

B. Natural Additives

1. Persimmon Color

Definition Persimmon Color is obtained by fermenting and heat treating fruits of persimmon of persimmon family (*Diospyros kaki* THUNB.). Major colorant is flavonoid. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Persimmon Color

Content Color value ($E_{1\text{cm}}^{10\%}$) of Persimmon Color should be higher than that is indicated value.

Description Persimmon Color is reddish brown~blackish brown liquid, lump, powder, or paste with a slight characteristic scent.

Identification (1) Test Solution obtained in Color Value for Persimmon Color becomes reddish brown.

(2) Take 0.5 g of the sample into 100 ml volumetric flask, add water to volume. When 10 ml of this solution is acidified with 1 ml of HCl, reddish brown~blackish brown precipitates are formed.

(3) Water is added to 0.5 g of Persimmon Color so that the total volume is 100 ml. When 2 ml of 2% ferric chloride solution(1→10) is added to 10 ml of this solution, blackish brown precipitates are formed.

Purity (1) Arsenic : 0.25 g of Persimmon Color is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath, and this is used as the Test Solution. This Test Solution is proceed as directed under Arsenic, it is appropriate and should not be more than 4ppm.

(2) Lead : When 5.0 g of Persimmon Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay(Color value) Appropriate amount of Persimmon Color is weighed so that the absorbance to be measured will within a range of 0.3~0.7. Citric acid-dibasic sodium phosphate buffer solution with pH 7.0 is added so that the total volume is 100 ml, and this is used as the Test Solution. If necessary, the solution is centrifuged and the

supernatant is used. Using citric acid–dibasic sodium phosphate buffer solution with pH 7.0 as a reference solution, absorption A is measured at 500 nm wavelength with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value (E}_{1\text{cm}}^{10\%}) = \frac{A \times 10}{\text{Weight of the sample (g)}}$$

Citric acid·dibasic sodium phosphate buffer solution (pH 7.0)

- Solution 1 : 0.1 M citric acid solution : 1 L of solution containing 21.01g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).
- Solution 2 : 0.2 M dibasic sodium phosphate solution : 1 L of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (35:165) and its pH is adjusted to 7.0.

2. Licorice Extract

Definition Licorice Extract includes purified licorice, and crude licorice. It is an extract from the roots and root stocks of licorice of leguminosae (*Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* FISCH.) or the same genus, which is extracted with water and purified. Its major component is glycyrrhizinic acid ($C_{42}H_{62}O_{16} = 822.94$). Purified licorice has two forms, a natural salt form and acid form (cation is removed).

Compositional Specifications of Licorice Extract

Content Purified licorice should contain more than 50.0% and crude licorice less than 50.0% as glycyrrhizinic acid, respectively.

Description Purified licorice is white~yellow crystal or powder and crude licorice is yellow~brown powder, thin platelet, granule, lump, liquid, or paste.

Identification 5~10 mg of Licorice Extract is dissolved in 10 ml of 50% alcohol, use the Test Solution. Separately, 5 mg of glycyrrhizinic acid standard is dissolved in 10 ml of 50% alcohol use the Standard Solution. Each of the solution proceed as directed under thin layer chromatography. 2 μ l of each solution drop-wise added on to a thin layer plate, which is prepared by using silica gel (with phosphor) for thin layer chromatography. Using a mixture of n-butyl alcohol : water : acetic acid (7:2:1) as a developing solvent, each plate is developed up to 10 cm, and then dried in air. When these plates are observed under UV light (major wavelength at 254 nm), one of the spots for test solution should have the same color tone and R_f against the dark violet spot for standard solution (glycyrrhizinic acid).

Purity (1) Arsenic : 0.25 g of Licorice Extract is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath, use the Test Solution. This Test Solution is proceed as directed under Arsenic, it is appropriate and should not be more than 4ppm.

(2) Lead : When 5.0 g of Licorice Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

Residue on Ignition 1 g of Licorice Extract is dried in a water bath, if necessary. When Residue on Ignition analysis is done, the amount of residue should be 2.5~15.0% for purified licorice extract (alkali type), should not be more than 1.0% for purified licorice extract (acid

type), and should be less than 15.0% for crude licorice extract.

Assay Licorice Extract which corresponds to approximately 20 mg as glycyrrhizinic acid is accurately weighed, dissolved in 50% alcohol, and the total volume is made to 100 ml, use the Test Solution. Separately, 20 mg of glycyrrhizinic acid standard is accurately weighed, dissolved in 50% alcohol, and the total volume is made to 100 ml, use the Standard Solution. 20 µl of each solution is injected into liquid chromatography as the following operation conditions. The content of licorice extract is obtained from the following equation.

$$\text{Content (\%)} = \frac{TG \times W_s}{SG \times W} \times 100$$

SG : Peak area of standard solution

TG : Peak area of test solution

W_s : Weight of standard (mg) (converted into an anhydrous form)

W : Weight of sample (mg) (converted into an anhydrous form)

Operation Conditions

-Detector : UV 254 nm

-Column : µ-Bondapak C18 (inner diameter 4~6 mm, length 15~30 cm) or its equivalent

-Column Temperature : 40°C

-Mobile Phase : 2% acetic acid : acetonitrile (20 : 11)

-Flow Rate : It is adjusted so that the retention time of glycyrrhizinic acid is approximately 10 minutes.

3. Cellulose, Microcrystalline

Cellulose Gel

Definition Cellulose, Microcrystalline is obtained by depolymerization of α -cellulose with inorganic acid followed by purification.

Compositional Specifications of Cellulose, Microcrystalline

Content If Cellulose, Microcrystalline is converted to an anhydrous form, it should contain 97.0~102.0% of hydrocarbons as cellulose.

Description Cellulose, Microcrystalline is white~grayish white fluidity crystalline powder. It is odorless and tasteless

Identification (1) 30 g of Cellulose, Microcrystalline is added to 270 ml of water, which is stirred for 5 minutes at approximately 3,000 rpm. This is transferred into a 100 ml mass cylinder, which is allowed to stand for 3 hours. The resulting liquid is white opaque dispersion without bubbles. Separation of phases should not be observed.

(2) 1 ml of phosphoric acid is added to 1 mg of Cellulose, Microcrystalline, which is heated for 30 minutes in a water bath. To this solution, 4 ml of catechol-phosphoric acid solution (1→500) is added and heated for 30 minutes. The color of the resulting solution brings out red.

Purity (1) Arsenic : 0.25 g of Cellulose, Microcrystalline, proceed as directed under Purity (1) in Guar Gum, its content should not be more than 4ppm.

(2) Lead : When 5.0 g of Cellulose, Microcrystalline is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Cellulose, Microcrystalline is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Cellulose, Microcrystalline is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Starch : When 2 drops of iodine TS are added to 30 ml of Test Solution in Identification (1), it should not become bluish violet~blue.

(6) pH : 5 g of Cellulose, Microcrystalline is well mixed for 20 minutes with 40 ml of freshly boiled and cooled water. It is then centrifuged. pH of the supernatant is measured using a glass electrode and should be 5.5~7.0.

(7) Water Solubles : 5 g of Cellulose, Microcrystalline is added to 80 ml of water, which is shaken for 10 minutes. This is filtered through a No.42 Whatman filter

paper or its equivalent into a beaker that make previously constant weight. The filtrate is evaporated, which is then dried for 1 hour at 105°C. The residue should not be more than 0.16%.

Loss on Drying When 1 g of Cellulose, Microcrystalline is dried for 3 hours at 105°C, the weight loss should not be more than 5%.

Residue on Ignition When Residue on Ignition is done with 2 g of Cellulose, Microcrystalline, the amount of residue should not be more than 0.05%.

Assay 125 mg of Cellulose, Microcrystalline is precisely weighed into a 300 ml Erlenmeyer flask using 25 ml of water. 50 ml of 0.5 N potassium bichromate solution is well mixed and 100 ml of sulfuric acid is carefully added, which is then boiled. After cooling for 15 minutes at room temperature and further cooled in a water bath. The resulting solution is transferred into a 250 ml volumetric flask and filled with water. 50 ml of the resulting solution is titrated with 0.1 N ammonium ferrous sulfate solution using 2~3 drops of o-phenanthroline as an indicator, its consumption is S (ml) Separately, a blank test is carried out and the consumption of 0.1N ammonium ferrous sulfate solution is B (ml). The content of cellulose in the sample is calculated from the following equation.

$$\text{Content of cellulose (\%)} = \frac{(B-S) \times 338}{W}$$

W : Weight of sample as a dehydrated form (mg)

4. Kaoliang Color

Definition Kaoliang Color is obtained by extracting kaoliang grains of rice family (*Sorghum nervosum* BESS.) with water or ethyl alcohol. Major colorant is apigenin ($C_{15}H_{10}O_5 = 270.27$) and luteolinidine of flavonoid origin. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Kaoliang Color

Content Color value of Kaoliang Color ($E_{10\%}^{1cm}$) should be more than the indicated value.

Description Kaoliang Color is brown liquid, lump, powder, or paste with a slight characteristic scent.

Identification (1) Test Solution obtained in Color Value of Kaoliang Color shows yellowish brown~reddish brown color. Visible absorption spectrum shows a gentle descending slope from short wavelength to long wavelength.

(2) When Test Solution of (1) is acidified with hydrochloric acid, brown precipitates are formed.

(3) When ferric chloride TS is added to Test Solution of (1), milky white precipitates are formed.

Purity (1) Arsenic : 0.25 g of Kaoliang Color is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate and should not be more than 4ppm.

(2) Lead : When 5.0 g of Persimmon Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay(Color value) Appropriate amount of Kaoliang Color is accurately weighed so that the absorbance to be measured will be within a range of 0.3~0.7 and dissolved in 30 ml of sodium carbonate solution (1→100). Citric acid-dibasic sodium phosphate buffer solution with pH 7.0 is added so that the total volume is to make 100 ml. Take 1 ml of this solution, add citric acid-dibasic sodium phosphate buffer solution with pH 7.0, and make to volume 100 ml and this is used as the Test Solution. If necessary, the solution is centrifuged and the supernatant is used. Using citric acid-dibasic sodium phosphate buffer solution with pH 7.0 as a reference solution, absorption A is

measured at 500 nm wavelength with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{10\%}^{1cm}) = \frac{A \times 1000}{\text{Weight of sample (g)}}$$

Citric acid·dibasic sodium phosphate buffer solution (pH 7.0)

- Solution 1 : 0.1 M citric acid solution : 1L of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).
- Solution 2 : 0.2 M dibasic sodium phosphate solution : 1 L of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (35:165) and its pH is adjusted to 7.0.

5. Guar Gum

Definition Guar gum is obtained by crushing endosperms of guar (*Cyamopsis tetragonolobus* TAUB.) seed of leguminosae or extracting by warm water or hot water. The major component is polysaccharides.

Compositional Specifications of Guar Gum

Description Guar gum is white~yellowish white powder. It is almost odorless.

Identification (1) 2 g of Guar gum is placed in a 400 ml beaker and wetted completely with 4 ml isopropyl alcohol. While stirring vigorously, 200 ml of water is added and homogenized. It becomes milky white viscous liquid.

(2) When 100 ml of Test Solution in (1) is boiled for 10 minutes in a water bath and cooled, the viscosity does not increase

(3) When small amount of borax is added to 100 ml of Test Solution in (1), it forms gel.

Purity (1) Arsenic : 0.25 g of Guar gum is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath, use the Test solution. When this test solution proceed as directed under Arsenic, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Guar gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Guar gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Guar gum is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Starch : 0.1 g of Guar gum is dissolved in 10 ml of water by boiling for 1 minute, which is then cooled. When 2 drops of iodine solution are added, it should not show blue.

(6) Isopropyl alcohol : 0.2 g of Guar gum is accurately weighed into a 300 ml round bottom flask, 200 ml of water is added, boiling chips and 1 ml of silicone resin are added and mixed well. Distillation column is connected to this, 4 ml of internal

standard solution is accurately weighed and added to a 100 ml flask. While caring for the bubbles not to overflow, distill the solution at the rate of 2~3 ml per 1 minute until the milky liquid becomes about 90 ml, and water is added to make 100 ml, test solution. However, tert-butyl alcohol (1→1,000) is used as internal standard solution. Separately, 0.5 g of isopropyl alcohol is accurately weighed and water is added to make 500 ml, 2 ml of this solution and 4 ml of internal standard solution is weighed again, water is added to make 100 ml, standard solution. 2μl of test solution and standard solution is taken respectively, and injected to gas chromatograph with the following operation condition. Then, ratio of isopropyl alcohol peak against tert-butyl alcohol peak in test Solution and standard solution, QT and QS, is calculated separately, and the content of isopropyl alcohol is calculated by following formula, the content should not be more than 1.0%.

$$\text{Content of Isopropyl alcohol(\%)} = \frac{\text{Weight of isopropyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_T}{Q_S} \times \frac{2 \times 100}{500 \times 100} \times 100$$

QT : Ratio of isopropyl alcohol peak against tert-butyl alcohol peak in Test Solution

QS : Ratio of isopropyl alcohol peak against tert-butyl alcohol peak in standard solution

Operation Conditions

Column : PLOT Q or equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Injection temperature : 200℃

Column Temperature : 120℃

Detector temperature : 300℃

Carrier gas : Nitrogen or Helium

- (7) Borates : 1 g of Guar Gum is accurately weighed and water is added to make 100 ml (Gel should not be formed). 10 ml of diluted hydrochloric acid is added and mixed and 1 drop of this solution is added to turmeric paper(Advantec 07810074 or its equivalent), it should not turn reddish brown.
- (8) Total Viable Aerobic Count : When Guar Gum is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 CPU per 1 g
- (9) E. Coli : When Guar Gum is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be

negative (-).

(10) *Salmonella* : When Guar Gum is tested by Microbe Test Methods for *Salmonella* in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

(11) Number of Fungi : When Guar Gum is tested by Microbe Test Methods for Number of Fungi in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 500 CFU per 1 g

Acid Insoluble substances 1.5 g of Guar gum is accurately weighed and dissolved it in a beaker containing 150 ml of water and 1.5 ml sulfuric acid, which is covered with a watch glass and heated for 6 hours in a water bath. Occasionally, it is stirred with a glass rod and water is added to supplement the loss. After heating is complete, it is filtered through a glass filter with constant weight, containing 500 mg of appropriate filtering aid, accurately weighed. The residue is washed thoroughly with hot water and dried for 3 hours at 105°C. The weight of the filtering aid is subtracted from the weight of the residue, which should not be more than 7%.

Total Ash When 3 g of Guar gum is accurately weighed and reduced to ash at 600°C, the content should not be more than 1.5%.

Loss on Drying When 3 g of Guar gum is dried for 5 hours at 105°C, the weight loss should not be more than 15%.

Protein When Guar gum proceed as directed under Kjeldahl Method in Nitrogen Determination, the amount should not be more than 10%. (Protein Factor : 6.25).

6. Koji

Definition There are nuruk, granular koji, coenzyme, and purified enzyme. Nuruk contains enzymes produced by naturally breeding fungus, yeast, and other microbes of *Aspergillus* genus and *Rhizopus* genus in raw grains. Granular koji contains enzymes produced by breeding fungus of *Aspergillus* genus and *Rhizopus* genus on steamed grains. Coenzymes are obtained from cultures of enzyme (germs, which produces 'saccharifying enzyme') in steamed or pasteurized material that contains cortex or starch. Purified enzyme is purified enzyme (by separation) obtained by culturing in solid or liquid culture medium. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Koji

Content When Koji is analyzed quantitatively, it contains 100~130% of the indicated amount as saccharogenic power, SP. However, nuruk, granular koji, coenzyme, liquid phase purified enzyme (liquid), and purified enzyme (powder) contains 300 SP, 60 SP, 600 SP, 10,000 SP, 15,000 SP, respectively.

Description Koji is yellow~yellowish gray or white~pale yellow~brown liquid, lump, or powder with a slight characteristic scent.

Purity (1) Arsenic : 0.25 g of Koji is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate(not more than 4ppm).

(2) Lead : When 5.0 g of Koji is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Various Germs (*Penicillium* Genus) : 0.15~0.2 g of Koji is placed in a pre-pasteurized culture medium (55 ml of water, 0.025 g of potassium phosphate, monobasic, and 1 g of dextrin are added in a 300 ml Erlenmeyer flask, which is then plugged with cotton and pasteurized for 20 minutes under 15 psi) and cultured for 5 days at 30°C in a thermostat. When it is observed with a microscope, it should be negative for various germs (*penicillium* genus). For the confirm on *Penicillium* genus, after culturing for 5 days in a 30°C in a thermostat, if *Penicillia*

are observed, it is positive. If *Penicillia* are not observed, it cultured for another 24 hours. If *Penicillia* are observed, it is positive. If not, it is negative. (In the case, this applies to granular koji only.)

- (4) Acid value : 20 g of Koji is leached in 100 ml of water for 3 hours at 30°C, which is then filtered. 2~3 drops of mixed indicator are added to the filtrate, which is titrated with 0.1 N sodium hydroxide solution until the solution becomes from pink to pale blue. Acid value is calculated by the following equation and should be more than 5.0. (In the case, this applies to granular koji only).

$$\text{Acid value} = a \times f$$

a : Consumption of 0.1 N sodium hydroxide solution (ml)

f : Factor of 0.1 N sodium hydroxide solution

Mixed indicator : 0.2 g of bromothymol blue and 0.1g of neutral red are dissolved in 300 ml of alcohol.

Loss on Drying

When 10 g of Koji is dried for 4 hours at 105°C, the weight loss should not be more than 12% for nuruk, not more than 30% for granular koji, not more than 10% for coenzyme, not more than 8% for purified enzyme (not applicable for liquid phase).

Assay (Saccharogenic Power)

Application and Principle : This test method is based on measuring the amount of reducing sugar produced by decomposition of soluble starch under a set of conditions of time, temperature, pH, and concentration.

Preparation of Test Solution

- Nuruk, granular koji, coenzyme : Test Solution is prepared so that 1 ml of the final dilution contains 1~2SP. Sample is accurately weighed into a Erlenmeyer flask (for nuruk, it is ground to 80~100mesh before weighing), which is isothermalized at 30°C. 200 ml of 1% sodium chloride solution is added to the flask, which is leached for 3 hours at 30°C while stirring gently in a 20 minute interval. It is then filtered and use the Test Solution.
- Purified enzyme : Test Solution is prepared so that 1 ml of the final dilution contains 1~2 SP.

Test Procedure

- ① Production of Reducing Sugar : 50 ml of substrate solution and 30 ml of acetate buffer solution are placed in a 100 ml volumetric flask, which is allow to stand for 10

minutes in a 55°C water bath. 10 ml of Test Solution is added to this solution and it is timed. The content is well mixed by shaking and it is allowed to stand in the water bath. After exactly 60 minutes, the reaction is stopped by adding 10 ml of 0.5 N sodium hydroxide solution. It is then cooled to room temperature in running water. Water is added to make the total volume to 100 ml. 10 ml each of this solution and the reference is tested for the amount of reducing sugar. Reference solution is prepared by following the same procedure as Test Solution with 10 ml of water instead of 10 ml of Test Solution.

② Measurement of Reducing Sugar : 10 ml of Fehling solution is added to a 250 ml Erlenmeyer flask, where 40 ml of water, 10 ml of the above solution obtained by production of reducing sugar, and 10 ml of glucose standard solution are added. The mixture is mixed with shaking gently. It is then boiled for 1 minute. While continuously boiling, it is titrated with glucose standard solution. If the blue color of copper sulfate almost disappears, 4~5 drops of methylene blue TS are added and the titration is continued. The end point is where the color of methylene blue disappears and the consumed amount of glucose standard solution is S (ml). Separately, a blank test is carried out with 10 ml of Fehling solution, 40 ml of water, 10 ml of reference solution, and 10 ml of glucose standard solution. The consumed amount (ml) of glucose standard solution is B (approximately 25 ml is consumed for a blank test).

③ Calculation of Saccharogenic Power

$$SP = \frac{(B-S) \times 2}{W \times 1}$$

2 : Factor of 20/10, which comes from the concentration of glucose standard solution (2 mg/ml) and used amount of standard solution (10 ml).

W : Weight of sample contained in 10 ml of Test Solution (g)

1 : Reaction time (hour)

Definition of Saccharogenic Power : 1 Saccharogenic power(SP) corresponds to production of 10 mg of glucose by 1 g of an enzyme in 1 hour under the test conditions above.

Solution

- 0.2 M Acetate Buffer Solution (pH 5.0) : 0.2 M sodium acetate solution is added to 0.2 M acetic acid with stirring continuously, where pH is adjusted to 5.0 ± 0.05 .
- Starch : Soluble starch (Lintner) or equivalent is used.

- Substrate Solution : 10 g of starch (as dried form) is dispersed in 100 ml of cold water, where 300 ml of boiling water is slowly added. It is then boiled for 1~2 minutes with stirring. After cooling, it is then transferred into a 500 ml volumetric flask, which is then filled with water to make 500 ml.
- Glucose Standard Solution : 2.0 g of glucose (anhydrous) is accurately weighed and dissolved in water, to make total volume 1,000 ml.
- Methylene Blue Solution : 1 g of methylene blue is dissolved in water to make total volume 100 ml.

7. Diatomaceous Earth

Definition Diatomaceous Earth is silicon dioxide originated from diatom. There are three types, dried, calcined, and flux-calcined. These will be named as diatomaceous earth (dried), diatomaceous earth (calcined), and diatomaceous earth (flux-calcined). Calcined diatomaceous earth is calcined at 800~1,200°C. Flux-calcined diatomaceous earth is calcined at 800~1,200°C with a small amount of alkali carbonates. If flux-calcined diatomaceous earth is washed with acid, specifications for calcined form are applied (except for characteristics).

Compositional Specifications of Diatomaceous Earth

Description Dried material is milky white to pale gray powder, calcined material is pale reddish brown powder, and flux-calcined material is white to pale reddish brown powder.

Identification (1) 0.2 g of Diatomaceous Earth is dissolved in 5 ml of hydrofluoric acid in a platinum crucible. When the solution is heated, almost all of it volatilizes.
(2) When examined with 100x to 200x microscope, typical diatom shapes are observed.

Purity (1) Water Solubles substances and pH : 10 g of Diatomaceous Earth is added to 100 ml of water. It is then boiled for 2 hours in a water bath, supplementing water with occasionally shaking. After cooling, it is filtered with a suction-filtering apparatus that is equipped with a 47 mm diameter membrane filter (pore size 0.45 μ m). If the filtrate is turbid, it is filtered again through the same filter. The residue on filter paper is washed with water and wash water is added to the previous filtrate. The total volume is make to 100 ml with water. Using a glass electrode, pH of the resulting filtrate is measured. pH should be 5.0~10.0 for dried or calcined material and 8.0~11.0 for flux-calcined material. 50 ml of the filtrate is evaporated to dryness and the residue is further dried for 2 hours at 105°C. The amount of water solubles should not be more than 15 mg, 10 mg, and 25 mg for dried, calcined, and flux-calcined material, respectively (should not be more than 0.3%, 0.2%, 0.5% for dried, calcined, and flux-calcined material, respectively).

(2) Hydrochloric acid soluble substances : 50 ml of diluted hydrochloric acid is added to 5 g of Diatomaceous Earth, which is shaken for 15 minutes at 50°C. It is then heated for 1 hour in a water bath, supplementing water with occasionally shaking. After cooling, it is filtered. The residue on filter paper is washed with water and wash water is added to the filtrate. The total volume is make to 100 ml with water and use the Solution A. 1 ml of diluted sulfuric acid (1→20) is added to 10 ml of

Solution A, which is evaporated to dryness and further dried at 550°C until the weight becomes constant. The amount of residue should not be more than 15 mg (should not be more than 3%).

(3) Arsenic : When 2 ml of liquid A in (2) proceed as directed under Arsenic, it should be appropriate (not be more than 10ppm).

(4) Lead : When solution A as test solution in (2) proceed is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Loss on Ignition When Diatomaceous Earth is dried at 105°C for 2 hours and heat treated at 1,000°C for 30 minutes, weight loss should not be more than 7.0% for dried material and should not be more than 2.0% for calcined, and flux-calcined material.

Hydrofluoric Acid Residue A platinum crucible is previously ignited for 30 minutes at 1,000°C. 0.2 g of Diatomaceous Earth is accurately weighed in a crucible, where 5 ml of hydrofluoric acid and 2 drops of diluted sulfuric acid (1→2) are added, which is evaporated to approximate dryness on a water bath. It is then heat treated for 1 hour at 550°C and gradually further heated to 1,000°C kept temperature for 30 minutes. It is then cooled in a desiccator and weighed accurately. The amount of residue does not exceed 50 mg(not more than 25%).

8. β -Glucanase

Definition β -Glucanase is an enzyme obtained from a culture of *Aspergillus niger* and its variety, *Bacillus subtilis* and its variety, and *Humicola insolens* and its variety. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of β -Glucanase

Content When β -Glucanase is analyzed quantitatively, it contains 90~130% of the indicated activity(activity) as β -Glucanase.

Description β -Glucanase is white to pale yellowish brown power or transparent to brown liquid.

- Purity**
- (1) Arsenic : 0.25 g of β -Glucanase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by gradually heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate (not more than 4ppm).
 - (2) Lead : When 5.0 g of β -Glucanase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.
 - (3) Coliform Group : When β -Glucanase proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods in Food Code, it should contain not more than 30 colonies per 1 g of this product.
 - (4) Salmonella : When β -Glucanase proceed as directed under Microbe Test Methods for Salmonella in General Test Methods in Food Code, it should be negative(-).
 - (5) E. Coli : When β -Glucanase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay (Activity) Application and Principle : This test is to measure the activity of β -glucanase in an enzyme that is obtained from a culture of *Aspergillus niger* and its variety, *Bacillus subtilis* and its variety, and *Humicola insolens* and its variety. Activity test is based on hydrolysis of Lichenin substrate for 15 minutes at pH 6.5, temperature 40°C. The increase in reducing power due to generated reducing matters is measured by Neocuproine method.

- Preparation of Test Solution : The final diluted solution is prepared so that it contains 0.01~0.02 β-Glucanase units per 1 ml. Sample is taken into a volumetric flask, dissolved and filled with phosphate buffer solution.
- Procedure : 2 ml each of substrate solution is added into four 25 ml volumetric flasks, which are then isothermalized in a water bath at 40°C for 10~15 minutes. 1 ml of phosphate buffer solution are added to test tube 1, 1 ml of glucose standard solution are added to test tube 2 (glucose standard), 4 ml of neocuproine solution A and 1 ml of Test Solution are added to test tube 3 (enzyme blank test), 1 ml of Test Solution added to test tube 4 (enzyme test), and 3 ml of phosphate buffer solution are added to test tube 5 (phosphate buffer solution blank test). Test tubes are isothermalized at 40°C for exactly 15 minutes. 4 ml each of neocuproine solution A is added to test tubes 1, 2, 4, and 5. After adding 4 ml each of neocuproine solution B to all test tubes, a glass stopper is placed on each tube (rubber stopper should not be used). It is then colorized by heating vigorously in a water bath. After cooling to room temperature, water is added to make the total volume to 25 ml. Using either parafilm or appropriate stopper, the content of each tube is mixed well by turning upside down a few times. Using phosphate buffer solution for blank test of test tube 5 as a reference solution, absorbance of each solution is measured at 450 nm with 1cm path length.

Enzyme activity is obtained from the following equation.

$$\text{BGU} = \frac{(A_4 - A_3) \times 36 \times 10^6 \times F}{(A_2 - A_1) \times 180 \times 15 \times S}$$

- A₄ : Absorbance of enzyme test solution (test tube 4)
- A₃ : Absorbance of enzyme blank test solution (test tube 3)
- A₂ : Absorbance of glucose standard solution (test tube 2)
- A₁ : Absorbance of substrate blank test solution (test tube 1)
- F : Dilution factor of test solution
- S : Weight of sample(μg)
- 36 : Content of glucose (μg) in glucose standard solution
- 106 : Conversion factor from μg to g
- 180 : Weight of 1μmol of glucose
- 15 : Reaction time (minutes)

Definition of Activity : 1 β-Glucanase unit (BGU) corresponds to the amount of

enzymes which produce 1 μ mol of glucose per 1 minute as reducing sugar, under the test conditions described above.

Solutions

- Phosphate Buffer Solution : 13.6 g of potassium phosphate, monobasic is added in 1,900 ml of water. pH is adjusted to 6.5 ± 0.05 with 70% sodium hydroxide solution. The total volume of the solution is make to 2,000 ml with water.
- Neocuproine Solution A : 40 g of anhydrous sodium carbonate, 16 g of glycine, and 450 mg of copper sulfate (5 hydrate) are dissolved in approximately 600 ml of water. The total volume is make to 1,000 ml with water.
- Neocuproine Solution B : 600 mg of neocuproine hydrochloride is dissolved in 400 ml of water and the total volume is make to 500 ml. If the solution becomes yellow, it is discarded.
- Substrate Solution : 150 mg of Lichenin is ground into fine powder using a mortar and pestle, which is dissolved in 50 ml of water at approximately 85°C. When it is dissolved completely (it takes 20~30 minutes), 90 mg of sodium borohydride is added. It is then heated at boiling point for 1 hour. 15 g of Amberlite MB-20 or equivalent ion exchange resin is added, which is then stirred continuously for 30 minutes. It is vacuum-filtered through Whatman No.1 filter or equivalent using a Buchner funnel, which is then washed with 20 ml of water. 680 mg of potassium phosphate, monobasic is added to the filtrate, which is then filtered through a 0.22 μ m Millipore filtration apparatus (or equivalent). The filtration apparatus is washed with 10 ml of water. pH of the filtrate is adjusted to 6.5 ± 0.05 with 1N sodium hydroxide solution or 1N hydrochloric acid. The total volume is then make to 100 ml with water. The solution should be kept at 2~4°C and used within 3 days.
- Glucose Standard Solution : 36.0 mg of anhydrous glucose is dissolved in 50 ml of phosphate buffer solution. The total volume is make to 1,000 ml with water.

Storage Standard of β -Glucanase

β -Glucanase is strongly hygroscopic, so should be stored in a hermetic container in a cold dark place.

9. Glucoamylase

Definition Glucoamylase is an enzyme obtained from a culture of *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety, and *Rhizopus oryzae* and its variety. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Glucoamylase

Content When Glucoamylase is analyzed quantitatively, it contains 90~130% of the indicated activity as Glucoamylase.

Description Glucoamylase. is white to pale yellowish brown power or transparent to brown liquid.

Purity (1) Arsenic : 0.25 g of Glucoamylase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by gradually heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate (not more than 4ppm).

(2) Lead : When 5.0 g of Glucoamylase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

(3) Coliform Group : Glucoamylase proceed as directed under Microbe Test Methods in Coliform Group in General Test Methods in Food Code. It should contain not more than 30 colonies per 1 g of this product.

(4) Salmonella : Glucoamylase proceed as directed under Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).

(5) E. Coli : When Glucoamylase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

◦ Application and Principle : This test is to measure the activity of Glucoamylase that is obtained from a culture of *Aspergillus niger* and its variety. It can also be modified to measure activity of an enzyme that is obtained from a culture of *Aspergillus oryzae* and its variety and *Rhizopus oryzae* and its variety. Activity test is carried out under a fixed set of conditions at time, temperature, pH, and

concentration and is measured as a reducing sugar generated by decomposition of hydrolyzed solution of corn starch.

- Preparation of Test Solution : Test Method described below is based on the use of sample that contains 0.1~0.2 units of activity of glucoamylase. This corresponds to an amount that produces 0.2~0.4 g of reducing sugar under the same test conditions. The most appropriate results can be achieved within this range. Liquid, solid, and liquid extract sample are prepared by the following table, where indicated amounts should be used.

Liquid Sample

Enzyme in sample (unit/ml)	Dilution Factor (ml)	Amount (ml)	Dilution Factor(F)
Not more than 0.05	—	5.0	0.2
0.06~0.1	—	2.0	0.5
0.11~0.25	—	0.80	1.25
0.3~0.5	—	0.40	2.5
0.6~1.0	—	0.20	5
1.1~2.0	—	0.10	10
2.1~4.0	5.0→100	1.00	20
4.1~5.0	4.0→100	1.00	25
5.1~7.0	3.0→100	1.00	33.3
7.1~10.0	2.0→100	1.00	50

Solid Sample and Liquid Extracts

Enzyme in sample (unit/ml)	Weight(g) [※]	Diluted to (ml)	Amount (ml)
Not more than 4	10	1,000	5.0
5~10	4	1,000	5.0
11~25	1.6	1,000	5.0
26~50	1.4	1,000	3.0
51~75	1.25	1,000	2.0
76~100	1.00	1,000	2.0
101~150	1.25	1,000	1.0
151~200	1.00	1,000	1.0
201~250	1.50	2,000	1.0
251~300	1.00	2,000	1.0

※ Sample is accurately weighed into a 1,000 ml volumetric flask and water is filled up to 2/3. It is then allow to stand for 30 minutes at room temperature, while the flask

is vigorously shaken at least 5 times. The flask is then filled with water. The solution is filtered through a Whatman No.12 or equivalent, use the Test Solution. Indicated amount is taken for the test.

Procedure

① Generation of Reducing Sugar : 50 ml of hydrolyzed starch solution and 5 ml of acetate buffer solution are added into a 100 ml volumetric flask, Test Solution. As a reference, water is taken into a volumetric flask and the same procedure is followed. These flasks are allow to stand for 10 minutes in water bath at 60°C.(Note : For enzymes generated from *Aspergillus oryzae* and *Rhizopus oryzae*, it is carried out at 55°C). Indicated amount of Test Solution is taken into a flask for Test Solution and it is timed simultaneously. (When multiple sample are tested, there can be an interval in sampling time considering the time taken for neutralizing the solution after 120 minute reaction time). The content is mixed completely by shaking and allow to stand for 120 minutes in a water bath. After 115~118 minutes of reaction time, 3 drops of phenolphthalein TS are added. The flask is removed from water bath when the reaction time reaches exactly 120 minutes. It is then quickly neutralized with 2% sodium hydroxide solution (approximately 3~7 ml) using a quick drawing burette. It is then cooled to room temperature in running water. The total volume is make to 10 ml with water. 10 ml each of this solution and reference solution is taken and tested for reducing sugar as follows

② Test for Reducing Sugar (Schoorl Method)

(Note : This test method is appropriate for measuring reducing sugar from protein free soluble substances. Sample with considerable amount of proteins are tested after treating with protein precipitating agent.). 10 ml each of Fehling solution A, B is taken into a 250 ml Erlenmeyer flask, where exactly 10 ml of the solution obtained from reducing sugar generation above. A reference solution is treated as same. (Note : When multiple sample are tested, Test Solution is taken into a series of flasks, diluted to 30 ml with water, and Fehling solution A is added. Fehling solution B is added just before heating). The total volume is make to 50 ml with water, which is mixed by gently shaking. Two glass balls are added and a small funnel is placed on top of the flask as a cover. It is then brought to boil within 3 minutes and heated for 2 more minutes. It is then quickly cooled in a ice bath or running water. The funnel is washed with small amount of water. 10 ml of 30% potassium iodide solution and 10 ml of 28% sulfuric acid are added to the solution, which is quickly titrated with 0.1 N sodium thiosulfate solution until the color of iodine disappears. 1 ml of starch TS is

added to the resultant solution, which is titrated by drop-wise adding 0.1 N sodium thiosulfate solution until the blue color disappears. The consumed amount (ml) of 0.1N sodium thiosulfate for Test Solution is S, while the consumption for the reference solution is C. Blank test for reagent is carried out twice with 30 ml of water instead of sample. The average amount of consumption ml is B. Using the titrant difference between B and S (in ml), T_s is obtained. (Subtract S from B and indicate the sample in ml consuming 0.1 N Sodium Thiosulfate to obtain the titration difference, taking it as T_s) Using the titrant difference between B and C (in ml), titrant difference of reference is obtained, T_c .

(Note : Refer to the following Table).

[illegible]

② Use of this table is based on the assumption that two test results are identical under the same test conditions. The risk of error can be avoided by standardization of careful repetition using known pure glucose (5 samples of 10~70 mg range). The calibration curve (reducing sugar content mg vs. titrant difference) is a slightly bent straight line. If a standardization curve is adopted, it is not necessary to obtain standardization for the sodium thiosulfate solution. By using 0.065 N sodium thiosulfate solution, titration value for a blank test is increased to 44~45 ml, thus more accurate result can be obtained.

③ Reducing Sugar Content : By referring to the conversion table (titrant difference to reducing sugar content), reducing sugar content (mg) corresponding to titrant difference (Ts) of sample is obtained, W_s . By the same method, reducing sugar content (mg) corresponding to titrant difference (Ts) of reference is obtained, W_c . The total reducing sugar (glucose) content generated by the Test Solution used is obtained by the following equation.

$$D_s/g = \frac{W_s \times 100}{1,000 \times 10}$$

The total reducing sugar (glucose) content generated by the reference solution is obtained by the following equation.

$$D_c/g = \frac{W_c \times 100}{1,000 \times 10}$$

Calculation of activity of liquid enzyme : Activity of analyzed liquid enzyme is obtained by the following equation.

$$\text{Glucoamylase, units/ml} = (D_s - D_c) \times \frac{F}{2h}$$

F : Dilution factor

Calculation of activity of solid and liquid extract enzyme : Activity of analyzed solid, liquid extract enzyme is obtained by the following equation.

$$\text{Glucoamylase, units/g} = \frac{(D_s - D_c) \times V}{(G \times A \times 2h)}$$

V : Total volume of dilution (ml)

A : Amount of Test Solution used for the test (ml)

(Should refer to the table for Solid sample and Liquid Extracts in Preparation of Test Solution)

G : Weight of sample (g)

Definition of Activity : 1 Glucoamylase unit(GAU) corresponds to the amount of enzyme which produces 1g of glucose as reducing sugar in 1 hour under the test conditions above.

Solutions

- Hydrolyzed Starch Solution (4%) : An amount of solidified corn syrup with 15~20 dextrose equivalent, DE, which corresponds to 40 g of dried form, is dissolved in water and the volume is make to 1,000 ml. This solution is freshly prepared before use.
- Acetate Buffer Solution : 60 g of glacial acetic acid is diluted to 1,000 ml with water. pH of this solution is adjusted to 4.2 with sodium acetate solution, which is 136 g of sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) is dissolved in water and the total volume is make to 1,000 ml with water. (For enzymes produced by *Aspergillus oryzae* and *Rhizopus oryzae*, pH is adjusted to 5.0)
- Fehling solution A : 34.66 g of copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) is dissolved in water, and the total volume is make to 500 ml. This solution is stored in a small container with a cap.
- Fehling solution B : 173 g of potassium sodium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) is dissolved in water, where 50 g of sodium hydroxide is added and the total volume is make to 500 ml with water. This solution is stored in a small container with a cap. Same amount of solution A and B are mixed for use. The theoretical titrant consumption for blank test is 27.8 ml, but 27.5~29.5 ml is appropriate.

Storage Standard of Glucoamylase

Glucoamylase is strongly hygroscopic, so should be stored in a hermetic container in a cold dark place.

10. Glucose Oxidase

Definition Glucose oxidase is an enzyme obtained from a culture of *Aspergillus niger* and its variety. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Glucose Oxidase

Content When Glucose oxidase is analyzed quantitatively, it contains 90~130% of the indicated activity as glucose oxidase.

Description Glucose oxidase is white to pale yellowish brown power or transparent to brown liquid.

- Purity**
- (1) Arsenic : 0.25 g of Glucose oxidase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by gradually heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate (not more than 4ppm).
 - (2) Lead : When 5.0 g of Glucose Oxidase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.
 - (3) Coliform Group : When Glucose oxidase proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods in Food Code, it should contain not more than 30 colonies per 1 g of this product.
 - (4) Salmonella : When Glucose oxidase proceed as directed under Microbe Test Methods for Salmonella in General Test Methods in Food Code. it should be negative (-).
 - (5) E. Coli : When Glucose oxidase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

- Application and Principle : This test is to measure the activity of glucose oxidase that is obtained from a culture of *Aspergillus niger* and its variety. Activity test is based on titration of gluconic acid which is produced under the presence of excess amount of substrate and air.
- Preparation of Test Solution : Sample is diluted with chloride acetate buffer solution

(pH 5.1), so that 1 ml of the solution contains 5~7 GOTu.

◦ Test Procedure : 25 ml of substrate solution is added in a 32 × 200 mm test tube and isothermalized for 20 minutes in a water bath at $35 \pm 1^\circ\text{C}$. 3 ml of Test Solution is added and mixed by shaking. A glass sparger, previously control air flow of 700~750 ml per minute, is inserted into the test tube. If excess bubbles are generated, 3 drops of octadecanol solution are added. Glass sparger is removed exactly after 15 minutes and washed with water, which is added into the test tube. 10 ml of 0.1 N sodium hydroxide solution and 3 drops of phenolphthalein TS are added immediately, which is then stirred with a magnetic stir bar. It is then titrated 0.05 N hydrochloric acid. The consumption of hydrochloric acid (ml) for test solution is S. Separately, a blank test is carried out with 25 ml of chloride acetate buffer solution (pH 5.1) instead of substrate solution and the consumption of hydrochloric acid (ml) is B. Activity of the enzyme is obtained by the following equation.

$$\text{GOTu/g} = \frac{(B-S) \times N \times 180 \times F}{3 \times W}$$

N : Normality of 0.05 N hydrochloric acid

F : Dilution factor

W : Weight of sample contained in 1 ml of Test Solution (g)

180 : Molecular weight of glucose

3 : Conversion factor to a defined unit

Definition of Activity : 1 Glucose oxidase titrimetric unit(GOTu) corresponds to the amount of enzyme which oxidizes 3 mg of glucose to gluconic acid under the test condition above.

Solutions

- Phenolphthalein TS : 2 g of phenolphthalein is dissolved in 100 ml of methyl alcohol.
- Octadecanol Solution : Saturated octadecanol solution in methyl alcohol.
- Chloride-acetate buffer solution (pH 5.1): 2.92 g sodium chloride and 4.1 g sodium acetate are dissolved in 900 ml of water. pH of the solution is adjusted to 5.1 with diluted acetic acid or sodium hydroxide solution. The total volume is make to 1,000 ml with water
- Substrate Solution : 30 g of glucose (anhydrous) is dissolved in chloride-acetate buffer solution (pH 5.1) so that the total volume is 1,000 ml.

Storage Standard of Glucose Oxidase

Glucose Oxidase is strongly hygroscopic, so should be stored in a hermetic container in a cold dark place.

11. Glucose Isomerase

Definition Glucose Isomerase is an enzyme obtained from a culture of *Actinoplanes missouriensis*, *Bacillus coagulans*, *Microbacterium arborescens*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, *Streptomyces rubiginosus*, *Streptomyces murinus*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Glucose Isomerase

Content When Glucose Isomerase is analyzed quantitatively, it contains 90~130% of the indicated activity as Glucose Isomerase.

Description Glucose Isomerase is white~pale yellow~yellowish green~blackish brown platelet, power or transparent~blackish brown liquid.

Purity (1) Arsenic : 0.25 g of Glucose Isomerase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate(not more than 4ppm).

(2) Lead : When 5.0 g of Glucose Isomerase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

(3) Coliform Group : When Glucose Isomerase proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods in Food Code, it should contain not more than 30 colonies per 1 g of this product.

(4) Salmonella : When Glucose Isomerase proceed as directed under Microbe Test Methods for Salmonella in General Test Methods in Food Code, it should be negative (-).

(5) E. Coli : When Glucose Isomerase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

◦ Application and Principle : This test is to measure the activity of glucose isomerase that is obtained from a culture of *Actinoplanes missouriensis*, *Bacillus coagulans*, *Microbacterium arborescens*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*,

Streptomyces rubiginosus, *Streptomyces murinus*. Activity test is based on measuring conversion rate from glucose to fructose in a layered reactor. Test Procedure can be explained as initial velocity analysis method. Notable conditions are 45% w/w glucose extract, pH of inlet 7.0~8.5 at room temperature, 60°C temperature, and magnesium concentration of 4×10^{-3} M.

- Preparation of Test Solution : Sample is accurately weighed (g or ml) into a vacuum flask so that it contains 2,000~8,000 Glucose isomerase unit (GlcU), where 200 ml of substrate solution are added and mixed for 15 seconds. The mixture is stirred in a 5 minute interval for 40 minutes. It is then degassed for 30 minutes in vacuum. In case where the sample is liquid, it is adsorbed on resin prior to use as follows. Strongly alkaline anion exchange resin (IRA 90) is washed with water for 4 hours and pH is adjusted to 7.0~9.0. This is taken 80 ml, packed into a glass column, and added again 50 ml of water to the column. Sample, corresponding to 10,000 GlcU, accurately weighed, and added. Finally, the column is washed with 10 ml of water. The flow rate is adjusted to 2 ml/minute before the sample is added. Once the sample is added, the effluent is re-entered into the top of the column and it is circulated for 8 hours at room temperature so that the sample is adsorbed on the resin. 40 ml of sample adsorbed on the resin is into a vacuum flask and proceed as directed under the rest of the procedure described here.
- Test Procedure : Based on the activity estimate of a sample, a substrate flow rate is adjusted to 0.2~0.3 per 1 fractional transformation. Fractional transformation is obtained from the value of specific rotation on the initial substrate and the effluent as the following equations. Once the exact flow rate is determined, the column is run for 1 full day (at least for 16 hours) and pH of the substrate solution is monitored. If necessary, flow rate is adjusted. Flow rate is measured and the effluent is collected, which is covered and allow to stand for 30 minutes at room temperature. Fractional transformation from glucose to fructose is obtained from the following equation. If the transformation is not more than 0.2 or not less than 0.3, the flow rate is adjusted so that it falls within this range .In case where flow rate needs to be adjusted, the column is re-equilibrated for 2 hours or more, the additional effluent is collected and fractional transformation is obtained. The flow rate is measured and the effluent is allowed to stand for 30 minutes with a cover and fractional transformation is obtained.
- Specific Rotation : Optical rotations for the effluent and the initial substrate are measured at 25°C, and specific rotations are calculated from the following equation.

$$\alpha = 100a / lpd$$

α : Observed optical rotation

l : Length of sample tube (dm)

p : Concentration of Test Solution (g/100g solution)

d : Specific gravity of the solution at 25°C

Fractional conversion : Fractional conversion X is calculated from the following equation.

$$X = (\alpha E - \alpha S) / (\alpha F - \alpha S)$$

αE : Specific Rotation of the column effluent

αS : Specific Rotation of glucose substrate

αF : Specific Rotation of fructose (in this case, -94.54)

Activity of enzyme is obtained from the following equation.

$$\text{GlcU/g or ml} = \frac{FS}{W} \times XE \times \ln \left[\frac{XE}{XE - X} \right]$$

F : Flow rate (ml/min)

S : Concentration of glucose ($\mu\text{mol/ml}$)

X : Fractional conversion

XE : Fractional conversion at equilibrium or 0.51

W : amount of sample(g, ml)

Definition of Activity : Activity of an enzyme is expressed as Glucose isomerase unit (GlcU, c indicates Column Treatment Test Method). 1 GlcU corresponds to the amount of enzyme that isomerizes glucose into fructose under the above conditions at a lowest ratio of 1 μmol per minute.

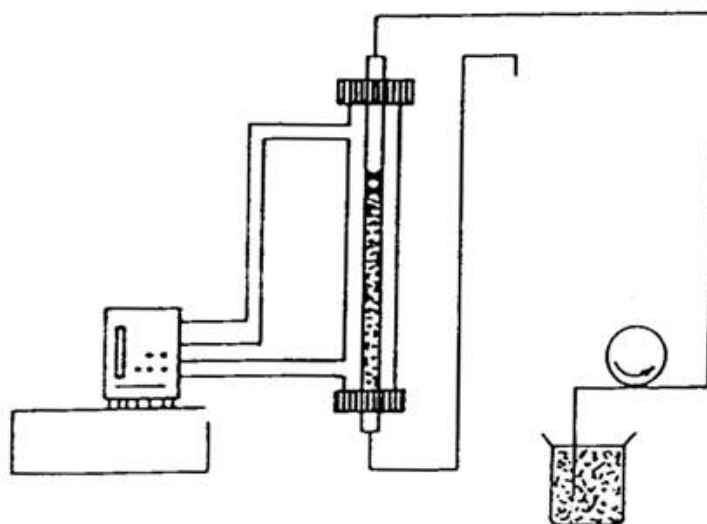
Apparatus

Column apparatus is depicted as below. A coarse glass filter is attached to the bottom and warm water is circulated by a circulation pump through a double tube (2.5cm inner diameter x 40cm length) that is connected to a water bath at 60°C. An

peristaltic pump with an inverter (maximum flow rate of 800 ml/h) is attached to the column. An inner diameter of the tube, that is connected to the peristaltic pump should be adjustable the flow rate as 60 to 150 ml/h. The connector of outlet of column is connected to a collection container. (Note : all the connecting parts should be made of glass or chemically inert plastic.)

Preparation of Column

Test Solution is transferred to the column using magnesium sulfate solution. The solution is allowed to stand so that enzymes are settled down, upon which a porous plate is placed. Air adsorbed on the plate should be removed. The top of the plate is filled with cotton ball to a thickness of 1~2 cm. This acts as a filter that removes bubbles generated from glucose substrate and keeps the solution temperature constant. The tube, that is connected to the peristaltic pump with an inverter, is connected to the column inlet and sealed connector to prevent to let air in. The connector to the pump inlet is inserted into the substrate solution. The flow rate is adjusted to at least 80 ml per hour with a pump. This is maintained for 1 hour at room temperature.



Column apparatus for analysis of glucose isomerizing enzyme

Solution

- Substrate Solution : 539 g of glucose and 1.0 g of magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) are dissolved in 700 ml of water (50~60°C). This solution is cooled to room temperature and the pH is adjusted to 7.0~8.5. The total volume make to 1,000 ml with water, which is degassed under a reduced pressure for 30 minutes.

- Magnesium Sulfate Solution : 1 g of magnesium sulfate is dissolved in water and pH is adjusted to 7.5~8.0 with 1 N sodium hydroxide solution. The total volume is brought up to 1,000 ml with water.

Storage Standard of Glucose Isomerase

Glucose Isomerase is strongly hygroscopic, hence should be stored in a hermetic container in a cold dark place.

12. Diastase (Diastatic Power, DP)

Definition Diastase is an enzyme obtained from malt. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Diastase

Content Diastase contains 90~130% of the indicated activity as Diastase.

Description Diastase is white~pale yellow~deep brown power, granule, lump or transparent~deep brown liquid.

Purity (1) Arsenic : 0.25 g of Diastase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should be appropriate (not more than 4ppm).

(2) Lead : When 5.0 g of Diastase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

(3) Coliform Group : Diastase is tested by Microbe Test Methods for Coliform Group in General Test Methods in Food Code. It should contain 30 colonies or less per 1 g of this product.

(4) Salmonella : Diastase is tested by Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).

(5) E. Coli : When Diastase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay (Activity)

Application and Principle : This test is to measure the activity of amylase in malt and other enzymes. Activity test is based on hydrolysis of starch substrate for 30 minutes at 20°C and pH 4.6. Reducing sugar obtained by hydrolysis is tested by the titration with alkaline ferricyanide.

Preparation of Test Solution

◦ Malt sample : 30 g of sample is finely ground with a mill. 25 g of the ground powder is accurately weighed into a 1,000 ml Erlenmeyer flask, which is leached in 500 ml of 0.5% sodium chloride solution for 2.5 hours at $20 \pm 0.2^{\circ}\text{C}$ while gently

shaking in a 20 minute interval (note : The flask should not be turned up side down. The amount of sample left on the inner wall of the flask should be minimized.) It is then filtered through a Whatman No.1 filter paper or equivalent on a 32 cm diameter funnel. First 50 ml of the filtrate is added back to the funnel and filtered again. To prevent evaporation during filtering, a watchglass is placed on top of the funnel and other opening (necks and mouth of receiving container) is covered appropriately. Filtrate is collected exactly for 30 minutes. 20 ml of the filtrate is diluted to 100 ml with 0.5% sodium chloride solution (Test Solution).

- Other Enzymes : The final diluted solution is prepared so that it contains Diastatic power(DP) value 2~150°C per 10 ml.

Test Procedure : 10 ml of Test Solution is accurately taken into a 250 ml volumetric flask, where 200 ml of substrate solution (isothermalized for 30 minutes at $20 \pm 0.2^\circ\text{C}$ prior to use) and time is recorded. The flask is cooled for 30 minutes in a water bath at 20°C . 20 ml of 0.5 N sodium hydroxide solution and water are added to bring the volume to 250 ml. 5 ml of the resultant solution is taken into a 125 ml Erlenmeyer flask, added 10 ml of alkaline ferricyanide solution, and mixed. This is heated exactly for 20 minutes in a boiling water bath. After cooling to room temperature, 25 ml of A.P.Z. solution, 1 ml of potassium iodide solution, and 2 ml of starch TS are added to the flask, which is then titrated with 0.05 N sodium thiosulfate solution until the blue color disappears completely (consumed amount in ml of sodium thiosulfate solution, S). Separately, blank test solution is prepared in a 250 ml volumetric flask with 20 ml of 0.5 N sodium hydroxide solution, 10 ml of Test Solution, 200 ml substrate solution, and water (total volume 250 ml) by following the same procedure. The consumed amount in ml of sodium thiosulfate solution for blank test is B.

Activity of diastase as expressed as DP°C is obtained using the following equations.

$$\text{DP}^\circ\text{C}(\text{as a base material}) = (B - S) \times 23 \times \frac{F}{100}$$

$$\text{DP}^\circ\text{C}(\text{dried form}) = \text{DP}^\circ\text{C}(\text{as a base material}) \times \frac{100}{(100 - M)}$$

23 : Conversion factor to a defined unit

M : Water Content (%)

F : Dilution Factor (Total Dilution/Weight of sample(g))

Definition of Activity : 1 Diastase activity unit expressed as DP°C (degrees of diastatic power) corresponds to an amount of enzymes contained in 0.1 ml of 5% solution of Test Solution which produces sufficient amount of reducing sugar that can reduce 5 ml of Fehling solution when 100 ml of substrate is processed for 1 hour at 20°C.

Apparatus

Mill : Laboratory mill is used.

Solutions

- Acetate Buffer Solutions : 68 g of sodium acetate is dissolved in 500 ml of 1N acetic acid. Water is added to bring the total volume to 1,000 ml.
- Starch : A starch that is specified in α -amylase(non microbial) is used.
- Substrate Solution : 20 g of starch (as dried form) is dispersed in 50 ml of water, and added slowly to 750 ml of boiling water. It is then boiled for 2 minutes. After cooling, it is mixed with 20 ml of acetate buffer solution. The total volume is brought up to 1,000 ml with water.
- Acetic Acid-Potassium Chloride-Zinc Sulfate Solution (A.P.Z.) : 70 g of potassium chloride and 20 g of zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) are dissolved in 700 ml of water, where 200 ml of glacial acetic acid is added. The total volume is brought up to 1,000 ml with water.
- 0.05 N alkaline ferricyanide solution : 16.5 g of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) and 22 g of sodium carbonate are dissolved in 800 ml of water. Water is added to bring the total volume to 1,000 ml.
- Potassium iodide solution : 50 g of potassium iodide is dissolved in 50 ml of water and diluted to 100 ml, where 2 drops of 50% sodium hydroxide solution are added. This solution should be colorless

Storage Standard of Diastase

Diastase is strongly hygroscopic, hence should be stored in a hermetic container in a cold dark place.

13. Lac Color

Definition Lac Color is extracted by water from the resinoid secreted by larvae of coccidium, *Laccifer lacca* KERR. The major component of this color is laccaic acid one of anthraquinones. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Lac Color

Content Color value ($[E]_{1cm}^{10\%}$) of Lac Color should be higher than the indicated value.

Description Lac Color is red~dark reddish brown liquid, lump, powder or paste with a slight characteristic scent.

Identification (1) Test Solution obtained in Color Value section for Lac Color is reddish violet. It has a maximum absorption near 490 nm.

(2) When adding 1 ml of hydrochloric acid to 10 ml of Test Solution in (1), it changes orange~orange red.

Purity (1) Arsenic : 0.25 g of Lac Color is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for Arsenic is carried out with this test solution, it should be appropriate (not more than 4ppm).

(2) Lead : When 5.0 g of Lac Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 8 ppm.

Assay (Color Value) Appropriate amount of Lac Color is accurately weighed so that the absorbance is within 0.3~0.7 and dissolved in 20 ml of sodium carbonate solution (1→200). Water is added so that the total volume is 100 ml. 1 ml of this solution is diluted to 100 ml with 0.1 N hydrochloric acid, Test Solution. If necessary, the solution is centrifuged and the supernatant is used. Using 0.1 N hydrochloric acid as a reference solution, absorbance A of the test solution is measured at the maximum absorption wavelength near 490 nm with 1 cm path length. Color value is obtained using the following equation.

$$\text{Color Value } [E]_{1cm}^{10\%} = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

14. Lactase

Definition Lactase is an enzyme obtained from a culture of *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety, *Bacillus circulans*, *Saccharomyces* genus. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Lactase

Content Lactase contains 90~130% of the indicated activity as lactase.

Description Lactase is white~pale yellow~deep brown power or transparent~deep brown liquid.

Purity (1) Arsenic : 0.25 g of Lactase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for Arsenic is carried out with this test solution, it should be appropriate (not more than 4ppm).

(2) Heavy Metals : 0.5 g of Lactase are carbonized by heating mildly in a quartz or porcelain crucible. After cooling, add 2 ml of nitric acid and 5 drops of sulfuric acid, it is heated until white smoke disappears, which is then reduced to ash by further heating at 450~550°C. After cooling, 2 ml of hydrochloric acid is added, which is then evaporated to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resultant residue, which is then heated for 2 minutes. After cooling, 1 drop of phenolphthalein TS is added, then ammonia TS is added until the color of the solution changes pale red. The resultant solution is transferred into a Nestler cylinder by rinsing with water. 50 ml of test solution is prepared by adding 2 ml of diluted acetic acid (1→20) and water. When this solution tested for Heavy Metals, the content should not be more than 40ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water are added to bring the total volume to 50 ml.

(3) Lead : 0.8 g of Lactase (if it is liquid, it is concentrated by evaporation in a

water bath) is carbonized by mildly heating, which is reduced ash by further heat treatment at a temperature below 500°C. Carefully 20 ml of dilute nitric acid is added to the ash, which is then gently boiled for 5 minutes and cool. It is then filtered (if necessary), the residue is washed with water, which is then added to the filtrate. Water is added so that total volume of this solution becomes 50 ml. This test solution is tested for lead. The detected amount of lead should not be more than 10ppm.

- (4) Coliform Group : Lactase is tested by Microbe Test Methods for Coliform Group in General Test Methods in Food Code. It should contain 30 colonies or less per 1 g of this product.
- (5) Salmonella : Lactase is tested by Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).

Assay(activity)

- Application and Principle : This test is to measure the activity of lactase obtained from a culture of *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety, *Bacillus circulans*, *Saccharomyces* genus. Activity test is based on hydrolysis of o-Nitrophenyl-β-D-Galactopyranoside (ONPG) substrate for 15 minutes at 37°C and specified pH (4.5 for *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety, 6.0 for *Bacillus circulans*, 6.5 for *Saccharomyces* genus).
- Preparation of Test Solution : A Test Solution is prepared so that 1 ml contains 0.15~0.65 lactase unit. Sample is accurately weighed into a mortar, added an appropriate buffer solution, and ground, which is then transferred to a volumetric flask and filled with a buffer solution.

Test Procedure : 4 ml of substrate solution is placed in a 20×150 mm test tube with a stopper, which is then isothermalized in a water bath at 37 ± 0.1°C. 1 ml of Test Solution is mixed by shaking. After exactly 15 minutes, 1 ml of the mixed solution is added to a test tube with 1 ml of 10% sodium carbonate solution, which is then diluted to 10 ml with water. Separately, a reference solution is prepared by following the same procedure with 1 ml of water. Absorbance at 420 nm is measured with 1 cm of path length. Activity of enzyme is calculated by the following equation.

$$\text{LacU/g} = \frac{A \times 5 \times 10}{e \times 15 \times W}$$

A : Average absorbance of Test Solution

5 : Amount of enzyme reaction mixture (ml)

10 : Final amount of diluted enzyme reaction mixture solution (ml)

ϵ : Extinction coefficient measured with standard o-nitrophenol solution

15 : Time for isothermalization (minute)

W : Amount of sample contained in 1 ml of Test Solution (g)

Definition of Activity : 1 Lactase unit (LacU) is an amount of enzyme that extricates 1 μ mol of o-nitrophenol per 1 minute under the above conditions.

Test Solution

- Acetate Buffer Solution (for *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety) : 800 ml of water is added to 50 ml of 2 N acetic acid and pH is adjusted to 4.5 ± 0.05 with 2 N sodium hydroxide solution (pH 6.0 ± 0.05 for *Bacillus circulans*). Then the solution is diluted to 1,000 ml with water.
- P-E-M Buffer Solution (for *Saccharomyces* genus) : 27.2 g of monobasic potassium phosphate, 37.2 mg of EDTA (2 hydrate), and 20.3 mg of magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) are dissolved in 800 ml of water. pH is adjusted to 6.5 ± 0.05 with 2 N sodium hydroxide solution. Then the solution is diluted to 1,000 ml with water
- Standard o-Nitrophenol Solution : 139.0 mg of o-Nitrophenol is dissolved in 10 ml of 95% alcohol in a 1,000 ml volumetric flask, which is then filled with water to mark. 2, 4, 6, 8, 10, 12 and 14 ml each of this solution is placed in a 100 ml volumetric flask, which is then filled with 1% sodium carbonate solution. 1 ml of each diluted solution contains 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, and 0.14 μmol of o-nitrophenol, respectively. Using water as a reference solution, absorption at 420 nm with 1cm path length is measured and a calibration curve of absorption vs. $\mu\text{mol/ml}$ of o-nitrophenol is obtained. Calibration curve is a straight line through zero point. Extinction coefficient obtained that absorbance of each diluted solution divided o-nitrophenol $\mu\text{mol/ml}$. Extinction coefficient (ϵ) should be approximately 4.65
- Substrate Solutions for *Aspergillus* and *Bacillus* : 370 mg of o-Nitrophenyl- β -D-galactopyranoside is dissolved in approximately 75 ml of acetate buffer solution in a 100 ml volumetric flask, which is then filled to 100 ml.
- Substrate Solution for *Saccharomyces* : 250.0 mg of o-Nitrophenyl- β -D-galactopyranoside is dissolved in approximately 75 ml of PEM buffer solution in a 100 ml volumetric flask, which is then filled to 100 ml.

Storage Standard of Lactase

Lactase is strongly hygroscopic, hence should be stored in a hermetic container in a cold dark place.

15. Lactoferrin Concentrates

Definition This is obtained by concentrating milk that is previously defatted and purified by separation. The major component is lactoferrin. It also contains whey protein.

Compositional Specifications of Lactoferrin Concentrates

Content Lactoferrin Concentrates should contain more than 90.0% of lactoferrin.

Description Lactoferrin Concentrates is pale orange red~pale reddish brown powder and scentless .

Identification When Lactoferrin Concentrates is quantitatively analyzed, a lactoferrin peak is observed at 280 nm.

Purity (1) Arsenic : 0.5 g of Lactoferrin Concentrates is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should be appropriate (not more than 2ppm).

(2) Lead : When 5.0 g of Lactoferrin Concentrates is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) pH : pH of this solution (2→100) should be 5.2~7.2.

(4) Coliform Group : Lactoferrin Concentrates is tested by Microbe Test Methods for Coliform Group in General Test Methods in Food Code. It should contain 30 or less per 1 g of this product.

Residue on Ignition When Residue on Ignition analysis is done with 1 g of Lactoferrin Concentrates, the amount of residue should not be more than 1.3%.

Assay Approximately 20 mg of Lactoferrin Concentrates is accurately weighed and dissolved in 0.5 M of sodium chloride solution and to make volume 10 ml. The solution is filtered through a 0.45 µm Millipore filter, Test Solution. Separately, a Standard Solution is prepared with 20 mg of lactoferrin standard following the same procedure. 20 µl each of Standard Solution and Test Solution is injected into liquid chromatograph and the content of lactoferrin is obtained from the following equation.

$$\text{Content (\%)} = \frac{Au \times Ws}{As \times Wu} \times 100$$

Au : Peak area of test solution

As : Peak area of standard solution

Ws : Amount of standard material (mg)

Wu : Amount of sample (mg)

Operation Conditions

-Detector : UV 280 nm

-Column : Ashaipak C4P 50(4.6 mm × 150 mm) or equivalent

-Column Temperature : Room temperature

-Mobile Phase : Solution A : Solution B (30 : 70)

Solution A : acetonitrile : 0.5M sodium chloride solution (1 : 9)

Solution B : acetonitrile : 0.5M sodium chloride solution (5 : 5)

Solutions A, B contains 0.03% of Trifluoroacetic acid.

-Flow rate : 0.8 ml/min

16. Lecithin

Definition Lecithin is prepared from oil seeds or yolk of egg. Its major component is phospholipid.

Compositional Specifications of Lecithin

Description Lecithin is transparent or semi transparent pale yellow~dark brown thick fluid, semi-solid, lump, powder or granule with slight characteristic scent and taste.

Identification (1) 1 g of Lecithin is dissolved in 5 ml of petroleum ether. Upon adding 15 ml of acetone, white~pale yellow precipitates are formed.

(2) 1 g of Lecithin is placed in a flask for decomposition, where 5 g of powdered potassium sulfate, 0.5 g of copper sulfate, and 20 ml of sulfuric acid are added. The flask is sloped to 45°C angle and gently heated so that it doesn't bubble. Then the temperature is raised to boil until the solution becomes transparent blue. It is then heated for 1~2 hours and cooled and the same amount of water is added. 10 ml of ammonium molybdate (1→5) is added to 5 ml of the resultant solution. Upon heating yellow precipitates are formed.

