and upper part of the flask are washed with 5ml alcohol. Excess acetic acid is titrated with alcoholic potassium hydroxide (indicator: 1ml of phenolphthalein TS). Blank test is conducted as the same manner.

Hydroxyl Value = (a-b) x 28.05/ sample weight (g) + acid value

a: Consumed amount of 0.5N alcoholic potassium hydroxide in blank test (ml)

b: Consumed amount of 0.5N alcoholic potassium hydroxide in sample solution (ml)




## 38. Test of Bactericidal Activity

Application and Principle : Test of Bactericidal Activity is for measuring whether food contact surface sanitizing solutions have or do not have a bactericidal activity. There are three tests : 1. test of bacterial suspension, 2. test of bacterial surface, and 3. test of spore suspension. Unless otherwise specified, measure by 1. test of bacterial suspension.

Definition of Bactericidal Activity : Reduction rate(%) in the number of viable bacterial cells(cfu/ml) corresponding to numbers of specified bacterial cells at the beginning (cfu/ml) under defined conditions regarding food contact surface sanitizing solutions.

### Test 1. Test of bacterial suspension

There are dilution-neutralization and membrane filtration in this test. The test of dilution-neutralization is as follows: add the suspension of test bacteria containing interfering substances to a prepared test solution, incubate at 20° for 5 minutes, immediately suppressed by a previously verified suitable neutralizer, determine the number of surviving bacteria in each sample and the reduction in viable counts is calculated. The test of membrane filtration can be used to suppress the bactericidal action by using filter film instead of neutralizer. In addition, verification test should be performed at the same time when this test is done.

1) Preparation of test solution

Test solution is prepared by taking a certain amount of sample into hard water at three different concentrations as follows. In addition, these test solutions should be prepared within 60 minutes for test..

- Solution A : The concentration of the solution is 1.25 times the required test concentration. However, in case of using undiluted solution itself, undiluted solution is solution A.
- Solution B and solution C : Solutions diluted 2 times and 4 times solution A.

2) Test Organisms

Use following 2 strains as standard organism.

- *Escherichia coli* ATCC 10536 or *Escherichia coli* ATCC 11229
- *Staphylococcus aureus* ATCC 6538

However, besides standard organisms above, additional strains can be chosen from, for example:

- *Bacillus cereus* ATCC 21772
- *Vibrio parahaemolyticus* ATCC 27969
- *Salmonella ctyphimurium* ATCC 13311
- *Listeria monocytogenes* ATCC 19111 (or *Listeria monocytogenes* ATCC 19115)

3) Working Cultures of Test Organisms

Test organisms spread on TSA medium and incubate at 36° for 18 to 24 hours. Prepare a second subculture in the same way and prepare third subculture, again. The second or third subculture designate as the working culture. However, for working culture of *V. parahaemolyticus a*mong additional test organisms, 10㎕ of test organisms inoculate on 10 ml of TSB medium, previously adjusted to 2% sodium chloride, and incubate at 36° for 18 to 24 hours. After culture, second and third subculture is done in 100 ml of TSB medium, previously adjusted to 2% sodium chloride.

4) Preparation and counting of bacterial test suspension

(1) Preparation : Take 10 ml of diluent(TSCS) and place in a 100 ml erlenmeyer flask with 5 g of glass beads (diameter 3~4㎜) and transfer *E. coli* working

culture using a platinum loop. The cells should be suspended in the diluent by immersing the platinum loop in the diluent(TSCS) and rubbing it against the side of the flask to dislodge the cells. Shake the flask for 3 minutes using a mechanical shaker. Take the suspension from inside of the glass beads and transfer to another tube. Adjust the number of viable cells in the suspension to $1.5 \sim 5 \times 10^8$ cfu/m*l* with the diluent(TSCS). Incubate the suspension in the constant-temperature water bath at 20° for 2 hours. This is *E. coli* bacterial test suspension.

Separately, prepare *S. aureus* working culture in the same manner as the above test preparation and prepare bacterial test suspension of *S. aureus*. However, for working culture of *V. parahaemolyticus a*mong additional test organisms, transfer working culture in 3) above into a sterilized 50 ml centrifuge tube. Centrifuge it at 20°, 5000×g (6,000rpm) for 5 minutes and discard the supernatant carefully. Add 25 ml of diluent(TSCS) to residual cells and stir it for 10 seconds with a mechanical shaker. After stirring, centrifuge it again and discard the supernatant. Add 2 ml of diluent(TSCS) and suspend the cells. Take 10 ml of diluent(TSCS) and place in a 100 ml erlenmeyer flask filled with 5 g of glass beads. Add previously prepared bacterial suspension. Shake the flask for 3 minutes with a mechanical shaker. Adjust the number of viable cells in the suspension to $1.5 \sim 5 \times 10^8$ cfu/m*l u*sing diluent (TSCS). Incubate the suspension in the constant-temperature water bath at 20° for 2 hours. This is bacterial test suspension.

(2) Counting : Prepare $10^{-6} \sim 10^{-7}$ dilutions of the *E. coli* bacterial test suspension with diluent(TSCS). Take 1 ml each of this fluid into the duplicate petri dishes and aseptically pipette approximately 15 ml of TSA medium maintaining 45°. After cooling and solidifying, add 3~5 ml of TSA medium and duplicated. Upside down the cooled and solidified petri dishes and incubate at 36° for 24 hours. Count the number of colony forming units in petri plates. Incubate the plates for a further 24 hours. When no longer plates show well separated colonies, count the highest number of colonies. Calculate the number of viable cells(N) in the *E. coli* test suspension with following equation.

