B. GENERAL TESTS

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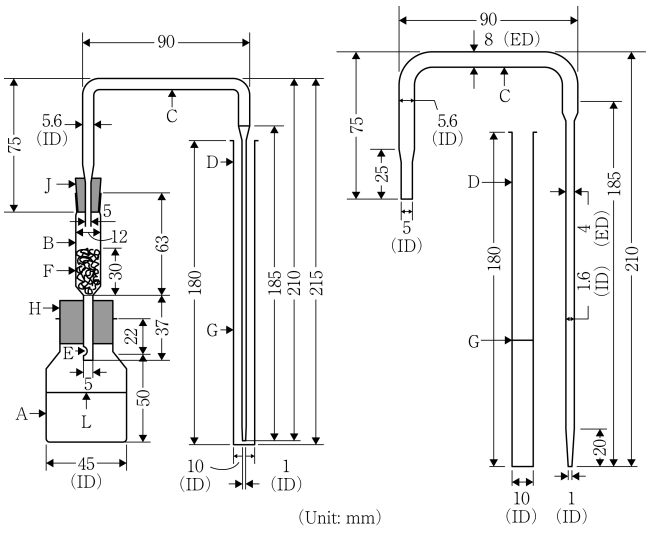
Arsenic Limit Test

The arsenic limit test is designed to demonstrate that the content of arsenic in an additive does not exceed the acceptable limit specified in the individual monograph.

In the Monographs, the specification "not more than 3 μg/g as As (0.50 g, Method 1, Standard Color: Arsenic Standard Solution 3.0 mL, Apparatus B)" for this test, for example, means that the arsenic content of the substance must be not more than 3 μg/g as As when determined using the following manner: *The test solution is prepared with 0.50 g of the test substance as directed in Method 1, the standard color is prepared with 3.0 mL of Arsenic Standard Solution, then the test is performed as directed under Method using Apparatus B.*

Apparatus B Use the apparatus illustrated in Fig. 1.

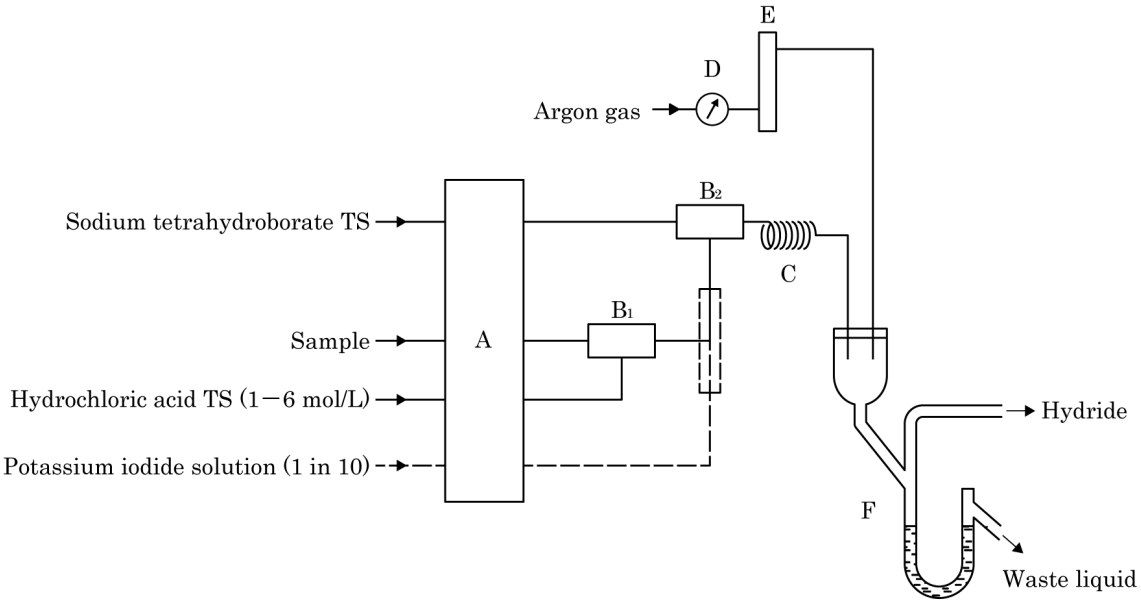


A: Generator bottle (about 70 mL capacity up to the shoulder)
B: Exit tube
C: Glass tube (5.6 mm internal diameter, with an end stretched into a capillary of 1-mm internal diameter)
D: Absorber tube (10 mm internal diameter)
E: Small perforation
F: Glass fiber (about 0.2 g)
G: A 5-mL mark
H and J: Rubber stoppers
L: A 40-mL mark

Fig.1

Stuff a plug of glass fiber (F) into exit tube B up to about 30 mm, moisten the glass fiber uniformly with a mixture of equal volumes of lead(II) acetate TS and water, and remove the excess of the mixture by gentle suction from the lower end. Insert the tube vertically into the center of rubber stopper H, and attach the stopper H with the tube to generator bottle A so that the small perforation E in the lower end of B extends slightly below stopper H. To the upper end of tube B, attach rubber stopper J fitted with tube C. Level the lower end of C with that of rubber stopper J in the exit tube.

Apparatus C Use the apparatus illustrated in Fig. 2.



A: Pump
B1, B2: Mixing joints
C: Reaction tube
D: Pressure gauge
E: Flow meter
F: Gas-liquid separator

Fig. 2

Preparation of Test Solutions Unless otherwise specified, proceed according to the most appropriate method selected from among the following methods.

Method 1 Weigh the specified amount of sample, and dissolve it by adding 5 mL of water and heating it if necessary.

Method 2 Weigh the specified amount of sample, add 5 mL of water, and then add 1 mL of sulfuric acid unless the sample is an inorganic acid. Add 10 mL of sulfurous acid, transfer into a small beaker, and evaporate the mixture to about 2 mL on a water bath. Dilute with water to make 5 mL.

Method 3 Weigh the specified amount of sample, and place it in a platinum, quartz, or porcelain crucible. Add 10 mL of a solution (1 in 50) of magnesium nitrate

hexahydrate in ethanol (95), ignite the ethanol, and heat gradually to char the sample. Then, incinerate it in an electric furnace at 450–550°C. If a charred mass still remains, moisten it with a small quantity of a solution (1 in 50) of magnesium nitrate hexahydrate in ethanol (95), and repeat the same steps to incinerate it. After cooling, add 3 mL of hydrochloric acid, and dissolve the residue by heating on a water bath.

Method 4 Weigh the specified amount of sample, and place it in a platinum, quartz, or porcelain crucible. Add 10 mL of a solution (1 in 10) of magnesium nitrate hexahydrate in ethanol (95), ignite the ethanol, and heat gradually to char the sample. Then, incinerate it in an electric furnace at 450–550° C. If a charred mass still remains, moisten it with a small quantity of a solution (1 in 50) of magnesium nitrate hexahydrate in ethanol (95), and repeat the same steps to incinerate the sample. After cooling, add 3 mL of hydrochloric acid, and dissolve the residue by heating on a water bath.

Method 5 Weigh the specified amount of sample, and place it in a platinum, quartz, or porcelain crucible. Add 10 mL of a solution (1 in 10) of magnesium nitrate hexahydrate in ethanol (95), ignite the ethanol, and heat gradually to char the sample. Then, incinerate it in an electric furnace at 450–550° C. If a charred mass remains, moisten it with a small quantity of a solution (1 in 10) of magnesium nitrate hexahydrate in ethanol (95), and repeat the same steps to incinerate the sample. After cooling, add 3 mL of hydrochloric acid, dissolve the residue by heating on a water bath, and use the resulting solution as the test solution. If the residue does not dissolve in hydrochloric acid, add 10 mL of water to suspend it, cool, and filter through filter paper for quantitative analysis (5C). Wash the residue in the crucible twice with 3 mL of warm water each time, filter the washings through the filter, and wash the filter and the residue on the filter with 5 mL of water. Use the resulting solution as the test solution.

Procedure Unless otherwise specified, proceed by either of the following methods.

Method using Apparatus B Transfer the test solution into the generator bottle, add a drop of bromophenol blue TS, neutralize with ammonia solution, ammonia TS, or diluted hydrochloric acid (1 in 4), add 5 mL of diluted hydrochloric acid (1 in 2) and 5 mL of potassium iodide TS, and allow to stand for 2 to 3 minutes. Add 5 mL of tin(II) chloride (acidic), allow to stand for 10 minutes at room temperature, then add water to make 40 mL. Add 2 g of zinc for arsenic analysis, and immediately connect the rubber stopper H, fitted with B and C, to the generator bottle. Insert the tip of C through the bottom of absorber tube D containing 5 mL of the absorbing solution for arsine, immerse the generator bottle up to the shoulder in water maintained at 25° C, and allow it to stand for 1 hour. Disconnect the absorber tube, add pyridine to make 5 mL, if necessary, and examine the color of the absorbing solution. The color produced is not deeper than the standard color.

The standard color is prepared as directed below in parallel with the procedure for the test directed above.

Standard Color Unless otherwise specified, measure exactly the specified volume of Arsenic Standard Solution, transfer into another generator bottle, add 5 mL of diluted hydrochloric acid (1 in 2) and 5 mL of potassium iodide TS, and allow to stand for 2 to 3 minutes. Add 5 mL of tin(II) chloride TS (acidic), and allow to stand at room temperature for 10 minutes. Proceed as directed for the test solution, beginning with "add water to make 40 mL", and designate the color of the absorbing solution as the standard color.

Method using Apparatus C Unless otherwise specified, to 4 mL each of the test solution and control solution prepared as directed in the individual monograph, add 1 mL of hydrochloric acid and 1 mL of potassium iodide solution (1 in 10), warm in a water bath of 70° C for 4 minutes, and add water to make 20 mL of each solution.

Passing argon gas through the apparatus, introduce the three solutions (the solution prepared with the test solution, an appropriate concentration (1–6 mol/L) of hydrochloric acid TS, and sodium tetrahydroborate TS) in succession into the apparatus at appropriate flow rates of 1–10 mL/min using volumetric pump A, and mix them sequentially successively in the apparatus to generate arsine. Then, introduce the three solutions (the solution prepared with the control solution, an appropriate concentration (1–6 mol/L) of hydrochloric acid, and sodium tetrahydroborate TS) in succession into the apparatus, and proceed as directed for the test solution to generate arsine.

If an approach in which potassium iodide solution (1 in 10) is continuously introduced by pump A into the apparatus is used, introduce the test solution directly or diluted with water, and then introduce an appropriate concentration (1–6 mol/L) of hydrochloric acid TS, potassium iodide solution (1 in 10), and sodium tetrahydroborate TS into the apparatus in the same manner as described above to mix in sequentially, and generate arsine. Then, using the control solution, proceed as directed for the test solution.

The arsine obtained is separated from the waste liquid by gas-liquid separator F, and the gas containing arsine is introduced into an atomic absorption spectrophotometer equipped with a heating absorption cell. Measure the atomic absorbance of the test solution and the control solution at 193.7 nm. The absorbance value of the test solution is not greater than that of the control solution.

Notes on Procedure

(1) The apparatus, reagents, and test solutions used in the test should contain little or no arsenic. Perform a blank test if necessary.

(2) If Apparatus C is used, the amount and concentration of hydrochloric acid and potassium iodide solution to be added to the test solution and control solution will

depend on the apparatus used. Also the flow rate and concentration of the test solution, control solution, hydrochloric acid, sodium tetrahydroborate TS, and potassium iodide solution to be added to the apparatus may depend on the apparatus used.

Ash and Acid-Insoluble Ash

1. Ash

The ash test is designed to measure the amount of residual substances when a sample is ignited under the conditions specified in the individual monograph.

Procedure Ignite a platinum, quartz, or porcelain crucible at 500–550° C for 1 hour, allow to cool in a desiccator, and weigh accurately. Unless otherwise specified, place 2 to 4 g of the sample in the crucible and accurately weigh it.

Cover the crucible loosely with a lid, if necessary, heat it gently first, then raise the temperature gradually, and char the sample. Place the crucible in an electric furnace, ignite it at 500–550° C for not less than 4 hours until the sample is incinerated, cool it in a desiccator, and accurately weigh. Again ignite it to constant weight.

If a charred mass still remains and a constant weight cannot be obtained with the above procedure, moisten the residue with hot water, filter it through a filter paper for quantitative analysis, and heat the insoluble residue on the filter paper together with the filter paper in the crucible to char them. Place the crucible in an electric furnace, and ignite it at 500–550° C until the residue is free from any charred mass. Add the filtrate to the residue, evaporate it to dryness, ignite at 500–550° C, cool it in a desiccator, and weigh accurately. If a charred mass still remains, moisten it with a small amount of ethanol (95), break up the ash with a glass rod, wash the glass rod with a small amount of ethanol (95) into the crucible, and carefully evaporate the ethanol. Proceed as directed previously, and weigh accurately.

2. Acid-insoluble Ash

The acid-insoluble ash test is designed to measure the amount of ash that does not dissolve in diluted hydrochloric acid (1 in 4).

Procedure Add carefully 25 mL of diluted hydrochloric acid (1 in 4) to the ash (obtained by the ash test), boil it gently for 5 minutes, collect the insoluble matter by filtration using a filter paper for quantitative analysis, wash it thoroughly with hot water, and dry the residue together with the filter paper. Place it in a tared platinum, quartz, or porcelain crucible, previously treated as directed for the ash test, and heat to char it. Place the crucible in an electric furnace, ignite it for 3 hours, and weigh accurately. Allow it to cool in a desiccator, and weigh accurately. If the value obtained

is larger than the specified value, ignite until a constant weight is obtained.

Atomic Absorption Spectrophotometry

Atomic absorption spectrophotometry is designed to determine the amount (concentration) of elements in a sample by utilizing the phenomenon that when a light beam passes through the atomic vapor layer of the element, the ground-state atoms absorb the light of the specific wavelength, characteristic to each element.

Apparatus The apparatus usually consists of a light source, a sample-atomizer, a spectroscopic system, a photometric system, and a recording system. Some are equipped with a background correction system. As the light source, a hollow cathode lamp or a discharge lamp is normally used.

There are three types of sample-atomizers: the flame type, electrothermal type, and the cold-vapor type. Cold-vapor atomizers target mercury-containing compounds. They atomize and vaporize mercury in samples. They are further categorized into two subtypes: one using the reduction vaporization method and the other using the thermal vaporization method. A flame atomizer is composed of a burner and a gas-flow regulator, an electrothermal atomizer is composed of an electric furnace and a power source, and a cold-vapor atomizer is composed of a mercury generator, such as a reduction-vaporizer and a thermal vaporizer, and an absorption cell.

For the spectroscopic system, a diffraction grating or interference filter is used. The photometric system consists mainly of a detector and a signal processing system. The recording system is composed of a display and a recording device. A background correction system is employed to correct background absorption by the measuring system. There are several types for background correction, including continuous spectrum sources, the Zeeman split spectrum, the nonresonance spectrum, and the self-reversal phenomena.

In addition, special devices, such as a hydride generator and a heat absorption cell, can be used for analyzing elements such as arsenic and selenium. Hydride generators employ the batch system or the continuous flow system. The heated absorption cell employs flame or an electric furnace for heating.

Procedure Unless otherwise specified, proceed by either of the following methods.

(1) Flame Atomic Absorption Spectrophotometry Fit the light source lamp specified in the individual monograph to the lamp housing, and switch on the photometric system. Light the source lamp, set the spectrometer to the analytical wavelength specified in the individual monograph, and select an appropriate current

value and slit-width. Ignite a mixture of the supporting gas and combustible gas specified, adjust the gas flow rate and pressure, and make a zero adjustment by nebulizing the solvent into the flame. Nebulize the test solution, prepared as specified in the individual monograph, into the flame, and measure the absorbance. Follow the same procedure for the standard solutions and control solution prepared as specified.

(2) Electrothermal Atomic Absorption Spectrophotometry Fit the light source specified in the individual monograph to the lamp housing, and switch on the photometric system. Light the source lamp, set the spectrometer to the analytical wavelength specified in the individual monograph, and select an appropriate electric current and slit-width. Inject a suitable amount of the test solution prepared as specified in the individual monograph into the furnace, and run an appropriate stream of the inert gas through the furnace. By heating at an appropriate temperature for an appropriate period of time and in an appropriate mode, dry and incinerate the specimen, and atomize the element included in the specimen. Next, measure the absorbance. Follow the same procedure for the standard solutions and control solution prepared as specified.

(3) Cold-vapor Atomic Absorption Spectrophotometry Fit a low-pressure mercury lamp to the lamp housing, and switch on the photometric system. Light the source lamp, set the spectrometer to the analytical wavelength specified in the individual monograph, and select an appropriate current value and a slit-width. If the reduction vaporizing method is used, transfer the test solution into a closed vessel, and reduce it to the element by addition of a proper reducing agent, and then vaporize. If the heat vaporizing method is used, vaporize the sample by heating. Next, measure the absorbance of the generated atomic vapor. Follow the same procedure for the standard solutions and control solution prepared as specified.

The determination can usually be performed using an appropriate method from among the methods given below. Possible interference and background should be considered in the determination.

(1) Calibration Curve Method Prepare at least three standard solutions containing different concentrations of the element to be determined, measure the absorbance of these standard solutions, and prepare a calibration curve from the values obtained. Then measure the absorbance of the test solution, adjusted to be within the concentration range of the standard solutions, and determine the amount (concentration) of the element in the test solution from the calibration curve.

(2) Standard Addition Method To equal volumes of at least three test solutions,

add suitable quantities of a standard solution containing the reference standard of the element to be determined so as to prepare a series of solutions containing stepwise increasing amounts of the element, and add a solvent to make a constant volume. Measure the absorbance of each solution, and plot the values obtained on a graph, with the added amounts (concentrations) of the reference standards of the element to be determined on the abscissa and the absorbance values on the ordinate. Extrapolate the regression line formed by joining the points on the graph, and determine the amount (concentration) of the element in the test solution from the distance between the origin and the intersection point of the regression line and the abscissa. This method is applicable only in the case that the calibration curve drawn as directed in (1) above passes through the origin.

(3) Internal Standard Method Prepare several standard solutions containing a constant amount of the internal standard element specified in the monograph, and known, graded amounts of the reference standard of the element to be determined. For these solutions, measure the atomic absorbance of the reference standard and the internal standard element at the analytical wavelength of each element under the same measuring conditions, and obtain the absorbance ratio of the reference standard to the internal standard element for each solution. Prepare a calibration curve by plotting the values obtained, with the amounts (concentrations) of the reference standard on the abscissa and the absorbance ratio on the ordinate. Next, prepare a test solution containing the same amount of the internal standard element as specified for the standard solutions. Proceed under the same conditions as for the preparation of the calibration curve, obtain the absorbance ratio of the element to be determined to the internal standard element, and determine the amount (concentration) of the element in the test solution from the calibration curve.

Note: In atomic absorption spectrophotometry, avoid the use of reagents, test solutions, and gases that may interfere with determination.

Boiling Point and Distillation Range

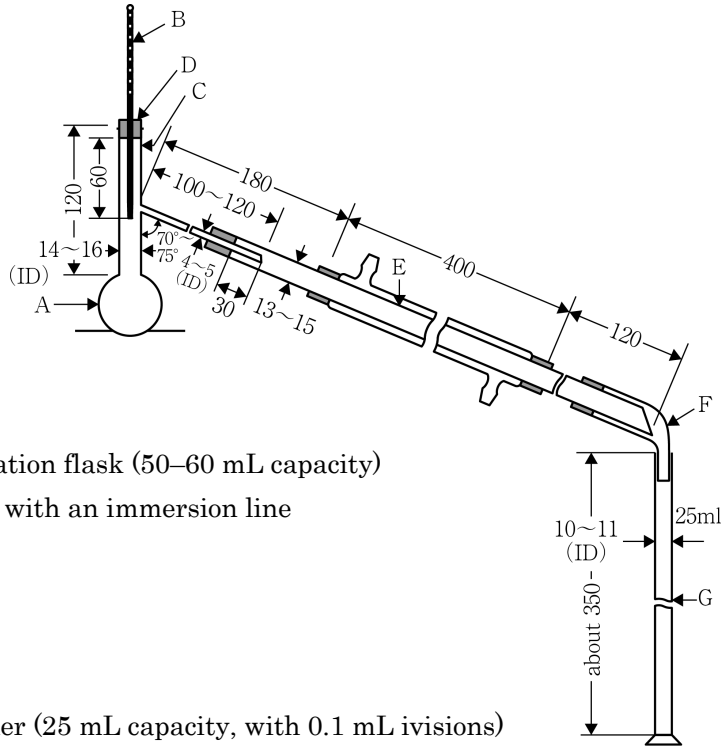
Unless otherwise specified, the boiling point and distillation range are determined by Method 1 or Method 2 below. Unless otherwise specified, the boiling point is expressed as the range between the minimum temperature, at which the first 5 drops of distillate leave the end of the condenser, and the maximum temperature, at which the liquid in the distillation flask is almost gone and a sufficient amount of vapor is no longer obtained. The distillation range test is conducted to determine the volume of distillate collected within the temperature range specified in the individual monograph.

In the Monographs, the specification "55.5–57.0° C (Method 1)" for these tests, for example, means that the boiling point of the substance must be 55.5–57.0° C when determined as directed in Method 1. Also, the specification "the amount of distillate at 64–70° C is not less than 95% vol. (Method 2)," for example, means that the amount of distillate of the substance at 64–70° C must be not less than 95% vol. when determined as directed in Method 2.

Method 1

This method is used to determine the boiling point and amount of distillate of a liquid when the specified temperature range is less than 5° C.

Apparatus Use the apparatus as illustrated below.



A: Hard glass distillation flask (50–60 mL capacity)
B: Rod thermometer with an immersion line
C: Immersion line
D: Stopper
E: Condenser
F: Adapter
G: Volumetric cylinder (25 mL capacity, with 0.1 mL ivisions)

(Unit: mm)

Dry thoroughly all the glass instruments before use. Insert thermometer B into the distillation flask so that immersion line C is on a level with the lower end of cork stopper D and the upper end of its mercury bulb is at the center of the delivery tube. Connect condenser E with distillation flask A and adapter F with condenser E, and then insert the open end of F into the mouth of cylinder G so that a little amount of air passes through.

Place boiling chips or a capillary tube into A, use a hood sufficiently high to shield A, and heat A with a suitable heat source. When an open flame is applied as the heat source, place A on a hole of a ceramic board (a 150-mm square wire gauze coated with a 6-mm thick ceramic layer having a hole 30 mm in diameter at its center), and then

heat.

Procedure Measure 25 mL of the sample, whose temperature was previously measured, using G, and transfer into A. Use G as the receiver without washing. When the apparatus is set up, pass water through E, and heat A so that distillation starts after about 10 minutes. Unless otherwise specified, distill the liquid sample by applying heat, at a rate of 4 to 5 mL per minute in the case of liquids whose boiling temperature to be determined is less than 200° C, or at a rate of 3 to 4 mL per minute in the case of liquids whose boiling temperature is not less than 200° C. Lower the temperature of the distillate to the temperature of the sample previously measured, and measure the volume of distillate. In the case of liquids that begin to distill at 80° C or below, cool to 10–15° C before measuring the volume, and keep receiving cylinder G immersed in ice up to a point 25 mm from the top during distillation.

Correct the measured temperature for any variation in the barometric pressure from the normal (101 kPa) by allowing 0.1° C for each 0.36 kPa of variation, adding if the pressure is lower than 101 kPa, or subtracting if higher than 101 kPa.

Method 2

This method is used to determine the boiling point and amount of distillate of a liquid when the specified range of temperature is not less than 5° C.

Apparatus The same apparatus as described in Method 1 is used. The distillation flask (A) should be 200 mL in capacity and 18–24 mm in internal neck diameter having a delivery tube of 5–6 mm in internal diameter. The ceramic board used for direct-flame heating should have a hole 50 mm in diameter at its center. Use a 100-mL volumetric cylinder graduated in 1-mL divisions as the receiver (G) in Method 2.

Procedure Measure 100 mL of the sample, whose temperature was previously recorded, using G, and carry out the distillation in the same manner as Method 1.

Calcium Salt Determination

Calcium salt determination is designed to determine the quantity of calcium salts contained in a sample by using disodium dihydrogen ethylenediaminetetraacetate. There are two methods: the direct titration method (Method 1) in which the test solution is titrated with a solution of disodium dihydrogen ethylenediaminetetraacetate and the back-titration method (Method 2) in which an excess solution of disodium dihydrogen ethylenediaminetetraacetate is added and titration is conducted with zinc acetate solution.

Procedure Unless otherwise specified, proceed using Method 1 or Method 2, whichever is appropriate.

Method 1 Measure exactly 10 mL of the specified test solution, add 50 mL of water and 10 mL of potassium hydroxide solution (1 in 10), and allow to stand for 1 minute. Add about 0.1 g of NN indicator, and titrate immediately with 0.05 mol/L disodium dihydrogen ethylenediaminetetraacetate until the red-purple color of the solution completely disappears and a blue color develops.

Method 2 Measure exactly 20 mL of the specified test solution and add exactly 25 mL of 0.02 mol/L disodium dihydrogen ethylenediaminetetraacetate, then add 50 mL of water and 5 mL of ammonium buffer (pH 10.7), and allow the mixture to stand for 1 minute. Add 25 mg of eriochrome black T–sodium chloride indicator, and titrate immediately the excess solution of disodium dihydrogen ethylenediaminetetraacetate with 0.02 mol/L zinc acetate until the blue color of the solution changes to blue-purple. Perform a blank test in the same manner as the sample.

Chloride Limit Test

The chloride limit test is designed to demonstrate that the content of chloride in an additive does not exceed the acceptable limit specified in the individual monograph.

In the Monographs, the specification "not more than 0.041% as Cl (0.30 g, Control Solution: 0.01 mol/L hydrochloric acid 0.35 mL)" for this test, for example, means that the chloride content of the substance must be not more than 0.041% as Cl when determined according to the following manner: *The test solution and control solution are prepared with 0.30 g of the test substance and 0.35 mL of 0.01 mol/L hydrochloric acid, respectively, as directed below. and the test is performed as directed in the procedure*.

Preparation of Test Solutions and Control Solutions Unless otherwise specified, proceed as directed below. Weigh the amount of sample specified in the individual monograph, transfer into a Nessler tube, and dissolve in about 30 mL of water. Neutralize the solution with diluted nitric acid (1 in 10) if the solution is alkaline. Add 6 mL of diluted nitric acid (1 in 10) and water to make 50 mL, and use this as the test solution. If the individual monograph requires the preparation of the sample solution, transfer the solution into a Nessler tube, and add 6 mL of diluted nitric acid (1 in 10) and water to make 50 mL. Use this as the test solution.

Measure the specified amount of 0.01 mol/L hydrochloric acid, and transfer into another Nessler tube. Add 6 mL of diluted nitric acid (1 in 10) and water to make 50 mL. Use this as the control solution. If the test solution is not clear, filter both solutions using the same procedure.

Procedure Unless otherwise specified, add 1 mL of silver nitrate solution (1 in 50) to each of the test and the control solutions, mix thoroughly, and allow them to stand for 5 minutes, protected from direct sunlight. Next, examine both Nessler tubes from the side and from above against a black background, and compare the turbidity. The turbidity developed in the test solution is not thicker than that of the control solution.

Clarity of Solution Test

The clarity of solution test is designed to scientifically and objectively determine the solubility of a sample in the solvent specified under Clarity of Solution in Purity in the individual monograph. By examining the state of the solution, the characteristic properties of a substance and the presence of impurities in that substance can be easily identified.

In the Monographs, the specification "almost clear (1.0 g, water 20 mL)" for this test, for example, means that a solution prepared by dissolving 1.0 g of the test substance in 20 mL of water must be almost clear.

Preparation of Test Solutions Unless otherwise specified, prepare the solution, as specified under Clarity of Solution in the individual monograph, in a Nessler tube or an appropriate container. If necessary, place 20 mL of it into a Nessler tube, and use as the test solution.

Preparation of Standard Solution

Standard Stock Solution Measure exactly 14.1 mL of 0.1 mol/L hydrochloric acid, and add water to make exactly 50 mL. One mL of this solution contains 1 mg of chlorine (Cl).

Standard Solution Measure exactly 1 mL of the Standard Stock Solution, and add water to make exactly 100 mL. One mL of this solution contains 0.01 mg of chlorine (Cl).

Preparation of Reference Solutions

Turbidity is identified by the solutions prepared as directed below.

Clear. Measure 0.2 mL of the Standard Solution, and add water to make 20 mL. Add 1 mL of diluted nitric acid (1 in 3), 0.2 mL of dextrin hydrate solution (1 in 50), and 1 mL of silver nitrate solution (1 in 50). Shake the mixture, and allow it to stand for 15 minutes, protected from direct sunlight.

Almost clear. Measure 0.5 mL of the Standard Solution, and add water to make 20 mL. Add 1 mL of diluted nitric acid (1 in 3), 0.2 mL of dextrin hydrate solution (1 in 50), and 1 mL of silver nitrate solution (1 in 50). Shake the mixture, and allow it to stand

for 15 minutes, protected from direct sunlight.

Very slightly turbid. Measure 1.2 mL of the Standard Solution, and add water to make 20 mL. Add 1 mL of diluted nitric acid (1 in 3), 0.2 mL of dextrin hydrate solution (1 in 50), and 1 mL of silver nitrate solution (1 in 50). Shake the mixture, and allow it to stand for 15 minutes, protected from direct sunlight.

Slightly turbid. Measure 6 mL of the Standard Solution, and add water to make 20 mL. Add 1 mL of diluted nitric acid (1 in 3), 0.2 mL of dextrin hydrate solution (1 in 50), and 1 mL of silver nitrate solution (1 in 50). Shake the mixture, and allow it to stand for 15 minutes, protected from direct sunlight.

Turbid. Measure 0.3 mL of the Standard Stock Solution, and add water to make 20 mL. Add 1 mL of diluted nitric acid (1 in 3), 0.2 mL of dextrin hydrate solution (1 in 50), and 1 mL of silver nitrate solution (1 in 50). Shake the mixture, and allow it to stand for 15 minutes, protected from direct sunlight.

Procedure Unless otherwise specified, proceed according to the follow the method: Place equal volumes of the test solution and the reference solution into separate Nessler tubes, shake them for 30 seconds to 5 minutes, and examine them from above and from the side, protected from direct sunlight; the turbidity of the test solution is not thicker than that of the reference solution corresponding to the specified turbidity. As for solutions specified as “clear” or “almost clear,” foreign matter, such as floating matter, should not be present practically.

Coloring Matter Tests

The tests specified below are applied as purity tests and assay for coloring matters.

1. Water-insoluble Substances

In the Monographs, the specification “not more than 0.20% (Coloring Matter Tests)” for this test, for example, means that the content of water-insoluble substances must be not more than 0.20% when determined as directed in the following procedure.

Procedure Dry a crucible type glass filter (1G4) at 135° C for 30 minutes, cool in a desiccator, and weigh accurately. Weigh 2.0 g of the sample, add 200 mL of boiling water, shake well, and cool. Filter the insoluble substances through the glass filter prepared as above, wash with water until the washings become colorless, dry together with the glass filter at 135° C for 3 hours, allow to cool in a desiccator, and weigh accurately.

2. Chloride and Sulfate

In the Monographs, the specification "not more than 5.0% as the total content (Coloring Matter Tests)" for this test, for example, means that the combined total content of sodium chloride and sodium sulfate must be not more than 5.0% when determined as directed below.

Test Solution Unless otherwise specified, weigh accurately about 0.1 g of the sample, and dissolve in water to make exactly 100 mL. Dilute exactly 10 mL of this solution with water to make exactly 50 mL.

Standard Solutions Place exactly 0.5 mL, 1 mL, 5 mL, and 10 mL of each of the Chloride Ion Standard Stock Solution and Sulfate Ion Standard Stock Solution into separate 100-mL volumetric flasks, dilute each to volume with water to prepare four standard solutions for each stock solution.

Procedure Proceed as directed under Ion Chromatography according to the following operating conditions, using a fixed amount each of the test solution and the standard solutions. Next, determine the peak areas or peak heights of the chloride ion and sulfate ion for each standard solution to make a calibration curves. Determine the peak areas or peak heights of chloride ion and sulfate ion for the test solution to obtain the content of chloride and sulfate ions from the calibration curves. Multiply the chloride content and the sulfate content by 1.65 and 1.48, respectively, to obtain the concentrations of sodium chloride and sodium sulfate in the test solution, and thus determine their contents in the sample. If the peak areas or peak heights of the chloride ion and sulfate ion in the test solution are beyond the range of the calibration curve, dilute the test solution, and derive the contents by multiplying the value obtained by the dilution ratio.

Operating Conditions

Detector: Electric conductivity detector.

Column: A stainless steel or plastic tube (4.6–6.0 mm internal diameter and 5–10 cm length).

Column packing material: Porous anion exchanger.

Guard column: A column with the same internal diameter and packing material as the above column.

Column temperature: 40° C.

Mobile phase: A solution prepared by dissolving 0.42 g of phthalic acid and 0.29 g of 2-amino-2-hydroxymethyl-1,3-propandiol in 1000 mL of water (pH 4.0).

Flow rate: 1.5 mL/min.

3. Iodide

In the Monographs, the specification "not more than 0.40% (Coloring Matter Tests)" for this test, for example, means that the content of sodium iodide must be not more than

0.40% when determined as directed below.

Test Solution Weigh accurately about 0.1 g of the sample, and dissolve in water to make exactly 100 mL. Dilute exactly 4 mL of this solution with water to make exactly 10 mL.

Standard Solutions Place exactly 0.5 mL, 1 mL, 2 mL, and 4 mL of the Iodide Ion Standard Stock Solution into separate 100-mL volumetric flasks, and dilute each to volume with water to prepare standard solutions.

Procedure Proceed as directed under Ion Chromatography according to the same conditions specified under Chloride and Sulfate, using a fixed amount of each of the test solution and standard solutions. Determine the peak areas or peak heights of iodide ion for the standard solutions to make a calibration curve. Determine the peak areas or peak heights of iodide ion for the test solution to obtain the content of iodide ion using the calibration curve. Multiply the content by 1.18 to obtain the concentration of sodium iodide in the test solution, and thus determine its content in the sample. Avoid direct sunlight during the procedure, use light-resistant containers for the preparation of the test solution, and perform the test immediately after its preparation.

4. Bromide

In the Monographs, the specification "not more than 1.0% (Coloring Matter Tests)" for this test, for example, means that the content of sodium bromide must be not more than 1.0% when determined as directed below.

Test Solution Weigh accurately about 0.1 g of the sample, and dissolve in water to make exactly 100 mL. Dilute exactly 4 mL of this solution with water to make exactly 10 mL.

Standard Solutions Place exactly 0.5 mL, 1 mL, 2 mL, and 4 mL of Bromide Ion Standard Stock Solution into separate 100-mL volumetric flasks, and dilute each to volume with water to prepare standard solutions.

Procedure Proceed as directed in Ion Chromatography according to the same conditions specified under Chloride and Sulfate, using a fixed amount each of the test solution and standard solutions. Determine the peak areas or heights of bromide ion for the standard solutions to make a calibration curve. Determine the peak area or height of bromide ion for the test solution to obtain its content of bromide ion using the calibration curve. Multiply the content by 1.29 to obtain the concentration of sodium bromide, and thus determine its content in the sample. Avoid direct sunlight during the whole procedure. Use light-resistant equipment in preparing the test solution, and perform the test immediately after the preparation of the solution.