(3) 5 ml of diluted hydrochloric acid (1→2) is added to 0.5 g of Lecithin, which is then heated for 2 hours in a water bath and filtered, Test Solution. 0.01 ml of Test Solution is tested by Method 1 in Paper Chromatography using a mixed solution of n butyl alcohol, acetic acid, and water (4 : 1 : 2) as a developing solution. An orange red spot corresponds to the spot obtained from the control solution is observed. For the filter paper, No.2 filter paper for chromatography is used. Development is stopped when the developing solvent rises about 25 cm, which is then dried in air. Colour is developed by spraying Dragendorf TS and observed in daylight. 0.01 ml of the reference solution is prepared by dissolving 0.1 g of choline chloride in water (total volume 20 ml).

Purity (1) Acid Value : About 2 g of Lecithin is accurately weighed and dissolved in 50 ml of petroleum ether. Then add 50 ml of alcohol, Test Solution. When it tested as Acid Value in Oil and Fat Test, the value should not be more than 36.

(2) Toluene Insoluble matter : About 10 g of Lecithin is accurately weighed and dissolved in 100 ml toluene by shaking in a 250 ml Erlenmeyer flask. Insoluble matter are filtered through a crucible type G3 glass filter (pore size 16~40 μm), previously weighed, and washed with 25 ml of toluene several times. It is then dried for 1 hour at 105°C and cooled in a desiccator and weighed. The content should not be more than 0.3%.

(3) Acetone Soluble Substances : Approximately 2 g of Lecithin is accurately

weighed into a 50 ml graduated centrifuge tube with a stopper, where 3 ml of petroleum ether and 15 ml of acetone. It is then well mixed by stirring and placed in an ice bath for 15 minutes. 50 ml of acetone, previously chilled to 0~5°C, is added to the solution, which is well mixed by stirring and placed in an ice bath for 15 minutes. It is then centrifuged for 10 minutes to 3,000rpm by the following procedure. The supernatant is taken into a previously weighed flask. Again, 0~5°C acetone is added to make 50ml, and cooled in an ice bath while stirring and mixing. It is then centrifuged under the following manner. The supernatant is transferred into a flask and distilled. The residue is dried for 1 hour at 105°C. The amount of the residue should not be more than 40%.

- (4) Peroxide Value : 5 g of Lecithin is accurately weighed into a 250 ml of Erlenmeyer flask with a stopper. It is then dissolved to a clear solution in 35 ml of a 3 : 2 mixture of glacial acetic acid and chloroform. Clean nitrogen is passed through to replace air in the flask. 1 ml of potassium iodide TS is added while nitrogen is bubbled through. A stopper is placed immediately and the flask is shaken for 1 minute. It is then allow to stand for 5 minutes in a dark place. 75 ml of water is added and shaken vigorously with a stopper. It is then titrated with 0.01 N sodium thiosulfate solution (indicator : starch TS). Peroxide value is obtained from the following equation. It should not be more than 10. Separately, a blank test is carried out for correction.

$$\text{Peroxide Value} = \frac{\text{consumed amount of 0.01N sodium thiosulfate solution (ml)}}{\text{weight of sample (g)}} \times 10$$

- (5) Arsenic : Should follow the procedure of Purity (1) in Guar Gum (Not more than 4ppm).
- (6) Lead : When 5.0 g of Lecithin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.
- (7) Mercury : When Lecithin accurately weighed 0.1g and tested by Mercury Test Method, its content should not be more than 1.0ppm.

Loss on Drying When Lecithin is dried for 1 hours at 105°C, the loss should not be more than 2.0%.

17. Rennet Casein

The specification of Rennet Casein is combined to 63. Casein in Natural Additives.

The date of notification : November 10, 2010 (Notification No. 2010-82)

18. Locust Bean Gum

Carob Bean Gum

Definition Locust Bean Gum is obtained by crushing endosperm of legumes and locust bean (*Ceratonia siliqua* LINNE). Crushed endosperms are dissolved in hot water and filtered. By adding isopropyl alcohol, precipitates are formed. Major component is polysaccharide.

Compositional Specifications of Locust Bean Gum

Description Locust Bean Gum is white~yellowish white powder with characteristic odor.

Identification (1) 2 g of Locust Bean Gum is placed into a 400 ml beaker. It is then wetted with 4 ml of isopropyl alcohol. 200 ml of cold water is added with vigorously stirring. When the solution is homogenized by stirring continuously, it becomes sticky solution.

(2) 100 ml of Test Solution in (1) is placed into a 400 ml beaker. When it is boiled for 10 minutes in a water bath, the viscosity increases significantly.

Purity (1) Arsenic : It is tested as directed under the procedure of Purity (1) in Guar Gum.

(2) Lead : When 5.0 g of Locust Bean Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Locust Bean Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Locust Bean Gum proceeded by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Starch : 1 g of Locust Bean Gum is dissolved in 10 ml of water and is boiled, which is then cooled. When 2 drops of iodine TS are added, it should not turn blue.

(6) Isopropyl alcohol : 0.2 g of Locust Bean Gum is accurately weighed into a 300 ml round bottom flask, 200 ml of water is added, boiling chips and 1 ml of silicone resin are added and mixed well. Distillation column is connected to this, 4 ml of internal standard solution is taken into a 100 ml flask. While caring for the bubbles not to overflow, distill the solution at the rate of 2~3 ml per 1 minute until the milky liquid becomes about 90 ml, and water is added to make 100 ml, Test Solution. However, tert-butyl alcohol (1→1,000) is used as internal standard solution. Separately, 0.5 g of isopropyl alcohol is accurately weighed and water is added to

make 500 ml, 2 ml of this solution and 4 ml of internal standard solution is taken again, water is added to make 100 ml, Standard Solution. 2µl of each of test solution and standard solution is taken respectively, and injected to gas chromatograph with the following operation condition. Then, ratio of isopropyl alcohol peak against tert-butyl alcohol peak in test Solution and standard solution, Q_T and Q_S , is calculated separately, and the content of isopropyl alcohol is calculated by following formula, the content should not be more than 1.0%.

$$\text{Content of Isopropyl alcohol(\%)} = \frac{\text{Weight of isopropyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_T}{Q_S} \times \frac{2 \times 100}{500 \times 100} \times 100$$

Q_T : Ratio of isopropyl alcohol peak against tert-butyl alcohol peak in Test Solution

Q_S : Ratio of isopropyl alcohol peak against tert-butyl alcohol peak in standard solution

Operation Conditions

Column : PLOT Q or equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Injection Temperature : 200°C

Column Temperature : 120°C

Detector temperature : 300°C

Carrier gas : Nitrogen or Helium

- (7) Total Viable Aerobic Count : When Guar Gum is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 per 1 g
- (8) E. Coli : When Locust Bean Gum is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (9) Salmonella : When Locust Bean Gum is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (10) Number of Fungi : When Locust Bean Gum is tested by Microbe Test Methods for Number of Fungi in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 500 per 1 g

Ash When it is tested for ash, the content should not be more than 1.2%.

Loss on Drying 3 g of Locust Bean Gum is dried for 5 hours at 105°C. The loss

should not be more than 15%.

Protein When Locust Bean Gum is tested by Kjeldahl Method in Nitrogen Determination, the amount should not be more than 8%. (Protein Factor : 6.25).

Acid Insoluble substances 1.5 g of Locust Bean Gum is accurately weighed and dissolved in 150 ml of water and 1.5 ml sulfuric acid into a beaker, which is covered with a watch glass and heated for 6 hours in a water bath. Beaker wall is washed with water so that the residue doesn't remain on the wall. After heating is complete, it is filtered through a glass filter (Glass filter is accurately weighed. 500 mg of appropriate filtering aid is added to the filter, which is then heated until the weight becomes constant.). The residue is washed thoroughly with hot water and dried for 3 hours at 105°C. The weight of the filtering aid is subtracted from the weight of the residue, which should not be more than 5%.

19. Lysozyme

Definition Lysozyme is an enzyme obtained by refined resin albumen. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Lysozyme

Content When Lysozyme is converted to a dried form and analyzed quantitatively, it contains 90~130% of indicated activity as lysozyme.

Description Lysozyme is white powder.

Identification (1) 50 mg of Lysozyme is dissolved in 100 ml of phosphate buffer solution (pH 6.2). 2 ml of this solution is diluted 100 ml with phosphate buffer solution (pH 6.2). 2 ml of this solution is diluted 50 ml with phosphate buffer solution (pH 6.2), Test Solution. 3 ml each of substrate solution is taken into 2 test tubes, which are heated for 3 minutes at 35°C. Separately, Test Solution and phosphate buffer solution (pH 6.2) are heated for 3 minutes at 35°C. 3 ml of each is added to the previous test tubes, which is then allow to stand for 10 minutes at 35°C. Turbidity of the solution with Test Solution should not be more than that with phosphate buffer solution (pH 6.2).

(2) A solution (1→10,000) of Lysozyme dissolved in acetic acid-sodium acetate buffer solution (pH 5.4) shows a maximum absorption at 279~281 nm..

Purity (1) Clarity of Solution : 5 ml of an aqueous solution (1→100) of Lysozyme is taken. pH is adjusted to 3.0 with dilute hydrochloric acid if necessary. The transmittance of the resultant solution at 660 nm should be more than 80.0%.

(2) Arsenic : 0.77 g of Lysozyme is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 1.3ppm.

(3) Lead : When 5.0 g of Lysozyme is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(4) Mercury : When 0.1 g of Lysozyme is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Chloride : Approximately 0.5 g of Lysozyme is accurately weighed and dissolved

in 50 ml of water, where 0.1 ml of 10% potassium chromate solution is added. It is then titrated with 0.1 N silver nitrate solution. The content of chlorides (as chlorine) should not be more than 3.0%.

1 ml of 0.1 N silver nitrate solution = 3.545 mg Cl

- (6) Nitrogen : When Lysozyme is tested by Kjeldahl Nitrogen Test in nitrogen determination method, the amount should be between 16.8 and 17.8%.
- (7) Total Viable Aerobic Count : When Lysozyme is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 per 1 g
- (8) E. Coli : When Lysozyme is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (9) Salmonella : When Lysozyme is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (10) Staphylococcus aureus : When Lysozyme is tested by Microbe Test Methods for Staphylococcus aureus in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Water Content Water content of Lysozyme is determined by direct titration in water determination (Karl-Fisher Titration) and should not be more than 6.0%.

Residue on Ignition When Lysozyme is done with Residue on Ignition, the amount of residue should not be more than 1.5%.

Assay

- Application : This test is to measure the activity of lysozyme that is obtained by refining albumen with resin.
- Preparation of Test Solution : 50 mg (activity) of Lysozyme is accurately weighed and dissolved in phosphate buffer solution (pH 6.2) (total volume 100 ml). 2 ml of this solution is diluted to 100 ml with in phosphate buffer solution (pH 6.2). 2 ml of the diluted solution is further diluted to 50 ml with in phosphate buffer solution (pH 6.2).
- Preparation of Standard Solution : Amount of being equivalent to 50 mg (activity) lysozyme standard (drying loss is previously measured by the same method as the sample) is accurately weighed and dissolved in phosphate buffer solution (pH 6.2) to make volume 100 ml. 2 ml of this solution is diluted to 100 ml with in phosphate buffer solution (pH 6.2). 2 ml of the diluted solution is further diluted to 50 ml with

in phosphate buffer solution (pH 6.2).

Test Procedure : 3 ml each of substrate solution is placed in three test tubes, which are heated for 3 minutes at 35°C. Separately, Standard Solution, Test Solution, and phosphate buffer solution are heated for 3 minutes at 35°C. Each solution is added to the previous 3 test tubes, which are then reacted for 10 ± 0.1 minutes at 35°C. Using water as a reference, absorbance at 640 nm is measured immediately (AS = Standard Solution, AT = Test Solution, and AO = phosphate buffer solution). The test is repeated three times and an average value is obtained. Activity of lysozyme is calculated from the following equation.

Activity of lysozyme mg(activity)/mg, as a dehydrated form

$$\text{Content (\%)} = \frac{\text{Amount of standard material} \left[\text{dehydrated form, mg(activity)} \right]}{\text{Weight of sample} \left[\text{dehydrated form (mg)} \right]} \times \frac{A_o - A_T}{A_o - A_s}$$

Solutions

◦ Phosphate Buffer Solution (pH 6.2)

Solution 1 : 10.4 g of sodium phosphate, monobasic is dissolved in water (total volume = 1,000 ml).

Solution 2 : 9.465 g of sodium phosphate, dibasic (anhydrous) is dissolved in water (total volume = 1,000 ml).

Solution 1 and Solution 2 (815 : 185) are mixed and pH is adjusted to pH 6.2.

◦ Acetic Acid Sodium Acetate Buffer Solution (pH 5.4)

Solution 1 : 13.6 g of sodium acetate is dissolved in water (total volume = 1,000 ml).

Solution 2 : 6 ml of glacial acetic acid is diluted to 1,000 ml with water.

Solution 1 and Solution 2 (800:100) are mixed and pH is adjusted to 5.4.

◦ Substrate Solution : Appropriate amount of dried biomass of *Micrococcus luteus* (*Micrococcus lysodeikticus*) is suspended in phosphate buffer solution (pH 6.2) by homogenizer. More phosphate buffer solution is added so that the transmittance at 640 nm becomes 10%. If there is a lot change in substrate, a calibration curve for the standard material is prepared and an optimum concentration in a straight line region is used. Usually, a straight line is observed in 0.2~0.6 µg(activity)/ml range.

Storage Standard of Lysozyme

Lysozyme is strongly hygroscopic, hence should be stored in a hermetic container in a cold dark place.

20. Lipase

Definition Lipase is an enzyme obtained from a culture of *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety, *Candida rugosa*, *Rhizopus oryzae*, and *Aspergillus oryzae* where the lipase gene of *Rhizomucor miehei*, *Aspergillus niger* where the lipase gene of *Thermomyces lanuginosus*, *Aspergillus oryzae* where the lipase gene of *Fusarium oxysporum* is inserted, and animal pancreas tissue or forestomach of animal. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Lipase

Content Lipase contains 90~130% of the indicated activity as lipase.

Description Lipase is white~pale yellow~brown power or transparent~brown liquid.

Purity (1) Arsenic : 0.25 g of Lipase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should be appropriate (not more than 4ppm).

(2) Lead : When 5.0 g of Lipase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Coliform Group : Lipase is tested by Microbe Test Methods for Coliform Group in General Test Methods 「Standards and Specifications for Foods」. It should contain 30 or less per 1 g of this product.

(4) Salmonella : Lipase is tested by Microbe Test Methods for Salmonella in General Test Methods 「Standards and Specifications for Foods」. It should be negative (-).

(5) E. Coli : When Lipase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Assay(activity)

◦ Application and Principle : This test is to measure the activity of lipase in an enzyme that is obtained from a culture of *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety, *Candida rugosa*, *Rhizopus oryzae*, and *Aspergillus oryzae* where the lipase gene of *Rhizomucor miehei*, *Aspergillus niger* where the lipase gene of *Thermomyces lanuginosus*, *Aspergillus oryzae* where the

lipase gene of *Fusarium oxysporum* is inserted, and animal pancreas tissue or forestomach of animal. Activity test is based on measuring the ratio of increasing hydrolysis rate of Tributyrin by potentiometric titration.

- Preparation of Test Solution : sample is diluted with glycine so that 1 ml of the solution contains 2,000~5,000 Lipase units. The resultant solution is further diluted with water so that 1 ml of the resulting Test Solution contains 0.5~1.5 Lipase units.

Test Procedure : The burette of the titrator is filled with 0.05 N sodium hydroxide solution and the scale mark is adjusted. Temperature and pH are set to 30°C and 7.0, respectively. 15.0 ml of substrate emulsifying solution is transferred into a reaction vessel of the titrator and a magnetic stir bar is placed. The reaction vessel is attached to the titrator and 1.0 ml of Test Solution is added. Then the titrator is switched on. The reaction is maintained while adjusting the pH at 7.0 with 0.05 N sodium hydroxide solution. A calibration curve is prepared vs. the consumed amount of 0.05 N sodium hydroxide solution per minute.

(Note : Reaction rate shown in the recorder for 5 minutes should be a straight line.)

Activity of the enzyme is obtained by the following equation.

$$\text{LU/g} = \frac{R \times N \times 1,000}{W}$$

R : Consumed amount of titrant per minute in the straight line region (ml/min)

N : Normality of sodium hydroxide solution

1,000 : Conversion factor from mol to μmol

W : Weight of sample in 1 ml of Test Solution (g)

Definition of Activity : 1 Lipase unit (LU) corresponds to the amount of enzyme which separates 1 μmol of butyric acid per minute from the substrate under the conditions above.

Solutions

- Emulsifying Solution : 17.9 g of sodium chloride and 0.41 g of mono potassium phosphate are added in 400 ml of water, where 540 ml of glycerol is added. 6.0 g of gum Arabic (Sigma, or its equivalent) is added to the above solution, which is then shaken vigorously until it dissolves. Water is added to bring the total volume to 1,000 ml.
- Glycine Buffer Solution (0.1 M) : 7.5 g of glycine and 3.8 g of sodium hydroxide are

dissolved in 900 ml of water. After adjusting the pH to 10.8, water is added to bring the total volume to 1,000 ml.

- Substrate Emulsifying Solution : 15.9 ml of Tributyrin (Sigma, or equivalent) placed in a homogenizer, where 50 ml of emulsifier and 235 ml of water are added. It is then homogenized for 15 minutes at a high speed. Solutions are isothermalized at 30°C for at least 15 minutes in a water bath prior to use. This solution should be used within 4 hours.

Storage Standard of Lipase

Lipase should be stored in a hermetic container in a cold dark place.

21. Lipase/Esterase(Forestomach)

The specification of Lipase/Esterase is combined to 20. Lipase in Natural Additives.

The date of notification : November 12, 2010 (Notification No. 2010-82)

22. Tagetes Extract

Definition Tagetes Extract is a pigment that is obtained by extracting flowers of marigold of chrysanthemum family (*Tagetes erecta* WILLD.) with hexane. Its major colouring component is lutein of carotinoids and lutein dipalmitate. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Tagetes Extract

Content Color value ($E_{1cm}^{10\%}$) of Tagetes Extract should not be less than declared value.

Description Tagetes Extract is orange yellow~yellowish brown liquid, lump, or paste with characteristic scent.

Identification (1) The solution, which Ethyl alcohol : n-Hexan (1:1) is added and dissolved in tagetes extract, exhibits maximum absorption at 469~475 nm and 441~447 nm. It exhibits maximum absorption at 420~426 nm in some cases.

(2) The solution, which tagetes extract is dissolved in acetone, becomes colorless when 5% sodium nitrite and 0.5M sulfuric acid solution are added in order.

Purity (1) Arsenic : 0.25 g of Tagetes Extract is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should be appropriate (not more than 4ppm).

(2) Lead : When 5.0 g of tagetes extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Residual Solvent : When Tagetes Extract is tested by Purity (4) for 「Paprika Extract Pigments」, residual hexane should not be more than 25ppm.

Assay(color value) Appropriate amount of Tagetes Extract is accurately weighed so that the absorbance is within 0.3~0.7 and dissolved in hexane (total volume 100 ml). 1 ml of this solution is diluted to 100 ml with hexane (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using ethanol : hexane(1:1) as a reference solution, absorbance A is measured at the maximum absorption at 441~447 nm with 1 cm path length. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

23. Beeswax

Definition There are two grades, beeswax (white) and beeswax (yellow). Honey comb of honey bee (*Apis mellifera* L., *Apis indica* Radoszkowski) is heated, pressure-filtered, and purified to obtain beeswax (yellow), which is then bleached to obtain beeswax (white).

Compositional Specifications of Beeswax

Description Beeswax (white) is yellowish white solid having a faint and characteristic odour. Beeswax (yellow) is yellowish brown~grayish brown solid with honey like odour.

Purity

- (1) Arsenic : 0.25 g of Beeswax is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should be appropriate (not more than 4ppm).
- (2) Lead : When 5.0 g of Beeswax is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.
- (3) Mercury : When Beeswax is tested by Mercury Test Method, its content should not be more than 1.0ppm.
- (4) Melting Point : Melting point should be in a temperature range of 62~65°C.
- (5) Acid value : 3 g of Beeswax is accurately weighed into a 200 ml Erlenmeyer flask with 25 ml of anhydrous alcohol, previously neutralized to phenolphthalein with potassium hydroxide, until the sample is melted, Test Solution. When is tested by Mercury Test in Oil and Fats Method, acid value should be 17~24 for beeswax (white) and 18~24 for beeswax (yellow).
- (6) Ester Value : 25 ml of 0.5 N alcoholic potassium hydroxide and 50 ml of alcohol are added to the Test Solution for acid value. A reflux condenser is attached and the solution is heated for 4 hours in a water bath. Excess alkali is titrated with 0.5 N hydrochloric acid and ester value is calculated by the following equation. Ester value should be 72~79 for beeswax (white) and 72~77 for beeswax (yellow). Separately, a blank test is carried out.

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

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

b : Consumed amount of 0.5 N hydrochloric acid for Test Solution (ml)

- (7) Carnauba Wax : 100 mg of the sample is weighed into a test tube, where 20 ml of n-butyl alcohol is added. The test tube is heated in a boiling water bath and shaken until it becomes transparent. The test tube is placed in a beaker with 60°C water, which is then allow it to cool to room temperature. Loose, fine, needlelike crystals are separated from the solution and observed under a microscope. Crystals appear as loose needle or stellate cllusters, no amorphous masses are observed.
- (8) Fat, Japan Wax, Rosin, and Soap : 35 ml of sodium hydroxide solution (1→7) is added to 1 g of Beeswax, which is boiled for 30 minutes while occasionally adding water to supplement loss. After cooling, wax is separated so that the liquid remain clear. This liquid is then filtered. When the filtrate is acidified with hydrochloric acid, precipitates should not form.
- (9) Saponification Cloud Test : 3 g of Beeswax is weighed into a 100 ml round bottom flask, where 30 ml of potassium hydroxide solution (saponifying solution) in aldehyde free alcohol (40→1,000) is added. After attaching a reflux condenser to the flask, gently heated for 2 hours in a water bath. The condenser is removed, and inserted a thermometer, and the flask is placed in a 80°C water bath. It is then cooled to 65°C by shaking. The solution shows no cloudiness or globule formation before the solution reaches 65°C.
- (10) Saponification Value : 5 g of Beeswax is accurately weighed into a flask, where 50 ml of 0.5 N alcoholic solution of potassium hydroxide is added. After attaching a reflux condenser, the solution is gently saponified for 30 minutes to 1 hour. The saponified solution is used as Test Solution. The test solution is proceeded as directed under saponification value in Oils and Fats Test. The saponification value of the solution should be 87 ~ 104.
- (11) Peroxides value : 5 g of Beeswax is accurately weighed into a 250 ml round bottom erlenmeyer flask, 35 ml of Acetic acid · Cloroform mixture (3:2) is added, and gently shaken to be dissolved transparently. Clean nitrogen is passed through this to substitute the air in the container. When nitrogen is passed, 1 ml of potassium iodide TS is accurately weighed into the container. Stop the nitrogen, a stopper is immediately placed, mixed for 1 minutes, and allow to stand for 5 minutes in a dark place. 75 ml of water is added, and vigorously shaken and it is

titrated with 0.01N sodium thiosulfate (indicator : starch standard TS). When Peroxides value is obtained with following equation, the value should not be more than 5. Separately, a blank test is done to correct.

$$\text{Peroxides value} = \frac{\text{consumed amount of 0.01N sodium thiosulfate (ml)}}{\text{Weight of sample(g)}} \times 10$$

- (12) Ceresin , paraffin and other wax : 3g of Beeswax is accurately weighed into a 100 ml round-bottomed flask, where 30 ml of 4% alcoholic solution of potassium hydroxide solution is added. A reflux condenser is attached to the flask. It is then heated for 2 hours in a water bath, condenser is removed and thermometer is equipped. The flask is continuously shaken and slowly cooled in a beaker where 80°C water is, precipitates should not be formed before it reaches 65°C.
- (13) Glycerol and other polyol : 0.2 g of Beeswax is accurately weighed into a round-bottomed flask, where 10 ml of 4% alcoholic solution of potassium hydroxide solution is added. A reflux condenser is attached to the flask. It is then heated in a water bath for 30 minutes, again 50 ml of 10% sulfuric acid is added, cooled, and filtered. The resultant solution diluted to 100 ml with 10% sulfuric acid, test solution. 1.0 ml of test solution is placed in test tube, 0.5 ml of 1.0% sodium periodate is added, mixed, and allow to stand for 5 minutes. 1.0 ml of fuchsin sulfurous acid TS is added and mixed. Test tube is placed 10~15 minutes in a beaker containing water at 40°C, the bluish-violet color in the solution is not more intense than a standard prepared at the same time in the same manner using 1.0 ml of a 0.001% solution of glycerol(dissolved in 10% sulfuric acid).(Not be more than 0.5 % as glycerol)

24. Kaolin

Definition Kaolin is obtained from kaolin and its major constituent is hydrated aluminum silicate.

Compositional Specifications of Kaolin

Description Kaolin is white or milky white powder.

Identification (1) 0.2 g of Kaolin is mixed with 0.5 g of mixture(1:1) of anhydrous sodium carbonate and anhydrous potassium carbonate. It is then heated until it melts completely in a platinum or nickel crucible. After cooling, 5 ml of water is added and allow to stand for 3 minutes. The bottom of the crucible is gently heated and then the solidified matter is transferred into a beaker together with water. Hydrochloric acid is slowly added until foaming stops. After adding 10 ml more of hydrochloric acid, it is evaporated to dryness in a water bath. 200 ml of water is added to the residue, which is boiled and filtered. Gel phase residue is transferred into a platinum crucible. When 5 ml of hydrofluoric acid is added, it is dissolved. Upon heating, it almost completely evaporated.

(2) The filtrate obtained under test (1) responded to all tests for Aluminum Salt in the Identification Tests.

(3) 5 ml of water is added to 8 g of Kaolin, and mix well. It becomes plastic.

Purity (1) Water Solubles and pH : 10 g of Kaolin is added to 100 ml of water. It is then boiled for 30 minutes while supplementing water for the loss. After cooling, water is added to bring the total volume to 100 ml, which is filtered using a glass filter (3G4). pH of the filtrate should be 6.0~8.0. 50 ml of the filtrate is evaporated to dryness, which is then dried for 1 hour at 105°C. The resultant residue should not be more than 15 mg.

(2) Acid Solubles : 20 ml of diluted sulfuric acid (1→15) is added to 1 g of Kaolin, is mixed with shaking for 15 minutes, which is then filtered. 10 ml of the filtrate is evaporated to dryness and is ignited to constant weight. The residue should not be more than 10 mg .

(3) Arsenic : 5 ml of dilute hydrochloric acid is added to 0.5 g of Kaolin. It is then heated at 70°C for 15 minutes while shaking. The mixture is quickly cooled and filtered. The residue is washed with 5 ml of diluted hydrochloric acid and 10 ml of water, which is added to the filtrate. Water is added to bring the total volume to 20 ml. 10 ml of the resultant solution is tested for Arsenic and the content should be appropriate (not more than 4ppm).

(4) Lead : When 5.0 g of Kaolin is tested by Atomic Absorption Spectrophotometry

or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

- (5) Foreign matter : 5 g of Kaolin is mixed in 300 ml of water by stirring, which is allowed to stand for 30 seconds. Most of the solution containing the fine particles is discarded by decantation the container. When the portion remaining at the bottom of the container is pressed using a glass rod with a flat end, there is no audible sound produced by the sand.

Loss on Ignition When the Loss on Ignition analysis is done, weight loss should not be more than 15%.

25. Berries Color

Definition This is a collective name for pigments which is originated from berries. Major component of this pigment is anthocyanin which is obtained from juice or water extract of berries. There are gooseberry color (origin : *Cucumis myriocarpus* NAUO), European dewberry color (origin : *Rubus caesius* L. etc), Raspberry color (origin : *Rubus idaeus* L. etc.), American red raspberry color (origin : *Rubus strigosus* MICHX), Red currant color (origin : *Ribes sativum* SYME.), Loganberry color (origin : *Rubus loganobaccus* BAILEY.), Mulberry color (origin : *Morus nigra* L., *M. alba* L.), Blackberry color (origin : *Rubus fruticosus* L.), Black currant color (origin : *Ribes nigrum* L.), Black huckleberry color (origin : *Gaylussacia baccata* C. KOCH.), Blueberry color (origin : *Vaccinium corymbosum* L.), Salmonberry color (origin : *Rubus spectabilis* PURSH.), Strawberry color (origin : *Fragaria ananassa* DUCHESNE.), Elder berry color (origin : *Sambucus caerulea* RAFIN, etc.), Uguisukagura color (origin : *Lonicera caerulea* L. var. *emphyllocalyx* NAKAI), Whortleberry color (origin : *Vaccinium myrtillus* L.), Cowberry color (origin : *Vaccinium Vitis Idaea* L.), Cranberry color (origin : *Oxycoccus macrocarpus* PERS.), Thimbleberry color (origin : *Rubus occidentalis* L.). Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Berries Color

Content Color value ($[E]_{1cm}^{10\%}$) of Berries Color should not be less than the indicated value.

Description Berries Color is dark red liquid, lump, powder, or paste with a slight characteristic scent.

Identification (1) Test Solution obtained in Color Value section of Berries Color shows red~dark blue color and a maximum absorption at 500~540 nm.

(2) When Test Solution in (1) is alkalized by adding sodium hydroxide TS, the color of the solution changes.

Purity (1) Arsenic : 0.25 g of Berries Color is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Berries Color is tested by Atomic Absorption

Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Sulfur Dioxide : When Berries Color is tested by Purity (3) for 「Grape Skin Extract」, the content should not be more than 0.005% per 1 color value $(E-10\%, \pm 1 \text{ cm})(E_{1 \text{ cm}}^{10\%})$.

(4) Residual Solvent : When berries color is tested by Purity (5) for 「Paprika Extract Pigments」, residual methanol should not be more than 0.1% (based on the product whose color value is 40).

Assay (Color Value) Appropriate amount of Berries Color is accurately weighed so that the absorbance is within 0.3~0.7 and dissolved in citric acid-dibasic sodium phosphate buffer solution with pH 3.0 so that total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citric acid-dibasic sodium phosphate buffer solution with pH 3.0 as a reference solution, absorption A is measured at a wavelength of maximum absorption at 500~540 nm with 1 cm path length. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1 \text{ cm}}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

Citric acid·dibasic sodium phosphate buffer solution (pH 3.0)

◦ Solution 1 : 0.1 M citric acid solution : 1 ℓ of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

◦ Solution 2 : 0.2 M dibasic sodium phosphate solution : 1 ℓ of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (59 : 41) and its pH is adjusted to 3.0.

26. Betaine

Definition Betaine is obtained by purifying Mulyeots(sugar solutions and syrups) (by separation) from beet (*Beta vulgaris* L. var. *rapa*) of *Chenopodiaceae*. The major component is betaine ($C_5H_{11}NO_2 = 117.15$).

Compositional Specifications of Betaine

Content Betaine contains 98.0~102.0% of betaine ($C_5H_{11}NO_2$).

Description Betaine is white crystallite with a slight odor and sweet taste.

Identification 10 $\mu\ell$ each of aqueous solution (1→100) of Betaine and Betaine standard solution (1→100) is tested by liquid chromatography as following operation conditions. The retention times for Test Solution and Standard Solution should be identical.

Operation Conditions

- Detector : Differential refractometer(RI Detector)
- Column : Carbohydrate(8 mm×300 mm) or equivalent
- Column Temperature : 80℃
- Mobile Phase : Water
- Flow Rate : 1 ml/min

Purity (1) Clarity of solution : 1 g of Betaine is dissolved in 10 ml of water. The solution should be colorless and clear.

(2) pH : The pH of Betaine solution(1→20) is 5.0~7.0.

(3) Chloride : When 1 g of Betaine is tested as directed under Chlorides Test, the content should not be more than that amount corresponds to 0.15 ml of 0.01 N hydrochloric acid.

(4) Sulfate : 1 g of Betaine is tested as directed under Sulfates Test, the content should not be more than that amount corresponds to 0.2 ml of 0.01 N sulfuric acid.

(5) Arsenic : 0.25 g of Betaine is dissolved in 5 ml of water, Test Solution. When The Test Solution is tested as directed under Arsenic Test, it should be appropriate (not more than 4ppm).

(6) Lead : When 5.0 g of Betaine is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Loss on Drying When Betaine is dried for 3 hours at 105℃, the loss should not be more than 2% or less.

Residue on Ignition Residue on ignition should not be more than 0.1%.

Assay Approximately 1 g of Betaine, previously dried, accurately weighed and dissolved in water to make volume 100 ml. 10 ml of this solution is passed through a column packed with 10 ml of ion exchange resin A mixture of weakly acidic ion exchange resin (H type) and strongly alkaline ion exchange resin (OH type) in 1 : 4 volume ratio. The column is washed by water. Washing water is added to the effluent, which is acidified to pH 1.0 with hydrochloric acid. The total volume is brought up to 100 ml with water. To 5 ml of this solution, 5 ml of Reinecke salt solution, previously cooled, is added, which is cooled for 3 hours in a refrigerator. Precipitates are filtered through a glass filter (3G4), washed with ether and dried in air. The resultant precipitates are dissolved in 70% acetone so that the total volume is 25 ml, Test Solution. Absorbance of the Test Solution is measured at 525 nm with 1cm path length. Separately, approximately 1g of Betaine Standard, previously dried at 105°C for 3 hours, accurately weighed, and dissolved in water so that the total volume is 100 ml, Standard Stock Solution. 10 ml and 20 ml of this solution are taken. pH of each solution is adjusted to 1.0 with hydrochloric acid and the total volume is brought up to 100 ml with water, Standard Solutions. 5 ml of each Standard Solution is precipitated following the same procedure as the Test Solution. The precipitates are dissolved in 70% acetone and absorption is measured as the Test Solution. A calibration curve is prepared. Using the calibration curve and the absorbance of the Test Solution, the content of betaine is calculated from the following equation.

$$\text{Content(\%)} = \frac{\text{amount of betaine obtained from calibration curve (mg)}}{\text{Weight of sample (mg)}} \times 100$$

Test Solutions

- Reinecke Solution : 1.5 g of Reinecke salt is dissolved in water. pH is adjusted to 1.0 with hydrochloric acid and the total volume is brought up to 100 ml with water.

27. Bentonite

Definition Bentonite is naturally occurring colloidal hydrated aluminum silicate.

Compositional Specifications of Bentonite

Description Bentonite is odorless white~pale yellowish brown or pale green fine powder or granule with a slight taste similar to soil.

Identification (1) 0.2 g of Bentonite is mixed with 1.5 g of 50 : 50 mixture of anhydrous sodium carbonate and anhydrous potassium carbonate. It is then heated until it melts completely in a platinum or nickel crucible. After cooling, 5 ml of water is added and allow to stand for 3 minutes. The bottom of the crucible is gently heated and then the solidified matter is transferred into a beaker along with water. Hydrochloric acid is slowly added until foaming stops. After adding 10 ml more of hydrochloric acid, it is evaporated to dryness. 200 ml of water is added to the residue, which is boiled and filtered. Gel phase residue is transferred into a platinum crucible. When 5 ml of hydrofluoric acid is added, and dissolved. Upon heating, it volatilized almost.

(2) The filtrate in (1) shows the reaction of aluminum salt in Identification.

(3) When Bentonite is immersed in water, the volume swells up to 5 times.

Purity (1) Foreign Substances : 2 g of Bentonite is weighed into a mortar, where 20 ml of water is added and let it swells. It is evenly dispersed using a glass rod and water is added to bring the total volume to 100 ml. The dispersion is passed through a No.7 mesh screen using water. When the mesh screen is rubbed with finger tips, there should not be any sand.

(2) Arsenic : 10 ml of dilute hydrochloric acid is added to 0.77 g of Bentonite and heated at 70°C for 15 minutes while shaking. After cooling immediately, it is filtered. The residue is washed with 10 ml of dilute hydrochloric acid followed by 20 ml of water, which are added to the filtrate. The total volume of the filtrate is brought up to 40 ml, Test Solution. 20 ml of the Test Solution is tested for arsenic. The content should not be more than 2.6ppm.

(3) Lead : 5.0 g of dried sample is weighed into a 250 ml beaker containing 100 ml of diluted hydrochloric acid (1→25). It is then stirred, covered with a watch glass, and boiled for 15 minutes. After cooling to room temperature, the beaker is allowed insoluble matter to settle. The supernatant is filtered through a filter paper. The filter paper is washed with four 25 ml portions of hot water, collecting the filtrate in the beaker. The combined filtrate is concentrated by gentle boiling to approximately 20 ml. If a precipitate are formed, 2~3 drops of nitric acid are added

and boiled again. After cooling to room temperature, the concentrated extracts is filtered through a rapid-flow filter paper. The beaker and the filter paper are washed with water and the washing water is added to the filtrate. The total volume is brought up to 50 ml with water, Test Solution. When this test solution is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 20 ppm.

- (4) Total Viable Aerobic Count : When Bentonite is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 1,000 cfu per 1 g.
- (5) E. Coli : When Bentonite is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Loss on Drying When 2 g of Bentonite is dried at 110℃ until the weight becomes constant, the loss should not be more than 5~10%.

Swelling Test 2 g of Bentonite is added ,in several divided portions, to a 100 ml graduated cylinder with a stopper containing water. After the previous portion added settles down, the next portion added. Leave undisturbed for 24 hours, the volume of the swollen lump on bottom should read 10 ml or higher.

28. Cellulose, Powdered

Definition Cellulose, Powdered is cellulose obtained by hydrolyzing pulp fiber or short fiber.

Compositional Specifications of Cellulose, Powdered

Content What Cellulose, Powdered is converted to a dehydrated form, contain 97.0% and not more than 102.0% of carbohydrate, calculated as cellulose.

Description Cellulose, Powdered is white odorless powder.

Identification

- (1) 10 g of Cellulose, Powdered in 90 ml of water is boiled for 5 minutes. While hot, it is filtered through ashless filter paper. When 2 drops of iodine TS are added to the filtrate, the color does not change from yellow-red.
- (2) 2 to 5 mg of Cellulose, Powdered is added to 20 ml of 0.1% solution of anthrone in 75% sulfuric acid and heated in a water bath. The solution turns blue-green within 5 minutes.
- (3) 30 g of Cellulose, Powdered is mixed in 270 ml of water with a high speed stirrer at 8,000~8,500 rpm for 5 minutes. The mixture will be either a free-flowing suspension or heavy, lumpy suspension. In the latter case, precipitates are slightly formed and the suspension contains inhomogeneously dispersed air bubbles. If the sample mixture is not free-flowing suspension, 100 ml of the mixture is transferred into a 100 ml graduated cylinder, which is then allow it to settle for 1 hour. Solid phase settles at the bottom and a supernatant liquid appears above the layer of cellulose .
- (4) When a few drops of sample mixture obtained from (3) are observed at 100 magnification with a microscope, fibers and fiber fragments are visible, regardless of the degree of fineness of the sample.

Purity

- (1) Arsenic : 0.5 g of Cellulose, Powdered is tested as directed under the Purity (1) for Guar Gum (not more than 2ppm).
- (2) Lead : When 5.0 g of Cellulose, Powdered is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.
- (3) Cadmium : When 5.0 g of Cellulose, Powdered is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.
- (4) Mercury : When 0.1 g of Cellulose, Powdered is tested by Mercury Test Method, its content should not be more than 1.0ppm.
- (5) Chloride : Approximately 5 g of Cellulose, Powdered is accurately weighed into a

500 ml Erlenmeyer flask, where 250 ml of water is added. It is then refluxed for 1 hour and filtered. The filtered sample with 200 ml of water is refluxed for 30 minutes and then filtered. This filtrate is combined to the previous filtrate and hot water rinses, where 1 ml of nitric acid is added. After boiling the resultant mixture, 5 ml of 5% solution of silver nitrate is slowly added. After the precipitate has coagulated, it is filtered through a glass filtering funnel. The precipitates are washed with diluted nitric acid (1→100) until free from silver nitrate and rinsed with water, dried at 130°C, and weighed. To obtain an corrected weight of the precipitate, a blank determination performed for correction. 1 mg of precipitate is equivalent to 0.247 mg of Cl. The content of Cl should not be more than 0.05%.

(6) pH : 10 g of Cellulose, Powdered is dissolved in 90 ml of water and allow to stand with occasional stirring for 1 hour. The pH of the supernatant liquid is measured with a glass electrode. It should be between 5.0 and 7.5.

(7) Water Solubles : 6 g of Cellulose, Powdered is mixed to 90 ml of recently boiled and cooled water and allowed to stand with occasional stirring for 10 minutes. It is then filtered. First 10 ml of filtrate is discarded and passed the filtrate through the same filter paper a second time, if necessary. 15 ml portion of the filtrate is evaporated to dryness. The residue is further dried at 105°C for 1 hour. The content should not be more than 1.5%.

Ash Approximately 3 g of Cellulose, Powdered is accurately weighed and heated until completely charred at $550 \pm 50^\circ\text{C}$. It is then ignited at $800 \pm 25^\circ\text{C}$ until free from carbon. The amount of ash should not be more than 0.3%.

Loss on Drying 3 g of Cellulose, Powdered is dried at 105°C. The loss on drying should not be more than 7%.

Assay Approximately 125 mg of Cellulose, Powdered is accurately weighed into a 300 ml Erlenmeyer flask. The weighing boat is rinsed with 25 ml of water, which is added to the flask. 50 ml of 0.5 N potassium dichromate solution is added to the flask and 100 ml of sulfuric acid is carefully added. It is then heated to boiling, allowed the solution to stand at room temperature for 15 minutes, and cooled it in a water bath. Water is added to bring the total volume to 250 ml. 50 ml of the resultant solution is titrated with 0.1 N ferrous ammonium sulfate solution (indicator : 3 drops of o-phenanthroline TS). Separately, a blank test is performed, where the consumed amount of 0.1 N ferrous ammonium sulfate solution is B(ml). The normality (N) of 0.1 N ferrous ammonium sulfate solution is obtained by the following formula.

$$\text{Normality (N)} = 0.1 \times 50/B$$

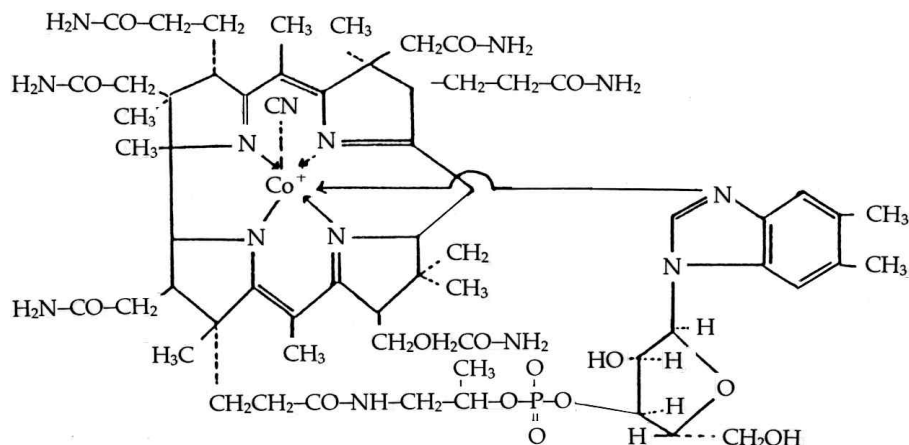
The content of the cellulose in the sample is obtained by the following formula.

$$\text{Cellulose content (\%)} = 6.75(B - S) \times \frac{N}{2W}$$

S : Volume(ml) of 0.1 N ferrous ammonium sulfate solution used in the sample titration

W : Weight(g) of the sample taken, on the dried basis

29. Cyanocobalamin



Chemical Formula $C_{63}H_{88}CoN_{14}O_{14}P$

Molecular Weight 1,335.40

Definition Cyanocobalamin is obtained by separating the cultures of *Streptomyces*, *Bacillus*, *Flavobacterium*, *Propionibacterium*, and *Rhizobium*. The major component is Cyanocobalamin.

Compositional Specifications of Cyanocobalamin

Content If Cyanocobalamin is converted to a dehydrated form, it should contain not less than 96.0% cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$).

Description Cyanocobalamin is hygroscopic dark red crystallite, amorphous or crystalline powder. 1 g of Cyanocobalamin dissolves in 80 ml of water. It is almost insoluble in ether, chloroform, or acetone.

Identification (1) When absorbance of the Test Solution in Assay is measured, absorbance maximum are observed at 277~279 nm, 360~362 nm, and 548~552 nm. The ratio of A_{361}/A_{550} is 3.15~3.40.

(2) Approximately 1 mg of Cyanocobalamin and 50 mg of potassium pyrosulfate is transferred into a crucible, are melted by heating. After cooling, the lump is broken into small pieces with a glass rod. It is then dissolved in 3 ml of water by heating. After adding 1 drop of phenolphthalein TS, sodium hydroxide TS (1→10) is drop-wise added until the solution shows pale red color. When 500 mg of sodium acetate, 0.5 ml of dilute acetic acid, and 0.5 ml of sodium nitroso-2-naphthol-3,6-disulfonate solution (1→500) are added to the resultant solution, red or orange red color appears immediately. When 0.5 ml of hydrochloric acid is added and the

solution is boiled for 1 minute, the red color persists.

(3) A 50 ml distillation flask (two neck / round bottom) is connected to a vertical condenser, of which the end is immersed in a test tube with 1 ml of 0.1 N sodium hydroxide solution. 1.5~2.0 mg of Cyanocobalamin is dissolved in 5 ml of water in the flask. 2.5 ml of hypophosphite is added to the flask, which is then gently boiled for 10 minutes under air. 1 drop of saturated ferrous ammonium sulfate solution is added to the small test tube and 30 mg of sodium fluoride is added, which is gently boiled and cooled. Diluted sulfuric acid (1→7) is drop-wise added until the solution becomes clear. When 3~5 drops of diluted sulfuric acid (1→7) are added additionally, the solution turns blue or bluish green within a few minutes.

Purity Analogous vitamin B₁₂ : 1 mg of Cyanocobalamin is dissolved in 20 ml of water, which is transferred into a small separatory funnel. 4 ml of mixture of carbon tetrachloride and m-cresol (50:50) is added and mixed well for 1 minute by shaking. It is allowed to stand to separate phases. The lower phase is transferred into another separatory funnel, where 5 ml of diluted sulfuric acid (1→7) is added. The mixture is vigorously shaken. Two phases are separated, centrifuged if necessary. The supernatant should be colorless or should not be darker than the mixture of 0.15 ml of 0.1 N potassium permanganate solution and 250 ml water.

Loss on Drying Approximately 25 mg of Cyanocobalamin is accurately weighed and dried for 2 hours at 105°C under a decompression of 5 mm Hg or less. The loss on drying should not be more than 12%.

Assay Approximately 30 mg each of Cyanocobalamin and vitamin B₁₂ standard, previously measured losses on drying as method used in sample, is accurately measured and dissolved in water so that the total volume is 1,000 ml, respectively (Test Solution & Standard Solution). Absorbance of ET and ES for Test and Standard Solutions are measured at 361 nm with 1 cm path length.

Amount of vitamin B₁₂(C₆₃H₈₈CoN₁₄O₁₄P) (mg)

$$= \text{Weight of vitamin B}_{12} \text{ standard (on dry basis) (mg)} \times \frac{E_T}{E_S}$$

30. Beet Red

Definition Beet Red is a pigment obtained from extracting roots of beet (*Beta vulgaris* Linné) of chenopodiaceae with water or ethyl alcohol. The major component is isobetanine and betanine. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Beet Red

Content Color value ($[E]_{1cm}^{10\%}$) of Beet Red should be higher than the indicated value.

Description Beet Red is reddish violet~dark violet liquid, lump, powder, or paste with a slight characteristic odor.

Identification (1) Test Solution of Beet Red obtained in Color Value section shows reddish violet and a absorbance maximum at about 535 nm.

(2) When 1 ml of sodium hydroxide solution(1→10) is added to 5 ml of Test Solution in (1), the colour changes yellow.

Purity (1) Arsenic : 0.25 g of Beet Red is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When the test for arsenic is carried out with this test solution, it should be appropriate (not more than 4ppm).

(2) Lead : When 5.0 g of Beet Red is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Beet Red is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Beet Red is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Nitrate : 0.1 g of Beet Red is accurately weighed into water to make 100 ml, Test Solution. Separately, take 0.2 ml, 1 ml, 10 ml and 50 ml of Nitrate standard stock solution and add water to make to 100 ml, standard solution respectively. Measure the peak area of nitrate ion of standard solution and standard stock solution and plot the calibration curve. When measure the peak area of nitrate ion of test solution and plot the calibration curve, the calculated content of nitrate(NO_3) should

not be more than 0.27%(based on the product whose color value is 11215).

Operation condition

Detector: Conductivity detector

Filler: Porous anion exchnager

Column: Inner diameter 4.6~6.0 mm length 5~10 cm Stainless steel tube

Temperature of column: 40 °C

Eluent: Aqueous solution(pH 4.0) contains phthalic acid(2.5 mmol/L) and
tris(hydroxymethyl)aminomethane(2.4 mmol/L)

Rate of discharge: 1.5mL/min

Solution

Standard nitrate stock solution : 1.631 g of potassium nitrate is accurately weighed into water to make 1,000 ml. 10ml of this solution is taken into water to make accurately 100ml(1 ml of this solution contains 0.1mg of nitrate(NO₃))

Assay(color value) Appropriate amount of Beet Red is accurately weighed so that the absorption is within the range of 0.3 to 0.7 and dissolved in acetic acid·sodium acetate buffer solution with pH 5.4 to make 100 ml. 1 ml of this solution is diluted to 100 ml with acetic acid·sodium acetate buffer solution with pH 5.4, Test Solution. If necessary, the solution is centrifuged and the supernatant is used. Using acetic acid·sodium acetate buffer solution with pH 5.4 as a blank, absorbance A is measured at the maximum absorption at about 535 nm with 1 cm path length. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

◦ Acetic acid · sodium acetate buffer solution (pH 5.4)

Solution 1 : 1,000 ml of solution containing 13.6 g of sodium acetate.

Solution 2 : 1,000 ml of solution containing 6 ml of glacial acetic.

Solution 1 and Solution 2 are mixed well (8:2) and its pH is adjusted to 5.4.

31. Acid Clay

Definition Acid Clay is obtained by purifying clay minerals such as monmorillonite. Major components are kaolinite.

Compositional Specifications of Acid Clay

Description Acid Clay is gray~pale yellow fine powder.

Identification (1) 1 g of Acid Clay is placed in a porcelain crucible, where 10 ml of water and 5 ml of sulfuric acid are added. It is then evaporated to dryness by heating and cooled. 20 ml of water is added to the crucible, which is then boiled again for 2~3 minutes. The mixture is filtered. The color of the residue is gray.

(2) The filtrate in (1) is showed the reaction of aluminum salts in Identification.

Purity (1) pH : 1 g of Acid Clay is suspended in 50 ml of water, which is then filtered. pH of the filtrate should be 3.0~5.0.

(2) Arsenic : 2.5 g of Acid Clay, previously dried, is placed in a 250 ml beaker, where 100 ml of diluted hydrochloric acid (1→25) is added and well mixed. The beaker is covered with a watch glass and boiled gently for 15 minutes with stirring not to form excessive foaming. The hot supernatant is filtered through a filter paper at a high flow rate into a 200 ml flask. The residue on the filter paper is washed 4 times with 25 ml each of hot diluted hydrochloric acid (1→25), which is added to the previous filtrate and washing water. The resultant filtrate is cooled to room temperature and the total volume is brought up to 200 ml with diluted hydrochloric acid (1→25), Test Solution. 20 ml of the Test Solution is tested for arsenic. The content should be appropriate (not more than 4ppm).

(3) Lead : 3.75 g of Acid Clay, previously dried, is placed in a 250 ml beaker, where 100 ml of diluted hydrochloric acid (1→25) is added and mixed well. It is then boiled covered with watch glass for 15 minutes. It is cooled and allowed to stand for settling the insoluble substances. It is then filtered through a filter paper at a high flow rate. The residues on the filter paper are washed four times with 25 ml each of hot water. Washing water is added to the previous filtrate, which is concentrated to approximately 20 ml by heating gently. If precipitates are formed, 2~3 drops of nitric acid are added and boiled again. After cooling to room temperature, the concentrated filtrate is filtered through a filter paper into a 50 ml beaker at a high flow rate. The beaker and the residue on the filter paper are washed with water and the washing water is added to the filtrate. The total volume is brought up to 50 ml with water, Test Solution. Absorbance of the Test Solution should not be higher than that of lead standard solution containing Pb 3 μg per ml

(not more than 40ppm).

Loss on Drying When 2 g of Acid Clay is dried at 105°C until the weight becomes constant, the loss should not be more than 10.0%.

32. Xanthan Gum

Definition Xanthan Gum is a high-molecular-weight polysaccharide gum produced by a pure culture fermentation of a carbohydrates with *Xanthomonas Campestris*, purified by isopropyl alcohol, dried, and milled. Xanthan Gum is a mixture of sodium, potassium, and calcium salt of glucose, mannose, and gluconates.

Compositional Specifications of Xanthan Gum

Content Xanthan Gum (on the dried basis) contains 4.2~5.0% of carbon dioxide (CO₂), which corresponds to 91.0~108.0% of xanthan gum.

Description Xanthan Gum is white to pale yellow powder and a little odor.

Identification 300 ml of water, previously heated to 80°C, is transferred into a 500 ml beaker and a stir with a mechanical stirrer. 1.5 g of Xanthan Gum and 1.5 g of locust bean gum are added and dissolved, which is then kept at 60°C or higher for 30 minutes. When it allowed to cool at room temperature for at least 2 hours, a rubbery gel is formed. When locust bean gum is not mixed, a rubbery gel does not form.

Purity (1) Arsenic : Xanthan Gum is tested according to Purity (1) for Guar Gum (not more than 4ppm).

(2) Lead : When 5.0 g of Xanthan Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Isopropyl Alcohol : 4 g of Xanthan Gum is accurately weighed into a 300 ml round bottom flask, 200 ml of water is added, boiling chips and 1 ml of silicone resin are added and mixed well. Fractionating column is connected to this, 4 ml of internal standard solution is accurately taken and added to a 100 ml flask. With adjusting the heat so that foam does not enter the column, distill the solution at the rate of 2~3 ml per 1 minute until the milky liquid becomes about 90 ml, and water is added to make 100 ml, test solution. However, tert-butyl alcohol (1→1,000) is used as internal standard solution. Separately, 0.5 g of isopropyl alcohol is precisely weighed and water is added to make 500 ml, again 2 ml of this solution and 4 ml of internal standard solution is taken, water is added to make 100 ml, standard solution. 2μl of test solution and standard solution is taken respectively, and injected to gas chromatograph as the following operation condition. Then, ratio of isopropyl alcohol peak against tert-butyl alcohol peak in test solution and standard solution, QT and QS, is calculated separately, and the content of isopropyl alcohol is calculated by following formula, the content should not be more than 0.05%.

$$\text{Content of Isopropyl alcohol(\%)} = \frac{\text{Weight of isopropyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_T}{Q_S} \times \frac{2 \times 100}{500 \times 100} \times 100$$

QT : Ratio of isopropyl alcohol peak against tert-butyl alcohol peak in Test Solution

QS : Ratio of isopropyl alcohol peak against tert-butyl alcohol peak in standard solution

Operation Conditions

Column : PLOT Q or equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Temperature at injection port : 200°C

Column Temperature : 120°C

Detector temperature : 300°C

Carrier gas : Nitrogen or Helium

- (4) Viscosity : Viscosity of 1% aqueous solution of Xanthan Gum is measured by 2. Rotational Type Viscosity Measurement in Viscosity Measurement. It should be 600 cps or higher.
- (5) Nitrogen : When Xanthan Gum is tested by Kjeldahl Nitrogen Test in nitrogen determination method, the amount should not be more than 1.5%.
- (6) Total Viable Aerobic Count : When Xanthan Gum is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 cfu per 1 g
- (7) E. Coli : When Xanthan Gum is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (8) Salmonella : When Xanthan Gum is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (9) Number of Fungi : When Xanthan Gum is tested by Microbe Test Methods for Number of Fungi in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 500 per 1 g

Loss on Drying When Xanthan Gum is dried for 2 hours and 30 minutes at 105°C, the loss should not be more than 15%.

Residue on Ignition When Residue on Ignition is done with accurately weighed 3 g of Xanthan Gum, the amount of residue should be between 6.5 and 16.0%.

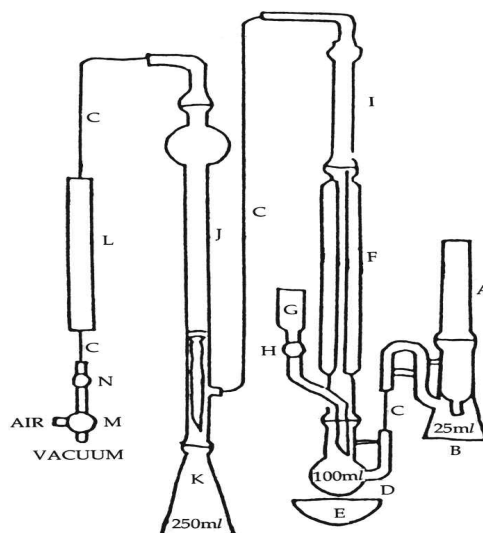
Pyruvic Acid (1) Test Solution : 600 mg of Xanthan Gum is precisely weighed and

dissolved in water to make 100 ml. 10 ml of this solution is placed in a 250 ml round bottom flask and 20 ml of 1N hydrochloric acid is added. Then record the whole weight and a reflux condenser is attached. It is then refluxed for 3 hours and cooled. The reflux condenser is removed and water is added to make up for any weight loss during refluxing. 2 ml of this solution is transferred into a 30 ml separatory funnel containing 1 ml solution of 1 g 2,4-dinitrophenylhydrazine prepared in 200 ml 2 N hydrochloric acid with a stop cock. It is then mixed and allowed to stand at room temperature for 5 minutes. The mixture is then extracted with 5 ml of ethylacetate and aqueous phase is discarded. The hydrazone is extracted three 5 ml portions of sodium carbonate TS. The total volume of the resultant extract is brought up to 50 ml with sodium carbonate TS.

- (2) Standard Solution : 45 mg of pyruvic acid is precisely weighed into 500 ml volumetric flask, diluted with water and mix. 10 ml of this solution is taken and continued as directed under sample solution.
- (3) Test Method : Absorbance of Test Solution and Standard Solution are determined at 375 nm using sodium carbonate TS as a blank. Absorbance value of Test Solution should be greater than that of Standard Solution (not less than 1.5%).

Assay

Experimental apparatus is as follows.



- (1) Experimental Apparatus

- A : Soda Water Tower (filled with calcium hydroxide granules)
B : Mercury Valve
C : Side Arm
D : 100 ml Reaction flask with a long neck
E : Heating Device
F : Reflux Condenser
G : 400 ml connection tube which is connected to the reaction tube
H : Stopcock
I : Trap (It is filled with approximately 25 g of 20 mesh zinc or tin and connected to an absorption tower, J).
J : Absorption Tower (It is consisted of a connection tube and a trap. There is a glass filter in-between.)
K : Erlenmeyer Flask (Connected to the bottom of the absorption tower)
L : Soda water tower
M : Three way stopcock
N : All the ground joints of capillary controller or needle valve (which controls air flow or vacuum) are 35/25.

(2) Experimental Method : Approximately 1.2 g of Xanthan Gum is precisely weighed into flask (D), where 25 ml of 0.1 N hydrochloric acid and boiling stones are added. The reaction flask is then connected (Vaseline is applied to the ground joints. Mercury in the inner tube of the mercury valve (B) is raised by approximately 5 cm and check for air leakage. The stop cock (M) is closed to hold the pressure. If the mercury does not drop in 1~2 minutes, there is no leak.). It is then heated for 2 minutes and cooled for 15 minutes in a flowing air (CO₂-free) at a flow rate of 3,000~6,000 ml per hour. 23 ml of hydrochloric acid is added through the connection tube (G) and the absorption tower (J) is disconnected. 25 ml of 0.25 N sodium hydroxide solution and 5 drops of butyl alcohol are added to the absorption tower, which is then re-connected. Then air (CO₂-free) flow at a rate of 2,000 ml per hour. Hydrochloric acid in the connection tube (G) is transferred to the flask (D), which is then heated for 2 hours and cooled. Using compressed air, sodium hydroxide solution in the absorption tower is transferred into the flask (K). The absorption tower is washed three 15 ml portion of water using compressed air. The flask is separated from the apparatus. 10 ml of 10% barium chloride solution is added to the flask, which is then plugged and mixed for approximately 2 minutes. It is then titrated with 0.1 N hydrochloric acid using phenolphthalein TS as an

indicator. Separately, a blank test is carried out by the same procedure.

0.25 N sodium hydroxide solution 1 ml = 5.5 mg CO₂

$$\text{CO}_2 \text{ (\%)} = \frac{5.5 \times (B - S)}{2.5 \times \text{Weight of sample (mg)}} \times 100$$

B : Consumed amount of 0.1 N hydrochloric acid for blank test (ml)

S : Consumed amount of 0.1 N hydrochloric acid for the test (ml)

33. Petroleum Wax

Refined Paraffin Wax : Microcrystalline Wax

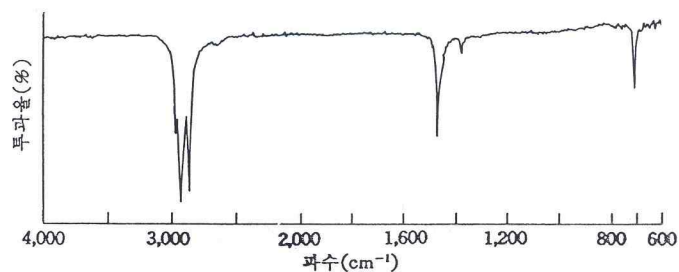
Definition Petroleum Wax is prepared by removing with propanol lake, lead, and oil from vacuum distillation residue oil of crude oil, in the cold. Or it can be prepared by treating with furfural, in the hot, and followed by removal of furfural. It consists of branched hydrocarbons ($C_{30} \sim C_{60}$).

Compositional Specifications of Petroleum Wax

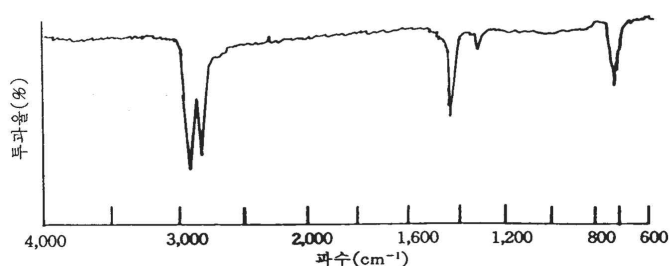
Description Petroleum Wax is translucent, tasteless, and odorless wax.

Identification Infrared spectra of Petroleum Wax (observed by IR spectrophotometry) shows the following pattern. Petroleum Wax is melted and determined using potassium bromide plate.

(1) Purified Petroleum Wax



(2) Microcrystalline Wax



Purity (1) Arsenic : 0.5 g of Guar gum is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath,

use the Test solution. When this test solution proceed as directed under Arsenic, it should not be more than 2ppm.

(2) Lead : When 5.0 g of Petroleum Wax is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 3.0 ppm.

(3) Melting Point : When Petroleum Wax determined it by melting point method, it should be within the indicated range (48~93°C).

34. Cellulase

Definition Cellulase is an enzyme obtained from cultures of *Aspergillus niger* and its variety, *Trichoderma reesei* and its variety, and *Humicola insolens* and its variety. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Cellulase

Content It contains 90~130% of indicated activity as Cellulase.

Description Cellulase is white~pale yellow~brown paste or transparent~brown liquid.

Purity (1) Arsenic : 0.25 g of Cellulase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should be appropriate (not more than 4ppm).

(2) Heavy Metals : 0.5 g of Cellulase are carbonized by heating mildly in a quartz or porcelain crucible. After cooling, add 2 ml of nitric acid and 5 drops of sulfuric acid, it is heated until white smoke disappears, which is then reduced to ash by further heating at 450~550°C. After cooling, 2 ml of hydrochloric acid is added, which is then evaporated to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resultant residue, which is then heated for 2 minutes. After cooling, 1 drop of phenolphthalein TS is added, then ammonia solution is added until the color of the solution becomes pale red. The resultant solution is transferred into a Nestler cylinder by rinsing with water. 50 ml of test solution is prepared by adding 2 ml of diluted acetic acid (1→20) and water. When this solution tested for heavy metals, the content should not more than 40ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water are added to bring the total volume to 50 ml.

(3) Lead : 0.8 g of Cellulase (if it is liquid, it is concentrated by evaporation in a

water bath) is slowly carbonized by heating, which is reduced ash by further heat treatment at a temperature below 500°C. Carefully 20 ml of dilute nitric acid is added to the ash, which is then gently boiled for 5 minutes. It is then filtered (if necessary), the residue is washed with water, which is then added to the filtrate. Water is added so that total volume of this solution becomes 50 ml. This test solution is tested for lead. The amount of lead should not be more than 10ppm.

(4) Coliform Group : Cellulase is tested by Microbe Test Methods for Coliform Group in General Test Methods in Food Code. It should not be more than 30 cfu per 1 g of this product.

(5) Salmonella : Cellulase is tested by Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).