Count of viable cells in bacterial test suspension *(cfu/ml) = $\frac{c}{(n_1 + 0.1n_2)d}$

c : The sum of the colonies counted on petri plates

$n_1$ : The number of petri plates counted at the first dilution

$n_2$ : The number of petri plates counted at the secondary dilution

d : The dilution rate of first dilution

* Only colony counts which are 15~300 cfu/plate should be used for calculation of viable counts. For a result to be valid, viable counts should be calculated using at least 2 or more plates. If plates from two dilutions fall within this range, calculate the number of cfu/ml as the calculation above. If plates from only one dilution fall within this range, calculate the arithmetic mean. Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is more than 5, the preceding figure is increased by one unit; if the last figure is equal to 5, round off the preceding figure to the next nearest even figure in case of the preceding figure is odd, and make 0 in case of the preceding figure is even. Repeat this process until valid two figures are obtained. As a result, the number of cfu/ml is expressed by a number between 1.0 and 9.9 multiplied by a multiple of 10.

Separately, prepare *S. aureus* test suspension in the same manner as test procedure above and calculate the number of viable cells in *S. aureus* test suspension. The number of viable cells in each test suspension should be $1.5\times10^8$ cfu/ml ~ $5\times10^8$ cfu/ml.

5) Preparation and counting of bacterial test suspended diluent

(1) Preparation : Dilute *E. coli* test suspended diluent with diluent (TSCS) to adjust the number of viable cells of *E. coli* to $6\times10^2$ ~ $3\times10^3$ cfu/m*l*. This is *E. coli* test suspended diluent.

Separately, prepare *S. aureus* test suspension in the same manner as above test procedure and prepare *S. aureus* test suspended diluent.

(2) Counting : Dilute *E. coli* bacterial suspended diluent 10 times with diluent(TSCS). Transfer 1 ml each of this solution into duplicate petri dishes and incubate in the same manner as (2) in 4) above. Count the highest number of

colonies on petri plate. Calculate the number of viable bacterial cells(Nv) in *E. coli* bacterial suspended diluent under following equation.

The number of viable bacterial cells in test procedure

and verification test (cfu/m*l*) = $\frac{c}{n \times d \times V}$

c : The sum of the colonies counted on petri plates

n : The number of petri plates counted

d : The dilution factor (In case the dilution neutralization test procedure and the bacterial suspended diluent, the dilution factor is $10^{-1}$)

V : The volume of sample (In case the dilution neutralization and verification test, and the bacterial suspended diluent, the volume is 1.0 ml. In case the membrane filtration test and validation procedure, the volume is 0.1 ml. )

Separately, prepare *S. aureus* bacterial suspended diluent in the same manner as test procedure above and calculate the number of viable cells in *S. aureus* bacterial suspended diluent. The number of viable cells in each bacterial suspended diluent should be $6 \times 10^2$ cfu/m*l*~ $3 \times 10^3$ cfu/m*l*

6) Test Procedure

The temperature of product test solution, bacterial test suspension, bacterial suspended diluent, test solution, and water maintain at 20° in a constant temperature water bath.

(1) Dilution-neutralization method

① Test Procedure

Transfer 1 ml of interfering substance and bacterial test suspensions into the test tube. Immediately mix it and incubate it in a constant temperature water bath at 20° for 2 minutes. 8 ml of test solution (solution A) is added, mixed, and incubate in a constant temperature water bath at 20° for 5 minutes. Pipette 1 ml of this mixture into a test tube filled with 8 ml of neutralizer and 1 ml of water. Neutralize it in a constant temperature water bath at 20° for 5 minutes. After neutralization, take 1 ml each of neutralized mixture into duplicate petri dishes each. Incubate them by adding TSA medium as (2) in 4) above. Count the highest number of colonies

on petri plate. Calculate the number of viable cells by sterilization (Na) with the formula (2) in 5) above.

Separately, proceed on *S. aureus* bacterial suspension and other test solutions (solution B and C) in the same manner as test procedure above. Calculate the number of viable cells by sterilization of test solution. However, for the sample with undiluted solution, add 9.8 ml of test solution(solution A) and add 0.1 ml of each of interfering substance and bacterial suspension with preparing their concentration 10 times higher than that used above. Proceed in the same manner as the test procedure above.

② Test for Validation

Ⓐ Validation of experimental conditions

Pipette 1 ml of interfering substance and 1 ml of bacterial suspended diluent into a test tube. Mix for a few seconds and incubate in a constant temperature water bath at 20° for 2 minutes and add 8 ml of hard water, mix, and maintain it in a constant temperature water bath at 20° for 5 minutes. Take 1 ml each of the mixture into duplicate petri dishes and incubate them under (2) in 4) above. Count the highest number of colonies on petri plate. Calculate the number of viable cells in test for validation of experimental condition under the formula (2) in 5) above.

Separately, proceed *S. aureus* suspended diluent in the same manner as test procedure above and calculate the number of viable cells. The number of viable cells in each test organism should be 0.05 times or more than the number of viable cells in test suspended diluent.