5. Lead

In the Monographs, the specification "not more than 2 μg/g as Pb (Coloring Matter Tests, Method 1)" for this test, for example, means that the content of lead must be not more than 2 μg/g as Pb when determined using the solutions prepared as directed in Method 1 given below.

Preparation of Test Solution, Control Solution, and Blank Test Solution

Unless otherwise specified, proceed as directed below.

Method 1

Test Solution Weigh 1.0 g of the sample into a platinum, quartz, or porcelain crucible, and add small portions of sulfuric acid to moisten it. Heat by gradually increasing the temperature in the range of 100–500° C until the sample is almost charred and white fumes of sulfuric acid are no longer evolved. If necessary, crush the contents with a glass rod while heating. Place the crucible in an electric furnace, heat by gradually increasing the temperature, and ignite at 500–600° C to incinerate the sample. If a charred mass remains, moisten it with sulfuric acid, heat until white fumes of sulfuric acid are no longer evolved, and ignite in the electric furnace to incinerate the residue. When incineration is done at 500–550° C, a heat-resistant glass beaker can be used. After cooling, add 10 mL of diluted hydrochloric acid (1 in 4) to the residue, cover the crucible with a lid if necessary, heat to dissolve the residue, and evaporate to dryness. Dissolve the residue by adding diluted nitric acid (1 in 100) to make 10 mL. Filter the resulting solution if necessary.

Control Solution To exactly 2 mL of Lead Standard Solution, add diluted nitric acid (1 in 100) to make exactly 10 mL.

Blank Test Solution Proceed as directed for the test solution without using the sample.

Method 2

Sample Solution Weigh 1.0 g of the sample in a platinum, quartz, or porcelain crucible, add 10 mL of a solution (1 in 10) of magnesium nitrate hexahydrate in ethanol (95), mix, and ignite and burn the ethanol. Cover with a lid if necessary because the contents in the crucible may sputter near the end of burning. After cooling, add small portions of sulfuric acid to moisten the sample, and proceed as directed in Method 1. If a charred mass remains, add 5 mL of a solution (1 in 10) of magnesium nitrate hexahydrate in ethanol (95), mix, and repeat the procedure directed in Method 1. When incineration is done at 500–550° C, a heat-resistant glass beaker can be used. Add 30 mL of diluted hydrochloric acid (1 in 4) to the residue, heat until the residue melts, and cool.

Test Solution To the sample solution, add 10 mL of diammonium hydrogen citrate solution (1 in 2). Add 1 mL of thymol blue TS as the indicator and then ammonia solution until the color of the solution changes from yellow to light yellow-green.

Transfer it to a separating funnel (or a centrifuge tube). Wash the container with a small amount of water into the separating funnel, and add water to make about 100 mL. Add 5 mL of a solution of ammonium pyrrolidine dithiocarbamate (3 in 100), and allow to stand for 5 minutes. Add exactly 10 mL of butyl acetate, shake for 5 minutes, and allow it to stand or centrifuge. Collect the butyl acetate layer and use it as the test solution.

Control Solution Measure exactly 2 mL of Lead Standard Solution, and proceed as directed for the test solution.

Blank Test Solution Proceed as directed in the preparation of the sample solution and the test solution without using the sample.

Procedure Determine the absorbance of the test solution, the control solution, and the blank test solution, as directed under Flame Atomic Absorption Spectrophotometry, using the following operating conditions. The difference between the absorbance values of the test solution and the blank test solution is not more than the value of the control solution.

Operating Conditions

Light Source: Lead hollow cathode lamp.

Wavelength: 283.3 nm.

Supporting gas: Air.

Combustible gas: Acetylene.

6. Zinc and Iron

In the Monographs, the specification "not more than 200 μg/g as Zn (Coloring Matter Tests, Zinc and Iron (1))" for this test, for example, means that the content of zinc as Zn must be not more than 200 μg/g when determined as directed in (1) given below using the sample solution prepared in the following manner.

Preparation of Sample Solution Weigh 1.0 g of the sample in a platinum, quartz, or porcelain crucible (or a heat-resistant glass beaker), add small portions of sulfuric acid to moisten it. Heat by gradually increasing the temperature in the range of 100–500°C until the sample is almost charred and white fumes of sulfuric acid are no longer evolved. If necessary, crush the contents with a glass rod while heating. Place the crucible in an electric furnace, heat by gradually increasing the temperature, and ignite at 450–550°C to incinerate the sample. If a charred mass remains, moisten it with sulfuric acid, and repeat the same procedure as described above. After cooling, add 3 mL of hydrochloric acid to the residue, and mix. Add 7 mL of water, shake, and filter through a filter paper (5C) for quantitative analysis. Wash the residue on the filter paper with 5 mL of diluted hydrochloric acid (1 in 4) and water, combine the washings with the filtrate, and add water to make 50 mL.

(1) Zinc

Test Solution Measure 2.5 mL of the sample solution, add 4 mL of diluted hydrochloric acid (1 in 4) and water to make 20 mL.

Control Solution To 1.0 mL of Zinc Standard Solution, add 4 mL of diluted hydrochloric acid (1 in 4) and water to make 20 mL.

Blank Test Solution Proceed as directed for the preparation of the sample solution and test solution without using the sample.

Procedure Determine the absorbance of the test solution, the control solution, and the blank test solution as directed under Flame Atomic Absorption Spectrophotometry, using the following operating conditions. The difference between the absorbance values of the test solution and the blank test solution is not more than the value of the control solution.

Operating Conditions

Light Source: Zinc hollow cathode lamp.

Wavelength: 213.9 nm.

Supporting gas: Air.

Combustible gas: Acetylene.

(2) Iron

Test Solution Measure 5 mL of the sample solution, add 4 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

Control Solution To 5.0 mL of Iron Standard Solution, add 10 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

Blank Test Solution Proceed as directed for the preparation of the sample solution and the test solution without using the sample.

Procedure Determine the absorbance of the test solution, the control solution, and the blank test solution as directed under Flame Atomic Absorption Spectrophotometry, using the following operating conditions. The difference between the absorbance values of the test solution and the blank test solution is not more than the absorbance of the control solution.

Operating Conditions

Light Source: Iron hollow cathode lamp.

Wavelength: 248.3 nm.

Supporting gas: Air.

Combustible gas: Acetylene.

7. Manganese and Chromium

In the Monographs, the specification “not more than 50 μg/g as Mn (Coloring Matter

Tests, Manganese and Chromium (1))" for this test, for example, means that the content of manganese as Mn must be not more than 50 μg/g when determined as directed in (1) given below using the sample solution prepared in the following manner.

Preparation of Sample Solution Weigh 1.0 g of the sample into a platinum, quartz, or porcelain crucible (or a heat-resistant glass beaker), and add small portions of sulfuric acid to moisten it. Heat by gradually increasing the temperature in the range of 100–500° C until the sample is almost charred and white fumes of sulfuric acid no longer evolve. If necessary, crush the contents with a glass rod while heating. Place the crucible in an electric furnace, heat by gradually increasing the temperature, and ignite at 450–550° C to incinerate the sample. If a charred mass remains, moisten it with sulfuric acid, and repeat the same procedure as described above. After cooling, add 3 mL of hydrochloric acid to the residue, and mix. Then, add 7 mL of water, shake, and filter through a filter paper (5C) for quantitative analysis. Wash the residue on the filter paper with 5 mL of diluted hydrochloric acid (1 in 4) and 5 mL of water, and combine the washings with the filtrate. Designate the resulting solution as Solution A. Put the residue with the filter paper into a crucible, dry at 105° C, heat by gradually increasing the temperature from 150 to 500° C until the residue is almost charred, and ignite at 450–550° C in the electric furnace to incinerate the residue. Add 0.8 g of sodium carbonate, and ignite at a temperature of 800° C or higher to melt. After cooling add 10 mL of water, and add hydrochloric acid dropwise to acidify the solution. Transfer the solution into a beaker, wash the crucible with a small amount of water, add the washings to the beaker, and shake vigorously. Add the resulting solution to Solution A, and add water to make 50 mL.

(1) Manganese

Test Solution Measure 10 mL of the sample solution, add 10 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

Control Solution To 1.0 mL of Manganese Standard Solution, add 10 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

Blank Test Solution Proceed as directed for the preparation of the sample solution and the test solution without using the sample.

Procedure Determine the absorbance of the test solution, the control solution, and the blank test solution as directed under Flame Atomic Absorption Spectrophotometry, using the following operating conditions. The difference between the absorbance values of the test solution and the blank test solution is not more than the absorbance of the control solution.

Operating Conditions

Light Source: Manganese hollow cathode lamp.

Wavelength: 279.5 nm.
Supporting gas: Air.
Combustible gas: Acetylene.

(2) Chromium

Test Solution Unless otherwise specified, measure 10 mL of the sample solution, add 10 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

Control Solution To 4.0 mL of Chromium Standard Solution, add 10 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

Blank Test Solution Proceed as directed for the preparation of the sample solution and the test solution without using the sample.

Procedure Determine the absorbance of the test solution, the control solution, and the blank test solution as directed under Flame Atomic Absorption Spectrophotometry, using the following operating conditions. The difference between the absorbance values of the test solution and the blank test solution is not more than the absorbance of the control solution.

Operating Conditions
Light Source: Chromium hollow cathode lamp.
Wavelength: 357.9 nm.
Supporting gas: Air.
Combustible gas: Acetylene.

8. Arsenic

In the Monographs, the specification "not more than 3 μg/g as As (Coloring Matter Tests)" for this test, for example, means that the content of arsenic as As must be not more than 3 μg/g when determined as directed in the procedure using the test solution prepared in the following manner.

Test Solution Weigh exactly 0.50 g of the sample, transfer into a porcelain crucible (or a heat-resistant glass beaker), add 20 mL of a solution (1 in 50) of magnesium nitrate hexahydrate in ethanol (95), and ignite and burn the ethanol. Cover the crucible with a lid if necessary because the contents in the crucible may sputter near the end of burning. Heat by gradually increasing the temperature in the range of 150–500° C until the sample is almost charred. If necessary, crush the contents with a glass rod while heating. Place the crucible in an electric furnace, heat gradually, and ignite at 450–550° C to incinerate the sample. If a charred mass remains, moisten it with a small amount of nitric acid, heat until white fumes no longer evolve, and ignite at 450–550° C in the electric finance to incinerate the sample. After cooling, add 6 mL of hydrochloric acid to the residue, and add about 10 mL of water if necessary. Cover the crucible with a lid, heat to dissolve the residue, and cool.

Add water to make exactly 25 mL.

Control Solution To 3.0 mL of Arsenic Standard Solution, add 6 mL of hydrochloric acid and water to make 25 mL.

Blank Test Solution Proceed as directed for the test solution without using the sample.

Procedure To 4 mL each of the test solution, control solution, and blank test solution, add 3 mL of hydrochloric acid and 1 mL of potassium iodide solution (1 in 10), and allow them to stand for 30 minutes at room temperature. To each solution, add 2 mL of L(+)-ascorbic acid solution (1 in 10) and water to make 20 mL. Proceed as directed under the Method using Apparatus C under the Arsenic Limit Test. The difference between the absorbance values of the solutions derived from the test solution and the blank test solution, respectively, is not more than the value of the solution derived from the control solution.

Depending on apparatus used, the quantity and concentration of hydrochloric acid, potassium iodide, and L(+)-ascorbic acid that should be added to the test solution, control solution, and blank test solution vary. Also the flow rate and concentration of the test solution, control solution, hydrochloric acid, potassium iodide, and sodium tetrahydroborate TS that should be added to the apparatus may vary.

9. Subsidiary Colors

In the Monographs, the specification "(Coloring Matter Tests, Subsidiary Colors (1))" for this test, for example, means that the test must be conducted as directed in the method specified in (1) below.

(1) *Test Solution* Weigh accurately about 0.1 g of the sample, add ammonium acetate TS (0.02 mol/L), dissolve it ultrasonically if necessary, and make exactly 100 mL with ammonium acetate TS (0.02 mol/L).

Standard Solutions Dry the specified subsidiary colors for 24 hours in a vacuum desiccator, weigh accurately about 10 mg of each subsidiary color, and separately dissolve them in ammonium acetate TS (0.02 mol/L) to prepare standard stock solutions of exactly 100 mL each. Prepare four standard solutions of different concentrations for each standard stock solution as follows: Place exactly 0.5 mL, 1 mL, 2 mL, and 5 mL of each standard stock solution in separate 100-mL volumetric flasks, and dilute each to volume with ammonium acetate TS (0.02 mol/L).

Procedure Analyze a fixed amount each of the test solution and the standard solution by liquid chromatography under the operating conditions given below. Next, measure the peak areas of the subsidiary colors for the standard solutions to prepare a calibration curve for each subsidiary color. Measure the peak area of each subsidiary color for the test solution. Obtain the content of each color using the calibration curves,

and calculate the total amount of the subsidiary colors.

Operating Conditions

Detector: Visible spectrophotometer or photodiode array detectors (use the wavelength specified in the individual monograph).

Column: A stainless steel tube (4.6 mm internal diameter and 25 cm length).

Column packing material: 5-μm octadecylsilanized silica gel for liquid chromatography.

Column temperature: A constant temperature of about 40° C.

Mobile phase

A: Ammonium acetate TS (0.02 mol/L).

B: A 7:3 mixture of acetonitrile/water.

Concentration gradient: Follow the requirement specified in the individual monograph.

Flow rate: 1 mL/min.

(2) *Test Solution* Unless otherwise specified, weigh accurately about 0.1 g of the sample, add ammonium acetate TS (0.02 mol/L), and dissolve it ultrasonically if necessary to make exactly 100 mL with ammonium acetate TS (0.02 mol/L). To exactly 2 mL of this solution, add ammonium acetate TS (0.02 mol/L) to make exactly 20 mL.

Procedure Analyze a fixed amount of the test solution by liquid chromatography under the operating conditions given below. Designate a 1000th of the main color peak area as A. Measure the areas of all peaks bigger than A that appear in the range specified in the individual monograph, and designate the total area as A_T. Measure the areas of all peaks, other than the main color peak, and designate as A_S. Calculate the amount of subsidiary colors by the formula:

$$\text{Amount (\%) of subsidiary colors} = \frac{A_S}{A_T} \times \text{content (\%)}$$

Operating Conditions

Detector: Visible spectrophotometer or photodiode array detectors (use the wavelength specified in the individual monograph).

Column: A stainless steel tube (4.6 mm internal diameter and 25 cm length).

Column packing material: 5-μm octadecylsilanized silica gel for liquid chromatography.

Column temperature: A constant temperature of about 40° C.

Mobile phase

A: Ammonium acetate TS (0.02 mol/L).

B: A 7:3 mixture of acetonitrile/water.

Concentration gradient: Follow the requirement specified in the individual

monograph.

Flow rate: 1 mL/min.

Range of measurement: Follow the requirement specified in the individual monograph.

10. Unreacted Raw Materials and Products of Side Reactions

Test Solution Weigh accurately about 0.1 g of the sample, and dissolve it in the specified solution to make exactly 100 mL.

Standard Solutions Dry the specified unreacted raw materials and products of side reactions for 24 hours in a vacuum desiccator, then weigh accurately about 10 mg of each substance, and dissolve them separately by adding ammonium acetate TS (0.02 mol/L), unless otherwise specified. If necessary, dissolve them ultrasonically. Add ammonium acetate TS (0.02 mol/L) to prepare standard stock solutions of exactly 100 mL each. Prepare four standard solutions of different concentrations for each standard stock solution. Place exactly 0.5 mL, 1 mL, 2 mL, and 5 mL of each standard stock solution in separate 100-mL volumetric flasks, and dilute each to volume with ammonium acetate TS (0.02 mol/L).

Procedure Analyze a fixed amount of each of the test solution and standard solutions by liquid chromatography using the operating conditions given below. Next, measure the peak areas of the unreacted raw materials and products of side reactions for standard solutions to prepare a calibration curve for each substance. Measure the peak area of each substance for the test solution. Determine the contents of the unreacted raw materials and products of side reactions using the calibration curves.

The amount to be injected should be adjusted so that linear calibration curves are produced. Designate the peak area derived from the standard solution of the lowest concentration as A. Measure the peak areas bigger than A for peaks of unreacted raw materials and products of side reactions in the test solution, and calculate the amount from the calibration curve.

Operating Conditions

Detector: Ultraviolet spectrophotometer or photodiode array detectors (use the wavelength specified in the individual monograph).

Column: A stainless steel tube (4.6 mm internal diameter and 25 cm length).

Column packing material: 5-μm octadecylsilanized silica gel for liquid chromatography.

Column temperature: 40° C.

Mobile phase

A: Ammonium acetate TS (0.02 mol/L).

B: A 7:3 mixture of acetonitrile/water.

Concentration gradient: Follow the requirement specified in the individual

monograph.
Flow rate: 1 mL/min.

11. Unsulfonated Primary Aromatic Amines

(1) Aniline

In the Monographs, the specification "not more than 0.01% as aniline (Coloring Matter Tests)" for this test, for example, means that the content of unsulfonated primary aromatic amines must be not more than 0.01% as aniline when determined as directed below.

Sample Solution Weigh 2.0 g of the sample, transfer it into a separating funnel containing 100 mL of water, and add 50 mL of water to dissolve it. Add 5 mL of sodium hydroxide solution (1 in 25) and 50 mL of ethyl acetate, and extract by shaking. Collect the ethyl acetate layer. Add 50 mL of ethyl acetate to the water layer, and extract by shaking. Combine the two ethyl acetate layers, and wash with sodium hydroxide solution (1 in 250) until the color of the solution disappears. Extract three times from the washed ethyl acetate with 10 mL of diluted hydrochloric acid (3 in 10) each time. Combine the hydrochloric acid extracts, and add water to make exactly 100 mL.

Test Solution Transfer exactly 10 mL of the sample solution into a test tube, and cool in ice for 10 minutes. Add 1 mL of potassium bromide solution (1 in 2) and 50 μL of sodium nitrite solution (1 in 30), mix, and allow the mixture to stand in ice for 10 minutes. Wash the mixture down with water into a Nessler tube containing 1 mL of disodium 3-hydroxy-2,7-naphthalenedisulfonate TS (0.05 mol/L) and 10 mL of sodium carbonate solution (1 in 10), and add water to make exactly 25 mL. Allow this solution to stand in a dark place for 15 minutes.

Control Solution Weigh 0.10 g of aniline, dissolve it in 30 mL of diluted hydrochloric acid (3 in 10), and add water to make exactly 100 mL. Measure exactly 2 mL of this solution, and add 30 mL of diluted hydrochloric acid (3 in 10) and then water to make exactly 100 mL. To exactly 10 mL of this solution, add 30 mL of diluted hydrochloric acid (3 in 10) and then water to make exactly 100 mL. Transfer exactly 10mL of this solution into a test tube, and proceed as directed for the test solution.

Reference Solution To measure the absorbance of the test solution, use the following reference solution: Transfer 10 mL of the sample solution into a Nessler tube, add 1 mL of disodium 3-hydroxy-2,7-naphthalenedisulfonate TS (0.05 mol/L) and 10 mL of sodium carbonate solution (1 in 10), and then add water to make exactly 25 mL. To measure the absorbance of the control solution, use the following reference solution: To 3 mL of diluted hydrochloric acid (3 in 10), add 1 mL of disodium 3-hydroxy-2,7-naphthalenedisulfonate TS (0.05 mol/L) and 10 mL of sodium carbonate solution (1 in 10), and then add water to make exactly 25 mL.

Procedure Measure the absorbance of each solution at a wavelength of 510 nm. The

absorbance value of the test solution is not more than that of the control solution.

(2) 1-Naphthylamine

In the Monographs, the specification "not more than 1.0 μg/g as 1-naphthylamine (Coloring Matter Tests)" for this test, for example, means that the content of 1-naphthylamine must be not more than 1.0 μg/g when determined as directed below.

Test Solution Weigh accurately about 2.5 g of the sample into a beaker, dissolve it by adding 25 mL of water, and transfer into a 50-mL volumetric flask containing 5 drops of sodium hydroxide solution (1 in 25) and 1 mL of methanol. Wash the beaker twice with 10 mL of water each time, add the washings to the volumetric flask, and make 50 mL by adding water. Designate this solution as the sample solution. Pour exactly 20 mL of the sample solution into a absorption column packed with 20-mL diatomaceous earth for chromatography to allow to flow. Allow to stand for 1 hour, pour 100 mL of hexane into the column, and collect the effluent into a 200-mL eggplant-shaped flask. Add 0.5 mL of diluted sulfuric acid (3 in 20,000), evaporate to about 1 mL under reduced pressure in a water bath at 40° C, and completely remove the hexane in the flask. Dissolve the residue by adding a 3:2 mixture of ammonium acetate TS (0.02 mol/L)/acetonitrile, and make exactly 2 mL.

Standard Solution Weigh accurately about 10 mg of 1-naphthylamine, dissolve in methanol to make exactly 100 mL. Use this solution as the standard stock solution. To exactly 5 mL of the standard stock solution, add a 3:2 mixture of ammonium acetate TS (0.02 mol/L)/acetonitrile to make exactly 50 mL. Dilute this solution with a 3:2 mixture of ammonium acetate (0.02 mol/L)/acetonitrile to prepare several solutions containing 0.05–1 μg of 1-naphthylamine in 1 mL.

Procedure Analyze a fixed amount each of the test solution and the standard solutions by liquid chromatography using the operating conditions given below. Measure the peak areas of 1-naphthylamine from the standard solutions to prepare a calibration curve. Next, measure the area of the peak corresponding to the retention time of 1-naphthylamine in the test solution, and calculate the content of 1-naphthylamine using the calibration curve.

Operating Conditions

Detector: Ultraviolet spectrophotometer or photodiode array detector (wavelength: 304 nm).

Column: A stainless steel tube (4.6 mm internal diameter and 15–25 cm length).

Column packaging material: 5-μm octadecylsilanized silica gel for liquid chromatography.

Column temperature: A constant temperature of around 40° C.

Mobile phase: A 3:2 mixture of ammonium acetate (0.02 mol/L)/acetonitrile.

Flow rate: 1 mL/min.

(3) 2-Methoxy-5-methylaniline

In the Monographs, the specification "not more than 10 μg/g as 2-methoxy-5-methylaniline (Coloring Matter Tests)" for this test, for example, means that the content of 2-methoxy-5-methylaniline must be not more than 10 μg/g when determined as directed below.

Test Solution Weigh accurately about 2.5 g of the sample into a beaker, add 25 mL of water to dissolve, and transfer into a 50-mL volumetric flask containing 5 drops of sodium hydroxide solution (1 in 25) and 1 mL of methanol. Wash the beaker twice with 10 mL of water each time, add the washings to the volumetric flask, and make 50 mL by adding water. Designate this solution as the sample solution. Pour exactly 20 mL of the sample solution into an absorption column packed with 20 mL of diatomaceous earth for chromatography to allow to flow. Allow to stand for 1 hour, pour 100 mL of hexane into the column, and collect the effluent into a 200-mL eggplant-shaped flask. Add 0.5 mL of diluted sulfuric acid (3 in 20,000), evaporate to about 1 mL under reduced pressure in a water bath at 40° C, and completely remove the hexane in the flask. To the residue, add a 3:2 mixture of ammonium acetate TS (0.02 mol/L)/acetonitrile to dissolve and make exactly 2 mL.

Standard Solutions Weigh accurately about 10 mg of 2-methoxy-5-methylaniline, and dissolve in methanol to make exactly 100 mL. Use this solution as the standard stock solution. To exactly 5 mL of the standard stock solution, add a 3:2 mixture of ammonium acetate TS (0.02 mol/L)/acetonitrile to make exactly 50 mL. Dilute this solution with a 3:2 mixture of ammonium acetate TS (0.02 mol/L)/acetonitrile to prepare several solutions containing 0.5–10μg of 2-methoxy-5-methylaniline in 1 mL.

Procedure Analyze a fixed amount each of the test solution and the standard solutions by liquid chromatography using the operating conditions given below. Measure the peak areas of 2-methoxy-5-methylaniline from standard solutions, and prepare a calibration curve. Measure the area of the peak corresponding to the retention time of 2-methoxy-5-methylaniline in the test solution, and calculate the content of 2-methoxy-5-methylaniline using the calibration curve.

Operating Conditions

Detector: Ultraviolet spectrophotometer or photodiode array detector (wavelength: 290 nm).

Column: A stainless steel tube (4.6 mm internal diameter and 15–25 cm length).

Column packaging material: 5-μm octadecylsilanized silica gel for liquid chromatography.

Column temperature: 40° C.

Mobile phase: A 3:2 mixture of ammonium acetate TS (0.02 mol/L)/acetonitrile.

Flow rate: 1 mL/min.

12. Color Precursor (Leuco Base)

Test Solution Use the test solution prepared for the unreacted raw materials and products of side reactions (Section 10).

Control Solution Dilute the color precursor standard stock solution specified in the individual monographs with ammonium acetate TS (0.02 mol/L) to prepare a solution containing 50 μg of the color precursor in 1 mL.

Procedure Analyze a fixed amount each of the test solution and the control solution by liquid chromatography using the operating conditions given below. The peak area of the color precursor in the test solution is not more than the peak area of that in the control solution. When there are plural color precursor peaks, use the sums of the peak areas.

Operating Conditions

Detector: Ultraviolet spectrophotometer or photodiode array detector (wavelength: 254 nm).

Column: A stainless steel tube (4.6 mm internal diameter and 25 cm length).

Column packaging material: 5-μm octadecylsilanized silica gel for liquid chromatography.

Column temperature: A constant temperature of about 40° C.

Mobile phase

A: Ammonium acetate TS (0.02 mol/L).

B: A 7:3 mixture of acetonitrile/water.

Concentration gradient: Follow the requirement specified in the individual monograph.

Flow rate: 1 mL/min.

13. Assay

(1) Titanium(III) Chloride Method Use the appropriate procedure based on the direction in the individual monograph.

(i) Measure exactly the specified volume of the test solution, and transfer into a 500-mL wide-mouthed Erlenmeyer flask. Add 15 g of trisodium citrate dihydrate and then water, and dissolve it ultrasonically if necessary to make about 200 mL with water. Titrate with 0.1 mol/L titanium(III) chloride while passing carbon dioxide gas or nitrogen gas through the solution and vigorously boiling. The endpoint of the titration is when the original color of the sample disappears.

(ii) Proceed as directed in (i) using 15 g of sodium hydrogen (+)-tartrate monohydrate, instead of trisodium citrate dihydrate.

(iii) Proceed as directed in (i) using 15 g of sodium hydrogen (+)-tartrate monohydrate, instead of trisodium citrate dihydrate. Using 10 mL of diluted Light Green SF Yellow (1 in 1000) as an indicator, perform a blank test in the same manner

as for the sample to make any necessary correction.

(iv) Proceed as directed in (i) using 20 g of sodium (+)-tartrate dihydrate instead of trisodium citrate dihydrate. The endpoint of the titration is when the original color of the sample disappears and an orange color appears.

(2) Mass Method Dry a crucible type glass filter (G4) at 135° C for 30 minutes, allow it to cool in a desiccator, and weigh accurately. Measure exactly the specified volume of the test solution, and transfer into a 500-mL beaker. Boil this solution, add 25 mL of diluted hydrochloric acid (1 in 50), and re-boil. Wash the inside of the beaker using about 5 mL of water, cover the beaker with a watch glass, heat on a water bath for about 5 hours, and then cool. Filter the precipitate through the glass filter prepared above, wash the beaker and precipitate three times with 10 mL of diluted hydrochloric acid (1 in 200) each time and then twice with 10 mL of water each time. Dry the precipitate together with the glass filter at 135° C for 3 hours, then cool in the desiccator, and weigh accurately.

Coloring Matter Aluminum Lake Tests

The tests specified below are applied to purity tests and assays for coloring matter aluminum lakes.

1. Hydrochloric Acid- and Ammonia-Insoluble Substances

In the Monographs, the specification "not more than 0.5% (Coloring Matter Aluminum Lake Tests)" for this test, for example, means that the content of the hydrochloric acid- and ammonia-insoluble substance must be not more than 0.5% when determined as directed in the following procedure.

Procedure Dry a crucible type glass filter (G4) at 135° C for 30 minutes, allow it to cool in a desiccator, and weigh accurately.

Weigh accurately about 2 g of the sample into a beaker, mix it with 20 mL of water, add 20 mL of hydrochloric acid, and stir well. Add 300 mL of boiling water, and shake well. Cover the beaker with a watch glass, heat on a water bath for 30 minutes, cool, and centrifuge. Filter the supernatant through the glass filter, previously prepared. If necessary, centrifuge in small amounts and filter each time. Transfer the insoluble substances in the beaker into the centrifuge tube with a small amount of water, add water to the tube to make about 50 mL, and centrifuge. Filter the supernatant through the glass filter, and transfer the insoluble substances in the beaker into the glass filter with a small amount of water. Wash the beaker and the insoluble substances on the filter twice with 5 mL of water each time, and then wash the insoluble substances on

the glass filter with diluted ammonia solution (1 in 25) until the washings become almost colorless. Then wash with 10 mL of diluted hydrochloric acid (1 in 35). If a long time is required for filtration during the water-washing process due to a large amount of residue, filter while dissolving the residue on the filter with ammonia solution (1 in 25). Then wash with water until the washings do not respond to silver nitrate solution (1 in 50). Dry the insoluble substances together with the glass filter at 135° C for 3 hours, allow to cool in a desiccator, and weigh accurately.

2. Iodide

In the Monographs, the specification "not more than 0.20% (Coloring Matter Aluminum Lake Tests)" for this test, for example, means that the content of sodium iodide must be not more than 0.20% when determined as directed below.

Test Solution Weigh accurately about 0.1 g of the sample, add exactly 25 mL of water, shake occasionally for about 30 minutes, and filter through a dry filter paper. To exactly 5 mL of the filtrate, add water to make exactly 50 mL.

Standard Solutions Transfer 0.5 mL, 1 mL, 2 mL, and 4 mL of Iodide Ion Standard Stock Solution into separate 100-mL volumetric flasks, and dilute each solution to volume with water.

Procedure Proceed as directed under Ion Chromatography according to the following operating conditions, using a fixed amount of each of the test solution and standard solutions. Determine the peak area or peak heights of the iodide ions of the standard solutions to make a calibration curve. Determine the peak area or peak height of the iodide ion of the test solution, and obtain the iodide ion content using the calibration curve. Multiply the iodide ion content by 1.18 to obtain the concentration of the sodium iodide of the test solution, and thus determine its content in the sample. Avoid direct sunlight during the procedure. Use a light-resistant container for the preparation of the test solution, and perform the test immediately after its preparation.

Operating Conditions

Detector: Electric conductivity detector.

Column: A stainless steel or plastic tube (4.6–6.0 mm internal diameter and 5–10 cm length).

Column packing material: Porous anion exchanger.

Guard column: A column with the same internal diameter and the same packing material as the above column.

Column temperature: 40° C.

Mobile phase: A solution (pH 4.0) prepared by dissolving 0.42 g of phthalic acid and 0.29 g of 2-amino-2-hydroxymethyl-1,3-propandiol in 1000 mL of water.

Flow rate: 1.5 mL/min.

3. Lead

In the Monographs, the specification "not more than 5 μg/g as Pb (Coloring Matter Aluminum Lake Tests, Lead)" for this test, for example, means that the content of lead must be not more than 5 μg/g as Pb when determined as directed below.

Preparation of Test Solutions, Control Solutions, and Blank Test Solutions

Sample Solution Weigh 1.0 g of the sample into a platinum, quartz, or porcelain crucible, and add small portions of sulfuric acid to moisten it. Heat by increasing the temperature gradually in the range of 100–500° C until the sample is almost charred and white fumes of sulfuric acid are no longer evolved. If necessary, crush the content with a glass rod while heating. Place the crucible in an electric furnace, heat by increasing the temperature gradually, and ignite at 500–600° C to incinerate the sample. If a charred mass remains, moisten it with sulfuric acid, heat until white fumes of sulfuric acid are no longer evolved, and ignite in the electric furnace to incinerate the sample. When incineration is done at 500–550° C, a heat-resistant glass beaker can be used. After cooling, add 30 mL of diluted hydrochloric acid (1 in 4), cover the crucible with a lid if necessary, heat to dissolve the residue, and cool.

Test Solution To the sample solution, add 10 mL of diammonium hydrogen citrate solution (1 in 2). Add 1 mL of thymol blue TS as the indicator, then add ammonia solution until the color of the solution changes from yellow to light yellow-green. Transfer it to a separating funnel (or centrifuge tube). Wash the crucible with a small amount of water into the separating funnel and add water to make 100 mL. Add 5 mL of a solution of ammonium pyrrolidine dithiocarbamate (3 in 5), and allow to stand for 5 minutes. Add exactly 10 mL of butyl acetate, shake for 5 minutes, and allow to stand or centrifuge, whichever may be appropriate. Use the butyl acetate layer as the test solution.

Control Solution Measure exactly 5 mL of Lead Standard Solution, and proceed as directed for the test solution.

Blank Test Solution Proceed as directed in the procedure of the sample solution and the test solution without using the sample.

Procedure Determine the absorbance of the test solution, the control solution, and the blank test solution as directed under Flame Atomic Absorption Spectrophotometry, according to the following operating conditions. The difference between the absorbance values of the test solution and the blank test solution is not more than the absorbance value of the control solution.

Operating Conditions

Light Source: Lead hollow cathode lamp.

Wavelength: 283.3 nm.

Supporting gas: Air.

Combustible gas: Acetylene

4. Zinc and Iron

In the Monographs, the specification "not more than 50 μg/g as Zn (Coloring Matter Aluminum Lake Tests, Zinc and Iron (1))" for this test, for example, means that the content of zinc must be not more than 50 μg/g as Zn when determined as directed in (1) using the sample solution prepared in the following manner.

Preparation of Sample Solution Weigh 1.0 g of the sample into a platinum quartz, or porcelain crucible or a heat-resistant glass beaker, moisten it with small quantities of sulfuric acid, heat by gradually increasing the temperature in the range of 100–500° C until the sample is almost charred and white fumes of sulfuric acid are no longer evolved. If necessary, crush the content with a glass rod while heating. Place the container in an electric furnace, heat by increasing the temperature gradually, and ignite at 450–550° C to incinerate the sample. If a charred mass remains, moisten it with sulfuric acid, and repeat the same steps. After cooling add 5 mL of hydrochloric acid and 1 mL of nitric acid, crush the lumps thoroughly, and heat to dryness. Add 5 mL of hydrochloric acid, crush the lumps, and heat to dryness. Add 10 mL of diluted hydrochloric acid (1 in 4) to the residue, dissolve by heating, cool, and filter through a filter paper (5C) for quantitative analysis. Wash the residue on the filter paper with about 30 mL of diluted hydrochloric acid (1 in 4), combine the filtrate and the washings, and heat to dryness. Add 10 mL of diluted hydrochloric acid (1 in 4) to the residue, dissolve by heating, cool, and filter. Wash the container and the residue on the filter paper with 5 mL of diluted hydrochloric acid (1 in 4) and with water, combine the filtrate and the washings, and add water to make 50 mL.

(1) Zinc

Test Solution Measure 10.0 mL of the sample solution, add 4 mL of diluted hydrochloric acid (1 in 4) and water to make 20 mL.

Control Solution Mix 1.0 mL of Zinc Standard Solution, 4 mL of diluted hydrochloric acid (1 in 4), and water to make 20 mL.