Assay(activity)

◦ Application and Principle : This test is to measure the activity of cellulase that is obtained from *Aspergillus niger* and its variety, *Trichoderma reesei* and its variety, *Humicola insolens* its variety. Activity test is based on enzymatic hydrolysis of glucosidic bonding within carboxymethylcellulose substrate (pH 4.5, 40°C). The decrease in viscosity of the substrate is measured by a viscometer with its scale corrected.

◦ Preparation of Test Solution : Test Solution is prepared by dilution so that 1ml of the final solution shows variation in relative fluidity of 0.18~0.22 in 5 minutes under the conditions below. Appropriate amount of sample is ground in a glass mortar and water is added. It is diluted in a volumetric flask. It is filtered through a Whatman No.1 filter paper or equivalent prior to use

Test Procedure : A viscometer, scale is previously corrected, is cleanly washed in water with sufficient detergent. It is then set up vertically in a glass water bath at $40 \pm 0.1^\circ\text{C}$. 20 ml of substrate solution and 4 ml of acetate buffer solution are added into a 50 ml Erlenmeyer flask with a stopper (2 for enzyme test and 1 for substrate blank test per sample). An enzyme test flask is plugged with a stopper and maintained for 15 minutes in a water bath, where precisely 1 ml of Test Solution is added and timed. It is then well mixed. Immediately, 10 ml of the mixed solution is added to the big branch of the viscometer. Approximately in 2 minutes, the reaction mixture is sucked in through the thin branch of the viscometer up to the upper scale using a rubber bulb. Time taken to reach the upper scale is measured in minutes (T_R). Again time taken to reach the lower scale (starting from the upper scale) is measured in seconds (T_T). By repeating the same procedure, T_R and T_T are measured again. This is repeated 4 times within 15minutes. Separately, a mixture of 20 ml substrate

solution, 4 ml acetate buffer solution and 1 ml water is added. 10 ml of the mixture is taken to the big branch of the viscometer. The time taken to reach the lower scale from the upper scale is measured five times and an average value is obtained T_S (seconds). A water blank test is carried out with 10 ml of water that is maintained at $40 \pm 0.1^\circ\text{C}$ by following the same procedure. An average value of 5 times is obtained, T_W (seconds). Using the following formula, relative fluidity and T_N values are obtained for each of 4 times of effluent time (TT) and reaction time (T_R).

$$F_R = \frac{T_S - T_W}{T_T - T_W}$$

$$T_N = \frac{1}{2} (T_T/60 \text{ seconds/minute}) + T_R = \frac{T_T}{120} + T_R$$

F_R : Relative fluidity for each reaction time

T_S : Average effluent time for substrate blank test (seconds)

T_W : Average effluent time for water blank test (seconds)

T_T : Effluent time for enzyme reaction solution (seconds)

T_R : Reaction time (minutes) (time taken from "adding the Test Solution" to "before the measurement of effluent time (T_T)")

T_N : Reaction time (T_R) (minutes) + one half of effluent time for Test Solution (T_T) (minutes)

A standard curve is prepared using the 4 relative fluidity (F_R) values for the 4 reaction times (T_N). This should be a straight line. The slope corresponds to the change in relative fluidity per minute and is proportional to the amount of enzyme. The optimum slope passing through a series of the test points is a better basis for the enzyme activity than a single value of relative fluidity. F_R values at 10 and 5 minutes are measured from the standard curve. The difference in fluidity should be 0.18~0.22. The enzyme activity is obtained from the following formula.

$$\text{CU/g} = \frac{1,000 (F_{R10} - F_{R5})}{W}$$

F_{R10} : Relative fluidity at reaction time of 10 minutes

F_{R5} : Relative fluidity at reaction time of 5 minutes

1,000 : Conversion factor (g to mg)

W : Amount of sample contained 1ml of Test Solution (mg)

Definition of Activity : 1 Cellulase unit(CU) is a activity which generates a change of 1 in relative fluidity in 5 minutes under the above test conditions on a carboxymethylcellulose substrate.

Apparatus

- Viscometer : Cannon Fenske Type Viscometer with size 100 corrected scale or its equivalent.
- Glass water bath : Isothermal glass water bath at $40 \pm 0.1^{\circ}\text{C}$ or its equivalent.

Agents and Solutions

- Acetate Buffer Solution (pH 4.5) : pH of 400 ml of 0.4 N acetic acid is adjusted to 4.5 ± 0.05 by adding 0.4 N of sodium acetate solution with stirring continuously.
- Sodium Carboxymethylcellulose : Sodium carboxymethylcellulose cellulose gum, Hercules(Aqualon) Type 7HF or its equivalent is used.
- Substrate Solution : 200 ml of water is added to a mixing container and the mixer is set at a low speed. 1g of sodium carboxymethylcellulose is carefully added so that it doesn't splash out and dispersed in water. Using a rubber policeman, contained wall is washed with warm water. The container is covered and the dispersion is mixed for 1 minute at high speed. The mixture is transferred into a 500 ml volumetric flask and the total volume is brought up to 500 ml with water. Substrate solution is filtered through a gauze prior to use.

Storage Standard of Cellulase

Cellulase is strongly hygroscopic, hence should be stored in a hermetic container in a cold dark place.

35. Shellac

Definition Shell is obtained from lac, the resinous secretion of the insect *Laccifer(Tachardia)*, Lacca Keer (Coccidae). White Shellac(White Shellac, Bleached Shellac, or Regular Bleached Shellac) is obtained by dissolving the lac in sodium carbonate solution, followed by bleaching with sodium hypochlorite, precipitation with dilute sulfuric acid solution, and drying; wax-free bleached shellac(refined bleached shellac, wax-free bleached shellac) is prepared by further treatment whereby is removed by filtration.

Compositional Specifications of Shellac

Description Shellac is grayish white~ light yellow, granular or fine particule, and odorless or having a slight characteristic odor.

Identification When a few drops of ammonium molybdate solution (1 g in 3 ml sulfuric acid) are added to 50 mg of Shellac, green color is produced. When this solution is neutralized with ammonia solution, it changed to lilac.

Purity (1) Arsenic : 0.5 g of Shellac is tested as directed under Purity (1) for Guar Gum (not more than 2ppm).

(2) Lead : When 5.0 g of Shellac is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Rosin : 2 g of Shellac is dissolved in 10 ml of dehydrated ethanol, where 50 ml of hexane is slowly added with shaking. The solution is transferred into a separatory funnel and washed two 50 ml portion of water. Take supernatant and filter it, and evaporate to dryness remaining solution on waterbath. Add 5 ml of acetic anhydride to residue, when it is necessary dissolve in water bath by heating. 20 ml of this solution is taken into bottle for colorimetry. When it is added 1 drop of sulfuric acid, it should not produce from reddish purple and purple to yellow ocher color.

(4) Wax : 10 g of Shellac is completely dissolved in 150 ml of hot water and 2.5 g of sodium carbonate into a beaker. It is then heated for 3 hours and cooled with cold water. If wax floats to the surface, it is filtered through a filter paper and washed with water. Then the wax is washed with 5~10 ml to accelerate drying. Filter paper is loosely folded and the top is bound with a piece of fine wire, which is then dried gently with the aid of gentle heat. It is then extracted with chloroform using a fat extracting soxhlet apparatus for 2 hours. After evaporating the solvent in the collector, the extract is dried for 2 hours at 105°C and weighed. The content of wax should not be more than 5.5% for white shellac and not be more than 0.2%

for refined bleached shellac.

- (5) Acid Value : Approximately 2 g of dried fine powder of Shellac is precisely weighed and dissolved in 50 ml of alcohol (neutralized with sodium hydroxide solution), test solution. When the sample is tested as directed under Acid Value in Oils Method, acid value should be 73~89 for while shellac and 75~91 for refined bleached shellac.

Loss on Drying When 3 g of fine powder of Shellac is dried for 4 hours at 40°C and then 15 hours in a vacuum desiccator (silica gel), the loss should not be more than 6% .

36. Steviol Glycoside

Stevioside

Rebaudioside A

Definition Steviol glycoside is obtained from *Stevia rebaudiana* Bertoni. The leaves are extracted with hot leaves and the aqueous extract is passed through an absorption resin and concentrate it. The product is recrystallized from methyl alcohol or ethyl alcohol and dried. Its major component is Steviol glucoside.

Compositional Specifications of Steviol Glycoside

Content When Steviol glycoside is dried and weighed, it should contain not less than 95.0% of whole Steviol glycoside.

Description Steviol glycoside is white or light yellow powder, flakes, or granules with strong sweet taste. It is odorless or having a slight characteristic odor.

Identification 0.5 g of Steviol glycoside is dissolved in 100 ml of water, test solution. 5 mg each of Stevioside for quantitative and Rebaudioside A is weighed and dissolved in 10 ml of water, standard solution. Liquid chromatography is carried out with test solution and standard solution under the operation conditions of assay. Retention time of the main peak of Test Solution is identical to the retention time of both peak of Stevioside and Rebaudioside or one peak of Standard Solution.

Purity (1) pH : pH of this aqueous solution (1→100) of Steviol glycoside should be 4.5~7.0 as determined by glass electrode method.

(2) Arsenic : 0.77 g of Steviol glycoside is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol(1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 21.3ppm.

(3) Lead : Accurately weigh 10 g of Steviol glycoside and place in a platinum or quartz crucible. Add minute amount of sulfuric acid, wet, gradually heat and preliminarily heat-treat the solution at the temperature as low as possible. Again add 1 ml of sulfuric acid, gradually heat, ignite until it is heat-treated at 450~550°C. After heat-treating, add minute amount of nitric acid(1→150) to the residue, again, add nitric acid(1→150) to make 10 ml, test solution. Separately, pipette 1 ml of lead standard solution , add nitric acid(1→150) to make 10 ml, reference solution.

When test solution and reference solution are tested by flame Atomic Absorption Spectrophotometry under following operation condition, the absorbance of test solution should not be higher than that of reference solution (not more than 1 ppm).

- (4) Residual solvent : 2 g of Steviol glycoside is precisely weighed into a 300 ml round bottom flask, 200 ml of water is added, boiling chips and 1 ml of silicone resin are added and mixed well. Receiver containing is connected to this, 4 ml of internal standard solution is precisely weighed and added to a 100 ml flask. While caring for the bubbles not to overflow, distill the solution at the rate of 2~3 ml per 1 minute until the milky liquid becomes about 90 ml, and water is added to make 100 ml, test solution. However, tert-butyl alcohol (1→1,000) is used as internal standard solution. Separately, 0.5 g of methyl alcohol is precisely weighed and water is added to make 500 ml, again 2 ml of this solution and 4 ml of internal standard solution is weighed, water is added to make 100 ml, standard solution. 2μl of test solution and standard solution is taken respectively, and injected to gas chromatograph with the following operation condition. Then, ratio of methyl alcohol peak against tert-butyl alcohol peak in test Solution and standard solution, Q_T and Q_S, is calculated separately, and the content of methyl alcohol is calculated by following formula, the content should not be more than 200ppm.

$$\text{Content of methyl alcohol(\%)} = \frac{\text{Weight of methyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_T}{Q_S} \times \frac{2 \times 100}{500 \times 100} \times 100$$

Q_T : Ratio of methyl alcohol peak against tert-butyl alcohol peak in Test Solution

Q_S : Ratio of methyl alcohol peak against tert-butyl alcohol peak in standard solution

Operation Conditions

Column : PLOT Q or its equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Temperature at injection hole : 200℃

Column Temperature : 120℃

Detector temperature : 300℃

Carrier gas : Nitrogen or Helium

Ash When 1 g of Steviol glycoside is tested by Ash Limit Test, it should not be more than 1.0%.

Loss on Drying When 2 g of Steviol glycoside is dried for 2 hours at 105℃, the loss

should not be more than 6%.

Assay Steviol glycoside is dried for 2 hours at 105°C, 60~120mg of stevioside is precisely weighed, dissolved in mobile phase to make 100 ml, test solution. Separately, stevioside and rebaudioside A standard are dried for 2 hours at 105°C, 50 mg of each is precisely weighed, dissolved in mobile phase to make 100 ml, standard solution. Each of test solution and standard solution is injected to liquid chromatograph with the following operation condition, and measure the content of whole steviol glycoside. Peak areas of dulcoside A, rubusoside, rebaudioside A, rebaudioside B, rebaudioside C, steviolvioside, stevioside of the sample are obtained. Also peak areas of dulcoside A, rubusoside, rebaudioside A, rebaudioside B, rebaudioside C, steviolvioside, stevioside of standard solution. The contents of the 7 components are obtained by the following formula. The sum of these contents is the content of steviolglycoside. When the peak of rebaudioside A is finished, the mobile phase is changed to 50 : 50 composition and the residuals in the column is washed out.

$$X \% = \frac{W_s}{W} \times \frac{A_x \times f_x}{A_s} \times 100$$

Ws : Amount of stevioside in standard solution(mg)

W : Weight of sample in test solution (mg)

As : Peak area of stevioside in standard solution

Ax : Peak area of X in test solution

fx : Ratio of molecular weight of X against stevioside (stevioside 1.00, dulcosideA 0.98, rebaudioside A 1.20, rebaudioside C 1.18, rubusoside 0.80, steviolvioside 0.80, rebaudioside B 1.00)

When test proceed with following operation condition, retention time is as follows. Against rebaudiosideA(1.00), rubusoside 0.12~0.16, dulcoside A 0.25~0.30, steviolvioside 0.35~0.41, stevioside 0.45~0.48, rebaudioside C 0.63~0.69, rebaudioside B 0.73~0.79.

Operation Conditions

-Detector : UV 210nm

-Column : Supelcosil LC-NH₂ or its equivalent

-Column Temperature : 235°C40°C

-Mobile phase : acetonitrile : water (80:20)

-Flow rate : adjust rebaudioside A is detected at about 21 minutes

37. Spirulina Color

Definition Spirulina Color is a pigment obtained by extracting *Spirulina Platensis* (NORD.) GEITLER, which is a blue-green algae. The major pigment is Phycocyanin. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Spirulina Color

Content Color value ($[E]_{1cm}^{10\%}$) of Spirulina Color should be higher than the indicated value.

Description Spirulina Color is blue powder having a slight characteristic odor.

- Identification**
- (1) The Test Solution obtained in Color Value section is blue and has a maximum absorption at about 618 nm.
 - (2) The Test Solution in (1) shows red fluorescence, which disappears after heating for 30 minutes at 90°C.
 - (3) When 3.9 g of ammonium sulfate is dissolved in 10 ml of the Test Solution in (1) and the solution is allowed to stand, blue precipitates are formed.
 - (4) When 1 ml of ferric chloride is added to 5 ml of the Test Solution in (1) and allow to stand for 20 minutes, the solution turns dark violet.
 - (5) When 0.1 ml of sodium hypochlorite solution (effective chlorine should not be less than 4%) is added to 5 ml of the Test Solution, the solution changes light yellow.

- Purity**
- (1) Arsenic : 0.25 g of Spirulina Color is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol(1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
 - (2) Lead : When 5.0 g of Spirulina Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 8.0 ppm.

Assay (Color Value) Appropriate amount of Spirulina Color is precisely weighed so that the absorbance is within 0.3~0.7 and dissolved in citric acid dibasic sodium phosphate buffer solution with pH 6.0 so that the total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citric acid dibasic sodium phosphate buffer solution with pH 6.0 as a reference

solution, absorption A is measured at the maximum absorption at about 618 nm with 1 cm path length. Color value is obtained using the following formula.

$$\text{Color Value } ([E]_{1\text{cm}}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

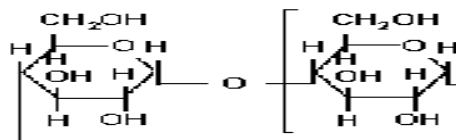
◦ Citric acid·dibasic sodium phosphate buffer solution (pH 6.0)

Solution 1 : 0.1 M citric acid solution : 1 l of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2 M dibasic sodium phosphate solution : 1 l of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

73.7 volume of Solution 1 and 126.3 volume of Solution 2 are mixed well and its pH is adjusted to 6.0.

38. Cyclodextrin



α -cyclodextrin ($C_6H_{10}O_5$)₆ molecular weight 972.85

β -cyclodextrin ($C_6H_{10}O_5$)₇ molecular weight 1134.99

γ -cyclodextrin ($C_6H_{10}O_5$)₈ molecular weight 1297.14

Definition (1) This is produced by the action of cyclodextrin producing enzyme on hydrolyzed starch. This is an cyclic oligosaccharide consisting of 6, 7, or 8 α -1,4 linked D-glucopyranosyl unit. There are α -cyclodextrin, β -cyclodextrin, and γ -cyclodextrin. Each of them is called α -cyclodextrin, β -cyclodextrin, and γ -cyclodextrin.

Compositional Specifications of Cyclodextrin

Content When Cyclodextrin is dried and analyzed quantitatively, it should contain not less than 98.0% of each α -cyclodextrin($C_6H_{10}O_5$)₆, β -cyclodextrin($C_6H_{10}O_5$)₇, and γ -cyclodextrin ($C_6H_{10}O_5$)₈, respectively.

Description Cyclodextrin is odorless, white crystallite or crystalline powder having a slight sweet taste.

Identification 0.2 g of Cyclodextrin is dissolved in 1 ml of 0.1 N iodine solution by heating in a water bath. When it allowed to stand at room temperature, α -cyclodextrin, β -cyclodextrin, γ -cyclodextrin, maltosyl- α -cyclodextrin, maltosyl- β -cyclodextrin, maltosyl- γ -cyclodextrin forms bluish violet, yellowish brown, reddish brown, bluish violet, yellowish brown, and reddish brown precipitates are formed, respectively.

Purity (1) Clarity of Solution : 0.5 mg of sample is dissolved in 50 ml water, it should be colorless and clear.

(2) Specific Rotation : Approximately 1 g of pre-dried sample is precisely weighed and dissolved in water so that the total volume becomes 100 ml. The optical rotation of the solution should be α -cyclodextrin $[\alpha]_D^{20} = +147.0 \sim +152.0^\circ$, β -cyclodextrin $[\alpha]_D^{20} = +160.0 \sim +164.4^\circ$, γ -cyclodextrin $[\alpha]_D^{20} = +173.0 \sim +178.0^\circ$

(3) Chloride : When 0.5 g of Cyclodextrin is tested for chlorides, the amount should not be more than or equal to the chloride content of 0.25 ml of 0.01 N hydrochloric

acid.

- (4) Arsenic : 5 ml of water and 1 ml of sulfuric acid are added to 1 g of Cyclodextrin in a small beaker, where 10 ml of sulfurous acid is added. It is then heated in a water bath until sulfurous acid is removed and the total volume becomes approximately 2 ml. Water is then added to bring the volume to 5 ml (Test Solution). The Test Solution is tested for arsenic and the content should not be more than 1ppm.
- (5) Lead : When 5.0 g of Cyclodextrin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.
- (6) Residual Solvent : 0.25 g of Cyclodextrin is precisely weighed, 10 ml of water is added and diffused with ultrasonic waves for 10 minutes, test solution. This solution is transferred into 25 ml of frit sparger, and analyzed with Purge and Trap and Gas chromatograph. To 10 ml of water, 0.25 ml of mixed standard solution is added, analyzed with Purge and Trap and Gas chromatograph, then limited to β -cyclodextrin, the amount of toluene and trichloroethylene should not be more than 1.0ppm, respectively.

Mixed standard solution : 50 mg each of toluene and trichloroethylene is precisely weighed and dissolved in methanol to make 50 ml, 0.1 ml of this solution is taken again, and water is added to make 100 ml, mixed standard solution.(1 ml of this solution contains 1 μ g of toluene, and 1 μ g of trichloroethylene, respectively).

Operation Condition

Purge and Trap

Trap : Tenax TA or its equivalent

Purge time : 11 minutes

Desorption temperature and time : 250°C, 4 minutes

Cryo focus temperature : -150°C

Bake temperature and time : 260°C, 10 minutes

Gas chromatography

Column : DB-1(30m \times 0.32 μ m) or its equivalent

Detector : (Hydrogen) Flame Ionization Detector (FID)

Column Temperature : hold at 40°C for 3 minutes and is raised to 220°C at a rate of 40°C per minute

Detector Temperature : 250°C

Carrier gas and flow rate : Nitrogen or Helium

Loss on Drying When Cyclodextrin is dried for 4 hours at 105°C under a reduced pressure of 5 mmHg, the loss should not be more than 12%.

Residue on Ignition When residue on ignition is done with 1 g of Cyclodextrin, the amount of residue should not be more than 0.1%.

Assay (1) After drying, approximately 0.1 g of Cyclodextrin is accurately weighed and dissolved in 10 ml of water (Test Solution). Separately, Standard for each of α -, β -, γ -cyclodextrin is dried. This standards are accurately weighed 0.1 g, and dissolved in 10ml of water, respectively (Standard Solution). 10 μ l each of Standard Solutions and Test Solution is injected into liquid chromatography under the following operation conditions. The content of cyclodextrin is calculated by the following formula.

$$\text{Content (\%)} = \frac{A_u \times W_s}{A_s \times W_u} \times 100$$

A_u : Peak area of Test Solution

A_s : Peak area of Standard Solution

W_s : Amount of standard taken (g)

W_u : Amount of sample taken(g)

Operation Conditions

-Detector : Differential refractometer (RI Detector)

-Column : Aminex HPX-42A (8 mm×300 mm) or its equivalent

-Column Temperature : a constant temperature at about 60°C

-Mobile Phase : Water

-Flow Rate : 0.6~1.0 ml/min

39. Curcumin

Turmeric Oleoresin

Definition This is a pigment obtained by extraction(ethanol, oils or organic solvent(extraction solvents for spices and oleo resins) of tumeric i.e., the ground rhizomes of *Curcuma longa* Linné. The major pigment is curcumin ($C_{21}H_{20}O_6 = 368.37$). Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Turmeric Oleoresin (Curcumin)

Content Color value ($[E]_{1cm}^{10\%}$) of Turmeric Oleoresin should not be less than the indicated value.

Description Turmeric Oleoresin is yellow~dark reddish brown liquid, lump, powder, or paste with a slight characteristic odor.

Identification Turmeric Oleoresin is dissolved in ethyl alcohol (if it is water soluble, it is dissolved in small amount of water and then ethyl alcohol is added). The concentration is adjusted so that it has almost same tone of color as potassium bichromate solution (1→1,000) (Test Solution).

- (1) Test Solution is characterized yellow color and a green fluorescence.
- (2) Test Solution produced red when 2 ml of sulfuric acid is added to 5 ml of test solution and stirred.
- (3) A piece of filter paper is dipped in Test Solution and dried. A few drops of hydrochloric acid, followed by a few drops of boric acid solution (1→100) are dropped onto the piece of filter paper. Upon drying by heating, it is developed cherry red. When a few drops of ammonia solution is added, it is changed blue.

Purity (1) Arsenic : 0.25 g of Turmeric Oleoresin is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol(1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450 ~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

- (2) Lead : When 5.0 g of Turmeric Oleoresin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2ppm.

- (3) Residual Solvents : When Turmeric Oleoresin is tested by Purity (5) for Paprika

Extract Pigments,

Methylene chloride, Trichloro ethylene	Not more than 30ppm(single or the sum of both)
Acetone	Not more than 30ppm
Isopropyl alcohol	Not more than 30ppm
Methyl alcohol	Not more than 50ppm
Hexane	Not more than 25ppm

Assay Appropriate amount of Turmeric Oleoresin is precisely weighed so that the absorbance is within 0.3~0.7 and dissolved in ethyl alcohol to make 100 ml. 1 ml of this solution is diluted to 100 ml with ethyl alcohol (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using ethyl alcohol as a blank solution, absorbance A of the sample is measured at the maximum absorption at 425 nm in a 1cm cell. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

40. Arabic Gum

Definition Arabic Gum is obtained from drying the secretion of acacia senegal (*Acacia senegal* WILLDENOW) of leguminosae or its other variety from the same genus. Or it can be obtained by de-salting the same. The major component is polysaccharides.

Compositional Specifications of Arabic Gum

Description Arabic Gum is white~light yellow powder, granules or light yellow~brown lump and odorless.

Identification 1 g of Arabic Gum is dissolved in 50 ml of cold water. Upon adding 0.2 ml of dilute lead nitrous acid solution to 10 ml of this solution, it forms agglomerates or white precipitates immediately.

Purity (1) Arsenic : Arabic Gum is tested as directed under the procedure in Purity (1) for Guar Gum.

(2) Lead : When 5.0 g of Arabic Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Arabic Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Arabic Gum is tested as directed under Mercury Test Method, its content should not be more than 1.0ppm.

(5) Acid Insoluble Ash : When Arabic Gum is tested for Ash by General Test Methods, it should not be more than 0.5%.

(6) Starch and Dextrin : 1 g of Arabic Gum is dissolved in 50 ml of water by boiling, which is then cooled. When a few drops of iodine solution are added, it should not change blue or red.

(7) Tannin Containing Gums : 1 g of Arabic Gum is dissolved in 50 ml of water. When 0.1 ml of ferric chloride TS is added to 10 ml of this solution, black matter or precipitates should not form.

(8) Water Insoluble substances : 5 g of Arabic Gum is placed into an Erlenmeyer flask containing about 100 ml of water. 10 ml of dilute hydrochloric acid is added to the flask, which is then gently boiled and filtered through a glass filter, previously made constant weight. The residue is washed with plenty of hot water. The residue is dried for 2 hours at 105°C. The content of water insoluble substances should not be more than 1%.

(9) E. Coli : When Arabic Gum is tested by Microbe Test Methods for E. Coli in

General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

- (10) Salmonella : When Arabic Gum is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Ash When Arabic Gum is tested for ash, it should not be more than 4%.

Loss on Drying When 1 g (uncrushed sample should be well mixed prior to weighing and passed through a standard mesh screen No.40) of Arabic Gum is dried for 5 hours at 105°C, the loss should not be more than 15%.

41. α-Amylase, Nonbacterial (DU)

Definition α-Amylase(nonbacterial) is an enzyme obtained from cultures of *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety, and *Rhizopus oryzae* and its variety, and malts. α-Amylase(bacterial) is an enzyme obtained from cultures of *Bacillus subtilis* and its variety, *Bacillus licheriformis* and its variety, *Bacillus stearothermophilus*, and *Bacillus licheriformis* containing the gene of alpha-amylase from *Bacillus stearothermophilus*. It is called α-Amylase(nonbacterial) and α-Amylase(bacterial), respectively. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

1. α-Amylase(nonbacterial)

Compositional Specifications of α-Amylase, Nonbacterial

Content α-Amylase, Nonbacterial (DU) contains 90~130% of the indicated activity(activity) as α-Amylase, Nonbacterial (DU).

Description α-Amylase, Nonbacterial (DU) is white~pale yellow~dark brown powder or transparent~dark brown liquid.

- Purity** (1) Arsenic : 0.25 g of α-Amylase, Nonbacterial (DU) is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
- (2) Lead : When 5.0 g of α-Amylase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
- (3) Coliform Group : α-Amylase, Nonbacterial is tested by Microbe Test Methods for Coliform Group in General Test Methods 「Standards and Specifications for Foods」. It should not be more than 30 cfu per 1 g of this product.
- (4) Salmonella : α-Amylase, Nonbacterial (DU) is tested by Microbe Test Methods for Salmonella in General Test Methods 「Standards and Specifications for Foods」. It should be negative (-).
- (5) E. Coli : When α-Amylase(nonbacterial) is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Assay (Activity)

- Application and Principle : This test is to measure the activity of enzyme obtained from cultures of *Aspergillus niger* and its variety, *Aspergillus oryzae* and its variety, and *Rhizopus oryzae* and its variety, and malts. Activity test is based on the time taken to reach standard degree of hydrolysis of starch solution with a certain concentration at $30 \pm 0.1^\circ\text{C}$. The degree of hydrolysis is measured by comparing iodine color of the hydrolyzed products with color standard.
- Preparation of Test Solution : Test Solution is prepared so that the end point of 5 ml of the finally diluted solution is reached in 10~30 minutes under the test conditions. In case of malt, 25.0 g of fine ground powder is weighed into a 1,000 ml Erlenmeyer flask, where 500 ml of 0.5% sodium chloride solution is added. The mixture is leached for 2.5 hours at $30 \pm 0.2^\circ\text{C}$ while stirring once in every 20 minutes (caution : The flask should not be turned upside down. Care must be minimized the amount of the content left on the inner wall.). After leaching, the mixture is filtered through a 32 cm Whatman No.1 filter paper using a 20 cm diameter funnel. First 50 ml of the filtrate is combined to sample solution and re-filtered through the same filter paper. Filtration is stopped in 3 hours since the point when the sample is mixed in sodium chloride solution. 20.0 ml of the filtrate is diluted to 100 ml with 0.5% sodium chloride solution (Test Solution).

Test Procedure : 5 ml of iodine solution is added to each of a set of 20 test tubes (13×100 mm), which are maintained at $30 \pm 0.1^\circ\text{C}$ in a water bath. 20 ml of substrate solution and 5 ml of 0.5% sodium chloride solution (previously maintained for 20 minutes in a water bath) are mixed in a 50 ml Erlenmeyer flask, which is then kept in a water bath. Upon starting the test, 5 ml of the Test Solution is added to the flask in the water bath. In 10 minutes, 1 ml of the reaction mixture in the 50 ml Erlenmeyer flask is taken and added to the test tube with iodine solution, which is well shaken and immediately compared to the color standard obtained from a comparator. A tube filled with water is used behind the comparator plate. (note : Care must be taken so that the tip of the pipette, which draws the reaction mixture, does not touch the iodine solution. If iodine solution is mixed with reaction mixture, the reaction may be affected.). By the same method, a comparison test is repeated at a same interval until the color of the Test Solution becomes the same as the color standard. Time at each pipetting is recorded.

Reference : If a previous test color is deeper and a subsequent (in a 30 second interval) test color is lighter than the color standard, the end point is obtained by adding 15 seconds to the time for the color nearer to the color standard. Comparator

tube (13 mm) is shaken after each observation. Difference in color judgment due to personal difference can be minimized by using a prism attachment and observing at a distance of 6~10" . Activity of an enzyme can be calculated by the following equation.

$$\text{DU (solution)} = \frac{24}{W \times T}$$

$$\text{DU (as a dried form)} = \text{DU (solution)} \times \frac{100}{100 - M}$$

W : Amount of enzyme in 5 ml of Test Solution (g)

T : Dextrinization time (minutes)

24 : Weight of starch substrate (0.4 g) multiplied by 60 minutes

M : Water content of sample (%)

Definition of Activity : 1 α -Amylase dextrinizing unit (DU) is an amount of enzyme that dextrinizes soluble starch at a rate of 1 g per hour at 30°C under the presence of sufficient amount of β -Amylase.

Apparatus

- Color standard for comparison : α -amylase color plate is used. Or 25 g of cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) and 3.84 g of potassium bichromate is dissolved in 0.01 N hydrochloric acid and is made 100 ml. (this is stable for a long period of time when is capped.).
- Comparator : Standard Hellige comparator or Pocket comparator is used along with prism attachment. A milky light (100 W) should be shone at a 6 inch distance from the back of the milky colored glass of the comparator. The light should be positioned so that it won't be shone directly on observer's eyes.
- Comparator Tubes : An angled tube with 13 mm path length or its equivalent should be used.

Solutions

- Acetate Buffer Solution (pH 4.8) : 164 g of anhydrous sodium acetate is dissolved in 500 ml of water and 120 ml of glacial acetic acid is added. pH of the solution is adjusted to 4.8 with glacial acetic acid. The total volume is brought up to 1,000 ml with water and well mixed.
- β -Amylase Solution : 250 mg of β -Amylase (free of α -Amylase) with 2,000 DP°C is

dissolved in 5 ml of water (note : Enzyme should be kept by refrigeration. Before opening the cap, it should be warmed to room temperature to prevent condensation of moisture.).

- Starch : Soluble starch (Lintner) or its equivalent is used. Before using a new lot, it should be confirmed that the quality is the same as the previous one. A new lot with a difference of diastatic power $\pm 3^\circ\text{C}$ or higher cannot be used.
- Substrate Solution : 10.0 g of starch (as a dried form) is dispersed in 100 ml of cold water, where 300 ml of boiling water is slowly added with stirring. After boiling for 1~2 minutes, it is cooled and transferred into a 500 ml volumetric flask. 25 ml of acetate buffer solution is added to the flask, where all of the β -Amylase solution is added. It is then saturated with toluene and diluted to 500 ml with water. The resultant solution is allowed to stand for 18~72 hours at $30 \pm 2^\circ\text{C}$ and then used.
- Iodine stock solution : 5.5 g of iodine and 11.0 g of potassium iodide are dissolved in 200 ml of water, which is then diluted to 250 ml with water. It is kept in a dark place. It should be freshly prepared in every 30 days.
- Iodine Solution : 20 g of potassium iodide is dissolved in 300 ml of water and 2.0 ml of iodine stock solution is added. The total volume of the solution is brought up to 500 ml with water.

Storage Standard of α -Amylase, Nonbacterial(DU)

α -Amylase, Nonbacterial (DU) is stored sealing tightly in a cold dark place.

2. α -Amylase(bacterial)

Compositional Specifications of α -Amylase, Bacterial

Content α -Amylase, Bacterial (BAU) contains 90~130% of the indicated activity as α -Amylase, Bacterial (BAU).

Description α -Amylase, Bacterial (BAU) is white~pale yellow~dark brown powder or transparent~dark brown liquid.

Purity (1) Arsenic : 0.25 g of α -Amylase, Bacterial (BAU) is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at $450\sim 550^\circ\text{C}$. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at $450\sim 550^\circ\text{C}$. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of α -Amylase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content

should not be more than 5.0 ppm.

- (3) Coliform Group : α-Amylase, Nonbacterial is tested by Microbe Test Methods for Coliform Group in General Test Methods 「Standards and Specifications for Foods」. It should not be more than 30 cfu per 1 g of this product.
- (4) Salmonella : α-Amylase, Nonbacterial (DU) is tested by Microbe Test Methods for Salmonella in General Test Methods 「Standards and Specifications for Foods」. It should be negative (-).
- (5) E. Coli : When α-Amylase(nonbacterial) is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Assay (Activity)

◦ Application and Principle : This test is to measure the activity of α-amylase in an enzyme (indicated as Bacterial amylase units (BAU)) that is obtained from a culture of *Bacillus licheniformis* and its variety, *Bacillus stearothermophilus*, *Bacillus licheniformis* that contains α-amylase genes of *Bacillus stearothermophilus*(This method can't apply to enzyme containing beta-amylase).

Activity test is based on the time taken to reach standard degree of hydrolysis of starch solution with a certain concentration at $30 \pm 0.1^{\circ}\text{C}$. The degree of hydrolysis is measured by comparing iodine color of the hydrolyzed products with color standard.

- Preparation of Test Solution : Test Solution is prepared so that the end point of 10 ml of the finally diluted solution is reached in 15~35 minutes under the test conditions.
- Test Procedure : It is tested as directed under the Test Procedure for α-Amylase, Nonbacterial (DU). However, 10 ml of Test Solution is added instead of sodium chloride solution.

Activity of an enzyme is calculated by the following equation.

$$\text{BAU/g} = 40\text{F/T}$$

F : Dilution factor (total amount of dilution/amount of sample(g))

T : Dextrinization time (minutes)

40 : A factor (400/10) that is calculated by dividing 400 mg (amount of starch in 20 ml of 2% substrate solution) with 10 ml (amount of Test Solution used).

- Definition of Activity : 1 Bacterial amylase unit(BAU) corresponds to an amount of

enzyme that dextrinizes 1 mg of starch per minute under the conditions above.

Apparatus

Color standard for comparison, Comparator, and Comparator Tubes are the same as in α -amylase, Nonbacterial(DU). However, daylight or daylight type fluorescent light is used as a light source.

Solutions

◦ Phosphate Buffer Solution (pH 6.6)

Solution A : 9.1 g of potassium phosphate, monobasic (anhydrous) is dissolved in plenty of water (total volume = 1,000 ml).

Solution B : 9.5 g of sodium phosphate, dibasic (anhydrous) is dissolved in plenty of water (total volume = 1,000 ml).

Solution A & B are mixed at a ratio of 400 ml : 600 ml. If necessary, pH of the solution is adjusted to 6.6 with Solution A or Solution B.

◦ Iodine Solution : Prepared same as α -amylase, Nonbacterial(DU).

Starch : Prepared same as α -amylase, Nonbacterial(DU).

Substrate Solution : 10 g of starch (as a dried form) is dispersed in 100 ml of cold water, where 300 ml of boiling water is slowly added with stirring. After boiling for 1~2 minutes, it is cooled and transferred into a 500 ml volumetric flask. 10 ml of phosphate buffer solution is added to the flask and the total volume is brought up to 500 ml with water.

Storage Standard of α -Amylase, Bacterial(BAU)

α -Amylase, Bacterial (BAU) is stored sealing tightly in a cold dark place.

42. α -Amylase, Bacterial(BAU)

The specification of α -Amylase (Bacterial) is combined to 41. α -Amylase (Nonbacterial) in Natural Additives.

The date of notification : November 10, 2010 (Notification No. 2010-82)

43. Annatto Extract

Definition There are types, oil soluble pigment and water dispersible pigment. Oil soluble pigment is obtained by extracting seed skin of *Bixa orellana* Linné. with oil and fat or organic solvents (extracting solvent for oleoresin spices). Its major component is bixin ($C_{25}H_{30}O_4 = 394.52$) of carotinoids. Water dispersible pigment is obtained by dispersing fine pigments contained in seed skins of *Bixa orellana* L.in with water or propylene glycol. It can also be obtained by hydrolysing bixin under pressure and heating. Its major component is bixin or norbixin ($C_{24}H_{30}O_4 = 380.49$) of carotinoids. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Annatto Extract

Content Color value ($[E]_{1cm}^{10\%}$) of Annatto Extract should be higher than the indicated value.

Description Annatto Extract is reddish brown~brown liquid, lump, powder or paste with a slight characteristic odor.

Identification (1) Test Solution obtained in Color Value section of Annatto Extract shows orange yellow color and a absorbance maximum at about 500 nm and 470 nm.

Purity (1) Arsenic : 0.25 g of Annatto Extract is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450 ~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Annatto Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Annatto Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Annatto Extract is tested by Mercury Test Method, its content should not be more than 1.0 ppm.

(5) Residual Solvents : When Annatto Extract is tested by Purity (4)(5) for Paprika Extract Pigments,the content of residual solvents should be.

Methylene chloride, trichloroethylene	Not more than 30ppm (individual or sum if used together)
Acetone	Not more than 30ppm
Isopropyl alcohol	Not more than 50ppm
Methyl alcohol	Not more than 50ppm
Hexane	Not more than 25ppm

Assay (Color Value) Annatto Extract is precisely weighed to showed absorbance within 0.3~0.7. It is dissolved in dimethylformamide for oil soluble pigment and in 0.1 N sodium hydroxide solution for water dispersible pigment and make 100 ml, respectively. A mixture of water, dimethylformamide, and acetic acid (50:50:1) is added to 5 ml of this solution and diluted to 100 ml with a mixture of water, dimethylformamide, and acetic acid (50:50:1), test solution. Using a mixture of water, dimethylformamide, and acetic acid (50:50:1) as a reference, a absorption maximum A of the Test Solution is measured at about 470 nm with 1 cm cell. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 200}{\text{Weight of sample (g)}}$$

44. Alginic Acid

Chemical Formula $(C_6H_7O_6)_n$

Equivalent Value 200.00

Definition Alginic Acid is a carbohydrate obtained from brown algae (Phaeophyceae). Chemically, it is a linear glycuronoglycan consisting mainly of pyranose ring type of β -(1 \rightarrow 4) linked D- mannuronic acid and α -1,4 -linked L- gluronic acid.

Compositional Specifications of Alginic Acid

Content On the dried basis, Alginic Acid contains not less than 20.0 and not more than of 23.0% carbon dioxide (CO₂), equivalent to not less than 91.0 and not more than of 104.5% alginic acid.

Description Alginic Acid is white, pale yellowish brown granule or filamentous powder having a slight characteristic odor and taste.

Identification (1) 1 g of Alginic Acid is dissolved in 150 ml of 0.1 N sodium hydroxide solution. When 1 ml of calcium chloride solution is added to 5 ml of this solution, voluminous gelatinous precipitates are formed.

(2) When 1 ml of diluted sulfuric acid is added to 5 ml of this solution, heavy gelatinous precipitates are formed.

(3) Approximately 5 mg of Alginic Acid is placed in a test tube, where 5 ml of water is added. 1 ml of freshly prepared solution of 1g naphtha resorcin in 100 ml alcohol and 5 ml of hydrochloric acid are added to the test tube, which is boiled for 3 minutes and cooled to 15°C. The content is transferred into a 30 ml separatory funnel with a stopper and the test tube is washed with 5 ml of water, which is added to the funnel. It is then extracted with 15 ml of isopropyl ether. Separately, a blank test is carried out. Isopropyl ether extract from the sample shows deeper violet color than that from the blank test.

Purity (1) Arsenic : It is tested as the procedure in Purity (1) for Guar Gum (not more than 4ppm).

(2) Lead : When 5.0 g of Alginic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Cadmium : When 5.0 g of Alginic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Alginic Acid is tested by Mercury Test Method, its

content should not be more than 1.0ppm.

- (5) Insoluble substances : 1 g of Alginate Acid is dissolved in 100 ml of 0.1 N sodium hydroxide solution. It is then centrifuged and the supernatant is discarded. The residue is washed five times with water by mixing, centrifuged and decanted. The residue is transferred by means of water to a tared glass filter, dried for 1 hour at 105°C, cooled and weighed. The amount of the residue should not be more than 10 mg.
- (6) pH : Suspension (3→100) of Alginate Acid should be pH 2.0~3.5.
- (7) Total Viable Aerobic Count : When Alginate Acid is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 colonies per 1 g
- (8) E. Coli : When Alginate Acid is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (9) Salmonella : When Alginate Acid is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (10) Number of Fungi : When Alginate Acid is tested by Microbe Test Methods for Number of Fungi in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 500 colonies per 1 g

Ash 3 g of Alginate Acid is weighed into a porcelain crucible that is previously made weight constant and tared. It is then reduced to ash at 650°C until black carbon disappears. It is then allowed to cool down in a desiccator (silica gel). The amount of ash should not be more than 4%.

Loss on Drying 3 g of Alginate Acid is dried for 4 hours at 105°C. The loss on drying should not be more than 15%.

Assay Alginate Acid is dried and analyzed as directed under the Content Analysis for Xanthan Gum.

1 ml of 0.25 N sodium hydroxide solution = 25 mg alginate acid (Equivalent Value : 200.00)

45. Oleoresin Capsicum

The specification of Oleoresin Capsicum is combined to 102. Spice Oleoresins in Natural Additives.

The date of notification : November 10, 2010 (Notification No. 2010-82)

46. Milk Clotting Enzyme

RENNET

Chymosin

Definition Milk Clotting Enzyme is an enzyme obtained from the stomach of cow, sheep and etc., or from the cultures of *Kluyveromyces lactis*, *Rhizomucor miehei*, *Rhizomucor pusillus*, *Mucor sp.*, *Irpex lacteus*, *Bacillus cereus*, *Cryphonectria parasitica*, *Escherichia coli* and an enzyme obtained from *Aspergillus awamori* in which chymosin gene of calf is inserted. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Milk Clotting Enzyme

Content Milk Clotting Enzyme contains 90~130% of the indicated activity as Milk Clotting Enzyme.

Description Milk Clotting Enzyme is white~light yellow~dark brown powder, granule, lump, or transparent~deep brown liquid.

Purity (1) Arsenic : 0.25 g of Milk Clotting Enzyme is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Milk Clotting Enzyme is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Coliform Group : Milk Clotting Enzyme is tested by Microbe Test Methods for Coliform Group in General Test Methods in Food Code. It should contain 30 colonies or less per 1 g of this product.

(4) Salmonella : Milk Clotting Enzyme is tested by Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).

(5) E. Coli : When Milk Clotting Enzyme is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay (Activity)

Application and Principle : This test is to measure the activity of milk clotting

enzyme that is obtained from the stomach of cow, sheep and etc, or from the cultures of *Kluyveromyces lactis*, *Rhizomucor miehei*, *Rhizomucor pusillus*, *Mucor sp.*, *Irpex lacteus*, *Bacillus cereus*, *Cryphonectria parasitica*, *Escherichia coli* and an enzyme obtained from *Aspergillus awamori* in which chymosin gene of calf is inserted. Activity test is based on clotting time of reaction mixture of rennet standard and enzyme using defatted dried milk at pH 6.5.

- Preparation of Test Solution : Test Solution is prepared using acetate buffer solution so that the clotting time falls within ± 40 seconds of mixed standard solution.
- Test Procedure : 25 ml each of substrate solution is placed in 2 flasks, which are set up in a rotational apparatus. The flasks are hung in a $32 \pm 0.2^\circ\text{C}$ water bath and tilted at an appropriate angle. While rotating, substrate solution is isothermalized for at least 12 minutes (maximum 20 minutes). 0.5 ml each of Test Solution and Standard Solution is added to each flask, which is then rotated and clotting time is measured. Clotting time is the time when fine particles are adsorbed on the inner wall of the flask. Activity is obtained by the following equation.

$$\text{IMCU/g or ml} = 1,000 \times \frac{T_s}{T_c} \times \frac{D_s}{D_c}$$

1,000 : activity of milk clotting enzyme standard

T_s : clotting time of mixed standard solution (seconds)

T_c : clotting time of test solution

D_s : concentration of mixed standard solution (g/ml)

D_c : concentration of test solution (g/ml)

Agents and Solutions

- Calf rennet standard (1,000 IMCU, IDF standard) : Should contain not less than 98% of chymosin and not more than 2% of bovine pepsin.
- Adult bovine rennet standard (1,000 IMCU, IDF standard) : Should contain not more than 1% of chymosin and no less than 99% of bovine pepsin.
- Substrate Solution : 110 g of defatted milk, dried at low temperature, is placed in a 2,000 ml beaker, where 100 ml of 0.05% calcium chloride solution is added and homogenized. 900 ml of 0.05% calcium chloride solution is added and stirred for 30 minutes (care must be taken to prevent excessive foaming). The resultant solution is allowed to stand for 30 minutes in a dark place. When pH is measured, it

should be approximately 6.5 (pH should not be adjusted). This solution should be used within 4 hours.

- Mixed Standard Solution : Mixed standard solution is prepared by obtaining a mixture ratio of calf rennet standard solution and adult bovine rennet standard solution, and mixing . Mixing ratio of calf rennet standard solution and adult bovine rennet standard solution is obtained by applying chymosin content(%) to Fig 1 in content of chymosin and pepsin in sample.
- Calf rennet Standard Solution : 2.5 g of calf rennet standard is precisely weighed and dissolved in 15~20 ml of acetate buffer solution in a 50 ml flask. The total volume is brought up to 50 ml with acetate buffer solution. 3 ml of this solution is further diluted to 50 ml with acetate buffer solution.
- Adult bovine rennet Standard Solution : It is prepared by the same method as calf rennet standard solution using adult bovine rennet standard.
- Acetate buffer solution (pH 5.5) : 10 ml of 1 M acetic acid and 10 g of sodium acetate (3 hydrate) 10 g are mixed and water is added to bring the total volume to 1,000 ml. pH of this solution should be 5.5 and is adjusted if necessary.

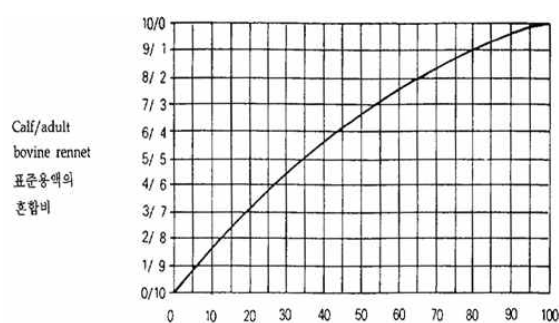


Fig. 1 Mixing ratio of calf rennet and adult bovine rennet standards against chymosin content (%) in the sample for mixed standard solution preparation

Storage Standard of Milk Clotting Enzyme

Milk Clotting Enzyme should be stored in a hermetic container in a cold dark place.

47. Liquid Paraffin

Definition Liquid Paraffin is a mixture of hydrocarbons derived from petroleum.

Compositional Specifications of Liquid Paraffin

Description Liquid Paraffin is colorless, clear, and viscous liquid having almost no fluorescence. It is odorless and tasteless.

Identification (1) Liquid Paraffin is placed in a porcelain dish. When ignited, it burns with a bright flame, which generates a characteristic odor of paraffin vapor.

(2) Approximately 0.5 g of Liquid Paraffin is mixed with the same amount of sulfur. When the mixture is heated, an odor of hydrogen sulfide is generated.

Purity (1) Free acid and free alkali : Approximately 10 ml of hot water and 1 drop of phenolphthalein are added to 10 ml of Liquid Paraffin. When the mixture is vigorously shaken, it should not develop red. When 0.2 ml of 0.02N sodium hydroxide solution is added and mixed by shaking, it should develop red.

(2) Arsenic : 0.25 g of Liquid Paraffin is tested according to Purity (5) for Sorbic Acid (Not more than 4ppm).

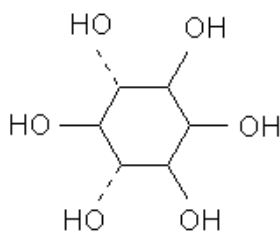
(3) Lead : When 5.0 g of Liquid Paraffin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Polynuclear aromatic hydrocarbons : 25 ml of Liquid Paraffin is precisely taken in a 25 ml measuring cylinder and transferred into a 100 ml separatory funnel, where 25 ml of n-hexane is added and well mixed by shaking. 5 ml of dimethyl sulfoxide is added to the solution, which is mixed by shaking vigorously for 2 minutes and settled for 15 minutes. The lower layer is transferred into a 50 ml separating funnel, where 2 ml of n-hexane is added, shaken vigorously for 2 minutes, and settled for 2 minutes. The lower layer is transferred into a centrifuge tube with a stopper and centrifuged at 2,500~3,000 rpm for about 10 minutes. The clear supernatant is transferred into a cell with a tight stopper (Test Solution). Separately, 25 ml of n-hexane is taken into a 50 ml separating funnel, where 5 ml of dimethyl sulfoxide is added, mixed by shaking vigorously for 2 minutes and settled for 2 minutes. The lower phase is centrifuged in a 10 ml centrifuge tube with a stopper at 2,500~3,000 rpm for 10 minutes. The clear supernatant is transferred into a cell with a tight stopper (Reference Solution). Absorption of the Test Solution is immediately measured with 1cm path length using the Reference Solution as a reference. The absorbance should not exceed 0.1 in at a wavelength range of 260~350 nm. N-hexane and dimethyl sulfoxide should be for UV

absorption spectrophotometry.

- (5) Readily Carbonizable Substances : 5 ml of Liquid Paraffin is taken into a Nestler tube, where 5 ml of 94.5~94.9% sulfuric acid. It is then heated for 2 minutes in a water bath. It is immediately taken out of the water bath and shaken (up and down) vigorously for 5 seconds. When this procedure is repeated 4 times, the color of the fluidal paraffin layer does not change color. The color of the layer of sulfuric acid should not be deeper than that of a solution that is prepared by mixing 3 ml of ferric chloride color standard solution, 1.5 ml of cobalt I chloride color standard solution, and 0.5 ml of copper sulfate color standard solution in a Nestler tube by shaking.

48. Inositol



Chemical Formula $C_6H_{12}O_6$

Molecular Weight 180.16

Definition Inositol is obtained from decomposition of phytic acid or by separating nectar or molasses of beet (*Beta vulgaris* LINNE var. *rapa* DUMORTIER) of chenopodiaceae. Its major component is inositol.

Compositional Specifications of Inositol

Content After drying, Inositol should contain not less than 97.0% of inositol ($C_6H_{12}O_6$).

Description Inositol is fine, white crystal or crystalline powder with odorless and sweet taste.

Identification (1) 6 ml of nitric acid is added to 1 ml of aqueous solution (1→50), which is then evaporated to dryness in a water bath. The residues are dissolved in 1 ml of water, where 0.5 ml of strontium acetate solution (1→10) is added. When this solution is again evaporated to dryness in a water bath, the resultant residue shows red color.

(2) Melting point of hexaacetyl inositol obtained in Assay is 212~216°C.

Purity (1) Melting Point : Melting point should be in a temperature range of 224~227°C.

(2) Chlorides : When 2 g of Inositol is tested for chlorides, the content should be equal to or less than the amount that corresponds to 0.3 ml of 0.01 N hydrochloric acid.

(3) Sulfates : When 4 g of Inositol is tested for sulfates, the content should be equal to or less than the amount that corresponds to 0.5 ml of 0.01 N sulfuric acid.

(4) Calcium : 1 g of Inositol is dissolved in 10 ml of water and 1 ml of ammonium hydroxide solution is added. The resultant solution remains clear for at least 1 minute.

(5) Arsenic : Arsenic : 0.5 g of Inositol transfer into a platinum, quartz, or porcelain crucible, add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) and then

alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with a small amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test by Arsenic Limit Test is carried out with this test solution, it should not be more than 2 ppm.

- (6) Lead : When 5.0 g of Inositol is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 4.0 ppm.

Loss on Drying When Inositol is dried for 4 hours at 105°C, the loss should not be more than 0.5%.

Residue on Ignition After drying at 105°C for 4 hours, it is tested by Residue on Ignition, the amount of residue should not be more than 0.1%.

Assay Inositol is dried for 4 hours at 105°C. Approximately 0.2 g of Inositol is precisely weighed into a 250 ml beaker, where a mixture of 1 ml dilute sulfuric acid and 50 ml anhydrous acetic acid is added. The beaker is covered with a watch glass and heated for 20 minutes in a water bath. It is then chilled in a ice bath. 100 ml of water is added to the beaker, which is boiled for 20 minutes. After cooling, the solution is transferred into a separatory funnel. The beaker is washed with a small amount of water, which is added to the funnel. The beaker is washed with six successive 30 ml, 25 ml, 20 ml, 15 ml, 10 ml, and 5 ml of chloroform, then extracted, combined all chloroform extracts, and washed with 10 ml of water. The extract is then filtered through a pledget of cotton, which is washed with 10 ml of chloroform. The filtrate and the washings are combined and evaporated to dryness in a water bath. The residue is dried of 4 hours at 105°C. After cooling, the residue is weighed to obtain the amount of hexaacetyl inositol ($C_{18}H_{24}O_{12}$). The content of inositol is calculated by the following equation

$$\text{Weight of Inositol } (C_6H_{12}O_6) \text{ (mg)} = \text{Weight of Hexaacetyl Inositol } (C_{18}H_{24}O_{12}) \text{ (mg)} \times 0.4167$$

49. Invertase

Definition Invertase is an enzyme that hydrolyzes sucrose. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Invertase

Content Invertase contains 90~130% of the indicated activity as invertase.

Description Invertase is white~pale yellow~brown powder or transparent~brown liquid.

Purity (1) Arsenic : 0.25 g of Invertase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Invertase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Cadmium : When 5.0 g of Invertase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 0.5 ppm.

(4) Coliform Group : Invertase is tested by Microbe Test Methods for Coliform Group in General Test Methods in Food Code. It should contain 30 colonies or less per 1g of this product.

(5) Salmonella : Invertase is tested by Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).

(6) Total Viable Aerobic Count : When Invertase is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 50,000 colonies per 1 g.

(7) E. Coli : When Invertase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Assay (Activity)

◦ Application and Principle : This test is to measure the activity of invertase. Activity

test is based on hydrolysis of sucrose (30 minutes, pH 4.5, 20°C). The degree of hydrolysis is measured by optical rotation of the solution using a polarimeter.

- Preparation of Test Solution : The test solution is prepared to obtain the 10 ml of final diluted solution for which the measured specific rotation using 2 dm cell will fall within 0~+20. When the sample is solid, it is ground in a mortar adding 5 times or more amount of water. This is transferred into a volumetric flask, which is then diluted to a proper concentration. Liquid sample is directly diluted with water.
- Test Procedure : 100 ml of substrate solution is placed in a 100~110 ml flask and equilibrated at $20 \pm 0.1^\circ\text{C}$ for 15 minutes in a water bath. Precisely 10 ml of Test Solution is added to the flask, which is turned upside down 5~6 times to ensure thorough mix and allowed to stand for 30 minutes in a water bath. If there are significant amount of insoluble substances, the flask is shaken every 10 minutes and mixed. When the time is up, approximately 2 g of sodium carbonate (1 hydrate) is added and dissolved by shaking. If the solution is not alkaline, sodium carbonate is added to alkalize the solution. 5 ml of this solution is transferred into a 100 ml volumetric flask, where 6 drops of neutral lead acetate solution are added and water is added to bring the total volume to 100 ml. 3 g of filtering aid such as cellulose type agglomeration agent is added to the solution, which is then filtered through Whatman No.1 filter paper. First 3 ml of filtrate is discarded. The filtrate should be completely clear. A blank test is carried out with 100 ml of water containing 10 ml of Test Solution by processing as the same as the enzyme digesting solution. Polarimeter tube is washed 3 times with the Test Solution and filled with Test Solution. It is then set up in the polarimeter and a thermometer (10~30°C range with 0.1°C scale) is inserted. It is then allowed to be equilibrated at 20°C. Each of solution is measured 5 times and measured value is averaged. The complete sample value is obtained by subtracting blank value from sample value. Polarimeter tube should be used Path length of 2dm. If 1 dm path length is used, it should be corrected. A standard curve is prepared by using the values of activity and specific rotation (in Ventzke degree : °Ventzke).

(note : specific rotation = °Ventzke \times 0.346).

Activity	Polarization Reading
0.960	0
0.735	+ 5
0.570	+ 10
0.420	+ 15
0.300	+ 20
0.190	+ 25
0.090	+ 30

Activity (A) of Test Solution is obtained from standard curve by interpolation. When the Test Solution is measured at 20°C or higher, 0.004 is subtracted from the activity per 1°C. When the Test Solution is measured at 20°C or lower, 0.004 is added to the activity per 1°C.

The activity of the enzyme is calculated using the following equation.

$$\text{IA, units/g} = A \times 2 \times \frac{1,000}{W}$$

2 : dilution factor

1,000 : conversion factor (mg to g)

W : Weight of sample in 10 ml of Test Solution (mg)

Definition of activity : 1 invertase unit corresponds to the amount of enzyme that hydrolyzes 77% of sucrose used in the above test conditions.

Solutions

- Phosphate Buffer Solutions : 115 g of sodium phosphate, monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) is dissolved in water and make 500 ml.
- Substrate Solution : 100 g of sucrose is dissolved in 300 ml of water, where 20 ml of phosphate buffer solution is added. The total volume of the solution is brought up to 1,000 ml with water.
- Neutral Lead Acetate Solution : 31 g of lead acetate ($\text{C}_4\text{H}_6\text{PbO}_4 \cdot 3\text{H}_2\text{O}$) is dissolved in 50 ml of water. pH of the solution is adjusted to 7.0 with sodium hydroxide solution and the total volume is brought up to 80 ml with water. The solution is filtered through a Whatman No.1 filter paper or its equivalent. The filtrate is stored in a container with a stopper.

Storage Standard of Invertase

Invertase is strongly hygroscopic, hence should be stored sealing tightly in a cold dark place.

50. Red Cabbage Color

Definition Red Cabbage Color is a pigment obtained by extracting red cabbage leaves (*Brassica oleracea* Linné) with slightly acidic solution. The major component is cyanidin acylglycoside. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Red Cabbage Color

Content Color value ($[E]_{1cm}^{10\%}$) of Red Cabbage Color should be higher than the indicated value.

Description Red Cabbage Color is deep red liquid, powder, or paste having a slight characteristic odor.

Identification (1) The Test Solution obtained in Color Value section shows red color and a absorption maximum at about 536 nm.

(2) When Test Solution in (1) is alkalinized by adding sodium hydroxide solution, colour changed deep green.

Purity (1) Arsenic : 0.25 g of Red Cabbage Color is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450 ~ 550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Red Cabbage Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 8 ppm.

Assay (Color Value) Appropriate amount of Red Cabbage Color is precisely weighed so that the absorbance will fall within 0.3~0.7 and dissolved citric acid · dibasic sodium phosphate buffer solution with pH 3.0 to make 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citric acid · dibasic sodium phosphate buffer solution with pH 3.0 as a reference solution, absorbance maximum A is measured at 536 nm with 1cm cell. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citric acid·dibasic sodium phosphate buffer solution (pH 3.0)

Solution 1 : 0.1M citric acid solution : 1 L of solution containing 21.01g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2M dibasic sodium phosphate solution : 1 L of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (159:41) and its pH is adjusted to 3.0.

51. Gelatin

Definition Gelatin is the product obtained from partial hydrolysis of collagen, the chief protein component of the bones and skins of animal. If it is prepared by treating collagen with acid processing, isoelectric point exhibited pH 7.0~9.0. If it is prepared by treating collagen with alkali processing, isoelectric point exhibited pH 4.6~5.2. If it is a mixture treated by both acid and alkali as well as Gelatin is produced by modification of the above mentioned process may exhibited isoelectric points outside of the stated ranges.

Compositional Specifications of Gelatin

Description Gelatin is colorless~faintly yellow plate, piece, or powder with a slight odor and taste of gravy. It is stable in dry air. It decomposes in damp air or solution by microbes. It is insoluble in cold water, but it swells slowly and softens to absorb 5~10 times its volume of water. It is soluble in hot water, acetic acid, or hot mixture of water and glycerin. It is almost insoluble in ethyl alcohol, ether, chloroform, fatty oil, or refined oil.

Identification (1) When chromium trioxide solution or picric acid solution is added to 5 ml of aqueous solution (1→100) of Gelatin, precipitates are formed.
(2) When tannic acid solution is added to 5 ml of aqueous solution (1→5,000) of Gelatin, it becomes turbid.

Purity (1) Other odor and Insoluble substances : A hot solution (1→40) of Gelatin should not generate unpleasant odor. The 2 cm liquid layer of this solution should be colorless and transparent. Even if it is turbid, it should not be deeper than the color of 50 ml of solution that is prepared by mixing 0.3 ml hydrochloric acid and 1 ml nitric acid adding 1 ml of 0.1 N silver nitrate solution and water, and allow to stand for 5 minutes.
(2) Sulfites : 20 g of Gelatin is dissolved in 150 ml of boiling water in a round bottom flask. 5 ml of phosphoric acid and 1 g of sodium bicarbonate are added and a condenser is attached. 50 ml of 0.1 N iodine solution is added to a collecting container. The end of the condenser is immersed in the iodine solution. It is then distilled and approximately 50 ml of distillate is collected. The distillate is acidified with 2~3 drops of hydrochloric acid. 2 ml of barium chloride solution is added to the distillate, which is then heated until it becomes colorless. The precipitates of barium sulfate are filtered and washed with water. After heat treatment, the remaining residue should not be more than 3 mg. Separately, a blank test is carried out by the same procedure.

- (3) Arsenic : 10 g of Gelatin is weighed into a flask, where 60 ml of dilute hydrochloric acid (1→4) is added. A stopper is placed on the flask, which is then heated to dissolve it. Approximately 15 ml of bromine solution is added to the solution, which is then heated to remove excess amount of bromine. It is then neutralized with ammonia solution. After adding 1.5 g of sodium phosphate, the solution is cooled. 30 ml of magnesia solution is added to the solution, which is then allowed to stand for about 1 hour. Resulting precipitates are collected by filtration and washed 5 times with 10 ml of a 3:1(v/v) mixture of ammonia solution : water. The precipitates are dissolved in dilute hydrochloric acid (1→4) to make 50 ml. 5 ml of the resulting solution is tested for arsenic, it should not be more than 1ppm.
- (4) Chromium : 5 g of gelatin is placed in decomposition flask. 50 ml of water and 10 ml of nitric acid are added and mixed to the flask and the solution in the flask is allowed to stand. The solution is mildly heated and it is cooled after stopping vigorous reaction. Then, 5 ml of sulfuric acid is added to the solution and the solution is mildly heated again. Add 2 ~ 3 ml of nitric acid to it when the content of the solution appears dark brown color. Heat continually it until the content of the solution appear light yellow ~ colorless, which means to finish the decomposition of the solution. After cooling the decomposition solution, add water to the solution and make up 50 ml as a test solution. Make blank test solution to the same procedure. Separately, take 20 ml of chrome standard stock solution(1000 ppm) and make up to 200 ml with 0.2 % of nitric acid solution. Then take again 20 ml in this solution and make up to 200 ml with 0.2 % of nitric acid solution. The concentration of this diluted solution is 10 $\mu\text{g/ml}$ (10 ppm). Take each of 1 ml and 5 ml from the diluted solution and each taken solution is made up to 10 ml with 0.2 % of nitric acid solution(1, 5, 10ppm). The amount of test solution and each of standard solution should not be more than 10 ppm when testing according to the saltless process of automatic absorption spectrophotometry.
- (5) Lead : When 5.0 g of Gelatin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.5 ppm.
- (6) Total Viable Aerobic Count : When Gelatin is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Food Code」, it should not be more than 1,000 colonies per 1 g
- (7) Salmonella : Gelatin is tested by Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).

(8) E. Coli : When Gelatin is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Residue on Ignition When Residue on Ignition analysis is done with 1 g of Gelatin, the amount of residue should not be more than 2%.

52. Gellan Gum

Definition Gellan Gum is a high molecular weight polysaccharide gum produced by pure culture and fermentation of a carbohydrates by *Pseudomonas elodea*, purified by recovery with isopropyl alcohol, dried, and milled. Heteropolysaccharide is principally composed of rhamnose, gluconic acid, and glucose (1:1:2). It can also contain acyl (glyceryl and acetyl) group as an O-glycosidically linked ester.

Compositional Specifications of Gellan Gum

Content Gellan Gum (on a dried basis) contains not less than 3.3 and not more than 6.8% of carbon dioxide (CO₂).

Description Gellan Gum is off-white powder.