Ⓑ Neutralizer toxicity validation

Place 8 ml of neutralizer, 1 ml of water, and 1 ml of *E. coli* suspended diluent into a test tube. Mix for a few seconds and incubate in a constant temperature water bath at 20° for 5 minutes. Take 1 ml each of the mixture into duplicate petri dishes and incubate them by adding TSA medium in the same manner as (2) in 4) above. Count the highest number of colonies on petri plate and calculate the number of viable cells (B) in neutralizer toxicity validation under the formula (2) in 5) above.

Separately, proceed *S. aureus* suspended diluent in the same manner as test procedure above and calculate the number of viable cells. At that time, the number

of viable cells in each test organism should be 0.05 times or more than the number of viable cells in test suspended diluent.

© Dilution-neutralization validation

Place 1 ml of interfering substance, 1 ml of diluent(TSCS), and 8 ml of test solution (solution A) into a test tube. Mix for a few seconds and incubate in a constant temperature water bath at 20° for 5 minutes. Take 1 ml of the mixture into a test tube filled with 8 ml of neutralizer and incubate it in a constant temperature water bath at 20° for 5 minutes. To this solution, add 1 ml of *E. coli* suspended diluent, mix, and incubate in a constant temperature water bath at 20° for 30 minutes. Take 1 ml each of the mixture into duplicate petri dishes and incubate them by adding TSA medium in the same manner as (2) in 4) above. Count the highest number of colonies on petri plate and calculate the number of viable cells (C) in dilution-neutralization validation under the formula (2) in 5) above.

Separately, proceed *S. aureus* suspended diluent in the same manner as test procedure above and calculate the number of viable cells. The number of viable cells in each test organism should be 0.5 times or more than the number of viable cells calculated in dilution-neutralization validation.

(2) Membrane Filtration Method

The temperature of product test solutions, bacterial suspension, test solution, and water is stabilized at 20° in a constant temperature water bath.

① Test Procedure

Pipette 1 ml of interfering substance and 1 ml of *E. coli* test suspension into a test tube. Immediately mix and place the test tube in the constant temperature water bath at 20° for 2 minutes. Add 8 ml of product test solution(solution A), mix, and leave in the constant temperature water bath at 20° for 5 minutes. 0.1 ml each of the test mixture and 50 ml of the rinsing liquid transfer to two separate membrane filtration apparatus and immediately filter. The time required for filtration should not exceed 1 minute. Filter by adding 150~500 ml of rinsing liquid and 50 ml of water to each membrane filtration apparatus. The surface filtered of membrane place toward, closely face on TSA medium and incubated at 36° for 24

hours. Care should be avoid let air in between the membrane and agar surface. Count the number of colonies on the petri plates and incubate the plates for a further 24 hours. When no longer plates show well-separated colonies, count the higher number of colonies in petri plates. Calculate the number of viable cells(Na) by sterilization in the test solution under the formula (2) in 5).

Separately, proceed *S. aureus* test suspension and other test solutions(solution B and C) in the same manner as test procedure above and calculate the number of viable cells by sterilization of test solution. However, for the sample with undiluted solution, add 9.8 ml of test solution(solution A) and add 0.1 ml each of interfering substance and bacterial suspension with preparing their concentration 10 times higher than that used above. Proceed in the same manner as the test procedure above.

② Test for Validation

Ⓐ Validation of experimental conditions

Proceed 1 ml of *E. coli* suspended diluent, 8 ml of hard water, and 50 ml of water instead of 1 ml of E. coli test suspension, 8 ml of test solution(solution A), and 150~500 ml of rinsing liquid from 6) (2) ① above, under the same manner as test above. Count the highest number of colonies for each petri plate and calculate the number of viable cells in the validation of experimental conditions under the formula (2) in 5) above.

Separately, proceed *S. aureus* suspended diluent in the same manner as test procedure above and calculate the number of viable cells in the validation of experimental conditions. The number of viable cells in each test organism should be 0.05 times or more than the number of viable cells in test suspended diluent.

Ⓑ Validation of the filtration procedure

Transfer 0.1 ml each of *E. coli* suspended diluent and 50 ml each of the rinsing liquid into two separate membrane filtration apparatus and filter immediately. Add 50 ml of water to each filtration apparatus, filter and incubate the filter paper in the same manner as 6) (2) ① above. Count the highest number of colonies on petri plates. Calculate the number of viable cells in the filtration control (B) in validation of the filtration procedure using the method (2) in 5) above.

Separately, proceed *S. aureus* suspended diluent in the same manner as test procedure

above and calculate the number of viable cells in validation of the filtration procedure. The number of viable cells in each test organism should be 0.05 times or more than the number of viable cells in test suspended diluent.

© Validation of the filtration method

Add 1 ml of interfering substance, 1 ml of diluent(TSCS), and 8 ml of test solution (solution A) into a test tube, mix and incubate in the constant temperature water bath at 20° for 5 minutes. Transfer 0.1 ml of the test mixture and 50 ml of the rinsing liquid to a separate membrane filtration apparatus equipped, filter immediately. Filter by adding 150~500 ml of water. Add 50 ml of rinsing liquid and 0.1 ml of *E. coli* suspended diluent to each membrane filtration apparatus and filter. Add 50 ml of water additionally, filter, and incubate the filter paper in the same manner as ①, (2) in 6) above. Count the highest number of colonies on petri plate and calculate the number of viable cells(C) on the filtration control in validation of the filtration method using the method (2) in 5) above.