Blank Test Solution Proceed as directed in the preparation of the sample solution and the test solution without using the sample.

Procedure Determine the absorbance of the test solution, the control solution, and the blank test solution as directed under Flame Atomic Absorption Spectrophotometry, according to the following operating conditions. The difference between the absorbance value of the test solution and the blank test solution is not more than the absorbance value of the control solution.

Operating Conditions

Light Source: Zinc hollow cathode lamp.
Wavelength: 213.9 nm.
Supporting gas: Air.
Combustible gas: Acetylene.

(2) Iron

Test Solution Measure 10 mL of the sample solution, and add 10 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

Control Solution Mix 5 mL of Iron Standard Solution, 10 mL of diluted hydrochloric acid (1 in 4), and water to make 50 mL.

Blank Test Solution Proceed as directed in the preparation of the sample solution and the test solution without using the sample.

Procedure Determine the absorbance of the test solution, the control solution, and the blank test solution as directed under Flame Atomic Absorption Spectrophotometry, according to the following operating conditions. The difference between the absorbance values of the test solution and the blank test solution is not more than the absorbance value of the control solution.

Operating Conditions
Light source: Iron hollow cathode lamp.
Wavelength: 248.3 nm.
Supporting gas: Air.
Combustible gas: Acetylene.

5. Barium

In the Monographs, the specification "not more than 500 μg/g as Ba (Coloring Matter Aluminum Lake Tests)" for this test, for example, means that the content of barium must be not more than 500 μg/g as Ba when determined as directed below.

Test Solution Weigh accurately about 0.10 g of the sample, add 5 mL of nitric acid, and heat at 100° C for 5 hours. After cooling, add water to make exactly 100 mL.

Control Solution To exactly 1 mL of Barium Standard Solution, add water to make exactly 100 mL. To exactly 5 mL of this solution, add about 50 mL of water and 5 mL of nitric acid, cool, and add water to make exactly 100 mL.

Blank Test Solution Proceed as directed in the preparation of the test solution without using the sample.

Procedure Determine the emission of the test solution, the control solution, and the blank test solution as directed under Inductively Coupled Plasma-Atomic Emission Spectrometry. The difference between the emission intensity of the test solution and the blank test solution is not more than the emission intensity of the control solution.

6. Arsenic

In the Monographs, the specification "not more than 3 μg/g as As (Coloring Matter Aluminum Lake Tests)" for this test, for example, means that the content of arsenic as As must be not more than 3 μg/g when determined as directed below.

Test Solution Weigh 0.50 g of the sample into a porcelain crucible or a heat-resistant glass beaker, add 20 mL of a solution (1 in 10) of magnesium nitrate hexahydrate in ethanol (95), and ignite and burn the ethanol. Cover the container with a lid if necessary because the contents may sputter near the end of burning. Heat by increasing the temperature gradually in the range of 150 to 500° C until the sample is almost charred. If necessary, crush the contents with a glass rod while heating. Place in an electric furnace, and heat gradually to incinerate at 450–550° C. If a charred mass still remains, moisten it with a small quantity of nitric acid, heat until white fumes no longer evolve, and ignite again in the electric furnace at 450–550° C to incinerate. Cool, add 6 mL of hydrochloric acid to the residue, add about 10 mL of water if necessary, cover the container with a lid, and heat to dissolve it. Cool, and add water to make 25 mL.

Control Solution Mix 3.0 mL of Arsenic Standard Solution, 6 mL of hydrochloric acid, and water to make 25 mL.

Blank Test Solution Proceed as directed for the test solution without using the sample.

Procedure To 4 mL each of the test solution, the control solution, and the blank test solution, add 3 mL of hydrochloric acid and 1 mL of potassium of iodide, allow to stand for 30 minutes at room temperature. To each solution, add 2 mL of L(+)-ascorbic acid solution (1 in 10) and water to make 20 mL. Proceed as directed in the Method using Apparatus C under the Arsenic Limit Test. The difference between the absorbance values of the solutions derived from the test solution and the blank test solution is not more than the absorbance value of the solution from the standard solution.

Depending on the apparatus used, the quantity and concentration of hydrochloric acid, potassium iodide, and L(+)-ascorbic acid that should be added to the test solution, control solution, and blank test solution will vary. Also the flow rate and concentration of the test solution, control solution, hydrochloric acid, potassium iodide, and sodium tetrahydroborate TS that should be added to the apparatus may vary.

7. Assay

Use the appropriate procedure based on the direction in the individual monograph.

(1) Weigh accurately the quantity of sample specified in the individual monograph, transfer into a 500-mL wide-mouth Erlenmeyer flask, and add 20 mL of diluted sulfuric acid (1 in 20). Shake well, add 50 mL of boiling water, and dissolve by heating. Add 150 mL of boiling water and 15 g of trisodium citrate dihydrate and dissolve them

ultrasonically, if necessary. While passing carbon dioxide gas or nitrogen gas through the solution and boiling this solution vigorously, titrate with 0.1 mol/L titanium(III) chloride until the original color of the sample disappears.

(2) Proceed as directed in (1) using 15 g of sodium hydrogen (+)-tartrate monohydrate, instead of trisodium citrate dihydrate.

(3) Proceed as directed in (1) using 15 g of sodium hydrogen (+)-tartrate monohydrate, instead of trisodium citrate dihydrate. Using 10 mL of diluted Light Green SF Yellow (1 in 1,000) as the indicator, perform a blank test in the same manner as the test solution, and make any necessary correction.

Coloring Matter Preparations Tests

The tests specified below are applied as identification tests and purity tests for preparations of tar colors.

1. Other Coloring Matters

Analyze 2-μL portions of the test solution by paper chromatography using a 6:3:2 mixture of 1-butanol/ammonia solution/ethanol (99.5) as the developing solvent. Use a No. 2 filter paper for chromatography. Stop the development when the developing solvent has ascended to a point about 15 cm above the original line. Air-dry the filter paper, and observe it on a white board from the top in daylight.

If the colors are not separated well from each other, use a 1:1 mixture of diluted ethanol (99.5) (1 in 4)/ammonia solution (1 in 5) as the developing solvent.

2. Other Color Matter Aluminum Lake

(1) Weigh the specified amount of sample, add 60 mL of diluted acetic acid (1 in 3), heat it until boiling, and cool. Add 100 mL of acetone, mix well, and collect the supernatant. Analyze 2-μL portions of the test solution by paper chromatography using a 6:3:2 mixture of 1-butanol/ammonia solution/ethanol (99.5) as the developing solvent. Use a No. 2 filter paper for chromatography. Stop the development when the developing solvent has ascended to a point about 15 cm above the original line. Air-dry the filter paper, and observe it on a white board from the top in daylight. If the colors are not separated well from each other, use a 1:1 mixture of diluted ethanol (99.5) (1 in 4)/ammonia solution (1 in 5) as the developing solvent.

(2) Proceed in the same manner as for (1), using ammonia solution (1 in 25) instead of diluted acetic acid (1 in 3). Use a 1:1 mixture of diluted ethanol (99.5) (1 in 4)/ammonia solution (1 in 5) as the developing solvent.

(3) Proceed in the same manner as for (1), using diluted acetic acid (1 in 20) instead of diluted acetic acid (1 in 3).

3. Heavy Metals

In the Monographs, the specification "not more than 20 μg/g as Pb (Coloring Matter Preparations Tests, Heavy Metals)" for this test, for example, means that the content of heavy metals must be not more than 20 μg/g as Pb when determined as directed below.

Preparation of Test Solutions and Control Solutions

(1) Tar color preparations not containing aluminum lake

Sample Solution Weigh 2.5 g of the sample into a platinum, quartz, or porcelain crucible or a heat-resistant glass beaker, and add small portions of sulfuric acid to moisten it. Heat by gradually increasing the temperature in the range of 100–500° C until the sample is almost charred and white fumes of sulfuric acid no longer evolve. If necessary, crush the content with a glass rod while heating. Place the container in an electric furnace, heat by gradually increasing the temperature, and ignite at 450–550° C to incinerate the sample. If a charred mass remains, moisten with sulfuric acid, and repeat the same steps. After cooling, add 3 mL of hydrochloric acid, stir, add 7 mL of water, shake, and filter through a filter paper for quantitative analysis (5C). Wash the residue on the filter paper with 5 mL of diluted hydrochloric acid (1 in 4) and water, and combine washings with the filtrate. Add water to make 50 mL.

Test Solution Place 20 mL of the sample solution in a Nessler tube, and add 1 drop of phenolphthalein TS, then add ammonia TS dropwise until the solution turns red. Add 2 mL of diluted acetic acid (1 in 4), filter if necessary, wash the filter paper with water into the tube, and add water to make 50 mL.

Control Solution Proceed as directed for the sample solution without the sample. Designate the resulting solution as Solution A. Place 20 mL of Solution A in a Nessler tube, add exactly 2.0 mL of Lead Standard Solution (for heavy metals limit test), add 1 drop of phenolphthalein TS, and proceed as for the test solution.

(2) Tar color preparations containing aluminum lake

Sample Solution Weigh 2.5 g of the sample into an appropriate crucible or heat-resistant glass beaker, and proceed as directed in (1) to incinerate the sample. After cooling, add 5 mL of hydrochloric acid and 1 mL of nitrate acid to the residue, crush the lumps well, and heat to dryness. If necessary, incinerate in an electric furnace at 450–550°C for 1 hour. Add 5 mL of hydrochloric acid to the residue, crush the lumps well, and heat to dryness. To the residue, add 10 mL of diluted hydrochloric acid (1 in 4), and dissolve it by heating, and allow to cool. Filter the dissolved residue through a filter paper for quantitative analysis (5C), wash the residue on the filter paper with about 30 mL of diluted hydrochloric acid (1 in 4), and combine washings with the filtrate, and heat to dryness. To the residue, add 10 mL of diluted hydrochloric acid (1 in 4), dissolve it by heating, allow to cool, and filter the dissolved residue through the filter paper. Wash the residue on the filter paper and the container with 5 mL of diluted hydrochloric acid (1 in 4) and water, combine the washings with the filtrate, add water

to make 50 mL.

Test Solution Place 20 mL of the sample solution in a Nessler tube, adjust the pH to about 4 with diluted ammonium acetate (2 in 15), and add water to make 50 mL.

Control Solution Proceed as directed for the sample solution without the sample. Designate the resulting solution as Solution A. Place 20 mL of Solution A in a Nessler tube, add exactly 2.0 mL of Lead Standard Solution (for heavy metals limit test), add 1 drop of phenolphthalein TS, and proceed as for the test solution.

Procedure Add 2 drops each of sodium sulfide TS to the test solution and control solution, shake, and allow to stand for 5 minutes. The color of the test solution is not darker than that of the control solution.

4. Manganese and Chromium

In the Monographs, the specification "not more than 50 μg/g as Mn (Coloring Matter Preparations Tests, Manganese and Chromium (1))" for this test, for example, means that the content of manganese must be not more than 50 μg/g as Mn when determined as directed in (1).

(1) Manganese

Sample Solution Weigh 2.5 g of the sample into a platinum, quartz, or porcelain crucible or a heat-resistant glass beaker, and add small portions of sulfuric acid to moisten it. Heat by gradually increasing the temperature in the range of 100–500°C until the sample is almost charred and white fumes of sulfuric acid are no longer evolved. If necessary, crush the content with a glass rod while heating. Place the container in an electric furnace, heat by gradually increasing the temperature, and ignite at 450–550°C to incinerate the sample. If a charred mass remains, moisten it with sulfuric acid, and repeat the same steps. After cooling, add 3 mL of hydrochloric acid, then stir, add 7 mL of water, shake, and filter through a filter paper for quantitative analysis (5C). Wash the residue on the filter paper with 5 mL of diluted hydrochloric acid (1 in 4) and 5 mL of water, and combine washings with the filtrate. Designate the resulting solution as Solution A. Place the residue with the filter paper in a platinum crucible, dry at 105° C, and incinerate about 450° C. Add 2 g of sodium carbonate, ignite at 800° C or higher to fuse. After cooling, add 10 mL of water, and add hydrochloric acid dropwise to acidify it. Transfer the resulting solution into a beaker, wash the crucible with a small volume of water, add washings to the beaker, agitate the solution, and add it to Solution A. Add water to make 50 mL, and use as the sample solution. Proceed as directed for the sample solution without the sample. Designate the resulting solution as Solution B.

Test Solution and Control Solution

When the color content exceeds 50%: Prepare the test solution by adding 10 mL of

diluted hydrochloric acid (1 in 4) to 4.0 mL of the sample solution and making up with water to 50 mL. Prepare the control solution by mixing 4 mL of Solution B, 1.0 mL of Manganese Standard Solution, and 10 mL of diluted hydrochloric acid (1 in 4) and making up with water to 50 mL.

When the color content is 50% or less: Prepare the test solution and the control solution in the same manner as directed above, using 8.0 mL of the sample solution and Solution B, respectively.

Procedure Determine the absorbance by atomic absorption spectrophotometry using the operating conditions given below. The absorbance value of the test solution is not more than that of the control solution.

Operating Conditions

Light Source: Manganese hollow cathode lamp.

Wavelength: 279.5 nm.

Supporting gas: Air.

Combustible gas: Acetylene.

(2) Chromium

Test Solution and Control Solution

When the color content exceeds 50%: Prepare the test solution by adding 10 mL of diluted hydrochloric acid (1 in 4) to 10 mL of the sample solution prepared in (1) and making up with water to 50 mL. Or prepare the control solution by mixing 10 mL of Solution B prepared in (1), 1.0 mL of Chromium Standard Solution, and 10 mL of diluted hydrochloric acid (1 in 4) and making up with water to 50 mL.

When the color content is 50% or less: Prepare the test solution and the control solution in the same manner as directed above, using 20 mL of the sample solution and Solution B prepared in (1), respectively.

Procedure Determine the absorbance by atomic absorption spectrophotometry using operating conditions given below. The absorbance value of the test solution is not greater than that of the control solution.

Operating Conditions

Light Source: Chromium hollow cathode lamp.

Wavelength: 357.9 nm.

Supporting gas: Air.

Combustible gas: Acetylene.

Color Value Determination

Color value determination is designed to measure the concentration (Color Value) of a colorant in a food color by determining the absorbance using ultraviolet-visible spectrophotometry. The color value is the absorbance of a solution of the food color, in terms of the absorbance of a 10% (w/v) solution, that is determined at the maximum absorption wavelength within the visible light region. It is generally expressed as the figure $\left(E_{1cm}^{10\%}\right)$.

Procedure Unless otherwise specified, proceed as directed below.

Weigh accurately the amount of sample equivalent to the labeled color value, shown in the Table below, transfer it into a volumetric flask, dissolve in about 10 mL of the specified solvent, and then add the same solvent to make exactly 100 mL. Centrifuge or filter it if necessary, and use this solution as the sample solution. Designate this solution as the test solution for absorbance measurement. The test solution should be adjusted so that its absorbance falls within the range of 0.2–0.7 or 0.4–1.4 when a single-beam absorption photometry or a double-beam absorption photometry is used, respectively. If necessary, dilute the sample solution according to the corresponding dilution factor given in the Table.

Determine the absorbance (A) at the specified wavelength in a 1-cm cell using the same solvent used for preparing the test solution as the reference. Calculate the color value by the formula below. Determination of the color value should be done promptly after the preparation of the test solution to avoid the influence of fading after preparation.

$$\text{Color value} = \frac{10 \times A \times F}{\text{Amount (g) of the sample}}$$

F = dilution factor of test solution used to adjust the absorbance to the appropriate range.

Color value	Concentration (%) for measurement	Absorbance	Dilution method	Volume (mL) of dilution	F
20	0.025	0.5	0.25 g →100 mL	100	1
50	0.10	0.5	0.1 g →100 mL	100	1
100	0.05	0.5	0.5 g →100 mL→10mL→100 mL	1000	10
200	0.03	0.6	0.6 g →100 mL→5 mL→100mL	2000	20
400	0.015	0.6	0.3 g →100 mL→5 mL→100 mL	2000	20
500	0.01	0.5	0.2 g →100 mL→5 mL→100 mL	2000	20
700	0.01	0.7	0.2 g →100 mL→5 mL→100mL	2000	20
800	0.00625	0.5	0.25 g →100 mL→5 mL→200 mL	4000	40
900	0.005	0.45	0.2 g →100 mL→5 mL→200 mL	4000	40
1000	0.006	0.6	0.3 g →100 mL→5 mL→250mL	5000	50
1500	0.003	0.6	0.4 g →100 mL→5 mL→50 mL→5 mL→50 mL	10,000	100
2000	0.003	0.6	0.3 g →100 mL→5 mL→50 mL→5 mL→50 mL	10,000	100
2500	0.002	0.5	0.2 g →100 mL→5 mL→50 mL→5 mL→50 mL	10,000	100

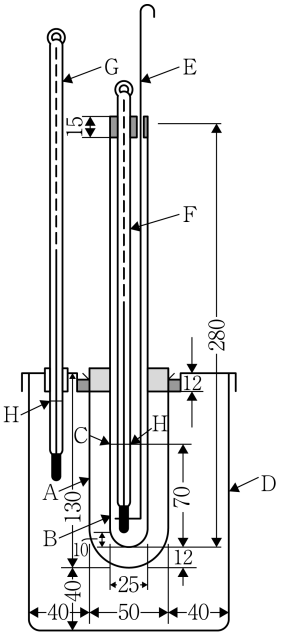
If the color value exceeds the maximum value in the above table, adjust the dilution factor.

Congealing Point

The congealing point is measured as directed below.

Apparatus Use the apparatus illustrated in Fig. 1.

A: Glass cylinder (apply silicone oil to the inner and outer surfaces of the cylinder to prevent clouding.)
B: Sample container (a hard-glass test tube. If necessary, apply silicone oil to the outer surface to prevent clouding. Insert into cylinder A, and fix with a cork stopper.)
C: Mark
D: Glass or plastic cooling bath
E: Glass or stainless steel stirrer (a 3 mm diameter wire with the lower end bent into a loop of about 18 mm diameter)
F: Rod thermometer with an immersion line
G: Auxiliary thermometer
H: Immersion line



(Unit: mm)

Fig. 1

Procedure Fill glass or plastic cooling bath D almost to the top with water that is about 5° C lower than the expected congealing point. If the sample is a liquid at ordinary temperature, fill bath D with water whose temperature is 10–15° C lower than the expected congealing point. Transfer the sample into sample container B up to mark C. If the sample is a solid, melt it by warming, taking care not to exceed 20° C above the expected congealing point, and transfer into B. Insert B into glass cylinder A, and adjust immersion line H of thermometer F so that it is at the same level as the meniscus of the sample. After cooling the sample to a temperature 5° C higher than the expected congealing point, move stirrer rod E vertically at a rate of 60 to 80 strokes per minute, and take the temperature readings at 30-second intervals. The temperature falls gradually. Stop stirring when an appreciable amount of crystals is formed and the temperature becomes constant, or begins to rise.

Ordinarily, the temperature becomes constant for a short time after rising. Record the maximum temperature (reading on the thermometer) that keeps constant for a whole (Fig. 2). If the temperature does not increase, record the temperature that has remained constant for a while (Fig. 3). The average of four or more consecutive readings between which the temperature difference is within 0.2° C should be taken as the congealing point is the.

If many impurities are present in the sample, the congealing point curve will not be as shown in Fig. 2, but rather as shown in Fig. 3, 4, or 5. In the case of Figs. 4 and 5, extend each of the curves of the solid and liquid phases on the graph, and take their intersection as the congealing point. In the case of Fig. 3, proceed as in Fig. 2.

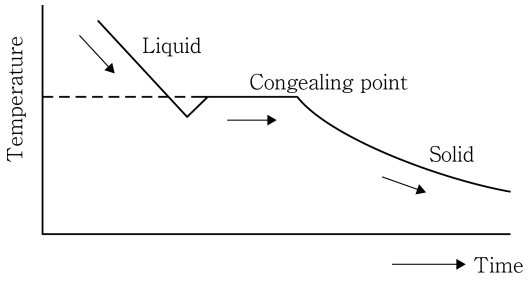


Fig.2

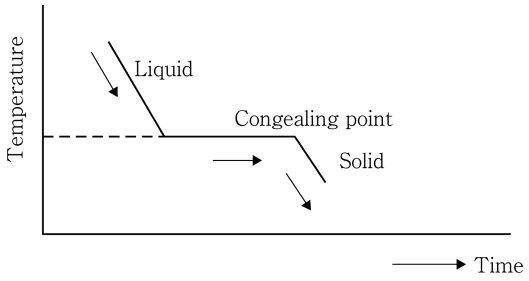


Fig.3

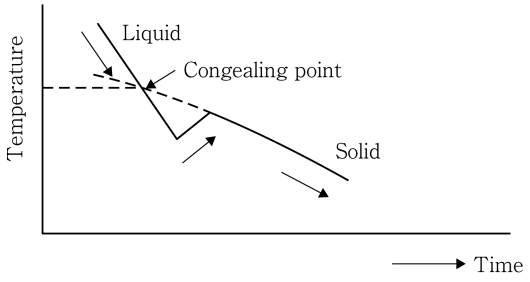


Fig.4

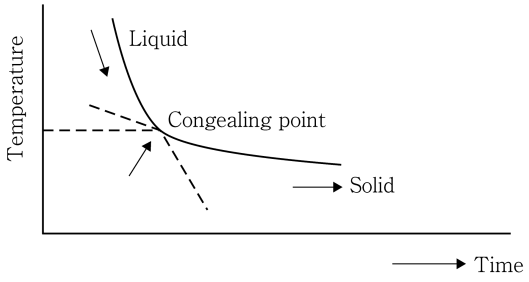


Fig.5

Note: If a state of supercooling is anticipated, rub the inner wall of bath B or put a small fragment of the solid sample into bath B to promote congealment when the temperature approaches the expected congealing point.

Fats and Related Substances Tests

The fats and related substances tests are designed to determine the ester value, saponification value, acid value, hydroxyl value, and iodine value of fats and related substances, other than flavoring substances, such as fatty acids, higher aliphatic alcohols, and fatty acid esters.

1. Ester Value

The ester value is the number of mg of potassium hydroxide (KOH) required to saponify the esters in 1 g of sample.

In the Monographs, the specification "125–164 (Fats and Related Substances Tests)" for this test, for example, means that the ester value must be 125–164 when determined as directed in the procedure below.

Procedure Unless otherwise specified, determine the saponification value and the acid value, and calculate the ester value by the following formula:

$$\text{Ester value} = \text{Saponification Value} - \text{Acid Value}$$

2. Saponification Value

The saponification value is the number of mg of potassium hydroxide (KOH) required to saponify the esters and neutralize the free acids in 1 g of sample.

Procedure Unless otherwise specified, proceed as follows: Weigh accurately about 1 g of sample, transfer into an Erlenmeyer flask, add 40 mL of ethanol (95), and dissolve while warming if necessary. Add 20 mL of 3.5% (w/v) potassium hydroxide–ethanol TS, accurately measured, equip the flask with a reflux condenser, and heat in a water bath for 30 minutes while shaking occasionally. Allow to cool, add a few drops of phenolphthalein TS, and immediately titrate the excess potassium hydroxide with 0.5 mol/L hydrochloric acid. If the solution is turbid when cold, titrate while warm. Perform a blank test, and calculate the saponification value by the formula:

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

a = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the blank test,

b = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the test.

3. Acid Value

The acid value is the number of mg of potassium hydroxide (KOH) required to neutralize 1 g of sample.

In the Monographs, the specification "not more than 15 (Fats and Related Substances Tests)" for this test, for example, means that the acid value must be not more than 15 when determined as directed in the following procedure.

Procedure Unless otherwise specified, proceed as follows: Weigh accurately the amount of sample specified in the Table, according to its expected acid value, and add 50 mL of a 1:1 mixture of ethanol (95)/diethyl ether. Dissolve while heating if necessary, and use this solution as the test solution. Cool, add 2 to 3 drops of phenolphthalein TS, titrate with 0.1 mol/L ethanolic potassium hydroxide to the first light red color that persists for 30 seconds, and calculate the acid value using the formula below. If the solution is turbid when cold, titrate while warm. To the solvent mixture used, add previously 0.1 mol/L ethanolic potassium hydroxide until its light red color persists for 30 seconds using 2 to 3 drops of phenolphthalein TS as the indicator.

$$\text{Acid value} = \frac{(\text{Volume (mL) of 0.1 mol/L ethanolic potassium hydroxide consumed}) \times 5.611}{\text{Weight (g) of the sample}}$$

Table

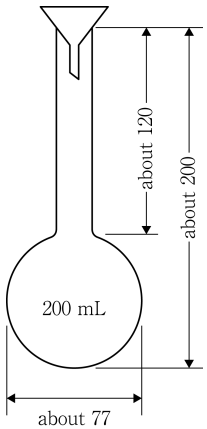
Acid value	Weight (g) of sample
Less than 5	10
5 to less than 15	5
15 to less than 50	3
50 to less than 120	1
Not less than 120	0.5

4. Hydroxyl Value

The hydroxyl value is the number of mg of potassium hydroxide (KOH) required to neutralize the acetic acid combined to hydroxyl groups, when 1 g of sample is acetylated under the following conditions.

In the Monographs, the specification "155–187 (Fats and Related Substances Tests), provided that the acid value is assumed to be 0" for this test, for example, means that the hydroxyl value must be 155–187 when determined as directed in the following procedure by assuming the acid value to be 0.

Procedure Unless otherwise specified, proceed as follows: Weigh accurately about 1 g of sample, transfer into a round-bottom flask as shown in the figure below, and add exactly 5 mL of acetic anhydride–pyridine TS. Place a small funnel on the mouth of the flask, and heat for 1 hour while immersing the flask to a depth of about 1 cm into an oil bath at 95–100° C. Cool, add 1 mL of water, shake well, and heat for 10 minutes. After cooling, rinse the funnel and the neck of the flask with 5 mL of ethanol (95) into the flask, and titrate the excess acetic acid with 0.5 mol/L ethanolic potassium hydroxide (indicator: 1 mL of phenolphthalein TS).



(Unit: mm)

Perform a blank test, and calculate the hydroxyl value by the formula:

$$\text{Hydroxyl value} = \frac{(a - b) \times 28.05}{\text{Weight (g) of the sample}} + \text{Acid value}$$

a = volume (mL) of 0.5 mol/L ethanolic potassium hydroxide consumed in the blank test,

b = volume (mL) of 0.5 mol/L ethanolic potassium hydroxide consumed in the test.

5. Iodine Value

The iodine value is defined as the amount of halogens, in terms of the number of g of iodine (I), that are absorbed by 100 g of sample under the following conditions.

Procedure Unless otherwise specified, weigh accurately the amount of sample specified in the Table, according to the expected iodine value of the sample, in a small glass container. Place this container in a 500-mL ground-glass stoppered Erlenmeyer flask, add exactly 20 mL of cyclohexane to dissolve the sample, followed by 25 mL of Wijs TS, and mix well. Stopper the flask tightly, and allow to stand, protected from light, at 20–30° C for 30 minutes (when the expected iodine value is 100 or more, for 1 hour) with occasional shaking. Add 20 mL of potassium iodine solution (1 in 10) and

100 mL of water, and shake. Next, titrate the freed iodine with 0.1 mol/L sodium thiosulfate VS (indicator: 1 mL of starch TS). Perform a blank test. Obtain the iodine content by the formula:

$$\text{Iodine value} = \frac{(a - b) \times 1.269}{\text{Weight (g) of the sample}}$$

a = amount (mL) of 0.1 mol/L sodium thiosulfate consumed in the blank test,

b = amount (mL) of 0.1 mol/L sodium thiosulfate consumed in the test with the sample.

Table

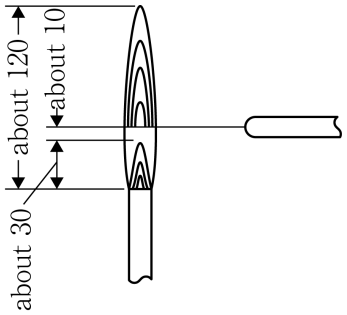
Acid value	Weight (g) of sample
Less than 30	1
30 to less than 50	0.6
50 to less than 100	0.3
Not less than 100	0.2

Flame Coloration Test

The flame coloration test is designed to determine the identity of an element based on the property that a certain type of element changes the flame of a Bunsen burner to a characteristic color.

Procedure Use a straight platinum wire about 0.8 mm in diameter for the test. In the case of a solid sample, make it into a paste by adding a small quantity of hydrochloric acid. Apply a small amount of the sample to the platinum wire along about 5 mm from the tip, and test immediately by putting the tip of the wire into a colorless flame, while keeping the platinum wire horizontal. In the case of a liquid sample, immerse the tip of the platinum wire into the sample about 5 mm below the liquid surface, remove it gently from the sample, and perform the test in the same manner as for a solid sample.

The description, "the flame coloration reaction persists," means that the reaction persists for 4 seconds.



(Unit: mm)

Flavoring Substances Tests

1. Alcohol Content

The alcohol content is the quantity of alcohol contained in a sample.

Procedure Measure exactly 10 mL of the sample, transfer into a 100-mL flask, and add 10 mL of acetic anhydride and 1 g of anhydrous sodium acetate. Connect the flask to an air condenser, and boil the mixture gently on a hot-plate for 1 hour. Allow to cool for 15 minutes, and then add 50 mL of water. Heat the mixture in a water bath for 15 minutes while shaking occasionally. Cool, transfer the mixture into a separating funnel, and remove the aqueous layer. Wash the oil layer with sodium carbonate solution (1 in 8) until the washings become alkaline, and wash further with sodium chloride solution (1 in 10) until the washings become neutral. Transfer the oil layer into a dry container, add about 2 g of sodium sulfate, shake well, allow to stand for about 30 minutes, and filter. Weigh accurately the specified amount of the acetylated oil thus obtained, and determine its ester value as directed under Ester Value in the Flavoring Substances Tests. Ester value, referred to as acetyl value here, is calculated by the formula:

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

Calculate the alcohol content by the formula:

$$\text{Alcohol content (\%)} = \frac{(\text{Molecular weight of alcohol}) \times (a - b) \times 0.5}{[(\text{Weight of acetylated oil}) - 0.02102\,(a - b)] \times 1000} \times 100$$

$$= \frac{\text{Acetyl value} \times \text{Molecular weight of alcohol}}{561.1 - (0.4204 \times \text{Acetyl value})}$$

a = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the blank test,
b = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the test.

2. Aldehyde or Ketone Content

The aldehyde or ketone content is obtained based on the property that aldehydes and ketones react with hydroxylamine (NH_2OH).

Procedure Unless otherwise specified, proceed as directed in Method 1 or Method 2, whichever is appropriate.

Method 1 Weigh accurately the specified amount of sample, add exactly 50 mL of 0.5 mol/L hydroxylammonium chloride, and shake well. Either allow to stand for the time specified in the individual monograph, or heat gently under a reflux condenser in a water bath for the time specified in the individual monograph, and then cool to room temperature. Titrate the liberated acid with 0.5 mol/L ethanolic potassium hydroxide. Confirm the endpoint using a potentiometer or observing the appearance of a green-yellow color. Perform a blank test, make any necessary correction, and calculate the content by the formula:

Aldehyde or ketone content (%)

$$= \frac{\text{(Molecular weight of aldehyde or ketone)} \times (a - b) \times 0.5}{\text{Weight (g) of the sample} \times 1000} \times 100$$

a = volume (mL) of 0.5 mol/L ethanolic potassium hydroxide consumed in the test,
b = volume (mL) of 0.5 mol/L ethanolic potassium hydroxide consumed in the blank test.

Method 2 Weigh accurately the specified amount of sample, add exactly 75 mL of hydroxylamine TS, and shake well. Either allow to stand for the time specified in the individual monograph, or boil gently under a reflux condenser in a water bath for the time specified in the individual monograph, and then cool to room temperature. Titrate excess hydroxylamine with 0.5 mol/L hydrochloride acid. Confirm the endpoint using a potentiometer or macroscopically observing the change in the color of the solution from purple to green-yellow. Perform a blank test, make any necessary correction, and calculate the content by the formula:

Aldehyde or ketone content (%)

$$= \frac{\text{(Molecular weight of aldehyde or ketone)} \times (a - b) \times 0.5}{\text{Weight (g) of the sample} \times 1000} \times 100$$

a = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the blank test,
b = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the test.

3. Ester Value

The ester value is the number of mg of potassium hydroxide (KOH) required to saponify the esters contained in 1 g of sample.

In the Monographs, the specification "not more than 3.0 (5 g, Flavoring Substances Tests)" for this test, for example, means that the ester value must be not more than 3.0 when determined as directed in the following procedure using about 5 g of the test substance.

Procedure Unless otherwise specified, proceed as follows: Weigh accurately the specified amount of sample, transfer into a 200-mL flask, add 10 mL of ethanol (95) and 3 drops of phenolphthalein TS, and then neutralize with potassium hydroxide solution (1 in 250). Add exactly 25 mL of 0.5 mol/L ethanolic potassium hydroxide, and heat gently under a reflux condenser in a water bath for 1 hour. Cool, and then titrate the excess potassium hydroxide with 0.5 mol/L hydrochloride acid. Confirm the endpoint using a potentiometer or 2 to 3 drops of phenolphthalein TS as the indicator. Perform a blank test in the same manner, and calculate the ester value by the formula:

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

a = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the blank test,
b = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the test.

4. Ester Content

The content of monobasic acid esters is obtained by performing the test as directed under Ester Value in Flavoring Substances Tests and then calculating by the formula:

$$\text{Ester content (\%)} = \frac{\text{(Molecular weight of ester)} \times (a - b) \times 0.5}{\text{Weight (g) of the sample} \times 1000} \times 100$$

$$= \frac{\text{Ester value} \times \text{Molecular weight of ester}}{561.1}$$

in which a and b are the respective a and b, defined under Ester Value.

5. Saponification Value

Saponification value is the number of mg of potassium hydroxide (KOH) required to saponify the ester and neutralize the free acid contained in 1 g of sample.

Procedure Unless otherwise specified, proceed as follows: Weigh accurately the specified amount of sample, and transfer into a 200-mL flask. Add exactly 25 mL of 0.5mol/L ethanolic potassium hydroxide, and heat gently under a reflux condenser in a

water bath for 1 hour. Cool, and then titrate the excess alkali with 0.5 mol/L hydrochloric acid. Confirm the endpoint using a potentiometer or 1 mL of phenolphthalein TS as the indicator. Perform a blank test in the same manner, and calculate the saponification value by the formula:

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

a = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the blank test,
b = volume (mL) of 0.5 mol/L hydrochloric acid consumed in the test.

6. Acid Value

The acid value is the number of mg of potassium hydroxide (KOH) required to neutralize 1 g of sample.

In the Monographs, the specification "not more than 6.0 (Flavoring Substances Tests)" for this test, for example, means that the acid value must be not more than 6.0 when determined as directed in the following procedure.

Procedure Unless otherwise specified, proceed as follows: To about 10 g of the sample, accurately weighed, add about 50 mL of ethanol (neutralized), and dissolve it by warming if necessary. Using a microburet, titrate, while shaking occasionally, with 0.1 mol/L potassium hydroxide. Use a potentiometer or an indicator (3 drops of phenolphthalein TS) to confirm the endpoint. When the indicator is used, the endpoint is when the first light pink color persists for 30 seconds. Calculate the acid value by the following formula:

$$\text{Acid value} = \frac{(\text{Volume (mL) of 0.1 mol/L potassium hydroxide consumed}) \times 5.611}{\text{Weight (g) of the sample}}$$

7. Gas Chromatographic Assay of Flavoring Agents

Apparatus Follow the directions given under Gas Chromatography in the General Tests.