Identification (1) 1 g of Gellan Gum is hydrated with 99 ml of water (1% solution). It is stirred for about 2 hours using a magnetic stirrer. Small amount of supernatant is drawn into a wide mouth pipette and transferred into a 10% calcium chloride solution. A tough worm-like gel will be formed immediately.

(2) 0.5 g of sodium chloride is added to 1% solution obtained in (1), which is heated to 80°C with stirring, and hold at 80°C for 1 minute. The solution is allowed to cool to room temperature. A firm gel is formed.

Purity (1) Arsenic : 0.25 g of Gellan Gum is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Gellan Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Gellan Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Gellan Gum is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Isopropyl alcohol : 5 g of Gellan Gum is precisely weighed into a 1,000 ml single neck round bottom distilling flask with 24/40 ground joint, where 1 ml of anti

foaming agent (Dow-Corning G 10 or its equivalent) and 200 ml of water are added. It is then stirred for 1 hour. A 400 ml reflux condenser, distilling head, and a collector are attached. Approximately 95 ml of distillate is collected (care must be taken so that bubbles does not enter into the collector. 4 ml of internal standard solution is added to the collected distillate, where water is added to bring the total volume to 100 ml, test solution. Test Solution and mixed standard solution are analyzed with gas chromatography and the amount of isopropyl alcohol is obtained by the following equation. The content should not be more than 750ppm. The response factor (f) is calculated by the area ratio(A_{IPA}/A_{TDA}) between peak areas of isopropyl alcohol and tert-butyl alcohol in the mixed standard solution.

$$\text{Content of isopropyl alcohol (ppm)} = \frac{a_{IPA} \times 4,000}{f \times a_{TBA} \times \text{Weight of sample (g)}}$$

aIPA : peak area of isopropyl alcohol in Test Solution

aTBA : peak area of tert-butyl alcohol in Test Solution

Operation Conditions

- Column : A stainless steel tube 3.2mm x 1.8m
- Column Filler : 80~100 Porapak QS (or its equivalent)
- Detector : (Hydrogen) Flame Ionization Detector (FID)
- Injection port temperature : 200°C
- Column Temperature : 165°C
- Detector Temperature : 200°C
- Carrier gas and flow rate : Nitrogen, Flow rate is controlled so that the retention time of isopropyl alcohol and tert-butyl alcohol is about 2 minutes and 3 minutes, respectively.

Solutions

- Mixed Standard Solution : 4 ml each of IPA standard solution and TBA standard solution is pipetted into a flask, and diluted to 100 ml with water. 1 ml of this solution contains approximately 40 µg each of isopropyl alcohol and tert-butyl alcohol per ml.
- IPA Standard Solution : Approximately 500 mg of isopropyl alcohol (chromatographic quality) is precisely weighed and diluted to 50 ml with water. 10 ml of this solution is further diluted to 100 ml with water.
- TBA Standard Solution : Approximately 500 mg of tert-butyl alcohol (chromatographic

quality) is precisely weighed and diluted to 50 ml with water. 10 ml of this solution is further diluted to 100 ml with water.

- (6) Nitrogen : When Gellan Gum is tested by Kjeldahl Nitrogen Test in nitrogen determination method, the amount should be not more than 3.0%.
- (7) Total Viable Aerobic Count : When Gellan Gum is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 10,000 cfu per 1 g
- (8) E. Coli : When Gellan Gum is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (9) Salmonella : When Gellan Gum is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (10) Number of Fungi : When Gellan Gum is tested by Microbe Test Methods for Number of Fungi in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 400 cfu per 1 g

Loss on Drying When Gellan Gum is dried for 2 hours and 30 minutes at 105°C, the loss should not be more than 15%.

Ash 3 g of Gellan Gum is precisely weighed and dried for 4 hours at 105°C. It is then reduced ash at 650°C until the carbon is completely removed. The amount of remaining ash should not be more than 4~12%.

Assay Approximately 1.2 g of Gellan Gum is precisely weighed and analyzed by Assay of Xantan Gum

$$0.25 \text{ N NaOH } 1 \text{ ml} = 5.5 \text{ mg CO}_2$$

53. Seed Malt

Definition There are crude seed malt and powdered seed malt. Crude seed malt is obtained from a culture where starter of *Aspergillus kawachii*, *Aspergillus oryzae*, *Aspergillus usamii*, *Aspergillus shiroyamii*, *Aspergillus awamori* or *Rhizopus* genus are separately or mixedly inoculated so that spores are inserted into a pasteurized raw material containing starch. Powdered seed malt is obtained by collecting pure spawn spores by a special method.

Compositional Specifications of Seed Malt

Description Seed Malt is yellow~blackish brown or yellow~green powder or granule having a slight characteristic odor.

Purity (1) Arsenic : 0.5 g of Seed Malt is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 2ppm.

(2) Lead : When 5.0 g of Seed Malt is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

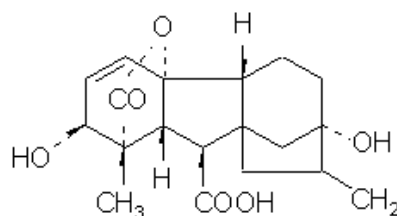
(3) Number of Spores : 1~2 g of Seed Malt is precisely weighed and mixed with 10 ml of 5% Tween 80 solution and 2~3 drops of 1% methylene blue solution, which is analyzed 5 times with blood cell counter and microscope. An average value of 5 times measurements is A, and the number of spores is calculated from the following equation. For crude seed malt, there should be 40×10^8 , 20×10^8 , 10×10^8 , 10×10^8 , and 10×10^8 or more starters per g for *Aspergillus kawachii*, *Aspergillus oryzae*, *Aspergillus usamii*, *Aspergillus shiroyamii*, and *Aspergillus awamori*, respectively (0 for *Rhizopus* genus). For powdered seed malt, there should be 200×10^8 , 100×10^8 , 50×10^8 , 50×10^8 , and 50×10^8 or more starters per g for *Aspergillus kawachii*, *Aspergillus oryzae*, *Aspergillus usamii*, *Aspergillus shiroyamii*, and *Aspergillus awamori*, respectively (0 for *Rhizopus* genus).

$$\text{Number of Spores} = \frac{A \times \text{dilution factor}}{\text{Weight of sample (g)}}$$

(4) Various Germs (*Penicillium* Genus) : 0.15~0.2 g of Seed Malt is cultured in pre-pasteurized liquid culture medium (55 ml of water, 0.025 g of mono potassium phosphate, and 1 g of dextrin are added to a 300 ml Erlenmeyer flask, which is plugged with cotton and pasteurized for 20 minutes under high pressure of 15 psi) for 5 days in a 30°C thermostat. When it is observed under microscope, test result for various germs (*Penicillium* genus) should be negative. If various germs(*Penicillium* genus) is observed (cultured for 5 hours as described above), it is positive. If not, it is cultured for additional 24 hours. If *penicillium* is observed, it is positive. If not, it is negative.

Loss on Drying When 5 g of Seed Malt is dried for 4 hours at 105°C, the loss should not be more than 10% for crude seed malt and more than 8% for powdered seed malt.

54. Gibberellic Acid



Chemical Formula $C_{19}H_{22}O_6$

Molecular Weight 346.37

Definition *Gibberella fujikuroi* is cultured, which is then filtered. The filtrate is concentrated under a reduced pressure. The concentrate is extracted and crystallized. Gibberellic Acid is obtained by purifying the crystallized precipitates.

Compositional Specifications of Gibberellic Acid

Content Gibberellic Acid should contain not less than 90.0% of gibberellic acid ($C_{19}H_{22}O_6 = 346.37$).

Description Gibberellic Acid is odorless white~pale yellow crystalline powder.

Identification A solution that dissolved in 2 ml of sulfuric acid a few milligrams of Gibberellic Acid is red color with green fluorescence.

Purity (1) Specific Rotation : Approximately 5 g of Gibberellic Acid is precisely weighed and dissolved in alcohol to make 50 ml. (This solution should not be heated during preparation.) The polarity of this solution should be $[\alpha]_D^{20} = +75.0 \sim +90.0^\circ$

(2) Arsenic : 0.25 g of Gibberellic Acid is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then ethanol is ignited. It is then reduced to ash by heating at $450 \sim 550^\circ\text{C}$. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at $450 \sim 550^\circ\text{C}$. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(3) Lead : When 5.0 g of Gibberellic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

Loss on Drying When Gibberellic Acid is dried at 100°C for 7 hours in vacuum of 20 mmHg, the loss should not be more than 3%.

Assay Approximately 40 mg is precisely weighed and dissolved in methyl alcohol (total volume = 50 ml). 10 ml of this solution is diluted to 100 ml with methyl alcohol (Test Solution). Separately, 25 mg of gibberellic acid standard is precisely weighed and dissolved in methyl alcohol (total volume = 50 ml). 10 ml of this solution is diluted to 50 ml with methyl alcohol (Standard Solution). Each of 5 ml Test Solution, 4 ml and 5 ml portions of the Standard Solution is separately added into 3 test tubes. The test tubes are evaporated to dryness and further dried at 90°C for 10 minutes. The tubes are allowed to cool to room temperature. The residue in each test tube is dissolved in 10 ml each of diluted sulfuric acid (8→10), which is then heated for 10 minutes in a water bath and cooled for 5 minutes in 10°C water bath. Absorbance of each solution is determined at 535 nm with 1 cm cells using dilute sulfuric acid as the blank. The content is measured using the following equation (Note the absorbance of the two solutions prepared from the 4 ml and 5 ml aliquots of the Standard Solution and use the nearest one to the solution prepared with the Sample solution, for the calculation).

$$\text{Content (\%)} = 500 \times C \times \frac{V}{5} \times \frac{A_u}{A_s} \times \frac{100}{\text{Weight of sample (mg)}}$$

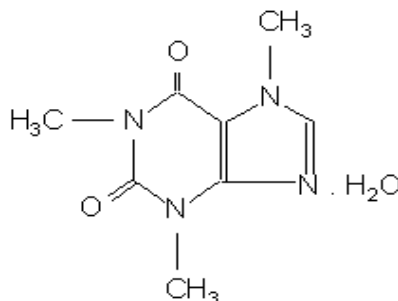
C : Concentration of the Standard Solution (mg/ml)

V : Amount of Standard Solution used

A_u : Absorbance of the Sample Solution

A_s : Absorbance of Standard Solution used

55. Caffeine



Chemical Formula $C_8H_{10}N_4O_2 \cdot nH_2O$ ($n=1$ and 0)

Molecular Weight hydrate : 212.21, anhydrous : 194.19

Definition Coffee beans (*Coffea arabica* LINNE) of rubiaceae or tea leaves (*Camellia sinensis* O. KZE) of camellia family are extracted with water or carbon dioxide. The extracts are separated and purified to obtain Caffeine. The major component is caffeine.

Compositional Specifications of Natural Caffeine

Content If Caffeine is converted to an anhydrous form, it should contain 98.5~101.0% of caffeine ($C_8H_{10}N_4O_2$).

Description Caffeine is odorless white crystalline powder having a bitter taste.

Identification (1) When small amount of tannic acid solution is drop-wise added to 2 ml of aqueous solution (1→500) of Caffeine, white precipitates are formed. When excess amount of this tannic acid is added, the precipitates are dissolved.

(2) 10 drops of hydrogen peroxide solution and 1 drop of hydrochloric acid are added to 0.01 g of Caffeine, which is then evaporated to dryness in a water bath. When the residue is exposed to ammonia gas, it acquires a purple colour. This color disappears when 2~3 drops of sodium hydroxide solution are added.

(3) 30 ml of 2% aqueous solution of Caffeine is placed in a Nestler tube. When a long wavelength UV lamp is shone from the side and the solution is observed from the top, it should not emit strong violet color. Natural caffeine should show no or slight yellowish green fluorescence.

(4) Theophylline and 8-chlorotheophylline : 0.01 g of Caffeine is dissolved in 5 ml of water, where 3 ml of ammonium chloride buffer solution (pH 8.0) and 1 ml of copper sulfate solution in pyridine are added and mixed. When 5 ml of chloroform is added and shaken, the chloroform phase should not turn green.

Purity (1) Melting Point : Caffeine is dried for 4 hours at 80°C. The melting point of this dried material should be 235~237.5°C.

- (2) Arsenic : 0.25 g of Caffeine is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
- (3) Lead : When 5.0 g of Caffeine is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.
- (4) Other Alkaloids : 20 mg of Caffeine is dissolved in 1 ml of water. When 2~3 drops of Meyer solution are added to this solution, it should not form precipitates.
- (5) Readily Carbonizable Substances : 0.5 g of Caffeine is dissolved in 5 ml of sulfuric acid, which is allowed to stand for 15 minutes. When the color of the solution is observed with a white background, the color should not be deeper than that of the color standard D.
- (6) Chloride: 2 g of Caffeine is dissolved in 80 ml of hot water and cooled quickly at 20°C. Then add water to make to 100ml and this is used as the test solution. The content should not be more than that amount corresponds to 0.25 ml of 0.01 N hydrochloric acid under Chloride Test. (it should not be more than 0.011%.)
- (7) Sulfate : When the test solution 40 ml of Purity (6) is tested for sulfates, the content should not be more than the amount that corresponds to 0.40 ml of 0.01 N sulfuric acid. (it should not be more than 0.024%.)
- (8) Caffeine-like substances: 0.1 g of caffeine is weighted and dissolved in 10 ml of Chloroform, which is used as test solution. And then Chloroform is added to 1 ml of test solution to make to 100 ml. Again, take 1 ml of this solution and make to 10 ml, it is used as standard solution. Take 10 μ l of test solution and standard solution, and spot respectively them to the prepared thin layer plate. The mixed solution of Chloroform:95% alcohol solution(9:1) is used as the solvent. When the solvent is developed up to 10 cm from base line, stop developing and air-dry the plate. The plate is observed under a UV light (wavelength 254nm). The spot except for a major spot which is obtained from the test solution should not thicker than a spot(this spot is obtained from the standard solution).

Water Content Water content of Caffeine is determined by direct titration method in water determination (Karl-Fisher Method) and should be 0.5% and 8.5% for anhydrous

form and mono hydrated form, respectively.

Residue on Ignition Residue on ignition should not be more than 0.1%.

Assay Approximately 0.4 g of Caffeine is precisely weighed and dissolved in 40 ml of anhydrous acetic acid with warming, and cooled. After 80 ml of benzene is added to the solution, which is then titrated with 0.1 N perchloric acid solution (indicator : 3 drops of crystal violet ·acetic acid solution). The end point is where the solution turns from violet to green and finally to yellow. Separately, a blank test is carried out by the same method.

0.1 N perchloric acid solution 1 ml = 19.42 mg $\text{C}_8\text{H}_{10}\text{N}_4\text{O}_2$

Storage Standard of Natural Caffeine

Caffeine should be stored in a light shielded well-closed container in a cool place.

56. Gardenia Red

Definition Gardenia Red is a pigment obtained by enzyme treating (with β -glucosidase, an enzyme used for food) on a mixture of hydrolyzed matter and decomposed protein from iridoid glycoside contained in fruit extract of gardeniae (*Gardenia augusta* Merrill or *Gardenia jasminoides* Ellis) of rubiaceae. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Gardenia Red

Content Color value ($[E]_{1cm}^{10\%}$) of Gardenia Red should be higher than the indicated value.

Description Gardenia Red is dark reddish violet liquid, lump, powder or paste having a slight characteristic odor.

Identification (1) Test Solution obtained in Color Value section shows reddish violet color and a absorbance maximum at about 535 nm.

(2) When pH of Test Solution in (1) is adjusted to 2.5 or less with dilute hydrochloric acid, its color almost doesn't change.

(3) When pH of Test Solution in (1) is adjusted to 2.0 or less with dilute hydrochloric acid, where 3 drops of sodium hypochlorite (effective chlorine should not be less than 4%), the solution decolorizes rapidly but doesn't form precipitates.

Purity (1) Arsenic : 0.25 g of Gardenia Red is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Gardenia Red is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 8 ppm.

Assay (Color Value) Appropriate amount of Gardenia Red is precisely weighed so that the absorbance is within 0.3~0.7 and dissolved in citric acid-dibasic sodium phosphate buffer solution with pH 4.0 so that the total volume is 100 ml. 1 ml of this solution is diluted to 100 ml with citric acid·dibasic sodium phosphate buffer solution with pH of 4.0 (Test Solution). If necessary, the solution is centrifuged and the

supernatant is used. Using citric acid–dibasic sodium phosphate buffer solution with pH 4.0 as a reference solution, absorbance A is measured at 535 nm wavelength with 1cm cell. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

◦ Citric acid–dibasic sodium phosphate buffer solution (pH 4.0)

Solution 1 : 0.1M citric acid solution : 1 ℓ of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2M dibasic sodium phosphate solution : 1 ℓ of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (123:77) and its pH is adjusted to 4.0.

57. Gardenia Blue

Definition Gardenia Blue is a pigment obtained by enzyme treating (with β -glucosidase, an enzyme used for food) on a mixture of hydrolyzed matter and decomposed protein from iridoid glycoside contained in fruit extract of gardeniae (*Gardenia augusta* Merrill or *Gardenia jasminoides* Ellis) of rubiaceae. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Gardenia Blue

Content Color value ($[E]_{1cm}^{10\%}$) of Gardenia Blue should be higher than the indicated value.

Description Gardenia Blue is deep blue liquid, lump, powder, or paste having a slight characteristic odor.

Identification (1) Test Solution obtained in Color Value section shows blue color and a absorption maximum at about 595 nm.

(2) The colour of test solution is blue. When Test Solution in (1) is alkalinized with a few drops of 1N sodium hydroxide solution, its color almost doesn't change.

(3) When Test Solution in (1) is acidified with a few drops of 1N hydrochloric acid, where 1~3 drops of sodium hypochlorite (effective chlorine should not be less than 4%), the solution decolorizes rapidly.

Purity (1) Arsenic : 0.25 g of Gardenia Blue is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Gardenia Blue is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Residual Solvent : When Gardenia Blue is tested by Purity (5) for 「Paprika Extract Pigments」, residual methanol should not be more than 0.1% (based on the product whose color value is 40).

Assay (Color Value)

Appropriate amount of Gardenia Blue is precisely weighed so that the absorbance is

within 0.3~0.7 and dissolved in citric acid dibasic sodium phosphate buffer solution with pH 6.0 so that the total volume is 100 ml. 1 ml of this solution is diluted to 100 ml with citric acid dibasic sodium phosphate buffer solution with pH 6.0 (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citric acid-dibasic sodium phosphate buffer solution with pH 6.0 as a reference solution, absorbance A is measured at 595 nm with 1cm cell. Color value is obtained using the following equation.

$$\text{Color value } ([E]_{1\text{cm}}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

◦ Citric acid·dibasic sodium phosphate buffer solution (pH 6.0)

Solution 1 : 0.1 M citric acid solution : 1 L of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2 M dibasic sodium phosphate solution : 1 L of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (73.7:126.3) and its pH is adjusted to 6.0.

58. Gardenia Yellow

Definition Gardenia Yellow is a pigment obtained by extracting and hydrolyzing fruit of gardeniae (*Gardenia augusta* Merrill or *Gardenia jasminoides* Ellis) of rubiaceae. Major pigments are crocin and crocetin of carotinoids. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Gardenia Yellow

Content Color value ($[E]_{1cm}^{10\%}$) of Gardenia Yellow should be higher than the indicated value.

Description Gardenia Yellow is yellow~orange yellowish red liquid, lump, powder or paste having a slight characteristic odor.

Identification (1) Test Solution obtained in Color Value section shows yellow color and a absorption maximum at about 440 nm or 420 nm.

(2) When 5 ml of sulfuric acid is added to 0.5 g of Gardenia Yellow (if necessary, evaporated to dryness by heating in a water bath and then cooled prior to use), it shows blue color, which changes to violet and then brown.

Purity (1) Arsenic : 0.25 g of Gardenia Yellow is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Gardenia Yellow is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay (Color Value) Appropriate amount of Gardenia Yellow is precisely weighed so that the absorbance is within 0.3~0.7 and dissolved in 50 v/v% alcohol (total volume 100 ml). 1 ml of this solution is diluted to 100 ml with 50 v/v% alcohol (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using 50v/v% alcohol as a blank solution, absorbance A is measured at the absorption maximum at about 440 nm or 420 nm with 1cm cell. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

59. Carnauba Wax

Definition Carnauba Wax is the refined wax obtained from leaves and shoots of the Brazilian tropical palm tree (*Copernicia cereferia* Mart).

Compositional Specifications of Carnauba Wax

Description Carnauba Wax is pale yellow~light brown powder, thin platelet, or hard and fragile lump.

- Purity**
- (1) Arsenic : 0.25 g of Carnauba Wax is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
 - (2) Lead : When 5.0 g of Carnauba Wax is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.
 - (3) Mercury : When 0.1 g of Carnauba Wax is tested by Mercury Test Method, its content should not be more than 1.0ppm.
 - (4) Melting Point : Melting point should be in a temperature range of 80~86°C.
 - (5) Acid value : 3 g of Carnauba Wax is precisely weighed into a 200 ml Erlenmeyer flask, where 25 ml of anhydrous alcohol (neutralized with potassium hydroxide solution using phenolphthalein solution as an indicator) is added. It is then heated until the sample dissolves, test solution. When the sample solution is tested Acid Value as directed under Acid Value in Oils and Fats Method, Acid Value should be 2~7.
 - (6) Ester Value : Ester value is obtained by Fatty Acid method, the value should be 70~80.
 - (7) Saponification Value : 5 g of Carnauba Wax is precisely weighed into a flask, where 50 ml of 0.5 N alcoholic solution of potassium hydroxide is added. After attaching a reflux condenser, the solution is gently saponified for 30 minutes~ 1 hour. The solution is proceeded as directed under Saponification Value in Oils and Fats Test, the value should be 78~95.
 - (8) Unsaponifiable matter : 5 g of Carnauba Wax is precisely weighed into a 250 ml flask, where 2 g of potassium hydroxide and 40 ml of alcohol are added and boiled

gently under refluxed for 1 hour with a reflux condenser. The content of flask is transferred to a glass-stoppered extraction cylinder (approximately 30 cm in length, 3.5 cm in diameter and graduated at 40, 80 and 130 ml). The flask is washed with sufficient alcohol to achieve a volume of 40 ml in the cylinder, and completed the transfer with warm and then cold water until the total volume is 80 ml. Finally, the flask is washed with a few ml of petroleum ether, which the washings is added to the cylinder. After cooling to room temperature, 50 ml of petroleum ether is added to the funnel. The funnel is shaken vigorously for at least 1 minute, and allowed both layers to become clear. The supernatant ether layer is collected in a 500 ml separatory funnel with a stopcock. The aqueous layer is repeated extraction at least 6 times with 50 ml portions of petroleum ether. These extracts are added to the first extract. The combined extracts are washed with 25 ml portions of 10% alcohol until the wash water is neutral to phenolphthalein, and discarded the washings. The ether extract is transferred to a tared beaker. With 10 ml of ether, the funnel is rinsed, which is added to the beaker. Ether layer is evaporated to dryness in a water bath, which is then dried at 100°C for 30 minutes to constant weight. Then the residue is cooled in a desiccator and weighed. The residue is dissolved in 50 ml of warm neutral alcohol and titrated with 0.02 N sodium hydroxide using phenolphthalein as an indicator. Each ml of 0.02 N sodium hydroxide is equivalent to 5.659 mg of fatty acid, calculated as oleic acid. The corrected weight of unsaponifiable matter is obtained by subtracting the calculated weight of fatty acid from the weight of the residues. The content of Unsaponifiable matter is calculated by the following equation and it should be 50~55%.

$$\text{Unsaponifiable matter (\%)} = \frac{\text{Amount of residue(mg)} - \text{amount of oleic acid(mg)}}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

Residue on Ignition When Residue on Ignition is done with 2 g of Carnauba Wax in a quartz or platinum crucible, the residue should not be more than 0.25%.

60. Carrageenan

Purified Carrageenan

Refined Carrageenan

Definition Carrageenan is obtained by extration and purification from seaweed of *Chondrus* genus, *Eucheuma* genus, *Gigartina* genus, *Hypnea* genus, and *Iridaea* genus into water or aqueous dilute alkali. The prevalent polysaccharides in carrageena are designated as ι(Iota)-Carrageenan, κ(Kappa)-Carrageenan, and λ (Lambda)-Carrageenan.

Compositional Specifications of Carrageenan

Description Carrageenan is white~pale brown powder or granula. It is either odorless or has a slight particular odor.

Identification (1) 4 g of Carrageenan is add to 200 ml of water, and heated the mixture in a water bath at 80°C, with constant stirring, until dissolved. Any water lost by evaporation is replaced, and allowed the solution to cool to room temperature. It becomes viscous and may form gel.

(2) 0.2 g of potassium chloride is added to 50 ml of the solution or gel obtained in (1), then , mixed well, and cooled. A compliant (elastic) gel indicates a carrageenan of predominantly ι-carrageenan, a short-textured(brittle) gel indicates a predominantly κ-carrageenan. If the solution doesn't gel, the carrageenan is of a predominantly λ-carrageenan.

(3) 20 ml of water is added to 0.1 g of Carrageenan, where 3 ml of barium chloride solution (3→25) and 5 ml of diluted hydrochloric acid (3→25) are added and mixed well. If necessary, precipitates are separated. When the separated solution is boiled for 5 minutes, while crystalline precipitates are appeared.

Purity (1) Arsenic : 0.25 g of Carrageenan is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Carrageenan is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

- (3) Acid Insoluble Ash : 3 g of Carrageenan is tested by the test method for Ash. When it is converted to a dried basis, the content of ash should not be more than 1.0%.
- (4) Sulfate : Carrageenan is dried for 4 hours at 105°C. Approximately 1 g of the dried basis is precisely weighed into a 100 ml round bottom flask, where 50 ml of dilute hydrochloric acid (1→10), a reflux condenser is fitted and reflux for 1 hour. 25 ml of 10 %(by volume) hydrogen peroxide is added to the flask, and resumed refluxing for about 5 hours. If necessary, the solution is filtered. The filtrate is transferred into a 500 ml beaker. While boiling, 10 ml of barium chloride solution (3→25) is slowly added to the beaker, which is heated for 2 hours in a water bath. After cooling, it is filtered through a quantitative filter paper (5 type C). The residue is washed with warm water until the filtrate is free from chloride. The filter paper and residue is dried in a drying oven. It is gently burned and heat at 800°C ash paper in a tared porcelain crucible until the ash is white. The remaining residue is weighed as barium sulfate. The weight of sulfate (SO₄) is calculated by the following equation. It should be 15.0~40.0%

$$\text{Content of Sulfate (SO}_4\text{) (\%)} = \frac{\text{Weight of barium sulfate (g)} \times 0.4116}{\text{Weight of sample (g)}} \times 100$$

- (6) Viscosity (viscosity of 1.5% solution) : 7.5 g of Carrageenan is weighed into a tared 600 ml tall-form beaker, where 450 ml of water is added. It is then dispersed for 10~20 minutes by agitation. Water is added to bring the final weight to 500 g, and heated in a water bath with continuous agitation, until a temperature of 80°C is reached. Water is added to adjust for loss by evaporation. It is then cooled to 76~77°C, and heated in a constant temperature bath at 75°C. It is then tested using a viscometer (Brookfield LVE, LTV type or its equivalent) with a No.1 spindle and capable of rotating at 30rpm. After six complete revolutions of the viscometer, the viscometer reading on 0~100 scale is taken. When the value is multiplied by 2, it should not be more than 5cps.
- (6) Residual solvent : 2 g of Carrageenan is precisely weighed into a 300 ml round bottom distilling flask, 200 ml of water is added, boiling chips and 1 ml of silicone resin are added and mixed well. A fractionating column is connected to flask, 4 ml of internal standard solution is precisely weighed and added to it. While adjusting the heat so that the foam does not enter the column, distill the solution at the rate of 2~3 ml per 1 minute until the milky liquid becomes about 90 ml, and water is added to make 100 ml, test solution. However, tert-butyl alcohol (1→1,000) is used

as internal standard solution. Separately, 0.5 g each of methyl alcohol and isopropyl alcohol is precisely measured and water is added to 500 ml. Again 2 ml of this solution and 4 ml of internal standard solution is weighed, water is added to make 100 ml, mixed standard solution. 2μl of test solution and mixed standard solution is taken respectively, and injected to gas chromatograph with the following operation condition. Then, Ratio of methyl alcohol and isopropyl alcohol peak area against tert-butyl alcohol peak area, Q_{T1} , Q_{T2} and Q_{S1} , Q_{S2} , is measured respectively, and measure the content of methyl alcohol and isopropyl alcohol under following equation, it should be not more than 0.1% as individual or sum if used together.

$$\text{Content of methyl alcohol(\%)} = \frac{\text{Weight of methyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_{T1}}{Q_{S1}} \times \frac{2 \times 100}{500 \times 100} \times 100$$

$$\text{Content of Isopropyl alcohol(\%)} = \frac{\text{Weight of isopropyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_{T2}}{Q_{S2}} \times \frac{2 \times 100}{500 \times 100} \times 100$$

Q_{T1} : Ratio of methyl alcohol peak area against tert-butyl alcohol peak area in Test Solution

Q_{T2} : Ratio of isopropyl alcohol peak area against tert-butyl alcohol peak area in Test Solution

Q_{S1} : Ratio of methyl alcohol peak area against tert-butyl alcohol peak area in mixed standard Solution

Q_{S2} : Ratio of isopropyl alcohol peak area against tert-butyl alcohol peak area in mixed standard Solution

Column : PLOT Q or its equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Injection Port Temperature : 200℃

Column Temperature : 120℃

Detector Temperature : 300℃

Carrier gas : Nitrogen or Helium

(7) Total Viable Aerobic Count : When Carrageenan is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」 (noticed by KFDA), it

should not be more than 5,000 colonies per 1 g

(8) E. Coli : When 25 g of Carrageenan is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

(9) Salmonella : When Carrageenan is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Ash 2 g of Carrageenan is precisely weighed and tested by test methods for Ash. When is converted to a dried material, the content of the ash should be not less than 15.0 and not more than 40.0%.

Loss on Drying When 3 g of Carrageenan is dried for 4 hours at 105°C, the loss should not be more than 12%.

61. Caramel Color

Definition There are caramel I, II, III, and IV. Each definition is as follows.

Caramel I: Caramel I is obtained by heating edible carbohydrates of sugars, hydrolyzed starch, and molasses. Or it can be obtained by treating with acids or alkalis (free of ammonium compounds and sulfites) followed by heat treatment.

Caramel II: Caramel II is obtained by treating edible carbohydrates of sugars, hydrolyzed starch, and molasses in the presence of sulfite compounds (ammonium compound free) followed by heat treatment. Or it can be obtained by treating with sulfites, acids, or alkalis (free of ammonium compounds) followed by heat treatment.

Caramel III: Caramel III is obtained by heating edible carbohydrates of sugars, hydrolyzed starch, and molasses in the presence of ammonium compounds, with or without acids or alkalis (free of sulfites).

Caramel IV: Caramel IV is obtained by heating edible carbohydrates of sugars, hydrolyzed starch, and molasses in the presence of both ammonium compounds and sulfite, with or without acids or alkalis (free of sulfites).

Compositional Specifications of Caramel Color

Description Caramel Color is black~dark brown liquid, solid or powder having an odor of burnt sugar and refreshing bitter taste.

Identification (1) Solution of Caramel Color(1→100) is brown~blackish brown

(2) Appropriate amount of Caramel Color is weighted so that the measured absorption in advance is about 0.5 and added 0.025N of hydrochloric acid to make to 100 ml. If necessary, the solution is centrifuged and the supernatant is used as A solution. 0.2 g of diethylaminoethyl cellulose anion exchange resin (DEAE cellulose) is dissolved in 20 ml of A solution. Then the solution is centrifuged and the supernatant is used as B solution. Using 0.025N hydrochloric acid as a reference solution, absorbance A_A and A_B of A and B solution are measured at the wavelength 560 nm with 1 cm path length. Value of $(A_A - A_B)/A_A$ should be as below.

Caramel Color I should be not more than 0.75, Caramel Color II and IV should be more than 0.75 and Caramel Color III should be not more than 0.5.

(3) (In the case, this applies to Caramel Color I and Caramel Color III only.)

0.2~0.3 g of Caramel Color is weighted and added 0.025N of hydrochloric acid to make to 100 ml. If necessary, the solution is centrifuged and the supernatant is used as C solution. After taking 40 ml of C solution, add 2 g of phosphoryl cellulose and mix it by shaking well. The solution is centrifuged and the supernatant is used as D solution. Using 0.025N hydrochloric acid as a reference

solution, absorbance A_C and A_D of C and D solution are measured at the wavelength 560 nm with 1 cm path length. Value of $(A_C - A_D)/A_C$ should be as below.

Caramel Color I should be not more than 0.5, Caramel Color III should be more than 0.5.

(4) (In the case, this applies to Caramel Color II and Caramel Color IV only.)

0.1 g of Caramel Color is weighted and added water to make to 100 ml. If necessary, the solution is centrifuged and the supernatant is used as A solution. After taking 5 ml of A solution, add water to make to 100 ml and the solution is used as B solution. When absorbance A_A of A solution is measured at the wavelength 560 nm with 1 cm path length, use water as a reference solution. Or when absorbance A_B of B solution is measured at the wavelength 280 nm with 1 cm path length, use water as a reference solution. Value of $A_B \times 20/A_A$ should be as below.

Caramel Color II should be more than 50, Caramel Color IV should be not more than 50.

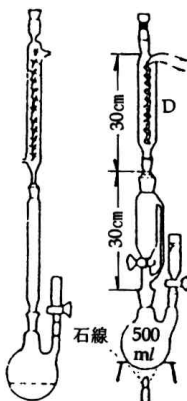
Purity (1) Arsenic : 0.77 g of Caramel Color is tested as directed under Purity (1) for Guar Gum (Not more than 1.3ppm).

(2) Lead : When 5.0 g of Caramel Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Caramel Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : 5 g of Caramel Color is taken into a flask for mercury decomposition, where 10 ml of water and 20 ml of nitric acid are added and then allowed to stand. 10 ml of sulfuric acid is slowly added. A reflux condenser is attached to the flask, which is then heated carefully until generation of NO_2 gas stops. If the decomposed liquid does not turn pale orange in color, it is cooled and 5 ml of nitric acid is added. It is then heated again. If necessary, this procedure is repeated until the decomposed liquid becomes transparent. After cooling, 50 ml of water and 10 ml of 10% urea solution are added to the decomposed liquid, which is boiled for 10 minutes. After cooling, 1 g of potassium permanganate is added and mixed for 10 minutes while shaking occasionally. If purple red color disappears, small amount of potassium permanganate is added to the solution, which is heated for 20 minutes. This is repeated until the purple red color persists. After cooling, 10% of hydrogen

peroxide solution is drop-wise added until the solution becomes colorless and transparent (Test Solution). The Test Solution is tested by reductive evaporation of atomic absorption spectrophotometry. The absorbance of the Test Solution should not be higher than that of 5 ml of mercury standard solution (Not more than 0.1ppm).



< Mercury Decomposition Apparatus >

- (5) Color Value : 100 mg of Caramel color is precisely weighed and dissolved in water to make 100 ml, test solution. If necessary, the solution is centrifuged and the supernatant is used. Absorbance A of this solution is measured with 1 cm cell at 610 nm, using water as a reference. Color value is obtained from the following equation (converted into a solid matter). The contents should be 0.01~0.6.

$$\text{Color Value} = \frac{A_{610} \times 100}{\text{Content of solid matter}(\%)}$$

Provided, the content of solid matter is obtained as follows. 30 g of quartzsand and glass rod are placed in a weighing bottle, which is dried at 60°C and under vacuum of 50 mm/Hg until the weight becomes constant. 1.5~2.0 g of the sample is precisely weighed into the weighing bottle, mixed well, and dried to constant weight. Solid matter content is calculated by the following equation. Quartz sand (purified quartzsand, particle size : No.40 ~ No.60 mesh) is decomposed by hydrochloric acid and washed with water until it doesn't appear acidity. It is then dried and heat treated before use.

$$\frac{(\text{weight of quartzsand and sample after drying}(g) - \text{weight of quartzsand}) \times 100}{\text{Weight of sample}(g)}$$

- (6) Total Nitrogen : When Caramel color is tested under Kjeldahl Method in Nitrogen Determination, the amount should not be more than 3.3% (as based on product with color value of 0.1).

※ Calculation of impurities based on color value of 0.1

Using the content of each impurity (nitrogen as ammonia, 4-methyl imidazole, sulfur dioxide, total nitrogen) obtained under the each Compositional Specifications, Cs (conversion to solid matter) is obtained. On the basis of color value 0.1, the content of each impurity is calculated by the following equation.

$$\frac{Cs \times 0.1}{\text{Color Value}}$$

$$Cs : \frac{\text{Content Impurities} \times 100}{\text{Content of Solid Matter}(\%)}$$

- (7) Total Sulfur : 1~3g of oxide of magnesium or 6.4~19.2g of acetic acid magnesium, 1 g of sugar, and 50ml of nitric acid are taken to evaporation dish, 5~10 g of Caramel Color is precisely weighed and concentrated in a water bath until it forms paste. Put evaporation dish into muffle's furnace, gently heat (not more than 525°C) until nitrogen dioxide smoke doesn't generate. After cooling evaporation dish, add hydrochloric acid(1→2.5) to this, dissolve, add 5 ml more after neutralizing, filter, and heated until it boils. 5 ml of 10% barium chloride solution is drop-wise added, contrate until it becomes 100 ml, and allowed to stand for 1 night. Filter this with filter paper(5C or its equivalent), put filter paper and residue into a previously weighed crucible, heat-treat until the weight becomes constant, and weigh as barium chloride. When measuring the content of total sulfur, the amount should not be more than 3.5%. (based on the substance whose color value is 0.1). Separately, perform a blank test.

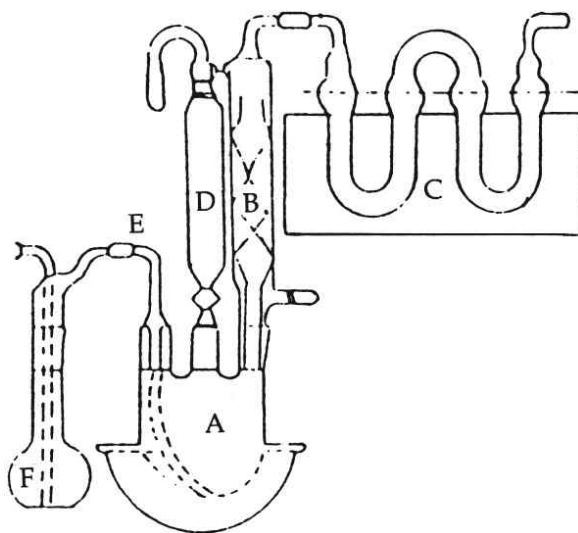
$$\text{Total Sulfur}(\%) = \frac{\text{Content of barium chloride(g)} \times 0.1374}{\text{Weight of Sample(g)}} \times 100$$

- (8) Ammoniacal nitrogen (In the case, this applies to Caramel Color III and Caramel Color IV only.) : 25 ml of 0.1 N sulfuric acid is added to a 500 ml Erlenmeyer flask. Kjeldahl distillation apparatus is set up so that the end of the condenser delivery tube is immersed beneath the surface of the sulfuric acid solution in the receiving flask. Separately, approximately 2 g of Caramel Color is precisely weighed into a 800 ml Kjeldahl flask for decomposition, where 2 g of magnesium oxide, 200

ml of water, and several boiling chips. The content is dissolved by shaking and the apparatus is quickly connected. It is then boiled and 100 ml of distillate is collected. The tip of the condenser is washed with water, which is added to the distillate. 4~5 drops of methyl red solution are added to the distillate, which is then titrated with 0.1 N sodium hydroxide, recording the volume, in ml, required as S. Separately, a blank determination is conducted and recorded the volume, in ml, of 0.1 N sodium hydroxide required to neutralized as B. Ammoniacal nitrogen is calculated by the following equation and it should not be more than 0.6%. (for a product with color value of 0.1).

$$\text{Content of Nitrogen as ammonia (\%)} = \frac{(B - S) \times 0.0014 \times 100}{\text{Weight of sample (g)}}$$

- (9) Sulfur Dioxide (In the case, this applies to Caramel Color II and Caramel Color IV only.): The following apparatus is used.



- A : 1,000 ml three neck round bottom flask
 B : 30 cm Allihn reflux condenser
 C : absorption tube
 D : 125 ml separatory funnel with a stopcock
 E : introduction tube
 F : 250 ml gas wash bottle connected to a nitrogen cylinder

4.5 g of pyrogallol and 5 ml of water are ground in a small mortar. The supernatant

is transferred into a wash bottle. The remaining residue is ground again and washed with water, which is then transferred into the wash bottle. It is passed nitrogen from the cylinder to the bottle to flush out air. Through a long stem funnel, a cooled solution of 65 g potassium hydroxide in 85 ml of water. The wash bottle is again flushed with nitrogen to remove air from the headspace. It is connected to the glass inlet tube of a distillation flask. Gas washing solution is prepared prior to use. Two pieces of glass rods (8 mm diameter \times 25 mm length) are placed in the absorption tube. 10 ml of glass beads, 10 ml of 3% hydrogen peroxide solution, and 1 drop of methyl red solution are added to the exit side. After the apparatus is connected, the stopcock of the separatory funnel is closed. A small amount of gas is passed through to the apparatus, let stand for a few minutes, and checked for gas leak by equalizing the liquid level.

Test Procedure : 25 g of Caramel color is dissolved in 300 ml of recently boiled and cooled water in a distillation flask. Water is added to bring the total volume to approximately 400 ml. 90 ml of 4 N hydrochloric acid is slowly added to the separator, and force the acid into the flask by blowing gently into the tube in the neck of the separator. Close the stopcock of the separator. In a flowing nitrogen, the solution is brought to a stable reflux and maintained for 2 hours. Then cooling water for the condenser is stopped. When vapor gets condensed in the first absorption tube and the tube gets warm, heating is stopped. When the top connection part of the condenser is cooled, it is disconnected. The first absorption tube is separated from the second one. The solution in the first absorption tube is titrated with 0.1 N sodium hydroxide solution. The connection tube is connected to the exit part of the second absorption tube. The solution is titrated again. The consumed amount of is S. Separately, a blank test is carried out and the volume of 0.1 N sodium hydroxide solution is B. When the content of sulfur dioxide is calculated by the following equation, it should not be more than 0.2% (Based on product with color value of 0.1).

$$\text{Content of Sulfur Dioxide(\%)} = \frac{(S - B) \times 0.0032 \times 100}{\text{Weight of sample (g)}}$$

(10) 4-methylimidazol (In the case, this applies to Caramel Color III and Caramel Color IV only) : 10 g of 4-methylimidazol is taken in 150 ml polypropylene beaker. Add 5 mL of 3N NaOH solution and mix it well to make to be more than pH 12. 20 g of diatomaceous earth(Johns-Manville Celite 545 or its equivalent) for

chromatography is taken in a beaker until it is a semi-dried mixture. Then insert it into a 2 cm inner diameter glass tube (including a teflon cock) whose bottom is blocked with a glass fiber and fill the contents to be about 25 cm high. While wash the beaker for previous sample, disembody ethyl acetate into a glass tube. When a solvent reaches the bottom of the glass tube, lock a cock and allowed to stand for 5 minutes. Then open the cock and put ethyl acetate in the glass tube and collect a effluent until the total volume of the effluent is about 200 mL. Add 1 mL of the internal standard solution to the effluent, transfer it into flask and concentrate ethyl acetate below 35 °C. Dissolve residue in acetone and take precisely 5 mL of solution, used as test solution. Separately, 0.02 g, 0.06 g, 0.1 g, 0.2 g of 4-methylimidazol is precisely weighted and added precisely 20 mL of the internal standard solution. Then add acetone so that the volume is to be 100 mL. used as separatel standard solution. However, the internal standard solution is used the solution which is added with ethyl acetate to 0.05 g of 2-methylimidazol to make precisely to 50 mL. Take 5 μ l of test solution and standard solution respectively and do test gas chromatography under below operation conditions. Then measure a peak area of respective standard solution and prepare a calibration curve. The peak area of test solution obtained in the calibration curve should not be more than 250 mg/kg (based on the product whose color value is 0.1)

Operation conditions

Detector: (Hydrogen) Flame ionization detector(FID)

Column: Column is filled with support material (150~160 μ m of diatomaceous earth for gas chromatography) which is mixed with 7.5% of Carbowax 20M and 2% of NaOH or its equivalent

Column temperature: 180 °C

Temperature at injection hole: 200°C

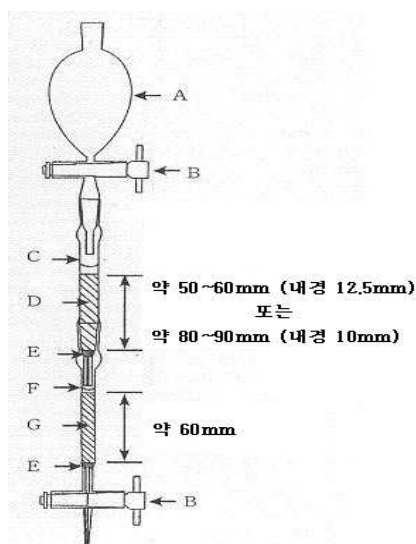
Carrier gas and flow rate: Nitrogen, 50 mL/min

- (11) 2-acetyl-4-tetrahydroxybutylimidazol (In the case, this applies to Caramel Color III): The following apparatus is used.

0.20~0.25 g of 2-acetyl-4-tetrahydroxybutylimidazol is precisely weighted and dissolved in 3 mL of water. Transfer this solution into top part C(connective part of column C and column F) and then wash

A : Separatory funnel (100mL)

B : Teflon cock



- C : Glass column inner diameter 12.5mm, length 150mm (including the connective part) or inner diameter 10mm, length 200mm (including the connective part)
- D : Weak acid cation exchange resin (particles)
- E : Cotton
- F : Glass column inner diameter 10mm, length 175mm (including the connective part)
- G : Strong acid cation exchange resin (particles)

it with 100 mL of water. Continually, remove column C and connect the separatory funnel A to the lower part column F and then elute column F with 0.5 N hydrochloric acid. The first effluent 10 mL is discarded and collect the subsequent effluent to 35 mL and concentrate it at 40 °C, 15 mmHG until it is dried condition. The syrup type residue without carbonyl group is dissolved in 250 μL of methanol. Add 2,4-dinitrophenylhydrazine hydrochloride solution, this reaction mixture is transferred into the glass bottle with cap. Keep it at room temperature for 5 hours and it is used as a test solution. After separately, stirring 0.5 g of 2,4-dinitrophenylhydrazine in 1 mL of hydrochloric acid, add 10 mL of ethanol. And then heat it in water bath until it is solution condition. Add 0.1 g of 2-acetyl-4-tetrahydroxybutylimidazol in hot solution. In a few minutes crystallization of 2-acetyl-4-tetrahydroxybutylimidazol-2,4-dinitrophenylhydrazone (THI-DNPH) begins, cool this until it is room temperature to make the crystallization completely. After adding the small quantity of ethanol to make it to suspension, do filtering it to separate. Refine the crystallized THI-DNPH with ethanol (added with 1 drop of hydrochloric acid per 5 mL of ethanol). After adding the small quantity of ethanol to make the refined crystal to suspension, do filtering it to separate and then dry it in desiccator. Weigh precisely 0.01 g of this item and add methanol without carbonyl group to make to 100 mL. Again this solution is diluted by methanol without carbonyl group to make a standard solution (1 mL of each solution includes 0, 20, 40, 60, 80, 100 μg). Test solution and standard solution respectively and do test liquid chromatography under below operation conditions. Then measure a peak area of respective standard solution and prepare a calibration curve. The peak area of test solution obtained in the calibration curve should not be more than 25 mg/kg (based on the product whose color value is 0.1). However, THI-DNPH 100 $\mu\text{g/mL}$

corresponds to THI 47.58 $\mu\text{g/mL}$.

Operation condition

Detector : UV 385 nm

Column : Capcell pak C₁₈(5 μm , 4.6 mm \times 250 mm) or its equivalent

Column temperature : Room temperature

Passing of solution : Methanol: 0.1 M of phosphoric acid (50:50)

Flow rate : 1.0 mL/min

Solution

2,4-dinitrophenylhydrazine hydrochloride solution : Put 10 mL of hydrochloric acid into erlenmeyer flask(100mL) and then add 5 g of 2,4-dinitrophenylhydrazine. Shake silently and mix it until free base(red color) is converted to hydrochloride(yellow color). Then after adding 100 mL of ethanol, do heating and melting in water bath. Cool and crystallize it at room temperature and filter it to remove hydrochloride and wash with ether. Dry it at room temperature and keep it in desiccator. This is used as 2,4-dinitrophenylhydrazine hydrochloride. Although hydrochloride can be slowly converted to free base, it can be removed by washing with 1,2-dimethoxyethane. 0.5 g of 2,4-dinitrophenylhydrazine hydrochloride is dissolved in 15 mL of 1,2-dimethoxyethane including 5% methanol. And keep it in a refrigerator.

Methanol without carbonyl group : Add 5 g of Girard P reagent and 0.2 mL of hydrochloric acid to 500 mL of methanol. After attaching a reflux condenser to it, distill it for 2 hours. Keep this solution which is sealed in glass bottle.

62. Karaya Gum

Sterculia Gum

Definition Karaya Gum is obtained by drying gummy secretion of *Sterculia urens* Roxburgh, *Sterculia* (Fam. *Sterculiaceae*) and its variety, *Cochlospermum Gossypium* A. P. De Condolle, or *Cochlospermum Kunth* (Fam. *Bixaceae*). It is a polysaccharide consisting mainly of galactose, rhamnose, and galacturonic acid.

Compositional Specifications of Karaya Gum

Description Karaya Gum is orange yellow~pale reddish brown lump or gray~pale reddish brown gray powder having a little odor of acetic acid.

Identification (1) When 2 g powder of Karaya Gum is mixed well with 50 ml of water, it becomes gluey and weakly acidic.

(2) When 0.4 g powder of Karaya Gum is mixed well with 10 ml of 60% alcohol, it swells.

(3) 1 g of Karaya Gum is added to 20 ml of water. Homogeneous mucilage is obtained by heating. After adding 5 ml of hydrochloric acid is added to the glue, it is boiled for 5 minutes. The resulting liquid becomes permanent pale red~pale reddish brown.

Purity (1) Arsenic : 0.25 g of Karaya Gum is placed in a flask for decomposition. After adding 5 ml of sulfuric acid and 5 ml of nitric acid, the flask is mildly heated. 2~3ml of nitric acid is added at a time and the flask is heated until the liquid becomes colorless~pale yellow. After cooling, 15 ml of saturated ammonium hydroxide is added to the flask, which is then heated and concentrated to 2~3 ml until thick white smoke is generated. The resulting concentrate is neutralized with ammonia water or ammonia TS. This test solution is tested for arsenic. It should be appropriate. Standard color is prepared with 1 ml of arsenic standard solution following the same procedure as the test solution (not more than 4ppm).

(2) Lead : When 5.0 g of Karaya Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Karaya Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Karaya Gum is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Acid Insoluble Ash : 3 g of Karaya Gum is carbonized at 550~600°C and reduced

to ash by further heating. The resulting ash are boiled for 5 minutes in 25 ml of dilute hydrochloric acid, which is filtered through an ashless filter paper. The residue on the filter paper is washed with a small amount of hot water. It is then carbonized to ash along with the filter paper in a crucible (previously weighed). The content of ash should not be more than 1%.

- (6) Insoluble matter : 5 g of Karaya Gum is precisely weighed into a 250 ml Erlenmeyer flask, where a 1:1 mixture of dilute hydrochloric acid and water. It is covered with a watch glass and boiled until the liquid is no longer viscous. The resulting liquid is filtered through a glass filter (previously weighed), which is washed with hot water until the washings are free from acid(pH paper). It is then heated at 105°C and weighed. The content of residues should not be more than 3%.
- (7) Starch : 0.1 g of Karaya Gum is dissolved in 100 ml of water by boiling, which is then cooled. When 2~3 drops of iodine solution are added, it should produced turn blue.
- (8) Other Gums : Karaya Gum swells in 60% alcohol.
- (9) Volatile Acidity : 1g of Karaya Gum is precisely weighed into a flask, 100 ml of freshly boiled and cooled water and 5 ml of phosphoric acid are added, and allowed to stand for several times until the gum is completely swollen. Under a reflux condenser, boil for 2 hours, steam-distilled until 800 ml of distillate. Use 20 ml of this solution as an indicator, titrate with 0.1N sodium hydroxide, the amount required for neutralizing should not less than 0.42 ml. (not less than 10% as acetic acid). Separately, perform a blank test.
- (10) E. Coli : When Karaya Gum is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
- (11) Salmonella : When Karaya Gum is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Loss on Drying Powder of Karaya Gum is sieved through a No.40 mesh screen. 3 g of sieved powder is dried for 5 hours at 105°C. The loss on drying should not be more than 20%.

Viscosity 4 g of Karaya Gum, fine powder, is placed in a container for stirrer. 10 ml of alcohol is added to wet the powder uniformly. After adding 390 ml of water, it is stirred for 7 minutes at 1,000 rpm. The suspension is transferred into a 500 ml bottle and maintained for 12 hours in a water bath at 25°C. Viscosity is measured using LVF Brookfield viscometer with an appropriate set of conditions such as spindle, rpm,

factor, etc. The viscosity should be higher than the indicated value or fall within the indicated range of values.

63. Casein

Definition There are casein and rennet casein. Respective definition is as follows.

Casein : It is obtained by treating a protein from milk or defatted milk with acid.

Rennet casein : It is obtained by treating a protein from milk or defatted milk with rennet.

1. Casein

Compositional Specifications of Casein

Content Casein, when dried, contains 13.8~16.0% of nitrogen ($N = 14.01$).

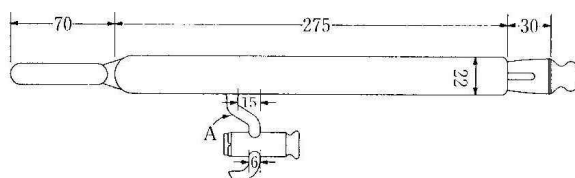
Description Casein is white~pale yellowish white powder, granules, or flakes. It is odorless and tasteless, or has a slight characteristic odor and taste.

Identification (1) 0.1 g of Casein is dissolved in 10 ml of sodium hydroxide solution, and added acetic acid to make weak acidity, white cotton-like precipitate is formed.
(2) 0.1 g of Casein is dissolved in 10 ml of sodium hydroxide solution, added 1 drop of cupric sulfate solution, and shake. A blue precipitates is formed, and the colour of the solution is purple.
(3) When 0.1 g of Casein is ignited, fume and a characteristic odor is developed. After the fume are no longer evolved, stop heating, and cooled. To the black residue, added 5 ml of dilute nitric acid, dissolved while warming, and filtered. To the filtrate, added 1 ml of ammonium molybdate TS, and warmed. A yellow precipitate is formed.

Purity

- (1) Clarity of Solution : Casein is dried in a vacuum desiccator for 4 hours and made into a fine powder. 0.1 g of dried fine powder is mixed by shaking in 30 ml of water, and allowed to stand for about 10 minutes, it is dissolved by adding 2 ml of 0.1 N sodium hydroxide solution, dissolved while warming by shaking at 60°C. After cooling, water is added to bring the total volume to 100 ml. The resulting solution should be colorless and slightly turbid.
- (2) pH : 1 g of Casein is mixed in 50 ml of water by shaking for 10 minutes. It is then filtered. pH of the filtrate measured by using a glass electrode should be 3.7~6.5.
- (3) Water Soluble Substances : 1.5 g of Casein is add 30 ml of water and shaking for 10 minutes, which is then filtered. 20 ml of filtrate is evaporated to dryness and the residue is dried at 100°C to constant weight. Its content should not be more than 10 mg.
- (4) Fat : About 2.5 g of Casein is precisely weighed, where 15 ml of diluted

hydrochloric acid (27→40) is added. It is then dissolved while gently heating directly, and heated in a water bath for 20 minutes. After cooling, 10 ml of alcohol is added, which is then transferred to a Rohrig tube, added 25 ml of ether, which is vigorously shaken for 1 minute. 25 ml of petroleum ether is added, which is then shaken vigorously for 30 seconds and allowed to stand. The supernatant taken from side branch tube (A) is filtered through a filter paper. The filtrate is collected into a flask that is previously weighed. It is repeated the extraction above two times, using 15 ml ether and 15 ml petroleum ether each time, transferred the upper-layer solution to the flask, and evaporated the ether and petroleum ether on a water bath. The residue is dried for 4 hours 98~100°C. The content of fat should not be more than 1.5%.



Leriche tube (standard : mm)

(5) Lead : When 5.0 g of Casein is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1 ppm.

Loss on Drying When Casein is dried for 3 hours at 100°C, the loss should not be more than 12%.

Residue on Ignition 1 g of Casein, previously dried, is accurately weighed. When Residue on Ignition is done with it, the amount of residue should not be more than 2.5%.

Assay About 0.15 g of Casein, previously dried, is precisely weighed and proceed as directed in the Kjeldahl Method under Nitrogen Determination.

$$0.1 \text{ N sulfuric acid } 1 \text{ ml} = 1.401 \text{ mg N}$$

2. Rennet Casein

Compositional Specifications of Casein

Content Rennet Casein, when dried, contains 13.5 % of nitrogen (N = 14.01).

Description Rennet Casein is white~pale yellowish white powder, flakes, or granules. It is odorless and tasteless, or has a slight characteristic odor and taste.

Identification (1) 0.2 g of Rennet Casein is dissolved in 10 ml of sodium hydroxide solution(1→100)(if necessary, heating), and added 4 ml of diluted acetic acid. A white cotton-like precipitate is formed.

(2) 0.2 g of Rennet Casein is dissolved in 10 ml of sodium hydroxide solution(1→100)(if necessary, heating), added 1 drop of copric sulfate solution, and shake. A blue precipitates is formed, and the colour of the solution is purple.

(3) When 0.1 g of Rennet Casein is ignited at 450~550°C, fume and a characteristic odor is developed. After the fume are no longer evolved, stop heating, and cooled. To the black residue, added 5 ml of dilute nitric acid, dissolved while warming, and filtered. To the filtrate, added 1 ml of ammonium molybdate TS, and warmed. A yellow precipitate is formed.

Purity (1) pH : 1 g of Rennet Casein is mixed in 50 ml of water by shaking for 10 minutes. It is then filtered. pH of the filtrate measured by using a glass electrode should be 6.0~7.8.

(2) Water Soluble Substances : Rennet Casein is tested as directed under in Purity (3) for Casein, its content should not be more than 10 mg.

(3) Fat : About 2.5 g of Rennet Casein, previously dried at 100°C for 30 minutes and cooled, is precisely weighed. It is tested as directed under in Purity (4) for Casein, its content should not be more than 1.5%.

(5) Lead : When 5.0 g of Rennet Casein is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1 ppm.

Loss on Drying When Rennet Casein is dried for 5 hours at 105°C, the loss should not be more than 13.0%.

Assay About 0.15 g of Casein, previously dried, is precisely weighed and proceed as directed in the Kjeldahl Method under Nitrogen Determination.

$$0.1 \text{ N sulfuric acid } 1 \text{ ml} = 1.401 \text{ mg N}$$

64. Cacao Color

Definition Cacao Color is a pigment obtained by fermenting and roasting cacao beans of cacao tree (*Theobroma cacao* Linné) of sterculiaceae followed by extracting with water. Its major pigment component is polymer of antocyanin. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Cacao Color

Content Color value ($[E]_{1cm}^{10\%}$) of Cacao Color should be higher than the indicated value.

Description Cacao Color is dark reddish brown liquid, lump, powder, or paste having a slight characteristic odor.

Identification (1) Test Solution obtained in Color Value section appears brown color.

(2) 0.1 g of Cacao Color is dissolved in water to make 100 ml. When 2~3 drops of hydrochloric acid are added to 5 ml of this solution, reddish brown precipitate is formed.

(3) 0.1 g of Cacao Color is dissolved in water to make 100 ml. When 2~3 drops of ferric chloride solution are added to 5 ml of this solution, it turns dark brown.

Purity (1) Arsenic : 0.25 g of Cacao Color is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with a small amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Cacao Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

(3) Residual Solvent : When berries color is tested by Purity (5) for 「Paprika Extract Pigments」, residual acetone should not be more than 30 ppm(based on the product whose color value is 50).

Assay (Color Value) Appropriate amount of Cacao Color is precisely weighed so that the absorbance is within 0.3~0.7 and dissolved in water to make 100 ml. 1 ml of this solution is diluted to 100 ml with citric acid·dibasic sodium phosphate buffer solution with pH 7.0. Use this solution as the test solution. If necessary, the solution is centrifuged and the supernatant is used. Using citric acid·dibasic sodium phosphate

buffer solution with pH 7.0 as a reference solution, absorbance A of test solution is measured at 520nm with 1cm cell. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

◦ Citric acid·dibasic sodium phosphate buffer solution (pH 7.0)

Solution 1 : 0.1M citric acid solution : 1L of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2M dibasic sodium phosphate solution : 1L of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (35:165) and its pH is adjusted to 7.0.

65. Catalase

Definition Catalase is an enzyme obtained from cultures of *Aspergillus niger* and its variety and *Micrococcus lysodeikticus*, or liver of cow. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Catalase

Content Catalase contains 90~130% of the indicated activity as catalase.

Description Catalase is white~pale yellow~dark brown powder, granule, lump, or transparent~dark brown liquid.

Purity (1) Arsenic : 0.25 g of Catalase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Catalase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Coliform Group : Catalase is tested by Microbe Test Methods for Coliform Group in General Test Methods in Food Code. It should not be more than 30 cfu per 1 g of this product.

(4) Salmonella : Catalase is tested by Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).

(5) E. Coli : When Catalase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay (Activity) Application and Principle : This test is to measure the activity of catalase, where the activity of an enzyme obtained from cultures of *Aspergillus niger* and its variety and *Micrococcus lysodeikticus*, or liver of cow is expressed in Baker units. Activity test is a type of consumption based on decomposition of hydrogen peroxide by catalase and simultaneously on decomposition of catalase by peroxide under the established conditions.

Test Procedure : Undiluted enzyme solution (or diluted enzyme solution) is transferred into a 200 ml beaker so that 1.0 ml or less of this solution contains about 3.5 Baker

units, where 100 ml of substrate solution (maintained at 25°C) is quickly added and stirred for 5~10 seconds. The beaker is covered and kept at $25 \pm 1^\circ\text{C}$ until the reaction completes. After stirring vigorously for 5 seconds, 4 ml of the solution is precisely taken and transferred into a 50 ml Erlenmeyer flask, where 5 ml of 2 N sulfuric acid is added and mixed. To this solution, 5 ml of freshly prepared 40% potassium iodide solution and 1 drop of 1% ammonium molybdate solution are added. While shaking continuously, the solution is titrated with 0.25 N sodium thiosulfate solution (consumed amount = S). Separately, a blank test is carried out with 4 ml of substrate solution (consumed amount of 0.25 N sodium thiosulfate solution = B). (Note : When the sample is derived from cow liver, the reaction is to complete in 30 minutes. When the sample is derived from *Aspergillus* and others, the reaction is to complete in 1 hour. If the origin is unknown, titration is carried out after 30 minutes at an interval of 10 minutes. If the titration values are same for the two consecutive titration, the reaction is complete.) Enzyme activity is calculated from the following equation.

$$\text{Baker units/g or ml} = 0.4(B - S) \times (1/C)$$

C : Dilution factor for ml of enzyme stock solution added in 100 ml of substrate solution or 1 ml of diluted enzyme solution

Definition of Activity : 1 Baker unit corresponds to the amount of catalase that decomposes 266 mg of hydrogen peroxide under the test conditions above.

Solutions

- 0.25 N Sodium Thiosulfate Solution : 62.5 g of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) is dissolved in 750 ml of water (previously boiled and cooled), where 3.0 ml of 0.2N sodium hydroxide solution is added as a stabilizer and water is added to bring the total volume to 1,000 ml. It is then standardized by 0.1 N sodium thiosulfate solution. If possible, the normality is adjusted to 0.250.
- Substrate Solution : 25.0 g of sodium phosphate, dibasic (anhydrous) or 70.8 g of sodium phosphate, dibasic (12 hydrates) is added in 1,500 ml of water. pH of the solution is adjusted to 7.0 ± 0.1 with 85% phosphoric acid. 100 ml of 30% hydrogen peroxide is carefully added to the solution and the total volume is brought up to 2,000 ml with water. The resulting solution is transferred into a

transparent brown glass bottle, where a cap is loosely placed. If the bottle is filled up to its neck and stored at 5°C, the solution is stable for 1 week or longer. (For a blank test with a freshly prepared substrate solution, approximately 16 ml of 0.25 N sodium thiosulfate solution is consumed. If the consumed amount of 0.25N sodium thiosulfate solution for blank test is 14 ml or less, the substrate solution is unstable. It needs to be prepared freshly. Consumption of enzyme solution should be in a range of 50~80% of the consumption in the blank).

Storage Standard of Catalase

Catalase is strongly hygroscopic, hence should be stored in sealing tightly at a cold dark place.

66. Curdlan

Definition Curdlan is obtained by separation and purification of polysaccharide produced from *Alcaligenes faecal* and *Agrobacterium*.

Compositional Specifications of Curdlan

Description Curdlan is white or nearly white powder.

Identification (1) When 10 ml of an aqueous suspension (1→50) of Curdlan is heated in a water bath, it forms a gel.

(2) 10 ml of sulfuric acid (2→5) is added to 10 ml of an aqueous suspension (1→50) of Curdlan, which is heated for 30 minutes in a water bath. After cooling, 1 ml of this suspension is diluted with 9 ml of water. While heating, it is neutralized with barium carbonate. When 2 ml of Fehling solution is added to 1 ml of the supernatant, which is boiled, red~dark red precipitates are formed.