Separately, proceed *S. aureus* suspended diluent in the same manner as test procedure above and calculate the number of viable cells in validation of the filtration method. The number of viable cells in each test organism should be 0.5 times or more than the number of viable cells calculated in validation of the filtration procedure.

7) Conclusion

For each of test organism and product test solution, calculate the reduction rate of the number of viable cells in the bacterial test suspension under following formula. When the reduction in viability of solution A is not less than 99.999%, it is appropriate.

$$Reduction\ in\ viability(\%) = \frac{N - 10N_a}{N} \times 100$$

$N_a$ – The number of viable cells by sterilization in test solution *(cfu/ml)

* Apply "$1.5\times10^2$ cfu/ml" as the number of viable cells(Na) when counted colonies is not more than 15 and apply "$3\times10^3$ cfu/ml" as the number of viable cells(Na) when counted colones is not less than 300 in the product test procedure.

## Test 2. Test of bacterial surface

A test suspension of bacteria in a solution of interfering substances is spread onto a

stainless steel surface and dried. A prepared sample of the product under test is applied on the dried film and is maintained at 20° for 5 minutes. The surface is transferred to a previously verified neutralization medium so that the action of the disinfectant is immediately neutralized. The reduction in viable counts is calculated by measuring he number of surviving organisms on each surface. However, main test proceed reference test using diluent instead of test solution, and perform test for validation at the same time.

1) Preparation of test solution

Product test solution is prepared in hard water at three different concentrations by taking a certain amount of sample as follows. In addition, these product test solutions should be prepared freshly and used within 60 minutes.

- Solution A : The solution is diluted to the required test concentration. However, in case of using undiluted solution itself, undiluted solution is solution A.
- Solution B and solution C : Solutions diluted 2 times and 4 times solution A.

2) Test Organisms

Use 2 strains as standard organism 2) in Test 1. Additional organisms are not used.

3) Working Cultures of Test Organisms

Follow 3) in Test 1.

4) Preparation and counting of bacterial test suspension

Follow 4) in Test 1.

5) Preparation for inoculating solution of test surfaces

Add 400㎕ of *E. coli* bacterial suspension and 100㎕ of interfering substance to a small test tube. Shake the test tube for 1 minute using a mechanical shaker. Maintain the suspension in the constant-temperature water bath at 20° for 30 minutes. This is *E. coli* inoculating of test surface.

Separately, prepare inoculated test surfaces for *S. aureus* test suspension in the same manner as test procedure above.

6) Preparation of test surfaces

Inoculate 10㎕ of *E. coli* inoculated test surfaces onto the middle of carrier (ø1㎝×0.07㎝ stainless steel disc ANSI 304 2B) and dry on 36° heat plate. This is *E. coli* test surface.

Separately, prepare test surface for *S. aureus* inoculated test surface in the same

manner as test procedure above.

7) Test Procedure

The temperature of product test solution, test solution, apparatus, etc. should be stabilized at 20°. Carrier and glass bottle are previously dried in desiccator.

① Test Procedure

Carefully place the dried inoculated surfaces upwards and put it into a glass bottle (bottom diameter 2~3 cm, volume 15~20ml) using sterilized tongs. Add 50㎕ of product test solution (solution A) on the middle of test surface in glass bottle. Maintain it at 20° for 5 minutes. Add 9.95 ml of neutralizer and 2~3g of glass beads, mix for 1 minute in a mechanical shaker, and filter with membrane filtration apparatus. When filtering, rinse 100~150 ml of diluent(TSCS) 2 times or 3 times. Separate filtration membrane from membrane filtration apparatus and incubate following ①, (2) in Test 1. Proceed the test procedure above five times with prepared *E. coli* test surface. Count the highest number of colonies in each petri plate. Calculate the number of viable cells by sterilization in the test solution(Nd) under the following equation.

The number of viable bacterial cells in test procedure

and test for validation (cfu/carrier) = $\frac{c}{n \times d}$

$c$ : the sum of the colonies counted on petri plates taken into account

$n$ : the number of petri plates taken into account

$d$ : the dilution factor corresponding to the dilution taken into account

Separately, calculate the number of viable cells(Nc) in reference test by using diluent (TSCS). Dilute the mixture by $10^{-2}$, $10^{-3}$, and $10^{-4}$ with diluent (TSCS) and filter with membrane filtration apparatus. Proceed the test procedure of surface 3 times. Proceed *S. aureus* test surface and other test solutions(solution B and C) in the same manner as test procedure above. Calculate the number of viable cells by sterilization of test solution. The number of viable cells in reference test of each cell should be more than $1.5 \times 10^5$ cfu/carrier under the formula above.