Procedure Unless otherwise specified, proceed as directed below. If the sample is a solid, dissolve it in the specified solvent before the test.

Peak Area Percentage Method This method applies to samples that do not produce any involatile component during storage and whose all components elute and are adequately separated into components being sought and impurities in the chromatogram. The sum of the areas of all peaks that appear within the specified time after the injection of the test solution is normalized to 100. The peak area percentage of the component being sought to the sum is determined as the content. When a solid sample is dissolved in a solvent, the same test should be conducted on the

solvent to confirm the peak from the solvent, and the sum of the areas of all peaks, other than the peak of the solvent, is normalized to 100.

Operating Conditions (1)

These conditions apply to samples whose boiling point is not lower than 150°C and lower than 200°C.

Detector: Flame ionization detector or thermal conductivity detector.

Column: Use a fused-silica capillary column (30–60 m length and 0.25–0.53 mm internal diameter) coated with a 0.25- to 1-μm thick layer of dimethylpolysiloxane or polyethylene glycol for gas chromatography.

Column temperature: Upon injection at 50°C, raise the temperature at a rate of 5°C/min to 230°C, and maintain it at 230°C for 4 minutes.

Inlet temperature: 225–275°C.

Detector temperature: 250–300°C.

Carrier gas: Helium or nitrogen.

Flow rate: Control the flow rate so that the peak of the component being sought appears 5–20 minutes after injection.

Injection: Split.

Split ratio: 1:30–1:250. Adjust the ratio so that no component exceeds the capacity of the column.

Measurement time: 40 minutes.

Operating Conditions (2)

These conditions apply to samples whose boiling point is lower than 150°C.

Follow operation conditions (1) for requirements of the detector, column, inlet temperature, detector temperature, carrier gas, flow rate, injection, split ratio, and measurement time.

Column temperature: Upon injection at 50°C, maintain the temperature at 50°C for 5 minutes, and then raise at a rate of 5°C/min to 230°C.

Operating Conditions (3)

These conditions apply to samples whose boiling point is lower than 150°C and whose assumed impurities have a boiling point higher than that of components being sought.

Follow operation conditions (1) for requirements of the detector, column, inlet temperature, detector temperature, carrier gas, injection, and split ratio.

Column temperature: Upon injection at 50°C, maintain the temperature at 50°C for 5 minutes, then raise at a rate of 5°C/min to 230°C, and maintain at 230°C for 19 minutes.

Flow rate: Adjust the flow rate so that the peak of the component being sought appears 5–10 minutes after injection.

Measurement time: 60 minutes.

Operating Conditions (4)

These conditions apply to samples whose boiling point is not lower than 200°C.

Follow operation conditions (1) for requirements of the detector, column, inlet temperature, detector temperature, carrier gas, injection, and split ratio.

Column temperature: Upon injection at 100°C, raise the temperature at a rate of 5°C/min to 230°C, and maintain at 230°C until the end of measurement. Adjust the initial temperature and the flow rate so that the peak of the component being sought appears 5–20 minutes after injection.

Measurement time: 60 minutes.

Gas Chromatography

Gas chromatography is a method to separate and analyze components of a mixture by virtue of differences in retention capacity against the stationary phase. The mixture to be analyzed is injected into a column packed with a stationary phase, developed in the gaseous state with the aid of a gaseous mobile phase (carrier gas), and then separated into components. This method is applicable to gases, liquids, and other samples that can be made into vapor. The method is applied to identification tests, purity tests, and assays.

Apparatus The apparatus consists generally of a carrier gas flow-rate controller, a sample injection port, a column, a column oven, a detector, and a data processor. If necessary, a flow-rate controller for a combustion gas, a combustion supporting gas, an accessory gas, and a headspace sampler are also used. The carrier gas flow-rate controller serves to deliver the carrier gas into the column at a constant flow rate and usually consists of a pressure regulation valve, a flow-rate regulation valve, and a pressure gauge. The sample injection port is used to deliver the sample to the flow line of carrier gas. Sample injection ports are roughly divided into two, depending on the column to be used: for a capillary column and for a packed column. There are two modes (split and splitless modes) to inject a sample to a capillary column. The column oven has enough capacity to store a column of required length and is equipped with a column-temperature control system to keep the column at the required temperature. The detector is a device to detect components with properties different from the carrier gas. Generally, a thermal conductivity, flame-ionization, electron-capture, nitrogen-phosphorous, flame photometric, or mass spectrometric detector is used. The data processor can record or output various data including chromatograms, retention times, and quantitative amounts of the components to be determined.

Procedure Unless otherwise specified, proceed as directed below.

Fix the apparatus in advance, and adjust the detector, the column, the temperature,

and the flow rate of the carrier gas to the operating conditions specified in the individual monograph. Inject the specified amount of the test solution prepared as specified in the individual monograph into the sample injection port. The separated components are detected by the detector, and the detector output is recorded as a chromatogram by the recorder. Follow the same procedure for the standard solutions and control solution prepared as specified in the same monograph. The test substance is identified by confirming that the retention time ("retention time" refers to the time from the injection of the test solution to the emergence of the peak maximum of the component) corresponds to that of the standard sample, or that the retention time does not change, nor does the peak width widen when the standard sample is added.

Determination is performed according to either of the following methods, generally using the peak area or peak height.

(1) Internal Standard Method Prepare several standard solutions containing a constant amount of the internal standard specified in the individual monograph and known, graded amounts of the reference standard of the compound to be determined. From each of the chromatograms obtained by injecting a constant volume of each standard solution, calculate the ratio of the peak area (or peak height) of the reference standard to that of the internal standard. Prepare a calibration curve by plotting the values obtained on a graph, with the peak area (peak height) ratio on the ordinate and the added amounts of the reference standard or the ratio of the amount of the reference standard to that of the internal standard on the abscissa. The calibration curve is usually a straight line through the origin. Next, prepare a test solution containing the same amount of the internal standard as specified in the individual monograph, record the chromatogram under the same conditions as for the preparation of the calibration curve, calculate the peak area (peak height) ratio of the compound to be determined to the internal standard, and then determine the concentration of the compound in the test solution from the calibration curve. In the individual monograph, generally, a standard solution with a concentration within the linear range of the calibration curve and a test solution with a concentration close to that of the standard solution are prepared, and specified volumes of these solutions are chromatographed under the fixed conditions to determine the amount of the compound.

(2) Absolute Calibration Curve Method Prepare standard solutions containing graded amounts of the reference standard, and inject a constant volume of each standard solution, exactly measured. Using each of the chromatograms obtained, prepare a calibration curve by plotting the values obtained on a graph, with the peak areas (or peak heights) of the reference standard on the ordinate and the amounts of the reference standard on the abscissa. The calibration curve is usually a straight line through the origin. Next, prepare the test solution as directed in the individual

monograph, record the chromatogram under the same conditions as for the preparation of the calibration curve, measure the peak area (or peak height) of the compound to be determined, and determine the concentration of the compound in the test solution from the calibration curve. The whole procedure from injection to measurement must be carried out under strictly constant conditions.

(3) Standard Addition Method Prepare at least four volumetric flasks. Place a constant volume of the sample solution in each flask, and to all but one of the flasks, add suitable quantities of a standard solution of the compound to be determined so as to prepare a series of solutions containing stepwise increasing concentrations of the compound to be determined. Dilute exactly all the solutions to a definite volume, and use these solutions as the test solutions. From each of the chromatograms obtained by exactly injecting a constant volume of individual test solutions so that high reproducibility is obtained, measure the peak areas (or peak heights) for the individual test solutions. Calculate the concentration of the compound to be determined added to each test solution, and prepare a regression line by plotting the values obtained on a graph, with the increment amounts of the compound to be determined on the abscissa and the peak areas (or peak heights) on the ordinate. Extrapolate the linear regression, and determine the amount of the compound in the test solution from the distance between the coordinate origin and intersection point of the regression line and the abscissa. This method is applicable only in the case that the calibration curve is a straight line passing through the coordinate origin when prepared by the absolute calibration curve method. The whole procedure from injection to measurement must be carried out under strictly constant conditions.

In any of the above methods, the peak area or peak height is measured generally by method (i) or (ii), whichever is appropriate.

(i) Method using the peak area Use either of the two methods.

Width at half-height method Multiply the peak width at half-height by the peak height.

Automatic integration method Measure the signal obtained from the detector using a data processor, and determine the peak area.

(ii) Method using the peak height Use either of the two methods.

Peak height method Measure the distance between the peak maximum and the intersection of a perpendicular line drawn from the peak maximum to the horizontal axis of the recording paper and a line linking both side inflection points of the lower end of the peak.

Automatic peak height method Measure the signal obtained from the detector using a data processor, and determine the peak height.

System Suitability Proceed as directed under System Suitability in Liquid

Chromatography. The operating conditions specified in the individual monograph may be changed in terms of the following requirements, within a range in which the system suitability is met: the internal diameter and length of the column, the particle size of the packing material, the concentration or thickness of the stationary phase, the column temperature, the temperature-rising rate, the type and flow rate of the carrier gas, and split ratio.

Terminology Apply the definitions specified under Liquid Chromatography.

Note: Avoid the use of reagents and test solutions containing substances that may interfere with the determination.

Heavy Metals Limit Test

The heavy metals limit test is designed to demonstrate that the content of heavy metals in an additive does not exceed the acceptable limit specified in the individual monograph. In this test, "heavy metals" mean the metallic substances that become darkened in the presence of sodium sulfide TS in an acidic medium, and the limit is expressed in terms of the quantity of lead (Pb).

In the Monographs, the specification "not more than 20 μg/g as Pb (1.0 g, Method 1, Control Solution: Lead Standard Solution (for heavy metals limit test) 2.0 mL)" for this test, for example, means that the content of heavy metals in the substance must be not more than 20 μg/g as Pb when determined according to the following manner: *The test solution and the control solution are prepared with 1.0 g of the test substance and 2.0 mL of Lead Standard Solution (for heavy metals limit test), respectively, as directed in Method 1 given below, and the test is performed as directed in the procedure below.*

Preparation of Test and Control Solutions Unless otherwise specified, proceed according to one of the following methods, as appropriate.

Method 1

Test Solution Weigh the specified amount of sample, transfer into a Nessler tube, dissolve it by adding about 40 mL of water, and add 2 mL of diluted acetic acid (1 in 20) and water to make 50 mL.

Control Solution Measure the specified amount of Lead Standard Solution (for heavy metals limit test), transfer into another Nessler tube, and add 2 mL of diluted acetic acid (1 in 20) and water to make 50 mL.

Method 2

Test Solution Weigh the specified amount of sample, transfer into a quartz or porcelain crucible, cover loosely with a lid, and char the sample by gently heating.

Allow to cool, add 2 mL of nitric acid and 5 drops of sulfuric acid, heat until white fumes no longer evolve, and ignite at 450–550° C to incinerate. Cool, add 2 mL of hydrochloric acid, evaporate to dryness on a water bath, add 3 drops of hydrochloric acid to the residue, add 10 mL of boiling water, and warm for 2 minutes. Cool, add 1 drop of phenolphthalein TS, and add ammonia TS until the solution becomes slightly red. Next, transfer the solution into a Nessler tube. Wash the crucible with water, and add the washings to the Nessler tube. Then add 2 mL of diluted acetic acid (1 in 20) and water to make 50 mL.

Control Solution Place 2 mL of nitric acid, 5 drops of sulfuric acid, and 2 mL of hydrochloric acid into a crucible of the same quality as that used for the sample, heat to evaporate to dryness, and add 3 drops of hydrochloric acid to the residue. Next, proceed as directed in the preparation of the test solution, and transfer the resulting solution into another Nessler tube. Wash the crucible with water, and add the washings to the Nessler tube. Then add the specified amount of Lead Standard Solution (for heavy metals limit test), 2 mL of diluted acetic acid (1 in 20), and water to make 50 mL.

If the test solution is not clear, filter both the test solution and the control solution under the same conditions.

Method 3

Test Solution Weigh the specified amount of sample, transfer into a quartz or porcelain crucible, heat gently and carefully to char, and then ignite to incinerate. Cool, add 1 mL of aqua regia, and evaporate to dryness on a water bath. Moisten the residue with 3 drops of hydrochloric acid, add 10 mL of boiling water, and warm for 2 minutes. Next, add 1 drop of phenolphthalein TS, ammonia TS until the solution becomes slightly red, and 2 mL of diluted acetic acid (1 in 20). Transfer the resulting solution into a Nessler tube, or alternatively, filter the solution into a Nessler tube if necessary, wash the filter with 10 mL of water, add washings to the Nessler tube. Then add water to make 50 mL.

Control Solution Place 1 mL of aqua regia into a crucible of the same quality as that used for the sample, and evaporate on a water bath. Proceed as directed in the preparation of the test solution, transfer the resulting solution into a Nessler tube, and add the specified amount of Lead Standard Solution (for heavy metals limit test) and water to make 50 mL.

Method 4

Test Solution Weigh the specified amount of sample, transfer into a platinum, quartz, or porcelain crucible, add 10 mL of a solution of magnesium nitrate hexahydrate in ethanol (95) (1 in 10), and mix. Ignite and burn the ethanol, and char the sample by heating gradually. Allow to cool, add 1 mL of sulfuric acid, heat carefully, and ignite at 500–600° C to incinerate. Moisten with a small amount of sulfuric acid if a charred mass remains, and ignite to incinerate. Cool, dissolve the residue with 3 mL

of hydrochloric acid, and evaporate to dryness on a water bath. Moisten the residue with 3 drops of hydrochloric acid, add 10 mL of water, and dissolve by warming. Add 1 drop of phenolphthalein TS, add ammonia TS until the solution becomes slightly red, and transfer it into a Nessler tube. Wash the crucible with water, and add the washings to the Nessler tube. Then add 2 mL of diluted acetic acid (1 in 20) and water to make 50 mL.

Control Solution Place 10 mL of a solution of magnesium nitrate hexahydrate in ethanol (95) (1 in 10) into a crucible of the same quality as that used for the sample, ignite, and burn the ethanol. Cool, add 1 mL of sulfuric acid, proceed as directed in the preparation of the test solution, and transfer the resulting solution into another Nessler tube. Wash the crucible with water, and add the washings to the Nessler tube. Then add the specified amount of Lead Standard Solution (for heavy metals limit test), 2 mL of diluted acetic acid (1 in 20), and water to make 50 mL.

If the test solution is not clear, filter both the test solution and the control solution under the same conditions.

Procedure Unless otherwise specified, add 2 drops of sodium sulfide TS to each of the test solution and the control solution, mix thoroughly, and allow them to stand for 5 minutes. Next, examine the tubes from above and from the side against a white background to compare the colors of the two solutions. The color of the test solution is not darker than that of the control solution.

Inductively Coupled Plasma-Atomic Emission Spectrometry

Inductively coupled plasma-atomic emission spectrometry is designed to quantify and qualify an analyte element in a sample by determining the intensity and the wavelength of the atomic emission spectral line that is obtained by nebulizing and exciting the element in inductively coupled plasma (ICP).

Apparatus Normally, the apparatus consists of an excitation source, a sample introduction system, an emission system, a spectrophotometric system, a data processing system, and a control system. The excitation source is composed of an electric power source circuit and a control circuit to supply and control the electric energy to maintain the spectroscopic system. The sample injection port, which is used to introduce the sample into the emission system, is composed of a nebulizer, a spray chamber, and a drain trap. The emission system, which is a unit that excites and emits the target element in the test solution, is composed of a torch and an induction coil. The torch usually consists of three concentric tubes, in which the test solution is

introduced from the innermost tube. Argon is used as the gas to form plasma. There are two ways to observe the light emitted: one is radial viewing, which observes the emission from the side of the plasma, and the other is axial viewing, which observes the emission from the central channel of the plasma. The spectrophotometric system is composed of a light-converging device to efficiently induce the emitted light into the spectroscopic system, a spectroscope to separate spectra, and a detector. There are two types of spectroscopes: monochrometer (a wavelength scanning type) and polychrometer (a fixed-wavelength type). If spectral lines in the vacuum UV region not more than 190 nm are measured, the interior of the spectroscope should be evacuated or the inside air should be replaced by argon or nitrogen. The data processing system, which processes detected signals, displays measurement results and calibration curves. The control system controls the gas flow rate, torch photometric position, and electric power of the excitation source to use the apparatus in the optimal conditions.

Procedure Confirm that all live parts are in proper conditions. Switch on the excitation source and the cooling system. When a vacuum-type spectroscope is used to measure the emission line in the vacuum-ultraviolet region, purge the air in the light-path between the emission system and the spectroscope with argon or nitrogen gas. Control the flow rate of argon or nitrogen gas to the specified rate and switch on the high frequency power to generate the plasma. Correct the wavelength of the spectroscope as directed for the apparatus used. Introduce the specified amount of the test solution prepared as specified in the individual monograph, and measure the intensity of an appropriate emission line of the analyte element. Follow the same procedure for the standard solutions and the control solution prepared as specified.

Usually, determination is done using one of the following methods. In the determination, the interference and background should be considered.

(1) Calibration Curve Method Prepare at least three standard solutions with different concentrations of the element to be determined, measure the emission intensities of these standard solutions, and prepare a calibration curve from the values obtained. Next, measure the emission intensity of the test solution, adjusted to a concentration within the concentration range that is measurable, and determine the amount (concentration) of the element from the calibration curve.

(2) Standard Addition Method To equal volumes of at least three test solutions, add suitable quantities of a standard solution containing the reference standard of the element to be determined so as to prepare solutions containing stepwise increasing amounts of the element, and then add a solvent to make a constant volume. Measure the emission intensity of each solution, and plot the values obtained on a graph, with the added amounts (concentration) of the reference standards on the abscissa and the

emission intensity on the ordinate. Extrapolate the regression line formed by joining the points on the graph, and determine the amount (concentration) of the element in the test solution from the distance between the origin and the intersection point of the regression line and the abscissa. This method is applicable only when the calibration curve drawn as directed in method (1) above is a straight line passing through the origin.

(3) Internal Standard Method Prepare several standard solutions containing a constant amount of the internal standard element specified in the individual monograph and known, graded amounts of the reference standard of the element to be determined. For these solutions, measure the emission intensity of the reference standard and internal standard element at the analytical wavelength of each element under the same measuring conditions, and obtain the ratio of the emission intensity of the reference standard to that of the internal standard element for each solution. Prepare a calibration curve by plotting the values obtained on a graph, with the amount (concentration) of the reference standard on the abscissa and the emission intensity ratio on the ordinate. Next, prepare a test solution containing the same amount of internal standard element as specified for the standard solutions. Proceed under the same conditions as for the preparation of the calibration curve, obtain the emission intensity ratio of the element to be determined to the internal standard element, and determine the amount (concentration) of the element in the test solution from the calibration curve.

Note: Avoid the use of reagents, test solutions, and gases that may interfere with the determination.

Infrared Spectrophotometry

Infrared spectrophotometry is designed to identify a substance from the absorption spectrum obtained when a sample is subjected to infrared radiation. An infrared spectrum is generally represented as a graph plotted with the wavenumber (cm^{-1}) on the abscissa and the transmittance (%) or absorbance on the ordinate.

Section C. 11 of JSFA-IX contains the Infrared Reference Spectra (in the range between 4000 cm^{-1} and 600 cm^{-1}) of substances for which the individual monographs specify identification tests by infrared spectrophotometry. These substances exclude those for which identification by absorption wavenumbers is specified.

Apparatus and Adjustment Use a dispersive infrared spectrophotometer or a Fourier-transform infrared spectrophotometer. Adjust the spectrophotometer as indicated for

the instrument, and confirm that the resolution, transmittance reproducibility, and wavenumber reproducibility comply with the test given below. When the spectrum is measured on a 0.04-mm thick polystyrene film, the transmittance difference of the obtained spectrum should be 18% or more between the minimum absorption at about 2870 cm^{-1} and the maximum absorption at about 2850 cm^{-1}. Also, it should be 12% or more between the minimum absorption at about 1589 cm^{-1} and the maximum absorption at about 1583 cm^{-1}.

The wavenumber scale is usually calibrated using some of the characteristic absorption wavenumbers (cm^{-1}) of a polystyrene film, shown below. The numbers in parentheses indicate the acceptable range of these values.

3060.0 (± 1.5)　2849.5 (± 1.5)　1942.9 (± 1.5)　1601.2 (± 1.0)
1583.0 (± 1.0)　1154.5 (± 1.0)　1028.3 (± 1.0)

When a dispersive infrared spectrophotometer is used, the acceptable range is ± 2.0 for 1601.2 cm^{-1} and ± 2.0 for 1028.3 cm^{-1}.

The transmittance reproducibility and wavenumber reproducibility should satisfy the following requirements when the absorption of a polystyrene film is measured twice at several wavenumbers in the range of 3000–1000 cm^{-1}: The difference in transmittance is within 0.5%, and the wavenumber difference is within 5 cm^{-1} at about 3000 cm^{-1} and within 1 cm^{-1} at about 1000 cm^{-1}.

Preparation of Samples and Measurement Unless otherwise specified, when individual monographs require the test to be conducted using a dried sample, dry the sample before use, according to the conditions specified for the loss on drying test in the corresponding monograph. Prepare a sample for measurement according to the most appropriate method among those given below so that the transmittance of the strongest absorption band (excluding absorption bands derived from liquid paraffin used in the paste method) of the sample will fall within a range of 5–10%. Potassium bromide or sodium chloride is usually used for optical plates. Generally, for double-beam instruments, the reference cell or material is placed in the reference beam and the spectrum is measured at the same time as the specimen. For single-beam instruments, the reference cell or material is placed in the same optical path as the specimen and the spectrum is measured separately from the specimen under the same operation conditions. The reference to be used depends on the sample preparation methods. The background absorption of the atmosphere may be utilized.

Unless otherwise specified in the individual monographs, the spectrum is usually determined between 4000–600 cm^{-1}. The spectrum should be scanned under the same operating conditions as those set to confirm the accuracy of resolution, wavenumber scale, and wavenumbers.

(1) Disk Method Powder 1 to 2 mg of a solid sample with an agate mortar, add 0.10–

0.20 g of potassium bromide for infrared spectrophotometry as the diluting agent, rapidly triturate the mixture while being careful to prevent moisture absorption, and transfer into a die (disk forming container). Press it at 50 to 100 kN (5000–10,000 kg)/cm^2 under reduced pressure of not more than 0.67 kPa for 5 to 8 minutes to make a transparent disk. Measure the spectrum against a disk prepared only with the diluting agent in the same manner as for the sample disk.

(2) Solution Method Place the sample solution prepared by the method directed in the individual monographs in a fixed cell for liquid, and measure the spectrum against the reference. Normally, the solvent used for preparing the sample solution is used as the reference. The solvent used in this method should be one that does not interact or chemically react with the sample to be examined and that does not damage the optical plate. The thickness of the fixed cell is generally 0.1 mm or 0.5 mm.

(3) Paste Method Powder 5–10 mg of a solid sample with an agate mortar, and unless otherwise specified, triturate the sample together with 1–2 drops of liquid paraffin to produce a paste. Spread the paste on the center of an optical plate, place another optical plate on top of the paste, making sure no air is trapped under the plate, and measure the spectrum against the optical plate.

(4) Liquid Film Method Hold 1 to 2 drops of a liquid sample between two optical plates, and examine the liquid layer between the plates. If a thicker liquid layer is needed, place aluminum foil or a similar material between the two optical plates to produce a thicker space for the sample between the plates. Usually, measurement is carried out against the optical plate.

(5) Thin Film Method Use a thin film as is or use a thin film sample prepared as directed in the individual monographs. Usually, measurement is carried out against the optical plate.

(6) Gas Sample Measurement Put a sample gas in a previously evacuated gas cell with a light path of 5 to 10 cm in length under pressure specified in the individual monograph, and measure its spectrum against the gas cell under reduced pressure (vacuumed). A long cell with a light path not shorter than 1 m can also be used if necessary.

Identification

When comparing the spectrum of a sample with the spectrum of the Reference Standard of the substance to be identified or the Reference Spectrum, which is given in section C.11 of JSFA-IX, if both spectra exhibit almost the same intensities of

absorption at the same wavenumbers, the sample is confirmed as identical to the substance. When the spectrum of the sample measured in the solid-state is different from the Reference Standard spectrum or the Reference Spectrum, measurement should be performed again, using the sample and the Reference Standard, both of them are treated according to the conditions specified in the individual monograph.

When comparing the sample spectrum with the Reference Spectrum, any difference in resolution between the instruments used should be taken into consideration because these two spectra are usually measured by different instruments. The wavenumber variation based on the difference in resolution between two instruments is the greatest in the wavenumber range between 4000 cm^{-1} and 2000 cm^{-1}. When Fourier-transform infrared spectrophotometers are used, the wavenumber accuracy is invariable through the scanning range because their resolution is constant, regardless of the wavenumber.

Ion Chromatography

Ion chromatography is a method to separate and analyze components of a mixture by virtue of differences in ion-exchange capability. The mixture to be analyzed is injected into a column packed with a stationary phase, such as ion exchanger, and then separated with the aid of an eluent as the mobile phase.

This method is applicable to liquids or substances that can be made into solutions. The method is usually applied to identification tests, purity tests, and assays.

Apparatus The apparatus consists generally of a pumping system to deliver the mobile phase, a sample injection port, a column, a detector, and a data processor. The column is maintained at a constant temperature, using a column chamber or other appropriate equipment. The pumping system serves to deliver the mobile phase into the column, connecting tube, and other devices at a constant flow rate. The sample injection port is used to introduce a constant quantity of the sample into the column with high reproducibility.

The detector detects components with properties different from those of the mobile phase. It generally produces electric signals proportional to the concentration of substances present in amounts of a few micrograms or less. Electric conductometers or UV spectrophotometers are commonly used as detectors.

When an electrical conductometer is used as the detector, a suppressor can be placed in front of the conductometer to reduce electric conductivity, which may cause background noise without failing to detect the ionic components to be determined. When a suppressor is used, the following basic solutions are generally used as eluents: potassium hydroxide, carbonate buffers, and borate buffers. The data processor can record or output various data including chromatograms, retention times, and

quantitative amounts of the components to be determined.

Procedure Fix the apparatus in advance, adjust the mobile phase, the column, the detector, and the flow rate of the mobile phase to the operating conditions specified in the individual monograph, and equilibrate the column at a specified temperature. Inject the specified amount of the test solution prepared as specified in the individual monograph into the sample injection port. The separated components are detected by the detector, and the detector output is recorded as a chromatogram on the data processor. Follow the same procedure for the standard solutions and control solution prepared as specified in the individual monograph.

The components are identified by confirming that the retention time (the time taken until the top of the sample peak appears after the sample is injected) of each component corresponds to that of the standard sample, or that the retention time does not change nor does the peak width widen when the standard sample is added.

Determination is usually performed by method (1) or (2) using the peak area or peak height.

(1) Internal Standard Method Prepare several standard solutions containing a constant amount of the internal standard specified in the individual monograph and known, graded amounts of the reference standard of the compound to be determined. From each of the chromatograms obtained by injecting a constant volume of each standard solution, calculate the ratio of the peak area (or peak height) of the reference standard of the compound to be determined to that of the internal standard. Prepare a calibration curve by plotting the values obtained on a graph, with the peak area (or peak height) ratio on the ordinate and the added amounts of the reference standard or the ratio of the amount of the reference standard to that of the internal standard on the abscissa. The calibration curve is usually a straight line through the origin. Next, prepare a test solution containing the same amount of the internal standard as specified in the individual monograph, record the chromatogram under the same conditions as for the preparation of the calibration curve, calculate the peak area (or peak height) ratio of the compound to be determined to the internal standard, and determine the concentration of the compound in the test solution from the calibration curve.

(2) Absolute Calibration Curve Method Prepare several standard solutions containing graded amounts of the reference standard of the compound to be determined, and inject a constant volume of each standard solution reproducibly. Using the chromatograms obtained, prepare a calibration curve by plotting the values obtained on a graph, with the peak areas (or peak heights) of the reference standard on the ordinate and the amounts of the reference standard on the abscissa. The calibration

curve is usually a straight line through the origin. Next, prepare the test solution as specified in the individual monograph, record the chromatogram under the same conditions as for the preparation of the calibration curve, and measure the peak area (or peak height) of the compound to be determined. Determine the concentration of the compound in the test solution from the calibration curve. The whole procedure from injection to measurement must be carried out under strictly constant conditions.

Use purified water for ion chromatography to prepare the solutions. Unless there is a particular problem, when an anion standard solution is prepared, use its sodium or potassium salt, and when a cation standard solution is prepared, use its chloride or nitrate.

For either method described above, the peak area or peak height is usually measured using method (i) or (ii), whichever is appropriate.

(i) Method using the peak area Use either of the two methods.

Width at half-height method Multiply the peak width at half-height by the peak height.

Automatic integration method Measure the signal from the detector, and determine the peak area using a data processor.

(ii) Method using the peak height Use either of the two methods.

Peak height method Measure the distance between the peak maximum and the intersection of a perpendicular line drawn from the peak maximum to the horizontal axis of the recording paper and a line linking both side inflection points of the lower end of the peak.

Automatic peak height method Measure the signal from the detector, and then determine the peak height using a data processor.

Iron Limit Test

The iron limit test is designed to demonstrate that the content of iron in an additive does not exceed the acceptable limit specified in the individual monograph.

In the Monographs, the specification "not more than 10 μg/g as Fe (1.0 g, Method 1, Control Solution: Iron Standard Solution 1.0 mL)" for this test, for example, means that the iron content of the substance must be not more than 10 μg/g as Fe when determined according to the following manner: *The test solution and the control solution are prepared with 1.0 g of the test substance and 1.0 mL of Iron Standard Solution, respectively, as directed in Method 1, and then the test is performed as directed in the procedure below.*

Preparation of Test Solutions and Control Solutions Unless otherwise specified, proceed according to Method 1 or Method 2, whichever is appropriate.

Method 1

Test Solution Weigh the amount of sample specified in the individual monograph, add 30 mL of acetic acid-sodium acetate buffer solution for iron limit test (pH 4.5), and dissolve by warming, if necessary.

Control Solution To the amount of Standard Iron Solution specified in the individual monograph, add 30 mL of acetic acid-sodium acetate buffer solution for iron limit test (pH 4.5).

Method 2

Test Solution Weigh the amount of sample specified in the individual monograph, add 10 mL of diluted hydrochloric acid (1 in 4), and dissolve by warming if necessary. Dissolve 0.5 g of L(+)-tartaric acid, and add one drop of phenolphthalein TS. Add ammonia TS until the solution develops a pale red color. Add 20 mL of acetic acid-sodium acetate buffer solution for iron limit test (pH 4.5).

Control Solution To the amount of Standard Iron Solution specified in the individual monograph, add 10 mL of diluted hydrochloric acid (1 in 4), and proceed as directed for the test solution.

Procedure Unless otherwise specified, transfer the test solution and the control solution into separate Nessler tubes, add 2 mL of a solution of L(+)-ascorbic acid (1 in 100) to each tube, mix well, and allow them to stand for 30 minutes. Add 1 mL of a solution (1 in 200) of 2,2′-bipyridyl in ethanol (95), add water to make 50 mL, and allow them to stand for 30 minutes. Finally, compare the colors of both solutions against a white background. The color of the test solution is not deeper than the control solution.

Lead Limit Test
(Atomic Absorption Spectrophotometry)

The lead limit test is designed to demonstrate that the content of lead in an additive does not exceed the acceptable limit specified in the individual monograph.

In the Monographs, for this test, the specification "not more than 2 μg/g as Pb (2.0 g, Method 1, Control Solution: Lead Standard Solution 4.0 mL, Flame Method)," for example, means that the lead content of the substance must be not more than 2 μg/g as Pb when determined according to the following manner: *The test solution and control solution are prepared with 2.0 g of the test substance and 4.0 mL of Lead Standard Solution, respectively, as directed in Method 1, and then the test is performed by flame atomic absorption spectrophotometry.*

Preparation of Test Solutions and Control Solutions Unless otherwise specified, proceed as directed below.

Method 1

Test Solution Weigh the amount of sample specified in the individual monograph, transfer it into a platinum, quartz, or porcelain crucible or a quartz beaker, and moisten with a small amount of diluted sulfuric acid (1 in 4). Heat it by gradually increasing the temperature until the sample is almost charred and white fumes of sulfuric acid are no longer evolved. If necessary, add diluted sulfuric acid (1 in 4) again, and heat until the sample is almost charred. For a liquid sample or a sample that is hard to char, concentrated sulfuric acid may be used, instead of diluted sulfuric acid (1 in 4). If the sample is an aqueous solution, gentle heating and evaporation may be done before sulfuric acid is added to char the sample. After it is charred, cover the container loosely with a lid if necessary, heat in an electric furnace by gradually increasing the temperature, and ignite at 450–600° C until the sample is incinerated. If a charred mass remains, crush with a glass rod if necessary, then moisten with 1 mL of diluted sulfuric acid (1 in 4) and 1 mL of nitric acid, heat until white fumes of sulfuric acid are no longer evolved and ignite in the electric furnace to incinerate the residue. To the residue, add 10 mL of diluted hydrochloric acid (1 in 4), heat on a water bath, and evaporate to dryness. Then add a small amount of diluted nitric acid (1 in 100) to the residue, and dissolve it by warming. After cooling, add diluted nitric acid (1 in 100) to make exactly 10 mL.

When incineration is done at 500° C or below, a heat-resistant glass beaker can be used.

Control Solution To the specified amount of Lead Standard Solution, exactly measured, add diluted nitric acid (1 in 100) to make exactly 10 mL.

Method 2

Test Solution Weigh the amount of sample specified in the individual monograph, and transfer it into a platinum, quartz, or porcelain crucible or a quartz beaker. Heat gradually, and stop heating before it starts to char, add 1 mL of sulfuric acid, heat by gradually increasing the temperature until the sample is charred and white fumes of sulfuric acid are no longer evolved. If necessary, add sulfuric acid again, and heat until the sample is almost charred. Cover the container loosely with a lid if necessary, heat in an electric furnace by gradually increasing the temperature, and ignite at 450–600° C to incinerate the sample. If a charred mass remains, crush it with a glass rod if necessary, moisten with 1 mL of diluted sulfuric acid (1 in 4) and 1 mL of nitric acid, heat until white fumes of sulfuric acid are no longer evolved, and ignite in the electric furnace to completely incinerate the sample. To the residue, add 10 mL of diluted hydrochloric acid (1 in 4), heat on a water bath, and evaporate to dryness. Then add a small amount of diluted nitric acid (1 in 100) to the residue, and dissolve it by warming. Add diluted nitric acid (1 in 100) again to make exactly 10 mL.

When incineration is done at 500° C or below, a heat-resistant glass beaker can be used.

Control Solution To the specified amount of Lead Standard Solution, exactly measured, add diluted nitric acid (1 in 100) to make exactly 10 mL.