Purity (1) Arsenic : 0.25 g of Curdlan is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Curdlan is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 0.5 ppm.

(3) Nitrogen : When Curdlan is tested by Kjeldahl Nitrogen Test in nitrogen determination method, the amount should not be more than 0.3%.

(4) Total Viable Aerobic Count : When Curdlan is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 1,000 colonies per 1 g

(5) E. Coli : When Curdlan is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

Residue on Ignition When Residue on Ignition is done with Curdlan, the amount of residue should not be more than 6.0%.

Loss on Drying When Curdlan is dried for 5 hours at 60°C under vacuum, the loss should not be more than 10.0%.

67. Cochineal Extract

Definition Dried bodies of *Dactylopius coccus costa* (*Coccus cacti*. Linnæus), which is female *coccus cati* parasitic on *cactus* (*Nopalea coccinellifera*), is extracted with aqueous alcohol. Cochineal Extract is the concentrated solution obtained after removing the alcohol from an aqueous, aqueous alcoholic or alcoholic extract of cochineal. The major pigment component is carminic acid. Dilutant, stabilizer, or solvent can be added for the purpose of content adjustment and quality preservation.

Compositional Specifications of Cochineal Extract

Content Cochineal Extract should contain not less than 1.8% of carminic acid ($C_{22}H_{20}O_{13} = 492.39$).

Description Cochineal Extract is red~dark reddish brown liquid, lump, powder, or paste having a slight characteristic odor.

Identification (1) Test Solution obtained in Content section shows a absorption maximum at about 495 nm.

(2) 1 g of Cochineal Extract is mixed with 50 ml of 0.1 N hydrochloric acid, which is filtered, if necessary. The filtrate is orange red in color. When it is alkalinized with sodium hydroxide solution, it produced violet~red.

Purity (1) Arsenic : 0.66 g of Cochineal Extract is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with a small amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 1.3ppm.

(2) Lead : When 5.0 g of Cochineal Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Cochineal Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Cochineal Extract is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Protein : When 3 g of Cochineal Extract is precisely weighed, proceed as directed under Kjeldahl Method in Nitrogen Determination, multiply the content of nitrogen

obtained from the test by nitrogen factor 6.25 to measure the content of protein, the amount should not be more than 2.2%.

(6) Methyl Alcohol : When Cochineal Extract is tested by Purity (5) for Paprika Extract Pigments, the content should not be more than 150ppm.

(7) Salmonella : Cochineal Extract is tested by Microbe Test Methods for Salmonella in General Test Methods, Food Code. It should be negative (-).

Assay About 800 mg of Cochineal Extract is precisely weighed and added with mixture of 2N hydrochloric acid and water(97:3), and made to volume 1,000 ml, Test Solution. Absorbance (A) of the Test Solution is measured using a mixture(97:3) of water and 2 N hydrochloric acid as the blank with 1cm cell at a maximum absorption about 495 nm. The content (%) is calculated using the following equation. The amount of sample is adjusted so that the absorbance of the Test Solution is within the range 0.2 to 0.25.

$$\text{Content (\%)} = \frac{15A}{\text{Weight of sample (mg)}} \times \frac{100}{0.262}$$

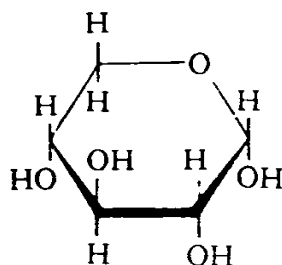
(0.262 : absorbance of carminic acid solution (15 mg/l))

68. Corn Color

○ Designed Cancellation

The date of cancellation : July.10.2009(Notification No. 2009-51).

69. D-Xylose



Chemical Formula $C_5H_{10}O_5$

Molecular Weight 150.13

Definition D-Xylose is obtained from hydrolysis with hot acidic aqueous solution and separation of wood, cotton (*Gossypium arboreum* LINNE) of malvaceae, rice (*Oryza sativa* LINNE) of gramineae, sugar cane (*Saccharum officinarum* LINNE) of gramineae, corn (*Zea Mays* LINNE) of gramineae or stems, fruits, or skins of other same genus. Its major component is D-xylose.

Compositional Specifications of D-Xylose

Content After drying, D-Xylose contains 98.0~101.0% D-xylose ($C_5H_{10}O_5$).

Description D-Xylose is colorless~white crystallite or white crystalline powder. It has odorless and sweet taste.

Identification (1) When 2~3 drops of aqueous solution (1→20) of D-Xylose are added to 5 ml of hot Fehling solution, red precipitate is formed.

(2) 1 g of xylose is dissolved in 25 ml of water (freshly boiled and cooled). This solution is dextrorotatory.

(3) 1 g of D-Xylose is dissolved in 3 ml of water by heating, where 3 ml mixture of 4 ml alcoholic solution of diphenyl amine (1→40) and 10 ml of diluted hydrochloric acid. When the solution is heated for 5 minutes in a water bath, it showed yellow~pale orange color.

(4) 0.5 g of D-Xylose is dissolved in 20 ml of water, where 30 ml of phenylhydrazine hydrochloride-sodium acetate solution and 10 ml of diluted acetic acid are added. When the solution is heated in a water bath, precipitate is formed, which are recrystallized in water. The melting point of the precipitate is 160~163°C.

Purity (1) Clarity of Solution : When 4 g of D-Xylose is dissolved in 200 ml of water, it is colorless and almost clear.

(2) Free acid : 1 g of D-Xylose is dissolved in 10 ml of water (freshly boiled and cooled). When 1 drop of phenolphthalein solution and 1 drop of 0.2 N sodium

hydroxide solution are added to this solution, it should turn red.

- (3) Sulfates : 1 g of D-Xylose is dissolved in 30 ml of water. When this Solution is tested for sulfates, the content should not be more than the amount that corresponds to 0.1 ml of 0.01 N sulfuric acid.
 - (4) Arsenic : 1 g of D-Xylose is dissolved in 5 ml of water. When test for arsenic is carried out with this test solution, it should not be more than 1ppm.
 - (5) Lead : When 5.0 g of D-Xylose is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.
 - (6) Other Saccharides : 0.5 g of D-Xylose is dissolved in water to make 1,000 ml. 0.1 ml of D-Xylose is tested by Method 1 in Filter Paper Chromatography. When the top of developing solution reaches 15cm from the Test Solution spot, stop. The position of the solution is marked. After the solvent is blow dried, it is developed with the same solvent until the front reaches the same point. This operation is repeated one more time. Colorizing solution is sprayed on to the filter paper, which is dried for 5 minutes at 100~125°C. There should be only one red spot under natural light. Reference solution is not used.
- Developing Solution : a mixture of n-butyl alcohol, pyridine, and water (6:4:3)
 - Colorizing Solution : 0.93 g of aniline and 1.66g of anhydrous phthalic acid are dissolved in 100 ml of n-butyl alcohol (saturated with water).
 - Filter Paper : No.2 filter paper for chromatography is used.

Loss on Drying When 3 g of D-Xylose is dried for 3 hours at 105°C, the loss should not be more than 1%.

Residue on Ignition When Residue on Ignition is done with precisely weighed 5 g of D-Xylose, the amount of residue should not be more than 0.05%.

Assay Approximately 1 g of dried D-Xylose is precisely weighed and dissolved in water to make 500 ml. 10 ml of this solution is added into an iodine bottle, where precisely 50 ml of sodium meta periodate solution (1→400) is added and 1 ml of sulfuric acid is added. It is then heated for 15 minutes in a water bath. After cooling, 2.5 g of potassium iodide is added and well mixed by shaking. After allowing to stand for 15 minutes in a cold dark place, it is titrated with 0.1 N sodium thiosulfate solution (indicator : starch solution). Separately, a blank test is carried out.

$$0.1 \text{ N sodium thiosulfate solution } 1 \text{ ml} = 1.877 \text{ mg } \text{C}_5\text{H}_{10}\text{O}_5$$

70. Chitosan

Definition Chitosan is obtained by alkali treating from chitin. Its component is polyglucosamine.

Compositional Specifications of Chitosan

Description This is white~pale yellow or red powder or scale shaped material having a slight characteristic odor.

Identification When 5 ml of anthrone solution and 1 ml of water are added to 0.2 g of Chitosan, which is heated in a water bath, it turns blue~green.

Purity (1) Arsenic : 0.25 g of Chitosan is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute a small of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Chitosan is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Degree of Deacetylation : 0.5 g of dried Chitosan is precisely weighed and dissolved in 5 v/v% acetic acid to make 100 ml. 1 ml of this solution is diluted with 30 ml of water in a 200 ml Erlenmeyer flask. It is titrated with 0.0025 N polyvinyl potassium sulfate solution (indicator: 2~3 drops of 0.1% toluidine blue solution). The degree of deacetylation is obtained from the following equation. It should not be less than 70.0%.

$$\text{Degree of Deacetylation (\%)} = \frac{X/161}{X/161 + Y/203} \times 100$$

$$X = \frac{1}{400} \times \frac{1}{1,000} \times f \times 161 \times v$$

$$Y = 0.5 \times \frac{1}{100} - X$$

v : Consumed amount of 0.0025 N polyvinyl potassium sulfate solution (ml)

f : Normality factor of 0.0025N polyvinyl potassium sulfate solution

Loss on Drying When Chitosan is dried for 4 hours at 105°C, the loss should not be more than 15%.

Residue on Ignition When Residue on Ignition is done with Chitosan (on dried basis), the amount of residue should not be more than 5%.

71. Tara Gum

Definition Tara Gum is a polysaccharide obtained by grinding endosperm of tara (*Caesalpinia spinosa* Kuntze) seeds of actinidiaceae.

Compositional Specifications of Tara Gum

Description Tara Gum is nearly odorless, white~pale yellow powder.

Identification (1) When a small amount of sodium borate is added to an aqueous solution of Tara Gum, a gel is formed.

(2) 2 g of Tara Gum is placed in a 400 ml beaker. It is then moisten thoroughly with about 4 ml of isopropyl alcohol. With vigorous stirring, 200 ml of water is added and further stirred until the gum is completely and uniformly dispersed. 100 ml of this solution is transferred into another 400 ml beaker, which is heated for 10 minutes in a water bath. When it is cooled to room temperature, the solution shows a marked increase in viscosity.

Purity (1) Arsenic : 0.25 g of Tara Gum is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with a small amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Tara Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Tara Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Tara Gum is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Starch : 0.1 g of Tara Gum is dissolved in 10 ml of water, which is heated and then cooled. When 2 drops of iodine solution are added, it should not produce blue.

(6) Protein : When 0.2 g of Tara Gum is precisely weighed and tested as directed in Kjeldahl Method under Nitrogen Determination, the amount should not be more than 3.5%. (Protein Factor : 6.25).

(7) Acid Insoluble substances : 0.5 g of Tara Gum is precisely weighed and dissolved

in 150 ml of water and 1.5 ml sulfuric acid in a beaker, which is covered with a watch glass and heated for 6 hours in a water bath. Beaker wall is washed with water so that sample is not left on the wall. 500 mg of appropriate filtering aid is added to the filter, previously make a constant weight . The residue is washed thoroughly with hot water and dried for 3 hours at 105°C. It is subtracted the weight of the filtering aid from the weight of the residue, the amount should not be more than 2%.

Loss on Drying When Tara Gum is dried for 5 hours at 105°C, the loss should not be more than 15%.

Ash When Tara Gum is tested as indicated under ash, the amount should not be more than 1.5%.

72. Tamarind Gum

Definition Tamarind Gum is obtained by extracting with endosperms of rice grain or tamarind (*Tamarindus indica* LINNE) seeds with warm water, hot water, or aqueous alkaline solution. It can also be obtained by treating the same with β -galactosidase. Its major component is polysacchiride.

Compositional Specifications of Tamarind Gum

Description Tamarind Gum is brownish gray- white powder having a slight odor.

Identification (1) 1 g of Tamarind Gum is dissolved in 100 ml of water at 80°C by stirring vigorously. When it is cooled to room temperature, it becomes slightly turbid and viscous neutral liquid. When 3 ml of saturated sodium sulfate solution is added to 5 ml of this liquid, it becomes a jelly phase.

(2) 1 g of Tamarind Gum is slowly added and dissolved in 100 ml of 50% sugar solution at 80°C by stirring vigorously. After boiling carefully for 5 minutes, and then allowed to stand. It becomes solid of jelly phase.

Purity (1) Arsenic : 0.5 g of Tamarind Gum is tested as directed under Purity (1) for Guar Gum (Not more than 2ppm).

(2) Lead : When 5.0 g of Tamarind Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Starch : 0.1 g of Tamarind Gum is dissolved in 10 ml of water, which is heated and then cooled. When 2 drops of iodine solution are added, it should not turn blue.

(4) Protein : When approximately 0.5 g of Tamarind Gum is tested as directed in Kjeldahl Method under Nitrogen Determination, the amount should not be more than 3%. (Protein Factor : 5.7).

(5) Crude Fat : 10 g of Tamarind Gum is precisely weighed into a cylindrical filter paper (Thimble Filter) and dried for 3 hours at 105°C. It is then extracted for 20 hours using a soxhlet extractor in a water bath. Then remove ether from the extract, and dried for 2 hours at 105°C. The content of crude fat should not be more than 1%.

Loss on Drying When 3 g of Tamarind Gum is dried for 3 hours at 100°C, the loss should not be more than 7%.

Ash 1 g of Tamarind Gum is tested for ash. The amount should not be more than 5%.

73. Tamarind Color

Definition Tamarind Color is obtained by roasting and extracting with water from tamarind seeds (*Tamarindus indica* L. of leguminosae, a bean family). Its major pigment component is flavonoid. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Tamarind Color

Content Color value ($[E]_{1cm}^{10\%}$) of Tamarind Color should be higher than the indicated value.

Description Tamarind Color is reddish brown~blackish brown liquid, lump, powder, or paste with a slight characteristic odor.

Identification (1) Test Solution obtained in Color Value section of Tamarind Color shows reddish brown.

(2) 0.5 g of Tamarind Color is dissolved in 100 ml of water. When 10 ml of this solution is acidified with 1 ml of hydrochloric acid, reddish brown precipitate is formed.

(3) 0.5 g of Tamarind Color is dissolved in 100 ml of water. When 2 ml of ferric chloride solution (1→50) is added to 10 ml of this solution, blackish brown precipitate is formed.

Purity (1) Arsenic : 0.25 g of Tamarind Color is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Tamarind Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay (Color Value) Appropriate amount of Tamarind Color is precisely weighed so that the absorbance is within 0.3~0.7 and dissolved in acetic acid·sodium acetate buffer solution with pH 7.0 so that total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using acetic acid·sodium acetate buffer solution with pH 7.0 as a reference solution, absorption A is measured at the maximum absorbance at 500 nm with 1 cm cell. Color value is

obtained using the following equation.

$$\text{Color Value } ([E]_{1\text{cm}}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citric acid • dibasic sodium phosphate buffer solution (pH 7.0)

Solution 1 : 0.1M citric acid solution : 1L of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2M dibasic sodium phosphate solution : 1L of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (35:165) and its pH is adjusted to 7.0.

74. Tannic Acid

Definition Tannic Acid is usually obtained from gallnut.

Compositional Specifications of Tannic Acid

Description Tannic Acid is yellowish white~pale brown amorphous powder, glistening scale shaped or spongy mass, odorless or with a faint, characteristic odor and astringent taste.

Identification (1) 1 g of Tannic Acid is dissolved 10 ml of water. When a small amount of ferric chloride solution is added, a bluish black colour or precipitate is formed.

(2) When alkaloid salts, albumin, or gelatin solution is added to the Test Solution obtained in (1), precipitate is formed.

Purity (1) Arsenic : 0.25 g of Tannic Acid is placed in a flask for decomposition. After adding 5 ml of sulfuric acid and 5 ml of nitric acid, the flask is slowly heated. 2~3 ml of nitric acid is added at a time and the flask is heated until the liquid becomes colorless~pale yellow. After cooling, 15 ml of saturated ammonium hydroxide is added to the flask, which is then heated and concentrated to 2~3 ml until thick white smoke is generated. The resulting concentrate is neutralized with ammonia water or ammonia solution. This test solution is tested for arsenic. Standard color reference is prepared with 1 ml of arsenic standard solution following the same procedure as the test solution. The amount of arsenic should not be more than 4ppm.

(2) Lead : When 5.0 g of Tannic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Residual Solvent : 1g of Tannic Acid is precisely weighed into a sample vial, 5 μ l of water is added, and seal it quickly with a septum, test solution. Proceed headspace-gas chromatography under operation conditions below and measure each amount of acetone and ethyl acetate from each calibration curve, it should be not more than 25ppm as individual or sum if used together

Operation Condition

Column : HP-1 or its equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Injection Port Temperature : 110°C

Column Temperature : 40°C

Detector Temperature : 110°C

Carrier gas : Nitrogen or helium

Head space sampler

Heating temperature : 80°C

Heating time : 40 minutes

Sample gas injection : 0.4ml

Mixed standard solution : 1 g of acetone and 1 g of ethyl acetate is precisely weighed into each flask, water is added to make 100 ml. 2, 20, 40 ml each of this solution is taken, water is added to make 100 ml, each mixed standard solution. (1 ml of each mixed standard solution contains 200, 2,000, 4,000µg of acetone and ethyl acetate, respectively).

Preparation of calibration curve : 1 g of tannic acid, free of acetone and ethyl acetate, is precisely weighed into a vial, 5µl each of 200, 2,000, 4,000ppm of mixed standard solution is added respectively, and seal it quickly with a septum. Proceed headspace-gas chromatography under operation conditions below and measure the peak area of acetone and ethyl acetate. From the peak area, prepare each calibration curve.

(4) Gums or Dextrins : 1 g of Tannic Acid is dissolved in 5 ml of water, which is then filtered. When 10 ml of alcohol is added to the filtrate, no turbid is produced within 15 minutes.

(5) Resinous substances : 1 g of Tannic Acid is dissolved in 5 ml of water, which is then filtered. When the filtrate is diluted to 15 ml with water, no turbid is produced

Loss on Drying When 3 g of Tannic Acid is dried for 2 hours at 105°C, the loss should not be more than 12%.

Residue on Ignition When Residue on Ignition is done with 1 g of Tannic Acid, the amount of residue should not be more than 1%.

75. Talc

Definition Talc is purified from natural hydrated magnesium silicate. It may contain small amount of aluminum silicate.

Compositional Specifications of Talc

Description Talc is odorless and white~greyish white crystalline powder with a slippery touch.

Identification 0.2 g of Talc is mixed with 0.9 g anhydrous sodium carbonate and 1.3 g of anhydrous potassium carbonate. It is then heated until it melts completely in a platinum or nickel crucible. After cooling, it is transferred into a beaker with approximately 5 ml of hot water. Hydrochloric acid is slowly added until foaming stops. After adding 10 ml of hydrochloric acid, it is evaporated to dryness. After cooling, 20 ml of water is added to the residue, which is boiled and filtered. Gel phase residue on the filter paper. When the filtrate shows the reaction of Magnesium Salts in Identification.

Purity (1) Water-soluble substances and pH : 10 g of Talc is added to 100 ml of water. It is then heated for 2 hours while adding water to supplement the loss and shaking occasionally. After cooling, it is filtered using a Millipore filter. If the filtrate is turbid, it is filtered again through the same filter. The beaker and the filter is washed with water, which is added to the filtrate. The total volume of the filtrate is brought up to 100 ml with water. Use this solution as the test solution. pH of the test solution should be 7.5~9.5. 50 ml of the test solution is evaporated to dryness, which is then dried for 2 hours at 105°C. The weight of residue does not exceed 10 mg.

(2) Hydrochloric acid soluble substances : 1 g of Talc is mixed with 20 ml of diluted hydrochloric acid, which is stir-mixed and heated for 15 minutes at 50°C. After cooling, it is filtered. The beaker and the residue on the filter is washed with water, which is added to the filtrate. The total volume of the filtrate is brought up to 20 ml with water. 1 ml of diluted sulfuric acid is added to 10 ml of the filtrate, which is evaporated to dryness and heat treated at 550°C until the weight becomes constant. The residue does not exceed 10mg.

(3) Arsenic : 5 ml of diluted hydrochloric acid (1→4) is added to 0.25 g of Talc. It is then slowly boiled while shaking. The mixture is quickly cooled and filtered. The residue is washed with 5 ml of diluted hydrochloric acid (1→4) followed by 10 ml of water, which is added to the filtrate. The resulting solution is tested for arsenic

and the content should not be more than 4ppm.

- (4) Water-soluble iron : 20 ml of test solution in (1) is slightly acidified with hydrochloric acid. When 1 drop of potassium ferrocyanide solution is added to this solution, the solution does not turn blue.
- (5) Lead : Accurately weigh 5 g of Talc, add 40 ml of diluted hydrochloric acid and 50 ml of water, mix well, mildly heat, cool, then filter. Wash the residue on the filter paper, combine the rinsings to the filtrate, then make up to 250 ml with water. Take 125 ml of this solution, evaporate to dry in the water bath, add 10 ml of diluted hydrochloric acid(1→10) to the residue, and make up to 10 ml, test solution. Separately, pipette 1 ml of lead standard solution, add diluted hydrochloric acid(1→10), then make up to 20 ml, reference solution. When test solution and reference solution are tested by flame Atomic Absorption Spectrophotometry under following operation condition, the absorbance of test solution should not be higher than that of reference solution (not more than 2ppm).

Operation Condition

Light source lamp : lead cathode lamp

Analysis curve wavelength : 283.3nm

Combustible support gas : air

Combustible gas : acetylene

- (6) Asbestos : Proceed test as directly under following (A) or (B), asbestos should not be detected. When asbestos is detected in the test by following (A) or (B), additionally test by (C), and asbestos should not be detected.

(A) Asbestos is measured by Potassium Bromide Disk Method in Infrared Spectrophotometry, absorption is identified at $600\sim650\text{cm}^{-1}$ (serpentine) or $757\sim759\text{cm}^{-1}$ (amphibole) of wave number. When absorption peak is at wave number $757\sim759\text{cm}^{-1}$, a certain amount of sample is ignited for 30 minutes at 850°C , cooled, again proceed under Infrared Spectrophotometry, and identify the absorption peak at wave number $757\sim759\text{cm}^{-1}$ which indicates tremolite in amphibole.

(B) When powder diffraction of Talc is measured with Powder X-Ray Diffractometer under following operation condition, the angle of diffraction 2θ identifies diffraction peak of $10.4\sim10.6^{\circ}$ (amphibole), $24.2\sim24.4^{\circ}$, and $12.0\sim12.2^{\circ}$ (serpentine).

Operation condition

X-ray light source : Cu Ka monochromator

Tube current and tube voltage : 24~30mA, 40kV

Incidence angle : 1°

measurement angle : 0.2°

Scanning speed : 0.1°/minute

Scanning range (angle of diffraction 2θ) : 10~13°, 24~26°

(C) Observe form and color of asbestos with optical microscope, asbestos is confirmed if the following criteria are met:

- ① The ratio of length and width of fiber is in the range of 20:1 to 100:1 or when the length of fiber is longer than 5 μm , the ratio of length and width is not less than 100:1.
- ② It can be split into very thin microfibrils.
- ③ if 2 or more of the following 4 criteria are met:
 - Ⓐ parallel fibers occurring in bundles
 - Ⓑ fiber bundles displaying worn or frayed ends
 - Ⓒ fibers in the form of thin needles
 - Ⓓ matted masses of individual fibres and/or fibres showing curvature

Loss on Drying When Talc is dried for 1 hour at 105°C, the loss should not be more than 0.5%.

Loss on Ignition When loss on Ignition is done, weight loss should not be more than 6%.

76. d-Tocopherol Concentrate, Mixed

Definition Mixed d-Tocopherol Concentrate is a concentrate of d-Tocopherol obtained from edible vegetable oil. Main ingredients are d- α -Tocopherol, d- β -Tocopherol, d- γ -Tocopherol, and d- δ -Tocopherol. Edible vegetable oil can be added to adjust the content.

Compositional Specifications of d-Tocopherol Concentrate, Mixed

Content Mixed d-Tocopherol Concentrate contains not less than 34.0% of total tocopherol.

Description Mixed d-Tocopherol Concentrate is reddish brown~red, transparent viscous liquid having a mild, characteristic odor and taste.

Identification (1) 50 mg of Mixed d-Tocopherol Concentrate is dissolved in 10 ml of anhydrous alcohol, where 2 ml of nitric acid is added. When this solution is heated at 75°C for 15 minutes, the solution developed bright red or orange.

(2) The retention time of major peak of chromatogram (obtained in Assay) for high α -type match with those of standard preparation as compared with the retention time of peak of internal standard. The retention time of the third major peak of chromatogram (obtained in Assay) for low α -type matches with that of standard preparation as compared with internal standard.

Purity (1) Acidity : 1 g of Mixed d-Tocopherol Concentrate is dissolved in 25 ml of mixture of equal volumes of alcohol and ether that has been neutralized to phenolphthalein TS with 0.1 N sodium hydroxide, 0.5 ml of phenolphthalein TS is added to this solution, which is then titrated with 0.1 N sodium hydroxide solution until the solution remains faintly pink after shaking for 30 seconds. Not more than 1 ml of 0.1 N sodium hydroxide is required.

(2) Lead : When 5.0 g of Mixed d-Tocopherol Concentrate is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Arsenic : 0.25 g of Mixed d-Tocopherol Concentrate is placed in a platinum, quartz, or porcelain crucible. 10 ml solution of magnesium nitrate in ethyl alcohol (1 \rightarrow 50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat-treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

- (4) Mercury : When 0.1 g of Mixed d-Tocopherol Concentrate is tested by Mercury Test Method, its content should not be more than 1.0ppm.
- (5) Specific Rotation : Mixed d-Tocopherol Concentrate is weighed, equivalent to about 100 mg of total tocopherols, to a separator and dissolved in 50 ml of ether. It is added 20 ml of a 10% solution of potassium ferricyanide in sodium hydroxide solution (1→125), and shake for 3 minutes. Ether solution is washed with 50 ml of water and dehydrated with anhydrous sodium sulfate. It is evaporated the dried ether solution on a water bath under reduced pressure until about 7~8 ml remain, and then removed the last traces of ether in a blowing nitrogen at a room temperature. The residue is immediately dissolved in 5 ml of isooctane. When specific rotation is calculated, it should be high α -type $[\alpha]_D^{25} = +24^\circ$ (or higher), low α -type $[\alpha]_D^{25} = +20^\circ$ (or higher).

Assay

Solutions

- Internal Standard Solution : About 600 mg of hexadecyl hexadecanoate is precisely weighed and dissolved in 2 parts of pyridine and 1 part of anhydrous propionic acid in a 200 ml volumetric flask, and diluted to volume with the solution.
- Preparation of Standard Solution : 12, 25, 37, and 50 mg portions of α -tocopherol standard is precisely weighed into each of 50 ml Erlenmeyer flask with a ground joint neck, where 25 ml each of internal standard solution is added. It is refluxed for 10 minutes under water-cooled condensers.
- Preparation of Test Solution : About 60 mg of Mixed d-Tocopherol Concentrate is precisely weighed into another Erlenmeyer flask, where 10 ml each of internal standard solution is added. It is refluxed for 10 minutes under water-cooled condensers. Gas chromatography is carried out under the following conditions.

Operation Conditions

- Column : Glass tube 4 mm inner diameter \times 2 m length
- Column Filler : 80 to 100 mesh Chromosorb W-DMCS coated with 2% to 5% methylpolysiloxane gum
- Detector : (Hydrogen) Flame Ionization Detector (FID)

- Temperature at injection port : 290°C
- Column Temperature : a constant temperature in a range of 240~260°C
- Detector Temperature : 300°C
- Carrier gas and its flow rate : Nitrogen, Flow rate is adjusted so that hexadecyl hexadecanoate is detected in 18~20 minutes.

System Suitability

Chromatograph a suitable number of injections of the Assay Preparation, as directed under Calibration, to assure that the resolution factor, R, between the major peaks occurring at retention times of approximately 0.5(δ -tocopheryl propionate) and 0.63(β - γ -tocopheryl propionate), relative of hexadecyl hexadecanoate at 1.0, is not less than 2.5.

Calibration Curve

Chromatograph successive 2 to 5 μ l portions of each Standard Preparation until the relative response factor, F, for each is constant(i.e. within a range of approximately 2%) for three consecutive injection. Measure the areas under the first (α -tocopheryl propionate) and the second (hexadecyl hexadecanoate) major peaks (excluding the solvent peak), and record the values as A_s & A_1 , respectively.

A factor "F" for each concentration of each Standard Solution is obtained from the following equation.

$$F = \frac{A_s}{A_1} \times \frac{C_1}{C_s}$$

C_1 : Exact concentration of internal standard solution (mg/ml)

C_s : Exact concentration of tocopherol standard solution (mg/ml)

Relative reaction coefficient curve is prepared by plotting peak area of α -tocopheryl propionate vs. relative reaction coefficient.

Test Procedure : 2~5 μ l of Test Solution is injected into chromatograph and measured the areas under the four major peaks occurring at relative retention times of 0.50, 0.63, 0.76, and 1.00. The content (mg) of each tocopherol type for δ -tocopheryl propionate, β - + γ -tocopheryl propionate, α -tocopheryl propionate and hexadecyl hexadecanoate in sample is calculated by the following equation.

$$\delta\text{-tocopherol} = \frac{10C_1}{F} \times \frac{a_\delta}{a_1}$$

$$\beta^- + \gamma\text{-tocopherol} = \frac{10C_1}{F} \times \frac{a_{\delta+\gamma}}{a_1}$$

$$\alpha\text{-tocopherol} = \frac{10C_1}{F} \times \frac{a_\alpha}{a_1}$$

F is obtained from the relative response factor curve for each the corresponding areas under the δ^- , $\beta^- + \gamma^-$, and α -tocopheryl propionate peak produced by the Sample Preparation. The relative response factor for δ -tocopheryl propionate and for $\beta^- + \gamma^-$ -tocopheryl propionate has been determined empirically to be the same as for δ -tocopheryl propionate.

77. d- α -Tocopherol Concentrate

Definition d- α -Tocopherol Concentrate is a form of vitamin E obtained from edible vegetable oil. Its major component is d- α -tocopherol. Edible vegetable oil can be added to adjust the required amount of total tocopherols.

Compositional Specifications of d- α -Tocopherol Concentrate

Content d- α -Tocopherol Concentrate should contain not less than 40.0% of total tocopherol, of which not less than 95.0% consists of d- α -tocopherol.

Description d- α -Tocopherol Concentrate is pale yellow~reddish brown, clear viscous oil with slightly characteristic odor.

Identification (1) 50 mg of d- α -Tocopherol Concentrate is dissolved in 10 ml of absolute alcohol, where 2 ml of nitric acid is added. When this solution is heated for 15 minutes at 75°C, bright red or orange colour developed.

(2) The retention time of the major peak in the chromatogram of the sample solution is the same as that of the standard solution, both relative to the internal standard, as obtained in the Assay.

Purity (1) Acidity : 1 g of d- α -Tocopherol Concentrate is dissolved in 25 ml of a mixture of equal volumes of alcohol and ether that has been neutralized to phenolphthalein TS with 0.1 N sodium hydroxide. 0.5 ml of phenolphthalein TS is added to this solution, which is then titrated with 0.1 N sodium hydroxide solution until the solution remains pale red color after shaking for 30 seconds. The consumed amount of the titrant should not be more than 1 ml.

(2) Lead : When 5.0 g of d- α -Tocopherol Concentrate is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Specific Rotation : d- α -Tocopherol Concentrate is weighed, equivalent to about 100 mg of total tocopherol, to a separator, and dissolved it in 50 ml of ether. It is added 20 ml of a 10% solution of potassium ferricyanide in sodium hydroxide solution (1→125) to the separator, and shake for 3 minutes. Ether solution is washed with 50 ml of water and dehydrated with anhydrous sodium sulfate. It is evaporated the dried ether solution on a water bath under reduced pressure until about 7~8 ml remain, and then removed the last traces of ether in a blowing nitrogen at a room temperature. The residue is immediately dissolved in 5 ml of isooctane. When specific rotation is calculated, it should be high α -type $[\alpha]_D^{25} = +24^\circ$ (or higher).

Assay Should follow the procedure in Assay for d-tocopherol (mixed).

78. Tragacanth Gum

Definition Tragacanth Gum is a polysaccharide obtained by drying the exuded secretion from stems of *Astragalus gummifer* LABILL. of leguminosae or allied species.

Compositional Specifications of Tragacanth Gum

Description Tragacanth Gum is white or whitish powder or white~pale yellowish white, semi-transparent flexible keratinous platelet or thin fragments.

Identification (1) When 50 ml of water is added to 1 g of Tragacanth Gum, it gradually forms almost uniformly dispersion.

(2) When iodine solution is added to the powder of Tragacanth Gum and examined under a microscope, a few number of blue starch grains are observed.

Purity (1) Arsenic : 0.25 g of Tragacanth Gum is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with a small amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Tragacanth Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Tragacanth Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Tragacanth Gum is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) E. Coli : When Tragacanth Gum is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

(6) Salmonella : When Tragacanth Gum is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

(7) Karaya Gum : 20 ml of water is added to 1 g of Tragacanth Gum, which is boiled until it forms a homogeneous viscous liquid. 5 ml of hydrochloric acid is add

to the this solution, which is then boiled for 5 minutes. its color should not develop light pink~red.

- (8) Viscosity : 4.0 g of fine powder is weighed into a stirring container. It is uniformly wetted with 10 ml of alcohol, added 390 ml of water, then stirred with for 7 minutes (care must be taken to prevent lump formation). The resulting suspension is transferred into a 500 ml bottle. It is then capped and allowed to stand for 24 hours in a 25°C water bath (Test Solution). Test Solution is tested by 2. Rotational Type Viscosity Measurement in Viscosity Measurement. It should not be less than 250 cps.

- (9) Acid Insoluble Ash : When Tragacanth Gum is tested as directed under Acid Insoluble Ash in Ash Test Method, the amount should not be more than 0.5%.

Ash When Tragacanth Gum is tested as directed under total ash in Ash Test, the amount of ash should not be more than 3.0%.

79. Trypsin

Definition Trypsin is an enzyme obtained from extracts of pancreas of pigs and cows. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Trypsin

Content Trypsin contains 90~130% of the indicated activity as Trypsin .

Description Trypsin is white~pale yellow~deep brown powder, granule, lump or transparent dark brown liquid.

- Purity**
- (1) Arsenic : 0.25 g of Trypsin is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with a small amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
 - (2) Lead : When 5.0 g of Trypsin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
 - (3) Coliform Group : Trypsin is tested by Microbe Test Methods for Coliform Group in General Test Methods in Food Code. It should not be more than 30 cfu per 1 g of this product.
 - (4) Salmonella : Trypsin is tested by Microbe Test Methods for Salmonella in General Test Methods in Food Code. It should be negative (-).
 - (5) E. Coli : When Trypsin is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

- Applications : This test is to measure the activity of trypsin in an enzyme obtained from extracts of pancreas of pigs and cows.
- Preparation of Test Solution : The final diluted solution in 0.001N hydrochloric acid is prepared so that it contains 3,000 trypsin units per 1 ml. A certain amount of this solution is diluted with 0.001N hydrochloric acid so that 0.2 ml of the solution contains 12, 18, and 24 Trypsin units. This solution is used as following Test Procedure.

◦ Test Procedure : This test is carried out with maintaining around the cell at $25 \pm 0.1^\circ\text{C}$. Temperature of the reaction cell should be checked before and after the measurement and the difference should not be more than 0.5°C . 0.2 ml of 0.001 N hydrochloric acid and 3 ml of substrate solution are placed in a 1cm cell. It is set up in a spectrophotometer. It is adjusted so that the absorbance at 253 nm is 0.050. In another cell, accurately pipetted 0.2 ml of Test Solution containing 12 Trypsin units, where 3 ml of substrate solution is added. Using a spectrophotometer, absorbance is measured in a 30 second interval for 5 minutes. This is repeated with the Standard Solutions containing 18 and 24 Trypsin units. Absorbance curve vs. time for each concentration is plotted. Only the values in straight line region are used. An average value of 3 concentrations (only in straight line region) is taken as Trypsin activity. Trypsin units/mg for each concentration is obtained from the following equation.

$$\text{Trypsin units} = \frac{A1 - A2}{T \times W \times 0.003}$$

A1 : The last absorbance on the straight line

A2 : The initial absorbance on the straight line

T : Time difference between the initial and the last time (min)

W : Amount of Trypsin used for absorbance measurement (mg)

Definition of Activity : 1 Trypsin unit corresponds to an activity that changes 0.003 absorbance unit per minute under the test conditions above.

Solutions

- 1/15M Phosphate Buffer Solution (pH 7.6) : 4.54 g of potassium phosphate, monobasic is dissolved in sufficient amount of water to make 500 ml. Separately, 4.73 g of anhydrous potassium phosphate, dibasic is dissolved in sufficient amount of water to make 500 ml. 13 ml of potassium phosphate, monobasic solution is mixed with 87 ml of potassium phosphate, dibasic solution
- Substrate Solution : 85.7 mg of N-benzoyl-L-arginine ethyl ester hydrochloride for Trypsin analysis is dissolved in water and the total volume is brought up to 100 ml with water (note : Using Trypsin reference standard, appropriateness of substrate and adjustment of spectrophotometer are checked). 10 ml of this solution is diluted to 100 ml with 1/15 M phosphate buffer solution (pH 7.6). Absorbance of this solution is measured at 253 nm with 1cm cell

at $25 \pm 0.1^\circ\text{C}$ using water as a reference. Absorbance is adjusted to a range of 0.575~0.585 using 1/15 M phosphate buffer solution if necessary. This solution should be used within 2 hours after it is prepared.

Storage Standard of Trypsin

Trypsin is strongly hygroscopic, hence should be stored in a cold dark place with sealing tightly.

80. Oleoresin Paprika

Definition Paprika Extract colorant is a carotinoid colorant that is obtained by extracting fruits of paprika (*Capsicum annum* L.) of solanaceae with organic solvents (extracting solvent for spices such as oleoresin). Its major colorant component is capsanthin. Dilutant, antioxidant, or other food additives (emulsifier, thickening agent, etc) can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Paprika Extract colorant

Content Color Value (ASTA) of Paprika Extract colorant should be higher than the labeled value.

Description Paprika Extract colorant is orange~dark brown liquid, paste, or powder with a slight characteristic odor.

Identification (1) Test Solution obtained in Color Value section shows the maximum absorption at about 453 nm or 470 nm.

(2) When 2 ml of sulfuric acid is added to 0.5 g of Paprika Extract colorant, the color of the liquid changes from orange to blue.

(3) When antimony trichloride solution is added to Paprika Extract colorant, it developed a blue.

Purity (1) Arsenic : 0.25 g of Paprika Extract colorant is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Paprika Extract colorant is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Cadmium : When 5.0 g of Paprika Extract colorant is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

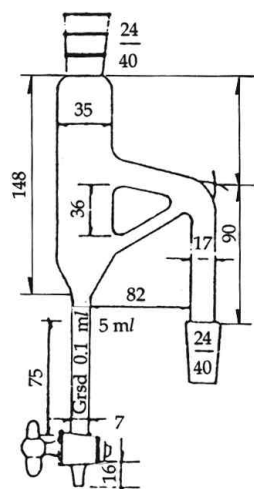
(4) Mercury : When 0.1 g of Paprika Extract colorant is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Residual Solvents : When Paprika Extract colorant is tested by the following test method, it should contain,

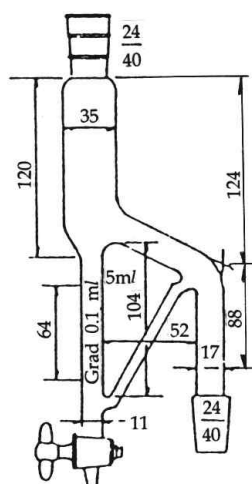
Methylene chloride, trichloroethylene	Not more than 30ppm (individual or sum if used together)
Acetone	Not more than 30ppm
Isopropyl alcohol	Not more than 50ppm
Methyl alcohol	Not more than 50ppm
Hexane	Not more than 25ppm

Test Method

- ① Distilling head : A clevenger trap, that is designed to used for oils (heavier than water), is used.



(a) For oils heavier than water



(b) For oils lighter than water

Distilling head : Clevenger Traps (Units : mm)

② Agents

- Toluene : The purity of toluene for this test should be such that it does not contain any solvents that are measured in this test as determined by gas chromatography using the following column or its equivalent.

- A) 35~80 mesh Chromosorb W Ucon 75 H 90,000
- B) 35~80 mesh Chromosorb W Ucon LB 135 20%
- C) 60~80 mesh Chromosorb W Ucon LB 1715 15%
- D) Porapak Q 50~60 mesh

Test proceed as directed under Test Procedure. The same amount of toluene is

injected as the amount used in solvent analysis. If any peaks for interfering impurities appear before the toluene peak, they should be removed by separation.

- Benzene : It should not contain any interfering impurities. Its purity is measured by the same method as toluene.
- Detergent & antifoam : It should not contain a volatile matter. If it does, an aqueous solution of the material should be heated until a volatile matter is removed.
- Reference Solution A : Toluene containing 2,500ppm of benzene is prepared. If the toluene contains benzene as the only impurity, the concentration should be adjusted so that it contains 2,500ppm of benzene as determined by gas chromatography.
- Reference Solution B : A solution containing 0.63% v/w of acetone in water is prepared.

③ Preparation of Test Solution

- Test Solution A (for solvents except methyl alcohol) : Small amount of detergent & antifoam, 50 ml of water, 10 g of anhydrous sodium sulfate, 1 ml of Reference Solution A, and 50 g of sample are added in a 250 ml single neck round bottom flask with a 24/40 ground joint. 400 mm reflux condenser, distilling head, and a collector is connected to the flask and 15 ml of distillate is collected. 15 g of anhydrous potassium carbonate is added to the distillate. It is then cooled while shaking. It is allowed to stand until layer is separated. The toluene layer contains all the solvents except methyl alcohol and is used in the following Test Procedure. The aqueous layer is used as Test Solution B.
- Test Solution B (for methyl alcohol only) : 50 ml of aqueous layer obtained in Test Solution A is transferred into a round bottom flask, where 2~3 glass balls and 1 ml of Reference Solution B are added. Approximately 1 ml of distillate is collected. This distillate contains acetone as internal standard substance and methyl alcohol in the sample. It is used in the following Test Procedure.

- ④ Test Procedure : Gas chromatography equipped with a thermally conductive detector and sample injection port is used. Under typical operation conditions, a 0.3 mm × 2 m column (isothermalized at 70~80°C) is used. Flow rate of a carrier gas is 50~80 ml/minute and injected amount of sample is 15~20 µl. The choice of a column depends on the components to be analyzed and is up to the analyzer to a certain degree. The columns described in A), B), C), and D) in Toluene section are used as follows.

- A) This column separates acetone and methyl alcohol from the aqueous layer. This also can be used to analyze or separate of hexane, acetone, and trichloro ethylene in the toluene layer obtained in Test Solution A. The order of effluence is acetone, methyl alcohol and water or hexane, acetone, isopropyl alcohol+methylene chloride, benzene, trichloro ethylene, and dichloro ethylene+toluene.
- B) This column separates methylene chloride, isopropyl alcohol, and dichloro ethylene. The order of effluence is hexane+acetone, methylene chloride, isopropyl alcohol, benzene, dichloro ethylene, trichloro ethylene, and toluene.
- C) This is the most generally used column except for methyl alcohol. The order of effluence is hexane, acetone, methylene chloride, isopropyl alcohol, benzene, and dichloro ethylene+trichloro ethylene, and toluene.
- D) This column is used to measure methyl alcohol, which appears right after a big peak of water.
- ⑤ Preparation of Calibration Curve : A mixture of benzene and solvent, previously known concentration, in toluene is injected into gas chromatography. The response of the detector for the previously known ratios of solvents is measured. The peak(area or height) of benzene and the solvents in toluene should be the same as those of the sample. Peaks areas for the solvents are calculated according to benzene and the weighing factor F is calculated as follows.

$$F(\text{solvent}) = \frac{\text{Wt}\% \text{ solvent}}{\text{wt}\% \text{ benzene}} \times \frac{\text{peak area of benzene}}{\text{peak area of solvent}}$$

When it is compared with benzene recovery rate, the recovery rates of various solvents from oleoresin sample are as follows.

hexane 52%, acetone 85%, isopropyl alcohol 100%, methylene chloride 87.5%, trichloro ethylene 113%, dichloro ethylene 102%, methyl alcohol 87%

- ⑥ Calculation : The concentration of residual solvents (except for methyl alcohol) is calculated by the following equation.

$$\text{Residual Solvent} = \frac{43.4 \times F(\text{solvent}) \times 100}{\text{Solvent recovery rate}\%} \times \frac{\text{peak area of solvent}}{\text{peak area of benzene}}$$

43.4 is the concentration(ppm) of internal standard benzene related to 50 g of oleoresin

sample used in the test.

$$\text{Methyl alcohol} = \frac{100 \times F(\text{methylalcohol})}{0.87} \times \frac{\text{peak area of methylalcohol}}{\text{peak area of acetone}}$$

100 is the concentration(ppm) of internal standard acetone related to 50 g of oleoresin sample used in the test.

(5) Spicy Taste : 400 mg of oleoresin paprika is weighed into a 100 ml volumetric flask, which is filled with alcohol. It is then mixed by shaking and settled to precipitate. 60 ml of 10% sugar solution in water is added to 0.15 ml of the supernatant. When 5 people consume 5 ml each of the resulting solution, should not be more than 3 people who feel the spicy taste.

Assay (Color Value) Appropriate amount of oleoresin paprika, the absorbance to be measured will be within a range of 0.2 to 0.7, is precisely weighed into a 100 ml volumetric flask and dissolved in acetone to make total volume 100 ml. (If it is water soluble, water is used instead of acetone). After settling for 2 minutes, 1 ml of this solution is diluted to 100 ml with acetone (For water soluble sample, diluting with acetone may cause severe turbidity. In this case, 1 ml of alkaline lead acetate solution (1→50) is added to the solution and then diluted to 100 ml with acetone. It is then centrifuged and the supernatant is used.). Absorbance(As) of the resulting solution is measured at 460 nm with 1 cm cell using acetone as a reference. Using the same method, absorbance of NBS (National Bureau of Standard) color standard glass plate 2030 (AF) is measured.

$$\text{Color Value (ASTA)} = \frac{As \times 164 \times F}{W} \times 10$$

W : Weight of sample(g)

ASTA : American Spice Trade Association

F : AN/AF, where AN is absorbance of glass filter defined by NBS.

Therefore, F is an activity for correcting of the instrument.

* If colorimetric glass plate is not available, a color standard solution is used.

◦ Color standard Solution : $\text{CoSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ is dried for 1 week in a desiccator with dried silica gel. 0.3005 g of $\text{K}_2\text{Cr}_2\text{O}_7$ and 34.960 g of dried $\text{CoSO}_4(\text{NH}_4)_2\text{SO}_4$ are dissolved in 1.8 M sulfuric acid to make 1,000 ml. The absorbance of this solution at 460 nm with 1 cm cell is 0.600.

81. Perlite

Definition Perlite is prepared by calcining mineral silicon dioxide at 800~1,200°C.

Compositional Specifications of Perlite

Description Perlite is white or light gray powder.

Identification Proceed as directed under Identification (1) for Diatomaceous Earth.

Purity (1) Water Solubles and pH : When Perlite proceed as directed under Purity (1) in Diatomaceous Earth, pH should be 5.0~9.0, and the residues should not be more than 10 mg.

(2) Hydrochloric Acid Solubles : When Perlite proceed as directed under Purity in Diatomaceous Earth, the content should not be more than 15 mg.

(3) Arsenic : Proceed as directed under Purity (3) in Diatomite. 5 ml of liquid A in (2) should be used as test solution and should not more than 4ppm.

(4) Lead : Proceed as directed under Purity (5) in Diatomaceous Earth and the content should not be more than 10ppm.

Hydrofluoric Acid Residue When Perlite proceed as directed under Hydrofluoric Acid Residue in Diatomaceous Earth, the content should not be more than 75 mg.

Loss on Ignition When Loss on Ignition is done at 1,000°C for 30 minutes, weight loss should not be more than 3%.

82. Pectinase

Definition Pectinase is an enzyme obtained from cultures of *Aspergillus niger*, cultures of *Aspergillus oryzae* where pectinase gene of *Aspergillus aculeatus* is inserted, and decompose pectin and pectin acid. Polygalacturonase, pectinesterase, and pectin lyase are included. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Pectinase

Content Pectinase contains 90~130% of the labeled activity as Pectinase.

Description Pectinase is white~pale yellow~brown powder or transparent~brown liquid.

Purity (1) Lead : Accurately weigh 2 g of Pectinase and place it in a platinum or quartz crucible. Add minute amount of sulfuric acid, wet, gradually heat and preliminarily heat-treated at the temperature as low as possible. Again add 1 ml of sulfuric acid, gradually heat, ignite until it is heat-treated at 450~550°C. After heat-treating, add minute amount of nitric acid(1→150) to the residue, again, add nitric acid(1→150) to make 10 ml, test solution. Separately, weigh 1 ml of lead standard solution, add nitric acid(1→150) to make 10 ml, reference solution. When test solution and reference solution are tested by flame Atomic Absorption Spectrophotometry under following operation condition, the absorbance of test solution should not be higher than that of reference solution (not more than 5 ppm)

(2) Coliform Group : When Pectinase proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods 「Standards and Specifications for Foods」 (noticed by KFDA), it should not contain more than 30 cfu per 1 g of this product.

(3) Salmonella : When Pectinase proceed as directed under Microbe Test Methods for Salmonella in General Test Methods 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

(4) E. Coli : When 25 g of Pectinase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity) Each of method 1, 2, and 3 is applied to polygalacturonase, pectinesterase, and pectin lyase, respectively.

Method 1

Principle : This test is to measure the amount of reducing sugar of Galacturonic acid

generated by hydrolysis of pectic acid at pH 4.0, temperature 40°C.

Preparation of Test Solution : Test Solution is prepared by dilution a certain amount of sample with citric acid buffer solution (pH 4.0) so that 1 ml contains 40~60 Pectinase units.

Test Procedure : 10 ml of substrate solution is maintained in a 100 ml Erlenmeyer flask in a water bath for 5 minutes at 40°C. 1 ml of Test Solution is added to the substrate solution, which is immediately shaken and mixed, allowed to stand for exactly 30 minutes at 40°C, 3ml of anhydrous sodium carbonate solution(106→1,000) is added, and reaction is stopped. 6 ml of 0.1 N iodine solution is added, which is then shaken, mixed, and allowed to stand for 30 minutes in a dark place. After adding 6 ml of 8 N sulfuric acid and the solution is quickly titrated with 0.02 N sodium thiosulfate solution until the color of iodine almost disappear. Again 1 ml of starch solution is added, 0.02 N sodium thiosulfate solution is drop-wise added, titrated until the blue color disappear(Aml). Separately, a blank test is carried out with 3 ml of anhydrous sodium carbonate solution(106→1,000) is transferred into a 100 ml erlenmeyer flask and 1 ml of test solution is added, shaken, and mixed. 10 ml of substrate solution is added, 6 ml of 0.1N iodine solution is added, shaken, mixed, allowed to stand for 30 minutes in a dark place, and titrated same as test solution. (Bml).

The enzyme activity is obtained by the following equation.

$$U/g = (B-A) \times 513 \times \frac{2}{100} \times \frac{60}{30} \times \frac{1}{W}$$

513 : Amount of 1mmol iodine corresponding to 513μmol of galacturonic acid

W : Weight of sample contained in 1 ml Test Solution (g)

Definition of Activity : 1 Polygalacturonase unit corresponds to an amount of enzyme that generates 1 μmol of galacturonic acid for 1 hour under the above conditions.

Solutions

- Substrate Solutions : 1.0g of pectic acids(Sigma P3889 or its equivalent) is precisely weighed, dried for 3 hours at 105°C, and measure the weight loss. Pectic acid corresponding to 0.55 g of anhydrous is precisely weighed, dissolved in 80 ml of citric acid buffer solution(pH 4.0). After dissolving, pH is adjusted to 4.0 with trisodium citrate solution(29.4→100) or hydrochloric acid(9→100), citric acid buffer

solution(pH 4.0) is added to make 100ml.

◦ Citric acid Buffer Solution (pH 4.0)

Solution A : 0.1N Hydrochloric acid

Solution B : 14.7 g of trisodium citrate is dissolved, and make to 1,000 ml with water.

pH of Solution B is adjusted to 4.0 with using solution A.

Method 2

Principle : This test is to measure the initial reaction rate from alkali consumption rate by titrating with alkali, which is generated by liberating of ester group in pectic acid at pH 4.8, temperature 30°C.

Preparation of Test Solution : Test Solution is prepared by dilution a certain amount of sample with water so that 1 ml contains 0.0007~0.006 unit.

Test Procedure : 20 ml of substrate solution is precisely weighed into a beaker, and maintained in water bath at 30°C. 0.05N of sodium hydroxide solution is added to substrate solution to bring pH 4.8, and 1 ml of test solution is added. While adjusting to pH 4.8 for 2 minutes, observe the reaction, and the consumed amount (ml) of 0.05N sodium hydroxide solution is A. Separately, for a blank test, 0.05N of sodium hydroxide solution is added to substrate solution to bring pH 4.8, and 1 ml of water is added. While adjusting to pH 4.8 for 2 minutes, the consumed amount (ml) of 0.05N sodium hydroxide solution is B. The enzyme activity is obtained by the following equation.

$$U/g = \frac{A-B}{20 \times 2 \times W}$$

20 : Titrated amount(μl) of 0.05N sodium hydroxide corresponding to 1μmol carboxyl group

2 : Reaction time (minute)

W : Weight of sample contained 1 ml of test solution (g)

Definition of Activity : 1 Pectinesterase unit is an amount of enzyme that liberates 1 μmol of carboxyl group from pectin for 1 minutes under the above conditions.

Solutions

Substrate Solutions : 5 g of Esterification pectinFluka 76282(pectin from apple, not less than 70% methoxylated) or equivalent is precisely weighed, gently mixed with 800 ml of water maintained to 40°C, and suspended. It is dissolved thoroughly at the

temperature not more than 60°C and cooled to room temperature. To this, 2.03g of magnesium chloride is added, pH is adjusted to 4.8 with sodium hydroxide solution, water is added to make 1,000 ml.

Method 3

Principle : This test is to measure the amount of unsaturated Galacturonic acid, which is generated from decomposition of pectin at pH 5.8, temperature 30°C, by absorbance.

Preparation of Test Solution : 0.5 g of Pectinase is dissolved in citric acid buffer solution(pH 5.8) to make 100 ml, 3 ml of this solution is diluted with citric acid buffer solution(pH 5.8) to make 25 ml, test solution.

Test Procedure: To 3 ml of substrate solution is maintained in a water bath at 30°C for 5 minutes, 0.1 ml of test Solution is added and shortly shaken. Using water as a reference solution, absorbance curve per minute of the test solution is prepared by measuring absorbance every 1 minute, for 10 minutes, at 235 nm with 1cm cell. Repeat the same procedure three times. Linear section should be maintained at least for 5 minutes (6 points). The change of absorbance should not be more than 0.03 per minute, the range of 0.02~0.03 is optimum.

The enzyme activity is obtained by the following equation.

$$U/mg = \frac{\Delta A_{235}/\Delta t}{0.01 \times 3.1 \times C}$$

3.1 : Final reaction solution (ml)

C : Concentration of final test solution (mg/ml)

Definition of Activity : 1 Pectin lyase unit is the amount of enzyme increasing absorbance 0.01 per minute under the above conditions.

Solutions

Substrate Solutions : 0.5 g of Pectin(Copenhagen Pectin X 2955, Sigma P9135 or its equivalent) is transferred into a beaker, stirred with 2 ml of ethanol, 80 ml of citric acid buffer solution(pH 5.8) is added with using magnetic stick, and stirred while caring bubbles are not generated. pH of the solution is adjusted to 5.8 with using solution A or solution B, and make 100ml with citric acid buffer solution(pH 5.8). This solution is allowed to settle in refrigerator for 1 night, next day, centrifuged for 10 minutes at 12,000×g, filtered, and used.

Citric acid buffer solution(pH 5.8)

Solution A : 35.6g of Disodium hydrogen phosphate (2 hydrates) is dissolved with water, and make to 1,000 ml.

Solution B : 21g of citric acid (1hydrate) is dissolved with water, and make to 1,000 ml.

57ml of Solution A and 43ml of Solution B are mixed well, and adjust to pH 5.8 with Solution A or Solution B.

Storage Standard of Pectinase

Pectinase is strongly hygroscopic, so should be stored in a cold dark place with sealing tightly.

83. Pectin

Definition

Pectin is a purified polymer of carbohydrates obtained by extracting with boiling water and acid aqueous solution citrus fruits or apple. The essential part of pectin chains consists of α -1, 4 bonding of D-galaturonic acid unit. A portion of carboxyl groups are methyl-esterified and the rest exist as free acid or salts of ammonium, potassium, and sodium. Depending on the usage, sugars can be added to standardize its characteristics or food additives, that are used as buffers to adjust acidity, can be added.

Compositional Specifications of Pectin

Description Pectin is odorless, yellowish white fine or coarse powder with mucus taste.

Identification (1) When 1% aqueous solution is mixed with a same volume of alcohol, transparent gelatinous precipitates are formed (distinguished from other gums).

(2) 1 ml of thorium nitrate solution (1→10) is added to 10 ml of 1% aqueous solution of pectin. After allowing to stand for 2 minutes, precipitate or gel is formed (distinguished from other gums).

(3) 1 ml of sodium hydroxide solution is added to 5 ml of 1% aqueous solution of pectin. After allowing to stand for 15 minutes at room temperature, a gel is formed (distinguished from tragacanth or other gums).

(4) The gel obtained in (3) is acidified by 1 ml of hydrochloric acid. When it is well shaken, colorless gelatinous voluminous precipitates are formed. Upon heating, white agglomerates are formed.

Purity (1) Arsenic : Proceed as directed under Purity (1) in Guar Gum.

(2) Lead : When 5.0 g of Pectin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Cadmium : When 5.0 g of Pectin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When 0.1 g of Pectin is tested by Mercury Test Method, its content should not be more than 1.0ppm.

(5) Residual solvent : 0.1g of pectin is precisely weighed, 10 ml of diluted internal standard solution(1→25) is added, a stopper is place, and stirred until a homogeneous dispersion is obtained. This solution is filtered by 0.45 μ m filter, and

the filtrate is used as test solution. However, tert-butyl alcohol (1→1,000) is used as internal standard solution. Separately, 0.1 g each of methyl alcohol and isopropyl alcohol is precisely measured and water is added to make 100 ml. Again 10 ml of this solution and 4 ml of internal standard solution is weighed, water is added to make 100 ml, mixed standard solution. 2 μ l of test solution and mixed standard solution is taken respectively, and injected to gas chromatograph with the following operation condition. Then, ratio of peak area of methyl alcohol and isopropyl alcohol peak against tert-butyl alcohol peak, Q_{T1}, Q_{T2} and Q_{S1}, Q_{S2}, is measured respectively, and measure the content of methyl alcohol and isopropyl alcohol under following equation, it should be not more than 1.0% as individual or sum if used together.

$$\text{Content of methyl alcohol (\%)} = \frac{\text{Weight of methyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_{T1}}{Q_{S1}}$$

$$\text{Content of isopropyl alcohol (\%)} = \frac{\text{Weight of isopropyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_{T2}}{Q_{S2}}$$

Q_{T1} : Ratio of methyl alcohol peak against tert-butyl alcohol peak in Test Solution

Q_{T2} : Ratio of isopropyl alcohol peak against tert-butyl alcohol peak in Test Solution

Q_{S1} : Ratio of methyl alcohol peak against tert-butyl alcohol peak in mixed standard Solution

Q_{S2} : Ratio of isopropyl alcohol peak against tert-butyl alcohol peak in mixed standard Solution

Column : PLOT Q or its equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Temperature at injection port : 200°C

Column Temperature : 120°C

Detector Temperature : 300°C

Carrier gas : Nitrogen or Helium

- (6) Galaturonic acid unit : 5 g of Pectin is precisely weighed into a beaker, 5 ml of hydrochloric acid and 100 ml of 60% ethyl alcohol are added, stirred for 10 minutes, filtered with a glass filter(1G3 or its equivalent). 60% of residue on a glass filter is washed with 15 ml each of 60% mixture of ethyl alcohol: hydrochloric acid(20:1) six times, washed solution is washed with 60% ethyl alcohol until it doesn't react for chloride, and washed with 20 ml of ethyl alcohol again. It is dried for 2.5 hours at

105°C, cooled in a desiccator, and weighed. The amount which corresponds to 1/10 of the weight of the dried substance is precisely weighed. Then the weight is W(mg). To this solution, 2 ml of ethyl alcohol is added and wetted, 100 ml of freshly boiled and cooled water is added, shaken, and mixed. 5 drops of phenolphthalein solution, titrated with 0.1N sodium hydroxide solution, and the consumed amount of the solution is V_1 (ml). 20 ml of 0.5N sodium hydroxide solution is precisely weighed, added, shaken well, mixed, and let stand for 15 minutes. Again, 20 ml of 0.5N hydrochloric acid is precisely weighed, added, titrated with 0.1N sodium hydroxide solution until the red color disappears, and the consumed amount of this solution is V_2 (ml). However, the final point is when the color of solution becomes slightly red after shaking vigorously. Titrated solution is transferred to 500 ml flask for decomposition, which is apparatus of Total Kjeldahl Nitrogen Test (nitrogen determination method). After distilling apparatus is attached, 20 ml of 0.1N hydrochloric acid and 150 ml of freshly boiled and cooled water are into flask for absorption. Tip of the condenser is submerged in the solution, 20 ml of sodium hydroxide(1→20) is transferred into a flask for decomposition, heated while caring generating bubbles, and 80~120 ml of distillate is obtained. It is titrated with 0.1N sodium hydroxide solution (indicator : Methyl red solution), the consumed amount of the solution is S(ml). Separately, perform the blank test, and the consumed amount of 0.1N sodium hydroxide is B(ml). When measure the content of Galaturonic acid with following equation, it should not be less than 65%.

$$\text{Content of Galaturonic acid(\%)} = \frac{19.41 \times V_1 + V_2 + (B - S)}{W} \times 100$$

(7) Sulfur dioxide : When Pectin is tested by Assay of sulfurous acid, hyposulfurous acid, and salts Test in General Test Method in 「Standards and Specifications for Foods」, its content should not be more than 50ppm.

(8) Acid Insoluble Ash : When 3 g of Pectin proceed as directed under Ash Test, the content should not be more than 1.0%.

Loss on Drying When 3 g of Pectin is dried for 2 hours at 105°C, the weight loss should not be more than 12%.

84. Pepsin

Definition Pepsin is an enzyme obtained from extracts of the stomach and intestines of pigs or other animals. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Pepsin

Content Pepsin contains 90~130% of the labeled activity as Pepsin.

Description Pepsin is white~pale yellow~deep brown powder, granule, lump, or transparent liquid.

Purity (1) Arsenic : 0.25 g of Pepsin is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate and should not be more 4ppm.

(2) Heavy Metals : 0.5 g of Pepsin are carbonized by heating mildly in a quartz or porcelain crucible. After cooling, add 2 ml of nitric acid and 5 drops of sulfuric acid, it is heated until white smoke disappears, which is then reduced to ash by further heating at 450~550°C. After cooling, 2 ml of hydrochloric acid is added, which is then evaporated to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. After cooling, 1 drop of phenolphthalein indicator solution is added, then ammonia solution is added until the color of the solution becomes pale red. The resulting solution is transferred into a Nestler cylinder by rinsing with water. 50 ml of test solution is prepared by adding 2 ml of diluted acetic acid (1→20) and water. When this solution proceed as directed under heavy metals, the content should not be more than 40ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water are added to make the total volume to 50 ml.

(3) Lead : 0.8 g of Pepsin (if it is liquid, it is concentrated by evaporation in a

water bath) is slowly carbonized by heating, which is reduced ash by further heat treatment at a temperature below 500°C. Carefully 20 ml of diluted nitric acid is added to the ash, which is then gently boiled for 5 minutes. It is then filtered (if necessary), the residue is washed with water, which is then added to the filtrate. Water is added so that total volume of this solution becomes 50 ml. When this test solution proceed as directed under lead, the amount of lead should not be more 10ppm.

- (4) Coliform Group : When Pepsin proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods in Food Code, it should not contain more than 30 per 1 g of this product.
- (5) Salmonella : When Pepsin proceed as directed under Microbe Test Methods for Salmonella in General Test Methods in Food Code, it should be negative (-).

Assay(activity)

- Applications : This test method is applied to an enzyme obtained from the stomach and intestines of pigs or other animals.
- Preparation of Test Solution : 100 mg of the sample or a certain amount of enzyme that contains activity slightly higher than or similar to the Standard Solution is dissolved in 150 ml of hydrochloric acid solution. This solution should be used within 1 hour after preparation.
- Test Procedure : 5.0 ml each of Standard Solution is placed in two bottles containing substrate solution. In two or more bottles, Test Solution is added so that one bottle contains the same amount of pepsin as 5.0 ml of Standard Solution and other bottles contain decrementally smaller amount of pepsin (for example, 5.0 ml, 4.9 ml, and 4.8 ml, etc.). When the amount of Test Solution should be not more than 5.0 ml, the difference is supplemented with hydrochloric acid solution. The bottles are capped and shaken upside down three times. The bottles are maintained in a water bath at $52 \pm 0.5^{\circ}\text{C}$ for 2 hours and 30 minutes, agitating the contents equally every 10 minutes by inverting the bottles once. Remove the bottles from the bath, the contents in the bottles are transferred into each test container. Undigested albumin attached on the inner walls of the bottles are washed with 50 ml of water and added to the test containers. The contents in the container are well mixed and allowed to stand for 30 minutes. The volume of undigested albumin is measured. The average volume of the precipitates in the two standard containers is obtained. The volume of the residues from the Test Solution, that is closest to this average value, is marked as V (ml).