② Test for Validation

Ⓐ Validation of neutralization

Add 9.95 ml of neutralizer and 50㎕ of test solution(solution A) into glass bottle. Mix

and maintain at 20° for 5 minutes. Then add *E. coli* test surface and 2~3 g of glass bead. Mix for 1 minute in a mechanical shaker. Dilute the mixture by $10^{-3}$, $10^{-4}$ and $10^{-5}$ with diluent (TSCS) and filter with membrane filtration apparatus. When filtering, rinse 100~150 ml of diluent (TSCS) 2 times or 3 times. Separate filtration membrane from membrane filtration apparatus and incubate following ①, (2) in Test 1. Proceed the described test procedure above two times with previously prepared *E. coli* test surface. Count the highest number of colonies in each petri plate. Calculate the number of viable cells (B) in validation of neutralization under the equation ① in 7) above.

Separately, proceed *S. aureus* test surface in the same manner as the test procedure above. Calculate the number of cfu/ml in validation of neutralization. The number of viable cells of each bacterial organism should be more than $1.5\times10^5$ cfu/carrier.

Ⓑ Neutralizer toxicity validation

Proceed under test for validation using diluent(TSCS) instead of test solution(solution A) in Ⓐ, ② in 7) above. Count the highest number of colonies in each petri plate. Calculate the number of viable cells(A) in Neutralizer toxicity validation under the equation ① in 7) above.

Separately, proceed *S. aureus* test surface in the same manner as test procedure above. Calculate the number of viable cells in neutralizer toxicity validation. The number of viable cells in each test organism should be not less than 0.5 times and not more than 2 times the number of cfu/ml counted in neutralizer toxicity validation.

8) Conclusion

For each test organism and product test solution, calculate the reduction of the number of viable cells in each test organism under following formula. When the reduction in viability of solution A is more than 99.99%, it is appropriate.

$$\textit{Reduction in viability}(\%) = \frac{N_c - N_d}{N_c} \times 100$$

$N_c$ - Viable counts in reference test (cfu/carrier)

$N_d$ - The number of viable cells by sterilization in test solution *(cfu/carrier)

* Apply "1.5×10 cfu/carrier" as the number of viable cells when counted colonies is not more than 15 and

apply "$3\times10^2$ cfu/carrier" as the number of viable cells when counted colones is not less than 300 in the product test procedure.

## Test 3. Test of spore suspension

There are dilution-neutralization and membrane filtration in this test. The test of dilution-neutralization is as follows : add a spore suspension in a solution of interfering substances to a prepared test solution, maintain at 20° for 60 minutes, immediately suppressed by a previously verified suitable neutralizer, determine the number of spore in each sample and the calculate reduction rate. If a suitable neutralizer cannot be found, membrane filtration can be used to suppress the bactericidal action by using filter film instead of neutralizer.

In addition, verification test should be performed at the same time when this test is done.

1) Preparation of test solution

Test solution is prepared in hard water at three different concentrations by taking a certain amount of sample as follows. In addition, these test solutions should be prepared freshly and used within 60 minutes.

- Solution A : The concentration of the solution is 1.25 times the required test concentration. However, in case of using undiluted solution itself, undiluted solution is solution A.
- Solution B and solution C : Solutions diluted 2 times and 4 times solution A.

2) Test Organisms

Use Bacillus subtilis ATCC 6633

3) Preparation of spore solution

Inoculate about $10^6$ spores on Nutrient Broth and incubate at 30° for 18~24 hours. Inoculate 2~3 ml of this culture medium into Roux bottle solidified by Nutrient Agar containing manganese sulfate and incubate at 30° for 14 days. Concentrate bacterial cells with sterilized glass beads and water. Centrifuge it at 10,000rpm for 20 minutes, discard the supernatant, float in the water, and rinse. Repeat this procedure 3 times. Float the residue in the water and heat at 75° for 10 minutes. Keep this spore solution in the refrigerator. For long-term preservation, keep it in a freezer.

4) Preparation and counting of spore suspension

(1) Preparation : Dilute spore solution with water to adjust the number of viable cells to $1.5\sim5\times10^6$ cfu/m*l*. Maintain it in a constant temperature water bath at 20°. This

is spore suspension. This solution should be used within 2 hours after preparation.

(2) Counting : Prepare $10^{-4}$ ~ $10^{-5}$ dilutions of the spore suspension with water. Add 1ml of each dilution to duplicate petri plates, add nutrient agar with 45°. Incubate under (2), 4) in Test 1 and calculate the number of spore (N) of spore suspension. Then, the number of spore in spore suspension should be 1.5 ~ $5\times10^{6}$ cfu/ml.

5) Preparation and counting of spore suspended diluent

(1) Preparation : Dilute spore suspended diluent with water to adjust the number of spores to $6\times10^{2}$ ~ $3\times10^{3}$ cfu/m*l*. This is spore suspended diluent.

(2) Counting : Dilute spore suspended diluent 10 times with water. Transfer 1 ml each of this solution into 2 separate petri dishes and add nutrient Agar stabilized at 45°. Incubate in the same manner as (2) in 4) above. Count the highest number of colonies on petri plate. Calculate the number of viable cells in spore suspended diluent (Nv) under the equation in (2) 5) in Test 1. The number of spores in spore suspended diluent should be $6\times10^{2}$ cfu/m*l* ~ $3\times10^{3}$ cfu/m*l*

6) Test procedure

Use following contact temperature($\Theta$) and contact time($t$).

- Contact temperature($\Theta$) : 20°
- Contact time($t$) : 60 minutes

However, following additional conditions may be chosen except conditions above.

- Contact temperature($\Theta$) : 4°, 10°, 40° or 75°, etc.
- Contact time($t$) : 5 minutes, 15 minutes or 30 minutes, etc.