Method 3

Sample Solution Weigh the amount of sample specified in the individual monograph, transfer it into a platinum, quartz, or porcelain crucible or a quartz beaker, and moisten with a small amount of diluted sulfuric acid (1 in 4) or sulfuric acid. Heat it by gradually increasing the temperature until the sample is almost charred and white fumes of sulfuric acid are no longer evolved. If necessary, add diluted sulfuric acid (1 in 4) again, and repeat this procedure. When the sample is hydrophobic or hard to char, it may be gradually heated until it melts, and after cooling, diluted sulfuric acid (1 in 4) or sulfuric acid may be added to then char it. Cover the container with a lid, heat it in an electric furnace by gradually increasing the temperature, and ignite at 450–600° C to incinerate the sample. If a charred mass remains, crush it with a glass rod if necessary, moisten with 1 mL of diluted sulfuric acid (1 in 4) and 1 mL of nitric acid, heat until white fumes of sulfuric acid are no longer evolved, and ignite in the electric furnace to incinerate the residue. Add 10 mL of diluted hydrochloric acid (1 in 4) to the residue, heat on a water bath, and evaporate to dryness. To the residue, add 20 mL of diluted hydrochloric acid, cover the container with a watch dish, and dissolve it by warming. Use this solution as the sample solution. If the residue does not dissolve, use a solution prepared by boiling for 5 minutes with the container covered with a watch dish and cooling.

Test Solution Add 10 mL of diammonium hydrogen citrate solution (1 in 2) to the sample solution. Add 1mL of thymol blue TS as the indicator, and then add ammonia solution until the color of the solution changes from yellow to light yellow-green. If the color change is not clear, adjust the pH to 8–9 using pH test paper or pH meter. Transfer the resulting solution into a separating funnel or centrifuge tube, and wash the container with a small amount of water or warm water into the funnel or tube, whichever is appropriate. If a precipitate is produced, add water again to make about 100 mL. Add 5 mL of ammonium pyrrolidine dithiocarbamate solution (3 in 100), allow to stand for 5 minutes, and add exactly 10 mL of butyl acetate. Shake it well for 5 minutes, and allow to stand or centrifuge. Collect the butyl acetate layer to use as the test solution.

Control Solution Measure exactly the specified amount of Lead Standard Solution, and proceed as directed for the test solution.

Method 4

Sample Solution Weigh the amount of sample specified in the individual monograph, transfer it into a Kjeldahl flask, heat-resistant glass beaker, or conical beaker, add 10 mL of nitric acid and 5 mL of sulfuric acid, and heat until red-brown fumes are no longer evolved. After cooling add 2 mL of nitric acid, and heat until the solution become transparent and heavy white fumes are no longer evolved. If the

contents become blackened while heating, add 2 mL of nitric acid and keep heating. After cooling, add 10 mL of diluted hydrochloric acid (1 in 4), cover the container with a watch dish, and heat until the precipitate dissolves. If necessary, add diluted hydrochloric acid (1 in 4) more, and cool.

Test Solution Add 10 mL of diammonium hydrogen citrate to the sample solution. Add 1mL of thymol blue TS as the indicator, and then add ammonia solution until the color of the solution changes from yellow to green. If the color change is not clear, adjust the pH to 8–9 using pH test paper or a pH meter. Transfer the resulting solution into a separating funnel or centrifuge tube, and wash the container with a small amount of water or warm water into the funnel or tube whichever is appropriate. If a precipitate is produced, add water again to make about 100 mL. Add 5 mL of ammonium pyrrolidine dithiocarbamate solution (3 in 100), allow to stand for 5 minutes, and add exactly 10 mL of butyl acetate. Shake well for 5 minutes, and allow to stand or centrifuge. Collect the butyl acetate layer to use as the test solution.

To prepare the sample solution, an automated wet incinerator can be used.

Control Solution Measure exactly the specified amount of Lead Standard Solution, and proceed as directed for the test solution.

Method 5

Test Solution First prepare a sample solution as specified in the individual monograph. To the sample solution, add 10 mL of a solution of diammonium hydrogen citrate (1 in 2). Add 1mL of thymol blue TS as the indicator, and add ammonia solution until the color of the solution changes from yellow to light yellow-green. If the color change is not clear, adjust the pH to 8–9 using pH test paper or pH meter. Transfer the resulting solution into a separating funnel or centrifuge tube, and wash the crucible (beaker) with a small amount of water into the funnel or tube. Add 5 mL of ammonium pyrrolidine dithiocarbamate solution (3 in 100), allow to stand for 5 minutes, and add exactly 10 mL of butyl acetate. Shake well for 5 minutes, and allow to stand or centrifuge. Collect the butyl acetate layer to use as the test solution.

Control Solution Measure exactly the specified amount of Lead Standard Solution, and proceed as directed for the test solution.

Procedure Unless otherwise specified, conduct the tests as directed below.

Flame Method

Measure the absorbance of the test solution and the control solution as directed under Flame Atomic Absorption Spectrophotometry using the conditions given below. The absorbance of the test solution does not exceed that of the control solution.

Operating Conditions

Light source: Lead hollow cathode lamp.

Analytical line wavelength: 283.3 nm.

Supporting gas: Air.

Inflammable gas: Acetylene.

Electrothermal Method

Perform the test as directed under the Standard Addition Method in Atomic Absorption Spectrophotometry (Electrothermal Atomic Absorption Spectrophotometry) using the operating conditions given below. The standard solution is prepared by measuring exactly a suitable volume of Standard Lead Solution and adding diluted nitric acid (1 in 100). Before injection, add the same volume of palladium nitrate TS to the solutions under test, and mix well. Perform a blank test with diluted nitric acid solution (1 in 100), and make any necessary correction.

Operating Conditions

Light source: Lead hollow cathode lamp.

Analytical line wavelength: 283.3 nm.

Temperature for drying: 110° C.

Temperature for incineration: 600° C.

Temperature for atomizing: 2100° C.

Liquid Chromatography

Liquid chromatography is a method to separate and analyze components of a mixture by virtue of differences in retention capacity against the stationary phase. The mixture to be analyzed is injected into a column packed with a stationary phase, and then separated with the aid of a liquid mobile phase passed through the column. This method is applicable to liquids or substances that can be made into solutions, and applied to many types of tests, including identification tests, purity tests, and assays.

Apparatus The apparatus consists generally of a pumping system to deliver the mobile phase, a sample injection port, a column, a detector, and a data processor. A mobile phase composition controller, a column oven, a pumping system for reaction reagents, and chemical reaction chamber are also used if necessary. The pumping system serves to deliver the mobile phase and reaction reagents to the column, connecting tube, and other devices at a constant flow rate. The sample injection port is used to introduce a constant quantity of the sample into the column with high reproducibility. The column is a tube with smooth interior surface, made of an inert material like metal, which is uniformly packed with a uniformly-sized material for liquid chromatography.

The detector detects components with different properties from the mobile phase, and produces signals proportional to the concentration of the substances in amounts of a few micrograms or less. Ultraviolet or visible spectrophotometers, differential refractometers, fluorescence spectrophotometers, photodiode array detectors, or mass

spectrometers are commonly used as detectors. The data processor can record or output various data including chromatograms, retention times, and quantitative amounts of the components to be determined. The mobile phase composition controller is used to vary the ratio of the mobile phase composition and there are two types of controllers: stepwise controllers and gradient controllers.

Procedure Fix the apparatus in advance, adjust the mobile phase, the column, the detector, and the flow rate of the mobile phase to the specified operating conditions as directed in the individual monograph, and equilibrate the column at the specified temperature. Inject the specified amount of the test solution prepared as directed in the individual monograph into the sample injection port. The separated components are detected by the detector and the detector output is recorded as a chromatogram on the data processor. If the components to be determined do not have physical properties, including absorption and fluorescence, that are suitable enough to be detected by the detector, convert them into suitable derivatives. For derivatization, either pre- or post-column method is used. Follow the same procedure for the standard solutions and control solution.

Identification of the substances is carried out by confirming that the retention time corresponds to that of the standard sample, or that the retention time does not change nor does the peak width widen when the standard sample is added. Determination is generally performed according to either of the methods below using the peak area or peak height.

(1) Internal Standard Method Prepare several standard solutions containing a constant amount of the internal standard specified in the individual monograph and known, graded amounts of the reference standard of the compound to be determined. From each of the chromatograms obtained by injecting a constant volume of each standard solution, calculate the ratio of the peak area (or peak height) of the reference standard to that of the internal standard. Prepare a calibration curve by plotting the values obtained on a graph, with the peak area (peak height) ratio on the ordinate and the added amounts of the reference standard or the ratio of the amount of the reference standard to that of the internal standard on the abscissa. The calibration curve is usually a straight line through the origin. Next, prepare a test solution containing the same amount of the internal standard as specified in the individual monograph, record the chromatogram under the same conditions as for the preparation of the calibration curve, calculate the peak area (peak height) ratio of the compound to be determined to the internal standard, and determine the concentration of the compound in the test solution from the calibration curve. In individual monographs, generally a standard solution with a concentration within the linear range of the calibration curve and a test solution with a concentration close to that of the standard solution are prepared, and

specified volumes of these solutions are chromatographed under the fixed conditions to determine the amount of the compound.

(2) Absolute Calibration Curve Method Prepare standard solutions containing graded amounts of the reference standard of the compound to be determined. Inject a constant volume of each standard solution reproducibly. Using the chromatograms obtained, prepare a calibration curve by plotting the values obtained on a graph, with the peak areas (or peak height) of the reference standard on the ordinate and the amounts of reference standard on the abscissa. The calibration curve is usually a straight line through the origin. Next, prepare a test solution as specified in the individual monograph, record the chromatogram under the same conditions as for the preparation of the calibration curve, measure the peak area (or peak height) of the component to be determined, and determine the concentration of the compound in the test solution from the calibration curve. The whole procedure from injection to measurement must be carried out under strictly constant conditions.

For either of the methods described above, the peak height or peak area is generally measured using method (i) or (ii), whichever is appropriate.

(i) Method using the peak area Use either of the following methods.

Width at half-height method Multiply the peak width at half-height by the peak height.

Automatic integration method Measure the signal from the detector and, then determine the peak area using a data processor.

(ii) Method using the peak height Use either of the following two methods.

Peak height method Measure the distance between the peak maximum and the intersection of a perpendicular line drawn from the peak maximum to the horizontal axis of the recording paper and a line linking both side inflection points of the lower end of the peak.

Automatic peak height method Determine the signal from the detector as the peak height using a data processor.

System Suitability System suitability tests are intended to verify that the chromatographic system used has suitable performance to carry out the analysis of food additives. No analysis carried out using the system is acceptable unless the requirements of system suitability have been met.

System suitability is basically evaluated by system performance and system reproducibility. For purity tests, confirmation of detectability is required in addition to the above two requirements.

(1) Confirmation of detectability This test is to confirm that the peaks of target impurities are distinctly detected at concentration levels equal to their specification limits, thereby verifying that the system to be used has adequate performance to

achieve its intended purpose.

(2) System performance This test is to confirm that the specificity of a component to be determined is ensured, thereby verifying that the system to be used has adequate performance to achieve its intended purpose.

In assay, the system performance is basically defined by the resolution of the component to be determine and the substance for the confirmation of resolution (a closely eluting substance is preferable), and when necessary, by the order of elution of them. In purity tests, the system performance is defined by both the resolution and the order of elution of the component to be determined and the substance for the confirmation of resolution. If necessary, the symmetry factor is also specified. If there is no suitable substance to be resolved, it is acceptable to specify the number of theoretical plates and the symmetry factor of the component to be determined.

(3) System reproducibility This test is to confirm that in repeated injections of a standard solution or solution for system suitability tests, the degree of variation (precision) of the responses of the component to be determined is at a level that meets the purpose of the analysis to be done, thereby verifying that the system used has adequate performance to achieve its intended purpose.

The acceptable limit of system reproducibility is normally defined as the relative standard deviation (RSD) of the responses of the component to be determined in repeated injections. It is acceptable to confirm the reproducibility of the system not only by repeated injections of the standard solution before sample injections, but also by divided injections of the standard solution before and after sample injections or by interspersed injections of the standard solution between sample injections.

In principle, the maximum number of repeated injections is six. However, in the case that one analysis requires a long time (for example, an analysis using a gradient method or an analysis of samples containing late eluting components), it is acceptable to decrease the number of injections by strictly setting the acceptable limit of variation to be achieved to guarantee a level of system reproducibility equivalent to that achieved with six injections.

The acceptable limit of system reproducibility should be set at an appropriate level based on the data used when suitability of the testing method of the target additive was verified and the precision required for the test.

The operating conditions specified in the individual monograph may be changed in terms of the following requirements, within a range in which the system suitability is met: the internal diameter and length of the column, the particle size of the packing material, the column temperature, the composition ratio of the mobile phase, the composition of buffer solutions in the mobile phase, the pH of the mobile phase, the concentration of ion-pair forming agents in the mobile phase, the salt concentration of the mobile phase, the flow rate of the mobile phase, the number and timing of change, gradient program and its flow rate, the composition and flow rate of derivatizing

reagents, reaction time, and the temperature of the chemical reaction chamber.

Note: Avoid the use of reagents and test solutions containing substances that may interfere with the determination.

Terminology

(1) SN ratio: It is defined by the following formula.

$$S/N = \frac{2H}{h}$$

H = peak height of the target substance from the baseline (the medial value of background noise),

h = width of background noise of the chromatogram of the sample solution or solvent blank before and after the peak of the target substance.

The baseline and background noise are measured in a range equivalent to 20 times the peak width at the center point of peak height of the target substance. When a solvent blank is used, it should be measured in almost the same range around the point where the target substance elutes.

(2) Symmetry factor: It indicates the degree of symmetry of a peak in the chromatogram and is defined as S in the following formula.

$$S = \frac{W_{0.05h}}{2f}$$

$W_{0.05h}$ = width of the peak at one-twentieth of the peak height from the baseline,

f = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height from the baseline,

where $W_{0.05h}$ and f are expressed in the same unit.

(3) Relative standard deviation: Generally, it is defined as RSD (%) in the following formula.

$$RSD\ (\%) = \frac{100}{\bar{X}} \times \sqrt{\frac{\sum_{i=1}^{n} (xi - \bar{X})^2}{n - 1}}$$

xi = measured value,

$\bar{X}$ = mean of measured value,

n = number of replicate measurements.

(4) Complete separation of peak: It means that the resolution between two peaks is not less than 1.5. It is also called as baseline separation.

(5) Peak-valley ratio: It indicates the degree of separation between two peaks in a

chromatogram when baseline separation cannot be attained. It is defined as p/v in the following formula.

$$p/_v = \frac{H_p}{H_v}$$

H_p = peak height from the baseline of the minor peak,

H_v = height from the baseline at the lowest point (peak valley) of the curve separating the major and minor peaks.

(6) Separation factor: It shows the relation between the retention times of peaks in a chromatogram and is defined as α in the following formula. The separation factor α indicates thermodynamic difference in partition of two compounds. It is basically the ratio of their partition equilibrium coefficients or of their mass distribution. It is obtained from the chromatogram as the ratio of retention times of the two compounds.

$$\alpha = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

t_{R1}, t_{R2} = retention times of two compounds used for the resolution measurement ($t_{R1} < t_{R2}$),

t_0 = time of passage of the mobile phase through the column (time measured from the time of injection of a compound with k = 0 to the time of elution at the peak maximum).

(7) Resolution: It shows the relation between the retention time and the peak width of peaks in a chromatogram and is defined as R_S in the following formula.

$$R_S = 1.18 \times \frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}}$$

t_{R1}, t_{R2} = retention times of two compounds used for the resolution measurement ($t_{R1} < t_{R2}$),

$W_{0.5h1}$, $W_{0.5h2}$ = peak widths at half peak height,

where t_{R1}, t_{R2}, $W_{0.5h1}$, $W_{0.5h2}$ are expressed in the same unit.

(8) Number of theoretical plates: It indicates the extent of band broadening of a compound in the column and generally defined as N in the following formula.

$$N = 5.54 \times \frac{{t_R}^2}{{W_{0.5h}}^2}$$

t_R = retention time of the compound,

$W_{0.5h}$ = width of the peak at half peak height,

where t_R, $W_{0.5h}$ are expressed in the same unit.

Loss on Drying

This test is designed to measure the amount of water and volatile matter lost from a sample when it is dried under the conditions specified in the individual monograph.

In the Monographs, the specification "not more than 0.5% (105° C, 3 hours)," for example, for this test, means that the loss in weight must be not more than 0.5% of the weight of the sample when determined according to the following manner: *1 to 2 g of the sample is accurately weighed, and dried at 105°C for 3 hours*. Also the specification "not more than 5.0% (reduced pressure, 24 hours)," for example, means that the loss in weight must be not more than 5.0% of the weight of the sample when determined according to the following manner: *1 to 2 g of the sample is accurately weighed, and dried in a desiccator with silica gel as desiccant under a reduced pressure of 2.0 kPa or less for 24 hours.*

Procedure Dry a weighing bottle for 30 minutes or more under the specified conditions, allow to cool in a desiccator if heated, and weigh accurately. If the sample consists of large crystals or lumps, promptly ground it into particles not larger than about 2 mm in diameter. Unless otherwise specified, place 1 to 2 g of the prepared sample into the weighing bottle, spread to a layer of not more than 5 mm thick, and weigh the bottle accurately. Place the bottle in a drying oven if the temperature is specified or in a desiccator if no temperature is specified, remove the stopper (placing it nearby), and dry under the specified conditions. Stopper the bottle, take the bottle out of the oven or the desiccator, and weigh again. If heated, unless otherwise specified, allow to cool in a desiccator, and weigh accurately. If the sample melts at a temperature lower than the specified drying temperature, dry the bottle with the sample at a temperature 5–10° C lower than the melting temperature for 1 to 2 hours, and then dry under the specified conditions.

Loss on Ignition

This test is designed to measure the amount of moisture and other impurities lost when a sample is ignited under the conditions specified in the individual monograph.

In the Monographs, the specification "18.0–24.0%," for example, for this test, means that the loss in weight must be 18.0–24.0% of the weight of the sample when determined by igniting 1 to 2 g of the sample, accurately weighed, at 450–550°C for 3 hours. Also, the specification "not more than 10% (0.5 g, 1000° C, 30 minutes)," for example, means that the loss in weight must be not more than 10% of the weight of the sample when determined by igniting about 0.5 g of the sample, accurately weighed, at 1000°C for 30 minutes. When the requirement "dried sample" is given in the

Monographs, the sample to be used for the test should be previously dried under the conditions specified for the loss on drying test in the individual monograph.

Procedure Ignite a platinum, quartz, or porcelain crucible under the conditions specified in the individual monograph for 30 minutes or more, allow it to cool in a desiccator, and weigh accurately.

If the sample consists of large crystals or lumps, rapidly grind them into particles not larger than about 2 mm in diameter. Unless otherwise specified, place 1 to 2 g of the sample in the crucible, and weigh accurately. Next, transfer the crucible into an electric furnace, and unless otherwise specified, ignite it at 450–550° C for 3 hours, allow it to cool in a desiccator, and weigh accurately.

Melting Point

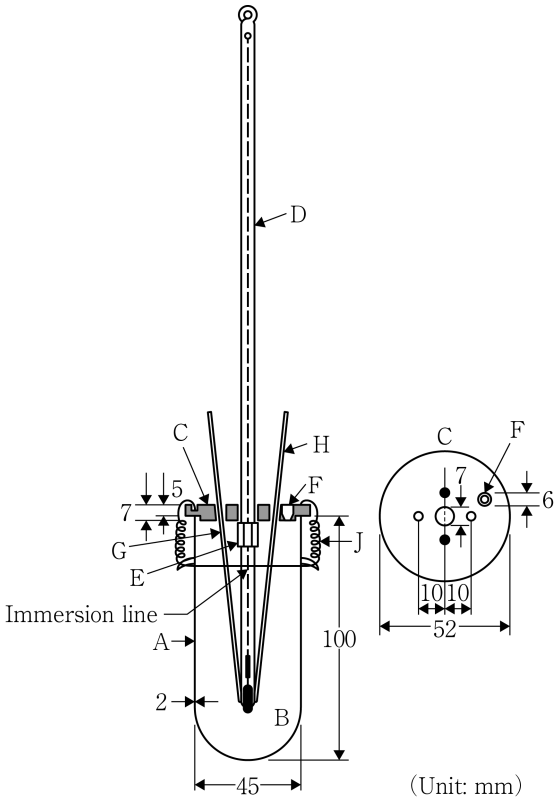
The melting point is defined as the temperature at which, or as the temperature range within which, a solid completely melts when determined by either of the methods given below. Method 1 is applied to substances that are comparatively high in purity and can be readily pulverized. Method 2 is applied to substances that are water-insoluble and cannot be readily pulverized.

Unless otherwise specified, measurement is performed by Method 1.

Method 1

This method is usually applied to those that can be readily pulverized.

Apparatus Use the apparatus illustrated in the figure.



A: Heating vessel (hard glass)

B: Bath fluid (use clear silicone oil with a viscosity of 50 to 100 mm²/s at ordinary temperature.)

C: Teflon stopper

D: Rod thermometer with an immersion line

Type 1 for a melting point lower than 50° C.

Type 2 for one not less than 40° C and less than 100° C.

Type 3 for one not less than 90° C and less than 150° C.

Type 4 for one not less than 140° C and less than 200° C.

Type 5 for one not less than 190° C and less than 250° C.

Type 6 for one not less than 240° C and less than 320° C.

F: Vent for adjustment of the bath fluid volume

G: Coiling spring

H: Capillary tube (a 120 mm long tube of 0.8 to 1.2 mm in an internal diameter, made of hard glass 0.2 to 0.3 mm thick, with one end sealed)

J: Spring to fasten the Teflon stopper

Procedure Finely pulverize the sample, and unless otherwise specified, dry in a desiccator for about 24 hours. When the stipulation "dried sample" is given in the Monographs, dry the sample under the conditions specified for the loss on drying test in the individual monograph. Place the sample in capillary tube H and pack it tightly

to form a 2.5- to 3.5-mm thick layer by dropping the capillary repeatedly, with its closed end down, in a glass tube about 70 cm long, held vertically on a glass or ceramic plate. When the stipulation "sealed tube" is given in the Monographs, close the open end of the tube. When the stipulation "vacuum sealed tube" is given, close the open end of the tube by heating gently under reduced pressure not exceeding 0.67 kPa.

Heat bath fluid B slowly until the temperature rises to about 10° C below the expected melting point, adjust the immersion line of thermometer D at the same level as the meniscus of the bath fluid, and insert capillary tube H containing the sample into coil spring G so that the position of the sample in H is on a level with the middle of the mercury bulb of thermometer D. Next, continue heating so that the temperature rises at a rate of about 3° C per minute until the temperature rises to about 5° C below the expected melting point, and continue heating to increase the temperature at a rate of 1° C per minute.

The temperature at which slight wetting or disintegration is observed in the contact surface of the sample and inside the wall of H is defined as the temperature at the start of melting. The temperature at which the sample melts completely and becomes transparent is defined as the temperature at the end of melting, which is referred to as the melting point.

Method 2

This method is usually applied to those that cannot be readily pulverized, such as fats, fatty acids, paraffins, and waxes.

Procedure Melt the sample at as low a temperature as practical it can be, and then aspirate it up into a capillary tube, taking care not to let bubbles in (use one specified in Method 1 with both ends open), to a height of about 10 mm. Allow to stand at about 10° C for 24 hours or cool it for at least 2 hours with ice. Attach the tube to the thermometer using a rubber band so that the sample in the tube is on a level with the middle the mercury bulb. Place it in a beaker of water to keep the upper end of the sample about 10 mm below the water surface. Heat the water with constant stirring until the temperature rises to about 5° C below the expected melting point, and continue heating to increase the temperature at a rate of 1° C per 2 minutes. The melting point is defined as the temperature at which the sample is observed to rise within the capillary tube H.

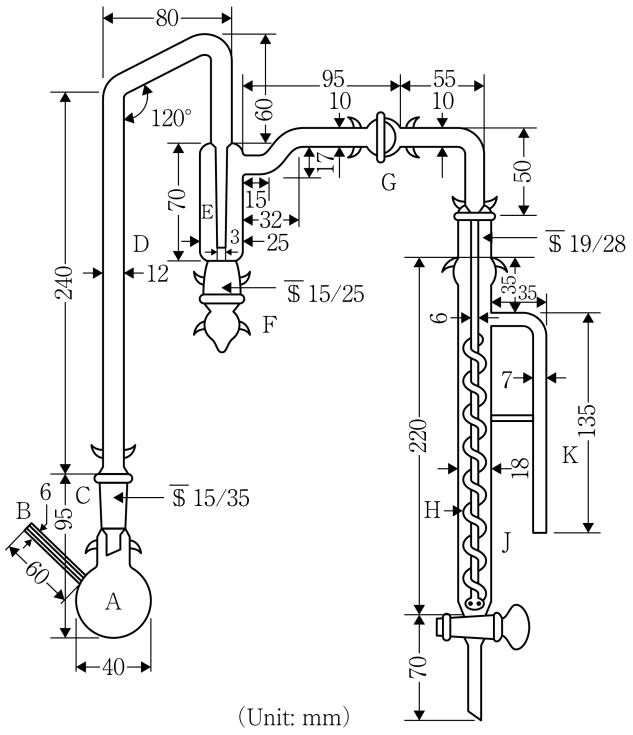
Methoxy Determination

Methoxy determination is designed to quantify the methoxy group in a sample by heating the sample with hydriodic acid, oxidizing the iodomethane produced with

bromine, adding potassium iodide and dilute sulfuric acid to the iodide acid produced, and titrating the iodine produced with sodium thiosulfate solution.

Apparatus Use the apparatus as illustrated in the figure.

A: Decomposition flask
B: Gas-inlet tube
C: Ground-glass joint
D: Air condenser
E: Gas scrubber
F: Glass stopper
G: Ground-glass ball joint
H: Gas duct
J: Absorption tube
K: Gas-exhaust tube



Preparation of Rinsing Solution and Absorbing Solution

Rinsing solution Weigh 1 g of red phosphorus, and suspend in 100 mL of water.

Absorbing solution Weigh 15 g of potassium acetate, and dissolve in 150 mL of a 9:1 mixture of acetic acid/acetic anhydride. To 145 mL of this solution, add 5 mL of bromine. Prepare fresh before use.

Procedure Put the rinsing solution into gas scrubber E up to about half the height of the scrubber, and transfer about 20 mL of the absorbing solution into absorption tube J. Weigh accurately an amount of the sample, equivalent to about 6.5 mg as methoxy group (CH_3O: 31.03), transfer to decomposition flask A, and add boiling chips and about 6 mL of hydriodic acid. Moisten ground-glass joint C of A with 1 drop of hydriodic acid, and connect A to condenser D. Assemble the apparatus by connecting ground-glass ball joint G using a suitable silicone resin. Pass nitrogen or carbon dioxide through gas-inlet tube B, and control the flow rate using a suitable pressure-regulating device so that bubbles appear in E at a rate of 2 bubbles per second. Place A in an oil bath, heat the flask so that the temperature of the bath reaches 150° C in 20 or 30 minutes, and continue to boil the liquid in A for another 60 minutes. Remove the oil bath, allow the flask to cool with the gas passing through it, and remove G after cooling. Drain the

contents of J into a 500-mL ground-glass stoppered Erlenmeyer flask containing 10 mL of a solution of sodium acetate trihydrate (1 in 5), wash the tube with water several times, add the washings to the flask, and dilute to about 200 mL with water. Add formic acid dropwise while shaking until the red color of the bromine disappears, and add another 1 mL of formic acid. Next, add 3 g of potassium iodide and 15 mL of diluted sulfuric acid (1 in 20), stopper, shake gently, allow to stand for 5 minutes, and titrate the liberated iodine with 0.1 mol/L sodium thiosulfate (indicator: 1 mL of starch TS). Add starch TS near the endpoint of the titration, when the solution is pale yellow. The endpoint is when the blue color of the solution disappears. Perform a blank test in the same manner, and make any necessary correction.

1 mL of 0.1 mol/L sodium thiosulfate = 0.5172 mg of CH_3O

Microbial Limit Tests

The microbial limit tests are used for the qualitative and quantitative estimation of specific viable microorganisms present in samples. They include total plate count, the enumeration of yeasts and molds, and tests for coliforms, *Escherichia coli,* and *Salmonella*. Great care must be taken when performing the tests to avoid microbial contamination from the outside. If the test samples have antimicrobial activity, which will have negative effects on the test results, the activity must be eliminated as far as possible by dilution, filtration, neutralization, inactivation, or other appropriate means. Samples should be prepared by mixing multiple portions randomly chosen from individual ingredients or products, and they should be tested by the method given below. Due attention must be paid to ensure effective quality control and prevent biohazard accidents.

1. Total Plate Count

This test determines mesophilic bacteria, yeasts, and molds that can grow in aerobic conditions. Psychrophilic, thermophilic, basophilic, anaerobic bacteria, and other microorganisms that require specific ingredients for growth may not form any colony, even if they exist in samples in a significant number. Alternative methods, like automated methods, may be used if they are comparable or superior in sensitivity and accuracy to the methods given under the Microbial Limit Tests.

Preparation of Sample Fluids Unless otherwise specified, use the most appropriate among the methods given below. Depending on the nature of samples, a volume of buffers larger than that specified may be required to disperse the samples or a quantity of samples different from that specified may be required. If necessary, a blender can be used for sample dispersion. Samples can be emulsified using an appropriate

surfactant, such as 0.1% (w/v) polysorbate 80. In the emulsification process, heating can be applied if the temperature does not exceed 45°C and the heating time does not exceed 30 minutes. Adjust the prepared sample fluid to pH 6–8, and use within one hour of preparation.

Method 1 Mix 10 g of the sample with 90 mL of phosphate buffer, 0.1% peptone solution, or sodium chloride-peptone buffer to disperse it uniformly.

Method 2 Mix 1.0 g of the sample with 100 mL of phosphate buffer, 0.1% peptone solution, or sodium chloride-peptone buffer to disperse it uniformly.

Method 3 Mix not less than 1.0 g of the sample with 9 or 100 times volume of phosphate buffer, 0.1% peptone solution, or sodium chloride-peptone buffer to disperse it uniformly.

If the method suitability is not achieved with the sample fluid prepared as directed above, use a sample fluid prepared by diluting 0.1 g of the sample with phosphate buffer, 0.1% peptone solution, or sodium chloride-peptone buffer to 200 times or more its original volume, or conduct the test using the membrane filter method, given in (2) below, taking method suitability into account.

Procedure Unless otherwise specified, use the method given in (1) below. If filtration is required for the removal of antimicrobial substances, unless otherwise specified, perform the test using membrane filters obtained after the filtration and washing steps specified in (2) below. Place the prepared membrane filters on the surface of standard agar medium and incubate under the conditions specified in (1).

(1) Pour Plate Method Use at least 2 petri dishes of 9–10 cm diameter for each dilution. Dispense 1 mL of the sample fluid or its dilution into each petri dish aseptically, add 15–20 mL of the standard agar medium, previously warmed and kept below 45° C, and mix. Allow the agar medium to solidify, incubate the plates at 35 ± 1° C for 48 ± 2 hours. Count the colonies developed on each dish, and calculate the total plate counts per gram of sample. If a large number of colonies develop, calculate the count based on the results from a plate with 25–250 colonies per plate.

(2) Membrane Filtration Method This method is applied to samples that contain antimicrobial substances to remove the substances through filtration before the test.

Use membrane filters of an appropriate material with a pore size of 0.45 μm or less. Filters of about 50 mm diameter are recommended, but other sizes may also be used. The membrane filters, filtration apparatus, media, and other elements to be used for the test should be well sterilized. Usually, 20 mL of the sample fluid (two 10-mL portions) is filtered using 2 filters, one for each 10-mL portion. If necessary, the sample fluid may be diluted. If the cell concentration is high, the pretreated sample fluid should be diluted; it is desirable that 10–100 colonies develop on each filter. After

filtration, wash each filter at least three times with an appropriate wash solution, such as phosphate buffer, 0.1% peptone solution, or sodium chloride-peptone buffer. The volume of the wash solution should be about 100 mL each time. If the diameter of the filter used is different from 50 mm, use an appropriate volume of wash solution, depending on the size of the filter. If the sample includes lipid, polysorbate 80 or other appropriate emulsifier may be added to the wash solution.

Effectiveness of Culture Media and Method Suitability

(1) Preparation of test organism suspensions Use the following strains or their equivalents for the test: *Escherichia coli* (NBRC 3972, ATCC 8739, or NCIMB 8545), *Bacillus subtilis* (NBRC 3134, ATCC 6633, or NCIMB 8054), *Staphylococcus aureus* subsp. *aureus* (NBRC 13276, ATCC 6538, or NCIMB 9518), *Candida albicans* (NBRC 1594, or ATCC 10231), and *Aspergillus brasiliensis* (NBRC 9455, or ATCC 16404). Incubate the above strains in the following medium under the following conditions: for bacteria, soybean-casein digest medium, soybean-casein digest agar medium, or standard medium at 35 ± 1° C for 18–24 hours; for *C. albicans,* soybean-casein digest medium, Sabouraud glucose liquid medium, Sabouraud glucose agar medium, or dichloran glycerol agar medium at 25 ± 1°C for 2–3 days; and for *A. brasiliensis*, Sabouraud glucose agar medium, potato-dextrose agar medium, or dichloran glycerol agar medium at 25 ± 1°C for 5–7 days or until adequate spore formulation is observed.

Dilute each of the prepared cultures with sodium chloride-peptone buffer or phosphate buffer to prepare test organism suspensions with an appropriate concentration. To suspend *A. brasiliensis* spores, polysorbate 80 may be added at 0.05% of the volume to the diluent used. The test organism suspensions should be used within 2 hours or 24 hours if stored in a refrigerator. For *B. subtilis* and *A. brasiliensis*, stable spore suspensions may be used.

(2) Media growth promotion test Adjust the test organism suspension so that the number of colonies to develop is not more than 100 per mL. Perform the test as directed under Procedure above, using 1 mL of the adjusted suspension instead of the sample fluid. Incubate a mixture of the test organism suspension and the specified medium at 35 ± 1°C for no longer than 46 hours. The medium is suitable if clearly visible growth of the applied microorganisms occurs and a sufficient number of microorganisms are recovered.

(3) Method suitability test Confirm method suitability as directed below. If there is any change that may affect the test results of ingredients, manufacturing processes, composition of the targeted product, suitability should be confirmed again.

To the sample fluid and the control, add the test organism suspension so that the number of colonies of the inoculated microorganisms to develop is not more than 100 per plate. The volume of the suspension to be inoculated should not exceed 1% of the volume of the sample fluid. As the control, use the solution employed for the sample

fluid preparation—phosphate buffer, 0.1% peptone solution, or sodium chloride-peptone buffer, whichever is appropriate.

Perform the test as directed under Procedure by strain. After incubation at 35 ± 1°C for no longer than 46 hours, count the colonies recovered, and compare the counts from the sample fluid with those from the control. The counts in the presence of the sample must be between 50% and 200% of those obtained from the control. If the requirement is not met, the effects must be eliminated to the utmost extent by an appropriate means, including dilution, filtration, neutralization, or inactivation. If the requirement is still not met through such means, conduct the test under the conditions that can give the counts closest to the above requirement, using the sample fluid at the lowest concentration that is commensurate with the growth of the target microorganism and the corresponding standard limit specified in the individual monographs.

2. Enumeration of Yeasts and Molds

This test determines mesophilic yeasts and molds that can grow in aerobic conditions. Alternative methods, like automated methods, may be used if they are comparable or superior in sensitivity and accuracy to the methods given in the Microbial Limit Tests.

Preparation of Sample Fluids Unless otherwise specified, prepare a sample fluid as directed under Preparation of Sample Fluids in 1. Total Plate Count.