The enzyme activity is calculated by the following equation.

$$\text{Pepsin units/mg} = 3,000 \times S/U \times 5.0/V$$

S : Weight of pepsin used in Standard Solution (mg)

U : Weight of sample(mg)

V : volume of the residues in Test Solution

Definition of Activity : 1 Pepsin unit corresponds to an amount of enzyme that digests the coagulated egg albumin that is 3,000 times the weight of enzyme under the above conditions.

Apparatus

Test Container : 100 ml conical container, that fits the following criteria, is used.

- (1) Diameter for the bottom part should not be more than 1 cm.
- (2) Appropriate for water and precipitate tube in ASTM standard method D 96-68.
- (3) There should be 0.05 ml scale marks in a range of 0~0.5 ml, 0.1 ml scale marks in a range of 2~3 ml, 0.2 ml scale marks in a range of 3~5 ml, 1 ml scale marks in a range of 5~10ml, 5 ml scale marks in a range of 10~25 ml, and scale marks at 50 ml, 75 ml, and 100 ml. (note : Any container with similar shape and scale marks can be used provided that the volume of residues can be measured with similar accuracy.)

Solutions

- Hydrochloric Acid Solution : 35 ml of 1 N hydrochloric acid is mixed with 385 ml of water.
- Substrate : 1~2 eggs are boiled for 15 minutes and rapidly cooled in cold water. Shells and yolks of the eggs are removed completely. Egg whites are sieved through No.40 mesh screen. First portion that passes through the sieve is discarded.
- Substrate Solution : 10.0 g of substrate is placed in a 100 ml wide mouth bottle (as many as needed for the test), where 35 ml of hydrochloric acid solution is added immediately. Egg white grains are finely ground by an appropriate method. It is isothermalized at 52°C before testing.
- Standard Solution : 100.0 mg of pepsin standard is precisely weighed and dissolved in 150 ml of hydrochloric acid solution. This solution is used within 1 hour after preparation.

Storage Standard of Pepsin

Pepsin is strongly hygroscopic, so should be stored in a cold dark place with sealing tightly.

85. Grape Skin Extract

Definition Grape Skin Extract is a pigment obtained by extracting skins of grapes (*Vitis vinifera* L., etc.) of vitaceae. Its major pigment component is enocyanin of anthocyanins. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Grape Skin Extract

Content Color value ($E_{10\%}^{1cm}$) of Grape Skin Extract should be less than the labeled.

Description Grape Skin Extract is red~dark purple liquid, lump, powder, or paste having a characteristic odor.

Identification (1) Test Solution obtained in Color Value section shows red color and a maximum absorption is at about 525 nm.

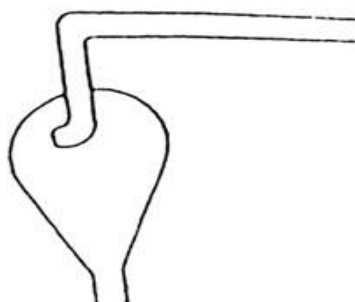
(2) When Test Solution in (1) is alkalinized with sodium hydroxide solution, the color of the solution becomes dark green.

Purity (1) Arsenic : 0.2 g of Grape Skin Extract is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Grape Skin Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Sulfur Dioxide : Approximately 1 g of Grape Skin Extract is precisely weighed into a distillation flask with the Wagner Tube as shown in the figure below. It is then refluxed in 100 ml of water and 25 ml of phosphoric acid (2→7). 25 ml of lead acetate solution (1→50) is placed in the collector. The condenser is arranged so that its lower end is immersed in the lead acetate solution. It is distilled until the liquid in the flask reaches about 100 ml. The end of the condenser is washed with a little amount of water. Washings is added to the distillate, where 5 ml of hydrochloric acid and 1 ml of starch solution. It is then titrated with 0.01 N iodine solution. The content of sulfur dioxide should not be less than 0.005% of 1 Color value ($E_{10\%}^{1cm}$) . Separately, a blank test is carried out.

0.01 N iodine solution 1 ml = 0.3203 mg SO₂



Wagner Tube

Assay (Color Value) An adequate amount of Grape Skin Extract so that the measured absorbance is between 0.3 and 0.7 is accurately weighed and added pH 3.0 citric acid-dibasic sodium phosphate buffer solution to make up a 100 ml solution. 1 ml of this solution is diluted to 100 ml with pH 3.0 citric acid-dibasic sodium phosphate buffer solution, the Test Solution. If necessary, the solution is centrifuged and the supernatant is used. Using pH 3.0 citric acid-dibasic sodium phosphate buffer solution as the blank, absorption A is measured at the wavelength of maximum absorption around 525 nm with 1cm cell. Color value is obtained using the following equation.

$$\text{Color Value } (E_{10\%}^{1\text{cm}}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

◦ Citric acid-dibasic sodium phosphate buffer solution (pH 3.0)

Solution 1 : 0.1 M citric acid solution : 1 ℓ of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2 M dibasic sodium phosphate solution : 1 ℓ of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (159:41) and its pH is adjusted to 3.0.

86. Pullulanase

Definition Pullulanase is an enzyme obtained from cultures of *Bacillus acidopullulyticus*, *Klebsiella aerogenes*, *Bacillus subtilis* where the pullulanase gene of *Bacillus deramificans* is inserted, and *Bacillus licheniformis* where the pullulanase gene of *Bacillus deramificans* is inserted. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Pullulanase

Content Pullulanase contains 90~130% of the labeled activity as Pullulanase.

Description Pullulanase is white~pale yellow~brown powder or transparent~deep brown liquid.

- Purity**
- (1) Arsenic : 0.25 g of Pullulanase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate and should not be more 4ppm.
 - (2) Lead : When 5.0 g of Pullulanase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
 - (3) Coliform Group : When Pullulanase proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods in Food Code, it should not contain more than 30 cfu per 1 g of this product.
 - (4) Salmonella : When Pullulanase proceed as directed under Microbe Test Methods for Salmonella in General Test Methods in Food Code, it should be negative (-).
 - (5) E. Coli : When Pullulanase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

- Application and Principle : This test is to measure the activity of Pullulanase in an enzyme that is obtained from a culture of *Bacillus acidopullulyticus*, *Klebsiella aerogenes*, *Bacillus subtilis* where the pullulanase gene of *Bacillus deramificans* is inserted, and *Bacillus licheniformis* where the pullulanase gene of *Bacillus deramificans* is inserted. Activity test is based on absorbance measurement of reaction mixture of

dinitrosalicylic acid and maltotriose which is a reducing sugar obtained by hydrolyzing α -1,6-glycosidic bond of pullulan at pH 5.0, temperature 50°C.

- Preparation of Test Solution : Test Solution is prepared so that the absorbance to be measured will be within a range of 0.2~0.5 under the following test method.
- Test Procedure : 1 ml of substrate solution is placed in a 17 × 1.5 cm test tube for enzyme test. It is allowed to stand for 5 minutes in a water bath at 50°C. 1 ml of Test Solution is added to the test tube and the mixture is reacted for 10 minutes. The reaction is stopped by adding 2 ml of 3,5-dinitrosalicylic acid solution. Separately, 1 ml of substrate solution and 2 ml of 3,5-dinitrosalicylic acid solution are added in a test tube for enzyme blank test, and 1 ml of Test Solution is added. Both test tubes are boiled for 5 minutes in a boiling water bath and cooled rapidly. 10 ml each of water is added to each test tube, which is then shaken. Using the blank enzyme test solution as a reference, absorbance of enzyme Test Solution is measured at 540 nm with 1 cm cell.

Standard Curve

1 g of maltose (standard) is precisely weighed and dissolved in water to make total volume to 100 ml. 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 ml each of this solutions diluted to 20 ml with water, use the Standard Solutions. Instead of 1 ml of Test Solution, with 1 ml of standard solution and 1 ml of water, the same procedure as the Test Solutions repeated. Using water as a reference, standard curve is prepared by plotting absorbance of each standard solution vs. concentration of standard solution (mg/ml)

Enzyme activity is calculated by the following equation

$$\text{Activity Pullulanase (U/g)} = C \times \frac{1,000}{\text{Concentration of Test Solution (mg/ml)} \times 10}$$

C : maltose concentration in enzyme Test Solution obtained from standard curve (mg/ml)

10 : reaction time

Definition of Activity : 1 Pullulanase unit is an activity which generates reducing sugar corresponding to 1 mg of anhydrous maltose per minute under the above conditions.

Solutions

- Substrate : 70 ml of water is added to 1 g of Pullulan standard, which is heated for 5 minutes and cooled. 10 ml of 1 M acetic acid sodium acetate buffer

solution is added to the solution, which is then diluted to 100 ml with water

- 1 M acetic acid sodium acetate buffer solution (pH 5.0)

Solution A : 60 g of acetic acid is diluted to 500 ml with water.

Solution B : 82 g of anhydrous sodium acetate is dissolved in water to make the total volume 500 ml.

148 ml of Solution A and 352 ml of Solution B are mixed. pH of the mixture is adjusted to 5.0 using Solution A or Solution B. The total volume is make to 1,000 ml with water.

- 3,5-Dinitrosalicylic acid, DNS Solution : 1 g of DNS is dissolved in 16 ml of 10% sodium hydroxide solution. 30 g of potassium sodium tartrate(4 hydrate) and 50 ml of water are added to the solution, which is heated and diluted to 100 ml with water. This solution should be stored at 5°C used within 5 days after preparation.

Storage Standard of Pullulanase

Pullulanase is strongly hygroscopic, so should be stored in a cold dark place with sealing tightly.

87. Protease

Definition Protease is an enzyme(Fungal) obtained from cultures of *Aspergillus niger* and its variety and *Aspergillus oryzae* and its variety, and an enzyme(Bacterial) obtained from cultures of *Bacillus subtilis* and its variety, *Bacillus licheniformis* and its variety and *Bacillus stearothermophilus* and its variety, and enzyme(Plant) obtained from plants such as papain, ficin, and bromelain etc., and called Protease(Fungal), Protease(Bacterial), and Protease(Plant), respectively. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

I . Protease(Fungal)

Compositional Specifications of Protease(Fungal)

Content Protease(Fungal) contains 90~130% of the labeled activity as Protease, Fungal.

Description Protease, Fungal, is white~pale yellow~dark brown powder, granule, lump or transparent~dark brown liquid.

Purity (1) Arsenic : 0.25 g of Protease, Fungal, is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Protease, Fungal, is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

(3) Coliform Group : When Protease, Fungal, proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods 「Standards and Specifications for Foods」, it should not contain more than 30 cfu per 1 g of this product.

(4) Salmonella : When Protease, Fungal, proceed as directed under Microbe Test Methods for Salmonella in General Test Methods 「Standards and Specifications for Foods」, it should be negative (-).

(5) E. Coli : When Protease, Fungal, proceed as directed under Microbe Test Methods for E. Coli in General Test Methods 「Standards and Specifications for Foods」, it should be negative (-).

Assay (Activity) The method is to measure the amount of Protease, and tested as directed under Method 1 SAP(Spectrometric acid protease unit) and Method 2 HUT(Hemoglobin unit on the throsine basis).

Method 1 SAP(Spectrometric acid protease unit)

- Application and Principle : This test is to measure the activity of protease (expressed as SAP: Spectrophotometric acid protease units) of an enzyme obtained from *Aspergillus niger* and its variety and *Aspergillus oryzae* and its variety. Activity test is based on hydrolysis of casein substrate for 30 minutes, at pH 3.0, 37°C. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The amount of casein dissolved in the filtrate is determined by the absorption measurement.
- Preparation of Test Solution : Test Solution is prepared so that the corrected absorption at 275 nm of isothermalized enzyme filtrate (defined as ΔA in this test) will be within a range of 0.200~0.500 using 2 ml of the final dilution with glycine hydrochloric acid buffer solution. Sample is precisely weighed and ground in a glass mortar with glycine hydrochloric acid buffer solution. It is then transferred into a volumetric flask and filled with glycine hydrochloric acid buffer solution.
- Test Procedure : 10 ml each of substrate solution is added to a 25×150 mm test tube, per 1 sample, test tubes for enzyme test should not be more than 2 , 1 for enzyme blank test, and 1 for substrate blank test. Each test tube is capped and maintained for 15 minutes in a water bath at $37 \pm 0.1^\circ\text{C}$. Precisely 2 ml of Test Solution is added to the test tube, well mixed, and allowed to settle in a water bath (note: Test Tube should be capped while isothermalizing.). For substrate blank test, 2 ml glycine hydrochloric acid buffer solution is added instead of Test Solution. After exactly 30 minutes, the reaction of enzyme is stopped by adding 10 ml of trichloroacetic acid solution. For enzyme blank test, 10 ml of substrate solution, 10 ml of trichloroacetic acid, and 2 ml of Test Solution are sequentially added. Protein is completely coagulated by heating all the test tubes in a water bath at $37 \pm 0.1^\circ\text{C}$. The test tubes are cooled for 5 minutes in an ice bath. The contents are filtered through Whatman No.42 filter paper or its equivalent. The filtrate should be completely clear. Absorbance of the filtrate is measured at 275 nm with 1 cm cell using the filtrate in the substrate blank test as a reference. Absorbance of enzyme Test Solution is corrected by subtracting the absorbance of enzyme blank test solution from the absorbance of enzyme test solution.

Standard Curve

181.2 mg of L-tyrosine (previously dried until the weight becomes constant) is precisely weighed and completely dissolved in 60 ml of 0.1 N hydrochloric acid. This solution is diluted to 1,000 ml with water. 1 ml of the resulting solution contains 1 μ mol of tyrosine. Using this solution, diluted solutions that contain 0.10, 0.20, 0.30, 0.40, and 0.50 μ mol each per 1 ml are prepared. Using water as a reference, absorbance of each solution is measured at 275 nm with 1 cm cell. An absorbance calibration curve for the amount(μ mol) of tyrosine per ml is prepared. This should be a straight line. The slope and intercept are obtained for the following calculation. It should be near 1.38. The slope and intercept is obtained by least square method as follows below.

$$\text{Slope (S)} = \frac{N\sum(MA) - \sum(M)\sum(A)}{n\sum(M^2) - (\sum M)^2}$$

$$\text{Intercept (I)} = \frac{\sum(A)\sum(M^2) - \sum(M)\sum(MA)}{n\sum(M^2) - (\sum M)^2}$$

n : Number of data points on the standard curve

M : Amount(μ mol) of tyrosine per ml for each data point

A : Absorbance for each concentration of Standard Solution

Enzyme activity is calculated by the following equation.

$$\text{SAP/g} = (\Delta A - I) \times \frac{22}{S \times 30 \times W}$$

ΔA : Corrected absorbance of isothermalized enzyme filtrate

I : Intercept of the standard curve

22 : Amount of final reaction liquid (ml)

S : Slope of the standard curve

30 : Reaction time (minutes)

W : Weight of sample contained in 2 ml of Test Solution (g)

Definition of Activity : 1 Spectrophotometric acid protease unit(SAP) corresponds to the activity that frees 1 μ mol of tyrosine per minute under the above test conditions.

Solutions

- Casein : Casein (Hammarsten) is used.
- Glycine Hydrochloric Acid Buffer Solution (0.05 M) : 3.75 g of glycine is dissolved in

about 800 ml of water, where pH is adjusted to 3.0 with 1 N hydrochloric acid. It is diluted to 1,000 ml with water.

- Trichloro Acetic Acid Solution : 18.0 g of trichloroacetic acid and 11.45 g of sodium acetate are dissolved in 800 ml of water, where 21.0 ml of glacial acetic acid is added. It is diluted to 1,000 ml with water.
- Substrate Solution : 8 ml of 1 N hydrochloric acid is added to 500 ml of water, where 7.0 g of casein (dried basis) is dispersed by stirring continuously. It is then heated for 30 minutes in a boiling water bath while stirring occasionally. After cooling to room temperature, 3.75 g of glycine is added to the solution. pH of the resulting is adjusted to 3.0 with 0.1 N hydrochloric acid. It is diluted to 1,000 ml with water.

Method 2 HUT(Hemoglobin units on the tyrosine basis)

- Application and Principle : This test is to measure the activity of protease (expressed as HUT: Hemoglobin units on the tyrosine basis) of an enzyme obtained from *Aspergillus niger* and its variety and *Aspergillus oryzae* and its variety using tyrosine on standard. Activity test is based on hydrolysis of Hemoglobin substrate for 30 minutes, pH 4.7 at 40°C. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The amount of Hemoglobin dissolved in the filtrate is determined by the absorbance measurement.
- Preparation of Test Solution : Test Solution is prepared by dissolving the sample in acetate buffer solution so that 1 ml of the final dilution contains 9~22 HUT (absorbance as measured by the Test Procedure will be within a range of 0.2~0.5).
- Test Procedure : 10 ml each of substrate solution is added to a 25 × 150 mm test tube for enzyme test and for substrate blank test. Each test tube is heated for 5 minutes in water bath at 40°C. 2 ml of Test Solution is added to the test tube for enzyme test and 2 ml of acetate buffer solution is added to the test tube for substrate blank test. It is placed the stopper on the test tube and diluted by tapping for 30 seconds on the palm. After heating for exactly 30 minutes in a water bath at 40°C, 10 ml of trichloroacetic acid solution is added to each tube (note : it should not be sucked in with mouth). Both tubes are capped and vigorously shaken for 40 seconds in every 10~12 minutes, which is repeated for 1 hour so that the tubes are cooled to room temperature. For enzyme blank test, 10 ml of substrate solution and about 5 ml of Test Solution are placed separately in two test tubes, which are heated for 30

minutes in a water bath. 10 ml of trichloroacetic acid solution is added to the test tube with 10 ml substrate solution, which is shaken for 40 seconds. To this solution, precisely 2 ml of the heated Test Solution is added. It is then shaken for 40 minutes in every 10~12 minutes. This is repeated for 1 hour so that the solution is cooled to room temperature. The 3 test tubes above are vigorously shaken and filtered through Whatman No.42 filter paper or its equivalent. First 3 ml of the filtrate is discarded. Absorbance of the filtrate is measured at 275 nm with 1 cm cell using the solution for the substrate blank test as a reference. A_u is subtracted the absorbance of enzyme blank test solution from the absorbance of enzyme test solution (if A_u does not will be within a range of this range, it is tested again with adjusted weight of sample).

Standard Curve

100.0 mg of L-tyrosine (previously dried until the weight becomes constant) is precisely weighed and completely dissolved in 60 ml of 0.1N hydrochloric acid. This solution is diluted to 1,000 ml with water. 1 ml of the resulting solution contains 1000 μ g of tyrosine. Using this solution, diluted solutions that contain 75.0, 50.0, and 25.0 μ g each per 1 ml are prepared. Using 0.006 N hydrochloric acid as a reference, absorbance of 4 each solution is measured at 275 nm with 1 cm cell. The slope of a curve is measured by plotting absorbance per 1 μ g of tyrosine. As is obtained by multiplying the slope with 1.10. This value should be approximately 0.0084.

Enzyme activity is calculated by the following equation.

$$\text{HUT/g} = \frac{A_u}{A_s} \times \frac{22}{30W}$$

22 : Amount of final reaction liquid (ml)

30 : Reaction time (minutes)

W : Weight of sample contained in 2 ml of Test Solution (g)

(Note : Under standardized conditions, A_s is obtained 0.0084. This value is used in usual tests instead of the value obtained from the standard curve. However, if an accuracy is an issue and there are any doubts, the value obtained from the standard curve should be used.)

Definition of Activity : 1 HUT unit corresponds to the amount of an enzyme that generates enzyme-decomposed matter in 1 minute that shows a similar absorbance (with 1 cm cell at 275 nm) as a solution containing 1.10 μ g of tyrosine per 1 ml of 0.006 N hydrochloric acid under the above test conditions.

Solutions

- Hemoglobin : Hemoglobin substrate powder or its equivalent, that is completely soluble in water, is used.
- Acetate Buffer Solution : 136 g of sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) is dissolved in plenty of water, which is then diluted to 500 ml with water. 25 ml of this solution and 50 ml of 1 M acetic acid are mixed and the total volume is made to 1,000 ml. pH of this solution should be 4.7 ± 0.02 .
- Substrate Solution : 4.0 g of Hemoglobin is dissolved in 100 ml of water by stirring for 10 minutes in a 250 ml beaker. pH of the solution is adjusted to 1.7 with 0.3 N hydrochloric acid while stirring (the electrode of the pH meter is immersed in the solution). After 10 minutes, pH is adjusted to 4.7 with 0.5M sodium acetate solution. The total volume is made to 200 ml with water. If this solution is stored in a refrigerator, it is effective for 5 days.
- Trichloroacetic acid solution : 140 g of trichloroacetic acid is dissolved in 75 ml of water, which is then diluted to 1,000 ml with water.

Storage Standard of Protease, Fungal (HUT)

Protease, Fungal (HUT) is stored in a cold dark place with sealing tightly.

II. Protease, Bacterial(PC)

Compositional Specifications of Protease, Bacterial(PC)

Content Protease, Bacterial (PC) contains 90~130% of the labeled activity as Protease, Bacterial (PC).

Description Protease, Bacterial (PC) is white~pale yellow~dark brown powder, granule, lump or transparent~dark brown liquid.

Purity (1) Arsenic : 0.25 g of Protease, Bacterial(PC) is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Protease, Bacterial, is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

(3) Coliform Group : When Protease, Bacterial(PC) proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods 「Standards and Specifications for Foods」, it should not contain more than 30 cfu per 1 g of this product.

(4) Salmonella : When Protease, Bacterial(PC) proceed as directed under Microbe Test Methods for Salmonella in General Test Methods 「Standards and Specifications for Foods」, it should be negative (-).

(5) E. Coli : When Protease, Bacterial, proceed as directed under Microbe Test Methods for E. Coli in General Test Methods 「Standards and Specifications for Foods」, it should be negative (-).

Assay(activity)

- Application and Principle : This test is to measure the activity of protease (expressed as PC unit) of an enzyme obtained from the culture of *Bacillus subtilis* and its variety, *Bacillus licheniformis* and its variety and *Bacillus stearothermophilus* and its variety. Activity test is based on hydrolysis of casein substrate for 30 minutes, at pH 7.0, 37°C. Unhydrolyzed casein is removed by filtration. The amount of casein dissolved in the filtrate is determined by the absorbance measurement.
- Preparation of Test Solution : Test Solution is prepared using Tris buffer solution so that 2 ml of the final dilution contains 10~44 PC units.

◦ Test Procedure : 10 ml each of substrate solution is added to a 25 × 150 mm test tube for enzyme test, enzyme blank test, and substrate blank test. These tubes are maintained for 15 minutes in a water bath at $37 \pm 0.1^\circ\text{C}$. For enzyme test, 2 ml of Test Solution is quickly added and shaken, which is then allowed to settle in the water bath. For substrate blank test, 2 ml of Tris buffer solution, instead of Test Solution, is added. After 10 minutes, 10 ml each of trichloroacetic acid solution is added to each test tube to stop the reaction. For enzyme blank test, 10 ml each of substrate solution and trichloroacetic acid solution are added, and mixed by shaking for 40 seconds, where 2 ml of Test Solution is then added (note : trichloroacetic acid should not be sucked in with mouth). These tubes are further heated for 30 minutes in a water bath to coagulate proteins completely. At the end point, the tubes are shaken vigorously and filtered through a Whatman No.42 filter paper. Initial 3 ml of the filtrate is discarded. Absorbance of the filtrate is measured at 275 nm with 1 cm path length using the solution for the substrate blank test as a reference. A_u is the value subtracted the absorbance of enzyme blank test solution from the absorbance of enzyme test solution.

Standard Curve

100.0 mg of L-tyrosine (previously dried until the weight becomes constant) is precisely weighed and completely dissolved in 60 ml of 0.1 N hydrochloric acid. This solution is diluted to 1,000 ml with water. 1 ml of the resulting solution contains 100 μg of tyrosine. Using this solution, diluted solutions that contain 75.0, 50.0, and 25.0 μg each per 1 ml are prepared. Using 0.006 N hydrochloric acid as a reference, absorbance of 4 each solution is measured at 275 nm with 1 cm cell. A standard curve is prepared using absorbance of tyrosine concentration. Absorbance of a solution that contains 60 μg of tyrosine per 1 ml is obtained by interpolation from the standard curve. This absorbance value is divided by 40, so that it represents an absorbance of a solution that contains 1.5 μg per 1 ml, A_s (which is approximately 0.0115.)

Enzyme activity is obtained by the following equation.

$$\text{PC/g} = \frac{A_u}{A_s} \times \frac{22}{30 W}$$

22 : Amount of final reaction liquid (ml)

30 : Reaction time (minutes)

W : Weight of sample contained in 2 ml of Test Solution (g)

Definition of Activity : 1 Bacterial protease unit(PC) corresponds to the amount of an

enzyme that generates 1.5 µg/ml of L-tyrosine per minute under the test conditions above.

Solutions

- Casein : Casein (Hammarsten) is used.
- Tris Buffer Solution (pH 7.0) : 12.1 g of Tris(Hydroxymethyl)aminomethane for enzyme test is dissolved in 800 ml of water. pH is adjusted to 7.0 with 1 N hydrochloric acid. Water is added to make the total volume to 1,000 ml.
- Trichloroacetic Acid Solution : 18 g of trichloroacetic acid and 19 g of sodium acetate (3 hydrate) are dissolved in 800 ml of water, where 20 ml of glacial acetic acid is added. It is diluted to 1,000 ml with water.
- Substrate Solution : 6.05 g of Tris(Hydroxymethyl)aminomethane for enzyme test is dissolved in 500 ml of water, where 8 ml of 1 N hydrochloric acid is mixed. 7 g of casein is added to this solution, which is heated for 30 minutes in a boiling water bath while shaking occasionally. After cooling to room temperature, pH is adjusted to 7.0 by slowly adding 0.2 N hydrochloric acid while shaking to prevent precipitation. The resulting solution is diluted to 1,000 ml with water.

Storage Standard of Protease, Bacterial(PC)

Protease, Bacterial(PC) is stored in a cold dark place with sealing tightly.

III. Plant Protease(PU)

Compositional Specifications of Plant Protease(PU)

Content Plant Protease (PU) contains 90~130% of the labeled activity as Plant Protease (PU).

Description Plant Protease (PU) is white~pale yellow~brown powder, granule, lump or transparent~brown liquid.

Purity (1) Arsenic : 0.25 g of Plant Protease (PU) is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°C. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Plant Protease, is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

(3) Coliform Group : When Plant Protease (PU) proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods 「Standards and Specifications for Foods」, it should not contain more than 30 cfu per 1 g of this product.

(4) Salmonella : When Plant Protease (PU) proceed as directed under Microbe Test Methods for Salmonella in General Test Methods 「Standards and Specifications for Foods」, it should be negative (-).

(5) E. Coli : When Plant Protease proceed as directed under Microbe Test Methods for E. Coli in General Test Methods 「Standards and Specifications for Foods」, it should be negative (-).

Assay(activity)

- Application and Principle : This test is to measure the protein-decomposing activity of papain, ficin, and bromelain. Activity test is based on protein hydrolysis of casein substrate for 60 minutes, at pH 6.0, 40°C. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The amount of casein dissolved in the filtrate is determined by the absorbance measurement.
- Preparation of Test Solution : The concentration of 2 ml of final diluted solution is adjusted so that the absorbance (measured as described in Test Procedure) to be measured will be within a range of 0.2 to 0.5. Sample is ground in a mortar with

phosphate cysteine EDTA buffer solution. It is then transferred into a volumetric flask and filled with the same buffer solution.

- Test Procedure : 5 ml each of casein substrate solution is added to a 25 × 150 mm test tube, 3 for enzyme test and 6 for papain standard curve). Tubes are maintained for 15 minutes in a water bath at $40 \pm 0.1^\circ\text{C}$. 2 ml of test solution and 2 ml of standard solution are added to each tube, which is mixed by shaking and again maintained for 60 minutes in a water bath. 3 ml of trichloroacetic acid solution is added to each solution. Separately, 5 ml of substrate solution and 3 ml of trichloroacetic acid solution are mixed in 9 test tubes for enzyme blank test. 2 ml of test solution and 2 ml of corresponding standard solution are added to each test tube. All the tubes are again maintained for 30 minutes in a water bath to coagulate the precipitated protein completely. It is then filtered through a Whatman No.42 filter paper or its equivalent. First 3 ml of the filtrate is discarded. Absorbance of the clear filtrate is measured at 280 nm with 1 cm cell using each blank test solution as a reference. A standard curve of absorbance of the filtrate vs. concentration of standard solution (mg/ml) is prepared. The concentration of the filtrate from test solution is obtained by interpolation on the standard curve. Enzyme activity is calculated from the following equation.

$$\text{PU/mg} = A \times C \times 10/W$$

A : Activity of USP papain standard (PU/mg)

C : Concentration of enzyme test solution obtained from standard curve (mg/ml)

W : Weight of sample contained in 2 ml of Test Solution (mg)

Definition of Activity : 1 Papain unit(PU) is an amount of enzyme that frees 1 μg equivalent of tyrosine in 1 hour under the above test conditions.

Solutions

- Sodium Phosphate Solution (0.05 M) : 7.1 g of sodium phosphate, dibasic (anhydrous) is dissolved in 500 ml of water, which is diluted to 1,000 ml with water. 1 drop of toluene is added as a preservative.
- Citric Acid (0.05 M) : 10.5 g of citric acid (1 hydrate) is dissolved in 500 ml of water, which is diluted to 1,000 ml with water. 1 drop of toluene is added as a preservative.

- Phosphate Cysteine EDTA Buffer Solution : 7.1 g of sodium phosphate is dissolved in about 800 ml of water, where 14.0 g of EDTA (2 hydrate) and 6.1 g of cysteine hydrochloride (1 hydrate) are added and dissolved. pH of the resulting solution is adjusted to 6.0 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide solution. The total volume of the solution is made to 1,000 ml with water.
- Trichloroacetic Acid : 30 g of trichloroacetic acid is dissolved in water to make total volume to 100 ml.
- Substrate Solution : 1 g of casein (Hammarsten) as a dried basis is dissolved in 50 ml of sodium phosphate solution, which is heated for 30 minutes in a boiling water bath while shaking occasionally. It is then cooled while continuously shaking and its pH is adjusted to 6.0 ± 0.1 with citric acid solution (note : if the solution is shaken continuously and rapidly, precipitates are not formed.). The resulting solution is diluted to 100 ml with water.
- Standard Solution, Stock : 100 mg of USP papain standard is dissolved in phosphate cysteine EDTA buffer solution to make total volume to 100 ml.
- Standard Solution : 2, 3, 4, 5, 6, and 7 ml each of Standard Solution(Stock) is placed in 100 ml volumetric flask. Each of the flask is filled with phosphate cysteine EDTA buffer solution.

Storage Standard of Plant Protease(PU)

Plant Protease(PU) is stored in cold dark place with sealing tightly.

88. Protease, Fungal(HUT)

The specification of Proteas, Fungal(HUT) is combined to 87. Protease in Natural Additives.

The date of notification : November 10, 2010 (Notification No. 2010-82)

89. Protease, Bacterial(PC)

The specification of Protease, Bacterial(PC) is combined to 87. Protease in Natural Additives.

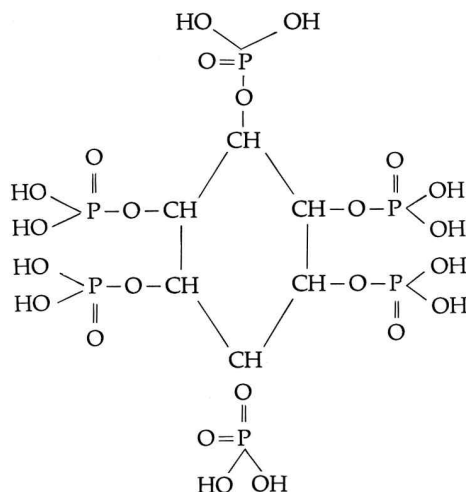
The date of notification : November 10, 2010 (Notification No. 2010-82)

90. Plant Protease(PU)

The specification of Plant Protease is combined to 87. Protease in Natural Additives.

The date of notification : November 10, 2010 (Notification No. 2010-82)

91. Phytic Acid



Chemical Formula $\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$

Molecular Weight 660.08

Definition Phytic Acid is obtained by extracting with water or acidic aqueous solution from rice (*Oryza sativa* LINNE) bran or corn (*Zea mays* LINNE) seeds of gramineae, followed by purification. It's major component is Inositol hexaphosphoric acid.

Compositional Specifications of Phytic Acid

Content Phytic Acid contains 48.0~52.0% of phytic acid ($\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6 = 660.08$).

Description Phytic Acid is clear scentless pale yellow syrup-phase liquid with a strongly acidic taste.

Identification (1) 3 drops of phenolphthalein TS is added to an aqueous solution (1→10) of this additive, which is neutralized by sodium hydroxide solution. When silver nitrate solution (1→100) is added to this solution, white colloidal precipitation is established.

(2) 3 ml of sulfuric acid is added to 1 ml of Phytic Acid, which is hydrolyzed by heating for 3 hours in a Kjeldahl flask. Add phenolphthalein TS, and neutralize the solution with sodium hydroxide solution. The neutralized solution shows the reaction (2) of Phosphates in Identification.

(3) Add 7 ml of 30% sulfuric acid, and hydrolyze by heating for 5 hours at 130°C in a sealed tube. Neutralize it with sodium hydroxide solution, and add some water to the 50 mL. After adding 0.5 g of activated carbon, it is stirred for 10 minutes and 30 mL of filtered solution is taken. 6 ml of nitric acid is added to 5 ml of this filtrate, which is evaporated to dryness in a water bath. 0.5 ml of barium chloride solution (1→10) is added to a small portion of the residue. When this is evaporated to dryness in a water bath, the residue becomes red color.

- Purity** (1) Chloride : 0.4 g of Phytic Acid is diluted to 10 ml with water. This solution is used as the test solution. The content should not be more than that amount corresponds to 0.45 ml of 0.01 N hydrochloric acid under Chloride Test.
- (2) Sulfate : 0.4 g of Phytic Acid is diluted to 10 ml with water. This solution is used as the test solution. The content should not be more than that amount corresponds to 0.6 ml of 0.01 N sulfuric acid under Sulfate Limit Test.
- (3) Arsenic : Dissolve 0.66 g of Phytic Acid in water to make the total volume is to 10 ml. This solution is used as the test solution. When 5 ml of this test solution proceed as directed under Arsenic Limit Test Test, it should not be more than 3ppm.
- (4) Lead : When 5.0 g of Phytic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.
- (5) Free Inorganic Phosphorus : Dissolve 1.0 g of Phytic Acid in 500 mL of water, add 5 ml of ascorbic acid solution (1→100) to 3 ml of this solution. Dilute the resulting solution to 50 ml with acetic acid·sodium acetate buffer solution (pH 4.0), which is set aside for 15 minutes ,use the Test Solution. Determine the absorbance of the Test Solution at 750 nm. Separately, dilute 5 ml of potassium phosphate monobasic standard solution to 1,000 ml with water. Add 5 ml each of ascorbic acid solution (1→100) to 5.0 ml, 10.0 ml, and 20.0 ml of this solution, respectively. A calibration curve is prepared by following the same procedure as as described test solution. A reference solution is prepared by mixing 5 ml of ascorbic acid solution (1→100) and 5 ml of molybdate solution, which is 1 g ammonium molybdate in 100 ml of 0.05 N sulfuric acid, and dilute to 50 ml with acetic acid·sodium acetate buffer solution (pH 4.0). The content of free inorganic phosphorus in the absorption of Test Solution and the calibration curve should not be more than 1.0%.
- Assay** (1) Total Phosphorus : 1.5 g of Phytic Acid is precisely weighted into a 200 ml Kjeldahl flask, Add 10 ml of sulfuric acid and 2.5 ml of nitric acid. This is hydrolyzed by heating until the liquid becomes transparent. Cool the solution, and dilute the resulting solution to 500 ml with water. Transfer 3 ml of this solution into a 100 ml volumetric flask, and neutralize with ammonia water (1→4), and weakly acidify with diluted nitric acid (1→10). 20 ml of metavanadate · molybdate solution are added to the resulting solution, which is diluted to 100 ml with water, mixed by shaking, and set aside for 30 minutes. This solution is used as the test solution. Measure the absorbance at 420 nm. Separately, dilute 5 ml of potassium phosphate monobasic standard solution to 1,000 ml with water. To determine the

calibration curve, place 5.0 ml, 10.0 ml, and 20.0 ml of this solution into a 100 ml volumetric flasks , respectively, and Proceed as directed under Test Solution. Calculate the content(%) of total phosphorus by using the absorption of Test Solution and the calibration curve.

- (2) Bonded Phosphorus : Calculate the content of bonded phosphorus from the difference between free inorganic phosphorus and total phosphorus, and calculate the content of phytic acid by the formula;

$$\text{Content of phytic acid (\%)} = \text{Content of bonded phosphorus(\%)} \times 3.552$$

Solutions

- Metavanadate·molybdate solution : Dissolve 1.12 g of ammonium metavanadate in excess amount of water, where 250 ml of nitric acid is added. Dissolve 27 g of ammonium molybdate in an appropriate amount of water. Two solutions are mixed so that the total volume is 1,000 ml. Store in light-resistant containers.

92. Hemicellulase

Definition Hemicellulase is an enzyme obtained from cultures of *Aspergillus niger* and its variety. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Hemicellulase

Content Hemicellulase contains 90~130% of the indicated activity as hemicellulase.

Description Hemicellulase is white~pale yellow~dark brown powder or transparent~dark brown liquid.

- Purity**
- (1) Arsenic : Place 0.25 g of Hemicellulase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.
 - (2) Lead : When 5.0 g of Hemicellulase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
 - (3) Coliform Group : When Hemicellulase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code, it should not contain more than 30 per 1 g of this product.
 - (4) Salmonella : When Hemicellulase proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in Food Code, it should be negative (-).
 - (5) E. Coli : When Hemicellulase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay (Activity)

- Application and Principle : This test is to measure the activity of hemicellulase that is obtained from a culture of *Aspergillus niger* and its variety. Activity test is based on enzymatic hydrolysis of Glycosidic bonding within locust bean gum substrate at pH 4.5, 40°C temperature. The decrease in viscosity of the substrate is measured by a viscometer with its scale corrected.
- Preparation of Test Solution : Test Solution is prepared by dilution so that 1 ml of

the final solution shows variation in relative fluidity of 0.18~0.22 under the conditions below in 5 minutes. Certain amount of sample is ground in a glass mortar and water is added. It is diluted in a suitable volumetric flask. Filter the solution through a Whatman No.1 filter paper or its equivalent prior to use

◦ Test Procedure : A viscometer (scale is previously corrected) is cleanly washed in water with sufficient detergent. It is then set up vertically in a glass water bath at $40 \pm 0.1^\circ\text{C}$. 20 ml of substrate solution and 4 ml of acetate buffer solution are added into a 50 ml Erlenmeyer flask with a stopper. Prepare 2 for enzyme test and 1 for substrate blank test per sample. An enzyme test flask is plugged with a stopper and isothermalized for 15 minutes in a water bath, where accurately 1 ml of Test Solution is added and well mixed measuring the time. Immediately, 10 ml of the mixed solution is added to the big branch of the viscometer. Approximately in 2 minutes, the reaction mixture is sucked in through the thin branch of the viscometer up to the upper scale using a rubber bulb. Time taken to reach the upper scale is measured in minutes (T_R). Again time taken to reach the lower scale (starting from the upper scale) is measured in seconds (T_T). By repeating the same procedure, T_R and T_T are measured again. This is repeated 4 times. Separately, a mixture of 20 ml substrate solution, 4 ml acetate buffer solution and 1 ml water is added to the big branch of the viscometer. The time taken to reach the lower scale from the upper scale is measured five times and an average value is obtained T_S (seconds). A blank water test is carried out with 10 ml of water that is isothermalized at $40 \pm 0.1^\circ\text{C}$ by following the same procedure. An average value of 5 measurements is obtained, T_W (seconds). Using the following equation, relative fluidity and T_N values are obtained for each of 4 measurements of effluent time (T_T) and reaction time (T_R).

$$F_R = \frac{T_S - T_W}{T_T - T_W}$$

$$T_N = \frac{1}{2} (T_T/60 \text{ 초} / \frac{1}{60}) + T_R = \frac{T_T}{120} + T_R$$

F_R : Relative fluidity for each reaction time

T_S : Average effluent time for blank substrate test (seconds)

T_W : Average effluent time for blank water test (seconds)

T_T : Effluent time for enzyme reaction solution (seconds)

T_R : Reaction time (minutes) (time taken from "adding the Test Solution" to "before the measurement of effluent time (T_T)")

T_N : Reaction time (T_R) (minutes) + one half of effluent time for Test Solution (T_T)

(minutes)

A standard curve is prepared using the 4 relative fluidity (F_R) values for the 4 reaction times (T_N). This should be a straight line. The slope corresponds to the change in relative fluidity per minute and is proportional to the amount of enzyme. The optimum slope of a series of measurements is a better basis for the enzyme activity than a single value of relative fluidity. F_R values at 10 and 5 minutes are measured from the standard curve. The difference in fluidity should be 0.18~0.22. The enzyme activity is obtained from the following equation.

F_{R10} : Relative fluidity at reaction time of 10 minutes

$$\text{HCU/g} = \frac{1,000(F_{R10} - F_{R5})}{W}$$

F_{R5} : Relative fluidity at reaction time of 5 minutes

1,000 : Conversion activity (g to mg)

W : Weight of sample in 1 ml of Test Solution (mg)

Definition of Activity : 1 hemicellulase unit (HCU) is the activity which generate a change of 1 in relative fluidity for 5 minutes under the above test conditions within a locust bean gum substrate.

Apparatus

- Viscometer : Cannon Fenske Type Viscometer with size 100 corrected scale or its equivalent.
- Glass water bath : Isothermal glass water bath at $40 \pm 0.1^\circ\text{C}$ or its equivalent.

Agents and Solutions

- Acetate Buffer Solution (pH 4.5) : pH of 400 ml of 0.2 N acetic acid is adjusted to 4.5 ± 0.05 by adding 0.2 N of sodium acetate solution while stirring continuously.
- Locust Bean Gum : Quality of powdered locust bean gum varies with substrate lots. Therefore, when a different lot is used, its quality should be checked. If each viscosity difference is more than $\pm 5\%$, it cannot be used for the same test.
- Substrate Solution : 12.5 ml of 0.2 N hydrochloric acid and 250 ml of warm water ($70 \sim 75^\circ\text{C}$) are added in a mixing container and the mixer is set at a low speed. 2 g of dried locust bean gum is carefully added so that it does not splash, and scatter in the container slowly.

Using a rubber police, container wall is scraped down with warm water. The container is then covered and the solution is mixed for 5 minutes at a high speed. It is then transferred into a 1,000 ml beaker and cooled to normal temperature. pH of the solution is adjusted to 6.0 with 0.2 N sodium hydroxide solution. Transfer the resulting solution into a 1,000 ml volumetric flask and diluted to 1,000 ml with water. It is filtered through a gauze before use.

Storage Standards of Hemicellulase

Hemicellulase is strongly hygroscopic. Store in a cold dark place and well-closed containers.

93. Hexane

Definition Hexane is obtained near the boiling point of n-hexane, which is petroleum ingredient, by distillation.

Compositional Specifications of Hexane

Description Hexane is colorless, transparent, volatile liquid with a characteristic scent.

Purity (1) Specific Gravity : Specific gravity of Hexane should be 0.665~0.687.

(2) Refractive Index : Refractive Index n_D^{20} of Hexane should be 1.374~1.386.

(3) Sulfur Compounds : 5 ml of Hexane and 5 ml of silver nitrate ammonia solution are well mixed by shaking. When the mixture heated for 5 minutes at 60°C in protected from light, it should not becomes brown.

(4) Readily carbonizable substances : 5 ml of Hexane is mixed with 5 ml of 94.5~95.5% sulfuric acid by shaking vigorously in a Nestler tube. The color of the sulfuric acid phase should not be deeper than that of the color standard solution B.

(5) Benzene : 50 ml of Hexane is mixed with 50 ml of internal standard solution, and use it as the Test Solution. Separately, 50 ml of benzene standard solution is mixed with 50 ml of internal standard solution, and use it as the Standard Solution. When these solutions are analyzed with gas chromatography, H/Hs should not be greater than H'/Hs', where H, Hs, H', and Hs' are indicated peak heights of Test Solution, internal standard (mixed with Test Solution), Standard Solution, and internal standard (mixed with Standard Solution), respectively. Internal standard solution is prepared by diluting 0.5 ml of methyl isobutyl ketone to 100 ml with n-hexane (UV absorption spectrum measurement grade). Benzene standard solution is prepared by diluting 0.05 ml to 100 ml with n-hexane (UV absorption spectrum measurement grade).

Operation Conditions

-Column : A glass or stainless tube with inner diameter of 3~4 mm and length of 2~3 m

-Column Filler : 177~250 μ porous support material. It is treated with chloroform solution that contains polyethylene glycol 6,000 of 10% in weight?? of the support material. After removing chloroform, it is dried for use

-Column Temperature : a constant temperature in a range of 50~70°C

-Detector : Flame Ionization Detector (FID)

-Carrier gas and flow rate : Nitrogen, Flow rate is adjusted so that benzene is detected in approximately 5 minutes.

- (6) Distillation Test : When Hexane proceed as directed under Method 2 in Boiling Point and Amount of Distillate, 95% or more should be extracted at 64~70°C.
- (7) Residue on Evaporation : 150 ml of Hexane is carefully evaporated by heating in a water bath. When the residue is dried for 30 minutes at 105°C, it's amount should not be more than 2 mg.
- (8) Lead : When 5.0 g of Hexane is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.
- (9) Polycyclic aromatic hydrocarbons : 25 ml of Polycyclic aromatic hydrocarbons is taken and transferred into a 125 ml separatory funnel. Add 25 ml of n-hexane and well mixed by shaking. 5 ml of dimethyl sulfoxide is added to the solution, which is mixed by shaking vigorously for 1 minute and settled until the phase is separated. Transfer the lower phase into a separatory funnel, where 2 ml of n-hexane is added, shaken vigorously for 2 minutes, and settled until the phase is separated. The lower phase is taken and used it as test solution. 5 ml of dimethyl sulfoxide and 25 ml of hexane are weighted, respectively. Shake and mix for 1 minutes, set aside, and the low phase is used as blank test solution. Absorption of the Test Solution is measured in a wavelength range of 260~420nm. Separately, 7.0 mg of naphthalene is accurately weighted. Dissolve in 1,000 ml of isooctane, then this solution is blank test solution. Absorption of the reference Solution is measured at 275nm wavelength. When the absorption of test solution is measured at a wavelength range of 260~420nm, the absorption should not exceed 1/3 of absorption of reference solution measured at 275 nm wavelength.

94. Monascus Color

Definition Monascus Color is a pigment obtained by extracting the cultures of *Monascus anka*(*Monascus purpureus*) with ethyl alcohol. Its major component is Monascorvbirin and Ankaflavin and so on. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Monascus Color

Content Color value ($E_{10\%}^{1cm}$) of Monascus Color should not be more than the indicated content.

Description Monascus Color is red~dark red liquid, solid, powder or paste with a slight characteristic scent.

Identification (1) 50 v/v% alcoholic solution of Monascus Color becomes red color and has a maximum absorption band near 500 nm.
 (2) Add 2 ml of ammonia water and 1 ml of acetone to 1 ml of Test Solution obtained in Color Value section of Monascus Color. When the solution is heated for 1 minute in an approximately 50°C water bath, it becomes yellowish green.
 (3) When 3 ml of nitric acid is added to 0.1 ml of Test Solution obtained in Color Value section, it becomes yellow and then changes to yellowish green.

Purity (1) Arsenic : Place 0.25 g of Monascus Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.
 (2) Heavy Metals : Carbonize 1 g of Monascus Color by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. This solution is used as the test solution. The

content should not be more 20ppm under Heavy Metal Limit Test. Standard color solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. Add 3 drops of hydrochloric acid to the residue, which is then transferred into another Nestler cylinder as described test solution. Then add 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water to make the total volume to 50 ml. This solution is used as the Standard color solution.

- (3) Citrinin : Wash methanol. Pack resin of acrylesters or styrene-divinylbenznes to make 10cm of height in the glass column with 1cm of inside diameter. 1g of *Monascus Color* (converted to color value 50) is accurately taken and packed in the upper layer of glass column. Developing solvent of mixture solution of methanol – water(7:3) in the column is flowed with speed of 2 –3 ml/min. 20 ml of initial eluted solution is splitted. Check the absorbed resin to know whether citrinin is splitted in the 20 ml of initial eluted solution. Filter the solution with membrane filter of no more than 0.5 μm pore size. The solution is used for Test Solution. Separately, 10.0 mg of Citrinin is precisely weighted. Volume up with methanol to make 100 ml. Add 1 ml of this solution to mixture of methanol–water(7 : 3) to make 100 ml. 10.0 ml, 5.0 ml and 1.0 ml are taken in this solution. These solutions are volumed up to make 100 ml with mixture solution of methanol–water(7 : 3). 5 μl of Test Solution and Standard Solution are taken, respectively. When the solutions are conducted by Liquid Chromatography according to following operation, the level should not be more than 0.2 $\mu\text{g/g}$ (Color value is converted to 50). Calculate the area of Citrinin in each peak. Draw standard curve. The level of Citrinin should be applied according to Curve line which is calculated by the area of tailed peak in order that the area of Citrinin in the Test Solution influence on tailing of other peaks

Operation Condition

Detector and wave length : Fluorescence Detector

(Excitation Wave : 330nm,

Fluorescence Wave: 500nm)

Column : The column which is packed with octadecylsilyled silicagel

(ODS Column, 5 μm , 4.6 mm \times 250mm or its equivalent)

Mobile phase: Acetonitril : water : Acetate tri-fluorine(TFA) Solution(100 : 100 : 1)

Flow rate : 1ml/min

Assay (Color Value) Appropriate amount of Monascus Color is precisely weighted to be measured the absorbance within a range of 0.3~0.7, and dissolve in 50 v/v% alcohol so that the total volume is 100 ml. Use the Test Solution. If necessary, the solution is centrifuged and the supernatant is used. Using 50 v/v% alcohol as a reference solution, absorption A is measured at the maximum absorption near 500 nm in 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{10\%}^{1cm}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

95. Monascus Yellow

Definition Monascus Yellow is a pigment obtained by drying, milling, and extracting the cultures of monascus (*Monascus pilosus* or *Monascus purpureus*) with acidic (hydrochloric acid) ethyl alcohol. Its major component is Xanthomonasins. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Monascus Yellow

Content Color value ($[E]_{1cm}^{10\%}$) of Monascus Yellow should not be less than the indicated value.

Description Monascus Yellow is red~yellowish brown liquid, solid, powder or paste with a slight characteristic scent.

Identification (1) 50 v/v% alcoholic solution of Monascus Yellow shows yellow color and fluorescent-green, which has a maximum absorption band near 460 nm.
(2) When an aqueous solution (1→5) of Monascus Yellow is alkalized with sodium hydroxide solution(1→25), its color changes to red~reddish brown.
(3) When 1~2 drops of sulfuric acid are added to an aqueous solution (1→5) of Monascus Yellow, yellow~yellowish brown precipitates are formed.

Purity (1) Arsenic : Place 0.25 g of Monascus Yellow in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.
(2) Lead : When 5.0 g of Monascus Yellow is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay (Color Value) Appropriate amount of Monascus Yellow is precisely weighted to be measured the absorbance within a range of 0.3~0.7, and dissolve in 50 v/v% alcohol so that the total volume is 100 ml. Use the Test Solution. If necessary, the solution is centrifuged and the supernatant is used. Using 50 v/v% alcohol as a reference solution, absorption A is measured at the maximum absorption near 460 nm in 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{10\%}^{1cm}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

96. Carthamus Red

Definition This is a pigment obtained by removing yellow pigment from ornamental flower of carthamus (*Carthamus tinctorius* Linné) of compositae followed by extracting with weakly alkaline water. Its major pigment component is carthamine. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Carthamus Red

Content Color value ($[E]_{1cm}^{10\%}$) of Carthamus Red should not be less than the indicated value.

Description Carthamus Red is dark red~dark violet crystallite or powder with a slight characteristic scent.

Identification (1) A solution of Carthamus Red in dimethylformamide shows red color and a maximum absorption band near 530 nm.

(2) When a mixture of 5 mg of Carthamus Red in 50 ml of water is alkalinized with sodium hydroxide solution (1→25), it shows dark yellow color. When it is acidified with hydrochloric acid, it changes to red.

Purity (1) Arsenic : Place 0.25 g of Carthamus Red in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(2) Lead : When 5.0 g of Carthamus Red is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

Assay (Color Value)

Appropriate amount of Carthamus Red is precisely weighted so that the absorption is within 0.3~0.7. Pigment is eluted with 100 ml of dimethylformamide, which is then filtered. The residue on the filter paper is washed with dimethylformamide, which is added to the filtrate. The filtrate is diluted to 200 ml with dimethylformamide, and use it as Test Solution. Using dimethylformamide as a reference solution, absorption A of Test Solution is measured at the maximum absorption near 530 nm with 1cm path

length. Color value is calculated from the following equation

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 20}{\text{Weight of the sample (g)}}$$

97. Carthamus Yellow

Definition This is a pigment obtained by extracting ornamental flower of carthamus (*Carthamus tinctorius* Linné) of compositae with water. Its major pigment component is safflower yellow. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Carthamus Yellow

Content Color value ($[E]_{1cm}^{10\%}$) of Carthamus Yellow should not be less than the indicated value.

Description Carthamus Yellow is yellow~dark brown liquid, solid, powder, or paste with a slight characteristic scent.

Identification (1) Test Solution obtained in Color Value section shows yellow color and a maximum absorption band near 403 nm.

(2) 3 ml of Fehling solution is added to an aqueous solution containing 0.1 g of Carthamus Yellow. When the solution is heated for 10 minutes in a water bath, red precipitates are formed.

Purity (1) Arsenic : Place 0.25 g of Carthamus Yellow in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(2) Lead : When 5.0 g of Carthamus Yellow is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

Assay (Color Value) Appropriate amount of Carthamus Yellow is precisely weighted so that the absorption is within 0.3~0.7 and dissolve the sample in acetic acid sodium acetate buffer solution with pH 5.28 (total volume 100 ml). 1ml of this solution is diluted to 100 ml with acetic acid sodium acetate buffer solution with pH 5.28 (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using acetic acid sodium acetate buffer solution with pH 5.28 as a reference solution, absorption A is measured at the maximum absorption near 403 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of the sample (g)}}$$

◦ Citric acid·dibasic sodium phosphate buffer solution (pH 5.28)

Solution 1 : Dissolve 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) into 1 ℓ of 0.1 M citric acid solution

Solution 2 : Dissolve 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) into 1 ℓ of 0.2 M dibasic sodium phosphate solution

Solution 1 and Solution 2 are mixed well (97:103) and its pH is adjusted to 5.28.

98. Active Carbon

Definition This is a obtained from the substance containing carbon, which include sawdust, wood pieces, fibers of palm tree barks, lignite, or petroleum, is carbonized and activated to prepare active carbon.

Compositional Specifications of Active Carbon

Description Active Carbon is scentless and tasteless black powder or solid.

Identification (1) 0.5 g of Active Carbon(as is for powder, ground for solid) is placed in a test tube. It is heated with a direct flam in a flowing air. It doesn't catch fire but does combust. Combustion gas is passed through an aqueous solution of potassium hydroxide, which turns the solution white and turbid.

(2) 0.1 g of Active Carbon (as is for powder, ground for solid) is well mixed with 10 ml of diluted methylene blue solution and 2 drops of hydrochloric acid by shaking. When the mixture is filtered through a quantitative filter paper (5 type C), the filtrate should be colorless.

Purity Preparation of Test Solution : Active Carbon (as is for powder, ground for solid) is dried for 3 hours at 110~120°C. 180 ml of water containing 0.1 ml of diluted nitric acid (1→100) is added to 4 g of the dried material, which is weakly boiled for 10 minutes. Cool the solution, and dilute the resulting solution to 200 ml with water and filtered through a quantitative filter paper (5 type C). Discard approximately initial 30 ml of the filtrate. Collected filtrate (Test Solution) is tested as follows.

- (1) Chloride : 1 ml of Test Solution is tested for Chloride. Test for chloride content should not be more than correspond to 0.3 ml of 0.01 N hydrochloric acid.
- (2) Sulfate : 2.5 ml of Test Solution is tested for sulfates. Test for sulfate content should not be more than correspond to 0.5 ml of 0.01 N sulfuric acid.
- (3) Arsenic : Evaporate 12.5 ml of Test Solution to dry in a water bath. 5 ml of water, 1 ml of sulfuric acid, and 10 ml of sulfurous acid are added to the residue. It is concentrated to approximately 2 ml by evaporating in a water bath. The concentrate is diluted to 5 ml with water, which is tested for Arsenic Limit Test. The content should not be more than 4ppm.
- (4) Lead : When test Solution is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
- (5) Zinc : When test Solution is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more

than 25 ppm.

- (6) Cyanogen compound : Weight 5g of Active Carbon is precisely into a flask. Add 50 ml of water and 2 g of tartaric acid, and attach distilling apparatus. 2 ml of 1N sodium hydroxide solution and 10 ml of water are added in the collector, and the end of condenser is immersed in this solution. It is distilled while being cooled with ice and collected in 25 ml of distillate. Water is added to distillate to make 50 ml and 1 ml of iron sulphate solution (1→20) is added to 25 ml of this solution. Heat until boiled, and then cool, filter. When adding 1 ml of hydrochloric acid and 0.5 ml of diluted iron chloride solution to this solution, blue color should not be showed.
- (7) Aromatic hydrocarbon : Add 12 ml of cyclohexane to 1 g of Active Carbon , and attach a reflux condenser. Heat for 2 hours in a water bath and cool to use test solution. Transfer Test solution into a nestler tube. When this solution is observed with irradiating ultraviolet rays, solution should not be thick than the color of the solution, which is obtained by processing 12 ml of solution made by dissolving 0.1 mg of quinine sulfate in 1,000 ml of 0.1N sulfuric acid in the same manner as test solution.

99. Yeast

Definition Liquid yeast is obtained by separating and washing cultures of yeast (*Saccharomyces sp.*). Raw yeast is obtained by dehydrating and forming. Dry yeast(active) or sterilized dry yeast(inactive) is obtained by removing water from raw yeast. Small amount of emulsifier can be added.

Compositional Specifications of Yeast

A. Dry Yeast

Description Dry Yeast is yellow~brown granule, powder, or solid with a characteristic scent.

Purity (1) Activation(In the case, this applies to active dry yeast only) : When 5 g of Dry Yeast is added to 50 ml of 1 % sugar solution and heated to 35~40℃, gas should be generated within 2 hours and 30 minutes.

(2) Arsenic : Test Solution is prepared with 1 g of Dry Yeast following the procedures in Preparation of Test Solution (1) in Purity for Diluted additives.

When 20 ml of this 100 ml test solution is tested for Arsenic Limit Test, it should be appropriate. A color standard is prepared by 20 ml of 100 ml solution which 5 ml of arsenic standard solution is processed following the same procedure as Test Solution.

(3) Heavy Metals : This test solution should be appropriate for Heavy Metals Test according to the test solution preparation method 1 in Purity for Mixed preparations with 2 g of Dry Yeast. It's content should not be more than 50ppm. Color standard solution is prepared by 20 ml of 100 ml solution which 5 ml of lead standard solution is processed following the same procedure starting with 10 ml of lead standard solution.

(4) Total Viable Aerobic Count(In the case, this applies to inactive dry yeast only) : When Guar Gum is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 7,500 per 1 g

(5) Coliform Group(In the case, this applies to inactive dry yeast only) : When yeast proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods in Food Code, it should contain not more than 10 colonies per 1 g of this product.

(6) Salmonella(In the case, this applies to inactive dry yeast only) : When Locust Bean Gum is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).

B. Raw Yeast

Description Raw Yeast is milky white~yellowish brown solid with a characteristic scent.

- Purity** (1) Activation : When 5 g of Raw Yeast is added to 50 ml of 10% sugar solution and heated to 30~35°C, gas should be generated within 1 hour.
- (2) Arsenic : Should follow the procedure in Purity (2) for A. Dry Yeast with 2 g of sample (not more than 3ppm). A color standard is prepared with 6 ml of arsenic standard solution following the same procedure as Test Solution.
- (3) Heavy Metals : Should follow the procedure in Purity (3) for A. Dry Yeast with 3.33 g of sample (not more than 30ppm). A color standard is prepared with 10 ml of lead standard solution following the same procedure as Test Solution.

C. Liquid Yeast

Description Liquid Yeast is white~yellowish brown liquid with a characteristic scent.

- Purity** (1) Activation : When 5 g of Liquid Yeast is added to 50 ml of 10% sugar solution and heated to 30~35°C, gas should be generated within 1 hour.
- (2) Arsenic : Should follow the procedure in Purity (2) for A. Dry Yeast with 2 g of sample (not more than 1.5ppm). A color standard is prepared with 3 ml of arsenic standard solution following the same procedure as Test Solution.
- (3) Heavy Metals : Should follow the procedure in Purity (3) for A. Dry Yeast with 3.33 g of sample (not more than 15ppm). A color standard is prepared with 5 ml of lead standard solution following the same procedure as Test Solution.

Storage Standards of Yeast

Dry yeast should be stored in light-resistant and shielded container.

100. Yeast Extract

Definition Yeast Extract consists of yeast cell components such as amino acids, peptides, carbohydrates, and water soluble salts. It is generated from hydrolysis of polypeptide bonds by yeast that is present in edible yeast, or added edible yeast. Salts can be added during manufacturing process

Compositional Specifications of Yeast Extract Place liquid or paste sample in a container, that is previously weighted. Evaporate the sample in the water bath to dry. For powder and granule, it is dried at 105°C until the weight becomes constant. Following each content specification is based on a dried form.

Content When Yeast Extract is Yeast Extract, it should contain not less than 42% of protein.

Description Yeast Extract is liquid, powder, granule, or paste.

Purity (1) Lead : When 5.0 g of Yeast Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(2) Sodium : Approximately 1.5 g (converted to a dried form) is precisely weighted into a porcelain crucible and reduced to ash for 2~4 hours at 246~260°C. Dissolve the ash with 5 ml of 20% hydrochloric acid. If the residue is needed to completely dissolve, heat the solution and filter through an acid washed filter paper into a 500 ml flask. Filter paper is washed with warm water to the flask. The volume of the filtrate is brought up to 500 ml with water. 1 ml of this solution is diluted to 100 ml with water (Test Solution). Separately, 0.5 ml of undiluted sodium standard solution is diluted to 100 ml with water (Sodium Standard Solution). Sodium Standard Solution and Test Solution is analyzed with atomic absorption spectrophotometer and the content of sodium in the sample is obtained not more than 20.0%.

(3) Potassium : Approximately 1 g (converted to a dried form) is precisely weighted into a porcelain crucible and reduced to ash for 2~4 hours at 246~260°C. Dissolve the ash with 5 ml of 20% hydrochloric acid. If the residue is needed to completely dissolve, heat the solution and filter paper into a 500 ml flask. Filter paper is washed with warm water to the flask. The volume of the filtrate is brought up to 500 ml with water. 0.8 ml of this solution is diluted to 100 ml with water (Test Solution). Potassium Standard Solution and Test Solution is analyzed with atomic absorption spectrophotometer and the content of potassium in the sample is obtained not more than 13.0%.

- Potassium Standard Solution : Potassium chloride is dried for 2 hours at 130°C. 9.534 g of dried potassium chloride is precisely weighted and dissolved in water (total volume = 1,000 ml). 0.4 ml of this solution is further diluted to 1,000ml (Standard Solution). 1 ml of this solution contains 2 µg of K.
- (4) Mercury : Weight accurately 0.1 g of Yeast Extract (converted to a dried form) into a flask for decomposition, where 10 ml of water and 20 ml of nitric acid are added and then set aside. 20 ml of sulfuric acid is slowly added. A reflux condenser is attached to the flask, which is then heated carefully until generation of NO₂ gas stops. If the decomposed liquid does not turn transparent pale orange in color, cool and add 5 ml of nitric acid. It is then heated again. If necessary, this procedure is repeated until the decomposed liquid becomes transparent and NO₂ generation stops, cool the solution, and add 50 ml of water and 10 ml of 10% urea solution to the decomposed liquid. Boil it for 10 minutes. Cool the solution. 1g of potassium permanganate is added and mixed for 10 minutes while shaking occasionally. If purple red color disappears, small amount of potassium permanganate is added to the solution and heated for 20 minutes. This is repeated until the purple red color persists. Boil the solution for 20 minutes, and if purple red color disappears, cool and add 1g of potassium permanganate, and boil for 20 minutes. When purple red color of this solution disappears, repeat adding potassium permanganate and heating twice. Cool and drop-wise add 10% of hydrogen peroxide solution carefully until the solution becomes colorless and transparent. Cool the solution, the inner walls and connections of the apparatus are washed with 20 ml of diluted sulfuric acid (1→100) to the flask making the total volume to 100 ml (Test Solution). The same procedure is repeated for the blank test solution to correct the Test Solution. 100 ml each of Test Solution and blank test solution is added to a test bottle of atomic absorption spectrophotometer, where 10 ml of stannous chloride solution is added. It is connected immediately to the atomic absorption spectrophotometer and a diaphragm pump is turned on to circulate the air. When the signal in the recorder rises suddenly and becomes constant, the absorption is measured. The absorption of the Test Solution should not be higher than that of the Standard Solution (not more than 3ppm). Atomic absorption of the mercury standard is measured by following the same procedure with 100 ml dilution of 3 ml of mercury Standard Solution.
- (5) Insoluble substances : Approximately 5 g (converted into a dried form) is precisely weighted into a 250 ml flask with a stopper, where 75 ml of water is added. It is covered with a watch glass and gently boiled for 2 minutes. The content is filtered through a porcelain type glass filter (previously weighted), which is then dried for 1

hour at 105°C. It is cooled in a desiccator and weighted. The content of insoluble substances should not be more than 2%.