All reagent should be stabilized at following temperature before test.

- contact temperature($\Theta$) (NMT 40°) : test solution, spore suspension, interfering substance are stabilized at $\Theta$° in constant temperature water bath. neutralizer and water are stabilized at 20°.
- contact temperature($\Theta$) (more than 40°) : test solution is stabilized at $\Theta$°. neutralizer, spore suspension, interfering, and water are stabilized at 20°.

(1) Dilution-neutralization method

① Test Procedure

Pipette 1 ml of interfering substance and 1 ml of spore suspensions into a test

tube. Immediately mix and maintain it in a constant temperature water bath at $\theta°$ for 2 minutes. 8 ml of test solution (solution A) is added, mixed, and maintained in a constant temperature water bath at $\theta°$ for 5 minutes. Pipette 1 ml of this mixture, transfer it into a test tube filled with 8 ml of neutralizer and 1 ml of water. Neutralize it in a constant temperature water bath at 20° for 5 minutes. After neutralization, take 1 ml of neutralized mixture into 2 separate petri dishes and incubate them by adding nutrient agar under (2) in 4) in Test 1. Count the highest number of colonies on petri plate and calculate the number of spores by sterilization (Na) with the formula (2), 5) in Test 1.

Separately, proceed other test solutions(solution B and C) in the same manner as test procedure above and calculate the number of spores by sterilization of test solution. However, for the sample with undiluted solution, add 9.8 ml of test solution(solution A) and add 0.1 ml each of interfering substance and bacterial suspension to prepared their concentration 10 times higher than that used above. Proceed in the same manner as the test procedure above.

② Test for Validation

Ⓐ Validation of experimental conditions

Pipette 1 ml of interfering substance and 1 ml of spore suspended diluent into a test tube. Mix for a few seconds and leave in a constant temperature water bath at $\theta°$ for 2 minutes. Add 8 ml of hard water, mix, and maintain it in a constant temperature water bath at $\theta°$ for t minutes. Take 1 ml each of the mixture and transfer into 2 separate petri dishes. Incubate them under (2), 4) in Test 1. Count the highest number of colonies on petri plate. Calculate the number of spores (A) in test for validation of experimental condition under the formula (2), 5) in Test 1. The number of spores in each test organism should be 0.05 times or more than the number of spores in spore suspended diluent.

Ⓑ Neutralizer toxicity validation

Place in a test tube, 8 ml of neutralizer, 1 ml of water, and 1 ml of spore suspended diluent. Mix for a few seconds and leave in a constant temperature water bath at 20° for 5 minutes. Take a sample of 1 ml each of the mixture and transfer into 2

separate petri dishes. Incubate them by adding nutrient agar in the same manner as (2) in 4) in Test 1. Count the highest number of colonies in petri plate. Calculate the number of viable cells in Neutralizer toxicity validation (B) under the formula (2) in 5) in Test 1. The number of spores should be 0.05 times or more than the number of spores in spore suspended diluent.

© Dilution-neutralization validation

Place in a test tube, 1 ml of interfering substance, 1 ml of water, and 8 ml of test solution (solution A). Mix for a few seconds and leave in a constant temperature water bath at $\theta°$ for t minutes. Transfer 1 ml of the mixture into a test tube filled with 8 ml of neutralizer. Leave it in a constant temperature water bath at 20° for 5 minutes. To this solution, add and mix 1 ml of spore suspended diluent and leave in a 20° constant temperature water bath for 30 minutes. Take a sample of 1 ml each of the mixture and transfer into 2 separate petri dishes. Incubate them by adding nutrient agar in the same manner as (2) in 4) in Test 1. Count the highest number of colonies in petri plate. Calculate the number of spores (C) in Dilution-neutralization validation under the formula (2) in 5) in Test 1. The number of spores should be 0.5 times or more than the number of spores calculated in Neutralizer toxicity validation.

(2) Membrane Filtration Method

① Test Procedure

Pipette 1 ml of interfering substance and 1 ml of spore suspended diluent into a test tube. Immediately mix and place the test tube in a constant temperature water bath at $\theta°$ for 2 minutes. Add 8 ml of product test solution(solution A), mix, and leave in a constant temperature water bath at $\theta°$ for t minutes. Pipette 0.1 ml each of the test mixture and transfer each of sample and 50 ml of the rinsing liquid into a separate membrane filtration apparatus and filter immediately. The time required for filtration should not exceed 1 minute. First, filter 150~500 ml of rinsing liquid and additionally filter 50 ml of water to each membrane filtration apparatus. The upper side of the membrane placed closely on the nutrient agar and incubated at 36° for 24 hours. Care should be avoid let air in between the membrane and agar surface. Count the number of colonies on petri plates and incubate the plates for a further 24 hours.

When no longer plates show well-separated colonies, count the highest number of colonies on petri plates. Calculate the number of spores by sterilization in the test solution under the formula (2), 5) in Test 1. However, for the sample with undiluted solution, add 9.8 ml of test solution(solution A) and add 0.1 ml of each of interfering substance and bacterial suspension with preparing their concentration 10 times higher than that used above. Proceed in the same manner as the test procedure above.