Procedure Use at least 2 petri dishes of 9–10 cm diameter for each dilution. Dispense 1 mL of the sample fluid or its dilution into each petri dish aseptically, add 15–20 mL of dichloran glycerol agar medium, previously warmed and kept below 45°C, and mix. Allow the agar medium to solidify, incubate the plates at 25 ± 1°C for 5–7 days. If reliable colony counts are considered to be obtained, the counts obtained after 5 days of incubation may be adopted. Count the duplicate plates, and calculate the total plate count per gram. If a large number of colonies develop, calculate the count based on the results from a plate with 10–150 colonies per plate.

If filtration is required for the removal of antimicrobial substances, unless otherwise specified, perform the test using membrane filters obtained after the filtration and washing steps specified in (2) of Procedure under Total Plate Count. Place the prepared membrane filters on the surface of dichloran glycerol agar medium, and incubate under the conditions specified for this procedure.

Effectiveness of Culture Media and Method Suitability

(1) Preparation of test organism suspensions Use the following strains or their equivalents for the test: *Candida albicans* (NBRC 1594 or ATCC 10231) and *Aspergillus brasiliensis* (NBRC 9455 or ATCC 16404). Prepare test organism suspensions as directed in (1) of Effectiveness of Culture Media and Method Suitability

under 1. Total Plate Count.

(2) Media growth promotion test Adjust the test organism suspension so that the number of colonies to develop is not more than 100 per mL. Perform the test as directed under Procedure above, using 1 mL of the prepared suspension instead of the sample fluid. Incubate a mixture of the suspension and the specified medium at 25 ± 1°C for no longer than 5 days. The medium is suitable if clearly visible growth of the applied microorganisms occurs and a sufficient number of microorganisms are recovered.

(3) Method suitability test Proceed as directed in (3) of Effectiveness of Culture Media and Method Suitability under 1. Total Plate Count. Incubate at 25 ± 1°C for no longer than 5 days.

3. Tests for Coliforms and *Escherichia coli*

This test is used to determine the presence or absence of coliforms and *Escherichia coli*. Coliforms and *Escherichia coli, the* target to be detected for this test, become important indices to evaluate microbial contamination of ingredients and intermediate products as well as of the finished products. They should not be present in any of ingredients or products.

In the Monographs, for this test, the specification "Coliforms are negative per test" means that the sample must be coliform negative when the confirmation test for coliforms is performed as directed under the Microbial Limit Test. Also, for this test, the specification "*Escherichia coli* is negative per test" means that the sample must be *Escherichia coli* negative when the confirmation test for *Escherichia coli* is performed as directed under the Microbial Limit Test.

Preparation of Pre-enrichment Culture Unless otherwise specified, use an appropriate method among those given below. Depending on the nature of samples, a volume of liquid medium larger than that specified can be used to disperse the samples. If necessary, a blender can be used for sample dispersion. Adjust the medium mixed with the sample to pH 6–8, and incubate within one hour after mixing.

If filtration is required to remove antimicrobial substances, unless otherwise specified, use membrane filters obtained by conducting the filtration and washing steps specified in (2) of Procedure under Total Plate Count. Place the membrane filters in lauryl sulfate broth, adjust to pH 6–8, and incubate at 35 ± 1°C for 48 ± 2 hours.

Method 1

Mix 10 mL of the sample fluid, prepared as directed under Method 1 in Preparation of Sample Fluids under 1. Total Plate Count, with 90 mL of lauryl sulfate broth, and incubate at 35 ± 1°C for 48 ± 2 hours.

Method 2

Mix 1.0 g of the sample with 100 mL of lauryl sulfate broth to disperse uniformly, and

incubate at 35 ± 1°C for 48 ± 2 hours.

Method 3

Mix 10 mL of the sample fluid, prepared as directed under Method 1 in Preparation of Sample Fluids under 1. Total Plate Count, with 90 mL of lauryl sulfate broth, and incubate at 35 ± 1°C for 48 ± 2 hours. If the amount of the sample is less than 1000 g, weigh an amount equivalent to 1% of the sample amount (not less than 1.0 g), mix with 9 volumes of phosphate buffer, 0.1% peptone solution, or sodium chloride-peptone buffer to disperse uniformly. Mix 10 mL of this fluid with 90 mL of lauryl sulfate broth to disperse uniformly, and incubate at 35 ± 1°C for 48 ± 2 hours. Use this as the pre-enrichment culture.

If the pre-enrichment culture prepared as directed above is found to be unsuitable for the method given in Procedure below, prepare the pre-enrichment culture as follows: Mix 0.20 g of the sample with 100 mL of lauryl sulfate broth to disperse uniformly, and incubate at 35 ± 1°C for 48 ± 2 hours. In this case, prepare five pre-enrichment cultures in the same manner, and perform the test for each culture. Or, perform the test taking the method suitability into consideration, using the membrane filter method or other appropriate method.

Procedure

(1) Confirmation Test for Coliforms Agitate gently the pre-enrichment culture, inoculate a loopful of it into BGLB medium, and incubate at 35 ± 1°C for 48 ± 2 hours. Examine for gas production. If there is no gas production, the sample is determined to be coliform negative. If there is gas production, smear a loopful of it from the gassing tube on standard agar medium plates, incubate at 35 ± 1°C for 18–24 hours, and examine the colonies. Perform gram stain. If Gram-negative non-spore-forming rods appear, the sample is determined to be coliform negative.

(2) Confirmation Test for *Escherichia coli* Agitate gently the pre-enrichment culture, inoculate a loopful of it into EC medium, and incubate at 45.5 ± 0.2°C for 24 ± 2 hours. Examine for gas production or turbidity. If neither is observed, continue incubation for up to 48 ± 2 hours. Examine again. If neither gas production nor turbidity is observed, the sample is determined to be *Escherichia coli* negative. If gas production or turbidity is observed, smear a loopful of it from the gassing or turbid test tube on EMB agar medium plates, and incubate at 35 ± 1°C for 18–24 hours. If no dark centered colonies (with or without metallic sheen) are not observed on the EMB plates, the sample is determined to be *Escherichia coli* negative. In case of suspicious colonies, perform the following two tests: A) Transfer at least two colonies to standard agar medium, incubate at 35 ± 1°C for 18–24 hours, and perform the gram stain. B) Inoculate at least two colonies into lauryl sulfate broth, incubate at 35 ± 1°C for 48 ± 2 hours, and confirm gas production. After these tests, if the observed colonies are Gram-

positive or no gas production is observed, the sample is determined to be *Escherichia coli* negative. For Gram-negative microorganisms with gas production, perform the IMViC tests (Indole production test, Methyl red reaction test, Voges-Proskauer test, and Citrate utilization test). If the microorganisms give IMViC patterns of ++ – –, they are considered as *Escherichia coli*. Alternatively, instead of performing the IMViC tests, use a commercially prepared *E. coli* identification kit.

Effectiveness of Culture Media and Method Suitability

(1) Preparation of test organism suspensions Use an appropriate *Escherichia coli* strain (NBRC 3972, ATCC 8739, or NCIMB 8545) or one of their equivalents. Prepare test organism suspensions as directed in (1) of Effectiveness of Culture Media and Method Suitability under 1. Total Plate Count. Adjust the test organism suspension so that the number of colonies to develop is not more than 1000 per mL.

(2) Media growth promotion test Prepare the pre-enrichment culture using 0.1 mL of the test organism suspension, instead of the sample fluid or the sample. Proceed as directed under Procedure in the Test for Coliforms and *Escherichia coli* above. Incubation should be done for the shortest time specified. The medium is suitable if a clearly visible growth of the applied microorganisms occurs and a sufficient number of microorganisms are recovered. Gas production must be observed in BGLB medium and lauryl sulfate broth.

(3) Method suitability test Confirm method suitability as directed below. If there is any change that may affect the test results in ingredients, manufacturing processes, composition of the targeted product, suitability should be confirmed again.
To a mixture of lauryl sulfate broth with the sample fluid or the sample and the control, add 0.1 mL of the test organism suspension, and pre-incubate as directed under Preparation of Pre-enrichment Culture. The volume of the suspension to be inoculated should not exceed 1% of the volume of the sample fluid . As the control, use lauryl sulfate broth or a mixture of lauryl sulfate broth with the solution used for sample fluid preparation-phosphate buffer, 0.1% peptone solution, or sodium chloride-peptone buffer, whichever is appropriate.

Perform the test as directed under Procedure in Tests for Coliform and *Escherichia coli*, according to the shortest incubation time specified. If in the presence of the sample, target microorganisms do not grow sufficiently when compared with the control, effects must be eliminated to the utmost extent by an appropriate means, including dilution, filtration, neutralization, or inactivation. If the requirement (counts should be between 50% and 200% of those obtained from the control), is still not met through such means, conduct the test under the conditions that can give counts as close as possible to the requirement, using the sample fluid at the lowest concentration that is commensurate with the growth of the target microorganism and the corresponding standard limit specified in the individual monographs.

4. Test for *Salmonella*

This test is used to determine the presence or absence of *Salmonella*. *Salmonella, the* target to be detected by this test, becomes an important index to evaluate microbial contamination of ingredients and intermediate products as well as of the finished products. They should not be present in any of them.

In the Monographs, for this test, the specification "*Salmonella* is negative per test" means that *Salmonella* must be negative when the *Salmonella* test is performed as directed under the Microbial Limit Test.

Preparation of Pre-enrichment Culture Unless otherwise specified, use an appropriate one among the methods given below. Depending on the nature of samples, a larger volume of liquid medium than specified can be used to disperse the samples. If necessary, a blender can be used for sample dispersion. Adjust the medium mixed with the sample to pH 6–8, and incubate within one hour after mixture.

If filtration is required to remove antimicrobial substances, unless otherwise specified, use membrane filters obtained the filtration and washing steps specified in (2) of Procedure under 1. Total Plate Count. Place the prepared membrane filters in lactose broth, adjust to pH 6–8, and incubate at 35 ± 1°C for 24 ± 2 hours. Use this as the pre-enrichment culture.

Method 1 Mix 25 g of the sample with 225 mL of lactose broth to disperse uniformly. Incubate at 35 ± 1°C for 24 ± 2 hours.

Method 2 Mix 25 g of the sample with 225 mL of lactose broth to disperse uniformly. Incubate at 35 ± 1°C for 24 ± 2 hours. If the amount of the sample is less than 2500 g, weigh an amount equivalent to 1% of the sample amount (not less than 1.0 g), mix with 9 volumes of lactose broth (not less than 100 mL) to disperse uniformly, and incubate at 35 ± 1°C for 24 ± 2 hours. Use this as the pre-enrichment culture.

If the pre-enrichment culture prepared as directed above is not considered suitable for the method described under Procedure below, prepare the pre-enrichment culture as follows: Mix 0.20 g of the sample with 100 mL of lactose broth to disperse uniformly, and incubate at 35 ± 1°C for 24 ± 2 hours. In this case, prepare five pre-enrichment cultures in the same manner, and perform the test for each culture. Or, perform the test taking method suitability into consideration, using the membrane filter method or other appropriate method.

Procedure

(1) Confirmation of *Salmonella* Colonies Gently agitate the pre-enrichment culture, inoculate 0.1 mL of it into 10 mL of Rappaport-Vassiliadis liquid medium, incubate 42 ± 0.2°C for 24 ± 2 hours. Separately, inoculate 1 mL of the pre-enrichment culture into 10 mL of tetrathionate broth, incubate for 24 ± 2 hours at 43 ± 0.2°C (where the amount of *Salmonella* is supposedly large) or 35 ± 0.2°C (where the amount of

Salmonella is supposedly small). Separately, smear an aliquot from each culture medium on bismuth sulfite (BS) agar medium, XLD agar medium, and Hektoen enteric (HE) agar medium, and incubate them at 35 ± 2°C for 24 ± 2 hours. Examine the presence or absence of the typical *Salmonella* colonies (see the table below) or suspicious *Salmonella* colonies. If typical *Salmonella* colony morphology or suspicious colonies are not observed, examine the presence or absence of atypical *Salmonella* colonies (see the table below). If atypical colonies or suspicious colonies are not observed after 24 ± 2 hours incubation on the bismuth sulfite agar medium, incubate for an additional 24 ± 2 hours. If no colony is observed on each medium, the sample is determined to be *Salmonella* negative.

Typical and Atypical *Salmonella* Colony Morphology

Selective medium	Typical colony morphology	Atypical colony morphology
BS agar	Typical *Salmonella* colonies may appear brown, gray, or black; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo effect. Some strains may produce green colonies with little or no darkening of surrounding medium.	
XLD agar	Pink colonies with or without black centers. Many cultures of *Salmonella* may have large, glossy black centers or may appear as almost completely black colonies.	A few *Salmonella* cultures produce yellow colonies with or without black centers on HE and XLD agars.
HE agar	Blue-green to blue colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.	A few *Salmonella* cultures produce yellow colonies with or without black centers on HE and XLD agars.

Note: BS: Bismuth sulfite, XLD: Xylose lysine desoxycholate, HE: Hektoen enteric

(2) Agar Slant Analysis Select two or more of typical colonies or colonies suspected to be *Salmonella* from each selective agar plate. Inoculate them into the butt and slant of triple sugar iron (TSI) agar and lysine iron agar (LIA), and incubate at 35 ± 1°C for 24 ± 2 hours. If BS agar plates after 48 ± 2 hour incubation or XLD or HE agar plates after 24 ± 2 hour incubation have no colonies typical or suspected to be

Salmonella, pick two or more atypical colonies, inoculate into the butt and slant of TSI agar and LIA, and incubate at 35 ± 1°C for 24 ± 2 hours. In TSI agar, *Salmonella* produces an acidic (yellow) reaction in the butt of the tube and an alkaline (red) reaction in the slant, with or without the production of hydrogen sulfide. *Salmonella* produces an alkaline (purple) reaction in the butt of the tube. Consider only distinct yellow in the butt of tube as an acidic (negative) reaction. Most *Salmonella* cultures produce hydrogen sulfide in LIA.

If test results show that *Salmonella* is likely to exist in the sample, detailed biochemical and serological tests (including commercially available kits) should additionally be performed to identify *Salmonella*.

Effectiveness of Culture Medium and Method Suitability

(1) Preparation of test organism suspensions Use the following strains or their equivalents: *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028) or *Salmonella enterica* subsp. *enterica* serovar Abony (NBRC 100797 or NCTC 6017).

Prepare test organism suspensions as directed in (1) of Effectiveness of Culture Media and Method Suitability under 1. Total Plate Count. Adjust the suspension so that the number of colonies to develop is not more than 1000 per mL.

(2) Media growth promotion test Prepare the pre-enrichment culture using 0.1 mL of the test organism suspension, instead of the sample. Proceed as directed under Procedure in the Test for S*almonella*. Incubation should be done for the shortest time specified. The medium is suitable if clearly visible growth of the applied microorganisms occurs and a sufficient number of microorganisms are recovered.

(3) Method suitability test Confirm method suitability as directed below. If there is any change that may affect the test results of ingredients, manufacturing processes, composition of the targeted product, or suitability should be confirmed again.

To a mixture of lactose broth with the sample and the control, add 0.1 mL of the test organism suspension. The volume of the suspension to be inoculated should not exceed 1% of the volume of the medium. As the control, use lactose broth.

Perform the test as directed under Procedure in the Test for *Salmonella* according to the shortest incubation time specified. If in the presence of the sample, the target microorganisms do not grow sufficiently when compared with the control, effects must be eliminated to the utmost extent by an appropriate means, including dilution, filtration, neutralization, or inactivation. If the requirement (counts are between 50% and 200% of those obtained from the control) is still not met through such means, conduct the test under the conditions that can give counts as close as possible to the requirement, using the sample fluid at the lowest concentration that is commensurate with the growth of the target microorganism and the corresponding standard limit specified in the individual monographs.

5. Buffer Solutions and Media

Use the buffer solutions and media given below for the microbial limit test. Other media may be used if they include similar nutritive ingredients and have similar selectivity and growth-promoting ability for the microorganism to be tested. For the preparation of media, use regents and solutions that are suitable for microbial tests. If autoclaved sterilization is done, previously, well mixed ingredients should be uniformly dispersed or melted, if necessary by heating or boiling them.

(1) Buffer Solutions

(i) *Phosphate buffer*

Stock solution: Dissolve 34 g of potassium dihydrogen phosphate in about 500 mL of water, add about 175 mL of sodium hydroxide TS (1 mol/L) to adjust to pH 7.1–7.3, and add water to make 1000 mL. Autoclave at 121° C for 15–20 minutes, and store in a cool place. Before use, dilute with water to 800 times its original volume, and autoclave at 121° C for 15–20 minutes.

(ii) *Sodium chloride-peptone buffer*

Peptone	1.0 g
Potassium dihydrogen phosphate	3.6 g
Disodium hydrogenphosphate dihydrate	7.2 g
Sodium chloride	4.3 g
Water	1000 mL

Mix all the ingredients, and autoclave at 121° C for 15–20 minutes. The pH should be 6.9–7.1 after sterilization.

(iii) *0.1% Peptone solution*

Peptone	1.0 g
Water	1000 mL

Mix all the ingredients, and autoclave at 121° C for 15–20 minutes.

(2) Media

(i) *Standard agar medium*

Tryptone	5.0 g
Yeast extract	2.5 g
D(+)-Glucose	1.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and autoclave at 121° C for 15–20 minutes. The pH should be 6.8–7.2 after sterilization.

(ii) *Soybean-casein digest medium*

Peptone (casein)	17.0 g
Peptone (casein)	3.0 g

D(+)-Glucose	2.5 g
Dipotassium hydrogenphosphate	2.5 g
Sodium chloride	5.0 g
Water	1000 mL

Mix all the ingredients, and autoclave at 121° C for 15–20 minutes. The pH should be 7.1–7.5 after sterilization.

(iii) *Soybean-casein digest agar medium*

Peptone (casein)	15.0 g
Peptone (soybean)	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, boil for 1 minute, and autoclave at 121° C for 15–20 minutes. The pH should be 7.1–7.5 after sterilization.

(iv) *Sabouraud glucose liquid medium*

Peptone	10.0 g
D(+)-Glucose	20.0 g
Water	1000 mL

Mix all the ingredients, and autoclave at 121° C for 15–20 minutes. The pH should be 5.4–5.8 after sterilization.

(v) *Sabouraud glucose agar medium*

Peptone	10.0 g
D(+)-Glucose	40.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and autoclave at 121° C for 15–20 minutes. The pH should be 5.4–5.8 after sterilization.

(vi) *Dichloran 18% glycerol (DG18) agar medium*

Peptone	5.0 g
D(+)-Glucose	10.0 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulfate	0.5 g
Dichloran	2.0 mg
Chloramphenicol	0.10 g
Agar	15.0 g
Water	1000 mL

Mix all ingredients, add 220 g of glycerol and autoclave at 121° C for15–20 minutes. The pH should be 5.4–5.8 after sterilization.

(vii) *Potato dextrose agar medium*

Potato extract	200 mL

D(+)-Glucose	20.0 g
Agar	20.0 g
Water	1000 mL

Mix all the ingredients, and autoclave at 121° C for15–20 minutes. The pH should be 5.4–5.8 after sterilization.

(viii) *Lauryl sulfate broth*

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Dipotassium hydrogenphosphate	2.75 g
Potassium dihydrogen phosphate	2.75 g
Sodium chloride	5.0 g
Sodium lauryl sulfate	0.1 g
Water	1000 mL

Mix all the ingredients, and autoclave at 121°C for15–20 minutes. If the broth is used for the confirmation of gas production, sterilize the mixture with a fermentation tube contained in it. The pH should be 6.6–7.0 after sterilization.

(ix) *BGLB (brilliant green lactose bile) medium*

Peptone	10.0 g
Lactose	10.0 g
Dried cattle bile	20.0 g
Brilliant green	13.3 mg
Water	1000 mL

Mix all the ingredients, and autoclave at 121° C for15–20 minutes with a fermentation tube contained in the mixture. The pH should be 7.0–7.4 after sterilization.

(x) *EC medium*

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salt	1.5 g
Dipotassium hydrogenphosphate	4.0 g
Potassium dihydrogen phosphate	1.5 g
Sodium chloride	5.0 g
Water	1000 mL

Mix all the ingredients and autoclave at 121°C for 15–20 minutes with a fermentation tube contained in the mixture. The pH should be 6.7–7.1 after sterilization.

(xi) *EMB (Eosin-methylene blue) agar medium*

Peptone	10.0 g
Lactose	10.0 g
Dipotassium hydrogenphosphate	2.0 g

Eosin Y	0.40 g
Methylene blue	65 mg
Agar	15.0 g
Water	1000 mL

Mix all the ingredients and autoclave at 121° C for15–20 minutes. Cool to 50°C, mix well, and dispense into petri dishes to make plates. The pH should be 6.9–7.3 after sterilization.

(xii) *Lactose broth*

Peptone	5.0 g
Meat extract	3.0 g
Lactose	5.0 g
Water	1000 mL

Mix all the ingredients and autoclave at 121° C for15–20 minutes. The pH should be 6.7–7.1 after sterilization.

(xiii) *Rappaport -Vassiliadis liquid medium*

Tryptone	5.0 g
Potassium dihydrogen phosphate	1.6 g
Sodium chloride	8.0 g
Water	1000 mL

Mix all the ingredients, add 100 mL of a mixture of 400 g of magnesium chloride hexahydrate and 1000 mL of water, then 10 mL of a mixture of 40 mg of malachite green oxalate and 100 mL of water, and mix well. Autoclave at 115°C for 15 minutes. The pH should be 5.3–5.7 after sterilization.

(xiv) *Tetrathionate liquid medium*

Polypeptone	5.0 g
Bile salt	1.0 g
Calcium carbonate	10.0 g
Sodium thiosulfate pentahydrate	30.0 g
Water	1000 mL

Mix all the ingredients, heat until boiling to make a uniform suspension, and cool to 45° C. Do not autoclave. The pH should be 8.2–8.6. Before use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 mL of water. Mix with 10 mL of a solution obtained by sterilizing a mixture of 0.1 g of brilliant green and 100 mL of water. Thereafter, do not apply heat to the medium.

(xv) *Bismuth sulfite (BS) agar medium*

Polypeptone	10.0 g
Meat extract	5.0 g
D(+)-Glucose	5.0 g
Disodium hydrogenphosphate	4.0 g
Iron(II) sulfate	0.3 g

Bismuth sulfite indicator	8.0 g
Brilliant green	25 mg
Agar	20.0 g
Water	1000 mL

Mix all the ingredients, boil to make a uniform suspension, and cool to 50° C. Do not autoclave. The pH should be 7.5–7.9. After cooling, mix well, and dispense into petri dishes to make plates.

(xvi) *XLD (xylose lysine desoxycholate) agar medium*

Yeast extract	3.0 g
L-Lysine	5.0 g
D-Xylose	3.75 g
Sucrose	7.5 g
Lactose	7.5 g
Sodium desoxycholate	2.5 g
Ammonium iron(III) citrate	0.8 g
Sodium thiosulfate	6.8 g
Sodium chloride	5.0 g
Phenol red	80 mg
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and heat until boiling to dissolve. Avoid excess heating. Do not autoclave. The pH should be 7.2–7.6. Cool to 50° C, mix well, and dispense into petri dishes to make plates.

(xvii) *Hektoen enteric (HE) agar medium*

Peptone	12.0 g
Yeast extract	3.0 g
Sucrose	12.0 g
Lactose	12.0 g
Bile salt	9.0 g
Ammonium iron(III) citrate	1.5 g
Sodium thiosulfate	5.0 g
Fuchsin Acid	0.1 g
Salicin	2.0 g
Sodium chloride	5.0 g
Bromothymol blue	64 mg
Agar	13.5 g
Water	1000 mL

Mix all the ingredients, and heat until boiling to dissolve. Do not boil for 1 minute or longer. Avoid excess heating. The pH should be 7.4–7.8 after dissolving. Cool to 50° C, mix well, and dispense into petri dishes to make plates.

(xviii) *TSI (Triple sugar iron) agar medium*

Polypeptone	20.0 g
D(+)-Glucose	1.0 g
Sucrose	10.0 g
Lactose	10.0 g
Ammonium iron(II) sulfate hexahydrate	0.2 g
Sodium thiosulfate	0.2 g
Sodium chloride	5.0 g
Phenol red	25 mg
Agar	13.0 g
Water	1000 mL

Mix all the ingredients, dispense into test tubes, and autoclave at 118°C for 12–15 minutes. The pH should be 7.1–7.5 after sterilization. It is used as a slant. Also, medium containing 3.0 g each of meat extract and yeast extract can be used. In this case, autoclave the medium at 121°C.

(xix) *LIA (Lysine iron agar) medium*

Peptone	5.0 g
Yeast extract	3.0 g
D(+)-Glucose	1.0 g
Ammonium iron(III) citrate	0.5 g
Sodium thiosulfate	40 mg
Bromocresol purple	20 mg
Agar	12.5 g
Water	1000 mL

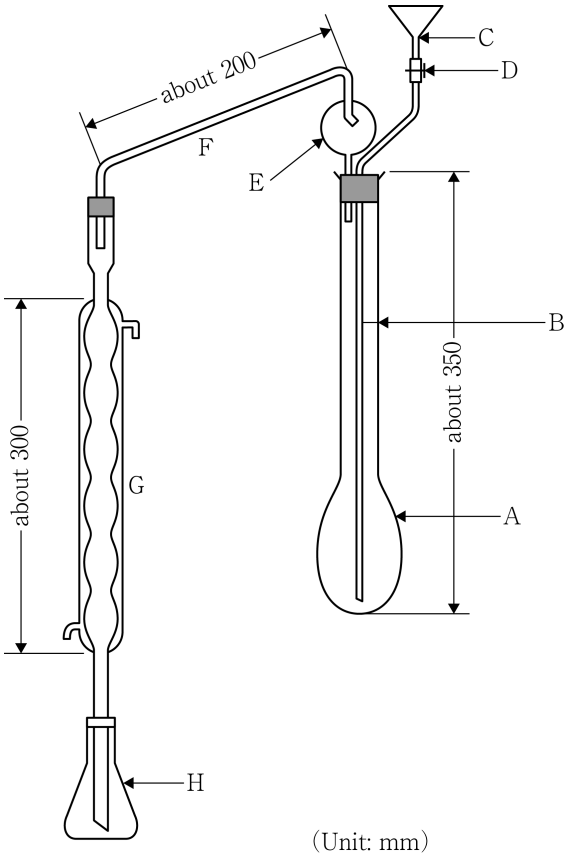
Mix all the ingredients, dispense into test tubes, and autoclave at 121°C for12–15 minutes. The pH should be 6.5–6.9 after sterilization. It is used as a slant.

Nitrogen Determination

Nitrogen determination is designed to determine nitrogen in organic compounds. Nitrogen in an organic compound is converted into ammonia nitrogen by thermal decomposition with sulfuric acid, and the ammonia liberated by alkali and trapped by steam distillation is determined by titration,

(1) Kjedldahl Method

Apparatus Use the apparatus as illustrated in Fig. 1. Ground glass may be used for joints.



A: Kjeldahl flask (made of hard glass, capacity of about 300 mL)
B: Glass tube
C: Funnel for the addition of alkaline solution
D: Rubber tube (connecting B and C, with a pinch cock attached)
E: Spray trap
F: Delivery tube
G: Condenser
H: Absorption flask (about 300 mL capacity)

Fig. 1

Procedure Unless otherwise specified, proceed as directed below.

Weigh accurately a quantity of sample equivalent to about 20 to 30 mg of nitrogen, place it into Kjeldahl flask A, and add 5 g of powdered potassium sulfate, 0.5 g of copper(II) sulfate pentahydrate, and 20 mL of sulfuric acid. Tilt flask A to about 45°, heat gently until effervescence almost stops, and raise the temperature to a boiling point. After the content becomes a clear, blue solution, heat for another 1 to 2 hours. Allow it to cool, and add gradually 150 mL of water, and cool again. Add 2 or 3 granules of boiling chips or granulated zinc, and assemble the apparatus.

Measure exactly 25 mL of 0.05 mol/L sulfuric acid, transfer into absorption flask H,

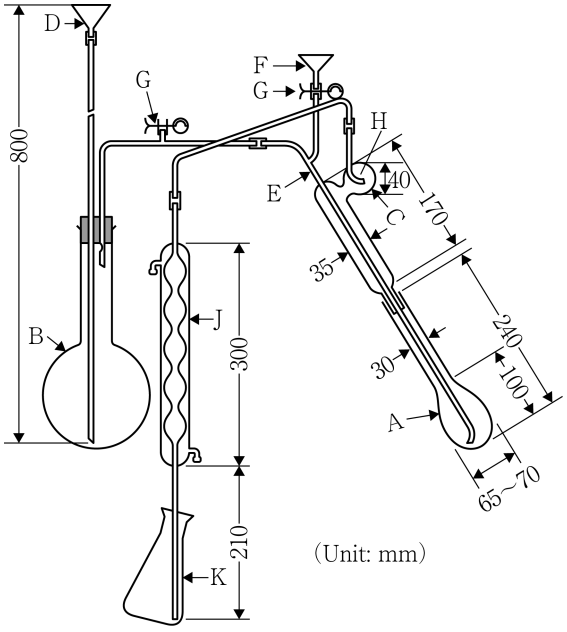
add about 50 mL of water, and immerse the lower end of condenser G into this solution. Add gradually 85 mL of sodium hydroxide solution (2 in 5) to flask A through funnel C, rinse the funnel with a small quantity of water, close the pinch cock on rubber tube D, mix the contents by lightly shaking flask A, and heat gently. When the solution starts to boil, turn up the heat, and distill until about two thirds of the contents are distilled. Lower H until the lower end of G is above the solution surface in H, continue the distillation for a short time, wash the lower end of G with a small quantity of water, and titrate the excess sulfuric acid in the solution in H with 0.1 mol/L sodium hydroxide. The endpoint is usually confirmed using a potentiometer or indicator (3 drops of bromocresol green–methyl red mixture TS). When the indicator is used, the endpoint is when the color of the solution changes from red-purple through pale grayish yellow to pale grayish green. Perform a blank test and make any necessary correction.

1 mL of 0.05 mol/L sulfuric acid = 1.401 mg of N

(2) Semi-micro Kjeldahl Method

Apparatus Use the apparatus illustrated in Fig. 2. The apparatus is made of hard glass and ground glass may be used for joints. All rubber parts used in the apparatus should be boiled in sodium hydroxide solution (1 in 25) for 10 to 30 minutes and then in water for 30 to 60 minutes, and finally washed thoroughly with water before use.

Automated devices can be used for some procedures, including decomposition of organic compounds, distillation of the liberated ammonia, and endpoint detection in titrimetry.



A: Kjeldahl flask

B: Steam generator (filled with water containing 2 to 3 drops of sulfuric acid and boiling chips to prevent bumping,)
C: Spray trap
D: Water supply funnel
E: Steam tube
F: Funnel for the addition of alkaline solution
G: Rubber tube with a pinch cock
H: Small hole (of a diameter approximately equal to the internal diameter of the tube)
J: Condenser (with a beveled lower end)
K: Receiver

Fig. 2

Procedure Unless otherwise specified, proceed as directed below.

Weigh accurately or pipet a quantity of the sample equivalent to 2 to 3 mg of nitrogen (N: 14.01), and place in Kjeldahl flask A. Add 1 g of a powdered mixture of 10 g of potassium sulfate and 1 g of copper(II) sulfate pentahydrate. Wash down the sample adhering to the neck of flask A with a small quantity of water. Add 7 mL of sulfuric acid, allowing it to flow along the inner surface of A.

Next, while shaking flask A, carefully add 1 mL of hydrogen peroxide drop by drop along the inner surface of A. Heat A gradually, and keep heating so that the vapor of sulfuric acid is condensed at the neck of the flask until the solution exhibits a clear vivid green color through a clear blue color and the inner surface is free from carbonaceous material. If necessary, cool, add a small quantity of hydrogen peroxide, and heat again. Allow it cool, and carefully add 20 mL of water, and cool the solution. Connect A to the distillation apparatus, washed in advance by passing steam through it. Add 15 mL of boric acid solution (1 in 25) to receiver K, and sufficient water to immerse the lower end of condenser tube J. Add 30 mL of sodium hydroxide solution (2 in 5) through funnel F, carefully wash the funnel with 10 mL of water, close the pinch cock attached to rubber tube G, and distill with steam until the distillate measures 80 to 100 mL. Remove the lower end of J from the solution, wash the lower end of J with a small quantity of water, and titrate the distillate with 0.005 mol/L sulfuric acid. The endpoint is usually confirmed using a potentiometer or indicator (3 drops of bromocresol green–methyl red mixture TS). When the indicator is used, the endpoint is when the color of the solution changes from green through pale grayish blue to pale grayish red-purple. Perform a blank test in the same manner, and make any necessary correction.

1 mL of 0.005 mol/L sulfuric acid = 0.1401 mg of N

If an automated apparatus is used, proceed as directed in the instructions provided for the apparatus.

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a method based on a phenomenon in which certain atomic nuclei of a substance placed in a static magnetic field absorb radio waves of a specific frequency when the nuclei are excited into the higher energy spin state from the lower energy spin state. NMR spectroscopy is applicable to various testing including identification tests, purity tests, and assays by utilizing the parameters (chemical shift, spin-spin coupling constant, signal intensity, and relaxation time) obtained from the NMR spectrum of the substance under test. The target nuclei include ^{1}H and ^{13}C.

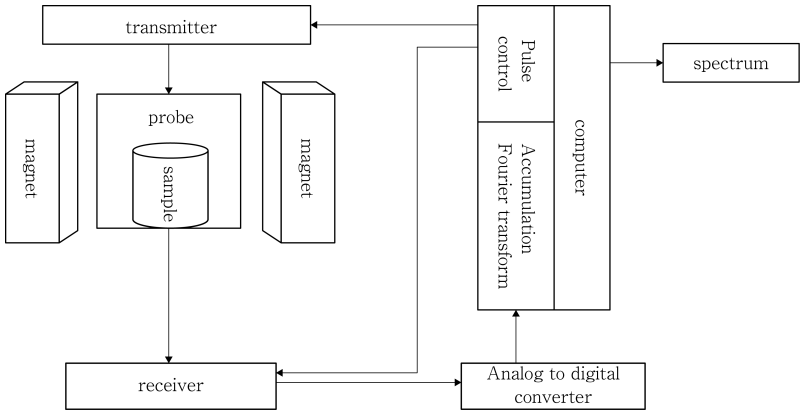
The chemical shift (δ) is given by

$$\delta = \frac{\nu_S - \nu_R}{\nu_R} + \delta_R$$

ν_S = the resonance frequency of the nucleus to be determined,
ν_R = the resonance frequency of the reference nucleus,
δ_R = the chemical shift of the reference nucleus (in the case the value is not 0).

The chemical shift is normally expressed, in ppm, by assuming the chemical shift of the reference compound (reference nucleus) as 0 ppm (on a scale relative to $\delta = 0$).

Apparatus Use a Fourier transform NMR (FT-NMR) spectrometer, outlined in the figure.



Measurement Prior to measurement, the sensitivity and resolution of the instrument must be adjusted to the optimum levels.

(1) Procedure The spectrum of a substance under test is measured by fitting the NMR tube prepared by either of the following methods (the internal reference method and external reference method) to the NMR probe. In the internal reference method, a small amount of reference compound is added to the sample dissolved in a suitable solvent, and the mixture is injected into an NMR tube. In external reference method, a

sealed capillary tube containing the reference compound is inserted into the NMR tube together with the test solution prepared as otherwise specified.