- (6) Ratio of Nitrogen in α -Amino Acid over Total Nitrogen : 7~25 g (converted into a dried form without sodium) is precisely weighted into a 500 ml volumetric flask using 50 ml of warm water (repeated several times). The total volume is brought up to 500 ml with water (Test Solution). 20 ml of Test Solution is neutralized with 0.2 N barium hydroxide solution or 0.2 N sodium hydroxide solution (indicator : phenolphthalein TS). Add 10 ml of freshly prepared phenolphthalein : formalin solution to this solution, which is titrated with 0.2 N barium hydroxide solution until it turns clear red. A small excess amount of 0.2 N barium hydroxide solution is precisely added to the resulting solution, which is back titrated with 0.2 N hydrochloric acid. Separately, a blank test is carried out by following the same procedure with 20 ml of water. The content of α -amino nitrogen is calculated by the following equation. The ratio (AN/TN) of α -amino nitrogen (AN) over total nitrogen (TN) should be 15~55%.

1 ml of 0.2 N barium hydroxide solution = 2.8 mg of α -amino nitrogen

◦ Phenolphthalein Formalin Solution : 50 ml of 40% formalin containing 1 ml of 0.05% phenolphthalein TS in 50% alcohol (neutralized to pH 7.0 with 0.2 N barium hydroxide solution or 0.2 N sodium hydroxide solution).

- (7) Glutamic Acid : 5 mg (converted to a dried form) of Yeast Extract is precisely weighted and added with 0.2 N sodium citrate buffer solution (pH 2.2, total volume 5 ml) (Test Solution). If there is any insoluble residue, it is filtered or centrifuged and the supernatant is used. 2 ml each of Test Solution and glutamic acid standard solution is analyzed by ion exchange amino acid analyzer. From the obtained chromatogram, the concentration of glutamic acid (C_A , mg/ml) in Test Solution is obtained. The content of glutamic acid in sample is obtained by the following equation and it should not be more than 12.0%. The content of glutamic acid in total amino acid should not be more than 28.0%.

$$\text{Content of glutamic acid (\%)} = \frac{C_A \times 5 \times 100}{\text{Weight of the sample (mg)}}$$

$$C_A \text{ (mg/ml)} = \frac{A_A \times C_S}{A_s}$$

A_A : Peak area of glutamic acid in Test Solution

A_S : Peak area of glutamic acid in glutamic acid Standard Solution

C_S : Concentration of glutamic acid Standard Solution (mg/ml)

$$\text{Glutamic acid content in total amino acid (\%)} = \frac{\text{content of glutamic acid (\%)}}{6.25N_T} \times 100$$

N_T : Total nitrogen content (%)

-Ion Exchange Amino Acid Analyzer : A sulfonated polystyrene column is attached. sample is eluted by reacting with ninhydrine solution. Absorptions at 440 nm and 570 nm are automatically measured by spectrophotometer.

- Glutamic Acid standard solution : 1,250 ± 2 mg of glutamic acid is precisely weighted into a 500 ml volumetric flask, and made 250 ml with water. dissolve undissolved amino acid by adding 5 ml of hydrochloric acid. The total volume is brought up to 500 ml with water. Exactly 1 ml of this solution is mixed with 4 ml of sodium citrate buffer solution (pH 2.2) (total volume = 5 ml). 2 ml of this solution contains 1.0 mg of glutamic acid.
- 0.2 N Sodium Citrate Buffer Solution (pH 2.2) : Dissolve weighted 10.52 g of sodium citrate in 150 ml of water. pH of this solution is adjusted to pH 2.2, which is then diluted to 200 ml with water.

- (8) Total viable aerobic count : Yeast Extract (converted to a dried form) is tested by Total viable aerobic count in Microbiological Methods in General Testing Methods in Food Code. It should not be more than 50,000 per 1 g.
- (9) Fungi : Yeast Extract (converted to a dried form) is tested by Fungi in Microbiological Methods in General Testing Methods in Food Code. It should not be more than 50 per 1 g.
- (10) Coliform Group : Yeast Extract (converted to a dried form) is tested by Microbiological Methods for Coliform Group in General Testing Methods in Food Code. It should not be more than 10 per 1 g.
- (11) Salmonella : Yeast Extract (converted to a dried form) is tested by

Microbiological Methods for Salmonella in General Testing Methods in Food Code. It should be negative (-).

Assay Approximately 0.3 g (converted to a dried form, nitrogen excluded) of Yeast Extract is analyzed by the procedure in Kjeldahl Method in Nitrogen Determination Method.

0.1 N sulfuric acid 1 ml = 1.401 mg N

101. Enzymatically Decomposed Lecithin

Definition Enzymatically Decomposed Lecithin is obtained by decomposing lecithin with enzyme. Its major components are rhizolecithin and phosphatidic acid.

Compositional Specifications of Enzymatically Decomposed Lecithin

Description Enzymatically Decomposed Lecithin is white~brown powder or granule, or pale yellow~dark brown viscous liquid with characteristic scent and taste.

Identification 1 g of Enzymatically Decomposed Lecithin is placed in a flask for decomposition. Add 5 g of powdered potassium sulfate, 0.5 g of copper sulfate, and 20 ml of sulfuric acid. The flask is tilted to 45°C angle and gently heated so that it doesn't bubble. Then the temperature is raised to boil until the solution becomes transparent blue. It is then heated for 1~2 hours and cooled and the same amount of water is added. 10 ml of ammonium molybdate (1→5) is added to 5 ml of the resulting solution. Upon heating yellow precipitates are formed.

Purity (1) Acid value : 2 g of Enzymatically Decomposed Lecithin is precisely weighted and dissolved in 50 ml of toluene. After adding 50 ml of alcohol previously neutralized with 0.1N potassium hydroxide solution while using phenolphthalein TS as indicator, heat until the sample is dissolved. This mixture is used as the test solution, and when it is proceeded as directed under Acid value in Fats Test, the tvalue of the solution should not be more than 45.

(2) Toluene Insoluble substances : Approximately 5 g of Enzymatically Decomposed Lecithin is precisely weighted and dissolved in 100 ml toluene. Insoluble substances are filtered through a glass filter (1G4) that is previously weighted. It is washed several times with 25 ml of toluene. The residue along with the filter is dried for 1 hour at 105°C and cooled in a desiccator and weighted. Or 5 g of Enzymatically Decomposed Lecithin is precisely weighted and dissolved in 100 ml of toluene in a Erlenmeyer flask. Transfer 50 ml of the solution into a centrifuge tube, which is then centrifuged for 15 minutes at 3,000 rpm. The supernatant is removed. The remaining 50 ml of the solution is centrifuged by the same method using the same tube. The inner wall of the flask is washed with 50 ml of toluene into the same tube, which is then centrifuged by the same method. The supernatant is discarded. This is repeated twice. The insoluble substances are dried in the tube for 2 hours at 105°C, cooled in a desiccator, and weighted. The content should not be more than 0.3%.

(3) Acetone Insoluble substances

Preparation of sample : If Enzymatically Decomposed Lecithin contains moisture, it

is dehydrated and dried by heating at 80°C and evaporating under vacuum. It is then dissolved in toluene and the solution is filtered through a filter paper to remove impurities. Toluene from the filtrate is removed by evaporation under a reduced pressure in a round bottom flask. The residue is crude sample.

Test Procedure : 10 g of crude sample is precisely weighted into a 300 ml beaker, and then 200 ml of acetone saturated with phospholipid that is cooled in ice water is added. It is thoroughly mixed and set aside for 30 minutes. Acetone insoluble substances settle down at the bottom of the beaker and the solution becomes clear. The supernatant is vacuum filtered with a glass filter, precisely weighted Acetone insoluble substances are washed three times with 30 ml of acetone saturated with phospholipid that is cooled in ice water. Acetone insoluble substances and wash acetone are transferred into a glass filter, which is then vacuum filtered. Acetone insoluble substances are dried for 1 hours under a reduced pressure. Or, 2.0 g of crude sample is precisely weighted into a 50 ml graduated centrifuge tube with a stopper (precisely weighted) and dissolved by heating in 5 ml of acetone saturated with phospholipid that is cooled in ice water. The tube is then cooled for 15 minutes in an ice bath (also a glass stirring rod is precisely weighted and cooled for 15 minutes in an ice bath). Then the tube is filled to 50 ml with acetone saturated with phospholipid, which is stirred thoroughly while hitting. It is cooled for 15 minutes in an ice bath and then stirred again. It is then centrifuged at 3,000 rpm for 15 minutes and the supernatant is discarded. This procedure is repeated twice. Acetone insoluble substances are dried along with the centrifuge tube for 2 hours at 105°C and cooled in a desiccator. The content of acetone insoluble substances is calculated by the following equation and it should not be less than 56%.

$$\text{Acetone insoluble substances (\%)} = \frac{\text{Insoluble substances (g)}}{\text{Weight of the sample (g)}} \times 100$$

Solutions

- Acetone Saturated with Phospholipid : Acetone insoluble substances (phospholipid) are obtained by treating crude sample with acetone (as described above). 1 g of the insoluble substances is placed in a 1,000 ml flask with a stopper and dissolved in acetone (total volume = 1,000 ml). The solution is cooled in an ice bath while shaking occasionally. This acetone is saturated with phospholipid. The supernatant is filtered before use.

- (4) Peroxide Value : 5 g of Enzymatically Decomposed Lecithin is precisely weighted into a 250 ml of Erlenmeyer flask with a stopper. It is then dissolved to a clear solution in 35 ml mixture of acetic acid and chloroform (3 : 2) by gently shaking. Clean nitrogen is passed through to replace air in the flask. 1 ml of potassium iodide solution is accurately added while nitrogen is passed through. A stopper is placed immediately and the flask is shaken for 1 minute. It is then set aside for 5 minutes in a dark place. 75 ml of water is added and shaken vigorously with a stopper. It is then titrated with 0.01 N sodium thiosulfate solution (indicator : starch solution). Peroxide value is obtained from the following equation. It should not be more than 10. Separately, a blank test is carried out for correction.

$$\text{Peroxide Value} = \frac{\text{Consumed amount of 0.01N sodium thiosulfate solution (ml)}}{\text{Weight of the sample (g)}} \times 10$$

- (5) Arsenic : Place 0.25 g of Enzymatically Decomposed Lecithin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.
- (6) Lead : When 5.0 g of Enzymatically Decomposed Lecithin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.
- (7) Mercury : When Enzymatically Decomposed Lecithin is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

Water Content Water content of Enzymatically Decomposed Lecithin is determined by direct titration method in water determination (Karl-Fisher Titration) and should not be more than 2.0%. However, chloroform : methyl alcohol mixture (4:1) is used instead of methyl alcohol for Karl-Fisher titration.

102. Spice Oleoresins

Definition Spice oleoresins are prepared by one of the following processes.

(1) Spice oleoresins is obtained by extracting each raw materials of spice with an appropriate solvent or a combination of solvents such as ethyl alcohol, methyl alcohol, trichloroethylene, acetone, isopropyl alcohol, methylene chloride, and hexane. Solvents should be removed according to the specifications in Residual Solvents.

(2) Spice oleoresins is a mixture of volatiles and non-volatiles from a spice. Volatiles in each raw materials of spice are fractionally distilled. Non-volatiles are extracted by the solvents listed in (1) and solvents are removed. In oleoresins, there are Oleoresin Thyme (origin : dried root cortex of *Thymus vulgaris* L.), Oleoresin Dill seed (origin : dried seeds of *Anethum graveolems* L.), Oleoresin Laurel Leaf (origin : dried leaves of *Laurus nobilis* L.), Oleoresin Marjoram (origin : dried root cortex of *Majorana hortensis* Moench), Oleoresin Basil, (origin : dried root cortex of *Ocimum basilicum* L.), Oleoresin Black and Oleoresin White Pepper (origin : dried fruits of *Piper nigrum* L.), Oleoresin Celery (origin : dried seeds of *Apium graveolens* L.), Oleoresin Anise (origin : dried fruits of *Pimpinella anisum* L.), Oleoresin Angelica Seed (origin : dried seeds of *Angelica archangelica* L.), Oleoresin Origanum (origin : dried leaves of *Origanum*), Oleoresin Ginger (origin : dried rootstocks of *Zingiber officinale* L.), Oleoresin Cardamom (origin : dried seeds of *Elettaria cardamomum* Maton), Oleoresin Caraway (origin : dried seeds of *Carum carvi* L.), Oleoresin Coriander (origin : dried seeds of *Coriandrum sativum* L.), Oleoresin Cumin (origin : dried seeds of *Cuminum cyminum* L.), Oleoresin Cubeb (origin : dried seeds of *Piper cubeba* L.), Oleoresin Parsley Leaf (origin : dried root cortex of *Petroselinum crispum* L.), oresin Parsley Seed (origin : dried seeds of *Petroselinum crispum* L.), Oleoresin Fennel (origin : dried leaves of *Foeniculum vulgare* P. Miller), Oleoresin Pimenta Berries (origin : dried fruits of *Pimenta officinalis* Lindl), Oleoresin Garlic (origin : bulbs or leaves of *Allium sativum* L.), Oleoresin Nutmeg (origin : seed kernel of dried mature seeds of *Myristica fragrans* Houttuyn), Oleoresin Rosemary (origin : juvenile leaves of *Rosmarinus officinalis* L.), Oleoresin Mace (origin : dried s pornioderm of dried mature seeds of *Myristica fragrans* Houtt.), Oleoresin Sage (origin : dried leaves of *Salvia officinalis* L.), Oleoresin Cinnamon (origin : dried inner barks of *Cinnamomum zeylanicum* nees), Oleoresin Onion (origin : bulbs of *Allium cepa* L.), Oleoresin Cassia (origin : dried barks of *Cinnamomum cassia* Blume), Oleoresin Capsicum (origin : dried fruits of *Capsicum annum* L. or *Capsicum frutescens* L.),

Oleoresin Clove (origin : dried flower buds of *Eugenia caryophyllata* Thunberg), and

Oleoresin Tarragon (origin : leaves, stems, and flowers of *Artemisia dracunculus* L.). Dilutant, antioxidant, or other food additives (emulsifier, etc.) can be added for quality preservation.

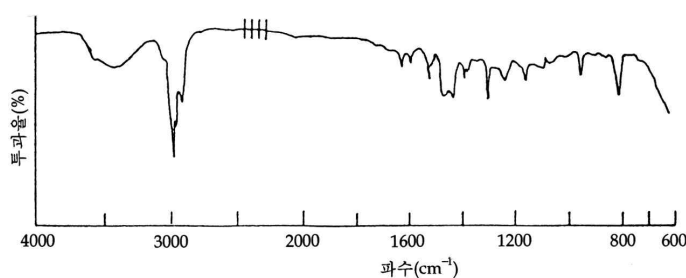
Compositional Specifications of Spice Oleoresins

Description Spice oleoresins is liquid, viscous liquid, or semi solid material. It has characteristic scent and taste of the corresponding spice (and its raw material).

Identification (1) Dissolve 50 mg of Spice oleoresins in 10 ml of ethyl-alcohol. If necessary, Centrifuge and use Test Solution. Separately, dissolve 1 mg of capsaisin with 10 ml of ethyl-alcohol to be used as a standard solution. Apply 10 ml of Test Solution and Standard Solution, separately, to silica gel plate for Thin Layer Chromatography. After developing the plate about 12 cm in the Developing solvent with a mixture of Ether : Ethylalcohol (19:1), and air-dry. Spray equally the plate with 2,6-Dibromoquinone-chloride solution. and set aside in ammonia gas. The spot of test solution should be same in the aspects of the color, developing distance comparing the blue spot of the standard solution(only apply for Oleoresin Capsicum).

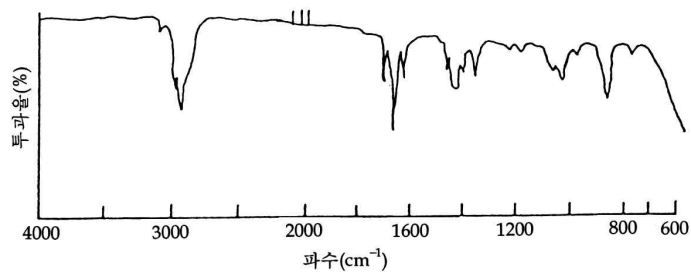
(2) Spice oleoresins is refined by Test Procedure in Purity (5) for Volatile Oil. It is tested by (2) Solution Method in Infrared Spectrophotometry and it shows the following characteristic spectrum, except Oleoresin Capsicum.

(1) Thyme Oil

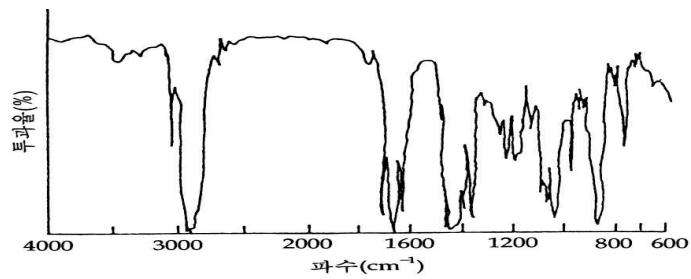


(2) Dill Seed Oil

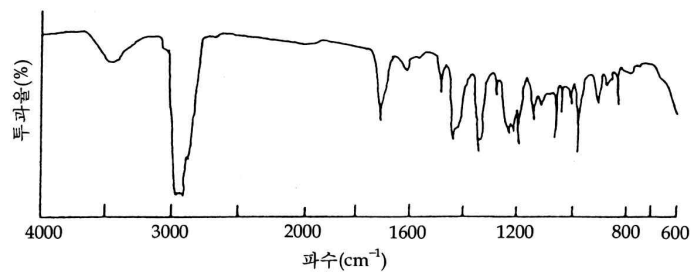
① Dill Seed Oil, European Type



② Dill Seed Oil, Indian Type

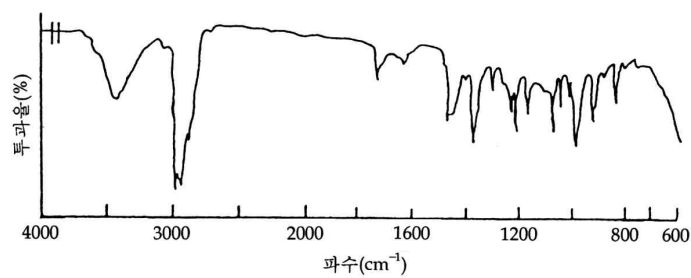


(3) Laurel Leaf Oil

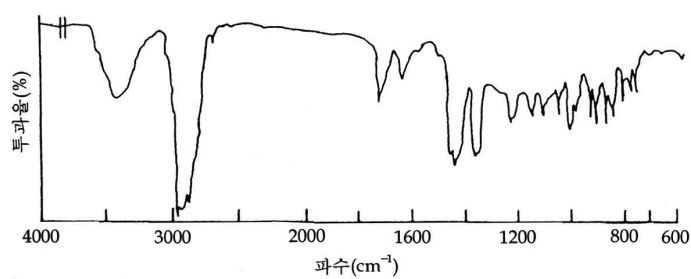


(4) Marjoram Oil

① Marjoram Oil, Spanish Type

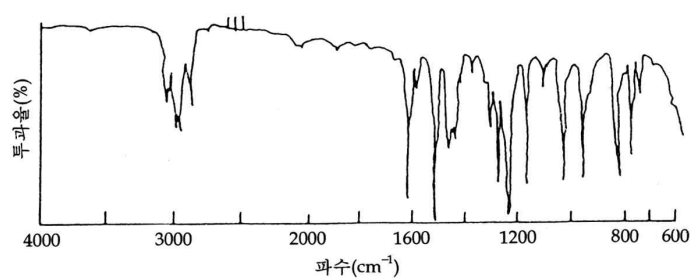


② Marjoram Oil, Sweet

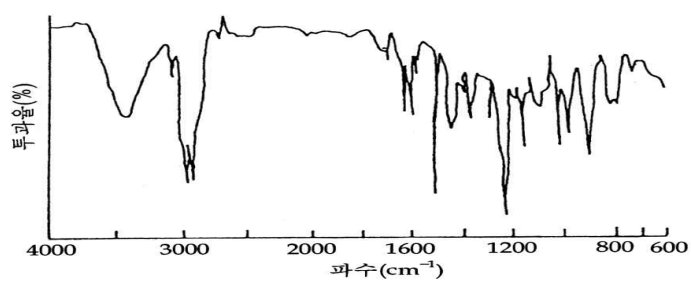


(5) Basil Oil

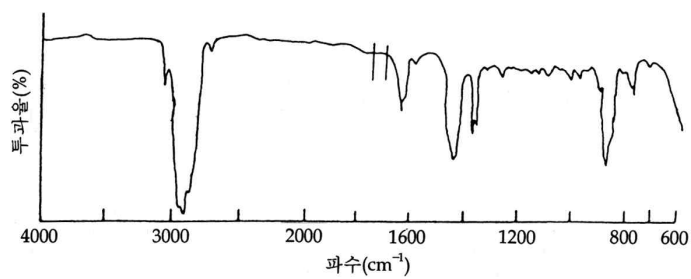
① Basil Oil, Comoros Type



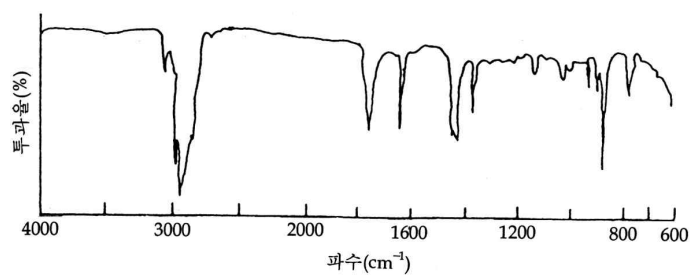
② Basil Oil, European Type



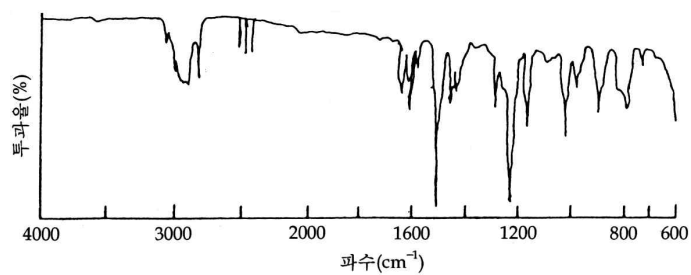
(6) Black and White Pepper Oil



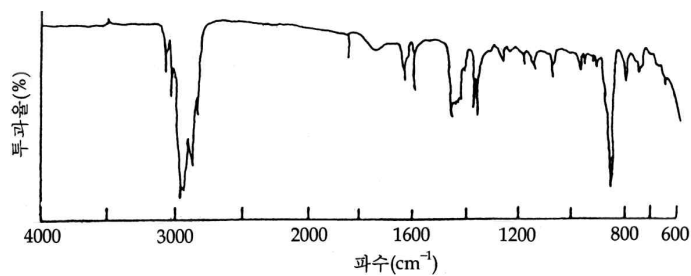
(7) Celery Seed Oil



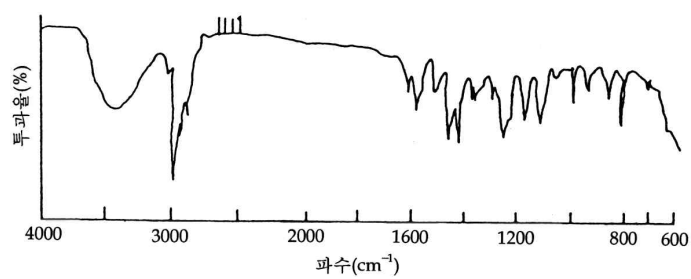
(8) Anise Oil



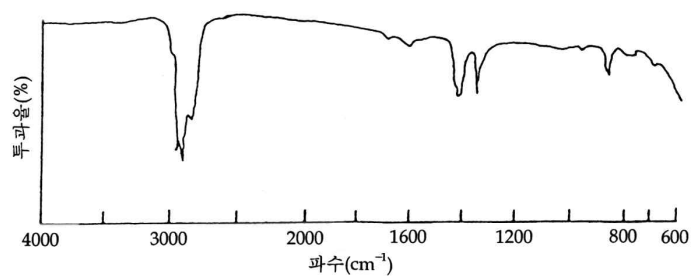
(9) Angelica Seed Oil



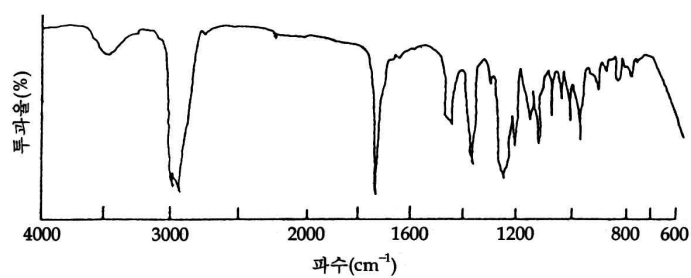
(10) Origanum Oil, Spanish Type



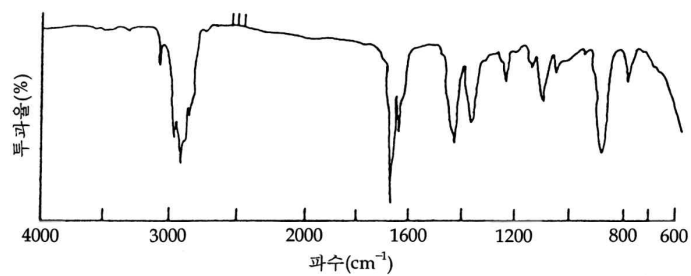
(11) Ginger Oil



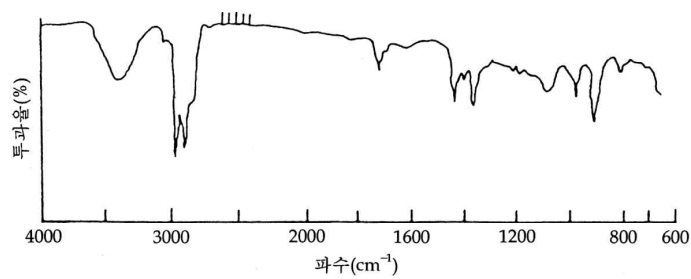
(12) Cardamom Oil



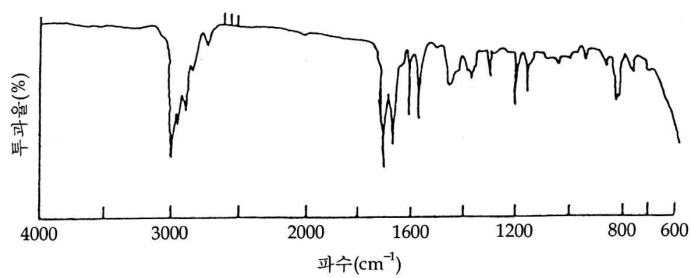
(13) Caraway Oil



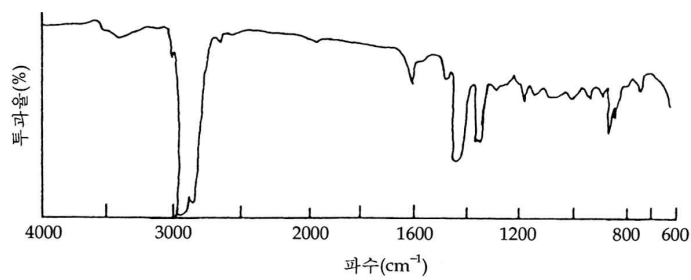
(14) Coriander Oil



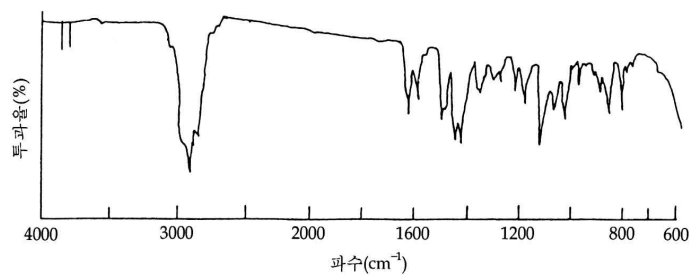
(15) Cumin Oil



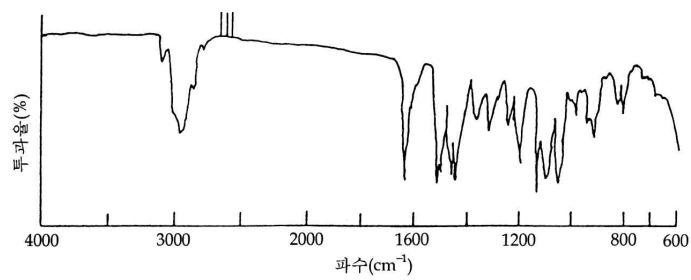
(16) Cubeb Oil



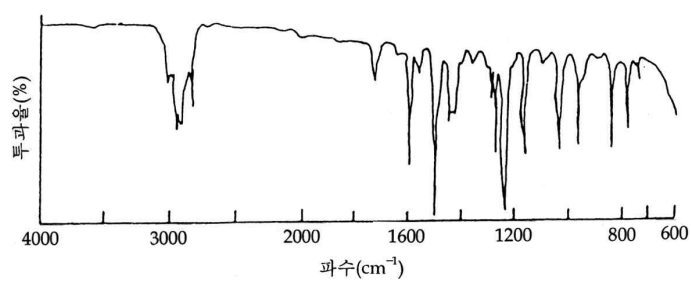
(17) Parsley Herb Oil



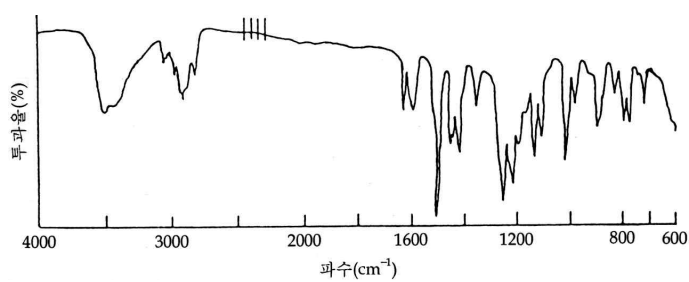
(18) Parsley Seed Oil



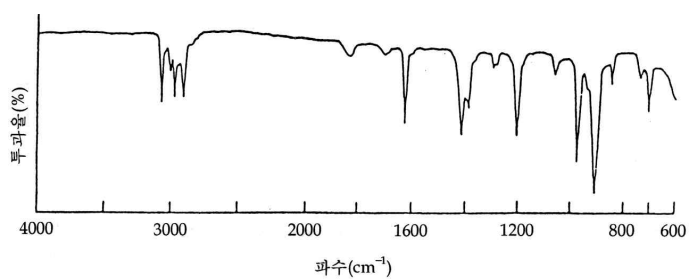
(19) Fennel Oil



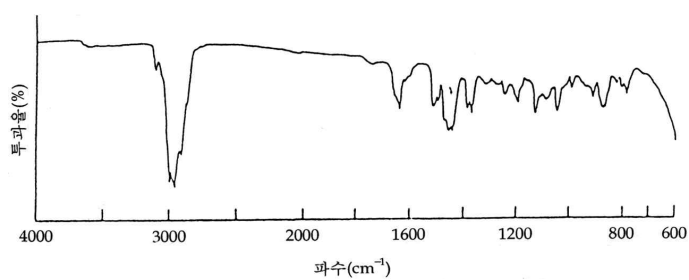
(20) Pimenta Oil



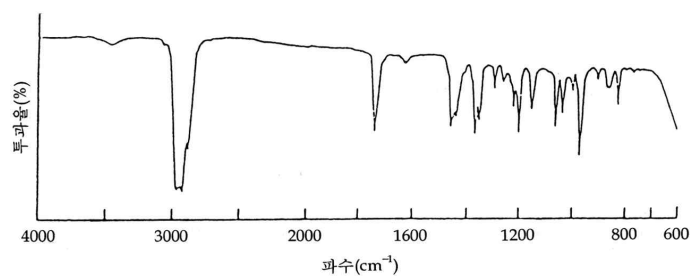
(21) Garlic Oil



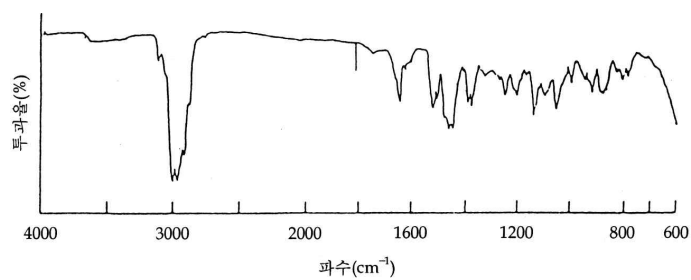
(22) Nutmeg Oil



(23) Rosemary Oil

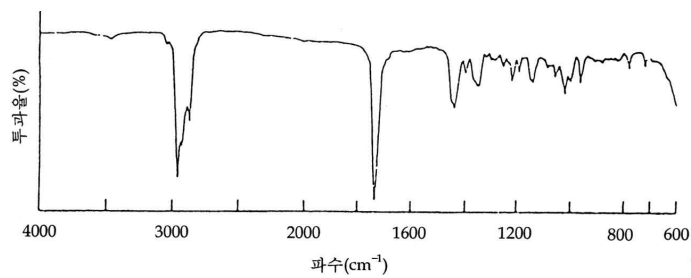


(24) Mace Oil

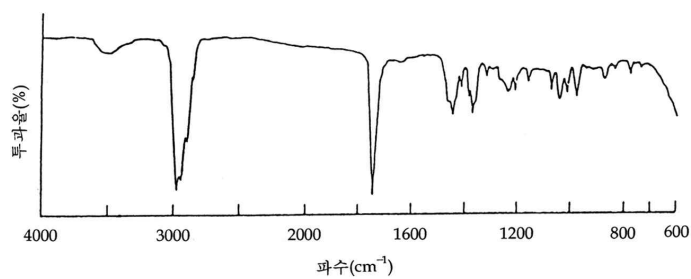


(25) Sage Oil

① Sage Oil, Dalmatian Type

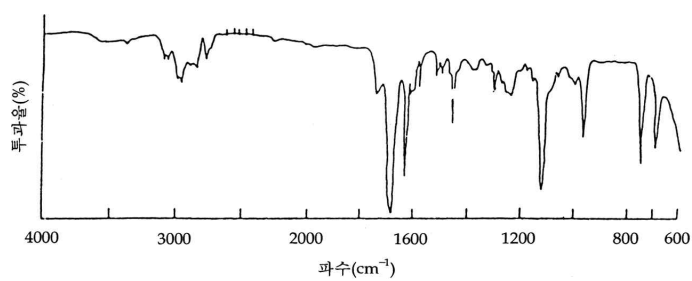


② Sage Oil, Spanish Type

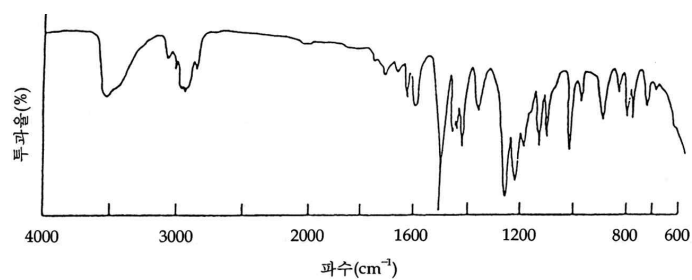


(26) Cinnamon Bark Oil

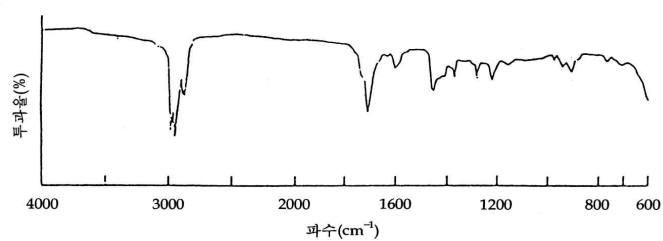
① Cinnamon Bark Oil, Ceylon Type



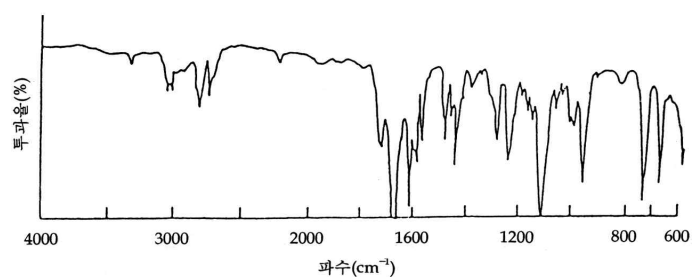
② Cinnamon Leaf Oil



(27) Onion Oil

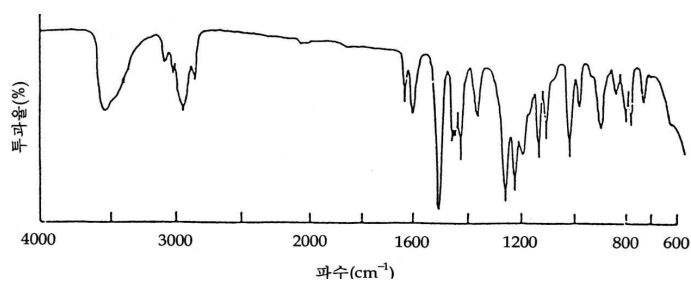


(28) Cassia Oil

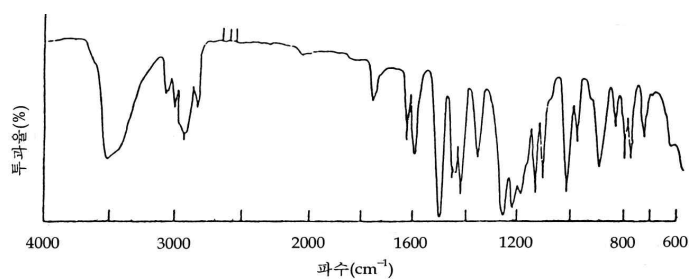


(29) Clove Leaf Oil

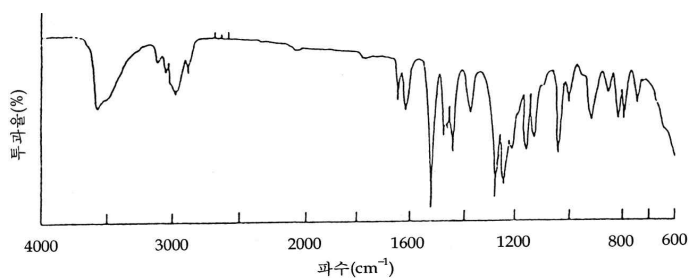
① Clove Leaf Oil



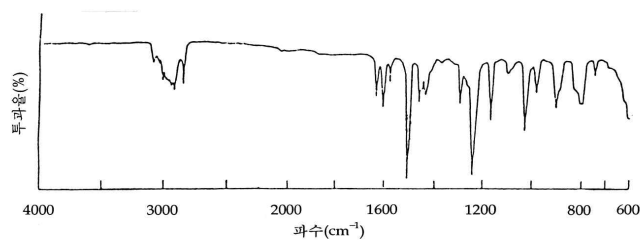
② Clove Oil



③ Clove Stem Oil



(30) Tarragon Oil



Purity (1) Arsenic : Place 0.25 g of Spice oleoresins in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of

hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(2) Lead : When 5.0 g of Spice oleoresins is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm.

(3) Residual Solvents : When Spice oleoresins is tested by Purity (5) for Paprika Extract Pigments, the content of residual solvents should be,

Methylene chloride, trichloroethylene	Not more than 30ppm (individual or total if combined))
Acetone	Not more than 30ppm
Isopropyl alcohol	Not more than 50ppm
Methyl alcohol	Not more than 50ppm
Hexane	Not more than 25ppm

(4) Volatile Oil : When Spice oleoresins is tested for the amount of volatile distillate by the following Test Procedure, it should be appropriate for the following specifications. Test Procedure. When it is diluted with an emulsifier (a spice oleoresin type), the mixing ratio of the oleoresins is taken into account in the specifications.

Oleoresin Thyme : 5~12 (v/w)%

Oleoresin Dill Seed : 10~20 (v/w)%

Oleoresin Laurel Leaf : 5~25 (v/w)%

Oleoresin Marjoram : 10~20 (v/w)%

Oleoresin Basil : 4~17 (v/w)%

Oleoresin Black Pepper and oleoresin White Pepper : 15~35 (v/w)%

Oleoresin Celery Seed : 7~20 (v/w)%

Oleoresin Anise : 9~22 (v/w)%

Oleoresin Angelica Seed : 2~7 (v/w)%

Oleoresin Origanum Oil : 20~45 (v/w)%

Oleoresin Ginger : 18~35 (v/w)%

Oleoresin Cardamom : 50~80 (v/w)%

Oleoresin Caraway : 10~20 (v/w)%

Oleoresin Coriander : 2~12 (v/w)%

Oleoresin Cumin : 10~30 (v/w)%

Oleoresin Cubeb : 50~80 (v/w)%

Oleoresin Parsley Herb : 2~10 (v/w)%

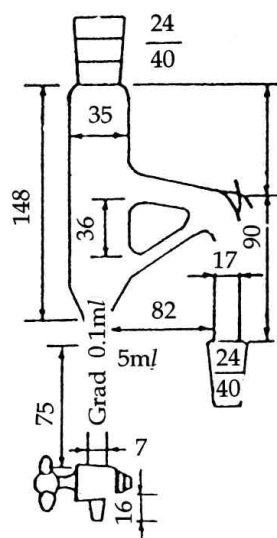
Oleoresin Parsley Seed : 2~7 (v/w)%

Oleoresin Fennel : 3~20 (v/w)%

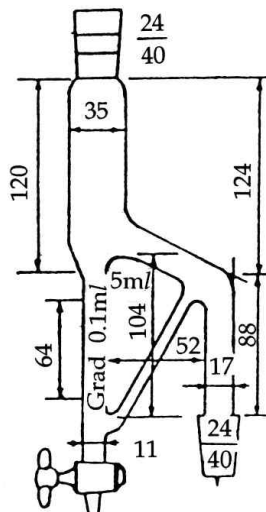
Oleoresin Pimenta Berries : 20~50 (v/w)%

Test Procedure : Sufficient amount of sample is precisely weighted (so that 2~5 ml of volatile oil can be collected) into a 1,000~2000 ml round bottom flask with a 24/40 ground joint neck, where a magnetic bar and 500 ml of water are added. A distilling head and reflux condenser are attached as shown in the figure. The flask is heated while stirring until the amount of the oil does not change. It is cooled to room temperature and set aside until the oil becomes clear. The volume of collected oil is measured (down to 0.02 ml) and its content is calculated by the formula;

$$\text{Content of volatile oil (v/w) \%} = \frac{\text{amount of collected oil (ml)}}{\text{Weight of the sample (g)}} \times 100$$



(a) for oils heavier than water



(b) for oils lighter than water

Distilling head : Clevenger Traps (unit : mm)

- (5) Piperin (only for oleoresin black pepper and oleoresin white pepper) : Preparation of Undiluted Standard Solution : Piperin is purified by recrystallization in isopropyl alcohol so that its melting point is 129~130°C. 100 mg of purified piperin as a crystal form is precisely weighted into a 100 ml flask and dissolved in dichloroethylene. Dilute it to 100 ml, and then 10 ml of this solution is rediluted to 100 ml with dichloroethylene (Undiluted Standard Solution).

- Preparation of Standard Solution : 1.0, 3.0, 5.0, and 10.0 ml (contains 0.1, 0.3, 0.5, and 1.0 mg of piperin) of undiluted Standard Solution is diluted to 100 ml with dichloroethylene (Standard Solutions).
- Preparation of Test Solution : Spice oleoresins is heated and stirred with glass rod in a 100°C steam bath or oven (hot plate should not be used). Approximately 100 mg is precisely weighted into a 100 ml flask and dissolved in dichloroethylene (total volume = 100 ml). 1 ml of this solution is further diluted to 100 ml with dichloroethylene (Test Solution).

Test Procedure : Absorptions of Test and Standard Solutions are measured at 342 nm with 1 cm path length using dichloroethylene as a standard. A standard curve of absorptions of 4 standard solutions using their concentrations (mg/100 ml) is obtained. Piperin concentration C (mg/100 ml) in the sample is obtained from the standard curve. The content of piperin in oleoresin black pepper and oleoresin white pepper is obtained by the following equation. It should not be less than 36%.

$$\text{Content of piperin (\%)} = 100 \times \frac{100C}{\text{Weight of the sample (mg)}}$$

(6) Hot taste (only apply for Oleoresin Capsicum)

The hot taste of Spice oleoresins should be more than indicated contents within 100,000 through 2,000,000 tested by the following procedure.

- test method : Transfer 200 mg of Spice oleoresins into a 50 ml volumetric flask, and dilute to volume with alcohol. Shake the mixture and set aside to use test solution. Dissolve 0.15 g of test solution in 140 ml of sugar solution (10w/v%), and mix. This test solution is tested by following procedure. 240,000 as Scoville Heat Units is the level that three or more people sense spicy tastes when separately 5 people eat 5 ml of test solution. Dilute the test solution according to a below table in case that the unit is higher than this level.

Scoville Heat Units	Test Solution (ml)	Sugar Solution (ml)
360,000	20	10
480,000	20	20
600,000	20	30
720,000	20	40
840,000	20	50
960,000	20	60

1,080,000	20	70
1,200,000	20	80
1,320,000	20	90
1,440,000	20	100
1,560,000	20	110
1,680,000	20	120
1,800,000	20	130
1,920,000	20	140
2,040,000	20	150

Make the test solution by taking the test solution according to a below table in case that the Heat Units is less than 240,000.

Scoville Heat Units	Test Solution (mℓ)	Sugar Solution (mℓ)
100,000	0.15	60
117,500	0.15	70
170,000	0.15	100
205,000	0.15	120

103. Dextranase

1, 6- α -D-glucan 6-glucanohydrolase

Definition Dextranase is an enzyme obtained from a culture of *Chaetomium gracile*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Dextranase

Content Dextranase contains 90~130% of the indicated activity as dextranase.

Description Dextranase is white~pale yellow~brown powder, or transparent~brown liquid.

- Purity**
- (1) Arsenic : Place 0.25 g of Dextranase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.
 - (2) Lead : When 5.0 g of Dextranase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
 - (3) Coliform Group : When Dextranase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code, it should not contain more than 30 per 1 g of this product.
 - (4) Salmonella : When Dextranase proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in Food Code, it should be negative (-).
 - (5) E. Coli : When Dextranase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity) Application and Principle : This test is to measure the activity of dextranase in an enzyme that is obtained from a culture of *Chaetomium gracile*. Activity test is based on hydrolysis of dextran substrate at pH 5.1, 40°C temperature.

- Preparation of Test Solutions : An appropriate amount of sample is diluted with phosphate buffer solution adjusting pH 7.0 so that 1 ml contains 8~12 Unit.
- Test Procedure : 2.0 ml of substrate solution placed in the test tube is isothermalized for 10 minutes in a 40 ± 1°C water bath. Exactly 1.0 ml of Test Solution is added to

this substrate solution and mixed well by shaking. After exactly 10 minutes, 0.5 ml of 2 N sulfuric acid is added to the solution, which is set aside for 10 minutes at room temperature. 1 drop of phenolphthalein TS is added to the resulting solution, which is then neutralized with sodium hydroxide solution. It is then well mixed with 0.5 ml of water and 5 ml of alkaline copper solution. For enzyme blank test, 2.0 ml of substrate solution and 0.5 ml of 2 N sulfuric acid are well mixed, where 1.0 ml of Test Solution is added. The same procedure as the Test Solution is followed for enzyme blank test. Separately, 1.0 ml and 2.0 ml of glucose standard solution are diluted to 5.0ml with water, where 5 ml each of alkaline copper solution is added. For a blank test for glucose standard solution, a mixture of 5.0 ml each water and alkaline copper solution is prepared. All the test tubes are boiled for 20 minutes in a boiling water bath. Cool the solution, and isothermalize the tubes in a $40 \pm 1^\circ\text{C}$ water bath and set aside until precipitates are formed at the bottom of the tubes. 2.0 ml of potassium iodide solution and 10 ml of 2 N sulfuric acid are added to the test tubes. It is quickly titrated with 0.005 N sodium thiosulfate solution until the color of Iodine disappears. After adding 1.0 ml of starch solution, it is again titrated by drop-wise adding 0.005 N sodium thiosulfate solution until the blue color disappears.

Enzyme activity is calculated by the following equation.

$$\text{Dextranase unit/g} = F \times (B - A) \times \frac{1}{\text{Weight of the sample (g)}} \times 2.775 \times 10^3$$

B : Consumed amount of 0.005 N sodium thiosulfate for enzyme blank test (ml)

A : Consumed amount of 0.005 N sodium thiosulfate for the test (ml)

F : factor of reducing sugar $\frac{2}{W-S_2}$

Linearity of the glucose standard solution is inspected as follows.

$$0.98 < \frac{2 \times (W - S_1)}{W - S_2} < 1.02$$

W : Consumed amount of 0.005 N sodium thiosulfate for blank test (ml)

S₁ : Consumed amount of 0.005 N sodium thiosulfate per 1.0ml of glucose standard solution (ml)

S₂ : Consumed amount of 0.005 N sodium thiosulfate per 2.0ml of glucose standard solution (ml)

If the measurement does not satisfy the above condition, it is discarded.

Definition of Activity : 1 Dextranase unit corresponds to an amount of enzyme that generates reducing sugar that corresponds to 1 μmol of glucose per minute under the above test conditions.

Solutions

- 0.1 M Acetate Buffer Solution : 0.1 M acetic acid is mixed with 0.1 M sodium acetate solution and pH is adjusted to 5.1.
- Phosphate Buffer Solution (pH 7.0) : 2.7 g of potassium phosphate, mono basic and 10.7 g of sodium phosphate, dibasic (12 hydrates) are dissolved in water (total volume = 500 ml). 100 ml of this solution is added with water to 1,000 ml.
- Potassium Iodide Solution : 2.5 g of potassium iodide is dissolve in water (total volume = 100 ml).
- Alkaline Copper Solution : 71 g of sodium phosphate, dibasic (12 hydrates) and 40 g of potassium sodium tartrate are dissolved in 650 ml of water, where 100 ml of sodium hydroxide solution is added. While stirring slowly, 80 ml of copper sulfate solution (10 \rightarrow 100) and 180 g of anhydrous sodium sulfate are added and dissolved. 25 ml of potassium iodate solution (3.567 \rightarrow 100) is added to the resulting solution, which is diluted to 1,000 ml with water.
- 0.005 N sodium thiosulfate solution : 0.1 N sodium thiosulfate solution is diluted to twenty times a capacity with freshly boiled and cooled water.
- Substrate Solution : 2.5 g of dextran (Dextran T 2000, Pharmacia-Fine Chemical AB Upsala Sweden, or its equivalent) is precisely weighted and dissolved in 100 ml of 0.1 M acetate buffer solution.

Storage Standards of Dextranase

Dextranase is strongly hygroscopic. Store in a cold dark place and well-closed containers.

104. Maltogenic Amylase

Definition Maltogenic Amylase is an enzyme obtained from a culture of *Bacillus subtilis* that contains amylase genes *Bacillus stearothermophilus*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Maltogenic Amylase

Content Maltogenic Amylase contains 90~130% of the indicated activity as Maltogenic Amylase.

Description Maltogenic Amylase is white~pale yellow~brown powder or transparent~brown liquid.

Purity (1) Arsenic : Place 0.25 g of Maltogenic Amylase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(2) Heavy Metals : Carbonize 1 g of Maltogenic Amylase by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. This solution is used as the test solution. The content should not be more 40ppm under Heavy Metal Limit Test. Standard color solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. Add 3 drops of hydrochloric acid to the residue, which is then transferred into another Nestler cylinder as described test solution. Then add 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water to make the total volume to 50 ml. This solution is used as the Standard color solution.

- (3) Lead : 0.8 g of Maltogenic Amylase (if it is liquid, it is concentrated by evaporation in a water bath) is mindly carbonized by heating, and ash at a temperature below 500°C. Carefully 20 ml of diluted nitric acid is added to the ash. Boil the sample for 5 minutes and cool. It is filtered (if necessary). The residue is washed with water, which is then added to the filtrate. Water is added so that total volume of this solution becomes 50 ml. This solution is used as the test solution. The detected amount of lead should not be more 10ppm under Lead Limit Test.
- (4) Coliform Group : When Maltogenic Amylase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code, it should not contain more than 30 per 1 g of this product.
- (5) Salmonella : When Maltogenic Amylase proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in Food Code, it should be negative (-).

Assay(activity)

- Application and Principle : This test is to measure the activity of Maltogenic Amylase in an enzyme that is obtained from a culture of *Bacillus subtilis* that contains amylase genes *Bacillus stearotherophilus*. Activity test is based on hydrolysis of maltoriose substrate at 37°C. Generated glucose is measured by using a mixture of Glucose dehydrogenase and NAD.
- Preparation of Test Solution : Sample is diluted with water so that 1 ml contains 0.015~0.075 MANU. Final diluted solution is prepared so that it contains 1% 1 M sodium chloride solution.
- Test Procedure : 0.5 ml of substrate solution is placed in a test tube, which is isothermalized in a $37 \pm 1^\circ\text{C}$ water bath. Exactly 0.5 ml of Test Solution is added to the test tube, which is mixed by shaking and set aside in a water bat. After exactly 30 minutes, the tube is taken out and the reaction is stopped by adding 1 ml of 0.06 N sodium hydroxide solution. 3 ml of GluDH solution is added to this solution, which is set aside for exactly 30 minutes at normal temperature. To this solution, 0.5 ml of substrate solution, 1 ml of 0.06 N sodium hydroxide solution, 0.5 ml of Test Solution are sequentially added. The resulting solution is set aside for 30 minutes (enzyme blank test solution). Using enzyme blank test solution as a reference solution, absorption of the test solution at 342 nm with 1 cm path length is measured and the concentration of glucose standard solution ($\mu\text{mol/L}$) is obtained from the standard curve.

Standard Curve

1.6 g of glucose is accurately weighted and dissolved in water (total volume = 1,000

ml). Using this solution, glucose standard solutions are prepared so that they contain 88.8 $\mu\text{mol/l}$, 177.6 $\mu\text{mol/l}$, 266.4 $\mu\text{mol/l}$, 355.2 $\mu\text{mol/l}$, 444.0 $\mu\text{mol/l}$, and 532.9 $\mu\text{mol/l}$ of glucose. 2 ml of each standard solution is placed in a test tube, where 3 ml of GluDH solution is added. Set it aside for 30 minutes at room temperature. Absorption of each resulting solution is measured at 340 nm with 1cm path length using water as a reference. A standard curve of absorption versus concentration ($\mu\text{mol/l}$) of glucose standard solution is prepared.

Enzyme activity is obtained from the following equation.

$$\text{MANU/g} = \frac{A \times 4 \times F}{30 \times W \times 1,000}$$

A : Concentration of glucose standard solution ($\mu\text{mol/l}$) in Test Solution obtained from the standard curve

F : Dilution factor of test solution

4 : Ratio of the amount of glucose standard solution (2 ml) vs. the amount of Test Solution (0.5 ml) used in the test

30 : Reaction time (minutes)

W : Weight of sample (g)

1,000 : Conversion factor from ℓ to ml

Definition of Activity : 1 Maltogenic Amylase Novo Unit(MANU) corresponds to an amount of enzyme that decomposes 1 μmol of maltotriose under the test conditions above.

Solutions

- Citric Acid Buffer Solution : 0.225 g of citric acid is added in 20 ml of water. pH of this solution is adjusted to 5.0 with 4 N or 1 N sodium hydroxide solution. It is then diluted to 250 ml with water.
- Substrate Solution : 1 g of Maltotriose is added in citric acid buffer solution (total volume = 50 ml).
- 1 M Sodium Chloride Solution : 29.22 g of sodium chloride is added in water (total volume = 500 ml).
- 0.06 N sodium hydroxide solution : 30 ml of 1N sodium hydroxide solution is diluted to 500 ml with water.
- GluDH Solution : Use a mixed solution (Thermo Fisher Scientific Code. 981304,

981779 or its equivalent) contained Glucose dehydrogenase.

Storage Standards of Maltogenic Amylase

Maltogenic Amylase is strongly hygroscopic. Store in a cold dark place and well-closed containers.

105. Exomaltotetrahydrolase (G4 Producing Enzyme)

1, 4- α -D-glucan 6-maltotetrahydrolase

Definition Exomaltotetrahydrolase is an enzyme obtained from a culture of *Pseudomonas stutzeri*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Exomaltotetrahydrolase (G4 Producing Enzyme)

Content Exomaltotetrahydrolase contains 90~130% of the indicated activity as Exomaltotetrahydrolase (G4 Producing Enzyme).

Description Exomaltotetrahydrolase is white~pale yellow~brown powder or transparent~brown liquid.

- Purity**
- (1) Arsenic : Place 0.25 g of Exomaltotetrahydrolase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.
 - (2) Lead : When 5.0 g of Exomaltotetrahydrolase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
 - (3) Coliform Group : When Exomaltotetrahydrolase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code, it should not contain more than 30 per 1 g of this product.
 - (4) Salmonella : When Exomaltotetrahydrolase proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in Food Code, it should be negative (-).
 - (5) E. Coli : When Exomaltotetrahydrolase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

- Applications : This test is to measure the producing enzyme activity of Exomaltotetrahydrolase (G4 Producing enzyme) in an enzyme that is obtained from a culture of *Pseudomonas stutzeri*.
- Preparation of Test Solution : sample is diluted with calcium chloride acetic acid

buffer solution (pH 6.0) so that 1 ml of the solution contains 0.5~0.9 Unit.

- Test Procedure : 0.5 ml of substrate solution and 0.4 ml of calcium chloride acetic acid buffer solution (pH 6.0) are placed in a 25 ml volumetric flask, which is isothermalized for 15 minutes in a $40 \pm 0.1^{\circ}\text{C}$ water bath. Exactly 0.1 ml of Test Solution is added to the solution, mixed well by shaking, and set aside in a water bath. After exactly 15 minutes, 2 ml of alkaline copper solution is added to the solution, which is sealed and heated for exactly 20 minutes in a boiling water bath. Cool the solution immediately. 2 ml of arsenic·ammonium molybdate solution is added to this solution and well mixed until red precipitates of copper suboxide are completely dissolved. After setting aside for 20 minutes at room temperature, water is added to bring the total volume to 25 ml. Using water as a reference, the absorption (As) is measured at 520 nm with 1cm path length. Separately, perform a blank test by adding 0.5 ml of substrate solution, 0.4 ml of calcium chloride acetate buffer solution (pH 6.0), 2 ml of alkaline copper solution, and 0.1 ml of Test Solution and well mixing. Its absorption (AB) is measured following the same procedure as the Test Solution.

Standard Curve

Glucose is dried for 6 hours at 105°C . 1.0 g of dried glucose is precisely weighted and dissolved in water (total volume = 100 ml). 1.0 ml, 2.0 ml, 3.0 ml, and 4.0 ml each of this solution is diluted to 100 ml with water. 1 ml of the each resulting solution contains 100 μg , 200 μg , 300 μg , and 400 μg of glucose. 1 ml of each glucose standard solution is placed in a 25 ml volumetric flask, where 2 ml of alkaline copper solution is added and well mixed. It is sealed, heated in a boiling water bath for exactly 20 minutes, and cooled immediately. 2 ml of arsenic·ammonium molybdate solution is added and well mixed until red precipitates of copper suboxide are completely dissolved. After setting aside for 20 minutes at room temperature, water is added to bring the total volume to 25 ml. Using water as a reference instead of the standard solution, the absorption of each standard solution is measured at 520 nm with 1cm path length. A standard curve of absorption versus the amount of glucose (μg) is prepared.

Enzyme activity is calculated by the following equation.

$$(\text{G4 Producing Enzyme}) \text{ unit/g} = \{(A_s - A_B)\} \times F \times \frac{1}{15} \times \frac{1.0}{0.1} \times \frac{1}{180} \times \frac{N}{W}$$

F : Amount of glucose (μg) when the difference in absorption is 1.0 (obtained from the standard curve).

- 15 : Reaction time (minutes)
180 : Molecular weight of glucose
N : Dilution factor of test solution
W : Weight of sample(g)

Definition of Activity : 1G4 producing enzyme unit is an amount of enzyme that produces reducing sugar that corresponds to 1 μ mol of glucose per minute under the conditions above.

Solutions

- Substrate Solution : 1.0 g of soluble starch (Lintner) is dispersed in 50 ml of water, where 50 ml of boiling water is slowly added while stirring. It is then boiled for 1~2 minutes. After cooling water is added to bring the total volume to 10 ml.
- Alkaline Copper Solution : 24.0 g of anhydrous sodium carbonate and 12.0 g of potassium sodium tartrate are dissolved in 200 ml of water. Separately, 18.0 g of sodium carbonate and 150 ml of water are added to a solution of 4.0 g copper sulfate in 50 ml of water and dissolved by heating. Cool the solution, and this solution is mixed with the previous solution. The total volume is brought up to 1,000 ml with water. The resulting solution is boiled for 10 minutes, which is set aside for 1 week and filtered through a glass filter.
- Arsenic · Ammonium Molybdate Solution : 3 g of sodium arsenate, dibasic (7 hydrate) is dissolved in 25 ml of water. 25 g of ammonium molybdate (4 hydrate) is dissolved in 450 ml of water, where 21 ml of sulfuric acid is added. Sodium arsenate, dibasic solution is slowly added to ammonium molybdate solution while stirring. It is set aside for 24 hours at 37°C. It is stored in a brown bottle.
- Calcium Chloride Acetate Buffer Solution (pH 6.0) : Prepare 0.1 M acetic acid and 0.1 M sodium acetate solution contained separately 5 mM calcium chloride. These two solutions are adjusted pH 6.0.

Storage Standards of Exomaltotetrahydrolase (G4 Producing Enzyme)

Exomaltotetrahydrolase is strongly hygroscopic. Store in a cold dark place and well-closed containers.

106. Tansglucosidase

1,4- α -D-Glucan 6- α -D-glucosyl transferase

Definition Tansglucosidase is an enzyme obtained from a culture of *Aspergillus niger*, its variety and the culture of *Bacillus* sp. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Tansglucosidase

Content Tansglucosidase contains 90~130% of the indicated activity as Tansglucosidase under quantitative analysis.

Description Tansglucosidase is white~pale yellow~brown powder or transparent~brown liquid.

- Purity**
- (1) Arsenic : Place 0.25 g of Tansglucosidase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.
 - (2) Lead : When 5.0 g of Tansglucosidase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
 - (3) Coliform Group : When Tansglucosidase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code, it should not contain more than 30 per 1 g of this product.
 - (4) Salmonella : When Tansglucosidase proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in Food Code, it should be negative (-).
 - (5) E. Coli : When Tansglucosidase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

- Application and Principle : This test is to measure the activity of Tansglucosidase in an enzyme that is obtained from a culture of *Aspergillus niger*, its variety and the culture of *Bacillus* sp. Activity test is based on substrate hydrolysis of methyl-D-glucoside at 40°C for 60 minutes and pH 5.0.

- Preparation of Test Solution : Test Solution (in water) is prepared so that the difference in absorption ($A_S - A_B$) is 0.15~0.32 under following test method.
- Test Procedure : 1 ml of substrate solution and 1 ml of acetic acid sodium acetate buffer solution (pH 5.0) are mixed in a test tube, which is isothermalized for 5 minutes in a $40 \pm 0.5^\circ\text{C}$ water bath. Exactly 0.5 ml of Test Solution is added to the tube, which is mixed by shaking and set aside for 60 minutes in a $40 \pm 5^\circ\text{C}$ water bath. It is then heated for 5 minutes in a boiling water bath and cooled in running water. 0.1 ml of this solution is added to a test tube, where 3 ml of colorizing solution is added. It is set aside for 20 minutes at $40 \pm 0.5^\circ\text{C}$. Absorption (A_S) of the resulting solution is measured at 505 nm with 1cm path length using water as a reference. Separately for enzyme blank test, 1 ml of acetic acid sodium acetate buffer solution (pH 5.0) and 0.5 ml of Test Solution are added to a test tube, which is set aside for 60 minutes at $40 \pm 0.5^\circ\text{C}$. Heat the test tube for 5 minutes in a boiling water bath, and cool it in running water. After adding 1 ml of substrate solution, absorption (A_B) is measured using the same procedure as Test Solution.

Standard Curve

Glucose is dried for 6 hours at 105°C . 1 g of dried glucose is precisely weighted and dissolved in water (total volume = 100 ml, 100 mg/ml). A set of glucose standard solutions are prepared so that each solution contains 100 μg , 200 μg , 300 μg , 400 μg , and 500 μg per 1 ml. 0.1 ml of glucose standard solution. Each glucose standard solution is placed in a test tube, where 3 ml of colorizing solution is added. It is then set aside for 20 minutes in a $40 \pm 0.5^\circ\text{C}$ water bath. Separately, a reference solution is prepared using water instead of standard solution. Absorption for each standard solution is measured. A standard curve of absorption versus concentration of glucose (μg).