② Test for Validation

Ⓐ Validation of experimental conditions

Proceed 1 ml of spore suspended diluent, 8 ml of hard water, and 50 ml of water instead of 1 ml of spore suspension, 8 ml of test solution(solution A), and 150～500 ml of rinsing liquid, under the same manner as test above. Count the highest number of colonies on each petri plate and calculate the number of spores in the validation of experimental conditions (A) under the formula given in 5), (2) in Test 1. The number of spores should be 0.05 times or more than the number of spores in spore suspended diluent.

Ⓑ Validation of the filtration procedure

Take 0.1 ml each of spore suspended diluent. Transfer each of sample and 50 ml of the rinsing liquid into two separate membrane filtration apparatus and filter immediately. After filtering 50 ml of water to each filtration apparatus and incubate the filter paper in the same manner as ①, (2), 6) in Test 1. Count the highest number of colonies on petri plates. Calculate the number of spores in the filtration control (B) in validation of the filtration procedure (2), 5) in Test 1. The number of spores should be 0.05 times more than the number of spores in spore suspended diluent.

Ⓒ Validation of the filtration method

Place in a test tube, 1 ml of interfering substance, 1 ml of water, and 8 ml of test solution (solution A). Mix and leave in a constant temperature water bath at $\Theta^{\circ}$ for t minutes. Pipette 0.1 ml each of the test mixture and transfer each of sample and 50 ml of the rinsing liquid into a separate membrane filtration apparatus and filter. Filter by adding 150～500 ml of water again. Add 50 ml of rinsing liquid and 0.1 ml of spore suspended diluent to each membrane filtration apparatus and filter. Add 50

ml of water additionally, filter, and incubate the filter paper in the same manner as ①, (2), 6) in Test 1. Count the higher number of colonies on petri plates. Calculate the number of spores in the filtration control (C) in validation of the filtration method under the formula 5) (2) in Test 1. The number of spores should be 0.5 times or more than the number of spores calculated in validation of the filtration procedure.

7) Conclusion

For each test solution, calculate the reduction rate under following formula, respectively. When the reduction rate of the number of spores of solution A is more than 99.9%, it is appropriate.

$$Reduction\ rate\ of\ spores(\%) = \frac{N - 10N_a}{N} \times 100$$

$N_a$ - The number of spores by sterilization in test solution *(cfu/m*l*)

* Apply "$1.5\times10^2$ cfu/carrier" as the number of viable cells when counted colonies is 15 or below and apply "$3\times10^3$ cfu/m*l*" as the number of viable cells when counted colones is more than 300 in the product test procedure.

Apparatus

Membrane filtration apparatus : It should have a usable volume 50 ml minimum, and suitable filtration membrane (diameter:47 ~ 50㎜, pore size:0.45㎛) should be used. In vacuum, the filtration rate should be equal so that microorganism can be distributed uniformly on the whole filtration membrane. To avoid long time filtration, it should be designed for 100 ml of rinsing liquid to be filtered between 20 seconds and 40 seconds.

Culture media

1) TSA (Tryptone Soya Agar)

| | |
|---|---|
| Tryptone, pancreatic digest of casein | 15.0g |
| Soya peptone, papaic digest of soybean meal | 5.0g |
| NaCl | 5.0g |
| Agar | 15.0g |

Dissolve above ingredients in 1,000 ml of distilled water. Adjust the pH to 7.2 and sterilize at 121° for 15 minutes.

2) TSB (Tryptone Soya Broth)

Tryptone, pancreatic digest of casein 15.0g

Soya peptone, papaic digest of soybean meal 5.0g

NaCl 5.0g

Dissolve above ingredients in 1,000 ml of distilled water. Adjust the pH to 7.2 and sterilize at 121° for 15 minutes.

3) Nutrient Broth

Peptone 10.0g

Beef Extract 3.0g

Dissolve above ingredients in 1,000 ml of distilled water. Adjust the pH to 7.2 and sterilize at 121° for 15 minutes.

4) Nutrient Agar

To 1,000 ml of nutrient agar, add 15.0g of refined agar, heat, dissolve, and correct the content of distilled water. Adjust the pH to 6.8 and sterilize at 121° for 15 minutes.

5) Amended Nutrient Agar

To Nutrient Agar, add manganese sulfate($MnSO_4 \cdot H_2O$) so that the content becomes 5$\mu$g/m*l*. Sterilize at 121° for 15 minutes.

Test solution

1) Sterilized phosphate buffer solution

Dissolve 34 g of anhydrous potassium dihydrogen phosphate in 500 ml of distilled water. Add 175 ml of 1N sodium hydroxide to adjust the pH to 7.2. Add distilled water to make 1,000ml, phosphate buffer solution. Sterilize this solution at 121° for 20 minutes. Dilute 1 ml of this solution in 800 ml of sterilized distilled water, sterilized phosphate buffer solution.

2) Water

The water shall be free from substances that are toxic or inhibiting to the bacteria. It shall be distilled water and not demineralized water. Sterilize at 121° for 15 minutes.

3) Diluent (TSCS)

Tryptone Sodium Chloride Solution:

| | |
|---|---|
| Tryptone, pancreatic digest of casein | 1.0g |
| NaCl | 8.5g |

Dissolve above ingredients in 1,000 ml of distilled water. Adjust the pH to 7.2 and sterilize at 121° for 15 minutes.