Test solutions should be completely homogenous. In particular, solid contaminants should be removed in order to obtain clear spectra. Some deuterated solvents for NMR spectroscopy are commonly used to dissolve the sample. Solvent selection should be done by considering that (i) the solvent signals do not overlap with the sample signals, (ii) the sample must be soluble in the solvent selected, and (iii) the solvent does not react with the sample. Furthermore, it should be noted that chemical shifts may depend upon the solvent employed, sample concentration and deuterium ion concentration, and that if test solutions are highly viscous, poorly resolve spectra are generated.

(2) Reference compounds Tetramethylsilane (TMS), DSS-d_6, or 1,4-BTMSB-d_4 is usually used as a reference compound, both for ^{1}H and ^{13}C. Furthermore, without adding a reference compound, residual protons in deuterated solvents or ^{13}C chemical shifts in the sample solvent can be used.

When the chemical shift of the reference compound is not assumed to be 0 ppm, the chemical shifts of the compound are corrected using the chemical shift previously established for the reference compound.

Record of apparatus and measurement conditions NMR Spectra change depending on measurement conditions. To allow appropriate comparison of spectra, conditions used for measurement should be recorded, including the name and frequency of the spectrometer, the solvent and temperature used for measurement, sample concentration, reference compound, and measurement method.

Optical Rotation

This method is designed to measure the angular rotation of a sample using a polarimeter.

Some substances or their solutions possess the inherent property to rotate the plane of incident polarized light to the right or to the left. This property is called optical activity or optical rotation property and its degree is related to the chemical structure of each substance.

Optical rotation is the angle through which the plane of polarization is rotated by an optically active substance or its solution.

Optical rotation is characterized as dextrorotatory or levorotatory, depending on whether the plane of the polarization is rotated to the right or to the left, respectively, when facing the direction of the polarized light. Optical rotation is expressed in angular degrees with the symbol (°), placed at the upper right of the number of degrees,

and a plus sign (+) or a minus sign (–) preceding the number for dextrorotation or levorotation, respectively.

The angle of rotation α_x^t is the value obtained when measured with specific monochromatic light x (expressed by the wavelength or the name) at temperature t° C. Usually, measurement is taken at 20° C or 25° C, using a polarimeter tube of 100 mm in length and the D line of the sodium spectrum as the light source. The thickness of the layer of a solution, i.e., the length of the polarimeter tube (the length of the layer of the solution measured) is also called optical path length or cell length.

The specific rotation $[\alpha]_x^t$ is expressed by the formula:

$$[\alpha]_x^t = \frac{\alpha}{lc} \times 100$$

t = the temperature of measurement,

x = the wavelength or the name of the specific monochromatic light of spectrum used (when D line is used, indicate as D),

α = the angle of the rotation, in degrees, of the plane of the polarization,

l = the length of the solution layer (mm),

c = the number of grams of the sample in 1 mL of the solution.

In the Monographs, the specification "$[\alpha]_D^{20}$: +20.5 to +21.5° (1g, freshly boiled and cooled water, 10 mL, on the dried basis)" for this test, for example, means that the specific rotation of the substance must be +20.5 to +21.5°, when determined according to the following manner: *About 1 g of the test substance, accurately weighed, is dissolved in newly boiled and cooled water to make exactly 10mL, and the specific rotation of the resulting solution is measured in a cell with 100 mm length at 20°, then the resulting value is calculated on the dried basis*.

Paper Chromatography

Paper chromatography is designed to develop a mixture in a mobile phase and to separate it into individual components using a sheet of filter paper. This method is applicable to identification tests and purity tests.

Procedure Unless otherwise specified, proceed as directed below.

Draw a horizontal line with a pencil across a sheet of the filter paper for chromatography specified in the individual monograph at a distance about 40 mm from one border of the filter paper. Using a micropipette or capillary tube, apply the specified amount of the test solution and the control solution on the line. The distance between the centers of the two spots applied should be about 25 mm. Air-dry the filter paper. Next, suspend it vertically from a stopper with a thread or wire in a developing

container of about 500-mm tall, containing the specified developing solvent and saturated with solvent vapor. This procedure should be done with care to avoid contact with the walls. Immerse the lower border of the paper 10 mm into the developing solvent, seal the container, and allow it to stand. When the solvent front has ascended from the sample spot to the specified position, remove the paper from the container, and air-dry. Examine as directed in the individual monograph, by comparing the spots between the test solution and the control solution in terms of location, color, and other characteristics.

pH Determination

pH is defined as the logarithm of the reciprocal of the hydrogen ion activity, which is the product of the hydrogen ion concentration (mol/L) and the activity coefficient of hydrogen. Practically, it is used as a scale of hydrogen ion concentration in a solution. The pH of a test solution is expressed by the following formula in relation to the pH of a standard solution (pH_S). It can be measured using a pH meter with a glass electrode.

$$pH = pH_S + \frac{E - E_S}{2.3026RT/F}$$

pH_S = pH value of a pH standard solution,

E = electromotive force (volts) of the following galvanic cell, composed of a glass electrode and a suitable reference electrode in a sample solution:

Glass electrode | sample solution | reference electrode

E_S = electromotive force (volts) of the following galvanic cell, composed of a glass electrode and a suitable reference electrode in a pH standard solution:

Glass electrode | pH standard solution | reference electrode

R = gas constant,

T = absolute temperature,

F = Faraday constant.

The values of 2.3026 RT/F (volts) at various temperatures are as follows:

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

In the Monographs, the specification "pH 6.0–7.5 (1.0 g, water 20 mL)" for this test, for example, means that the pH of the solution must be 6.0–7.5, when determined in a solution of 1.0 g of the test substance in 20 mL of water.

pH Standard Solutions The pH standard solutions are used as references for pH. To prepare pH standard solutions, use water whose electroconductivity does not exceed 2μS/cm (25° C). To prepare Borate pH Standard Solution, Carbonate pH Standard Solution, and Calcium Hydroxide pH Standard Solution, use carbon dioxide-free water obtained by boiling the water specified above for 15 minutes and cooling it with a carbon dioxide absorbing tube (soda lime tube) fitted. pH standard solutions are prepared as directed below but those that are specified in the Measurement Act may be used. Store them in sealed, hard-glass or polyethylene bottles. Since the pH value may change during storage, it is necessary to ascertain, before use, that the solution does have the expected pH value, by comparing it with newly prepared standard solutions.

Oxalate pH Standard Solution Weigh 12.606 g of potassium trihydrogen dioxalate dihydrate for pH determination, previously pulverized in an agate mortar and allowed to stand in a desiccator for at least 18 hours. Dissolve it in a small amount of water, transfer into a 1000-mL volumetric flask, and dilute with water to volume.

Phthalate pH Standard Solution Weigh exactly 10.119 g of potassium hydrogen phthalate for pH determination, previously heated at 120° C for 1 hour and cooled in a desiccator. Dissolve it in a small amount of water, transfer into a 1000-mL volumetric flask, and dilute with water to volume.

Neutral Phosphate pH Standard Solution Weigh 3.390 g of monopotassium dihydrogen phosphate for pH determination, previously heated at 105° C ± 2° C for 2 hours and cooled in a desiccator, and 3.536 g of disodium hydrogenphosphate for pH determination, previously heated at 110° C for 2 hours and cooled in a desiccator. Dissolve them together in a small amount of water, transfer into a 1000-mL volumetric flask, and dilute with water to volume.

Phosphate pH Standard Solution Weigh 1.179 g of monopotassium dihydrogen phosphate for pH determination, previously heated at 105° C ± 2° C for 2 hours and cooled in a desiccator, and 4.302 g of disodium hydrogenphosphate for pH determination, previously heated at 110° C for 2 hours and cooled in a desiccator. Dissolve them together in a small amount of water, transfer into a 1000-mL volumetric flask, and dilute with water to volume.

Borate pH Standard Solution Weigh 3.804 g of sodium tetraborate decahydrate for pH determination, previously pulverized in an agate mortar and left to stand in a desiccator (containing a mixture of saturated sodium bromide solution and sodium bromide) to constant weight. Dissolve it in a small amount of carbon dioxide-free water, transfer into a 1000-mL volumetric flask, and dilute with water to volume.

Carbonate pH Standard Solution Weigh 2.92 g of sodium hydrogen carbonate for

pH determination, previously allowed to stand in a desiccator for 3 hours. Weigh 2.640 g of sodium carbonate for pH determination, previously heated in a platinum crucible at 600° C to constant weight. Dissolve them together in a small amount of carbon dioxide-free water, transfer into a 1000-mL volumetric flask, and dilute with water to volume.

Calcium Hydroxide pH Standard Solution Transfer 5 g of calcium hydroxide for pH determination, previously pulverized, into a flask, add 1000 mL of carbon dioxide-free water, and shake well. Maintain the flask at 23–27° C to saturate thoroughly with calcium hydroxide, and filter the supernatant at the same temperature. Use the clear filtrate (about 0.02 mol/L).

The pH values of these pH standard solutions at various temperatures are shown in the Table below. The pH values at temperatures not indicated in the Table can be calculated from the value in the Table by the interpolation method.

pH values of pH standard solutions

Temperature	Oxalate pH Standard Solution	Phthalate pH Standard Solution	Neutral Phosphate pH Standard Solution	Phosphate pH Standard Solution	Borate pH Standard Solution	Carbonate pH Standard Solution	Calcium Hydroxide pH Standard Solution
0°C	1.67	4.01	6.98	7.53	9.46	10.32	13.43
5°C	1.67	4.01	6.95	7.50	9.39	10.25	13.21
10°C	1.67	4.00	6.92	7.47	9.33	10.18	13.00
15°C	1.67	4.00	6.90	7.43	9.27	10.12	12.81
20°C	1.68	4.00	6.88	7.43	9.22	10.07	12.63
25°C	1.68	4.01	6.86	7.41	9.18	10.02	12.45
30°C	1.69	4.01	6.85	7.40	9.14	9.97	12.30
35°C	1.69	4.02	6.84	7.39	9.10	9.93	12.14
40°C	1.70	4.03	6.84	7.38	9.07		11.99
50°C	1.71	4.06	6.83	7.37	9.01		11.70
60°C	1.73	4.10	6.84		8.96		11.45

Apparatus A pH meter generally consists of a detection unit, which is made up of a glass electrode and a reference electrode, an amplifier to amplify the electromotive force detected, and an indication unit to display the pH value measured. The indication unit is usually fitted with dials for zero and span (sensitivity) adjustments. A temperature compensation dial may be included in some pH meters.

The reproducibility of five consecutive measurements should be within ± 0.05 when the pH is measured for an arbitrary pH standard solution after the detecting unit is washed well with water before each measurement.

Procedure

Immerse the glass electrode in water for several hours before measurement. Switch on the pH meter, and confirm that the system is stable before the start of measurement. Rinse the detecting unit (electrodes) well with water, and blot the water gently with a piece of filter paper.

Calibration The pH meter is calibrated using two pH standard solutions usually as follows: Immerse the detection unit in the neutral phosphate pH standard solution (designated as the first pH standard solution) to adjust the indicated pH to the value corresponding to the temperature with the zero adjustment dial. Next, immerse the detection unit in the second pH standard solution, which should be selected so that the expected pH of the sample solution is between the pH values of the first and second pH standard solutions, and measure the pH in the same manner as for the first pH standard solution. Adjust the indicated pH to the specified pH value using the span adjustment dial when it is not identical to the value corresponding to the temperature. Repeat the above procedure until both pH solutions give pH values within ±0.05 of the corresponding specified values without further adjustment. When a pH meter with a temperature compensation dial is used, set the dial to the temperature of the pH standard solution to be used before starting the calibration procedure. In the case of an apparatus with auto-calibration function, it is necessary to confirm periodically that the pH values of two pH standard solutions are within ± 0.05 of their corresponding specified values.

Measurement After calibration, rinse the detection unit well with water, and blot the water gently with a piece of filter paper. Immerse the detection unit in the test solution, confirm that a stable value is obtained, and read the indicated value.

Notices on Procedure

(1) The structure and operating procedure vary among different pH meters.

(2) Because solutions above pH 11 containing alkali metal ions may give rise to large measurement errors, use an electrode with less alkali error, and make any necessary correction.

(3) The temperature of the test solution must be controlled to be the same as that of the pH standard solution that was used for calibration of the pH meter (within ± 2° C).

Qualitative Tests

The qualitative tests are mainly applied as identification tests. Unless otherwise specified, the concentration of the sample solutions used is about 1%. Generally, tests are done using 2–5 mL of the specified solution in a test tube with an internal diameter

of 8.0–18 mm.

Acetate

(1) Solutions of acetates, when warmed with diluted sulfuric acid (1 in 2), evolve the odor of acetic acid.

(2) Acetates, when heated with sulfuric acid and a small quantity of ethanol (95), evolve the odor of ethyl acetate.

(3) With a neutral solution of an acetate salt (1 in 20), solutions of iron(III) chloride hexahydrate (1 in 10) exhibit a red-brown color and yield a red-brown precipitate by boiling. The precipitate dissolves upon the addition of hydrochloric acid, and the solution turns yellow.

Aluminum Salt

(1) Solutions of aluminum salts (1 in 20) yield with a solution of ammonium chloride (1 in 10) and ammonia TS a white, gelatinous precipitate that does not dissolve in an excess of ammonia TS.

(2) Solutions of aluminum salts (1 in 20) yield with a solution of sodium hydroxide (1 in 25) a white, gelatinous precipitate that dissolves in an excess of sodium hydroxide solution (1 in 25).

(3) When ammonia TS is added to a solution of an aluminum salt until a precipitate is slightly formed, and then 5 drops of a solution of alizarin red S (1 in 1000) are added, the precipitate turns red.

Ammonium Salt

Ammonium salts, when warmed with an excess of sodium hydroxide solution (1 in 25), yield a gas having the odor of ammonia. This gas changes litmus paper (red) moistened with water to blue.

Benzoate

(1) Solutions of benzoates (1 in 20), when acidified by diluted hydrochloric acid (1 in 4), yields a crystalline precipitate. The separated precipitate, when washed well with cold water and dried, melts at about 120–124°C.

(2) Neutral solutions of benzoates (1 in 20) yield with a solution of iron(III) chloride hexahydrate (1 in 10) a light yellow-red precipitate that turns white upon the addition of diluted hydrochloric acid (1 in 4).

Bicarbonate

(1) Bicarbonates effervesce with diluted hydrochloric acid (1 in 4), evolving a gas that yields a white precipitate immediately when passed into calcium hydroxide TS (common with carbonates).

(2) Solutions of bicarbonates (1 in 20) yield with a solution of magnesium sulfate heptahydrate (1 in 10) no precipitate at ordinary temperature but a white precipitate when boiled.

(3) Solutions of bicarbonates remain unchanged or exhibit a faint pink color upon the addition of 1 drop of phenolphthalein TS (distinction from carbonates).

Bromate

(1) Solutions of bromates (1 in 20) acidified with nitric acid yield with 2 to 3 drops of a solution of silver nitrate (1 in 50) a white, crystalline precipitate that dissolves when heated. The resulting solution yields a light yellow precipitate upon the addition of 1 drop of a freshly prepared solution of sodium nitrite (1 in 10).

(2) Solutions of bromates (1 in 20) acidified with nitric acid exhibit a yellow to red-brown color upon the addition of 5 to 6 drops of a freshly prepared solution of sodium nitrite (1 in 10).

Calcium Salt

(1) When the flame coloration test is performed, calcium salts impart a yellowish red color to a colorless flame.

(2) Solutions of calcium salts yield a white precipitate with a solution of ammonium oxalate monohydrate (1 in 30). The separated precipitate does not dissolve in diluted acetic acid (1 in 20) but dissolves upon the subsequent addition of diluted hydrochloric acid (1 in 4).

Carbonate

(1) Carbonates effervesce with diluted hydrochloric acid (1 in 4), evolving a gas that immediately yields a white precipitate when passed into calcium hydroxide TS (common with bicarbonate).

(2) Solutions of carbonates (1 in 20) yield with a solution of magnesium sulfate heptahydrate (1 in 10) a white precipitate that dissolves upon the subsequent addition of diluted acetic acid (1 in 20).

(3) Solutions of carbonates exhibit a red color upon the addition of 1 drop of phenolphthalein TS (distinction from bicarbonates).

Chloride

(1) Solutions of chlorides (1 in 20), when heated with sulfuric acid and potassium permanganate, evolve a gas having the characteristic odor of chlorine. This gas changes the color of potassium iodide–starch paper moistened with water to blue.

(2) Solutions of chlorides yield a white precipitate with a solution of silver nitrate (1 in 50). The separated precipitate that does not dissolve in diluted nitric acid (1 in 10), but dissolves in an excess of ammonia TS.

Chlorite

(1) When 5 mL of diluted hydrochloric acid (1 in 4) is added to 5 mL of a solution of a chlorite (1 in 20), the resulting solution shows a yellow-brown color, evolving a yellow gas.

(2) When 0.1 mL of a solution of potassium permanganate (1 in 300) is added to 5 mL of a solution of a chlorite (1 in 20), and then 1 mL of diluted sulfuric acid (1 in 20) is added to the mixture, the red-purple color of the solution disappears.

Citrate

(1) When 20 mL of a 3:1 mixture of pyridine/acetic anhydride is added to 1 to 2 drops of a solution of a citrate (1 in 20) and the mixture is allowed to stand for 2–3 minutes, the solution shows a red-brown color.

(2) A white precipitate is produced when solutions of citrates are treated in the following manner: Neutralize a solution of a citrate salt (1 in 10), add an equal volume of 10% sulfuric acid TS, then two-thirds volume of a solution of potassium permanganate (1 in 300), heat the mixture until its color disappears, and add dropwise an amount of bromide TS equivalent to one tenth of the total volume.

Copper(II) Salt

(1) When a well-polished iron fragment is allowed to stand in acidic solutions of copper(II) salts in hydrochloric acid, a yellow-red metallic film is deposited on the surface of the fragment.

(2) Solutions of copper(II) salts yield with a small quantity of ammonia TS a light blue precipitate that dissolves in an excess of ammonia TS, producing a dark blue solution.

(3) Solutions of copper(II) salts, when treated with a freshly prepared solution of potassium hexacyanoferrate(II) trihydrate (1 in 10), yield a red-brown precipitate. The precipitate does not dissolve in diluted acetic acid (1 in 20), but dissolves in ammonia TS, producing a dark blue solution.

Glycerophosphate

(1) Solutions of glycerophosphates yield with ammonium molybdate TS no precipitate at cold temperatures, but yield a yellow precipitate when boiled for a long time.

(2) Glycerophosphates evolve the pungent odor of acrolein, when treated in the following manner: Add an equal volume of powdered potassium hydrogen sulfate to glycerophosphates and gently heat the resulting mixture over an open flame.

Hypochlorite

(1) When 2 mL of hydrochloric acid is added to 5 mL of a solution of a hypochlorite,

effervescence occurs with the evolution of a gas.

(2) When 1 mL of a solution of sodium hydroxide (1 in 2500) and 0.2 mL of potassium iodide TS are added to 5 mL of a solution of a hypochlorite (1 in 1000), the resulting solution is yellow. The color of the solution changes to a deep blue upon the subsequent addition of 0.5 mL of starch TS.

(3) When 5 mL of a solution of a hypochlorite (1 in 4) is treated with 0.1 mL of a solution of potassium permanganate (1 in 300) and then with 1 mL of a diluted sulfuric acid (1 in 20), the red-purple color of the solution does not fade (distinction from chlorites).

Iron(II) Salt

(1) Weakly acidic solutions of iron(II) salts, when treated with a freshly prepared solution of potassium hexacyanoferrate(III) (1 in 10), yield a blue precipitate that does not dissolve upon the addition of diluted hydrochloric acid (1 in 4) nor diluted nitric acid (1 in 10).

(2) Solutions of iron(II) salts yield a white, gelatinous precipitate (the color changes rapidly to grayish green and then gradually to red-brown when shaken) with a solution of sodium hydroxide (1 in 25) or ammonia TS. Upon the subsequent addition of sodium sulfide TS, a black precipitate is produced, and the separated precipitate dissolves in diluted hydrochloric acid (1 in 4).

Iron(III) Salt

(1) Weakly acidic solutions of iron(III) salts, when treated with a freshly prepared solution of potassium hexacyanoferrate(II) trihydrate (1 in 10), yield a blue precipitate that does not dissolve upon the addition of diluted hydrochloric acid (1 in 4) nor diluted nitric acid (1 in 10).

(2) Solutions of iron(III) salts yield a red-brown, gelatinous precipitate with a solution of sodium hydroxide (1 in 25) or ammonia TS. The color of the precipitate changes to black upon the subsequent addition of sodium sulfide TS. The separated precipitate dissolves in diluted hydrochloric acid (1 in 4), producing a white turbidity.

(3) Neutral to weakly acidic solutions of iron(III) salts exhibit with a solution of ammonium thiocyanate (2 in 25) a red color that remains unchanged upon the subsequent addition of hydrochloric acid.

Lactate

When a solution of potassium permanganate (1 in 50) is added to a solution of a lactate salt (1 in 20) acidified with sulfuric acid, and the mixture is heated, the odor of acetaldehyde is evolved.

Magnesium Salt

Solutions of magnesium salts yield no precipitate with a solution of ammonium chloride (1 in 10) and ammonium carbonate TS, but yield a white, crystalline precipitate upon the subsequent addition of a solution of disodium hydrogenphosphate dodecahydrate (1 in 10). The separated precipitate does not dissolve in ammonia TS.

Nitrate

(1) When a solution of nitrate is mixed well with an equal volume of sulfuric acid, then cooled, and iron(II) sulfate TS is superimposed, a dark brown ring is produced at the junction of the two liquids.

(2) Acidic solutions of nitrates in sulfuric acid do not decolorize the red-purple color of a solution of potassium permanganate (1 in 300) (distinction from nitrites).

Nitrite

(1) Solutions of nitrites (1 in 20), when acidified with diluted sulfuric acid (1 in 20), yield a yellow-brown gas having a characteristic odor. The resulting solutions produce a dark brown color upon the subsequent addition of a small amount of iron(II) sulfate heptahydrate crystals.

(2) Solutions of nitrites exhibit a yellowish-brown color and then yield a blackish-purple precipitate, when 2 to 3 drops of potassium iodide TS are added and then diluted hydrochloric acid (1 in 4) is added dropwise. The resulting solutions exhibit a deep blue color upon the subsequent addition of starch TS.

Peroxide

(1) When solutions of peroxides are mixed with an equal volume of ethyl acetate and 1 or 2 drops of a solution of potassium dichromate (3 in 40), and the mixture is acidified with diluted sulfuric acid (1 in 20) shaken immediately, and allowed to stand, the ethyl acetate layer exhibits a blue color.

(2) Acidic solutions of peroxides in sulfuric acid decolorize a solution of potassium permanganate (1 in 300) added dropwise, producing effervescence.

Phosphate (Orthophosphate)

(1) Neutral solutions of phosphates yield with a solution of silver nitrate (1 in 50) a yellow precipitate that dissolves upon the addition of diluted nitric acid (1 in 10) or ammonia TS.

(2) Neutral solutions of phosphates or acidic solutions of phosphates in nitric acid yield with ammonium molybdate TS a yellow precipitate upon warming. This precipitate dissolves upon the addition of a solution of sodium hydroxide (1 in 25) or ammonia TS.

Potassium Salt

(1) When the flame coloration test is performed, potassium salts impart a pale purple color to a colorless flame. When the flame exhibits a yellow color, a red-purple color is seen through cobalt glass.

(2) Neutral solutions of potassium salts (1 in 20), when treated with a freshly prepared solution of sodium hydrogen (+)-tartrate monohydrate (1 in 10), yield a white, crystalline precipitate (the formation of the precipitate is accelerated by rubbing the inside wall of the test tube with a glass rod). The separated precipitate dissolves in ammonia TS, in a solution of sodium hydroxide (1 in 25), or in a solution of sodium carbonate (1 in 8).

Sodium Salt

(1) When the flame coloration test is performed, sodium salts impart a yellow color to a colorless flame.

(2) Neutral solutions of sodium salts (1 in 20) yield a white, crystalline precipitate with potassium hexahydroxoantimonate(V) pyroantimonate TS (the formation of the precipitate is accelerated by rubbing the inside wall of the test tube with a glass rod).

Succinate

When 1 mL of a solution of iron(III) chloride hexahydrate (1 in 10) is added to 5 mL of a solution of a succinate (1 in 20), previously adjusted to pH 6 to 7, a brown precipitate is formed.

Sulfate

(1) Solutions of sulfates yield with a solution of barium chloride dihydrate (3 in 25) a white precipitate that does not dissolve upon the addition of hydrochloric acid or diluted nitric acid (1 in 10).

(2) Neutral solutions of sulfates yield with lead(II) acetate TS a white precipitate that dissolves upon the addition of a solution of ammonium acetate (1 in 10).

(3) When an equal volume of diluted hydrochloric acid (1 in 4) is added to solutions of sulfates, no white turbidity (distinction from thiosulfates) nor the odor of sulfur dioxide is produced (distinction from sulfites).

Sulfite and Bisulfite

(1) When iodine–potassium iodide TS is added dropwise to acidic solutions of sulfites or bisulfites in acetic acid, the color of the TS disappears.

(2) Solutions of sulfites or bisulfites (1 in 20) acidified with acetic acid, when treated with an equal volume of diluted hydrochloric acid (1 in 4), evolve a sulfur dioxide odor and the resulting solutions yield no turbidity. Upon the subsequent addition of 1 drop of sodium sulfide TS, the solutions immediately yield a white turbidity that changes to

a yellow precipitate.

Tartrate

(1) Neutral solutions of tartrates (1 in 20) yield a white precipitate with a solution of silver nitrate (1 in 50). The separated precipitate dissolves in nitric acid. This precipitate dissolves when warmed with ammonia TS, and metallic silver is deposited gradually.

(2) Solutions of tartrates exhibit a red-purple to purple color when treated in the following manner: To a solution of a tartrate salt (1 in 20), add 2 drops of diluted acetic acid (1 in 4), 1 drop of iron(II) sulfate TS, 2 to 3 drops of hydrogen peroxide TS, and then an excess of a solution of sodium hydroxide (1 in 25).

(3) When 5 mL of sulfuric acid, previously mixed with 2 to 3 drops of a solution of resorcinol (1 in 50) and 2 to 3 drops of a solution of potassium bromide (1 in 10), is added to 2 to 3 drops of a solution of a tartrate salt (1 in 20), and then the mixture is heated for 5 to 10 minutes on a water bath, a dark blue color is produced. Then, when the resulting dark blue solution, previously cooled, is poured into an excess of water, a red color is produced.

Thiocyanate

(1) Solutions of thiocyanates yield a white precipitate with an excess of a solution of silver nitrate (1 in 10). The separated precipitate does not dissolve in diluted nitric acid (1 in 10), but dissolves in ammonia solution.

(2) Solutions of thiocyanates exhibit with a solution of iron(III) chloride hexahydrate (1 in 10), a red color that does not fade with hydrochloric acid.

Zinc Salt

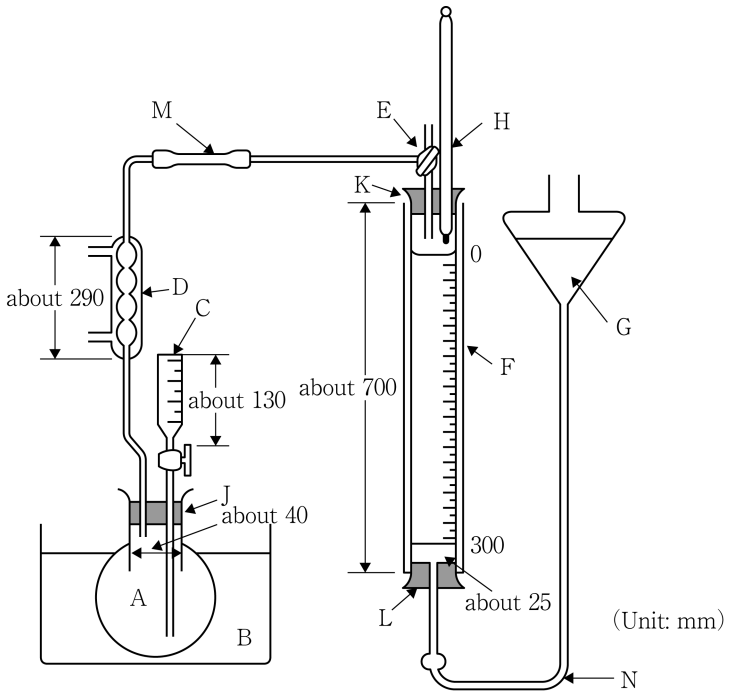
(1) Neutral to alkaline solutions of zinc salts yield a whitish precipitate with ammonium sulfide TS or sodium sulfide TS. The separated precipitate does not dissolve in diluted acetic acid (1 in 20), but dissolves upon the subsequent addition of diluted hydrochloric acid (1 in 4).

(2) Solutions of zinc salts, when treated with a freshly prepared solution of potassium hexacyanoferrate(II) trihydrate (1 in 10), yield a white precipitate. The separated precipitate does not dissolve in diluted hydrochloric acid (1 in 4), but dissolves in sodium hydroxide solution (1 in 25).

Quantitative Test for Generated Gas

The quantitative test for generated gas is designed to measure the quantity of gas generated from Baking Powder.

Apparatus Use the apparatus as illustrated in the figure



A: Round-bottom flask for gas generation (about 300 mL capacity)
B: Water bath
C: Acid-dropping funnel
D: Condenser
E: Three-way stopcock
F: Gas buret with outer tube (about 300 mL capacity, with 1 mL divisions)
G: Leveling bottle (about 400 mL capacity)
H: Thermometer
J, K, and L: Rubber stoppers
M and N: Rubber tubes

Preparation of Replacing Solution Weigh 100 g of sodium chloride, dissolve in 350 mL of water, add 1 g of sodium hydrogen carbonate, then add diluted hydrochloric acid (1 in 3) until the solution shows slight acidity to methyl orange TS.

Procedure Wrap 2.0 g of the sample (in the case of Duplex Baking Powder, use the mixture in the same ratio as specified for use) in paper that does not inhibit measurement, like *washi* (which is traditional Japanese paper), and place into flask A for gas generation containing 100 mL of water. Connect the apparatus, open three-way stopcock E, and move leveling bottle G vertically to adjust the level of the replacing solution inside to the zero mark on the scale of gas buret F. Allow water to flow through condenser D, turn three-way stopcock E and, after the path is opened between

condenser D and gas buret F, add 20 mL of diluted hydrochloric acid (1 in 3) dropwise from dropping funnel C, and then immediately close the cock of the dropping funnel. While occasionally shaking the flask gently, heat it in a water bath at 75° C, and lower leveling bottle G according to the level of gas buret F. After 3 minutes, when the levels of the replacing solution in gas buret F and leveling bottle G are equal, read the level on scale V (mL), and read the temperature (t° C) of the generated gas with thermometer H. Calculate the volume of generated gas V_0 (mL) in a normal state by the formula given below. Determine the blank value v (mL) in the same manner, and make any necessary correction.

$$V_0 \text{ (mL)} = (V - v) \times \frac{P - p}{101} \times \frac{273}{273 + t}$$

P = atmospheric pressure (kPa) at the time of measurement,
p = vapor pressure (kPa) of water at t° C.

Readily Carbonizable Substances Test

This test is designed to demonstrate that the content of impurities in a sample which are readily colored by the addition of sulfuric acid does not exceed the acceptable limit specified in the individual monograph.

Procedure Unless otherwise specified, proceed as directed below.

Before use, wash thoroughly a colorless, hard-glass test tube with sulfuric acid for the readily carbonizable substances test. Unless otherwise specified, when the sample is a solid, place 5 mL of sulfuric acid for the readily carbonizable substances test into the test tube, add the specified amount of powdered sample in small portions, and dissolve completely by stirring with a glass rod. When the sample is a liquid, measure the specified amount of the sample, and place into the test tube. Add 5 mL of sulfuric acid for the readily carbonizable substances test, and mix by shaking. Cool the tube if the temperature of the content in the tube rises. If the reaction may be affected by the temperature, maintain it at the standard temperature. Allow to stand for 15 minutes. Place the Matching Fluid, specified in the individual monograph, into another test tube of the same quality and form as used for the sample, and use this solution as the control solution. Compare the color with that of the control solution against a white background by examining from above and from the side. The color of the sample is not deeper than that of the control solution.

When the specification “to dissolve the sample in sulfuric acid while heating” is given, place the sample and sulfuric acid in the test tube, heat as directed in the individual monograph, and compare the color with that of the control solution.

Refractive Index

This test is designed to measure the ratio of the velocity of light in air to that in a sample. Generally, when light passes through air into the sample, the direction changes at the boundary surface. This phenomenon is called refraction. The refractive index is the ratio of the sine of the angle of incidence, *i*, to that of the angle of refraction, *r*, in the refraction phenomenon. "In air" means a place where air with atmospheric pressure exists. The D line of the sodium spectrum is used for measurement. The refractive index, which varies with the wavelength of the light used and with the temperature, is expressed as n_D^t, where t is the temperature (° C) at measurement and D refers to the D line. In isotropic substances, the refractive index is a constant unique to each substance at a definite wavelength, temperature, and pressure. Therefore, this measurement is applicable to purity tests of substances.

A refractometer with a measurement range from 1.300 to 1.700 and an attainable precision of 0.0001 is used to measure the refractive index, usually an Abbe refractometer. Measurement is taken at a temperature in the range of ± 0.2° C of that specified in the individual monograph.

Residue on Ignition

This test is designed to measure the amount of the substance left when a sample is ignited with sulfuric acid.

In the Monographs, the specification "not more than 0.5%" for this test, for example, means that the residue in weight must be not more than 0.5% of the weight of the sample when determined using 1 to 2 g of the sample, accurately weighed, according to the procedure given below. Also, the specification "not more than 7.0% (3 g, 800° C, 15 minutes, on the dried basis)" means that the residue in weight must be not more than 7.0% of the weight of the sample on the dried basis when determined according to the following manner: *About 3 g of the sample is accurately weighed, the test is performed as directed under Procedure below, and the residue is ignited in the* electric furnace *at 800° C for 15 minutes, instead of 600 ± 50°C for 3 hours*." When the stipulation "dried sample" is given in the Monographs, the sample to be used for the test should be previously dried under the conditions specified for the loss on drying test in the individual monograph.

Procedure Ignite a platinum, quartz, or porcelain crucible at 600 ± 50° C or under the conditions specified in the individual monograph for 30 minutes or more, allow to cool in a desiccator, and weigh accurately.

If the sample consists of large crystals or lumps, quickly grind them to a size not

exceeding about 2 mm in diameter. Unless otherwise specified, place 1 to 2 g of the ground sample in the crucible, and weigh accurately. Moisten the sample with a small amount (usually 1 mL) of sulfuric acid, ignite slowly at as low a temperature as practicable until the sample is almost charred, and allow to cool. Add 1 mL of sulfuric acid, and heat slowly until white fumes are no longer evolved. Transfer the crucible into an electric furnace, and unless otherwise specified, ignite it at 600 ± 50° C for 3 hours. Cool the crucible in a desiccator, and then weigh accurately. When the amount of the residue such obtained exceeds the limit specified in the individual monograph, unless otherwise specified, repeat the above procedure (moistening with sulfuric acid, heating, and 30-minute ignition). When the difference between two consecutive weighings is not more than 0.5 mg or not more than the standard value specified in the individual monograph, finish the procedure.

Specific Gravity

Specific gravity refers to the ratio of the mass of a substance to that of an equal volume of a standard substance. The specific gravity $d_t^{t'}$ means the ratio of the mass of sample at t′° C to that of an equal volume of water at t° C. Unless otherwise specified, the measurement of specific gravity is performed using Method 1, Method 2, or Method 4. When the figure specified is accompanied by the word "about," Method 3 is also applicable.

Method 1. Measurement by Pycnometer

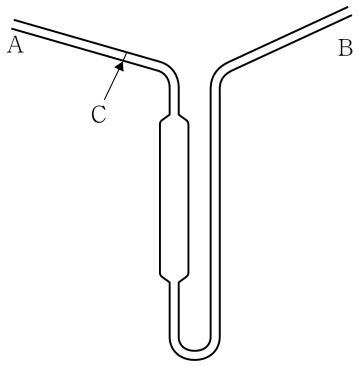
A pycnometer is a glass container with a capacity of usually 10 mL to 100 mL. It has a ground-glass stopper fitted with a thermometer and a side inlet-tube with a marked line and a ground-glass cap.

Weigh accurately a pycnometer, previously cleaned and dried, and record the mass (M). Remove the stopper and the cap, fill the pycnometer with the sample, keep at a temperature 1–3° C lower than the specified temperature (t′° C), and close the stopper, taking care not to leave any bubbles. Raise the temperature gradually until the thermometer shows the specified temperature. Remove the sample above the mark from the side tube, and close the cap. Wipe the outside surface thoroughly, weigh accurately, and record the mass (M_1). Perform the same procedure with water using the same pycnometer, and record the mass (M_2) at the specified temperature (t° C). Calculate the specific gravity ($d_t^{t'}$) by the formula:

$$d_t^{t'} = \frac{M_1 - M}{M_2 - M}$$

Method 2. Measurement by Sprengel-Ostwald Pycnometer

The Sprengel-Ostwald pycnometer (shown in the figure) usually has a capacity of 1 to 10 mL, and both ends are thick-walled fine tubes, one (A) of which has the marked line (C).



Weigh accurately a cleaned and dried pycnometer, and record the mass (M). Immerse fine tube B without a marked line in the sample kept at a temperature 3–5° C lower than the specified temperature. Attach a rubber tube or a ground-glass tube to the end of A, and suction up the sample gently until it comes up above marked line C, taking care to prevent bubble formation. Immerse the pycnometer in a water bath kept at the specified temperature (t′° C) for 15 minutes, and by attaching a piece of filter paper to the end of B, adjust the end of the sample to marked line C. Remove the pycnometer from the water bath, wipe the water off, weigh accurately, and record the mass (M_1). Perform the same determination with water using the same pycnometer. Weigh accurately the pycnometer containing water at the specified temperature (t° C), and record the mass (M_2). Calculate the specific gravity ($d_t^{t'}$) by the formula:

$$d_t^{t'} = \frac{M_1 - M}{M_2 - M}$$

Method 3. Measurement by Hydrometer

Use a hydrometer for the specified temperature with the required precision. Before using, clean the hydrometer with ethanol (95) or diethyl ether.

Shake the sample well. After the bubbles disappear, place the hydrometer in the sample. When the hydrometer comes to a standstill at the specified temperature, read the specific gravity at the upper brim of the meniscus. If a reading instruction is provided by the manufacturer, follow those instructions.

Method 4. Measurement by Oscillating Transducer Density Meter

This method is designed to determine the specific gravity of a liquid or gaseous sample from the mass of reference substances by measuring the density of the sample from the intrinsic oscillation period (T (s)) of a cell filled with the sample. When a cell containing

the sample to be examined is oscillated, it undergoes an oscillation with an intrinsic oscillation frequency, depending on the mass of the sample. If the volume of the oscillating part of the sample cell is constant, the relation of the square of the intrinsic oscillation period and density of the sample is linear.

To measure the sample density using this method, the respective intrinsic oscillation periods (T_{Sl} and T_{S2}) for two reference substances (density: ρ_{Sl}, ρ_{S2}) should be previously measured at the specified temperature (t′° C), and the cell constant $K_{t'}$ ($g \cdot cm^{-3} s^{-2}$) should be determined by the formula:

$$K_{t'} = \frac{\rho_{S1}^{t'} - \rho_{S2}^{t'}}{T_{S1}^{\ 2} - T_{S2}^{\ 2}}$$

Usually, water and dried air are used as reference substances. Here, the density of water ($\rho_{S1}^{t'}$) at the temperature (t′° C) is obtained from the table above, and that of dried air ($\rho_{S2}^{t'}$) is calculated by the formula:

$$\rho_{S1}^{t'} = 0.0012932 \times \frac{273.15}{273.15 + t'} \times \frac{p}{101.325}$$

in which the pressure of dried air is at p kPa.

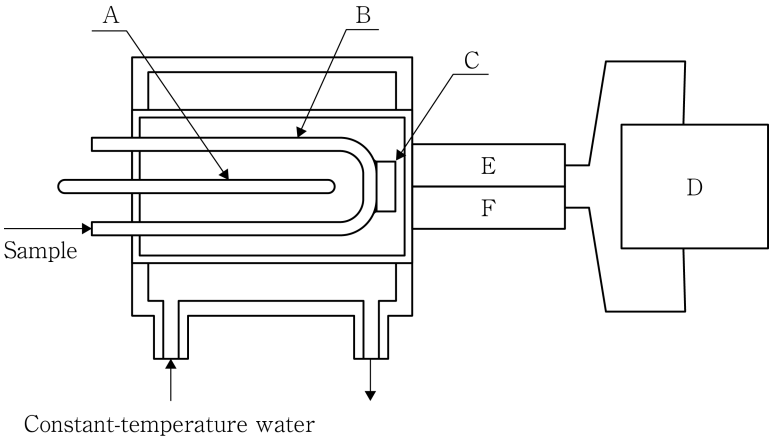
When the sample is introduced into a sample cell having a known cell constant and the intrinsic oscillation period (T_T) of the sample is measured under the same operating conditions as used for the reference substances, the density of the sample ($\rho_T^{t'}$) can be determined by the following formula using the intrinsic oscillation period of the reference substance (T_{Sl}) and the density of water ($\rho_{S1}^{t'}$) at the specified temperature (t′° C).

$$\rho_T^{t'} = \rho_{S1}^{t'} + K_{t'}(T_T^{\ 2} - T_{S1}^{\ 2})$$

The specific gravity of the sample $\left(d_t^{t'}\right)$ corresponding to water of a temperature of t° C can be determined by the formula below, using the density of water (ρ_{S1}^{t}) at the temperature (t° C) indicated in the table.

$$d_t^{t'} = \frac{\rho_T^{t'}}{\rho_{S1}^{t}}$$

Apparatus An oscillating transducer density/specific gravity meter is usually composed of a U-shaped glass sample cell, an oscillator to give an initial vibration to the sample cell, a detection system to measure the intrinsic vibration period, and a temperature controlling system. The sample cell is of an inner volume of about 1 mL and is fixed by its closed end. The structure of the sample cell chamber and its surrounding is illustrated in the figure.



A: Thermometer　　D: Amplifier
B: Sample cell　　E: Detector
C: Vibration plate　　F: Vibrator

Procedure　Adjust the sample cell, water, and the sample to the specified temperature (t′° C). Wash the sample cell with water or an appropriate solvent, and then dry thoroughly with a stream of dried air. Stop the flow of dried air, confirm that the temperature is maintained constant, and then measure the intrinsic oscillation period (T_{S2}) given by the dried air. Separately, measure the atmospheric pressure (p kPa) at the place of determination. Next, introduce water into the sample cell and measure the intrinsic oscillation period (T_{S1}) of the water. Determine the cell constant ($K_{t'}$) using the intrinsic oscillation periods of water and dried air obtained by the above-mentioned formula.

Next, introduce the sample into the glass cell, confirm that the temperature is maintained constant, and measure the intrinsic oscillation period T_T of the sample. Obtain the density of the sample ($\rho_T^{t'}$), using the intrinsic oscillation periods of water and the sample, the density of water ($\rho_{S1}^{t'}$), and the cell constant ($K_{t'}$). If necessary, calculate the specific gravity of the sample ($d_t^{t'}$) corresponding to water at the temperature (t° C), using the density of water ($\rho_T^{t'}$) given in the table.

When introducing the sample or water into the cell, take care to avoid the formation of bubbles in the sample cell.

Temperature °C	Density g/cm^3	Temperature °C	Density g/cm^3	Temperature °C	Density g/cm^3	Temperature °C	Density g/cm^3
0	0.99984	10	0.99970	20	0.99820	30	0.99565
1	0.99990	11	0.99961	21	0.99799	31	0.99534
2	0.99994	12	0.99950	22	0.99777	32	0.99503
3	0.99996	13	0.99938	23	0.99754	33	0.99470
4	0.99997	14	0.99924	24	0.99730	34	0.99437
5	0.99996	15	0.99910	25	0.99704	35	0.99403
6	0.99994	16	0.99894	26	0.99678	36	0.99368
7	0.99990	17	0.99877	27	0.99651	37	0.99333
8	0.99985	18	0.99860	28	0.99623	38	0.99297
9	0.99978	19	0.99841	29	0.99594	39	0.99259

Sulfate Limit Test

The sulfate limit test is designed to demonstrate that the content of sulfate in an additive does not exceed the acceptable limit specified in the individual monograph.

In the Monographs, the specification "not more than 0.024% as SO_4 (1.0 g, Control Solution: 0.005 mol/L sulfuric acid 0.50 mL)" for this test, for example, means that the sulfate content of the substance must be not more than 0.024% as SO_4 when determined according to the following manner: *The test solution and the control solution are prepared with 1.0 g of the test substance and 0.50 mL of 0.005 mol/L sulfuric acid, respectively, as directed below, and the test is performed as directed in the procedure.*

Preparation of Test Solutions and Control Solutions Unless otherwise specified, proceed as directed below.

Test Solution When the quantity of sample is only specified, measure the specified amount of sample, transfer it into a Nessler tube, and dissolve in about 30 mL of water. Neutralize the solution with diluted hydrochloric acid (1 in 4) if the solution is alkaline. Add 1 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

When the test solution is prepared using the sample solution prepared as directed in the individual monograph, transfer the specified quantity of the sample solution into a Nessler tube, add 1 mL of diluted hydrochloric acid (1 in 4) and then water to make 50 mL.

Control Solution Measure the specified quantity of 0.005 mol/L sulfuric acid, and transfer it into another Nessler tube. Add 1 mL of diluted hydrochloric acid (1 in 4) and water to make 50 mL.

If the test solution is not clear, filter both the test and control solutions under the

same conditions.

Procedure Unless otherwise specified, add 2 mL of barium chloride dihydrate solution (3 in 25) to each of the test and control solutions, mix thoroughly, and allow to them stand for 10 minutes. Examine both Nessler tubes from above and from the side against a black background, and compare the turbidity. The turbidity developed in the test solution is not thicker than that of the control solution.

Sulfite Determination

Sulfite determination is designed to determine the quantity of sulfites from the quantity of iodine required to react with sulfites. In the test, sulfites are made to react with iodine, the excess iodine is back-titrated with sodium thiosulfate, and the amount of iodine required for the reaction is calculated.

Procedure Unless otherwise specified, proceed as directed below.

Weigh accurately the specified amount of sample, transfer it into a ground-glass stoppered Erlenmeyer flask containing exactly 50 mL of 0.05 mol/L iodine, and dissolve. Stopper the flask, allow to stand for 5 minutes, and add 2 mL of diluted hydrochloric acid (2 in 3). Titrate the excess iodine with 0.1 mol/L sodium thiosulfate (indicator: 1–3 mL of starch TS). Starch TS should be added when the solution turns light yellow near the endpoint. The endpoint is when the blue color of the solution disappears. Separately perform a blank test.

Thin-Layer Chromatography

Thin-layer chromatography is designed to separate the individual components of a mixture by developing in a mobile phase, using a thin-layer made of a suitable immobile phase. This method is generally applicable to identification tests and purity tests.

Preparation of Thin-Layer Plate Unless otherwise specified, prepare a thin-layer plate by the following method.

Using suitable instruments, make a suspension by adding an adequate amount of water to the specified solid support. Apply this suspension on a 50 mm×200 mm or 200 mm×200 mm, uniform-thickness, smooth glass plate to make a uniform layer of 0.2–0.3 mm in thickness. After air-drying, dry further under the specified conditions. Store plates, protected from moisture. If necessary, heat the plates before use to dry. A

suitable plastic plate may be used instead of a glass plate. Commercially available thin-layer plates prepared by applying solid supports specified in the individual monographs on glass plates, plastic plates, or aluminum sheets may also be used.

Procedure Unless otherwise specified, proceed as directed below.

Designate a line about 20 mm distant from one end of the thin-layer plate as the starting line. Using a suitable tool like micropipette, apply the specified volumes of the test solution and control solution on the starting line at least 10 mm apart from each other and at least 10 mm distant from both edges of the plate so that spots with about 3 mm in diameter are produced. Air-dry the plates. Place the plate in a developing container with the starting line down, and tightly seal the container. The developing container should contain the specified developing solvent up to a depth of 10 mm, and should be saturated with the vapor of the solvent. When the solvent front has ascended from the starting line to the specified distance, take the plate out of the container, and air-dry. Examine and compare the location, color, and other characteristics of each spot obtained from the test solution and the control solution by the specified method. Calculate the R_f value by the formula:

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

Ultraviolet-Visible Spectrophotometry

Ultraviolet-visible spectrophotometry is designed to measure the degree of absorption of light, wavelength of 200 nm to 800 nm, by a substance. This method is used for the identification of substances and for purity and assay tests. When an atomic absorption spectrophotometer is used for these purposes, proceed as directed under Atomic Absorption Spectrophotometry. The ultraviolet and visible absorption spectra of a solution of a substance depend on the chemical structure of the substance. Spectrophotometry is used to identify a substance by measuring the absorption of the substance at various wavelengths. This method is applicable to identification tests, purity tests, and assays in which the absorbance of a solution at a given concentration is usually measured at the maximum absorption wavelength (λ_{max}) or the minimum absorption wavelength (λ_{min}).

When monochromatic light passes through a solution, the ratio of the transmitted light intensity (I) to the incident light intensity (I_0) is called transmittance (t), the transmittance expressed in percentage is called transmission rate (T), and the common logarithm of the reciprocal of transmittance is called absorbance (A).

$$t = \frac{I}{I_0} \qquad T = \frac{I}{I_0} \times 100 = 100t \qquad A = \log (I_0/I)$$

Absorbance (A) is proportional to the concentration (c) of the solution and the length (*l*) of the layer of the solution through which the light passes. The length of the layer (the length of the layer of the solution measured) is also called light path length or cell length.

$A = kcl$ (k: constant)

Calculated on the basis that *l* is 1 cm and c is a 1% (w/v) solution of a light-absorbing substance, the absorbance is called specific absorbance ($E^{1\%}_{1cm}$); calculated on the basis that *l* is 1 cm and c is a 1 mol/L solution of a light-absorbing substance, the absorbance is called molar absorption coefficient (ε). The molar absorption coefficient at the wavelength of maximum absorption is expressed as ε_{max}.

Use the following formulae to obtain $E^{1\%}_{1cm}$ or ε.

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

l = the length of the solution layer (cm),

a = the measured absorbance value,

c (%) = the concentration of the solution (% (w/v)),

c (mol) = the molarity of the solution (mol/L).

In the Monographs, the specification "$E^{1\%}_{1cm}$ (265 nm): 445–485" for this test, for example, means that $E^{1\%}_{1cm}$ is 445–485 when determined at a wavelength of 265 nm by the specified spectrophotometric procedure.

Apparatus and Adjustment Absorption is measured using a spectrophotometer or a photoelectric photometer. There are two types of light measurement modes: the single-beam mode and the double-beam mode. The single beam spectrophotometer takes measurements in the order of the reference substance and the sample in the single beam. The double beam spectrophotometer takes measurements at the same time on the reference and the sample placed in separate light paths.

After the spectrophotometer or photoelectric photometer is adjusted according to the operation manual of the instrument, it should be confirmed that the wavelength and the transmission rate meet the specifications for tests given below.

Calibration of the wavelength should be carried out as follows: Using an optical filter for wavelength calibration, determine the transmission rate in the vicinity of the standard wavelength value given in the test certificate accompanying each filter, under the test conditions given in the test certificate. Read a wavelength value at which the minimal transmission rate is exhibited. The difference between the measured wavelength and the standard wavelength should be within ± 0.5 nm. When the measurement is repeated three times, each value obtained should be within a mean of

± 0.2 nm. Testing can be carried out, using a deuterium discharge lamp at bright line wavelengths of 486.00 nm and 656.10 nm or a low-pressure mercury lamp at bright line wavelengths of 253.65 nm, 365.02 nm, 435.84 nm, and 546.07 nm. In the case of these tests, the difference between the measured wavelength and the wavelength of the bright line should be within ± 0.3 nm. When the measurement is repeated three times, each value obtained should be within a mean of ± 0.2 nm.

Calibration of the transmission rate or absorbance should be carried out as follows: Using an optical filter for transmission rate calibration, determine the transmission rate at the standard wavelength value given in the test certificate accompanying each filter under the test conditions given in the test certificate. The difference between measured transmission rate and the standard transmission rate should be within a range of that goes from 1% larger of the upper limit to 1% smaller of the lower limit for the relative accuracy shown in the test certificate. When the measurement is repeated three times, each absorbance value obtained (or value calculated from the transmission rate) should be within a mean of ± 0.002 when the absorbance is not more than 0.500 and within a mean of ± 0.004 when the absorbance is more than 0.500. In addition, it will be desirable to confirm the linearity of the transmission rate at the same wavelength using several optical filters for transmission rate calibration with different transmission rate values.

Procedure After the apparatus is adjusted as directed under the heading of Apparatus and adjustment, select and set the light source, detector, mode of measurement, measurement wavelength or wavelength range, spectrum width, and scan speed.

Subsequently, allow the apparatus to stand for a certain time to confirm its stability. Then, adjust the apparatus so that the transmission rate is 0% at a measurement wavelength or over the measurement wavelength range after shutting the sample side of light path. Then open the shutter and adjust the transmission rate to 100% (the absorbance is zero).

Usually, prior to the testing of samples, the transmission rate is adjusted to 100% (the absorbance is zero) by putting the blank (cells containing the reference solution) the light path. For the reference solution, unless otherwise specified, the solvent used in the test is used.

Then perform the measurement with the cell containing the sample solution, and record the absorbance reading at the measurement wavelength, or measure the spectrum over the measurement wavelength range. Unless otherwise specified, a cell with a path length of 1 cm, made of quartz for ultraviolet range and of quartz or glass for visible range, is used. Special consideration should be given to the absorption by solvents in the ultraviolet range; use a solvent which does not disturb measurement.

The concentration of the solution to be used should be such that the absorbance

obtained is in the range of 0.2–0.7 when a single-beam absorption photometer is used or in the range of 0.4–1.4 when a double-beam absorption photometer is used. If its absorbance is higher than the upper limit of the range, dilute it with the solvent to an appropriate concentration and measure again.

Viscosity

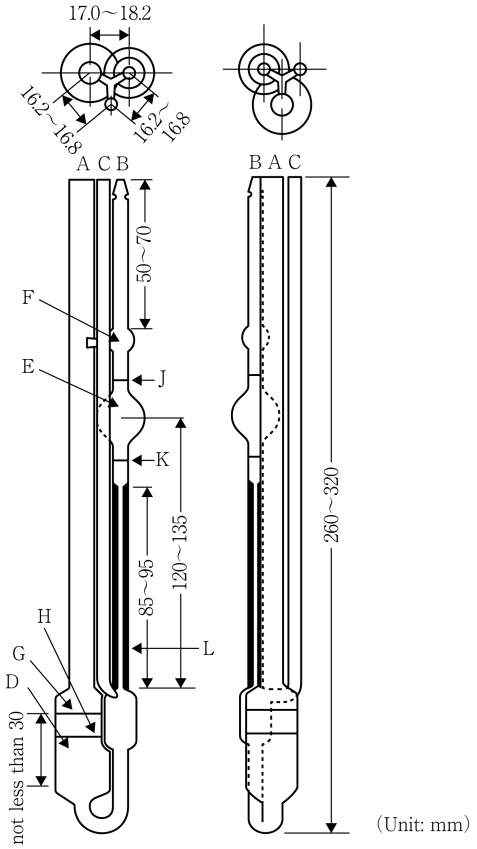
This test is designed to determine the kinematic viscosity and (absolute) viscosity of a sample, using a viscometer. The units are usually millimeters squared per second (mm^2/s) and milli-Pascal second (mPa・s), respectively.

Method 1　Viscosity Measurement by Capillary Tube Viscometer

The method is used to determine the kinematic viscosity of Newtonian liquids. Kinematic viscosity is calculated by measuring the time required for a definite volume of a liquid to flow down through a capillary tube.

Apparatus　Unless otherwise specified, use an Ubbelohde-type viscometer, outlined below.

A, B, C: Tube
D, E, F: Bulb
G, H, J, K: Mark
L: Capillary



The table below gives the approximate relations between the internal diameters of the

capillary tubes and the kinematic viscosity ranges suitable for measurements.

Although the internal diameters of the capillary tubes need not be exactly the same as shown in the Table, a viscometer should be selected so that the flow time ranges between 200 seconds and 1000 seconds.

Internal diameter of capillary tubes (mm) (Permissible tolerance: ± 10%)	Range of kinematic viscosity (mm^2/s)
0.58	2–10
0.73	6–30
0.88	10–50
1.03	20–100
1.36	60–300
1.55	100–500
1.83	200–1000
2.43	600–3000
2.75	1000–5000
3.27	2000–10,000
4.32	6000–30,000
5.20	10,000–50,000
6.25	20,000–100,000

Procedure Transfer the sample into tube A, being careful to prevent the formation of bubbles in the sample solution, and adjust the meniscus of the sample so that the upper surface comes to between marks G and H on bulb D, when the viscometer is placed upright.

Place the viscometer in a thermostatic water bath maintained at the specified temperature (± 0.1° C) so that bulb F of tube B is completely immersed in water.

Fix the viscometer vertically, and allow to stand for about 20 minutes until the sample reaches the specified temperature. Close tube C with a finger, draw the sample to tube B by gentle suction until the meniscus of the sample rises to the middle of bulb F, open the inlet of tube C, and immediately close the inlet of tube B with a finger. After confirming that the liquid column is cut off at the lowest end of the capillary tube open the inlet of tube B, and measure the time (t), in seconds, required for the meniscus of the sample to pass from mark J to mark K in tube B. Calculate the kinematic viscosity (ν) by the formula:

$$n = Kt$$

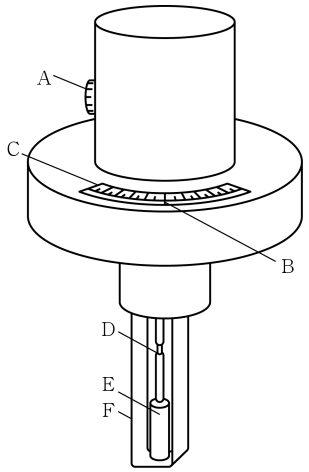
where K (mm^2/s^2) is the viscometer constant, previously determined using water or a reference standard solution with known viscosity in the same manner as for the sample. The temperature during this measurement may differ from that during the measurement of the sample.

Method 2　Viscosity Measurement by Rotational Viscometer

This method is applied to both Newtonian and non-Newtonian liquids. The method is based on the principle that when a rotor rotates at a constant angular velocity in a liquid, the force (torque) acting on the rotor is detected as the degree of torsion of the spring in the viscometer and calculated as the viscosity of the liquid.

Apparatus　Use the Brookfield-type viscometer, illustrated above. Because the type of rotor and the rotational frequency are not specified, select those that are appropriate for the sample used.

A: Rotational-frequency changing dial
B: Indicator
C: Scale
D: Immersion mark
E: Rotor
F: Guard



Procedure　Attach rotor E and guard F (except when an adapter for low viscosity is used) specified in the individual monograph. Adjust rotational-frequency changing dial A to the specified frequency. Immerse rotor E slowly into the sample liquid, and adjust immersion mark D to the surface of the sample. Switch on the viscometer to rotate E. Indicator B starts to move from zero. Either when the readings indicated by B stabilize or after the specified amount of time has elapsed, as directed in the individual monograph, stop the rotor and take the reading on scale C. To obtain the viscosity of the sample, multiply the reading by the appropriate conversion factor, given in the Table, which is determined from the type of rotor used and the rotational frequency selected.

In the Monographs, the specification "1500–2500 mPa・s (No. 2, 12 rotations, 30 seconds)" for this test, for example, means that when a No. 2 rotor is rotated at 12 rotations/min, the viscosity observed 30 seconds later must be 1500–2500 mPa・s. Also, the specification "30,000–40,000 mPa・s (No. 4, 12 rotations, stable)" means that when

a No. 4 rotor is rotated at 12 rotations/min, the viscosity must be 30,000–40,000 mPa・s when the readings have stabilized on the scale.

Conversion factor

Rotor \ Rotational frequency	60	30	12	6
Adapter	0.1	0.2	0.5	1.0
No. 1	1	2	5	10
No. 2	5	10	25	50
No. 3	20	40	100	200
No. 4	100	200	500	1000

Water Determination (Karl Fischer Method)

Water determination is designed to determine water, utilizing the quantitative reaction of water with iodine and sulfur dioxide in the presence of a lower alcohol, such as methanol, and an organic base, such as pyridine, as shown in the following formulae:

$$H_2O + I_2 + SO_2 + 3\ C_5H_5N + CH_3OH \rightarrow 2\ C_5H_5N \cdot HI + C_5H_5N \cdot HSO_4CH_3$$

There are two determination methods: the volumetric titration method and the coulometric titration method.

In the volumetric titration method, the iodine required for the reaction with water is dissolved beforehand in water determination TS, and the water content is determined by measuring the amount of iodine consumed as a result of the reaction with water in a sample. In the coulometric titration method, first, iodine is produced by electrolysis of an anolyte solution for water determination containing iodide ion. Next, the water content in a sample is determined by measuring the quantity of electricity that is required for electrolysis, based on the quantitative reaction of the generated iodine with water.

In the Monographs, the specification “not more than 4.0% (0.5 g, Volumetric Titration, Back Titration)” for this test, for example, means that when the test is conducted by accurately weighing about 0.5 g of the sample and performing back titration as directed under Volumetric Titration, the water content must be not more than 4.0% of the weight of the sample.

Method 1. Volumetric Titration

Apparatus Generally, the apparatus consists of an automatic burette, a titration vessel, a stirrer, and a constant-voltage amperometric titration system or constant-current potentiometric titration system.

Because water determination TS is extremely hygroscopic, the apparatus should be protected from atmospheric moisture. A suitable desiccant such as silica gel or calcium chloride for water determination is usually used for protection against moisture.

Procedure There are two titration methods: amperometric titration at a constant voltage and potentiometric titration at a constant current. As a rule, titration with water determination TS should be performed at the same temperature as that at which the TS has been standardized with protection from exposure to moisture.

Amperometric titration at a constant voltage: The apparatus is equipped with a variable resistor in the circuit, and the resistor is adjusted to apply a constant voltage (mV) between a pair of platinum electrodes or double platinum electrodes immersed in the solution to be titrated. The current (μA) that changes with the dropwise addition of water determination TS is measured. As titration progresses, the current in the circuit changes abruptly but returns to the original state within several seconds. The change in current persists for a certain time (usually, 30 seconds or longer). The endpoint of titration is determined when this electric state has been attained.

Potentiometric titration at a constant current: The resistor is adjusted to pass a constant current between the two platinum electrodes, and the potential (mV) that changes with the dropwise addition of water determination TS is measured. With the progress of titration, the value indicated by the potentiometer in the circuit decreases suddenly from a polarization state of several hundred mV to a non-polarization state, but it returns to the original state within several seconds. The non-polarization state persists for a certain time (usually, 10–30 seconds or longer). The endpoint of titration is determined when this electric state has been attained.

In the case of back titration, when the amperometric titration method is used at a constant voltage, the microammeter needle is out of scale in the presence of an excessive quantity of water determination TS. It returns rapidly to the original position when the titration reaches the endpoint. Similarly, when using the potentiometric titration method at a constant current, the reading on the voltmeter indicates the original reading in the presence of an excessive quantity of water determination TS. A definite voltage is applied when titration reaches the endpoint.

Unless otherwise specified, titration with water determination TS is performed by either of the methods described below. Usually, the endpoint of titration can be observed more clearly in the back titration method than in the direct titration method.

(1) Direct Titration Unless otherwise specified, proceed as directed below.

Put an appropriate volume of methanol for water determination in a dried titration vessel, and add water determination TS to the endpoint to keep the inside of the vessel free from water. Unless otherwise specified, Weigh accurately a quantity of the sample estimated to contain 5 to 30 mg of water, transfer it quickly into the titration vessel,

and dissolve by stirring. Titrate with water determination TS to the endpoint with vigorous stirring.

When the sample is insoluble in the solvent, powder the sample quickly, and weigh accurately a quantity of sample estimated to contain 5 to 30 mg of water. Transfer it quickly into a titration vessel, and stir the mixture for 5–30 minutes, protected from exposure to moisture. Titrate while stirring vigorously.

Also, when the sample is insoluble in the solvent or it interferes with the Karl Fisher reaction, an evaporation device may be used to heat the sample and to introduce the water in the sample evaporated into the titration vessel by using dry air or nitrogen gas as carrier.

Titration must be conducted in an atmosphere of low humidity. If titration is likely to be affected by atmospheric moisture—for instance, cases where titration takes a longer time—perform a blank test in the same manner as for determination of the sample and make any necessary correction.

$$\text{Water } (H_2O) = \frac{(\text{Volume (mL) of TS for water determination consumed}) \times f}{\text{Weight (mg) of the sample}} \times 100$$

f = the number of mg of water (H_2O) corresponding to 1 mL of water determination TS.

(2) Back Titration Unless otherwise specified, proceed as directed below.

Put an appropriate volume of methanol for water determination in a dried titration vessel, and add water determination TS dropwise to the endpoint to keep the inside of vessel free from water. Weigh accurately a suitable quantity of sample estimated to contain 5–30 mg of water, transfer the sample quickly into the titration vessel, add a definite volume of excess water determination TS, and stir to dissolve it. Then titrate the solution with Water–Methanol Standard Solution and vigorous stirring. When the sample is insoluble in the solvent, powder the sample quickly, and weigh accurately a quantity of sample estimated to contain 5 to 30 mg of water. Transfer it quickly into a titration vessel, and stir the mixture for 5–30 minutes, protected from exposure to moisture. Titrate while stirring vigorously.

$$\text{Water } (H_2O)\ (\%)$$

$$\frac{\left[\left(\begin{array}{c}\text{Volume (mL) of}\\ \text{TS for water determination added}\end{array}\right) \times f\right] - \left[\left(\begin{array}{c}\text{Volume (mL) of}\\ \text{Water – Methanol Standard Solution consumed}\end{array}\right) \times f'\right]}{\text{Weight (mg) of the sample}} \times 100$$

f = the number of mg of water (H_2O) equivalent to 1 mL of water determination TS,

f′ = the number of mg of water (H_2O) in 1 mL of Water–Methanol Standard Solution.

Method 2. Coulometric Titration

Apparatus Usually, the apparatus is comprised of a titration vessel equipped with an electrolytic cell for iodine production, a stirrer, and a constant-current potentiometric titration system. The iodine production device is composed of an anode and a cathode, separated by a diaphragm. The anode is immersed in the anolyte for water determination and the cathode is immersed in the catholyte for water determination. Both electrodes are usually made of platinum-mesh.

Because both the anolyte and catholyte for water determination are extremely hygroscopic, the apparatus should be protected from exposure to atmospheric moisture. For this purpose, an appropriate desiccant such as silica gel or calcium chloride for water determination is usually used.

Preparation of Anolyte and Catholyte for Water Determination

In the coulometric titration method, an anolyte and a catholyte are used in pairs. The solutions should be prepared by any of the methods given below. An anolyte and catholyte prepared by other methods can be used if they are equal or superior in accuracy to those prepared by the methods given below.

Preparation 1

Anolyte for Water Determination Dissolve 102 g of imidazole for water determination in 900 mL of methanol for water determination, cool the solution in ice, and pass dried sulfur dioxide gas through the solution while maintaining its temperature below 30° C. When the mass increase of the solution has reached 64 g, add and dissolve 12 g of iodine in the solution. Add water dropwise while stirring until the solution changes from brown to yellow. Finally, add methanol for water determination to make 1000 mL.

Catholyte for Water Determination Dissolve 24 g of 2-2′-iminodiethanol hydrochloride in 100 mL of methanol for water determination.

Preparation 2

Anolyte for Water Determination Dissolve 40 g of 1,3-di-(4-pyridyl)propane and 30 g of diethanolamine in about 200 mL of methanol for water determination, and pass dried sulfur dioxide gas through the solution until the mass increase of the solution has reached 25 g. Add 50 mL of propylene carbonate, and dissolve 6 g of iodine in the solution. Add methanol for water determination to make 500 mL, and then add water dropwise until the solution changes from brown to yellow.

Catholyte for Water Determination Dissolve 30 g of choline chloride for water determination in 100 mL of methanol for water determination.

Preparation 3

Anolyte for Water Determination Dissolve 100 g of diethanolamine in 900 mL of methanol for water determination or a 3:1 mixture of methanol for water determination/chloroform for water determination, and pass dried sulfur dioxide gas through the solution while cooling. When the mass increase of the solution has reached 64 g, add and dissolve 20 g of iodine in the solution. Add water dropwise while stirring until the solution changes from brown to yellow.

Catholyte for Water Determination Dissolve 25 g of lithium chloride in 1000 mL of a 4:1 mixture of methanol for water determination/nitromethane.

Procedure Place a suitable volume of an anolyte for water determination in a titration vessel, and immerse in this solution a pair of platinum electrodes or double platinum electrodes of the constant-current potentiometric titration system. Next, immerse the electrolytic cell for iodide production filled with a catholyte for water determination in the anolyte.

Switch on the electrolytic system, and make the inside of the titration vessel free from water. Next, weigh accurately an amount of sample so that 0.2–5 mg of water is contained in it, transfer quickly into the vessel, and dissolve by stirring. Perform the titration to the endpoint with vigorous stirring. If the sample is insoluble in the anolyte, powder it quickly, and transfer an amount of sample, accurately weighed so that 0.2–5 mg of water is contained in it, into the vessel. Titrate with vigorous stirring after stirring the mixture for 5–30 minutes, while protecting from exposure to atmospheric moisture. When the sample is insoluble in the solvent or it interferes with the Karl Fisher reaction, an evaporation device may be used to heat the sample and to introduce the water in the sample evaporated into the titration vessel by using dry air or nitrogen gas as carrier.

Determine the quantity of electricity (C) [electric current (A) × time (seconds)] required for the production of iodine during the titration, and calculate the content (%) of the water in the sample by the formula below.

Titration must be conducted in an atmosphere of low humidity. If titration is likely to be affected by atmospheric moisture—for instance, a case where titration takes a longer time—perform a blank test in the same manner as for the determination of the sample and make any necessary correction.

$$\text{Water } (H_2O)\ (\%) = \frac{\text{Quantity (C) of electricity required for iodine production}}{10.72 \times \text{Weight (mg) of the sample}} \times 100$$