4) Neutralizer

Choose proper one of the following neutralizers and use sterile one. The neutralizer should be validated in the test for validation.

(1) Neutralizers

| | |
|---|---|
| lecithin | 3g |
| polysorbate 80 | 30g |
| sodium thiosulfate | 5g |
| L-histidine | 1g |
| saponine | 30g |

Dilute above ingredients to 1,000 ml by adding 1% sterilized phosphate buffer solution or diluent.

(2) Sterilized phosphate buffer solution

Dissolve 34 g of potassium dihydrogen phosphate($KH_2PO_4$) in 500m*l* of water. Adjust the pH to 7.2 using 1N sodium hydroxide. Dilute this solution to 1,000 ml with water.

(3) 5% or 0.5%(V/V) egg yolk solution

Dilute 10 ml or 1 ml of sterilized egg yolk solution (the same amount of sterilized physiological saline is added to egg yolk solution) to 100 ml with water.

(4) Solution containing 3%(V/V) polysorbate 80, sodium lauryl sulfate at 4g/*l* , and lecithin at 3g/*l*.

(5) Solution containing 5%(V/V) egg yolk and 4%(V/V) polysorbate 80

(6) Solution containing 7%(V/V) ethylene oxide condensate of fatty alcohol, lecithin at 20g/*l* , and 4%(V/V) polysorbate 80

(7) Solution containing 4%(V/V) ethylene oxide condensate of fatty alcohol and lecithin at 4g/*l*.

(8) Solution containing 3%(V/V) polysorbate 80, lecithin at 3g/*l* , and histidine at 1g/*l*.

(9) Glycine

(10) Solution containing 3%(V/V) polysorbate 80 and lecithin at 3g/*l*.

(11) Solution containing phospholipid emulsion at 50㎎/m*l* and lecithin at 4g/*l*.
(12) Sodium thioglycollate solution at 0.05g/*l* or 0.5g/*l*
(13) Cysteine solution at 0.8g/*l* or 1.5g/*l*
(14) Sodium thiosulfate solution at 0.5g/*l*

With the exception of list above, other suitable neutralizer can be used.

5) Rinsing solution

Sterilized rinsing liquid should be used. Rinsing liquid should be filtered through membrane. One of the following rinsing liquid can be used.

(1) water
(2) diluent (TSCS)
(3) solution of 0.1%(V/V) polysorbate 80
(4) solution of 0.5%(V/V) polysorbate 80
(5) solution of 0.5%(V/V) polysorbate 80 and lecithin at 0.7g/*l*
(6) neutralizer
(7) sterilized phosphate buffer solution

The list above is not exhaustive and other liquids can be used.

6) Hard water

Hard water for dilution of products is prepared as follows.

- solution A : Dissolve 19.84g of anhydrous magnesium chloride and 46.24g of anhydrous calcium chloride in water and make to 1*l*.
- solution B : Dissolve 35.02g of sodium hydrogen carbonate in water and make to 1,000m*l*.

Add 3.0 ml of solution A and at least 600 ml of water into a 1,000 ml volumetric flask, then add 8.0 ml of solution B and dilute to 1,000 ml with water. Adjust the pH of the solution to 7.0. Sterilize by passing through a filter with a maximum effective pore size of 0.45㎛ or below. The solution can be stored at 4 ~ 8° for a maximum one month.

7) Interfering substances in Test of bacterial suspension or Test of spore suspension. Bovine albumin solution(clean condition or dirty condition) should be used as interfering substance. When precipitate is generated by the action between interfering substance and test solution, the following suitable interfering substance

can be chosen to be tested.

(1) Bovine albumin solutions

Bovine albumin solutions for the test conditions is prepared as follows :

- Preparation for clean conditions

Dissolve 0.3g of bovine albumin (Cohn fraction V for Dubos medium) in 100m*l* of water and sterilize by membrane filtration.

- Preparation for dirty conditions

Dissolve 3g of bovine albumin (Cohn fraction V for Dubos medium) in 100m*l*에 of water and sterilize by membrane filtration.

(2) Milk

100 g of powdered milk, guaranteed free of antibiotics or additives, is dissolved in 1*l* of water and prepared to 10%(V/V) solution. Sterilize for 30 minutes at 105°(or for 5 minutes at 121°).

(3) Yeast extract

Dissolve dehydrated yeast extract to 100g/*l* with water. Adjust the pH to 7.0±0.2 using NaOH. Sterilize at 121° for 15 minutes.

(4) Sucrose

Dissolve sucrose to 100g/*l* with water. Sterilize it using filtration membrane.

(5) pH 5 and pH 9 buffer solutions

(6) Sodium lauryl sulfate

Dissolve sodium lauryl sulfate to 50g/*l* with water. Sterilize at 121° for 15 minutes.

8) Interfering substance of test of bacterial surface

Interfering substance used for reflecting real use condition is prepared by mixing bovine albumin solution and tryptone solution. Prepare and store under following preparation method.

- Bovine albumin solutions : Add 0.3 g of albumin to 10 ml of sterilized phosphate buffer solution, filter and sterilize it.
- Tryptone soluton : Add 0.1 g of tryptone to 10 ml of sterilized phosphate buffer solution. filter and sterilize it.

Mix 50㎕ each of filtered and sterilized solutions(1:1) just before use and store it at 20°.