Enzyme activity is obtained using the following equation.

$$\text{Transglucosidase unit/g} = (A_S - A_B) \times G \times \frac{2.5}{0.5} \times \frac{n}{0.1 \times W}$$

G : Amount of glucose (μg) where the difference in absorption is 1 (obtained from the standard curve).

n : Dilution factor of test solution

W : Weight of sample(g)

Definition of Activity : 1 Transglucosidase unit corresponds to an amount of enzyme that produces 1 μg of glucose in 60 minutes under the test conditions above.

Solutions

- Substrate Solution : 2 g of α -Methyl-D-glucoside is weighted and dissolved in water (total volume = 100 ml).
- Acetic Acid · Sodium Acetate Buffer Solution (pH 5.0) : 0.02 M acetic acid are added to 0.02 M sodium acetate solution so that pH becomes 5.0.
- Colorizing Solution : After dissolve Glucose oxidase 550 unit, peroxidase 125 unit in 40 ml of tris·phosphate buffer solution(pH 7.2), add 1 ml of 0.4% of 4-aminoantipyrine solution and 1.4 ml of phenol solution(5%) and tris·phosphate buffer solution(pH 7.2) to make to 50 ml.

Storage Standards of Transglucosidase

Tansglucosidase is strongly hygroscopic. Store in a cold dark place and well-closed containers.

107. Phospholipase

Definition Phospholipase includes Phospholipase A₂ and Phospholipase D. Phospholipase A₂ is an enzyme obtained from an extract of pig pancreas tissues. Phospholipase D is obtained from the culture of *Streptomyces griseus*. Separately these are called Phospholipase A₂ Phospholipase D. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Phospholipase

Content Phospholipase contains 90~130% of the indicated activity as Phospholipase A₂, or 90~130% of the indicated activity as Phospholipase D, under quantitative analysis.

Description Phospholipase is white~pale yellow~brown powder or transparent~brown liquid.

Purity (1) Arsenic : Place 0.25 g of Phospholipase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(2) Lead : When 5.0 g of Phospholipase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Coliform Group : When Phospholipase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code, it should not contain more than 30 per 1 g of this product.

(4) Salmonella : When Phospholipase proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in 「Standards and Specifications for Foods」, it should be negative (-).

(5) E. coli : When Phospholipase proceed as directed under Microbiological Methods for E. coli in General Testing Methods in 「Standards and Specifications for Food s」, it should be negative (-).

Assay(activity) Phospholipase A₂ is done by Method 1, and Phospholipase D is done by Method 2.

Method 1

- Application and Principle : This test is to measure the activity of Phospholipase A₂ in an enzyme that is obtained from an extract of pig pancreas tissues. Activity test is based on hydrolysis of the substrate at 40°C for 5 minutes and pH 8.0.
- Preparation of Test Solution : A suitable amount of sample is diluted with water so that the solution contains 3~5 IU per 0.5 ml.
- Test Procedure : 25 ml of substrate solution is added to a beaker, which is maintained in a 40°C water bath for 10 minutes to equilibrate. 0.5 ml of Test Solution is added to the 40°C substrate solution. After exactly 5 minutes, 10 ml of modified alcohol is added and stirred immediately to stop the reaction. It is then taken out of the water bath and titrated with 0.02 N sodium hydroxide solution to pH 8.0. The consumed amount is S (ml). Separately, 25 ml of substrate solution, 10 ml of modified alcohol, and 0.5 ml of Test Solution is sequentially mixed. This solution is tested by the Test Procedure above and the consumed amount of 0.02 N sodium hydroxide solution is B (ml).

Activity of an enzyme(phospholipase A₂) is calculated by the following equation.

$$\text{The phospholipase A}_2 \text{ Activity (U/g)} = \frac{(S - B)}{5} \times \frac{N \times 10^3 \times F}{W}$$

N : Normality of sodium hydroxide solution

10³ : Conversion factor from mmol to μmol for acid

W : Weight of sample(g)

5 : Reaction time(minutes)

F : Dilution factor of test solution

Definition of Activity : 1 Phospholipase A₂ Unit(U) corresponds to an amount of enzyme that frees 1μmol of acid (H⁺) from substrate per minute under the test conditions above.

Solutions

- 0.016 M Sodium Deoxy Cholate Solution : 6.7 g of sodium deoxy cholate (C₂₄H₃₉NaO₄) is dissolve in water (total volume = 1,000 ml).
- 0.32M Calcium Chloride Solution : 4.7 g of calcium chloride (CaCl₂ · 2H₂O) is dissolve in water (total volume = 100 ml).
- Substrate Suspension : One egg yolk is homogenized in 100 ml of water, which is filtered through a twofold gauze. 5 ml of 0.32 M calcium

chloride solution is added to the filtrate.

- Substrate Solution : 100 ml of substrate suspension and 50 ml of 0.016 N sodium deoxy cholate solution are mixed, where water is added to bring the total volume to 100 ml. pH of the mixture is adjusted to 8.0 with 0.5 N sodium hydroxide solution.

Method 2

- Application and Principle : This test is to measure the activity of Phospholipase D in an enzyme that is obtained from the culture of *Streptomyces griseus*. Activity test is based on hydrolysis of Lecithin at 37°C, pH 5.5.
- Preparation of Test Solution : A suitable amount of sample is diluted with Tris-maleic acid buffer so that the solution contains 0.1~0.2 Units per 1 ml.
- Test Procedure : Weigh accurately 0.1 ml of substrate solution, 0.1 ml of Tris-maleic acid buffer, 0.05 ml of 0.1 M Calcium Chloride, 0.15 ml of 7.5% Triton X-100 solution, and mix well. Equilibrate the mixture at 37°C for 5 minutes. Add accurately 0.1 ml of test solution to this solution, and shake immediately. Set it aside at 37°C for 10 minutes precisely, and add 0.2 ml of Tris-EDTA solution accurately. Mix this solution, and heat for 5 minutes in a boiling water bath precisely. After cooling, add 4 ml of colorizing solution accurately and shake. It is set aside for 20 minutes at 37°C. Absorption of the this solution is measured at 500 nm using water as a reference. Separately, absorption (A_B) is measured using water instead of test solution under same procedure. Separately, absorption (A_S) is measured using 0.1 ml of Choline Chloride standard solution instead of test solution by proceeding same procedure.

Activity of an enzyme(phospholipase D) is calculated by the following equation.

$$\text{the phospholipase D Activity (U/g)} = \frac{A - A_B}{A_S - A_B} \times \frac{1.43}{10} \times \frac{1}{W}$$

A : Absorbance of enzyme test solution

A_B : Absorbance of enzyme blank test solutio

A_S : Absorbance of Choline Chloride standard solution

1.43 : Content of Choline Chloride standard solution(mmol/L)

10 : Reaction time(minutes)

Definition of Activity : 1 Phospholipase D Unit(U) corresponds to an amount of enzyme that 1 μ mol of Choline from substrate per minute under the test conditions above.

Solutions

- Standard Solution : Dissolve 0.2 g of choline chloride exactly in water to make 1,000 ml(1.43mM).
- Substrate Solution : Dissolve 0.5 g of Lecithin(Epikuron 200 of Cargill Inc., or its equivalent) in 9.5 ml of water. Set it aside for a day.
- Tris-maleic acid Buffer(pH 5.5) : Weight separately 1.12 g of Tris(hydroxy ethyl)aminomethane and 1.16 g of Maleic acid, and dissolve each samples in water to make 1,000 ml. Measure 25 ml of the solution, and add 0.1N Sodium Hydroxide solution to adjust pH 5.5. Add water to make 100 ml.
- 0.1M Calcium Chloride Solution : Weight 1.47 g of Calcium Chloride in water to make 100 ml.
- Triton X-100 Solution : Weight 7.5 g of Triton- 100polyoxyethylene(10) octyl phenyl ether in water to make 100 ml.
- Tris-EDTA solution : Dissolve 22.6 g of Ethylenediaminetetraacetic acid in Tris-Chloride Buffer Solution(pH 8.0) to make 1,000 ml.
- Tris-Chloride Buffer Solution : Dissolve 12.1 g of Tris in water to make 100 ml, and add 32 ml of 2M Hydrochloric acid and 800 ml of water. If necessary, adjust pH 8.0 by adding Sodium Hydroxide solution or Hydrochloric acid, and make 1,000 ml with water.
- colorizing solution : Dissolve 3 Unit of choline oxidase, 6 Unit of peroxidase, 0.001 g of phenol, 0.0006 g of 4-aminoantipyrine in 4 ml of HEPES Buffer Solution(pH 7.4).
- HEPES Buffer Solution(pH 7.4) : Weight 11.9 g of *N*-2-hydroxy ethylpiperazine -*N'*-2-ethanesulfonic acid and dissolve it in water to make 600 ml. Adjust pH 7.4 with 0.05 N Sodium Hydroxide solution, and make 1,000 ml with water.

Storage Standards of Phospholipase

Phospholipase should be stored in cold and dark container.

108. Glucomannan

Definition Glucomannan is a polysaccharide (that is purified with isopropyl alcohol and crushed) contained in root stems of dendrobium and Konjac (*Amorphophallus konja*). It is a mixture that consists of glucose and mannose.

Compositional Specifications of Glucomannan

Content Glucomannan (converted to a dried form) should contain not less than 90% of glucomannan.

Description Glucomannan is white~pale yellow powder

Identification (1) 6 g of Glucomannan is added to a 500 ml beaker. It is wetted with 10 ml of isopropyl alcohol. While stirring immediately, 200 ml of water is added. When it is set aside for 1 hour, it swells and becomes viscous solution.

(2) When 200 ml of 5% calcium hydroxide solution is added to viscous solution in (1), which is well mixed and then set aside, a gel is formed.

Purity (1) Arsenic : Place 0.25 g of Glucomannan in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(2) Lead : When 5.0 g of Glucomannan is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Isopropyl alcohol : 5 g of Glucomannan is precisely weighted into a 1,000 ml single neck round bottom flask with 24/40 ground joint, where 1 ml of anti foaming agent (Dow Corning G-10 or its equivalent) and 200 ml of water are added. It is then stirred for 1 hour. A 400 ml reflux condenser, distilling head, and a collector are attached. Approximately 95 ml of distillate is collected (care must be taken so that bubbles are not introduced into the collector). 4 ml of internal standard solution is added to the collected distillate, where water is added to bring the total volume to 100 ml (Test Solution). Test Solution and mixed standard solution are analyzed with gas chromatography and the amount of isopropyl alcohol is obtained by the following equation. The content should not be more than 500ppm. The reaction factor (f) is obtained by the ratio (A_{IPA}/A_{TBA}) of peak areas of isopropyl alcohol to

tert-butyl alcohol in the mixed standard solution.

$$\text{Content of isopropyl alcohol (ppm)} = \frac{A_{\text{IPA}} \times 4,000}{f \times A_{\text{TBA}} \times \text{Weight of sample(g)}}$$

A_{IPA} : Peak area of isopropyl alcohol in Test Solution

A_{TBA} : Peak area of *tert*-butyl alcohol in Test Solution

Operation Conditions

- Column : A stainless steel tube 3.2 mm × 1.8 m
- Column Filler : Porapak QS of 80~100 mesh (or its equivalent)
- Detector : Flame Ionization Detector (FID)
- Temperature at injection hole : 200℃
- Column Temperature : 165℃
- Detector Temperature : 200℃
- Carrier gas and flow rate : Nitrogen, Flow rate is controlled so that isopropyl alcohol and *tert*-butyl alcohol is detected in 2 minutes and 3 minutes, respectively.

Solutions

- Mixed Standard Solution : A mixture of 4 ml IPA standard solution and 4 ml TBA standard solution is diluted to 100 ml with water. 1 ml of this solution contains 40μg each of isopropyl alcohol and *tert* butyl alcohol.
 - IPA Standard Solution : Approximately 500 mg of isopropyl alcohol (chromatography grade) is precisely weighted and diluted to 50 ml with water. 10 ml of this solution is further diluted to 100 ml with water.
 - TBA Standard Solution : Approximately 500 mg of *tert*-butyl alcohol (chromatography grade) is precisely weighted and diluted to 50 ml with water. 10 ml of this solution is further diluted to 100 ml with water.
- (4) Viscosity : Viscosity of 1% aqueous solution of Glucomannan is measured by 2. Rotational Type Viscosity Measurement in Viscosity Measurement. It should be 500 cps or higher.
- (5) Salmonella : When Glucomannan proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in 「Standards and Specifications for Foods」, it should be negative (-).
- (6) E. coli : When Glucomannan proceed as directed under Microbiological Methods for

E. coli in General Testing Methods in 「Standards and Specifications for Foods」, it should be negative (-).

Loss on Drying When Glucomannan is dried for 3 hours at 105°C, the loss should not be more than 15%.

Ash When Glucomannan is tested for ash content, it should not be more than 4%.

Assay Same amount (0.5~1.0 g) each of Glucomannan (remove fat with ether if necessary) is placed separately in two 400 ml beakers. 50 ml each of phosphate buffer solution (pH 6.0) is added. Check the pH of the solution, and pH is adjusted to 6.0 ± 0.2 , if necessary. 0.1 ml of Termamyl solution is added to each beaker, which is covered with aluminum foil and heated for 30 minutes in a boiling water bath (shaken every 5 minutes). Using a thermometer, the temperature inside the beaker is maintained at 85~100°C for 15 minutes. Cool, and 10 ml of 0.275 N sodium hydroxide solution is added to each beaker and pH is adjusted to 7.5 ± 0.2 . 5 mg of protease (or 0.1 ml solution containing 50 mg of protease in 1 ml water) is added to the resulting solution. It is covered with aluminum foil and isothermalized for 30 minutes at 60°C while shaking continuously. Cool the solution, and pH is adjusted to 4.0~4.6 with 10 ml of 0.325 M hydrochloric acid and 0.3 ml of amylo glucosidase is added, which is then covered with aluminum foil and isothermalized for 30 minutes at 60°C while mixing by shaking. 280 ml of 95% alcohol (heated to 60°C) is added to the beaker and well mixed by shaking. It is then set aside for 1 hour at normal temperature to settle down glucomannan. A glass filter containing cellite (previously weighted) is wetted with 78% alcohol so that cellite is evenly distributed. It is vacuum filtered to evenly distribute the cellite. Test Solution (treated with enzyme) is then vacuum filtered through the glass filter. The residue is washed 3 times with 20 ml each of 78% alcohol, twice with 20 ml each of 95% alcohol, and twice with 10 ml each of acetone, in sequence. If a film is formed, it is broken with a reagent spoon to facilitate the filtration. Filtering time can be shortened if filtration is stopped occasionally. The filter is dried over night at $105 \pm 5^\circ\text{C}$, cooled in a desiccator, and weighted. The weight of the residue is obtained by subtracting the weight of glass filter. From the residue of one glass filter, the amount of proteins is obtained (Protein Factor : 6.25). The residue from another glass filter is ashed by heating for 5 hours at 525°C and ash content is obtained. Separately, a blank test is carried without sample. The content of glucomannan is obtained by the following equation.

$$\text{Blank Test Value B(mg)} = \text{Average weight of residue for blank test (mg)} - P_B - A_B$$

P_B : Amount of proteins for blank test (mg)

A_B : Amount of ash for blank test (mg)

$$\text{Content (\%)} = \frac{\text{Average weight of residue for blank test (mg)} - P - A - B}{\text{Average weight of sample (mg)}} \times 100$$

P : Weight of proteins (mg)

A : Weight of ash (mg)

B : Blank test value (mg)

Reagents and Solutions

- Phosphate Buffer Solution (pH 6.0) : 1.4 g of sodium phosphate, dibasic (anhydrous) and 9.68 g of sodium phosphate, monobasic (1 hydrate) are dissolved in 700 ml of water, which is diluted to 1,000 ml with water.
- 0.275 N sodium hydroxide solution : 11 g of sodium hydroxide is dissolved in 700 ml of water, which is diluted to 1,000 ml with water.
- 0.325 M hydrochloric acid : 325 ml of 0.1 M hydrochloric acid is diluted to 1,000 ml with water.
- Termamyl (thermostable- α -amylase) solution : No.120 L, Novo (refrigerated for storage)
- Protease : No.P-3910, Sigma (refrigerated for storage)
- Amylo glucosidase : No. A-9913, Sigma (refrigerated for storage)
- Cellite C-211(Fischer) : washed with acid solution

109. Rosin

Definition Rosin is obtained by filtering and purifying secretion from barks of pine trees (*Pinus* sp.) of pinacea.

Compositional Specifications of Rosin

Description Rosin is pale yellow powder or solid..

Identification (1) 0.1 g of Rosin is dissolve in 10 ml of anhydrous acetic acid by heating in water bath. Cool the solution. When sulfuric acid is added to this solution, the color of the solution becomes reddish violet.

Purity (1) Acid Value : Approximately 1 g of Rosin is precisely weighted and dissolved in approximately 50 ml of a mixture(1:1) of alcohol and ether (neutralized with 0.1 N potassium hydroxide solution using phenolphthalein TS) to use test solution. The test solution is proceeded as directed under Acid value method in Fats and Related substances tests, and the value should be 170~190.

(2) Arsenic : Place 0.25 g of Rosin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(3) Lead : When 5.0 g of Rosin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Residue on Ignition When thermogravimetric analysis is done with accurately weighted 1 g of Rosin, the amount of residue should not be more than 0.1%.

110. Mucin

Definition Mucin is glycoprotein obtained by precipitating (with ethyl alcohol) the water soluble extracts from pig stomach.

Compositional Specifications of Mucin

Content Mucin (converted to a dried form) contains 73~90% of Mucin.

Description Mucin is grayish white or pale yellow powder.

Purity (1) Acidity : pH of aqueous solution (2→100) of Mucin should be 3.7~6.5 (measured with glass electrode).

(2) Arsenic : Place 0.25 g of Mucin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(3) Lead : When 5.0 g of Mucin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(4) Nitrogen in Mucin : Extract with 70% alcohol based on Assay, and dry-mill the residue. The content should be 7~9% under Nitrogen Determination.

Total Nitrogen 250 mg of Mucin is precisely weighted and tested by nitrogen determination method. The content should not be less than 8.0%.

Ash 2 g of Mucin precisely weighted and tested by Ash and Acid-Insoluble Ash Limit, the amount of ash should not be more than 6.5%.

Loss on Drying When Mucin is dried for 5 hours at 105°C, the loss should not be more than 6%.

Assay 10 g of Mucin is precisely weighted into a 200 ml Erlenmeyer flask. It is extracted for 30 minutes with 100 ml of 70% alcohol and only the supernatant is decanted. This is repeated 5 times. All the extracts are mixed together and the total volume is brought up to 600 ml. It is then filtered. Transfer 50 ml of the filtrate into a beaker (previously weighted) and evaporated to dryness in a water bath. It is further dried for 5 hours at 105°C. The weight of the residue (S) is obtained and the content of mucin is calculated by the following equation.

$$\text{Content (\%)} = \frac{\text{Weight of sample (g)} - S \text{ (g)} \times 600/50}{\text{Weight of sample (g)}} \times 100$$

111. Mutastein

○ Designed Cancellation

The date of cancellation: 27.3.'12 (Notification No. 2012-10).

112. Alfalfa Extract

Definition Alfalfa Extract is a pigment prepared by the following procedure. Alfalfa is extracted with organic solvents such as acetone, isopropyl alcohol, ethyl alcohol, methyl alcohol, hexane, and methylene chloride. The extract is saponified to remove chlorophyll. Carotinoid is extracted and purified from the resultant with organic solvents. Its major pigment component is Lutein ($C_{40}H_{56}O_2$ —566.88). Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Alfalfa Extract

Content Color value ($E_{1cm}^{10\%}$) of Alfalfa Extract should be more than the indicated value.

Description Alfalfa Extract is deep yellowish brown liquid with a slight characteristic scent.

Identification Test Solution obtained in Color Value section have a maximum absorption band near 445 nm.

Purity (1) Arsenic : Place 0.25 g of Alfalfa Extract in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(2) Lead : When 5.0 g of Alfalfa Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Residual Solvents : When Alfalfa Extract is tested by Purity (4)(5) for Paprika Extract Pigments, the content of residual solvents should be,

Acetone	}	Not more than 50ppm (individual or total if combined)
Isopropyl alcohol		
Methyl alcohol		
Hexane		
Methylene chloride		Not more than 10ppm

Assay (Color Value) Appropriate amount of Alfalfa Extract is precisely weighted so

that the absorption is within 0.3~0.7 and dissolved in chloroform so that the total volume is 100 ml (if it is water soluble, water is used). 1 ml of this solution is diluted to 100 ml with chloroform (Test Solution). Using chloroform as a reference solution, absorption A is measured at the maximum absorption near 445 nm with 1 cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

113. Sepia Color

Definition Sepia Color is a pigment obtained from the contents of ink sac of cuttlefish (*Sepia officinalis* Linnaeus). Its major component is Eumelanin. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Sepia Color

Description Sepia Color is blackish brown~black powder or dispersion with a characteristic scent.

Identification When 0.1 g of Sepia Color is added to 10 ml of mixture (1:1) of sulfuric acid and nitric acid, it becomes yellowish brown.

Purity (1) Arsenic : Place 0.25 g of Sepia Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4ppm.

(2) Lead : When 5.0 g of Sepia Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

114. Yucca Extract

Definition Yucca Extract is obtained by extracting roots of yucca (*Yucca brevifolia* Engelm, *Yucca schidigera*) of agavaceae with water. Dilutant or other food additives can be added for the purpose of quality preservation.

Compositional Specifications of Yucca Extract

Description Yucca Extract is a dark brown liquid with a characteristic scent.

Identification 1 g of Yucca Extract is dissolved in water (total volume = 1,000 ml). This solution has a maximum absorption band at 250~300 nm.

Purity (1) Acidity : pH of Yucca Extract should be 3.8~4.0.

(2) Coliform Group : When Yucca Extract proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code, it should be negative (-).

(3) Formability : 1.9 L of water is added to a 3.8ℓ glass bottle (16.3cm diameter), where 6 drops of 85% phosphoric acid are added. Add 60 ml of aqueous solution of Yucca Extract (1.1→1,000), the bottle is shaken vigorously 100 times. The height of foam layer should be maintained at 1.2 cm or more for 30 seconds.

(4) Arsenic : Place 0.5 g of Grapefruit Seed Extract in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550℃. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550℃. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 2 ppm.

(5) Lead : When 5.0 g of Yucca Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Ash 5 g of Yucca Extract is precisely weighted and tested by Ash and Acid-Insoluble Ash Limit, the amount of ash should not be more than 10%.

115. Grapefruit Seed Extract

Definition Grapefruit Seed Extract is obtained by extracting seeds of grapefruit (*Citrus paradisi* MACF.) of rutaceae with water, ethyl alcohol, or glycerin. Its components are fatty acids and flavonoids.

Compositional Specifications of Grapefruit Seed Extract

Content Grapefruit Seed Extract contains 90~130% of the indicated activity as naringin.

Description Grapefruit Seed Extract is colorless~yellow viscous liquid with a slight characteristic scent and slightly bitter taste.

Identification (1) When Grapefruit Seed Extract is tested by Assay, vitamin C peak is observed at 254 nm.

(2) When Grapefruit Seed Extract is tested by Assay, naringin peak is observed at 280 nm.

Purity (1) Arsenic : Place 0.5 g of Grapefruit Seed Extract in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 2 ppm.

(2) Lead : When 5.0 g of Grapefruit Seed Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

Assay Approximately 15 g is precisely weighted and dissolved in 40 ml of 0.1 N hydrochloric acid, which is extracted with 50 ml of ether by shaking. Hydrochloric acid layer is diluted to 50 ml with 0.1 N hydrochloric acid (Test Solution). Separately, approximately 25 mg of naringin standard is dissolve in 0.1 N hydrochloric acid so that the total volume is 50 ml (Standard Solution). 3 µl each of both solutions is injected into liquid gas chromatography under the following Operation Conditions and the content of naringin is obtained by the following equation.

$$\text{Content (\%)} = \frac{A \times \text{Weight of naringin standard (g)}}{A_s \times \text{Weight of sample (g)}} \times 100$$

A : peak area of Test Solution

As : peak area of Standard Solution

Operation Conditions

- Detector : UV 280nm
- Column : μ -Bondapak C18 or its equivalent
- Column Temperature : room temperature
- Mobile Phase : acetonitrile : water (70 : 30)
- Flow Rate : 1.0 ml/min

116. Maize Morado Color

Purple Corn Color

Definition Maize Morado Color is a pigment obtained by extracting seeds of corn (*Zea mays* Linné of gramineae with water or ethyl alcohol. Its major pigment component is anthocyanin. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Maize Morado Color

Content Color value ($E_{1cm}^{10\%}$) of Maize Morado Color should be more than the indicated value.

Description Maize Morado Color is dark red powder, paste, or liquid with a slight characteristic scent.

Identification (1) Test Solution obtained in Color Value section shows red color and a maximum absorption band near 515 nm.

(2) When Test Solution in (1) is alkalinized with sodium hydroxide solution (1→25), it becomes dark green.

Purity (1) Arsenic : Place 0.25 g of Maize Morado Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 2 ppm.

(2) Lead : When 5.0 g of Maize Morado Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) FumonisinB₁: Weigh precisely 5 g which is converted to 30 of color value from indicated value of Maize Morado Color. Mix 80 ml solution of methanol·water(3:1) and add sodium hydroxide solution (1→10) to adjust pH 8~9. Add mixed solution of methanol·water(3:1) to make to 100 ml. Fill 2 g of trimethylaminopropylated silicagel in approximately 15 mm of glass or polypropylene column and wash the column the methanol and mixed solution of methanol·water(3:1) step by step. Add 10 ml of this solution in column and discard effluent. And wash the column with 20 ml of methanol·water(3:1) and 10 ml of methanol step by step. Elute with 20 ml mixed solution of methanol·acetic acid(99:1). Effluent is dried by reduced-pressure

drying below 40°C and dissolve in 0.2 ml of water·acetonitrile(1:1). After mixing respectively 0.1ml of test solution and standard solution with 0.1 ml of phthalaldehyde solution, in 1 minute test liquid chromatography under operation condition. Measure amount of FumonisinB₁ from calibration curve, it should not more than 0.3 ppm.

Standard solution: Weigh precisely 0.01 g of FumonisinB₁ and then dissolve in mixed solution of water·acetonitrile(1:1) to make to 100 ml. And respectively 1, 5, 10 ml of this solution with mixed solution of water·acetonitrile(1:1) make precisely to 200 ml to use as standard solution.

Preparation of calibration curve: Proceed liquid chromatography with 3 standard solutions under operation conditions below and prepare calibration curve.

Operation condition

Detector: Fluorescence detector (excitation wavelength 335 nm, fluorescence wavelength 440 nm)

Column filler: 5 μm of octadecylsilylated silicagel for liquid chromatography

Column tube: inner diameter 4.6 mm, length 15 cm stainless tube

Mobile phase: A solution: B solution = 3:7

A solution: phosphate buffer(Dissolve 12 g of sodium phosphate in water and make to 1,000 ml. Then adjust pH to 3.3 with phosphoric acid)

B solution: methanol

Assay (Color Value) Appropriate amount of Maize Morado Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in acetic acid · sodium acetate buffer solution with pH 3.0 so that total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using acetic acid sodium acetate buffer solution with pH 3.0 as a reference solution, absorption A is measured at the maximum absorption near 515 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1\text{cm}}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citric acid · dibasic sodium phosphate buffer solution (pH 3.0)

Solution 1 : 0.1M citric acid solution : 1 ℓ of solution containing 21.01 g of citric acid (C₆H₈O₇ · H₂O).

Solution 2 : 0.2M dibasic sodium phosphate solution : 1 ℓ of solution containing 71.63g of dibasic sodium phosphate (Na₂HPO₄ · 12H₂O).

Solution 1 and Solution 2 are mixed well (159:41) and its pH is adjusted to 3.0.

117. Tea Extract

Definition Tea Extract is obtained by extracting tea leaves of *Camellia sinensis* O. KZE. of Theaceae with water or ethyl alcohol and its major component is catechin.

Compositional Specifications of Tea Extract

Content Tea Extract (converted to anhydrous) are more than 20% as catechin, and should be 90~120% of the marked amount.

Description Tea Extract is pale yellow to dark brown power, paste, or liquid with a slight characteristic scent.

Identification (1) 0.1 g of Tea Extract in 10 ml of 50% ethyl alcohol. When 2~3 drops of ferric chloride(1→50) are added to the solution, it becomes greenish purple~darkish purple appear.

(2) The aqueous solution of Tea Extract show a maximum absorption peak at 265~280 nm.

Purity (1) Arsenic : Place 0.25 g of Tea Extract in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Tea Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Loss on Drying When Tea Extract is dried for 2 hours at 100°C, the loss should not be more than 5%. (For powder only).

Assay 0.5 g of the sample is previously weighted and the amount of water (W%) is measured beforehand.

(1) Green tea extract : The amount that corresponds to about 30 mg of catechin of Tea Extract is accurately weighted, to which water is added. If necessary, it is heated for dissolution. The volume is made precisely 100 ml by adding water. To 5 ml of this solution, 5 ml of ferrous tartarate is added and then phosphate buffer (pH 7.5) is added to make precisely 25 ml for the test solution. With the control solution being water, absorbance is measure at 540 nm. Separately, the standard solution containing 5, 10, 15, 20, 25 mg of ethyl gallate (standard) per 100 ml are made. Using 5 ml of

each of these standard solution, the same procedure as the test solution is performed to generate color. Then at 540 nm, absorbance is measured to determine the standard curve. From the absorbance of the test solution and the standard curve, calculate the content (mg) of catechin in 100 ml of the test solution by the following formula.

$$\text{Catechin Content (\%)} = \frac{C \times 1.5 \times 100}{\text{Weight of Sample (mg)} \times (100 - W)} \times 100$$

C : Concentration of ethyl gallate in the test solution obtained from the standard solution (mg/100ml)

1.5 : The absorbance of 1 mg of ethyl gallate corresponds to that of 1.5 mg of tea catechin.

W : Water content (%)

(2) Woorong tea and red tea extracts : The amount of Tea Extract that corresponds to about 10 mg of catechin is weighted accurately and dissolved in 1 ml of 50% ethanol, and made precisely 100 ml by adding water. This is the test solution. Separately, about 25 mg of (+) catechin (for assay) that is dried at 100 °C for 1 hour is accurately weighted and dissolved in 1 ml of 50% ethanol, and made precisely 100 ml by adding water. 5, 7.5, 10, 12.5, and 15 ml of this solution are respectively taken and diluted precisely to 25 ml by adding water. These are the standard solutions. To 0.15 ml of each standard solution and the test solution, 1.35 ml of water and 0.5 ml of Folin-Denis' solution are added and mixed. As for the control solution of the test solution, 0.5 ml of water is used. After 3 mins, 1 ml of sodium carbonate (1→10) is added. Place in a thermostatic water bath of 30°C for 1 hour and measure the absorbance at 700 nm. The standard curve is made from the measured values of the standard solutions of (+) catechin. Calculate the content of catechin in 100 ml of the test solution. The content of catechin is determined by the following formula.

$$\text{Content of Catechin} = \frac{C \times 100}{\text{Weight of Sample (mg)} \times (100 - W)} \times 100$$

C : Concentration (mg/100 ml) ethyl gallate in the test solution obtained from the standard curve

W : Water content (%)

Solutions

- Folin-Denis's solution : Add 180 ml of water to 25 g of sodium tungstate, 5 g of phosphomolybdic acid, and 15.5 ml of phosphoric acid. Attach a reflux condenser, heat the solution gently for 2 hours. Cool the solution and add water to make 1,000 ml.

118. Masticatory Substances, Natural

Definition There are Sorva (*Couma macrocarpa* BARB. RODR.), Sorvinha (*Couma utilis* MUELL.), Jelutong (*Dyera costulata* Hook. F. and *Dyera lowii* Hook. F.), Chicle (*Manikara zapotilla* Gilly and *Manikara chicle* Gilly), and Natural rubber (*Hevea brasiliensis*).

Compositional Specifications of Masticatory Substances, Natural

Description Masticatory Substance(Natural) is white~gray, pale brown~dark brown solid or viscoelastic solid with a slight characteristic scent.

Purity (1) Arsenic : Place 0.25 g of Masticatory Substance(Natural) in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of General Provisions is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 3 ppm.

119. Quillaia Extract

Definition Quillaia Extract is obtained by extracting barks of quillaia (*Quillaia saponaria* MOLINA) of rosaceae with water followed by purification. Its major component is saponin. Dilutant or other food additives can be added for the purpose of quality preservation.

Compositional Specifications of Quillaia Extract

Content When Quillaia Extract is quantitatively analyzed, it should contain not less than (as a partially hydrolyzed saponin) the indicated amount.

Description Quillaia Extract is pale yellow~brown powder or liquid with a characteristic taste.

Identification (1) 0.5 g of Quillaia Extract is dissolved in 10 ml of water. 2 μ l of this solution is spotted at 2 cm position from the bottom of thin plate of silica gel 60 (Kiesel gel 60, Merck) and dried. It is then developed up to 3 cm from the plate top using a mixture of chloroform : methyl alcohol : water : acetic acid (15:10:3:1) as a developing solvent. It is then air-dried and sprayed with anisaldehyde · sulfuric acid solution, which is heated for 10 minutes at 110°C. Brown spots (with violet tint) are observed at R_f values of 0.22, 0.26, 0.29, and 0.30. The largest spot is at R_f value of 0.29.

◦ Anisaldehyde · sulfuric acid : 9 ml of alcohol is stir-mixed with 0.5 ml of p-anisaldehyde and 0.5 ml of sulfuric acid.

(2) 2 g of Quillaia Extract is added to a 100 ml flask, where 25 ml of 1% potassium hydroxide solution is added, a reflux condenser is attached, and heated for 2 hours. Cool and transfer the content into a beaker and neutralize to pH 5 with hydrochloric acid (1→4). It is then diluted to 50 ml with water (Test Solution). Separately, 10 mg of partially hydrolyzed saponin used in Assay is dissolved in 5 ml of water (Standard Solution). 2 μ l of each solution is tested using Thin Plate Chromatography following the procedure under Identification (1). One of the spots from the Test Solution has the same color and R_f value from the bluish gray spot of the Standard Solution.

Purity (1) Acidity : An aqueous solution (1→100) of Quillaia Extract should have a pH of 4.5~5.5 (for powder only).

(2) Arsenic : Place 0.5 g of Quillaia Extract in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and

reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 2 ppm.

(3) Lead : When 5.0 g of Quillaia Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(4) Mercury : When Quillaia Extract is tested by Mercury Limit Test, its content should not be more than 1.0ppm

Loss on Drying When 1 g of Quillaia Extract is dried for 2 hours at 105°C in water, the loss should not be more than 6% (for powder only).

Residue on Ignition Residue on Ignition of Quillaia Extract should not be more than 10%.

Assay Approximately 2 g (approximately 5 g for liquid) is added to a 100 ml volumetric flask, which is filled with water. Precisely 10 ml of this solution is taken into a 100 ml volumetric flask, where 10 ml of 2% potassium hydroxide solution is added, a reflux condenser is attached, and heated for 2 hours in a water bath. Cool and transfer the content into a 50 ml volumetric flask using 25 ml of ethyl alcohol. Add 0.5 ml of phosphoric acid and dilute the solution to 50 ml with water (Test Solution). Separately, 20 mg of partially hydrolyzed saponin standard is precisely weighted into a 50 ml volumetric flask and dissolved in 50v/v% ethyl alcohol. The total volume is brought up to 50 ml with 50 v/v% ethyl alcohol (Standard Solution). Inject each 20 µl of test solution and standard solution to high speed liquid chromatography under the following operation conditions. The content of partially hydrolyzed saponin is obtained by the following equation.

$$\text{Content(\%)} = \frac{B}{A} \times \frac{(S_1+S_2) \times 10}{S_T} \times 100$$

A : Amount of sample(mg)

B : Amount of standard(mg)

St : Peak area of partially hydrolyzed saponin in Standard Solution

S₁ : Peak area of partially hydrolyzed saponin in Test Solution

S₂ : Peak area of saponin-like matters appeared before peak of partially hydrolyzed saponin in Test Solution

Operation Conditions

-Detector : UV 210 nm

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- Column : stainless steel tube with 4~6 mm inner diameter and 15~30 cm length, which is filled with 5~10 μm silylated silica gel with octadecyl group (for liquid chromatography)
- Column Temperature : 40°C
- Mobile Phase : 0.1% phosphoric acid : acetonitrile (65 : 35)
- Flow Rate : Adjusted so that the retention time of partially hydrolyzed saponin is approximately 10 minutes

120. Defatted Ricebran Extract

○ Cancellation of designation

The date of cancellation : 11.12.10(Notification No. 2010-82).

121. Heme Iron

Definition Heme Iron is obtained by separating hemoglobin enzymatically. Its component is Heme iron.

Compositional Specifications of Heme Iron

Content Dried Heme Iron contains 9.0~27.0% of protoheme ($C_{34}H_{32}FeN_4O_4 = 616.48$) and 1.0~2.6% of iron ($Fe = 55.85$).

Description Heme Iron is blackish brown powder or granule. It can be scentless or have a slight characteristic scent.

Identification (1) 100 mg of Heme Iron is dissolve in 500 ml of pyridine sodium hydroxide solution. When 15 mg of sodium hyposulfite is added to 5 ml of this solution, it becomes red.

(2) 10 mg of Heme Iron is added to a 100 ml flask for decomposition, where 5 ml of nitric acid is added. When it is heat treated, it becomes yellow. Cool and alkalize it with ammonia water. The color becomes orange yellow.

Purity (1) Arsenic : Place 0.25 g of Heme Iron in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Heme Iron is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Loss on Drying When Heme Iron is dried for 3 hours at 105°C, the loss should not be more than 6%.

Residue on Ignition When thermogravimetric analysis is done with precisely weighted 2 g of Heme Iron, the amount of Residue on Ignition should not be more than 8%.

Assay (1) Protoheme : 100 mg of Heme Iron is precisely weighted into a 250 ml flask and dissolved in pyridine sodium hydroxide solution so that the total volume is exactly 250 ml (Test Solution). 1 ml of Test Solution is accurately sodium hyposulfite transferred into a test tube, where 2 ml of pyridine sodium hydroxide solution is added and 3 mg of sodium hyposulfite is added immediately. Absorption of the resulting solution is measured at 557 nm using pyridine sodium hydroxide solution as

a reference. Separately, 1 ml of each hematin Standard Solution is added to a test tube, where 2 ml of pyridine sodium hydroxide solution is added and 3 mg of sodium hyposulfite is added immediately. Absorption of each solution is measured by following the same procedure as Test Solution and a calibration curve is prepared. The content of protoheme is obtained from the calibration curve and the absorption of Test Solution.

(2) Iron : 20~50 mg of this additive is precisely weighted into a 100 ml flask for decomposition, where 5~10 ml of nitric acid is added. It is gently heated until the evolution brown nitrogen oxide gas subsides. Cool the flask to room temperature, add 2 ml of perchloric acid to the flask, which is heated gently and then strongly until the solution becomes colorless and white smoke subsides. Cool and transfer the reaction mixture into a flask(Recovery). The flask for decomposition is washed with water, which is added to this flask. pH of the solution is adjusted to 3~8 with ammonia solution. Dilute the resulting solution to exactly 100 ml with water (Test Solution). 10 ml of Test Solution is added to a 100 ml flask and diluted to approximately 50 ml with water. To this solution, 1 ml of hydroxylamine hydrochloride solution (1→4), 5 ml of o-phenanthroline solution in hydrochloric acid (0.12→100), and 20 ml of acetate buffer solution (pH 4.2) are added. The total volume is brought up to 100 ml with water. Set it aside for 1 hour at room temperature, measure absorption at 510nm. Separately, 0.5, 1, 5, 10, 20 ml each of iron standard solution is placed in 100 ml flask, where 3 ml hydrochloric acid (1→4) and water are added to bring the total volume to approximately 50 ml. The same procedure as Test Solution is followed with these standard solutions. Absorption is measured to prepare a calibration curve. The content of iron is obtained from the calibration curve and the absorption of the Test Solution.

Solutions

- Pyridine Sodium Hydroxide Solution : 100 ml of pyridine and 30 ml of 1 N sodium hydroxide solution are mixed and the total volume is brought up to exactly 300 ml with water.
- Acetate Buffer Solution (pH 4.2) : 250 g of ammonium acetate is dissolve in 120 ml of water and 700 ml of acetic acid. The solution is diluted with water to 1,000 ml.
- Hematin Standard Solution : Hematin is dried for 3 hours at 100°C. 100 mg is precisely weighted and dissolved in pyridine sodium hydroxide solution so that the total volume is exactly

100 ml (hematin standard solution, undiluted). 1, 5, 10, and 20 ml. each of undiluted standard solution is diluted to 100 ml with pyridine sodium hydroxide solution. It is further diluted so that 1 ml of the final dilution contains 1, 5, 10, and 20 μg of hematin (Hematin Standard Solution).

- Iron Standard Solution (for heme iron) : 7.0213 g of ferrous ammonium sulfate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$) is precisely weighted and dissolved in a small amount of water. 3 ml of dilute hydrochloric acid (1→4) and water are added to bring the total volume to exactly 1,000 ml (Iron Standard Solution). From this solution, 10 ml is taken and made 100 ml by adding water. This is the standard solution (1 ml of this solution contains 100 μg of Fe).

122. Gum Ghatti

Definition Gum Ghatti is a polysaccharide obtained by drying sap that is leached from stems of *Anogeissus latifolia* WALL. or plants of the same genus.

Compositional Specifications of Gum Ghatti

Description Gum Ghatti is powder or granule with gray~reddish gray, or pale brown~dark brown amorphous solid. Gum Ghatti is almost scentless.

Identification (1) When 1 g of Gum Ghatti is dissolved in 5 ml of water, it becomes a viscous liquid.

(2) To 5 ml of the filtrate which aqueous solution of Gum Ghatt (1→100) is filtered with diatomite, 0.2 ml of diluted alkaline lead acetate solution(1→5) is added, then precipitate is not formed or slight precipitate is formed. When 0.5 ml of ammonia solution is added to this solution, opaque wool shaped precipitate is formed.

(3) The filtrate which aqueous solution of Gum Ghatt(1→50) is filtered with diatomite is levorotatory.

Purity (1) Arsenic : Place 0.25 g of Gum Ghatt in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2)Lead : When 5.0 g of Gum Ghatti is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Acid Insoluble Ash : When Gum Ghatti proceed as directed under Ash and Acid-Insoluble Ash Limit, the content should not be more than 1.75 %.

(4) Salmonella : When Gum Ghatti proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in 「Standards and Specifications for Foods」, it should be negative (-).

(5) E. coli : When Gum Ghatti proceed as directed under Microbiological Methods for E. coli in General Testing Methods in 「Standards and Specifications for Foods」, it should be negative (-).

Loss on Drying When Gum Ghatti is dried for 5 hours at 105°C, the loss should not be more than 14%.

Ash When Gum Ghatti is tested for ash content, it should not be more than 6%.

123. Madder Color

○ Cancellation of designation

The date of cancellation : 7.16.04(Notification No. 2004-50).

124. Kusagi Color

○ Cancellation of designation

The date of cancellation : 7.10.09(Notification No. 2009-51).

125. Peanut Color

○ Cancellation of designation

The date of cancellation : 7.10.09(Notification No. 2009-51).

126. Rutin

Definition Rutin is an extract (with water or ethyl alcohol) from flower or flower bud of Japanese pagoda tree (*Sophora japonica* L.) of leguminosae, or root cortex of buck wheat (*Fagopyrum esculentum* MOENCH.) of polygonaceae, or root cortex of red bean of (*Phaseolus angularis* CW. WIGHT.). Its major component is rutin ($C_{27}H_{30}O_{16}$ = 610.51) of flavonoids. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Rutin

Content Color value ($E_{1cm}^{10\%}$) of Rutin should be more than the indicated value.

Description Rutin is yellow~pale yellowish green liquid or powder with a slight characteristic scent.

Identification (1) Rutin is dissolve in 10 ml of ethyl alcohol. When 1~2 drops of ferric chloride solution (1→50) are added, the solution becomes greenish brown.

(2) Rutin is dissolve in 5 ml of ethyl alcohol by heating. When 2 ml of hydrochloric acid and 0.05 g of magnesium powder are added, the solution slowly becomes red.

(3) Add 500 ml of ethyl alcohol to Rutin. This solution has maximum absorption bands near 255 nm and 375 nm.

(4) 5 ml of the solution in (3) is neutralized with sodium hydroxide solution, where 3 ml of Fehling solution is added. When the resulting solution is heated for 10 minutes in a water bath, red precipitates are formed.

Purity (1) Arsenic : Place 0.25 g of Rutin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Rutin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Assay (Color Value) Appropriate amount of Rutin is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in 10 ml of ethyl alcohol by heating. If necessary, this solution is filtered through a glass filter, which is washed with hot ethyl alcohol. The filtrate and the wash are mixed and diluted to 200 ml with ethyl alcohol. To 10 ml of this solution, 1 ml of acetic acid solution in ethyl alcohol (1.2→

1,000) is added and the total volume is brought up to 100 ml with ethyl alcohol (Test Solution). A reference solution is prepared by diluting 1 ml of acetic acid solution in ethyl alcohol (1.2→1,000) to 100 ml with ethyl alcohol. Absorption A for Test Solution is measured at 375 nm with 1cm path length. Color value is obtained by the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 200}{\text{Weight of sample (g)}}$$

127. D-Ribose

Definition D-Ribose is obtained by the following process. Glucose is fermented by *Bacillus* (*Bacillus pumilus*). The resulting material is separated and purified. Its component is D-Ribose.

Compositional Specifications of D-Ribose

Content D-Ribose (converted to an anhydrous form) contains 90.0~102.0% of D-ribose ($C_5H_{10}O_5 = 150.13$).

Description D-Ribose is white~pale brown crystalline powder. It may be scentless or have a slight characteristic scent.

Identification (1) When 2~3 drops of an aqueous solution (1→20) of D-Ribose is added to 5 ml of warm Fehling solution, red precipitates are formed.

(2) An aqueous solution of D-Ribose (1→25) is levorotatory.

Purity (1) Arsenic : Dissolve 0.25 g of D-Ribose in 5 ml of water. If necessary, this solution is dissolved by heating to use Test Solution. When test for Arsenic Limit Test is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of D-Ribose is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Other Saccharide : Liquid chromatography is carried out according to Assay. All the peaks until a double retention time of D-ribose are observed. Peak area Apart from peak area of D-ribose in Test Solution, peak area should not be more than 10% of the sum of areas of all the peak.

Water Content Water content of D-Ribose is determined by direct titration method in Water Determination (Karl Fisher Method) and should not be more than 5.0%.

Residue on Ignition When thermogravimetric analysis is done with 1 g of D-Ribose, the amount of the amount of Residue on Ignition should not be more than 1.0%.

Assay Weight accurately 1.0 g of D-Ribose and 1.0 g of D-ribose standard. Dissolve each samples in water, and dilute to 50 ml with water (Test Solution and Standard Solution). 10 μ l of each solution is injected into liquid chromatography under the following Operation Conditions and the content of D-ribose is obtained by the following equation.

$$\text{Content (\%)} = \frac{\text{amount of standard converted to an anhydrous form(g)}}{\text{amount of sample converted to an anhydrous form(g)}} \times \frac{A_T}{A_S} \times 100$$

A_T : peak area of Test Solution

A_S : peak area of Standard Solution

Operation Conditions

- Detector : Differential refractometer (RI detector)
- Column : Shodex SUGAR SC1011(8 × 300 mm) or its equivalent
- Column Temperature : 80°C
- Mobile Phase : Water
- Flow Rate : 1.0 ml/min

128. Gallic Acid

Definition Gallic Acid is obtained by hydrolyzing tannin that is extracted from gallnut of lacquer tree (*Rhus javanica* L.) of anacardiaceae or gall of fagaceae (*Quercus infectoria* ol IV) with water, ethyl alcohol, or organic solvents. Its component is gallic acid.

Compositional Specifications of Gallic Acid

Description Gallic Acid is scentless white~whitish yellow needle-shaped crystalline powder with an astringent and a slight acidic taste.

Identification 20 ml of water is added to 1 g of Gallic Acid, which is mixed by shaking for 1 minute and filtered. When 2~3 drops of ferric chloride solution (1→10) are added to the filtrate, bluish black precipitates are formed.

Purity (1) Clarity of Solution : A solution of 1 g of Gallic Acid in 20 ml of water should be pale yellow and almost clear (or better).

(2) Tannin Acid : 20 ml of water is added to 1 g of Gallic Acid, which is mixed by shaking and filtered. When 5~6 drops of 1% warm gelatin solution, it should not turn turbid.

(3) Arsenic : Place 0.25 g of Gallic Acid in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(4) Lead : When 5.0 g of Gallic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Loss on Drying When Gallic Acid is dried for 2 hours at 105°C, the loss should not be more than 10%.

Residue on Ignition

Residue on Ignition of Gallic Acid should not be more than 0.1%.

129. Hibiscus Color

Definition Hibiscus Color is a pigment obtained by extracting flowers of rose of Sharon (*Hibiscus sabdariffa* Linné) of malvaceae with water. Its major pigment component is delphinidin-3-sambubioside. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Hibiscus Color

Content Color value ($E_{1cm}^{10\%}$) of Hibiscus Color should be more than the indicated value.

Description Hibiscus Color is dark red liquid, powder or paste with a slight characteristic scent.

Identification (1) A solution (1→100) of Hibiscus Color in citric acid buffer solution (pH 3.0) is red in color and has a maximum absorption band near 520 nm.

(2) When the solution in (1) is alkalinized with sodium hydroxide solution (1→25), its color becomes dark green.

Purity (1) Arsenic : Place 0.25 g of Hibiscus Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Hibiscus Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm. .

Assay (Color Value) Appropriate amount of Hibiscus Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved citrate buffer solution with pH 3.0 so that the total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citrate buffer solution with pH 3.0 as a reference solution, absorption A is measured at 520 nm wavelength with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citrate buffer solution (pH 3.0)

1390 Hibiscus Color

Solution 1 : 1 ℓ of solution containing 121 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 1 ℓ of solution containing 71.6 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (159:41) and its pH is adjusted to 3.0.

130. Microfibrillated Cellulose

Definition Microfibrillated Cellulose is microfibrillated cellulose obtained by homogenizing fibers such as pulps.

Compositional Specifications of Microfibrillated Cellulose

Description Microfibrillated Cellulose is white and wet fiber.

Identification (1) When 30 g of Microfibrillated Cellulose (converted to a dried form) is dispersed in water (total weight = 300 g) and homogenized at 3,000~5,000 rpm for 5 minutes, it becomes a suspension without fluidity. Suspension is maintained in 3 hours without separation.

(2) 1 ml of phosphoric acid is added to 1 mg (converted to a dried form) of Microfibrillated Cellulose, which is heated for 30 minutes in a water bath. When 4 ml of catechol phosphate solution (1→500) is added to this solution and heated for 30 minutes, it becomes red.

(3) When 2 ml of iodine solution (1→5) is added to 0.05 g (converted to a dried form) and set aside for 5 minutes, the color of the solution is maintained. The supernatant is discarded by leaning. When 1 drop of diluted sulfuric acid (1→2) is added to the residue, the color becomes bluish violet.

Purity (1) Acidity : 2 g (converted to a dried form) of Microfibrillated Cellulose is dispersed in 100 ml of water (freshly boiled and cooled). pH of this suspension should be 5.0~8.0 (using glass electrode).

(2) Arsenic : Place 0.5 g of Microfibrillated Cellulose in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 2 ppm.

(3) Lead : When 5.0 g of Microfibrillated Cellulose is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(4) Starch : The suspension in Purity (1) is filtered. When 2~3 drops of 0.1 N iodine solution is added to 20 ml of this filtrate, it is not appeared blue~bluish violet in color.

(5) Water Solubles : 100 ml of water is added to 10 g (converted to a dried form)

of Microfibrillated Cellulose, which is heated (with a reflux condenser) for 30 minutes in an oil bath. Cool the solution. It is vacuum filtered through a glass filter(G4). 50 ml of the filtrate is evaporated to dryness. The residue is dried for 1 hour at 105°C. The amount of residue should not be more than 50 mg.

(6) Total Viable Aerobic Count : When Microfibrillated Cellulose is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 per 1 g

(7) E. Coli : When Microfibrillated Cellulose is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Loss on Drying When Microfibrillated Cellulose is dried for 3 hours at 100°C, the loss should not be more than 80%.

Residue on Ignition When Residue on Ignition is done with precisely weighted material, the amount of residue should not be more than 0.3%.

131. Sandalwood Red

Definition Sandalwood Red is a pigment obtained by extracting tree of sandalwood (*Pterocarpus santalinus* Linné) of salicaceae with water. Its major pigment component is Santalin of flavonoids. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Sandalwood Red

Content Color value ($E_{1cm}^{10\%}$) of Sandalwood Red should be more than the indicated value.

Description Sandalwood Red is dark red~reddish violet liquid or powder with a slight characteristic scent.

Identification (1) When 0.1 g of Sandalwood Red is mixed with 100 ml, it is turbid. When the mixture is alkalinized with sodium hydroxide solution, it becomes clear reddish violet solution.

(2) When 10 ml of ammonium chloride solution (1→50) is added to a solution of Sandalwood Red (0.1 g dissolved in 100 ml of 80 v/v% ethyl alcohol), it becomes turbid and venetian red color.

(3) When 1 ml of ferric sulfate solution (1→10) is added to a solution of Sandalwood Red (0.1 g dissolved in 100 ml of 80 v/v% ethyl alcohol), it becomes brown with bluish tint and precipitates are formed.

(4) A solution of Sandalwood Red in 80 v/v% ethyl alcohol (pH 6.0) shows maximum absorption bands at 475 nm and 503 nm.

Purity (1) Arsenic : Place 0.25 g of Sandalwood Red in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Sandalwood Red is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay (Color Value) Appropriate amount of Sandalwood Red is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in 80 v/v% ethyl alcohol so that the total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged

and the supernatant is used. Using 80 v/v% ethyl alcohol as a reference solution, absorption A is measured at the maximum absorption near 500 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

132. Psyllium Seed Gum

Definition Psyllium Seed Gum is a polysaccharide obtained by crushing the outer shells of seeds of psyllium plant (*Plantago ovata* FORSK.) of plantaginaceae or its same species.

Compositional Specifications of Psyllium Seed Gum

Description Psyllium Seed Gum is pale light gray~yellowish brown powder with a slight characteristic scent.

Identification (1) Psyllium Seed Gum is wetted with cresol and observed under a microscope. Polygonal pillar cells surrounded by cell walls (4~6 sides) are observed. (2) Psyllium Seed Gum is wetted with ethyl alcohol and observed under a microscope. When a few drops of water is drop-wise added, polygonal pillar cells swell quickly and mucilage migrates into the solution.

Purity (1) Arsenic : Place 0.25 g of Psyllium Seed Gum in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm. (2) Lead : When 5.0 g of Psyllium Seed Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm. (3) Total Viable Aerobic Count : When Psyllium Seed Gum is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 per 1 g (4) E. Coli : When Psyllium Seed Gum is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-). (5) Protein : When 3 g of Psyllium Seed Gum is precisely weighed, proceed as directed under Kjeldahl Method in Nitrogen Determination, the amount should not be more than 2.0%.

1 ml of 0.01 N sulfuric acid = 0.8754 mg protein

Loss on Drying When Psyllium Seed Gum is dried for 6 hours at 105°C, the loss

should not be more than 12%.

Ash When Psyllium Seed Gum is tested by Ash and Acid-Insoluble Ash Limit, the amount of ash should not be more than 4.0%.

133. Saffron Color

Definition Saffron Color is a pigment obtained by extracting dried stigma of flower of saffron (*Crocus sativus*) of iridaceae with ethyl alcohol. Its major pigment component is Crocin and Crocetin of carotenoids. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Saffron Color

Content Color value ($E_{1\text{cm}}^{10\%}$) of Saffron Color should be more than the indicated value.

Description Saffron Color is yellow~venetian red liquid, solid, powder, or paste with a slight characteristic scent.

Identification (1) 50 v/v% ethyl alcohol solution (1→500) of this additive shows yellow color and a maximum absorption band near 430 nm.

(2) When 5 ml of sulfuric acid is added to 0.5 g of Saffron Color (if necessary, evaporated to dryness in a water bath and then cooled), it becomes deep green color, which changes to violet then to brown.

Purity (1) Arsenic : Place 0.25 g of Saffron Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Saffron Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Nitrogen : When Saffron Color is tested by Kjeldahl Method of Nitrogen Determination, the amount should not be more than 2.0%.

(4) Water Insoluble substances : 3 g of Saffron Color is placed into an Erlenmeyer flask, which is added with approximately 100 ml of water in advance. 10 ml of dilute hydrochloric acid is added to the flask, which is then gently boiled for 15 minutes, and filtered through a glass filter (previously dried until the weight does not change). The residue on the glass filter is fully washed with hot water. The residue is dried for 2 hours at 105°C, cooled in a desiccator, and measure the content of insoluble substance. The content of water insoluble substances should not

be more than 45%.

(5) Acid Insoluble Ash : When Saffron Color is tested for ash by acid insoluble ash methods in Ash and Acid-Insoluble Ash Limit, it should not be more than 8.0%.

Loss on Drying When Saffron Color is dried for 4 hours at 105°C, the loss should not be more than 14%.

Ash When Saffron Color is tested by total ash in Ash and Acid-Insoluble Ash Limit, the amount of ash should not be more than 8.0%.

Assay (Color Value) Appropriate amount of Saffron Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in 50 v/v% ethyl alcohol (total volume 100 ml). 1 ml of this solution is diluted to 100 ml with 50 v/v% ethyl alcohol (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using 50 v/v% ethyl alcohol as a reference solution, absorption A is measured at the maximum absorption near 430 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

134. L-Sorbose

○ Designed Cancellation

The date of cancellation: 27.3.'12 (Notification No. 2012-10).

135. Shea Nut Color

Definition Shea Nut Color is a pigment obtained by extracting (with water) fruits or spermoderms of *Butylospermum parkii* KOTSCHY. Its major pigment component is flavonoid. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Shea Nut Color

Content Color value ($E_{1cm}^{10\%}$) of Shea Nut Color should be more than the indicated value.

Description Shea Nut Color is brown~dark brown liquid, paste, powder, or paste with a slight characteristic scent.

Identification (1) Citrate buffer(pH 7.0) solution(1→100) of Shea Nut Color is brown color.

(2) When the solution in (1) is acidified by hydrochloric acid, the pigment becomes insoluble and yellowish brown precipitates are formed.

Purity (1) Arsenic : Place 0.25 g of Shea Nut Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Shea Nut Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay (Color Value) Appropriate amount of Shea Nut Color is precisely weighted so that the absorption is within 0.3~0.7, and dissolved in 30 ml of anhydrous sodium carbonate solution (1→200). Citric acid-dibasic sodium phosphate buffer solution with pH 7.0 is added so that the total volume is 100 ml (Test Solution). 1 ml of this solution is diluted to 100 ml with citric acid-dibasic sodium phosphate buffer solution with pH 7.0. If necessary, the solution is centrifuged and the supernatant is used. Using a mixture of 30 ml anhydrous sodium carbonate solution (1→200) and 100 ml citric acid-dibasic sodium phosphate buffer solution with pH 7.0 as a reference solution, absorption A is measured at 490 nm wavelength with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

136. Cyclodextrin Syrup

Definition Cyclodextrin Syrup is a starch hydrolysate by purifying and concentrating aqueous solution (containing cyclodextrin), which is prepared by treating starch latex with cyclodextrin producing enzyme. It contains sugars such as α -cyclodextrin, β -cyclodextrin, γ -cyclodextrin, glucose, and maltose, where 6, 7, or 8 molecules of glucose form a ring via α -1,4 glucoside bonding. Dried cyclodextrin syrup also falls within this category.

Compositional Specifications of Cyclodextrin Syrup

Content Dried Cyclodextrin Syrup should be more than the indicated content of cyclodextrin under Assay.

Description Cyclodextrin Syrup is colorless transparent viscous liquid or white powder. It is sweet but scentless. It tends to form white precipitates and turn turbid in cold places.

Identification (1) When 0.5 g of Cyclodextrin Syrup is dissolved in 1 ml of 0.1 N iodine solution by heating in a water bath and set aside at room temperature, yellowish brown precipitates are formed.
(2) 0.5 g of Cyclodextrin Syrup is dissolved in 3 ml of water by heating in a water bath. When 1 ml of trichloroethylene is added to this solution and stirred vigorously, it turns white and turbid.

Purity (1) Clarity of Solution : 2 g of Cyclodextrin Syrup is dissolved in 50 ml of water by heating. The resulting should be colorless and clear (or better).
(2) Arsenic : Place 1 g of Cyclodextrin Syrup in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 1 ppm.
(3) Lead : When 5.0 g of Cyclodextrin Syrup is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.
(4) Chloride : Weight 0.5 g of Cyclodextrin Syrup for Chloride Limit Test. The content of chloride should be equal to or less than the amount that corresponds to 0.4 ml of 0.01 N hydrochloric acid.

Loss on Drying When Cyclodextrin Syrup is dried for 4 hours at 105°C under a reduced pressure less than 5 mmHg, the loss should not be more than 25%.

Residue on Ignition When thermogravimetric analysis is done with 1 g of Cyclodextrin Syrup, the amount of residue should not be more than 0.05%.

Assay Cyclodextrin Syrup (corresponding to 0.5 g of cyclodextrin) is precisely weighted and diluted to 50 ml with water. 20 ml of this solution is heated for 10 minutes in a water bath and cooled. 2 ml of glucoamylase (10 IU/ml) is added to the solution, which is then reacted for 1 hour in a 40°C water bath. The reaction mixture is heated for 10 minutes in a water bath and filtered. The filtrate is cooled to room temperature and diluted to 25 ml with water (Test Solution). Separately, α -, β -, γ -cyclodextrin standards are dried. 0.1 g each is weighted and dissolved in water to bring the total volume to 20 ml (Mixed Standard Solution). 10 μ each of Test Solution and Mixed Standard Solution is injected into a high speed liquid chromatography under the following operation conditions. Peak areas of α -, β -, γ -cyclodextrin are obtained for Test Solution and Mixed Standard Solution. The contents of three components are obtained by the following equation and the content of cyclodextrin is obtained from the sum of the three.

Content of cyclodextrin (CD) (%) = Content sum of α -CD + β -CD + γ -CD (%)

$$\text{Content of } \alpha\text{-CD (\%)} = \frac{\text{Concentration of } \alpha\text{-CD standard solution (ppm)} \times 50 \times 25}{\text{Weight of sample (g)} \times 20} \times$$

$$\frac{\text{peak area of } \alpha\text{-CD Test Solution}}{\text{peak area of } \alpha\text{-CD Mixed Standard Solution}} \times \frac{100}{10^6}$$

$$\text{Content of } \beta\text{-CD (\%)} = \frac{\text{Concentration of } \beta\text{-CD standard solution (ppm)} \times 50 \times 25}{\text{Weight of sample (g)} \times 20} \times$$

$$\frac{\text{peak area of } \beta\text{-CD Test Solution}}{\text{peak area of } \beta\text{-CD Mixed Standard Solution}} \times \frac{100}{10^6}$$

$$\text{Content of } \gamma\text{-CD (\%)} = \frac{\text{Concentration of } \gamma\text{-CD standard solution (ppm)} \times 50 \times 25}{\text{Weight of sample (g)} \times 20} \times$$

$$\frac{\text{peak area of } \gamma\text{-CD Test Solution}}{\text{peak area of } \gamma\text{-CD Mixed Standard Solution}} \times \frac{100}{10^6}$$

Operation Conditions

- Detector : Differential refractometer (RI Detector)
- Column : Aminex HPX-42A (8 mm × 300 mm) or its equivalent
- Column Temperature : A constant temperature near 80°
- Mobile Phase : Water
- Flow Rate : 0.6~1.0 ml/min

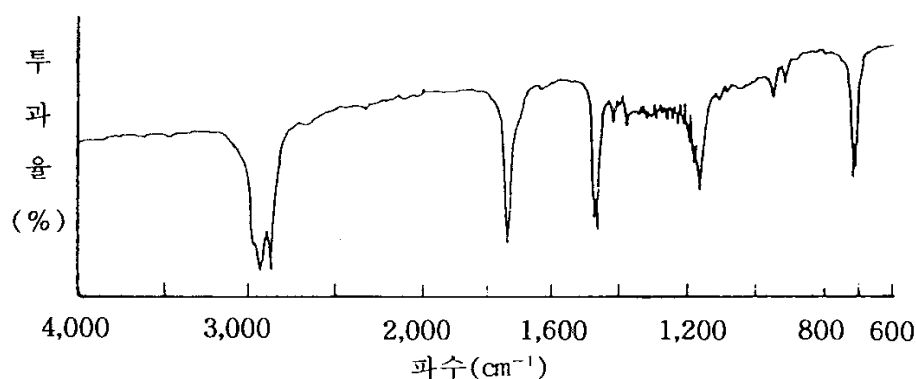
137. Rice Bran Wax

Definition Rice Bran Wax is obtained by separating and purifying rice bran oil of rice (*Oryza sativa* L.) of gramineae.

Compositional Specifications of Rice Bran Wax

Description Rice Bran Wax is pale yellow~pale brown flakes or solid with a slight characteristic scent.

Identification (1) 1~2 mg of Rice Bran Wax is analyzed by Potassium Bromide fining process in Infrared Spectrophotometry (1). Its spectrum is shown below.



Purity (1) Melting Point : Melting point of rice bran oil should be within 70~83°C.

(2) Free Fatty Acid : Approximately 7 g of is precisely weighted into a 250 ml of Erlenmeyer flask, where 75 ml of warm neutralized ethanol and 2 ml of phenolphthalein TS are added. It is titrated with 0.25 N sodium hydroxide solution until the red color persists for 30 seconds. The amount of free fatty acid (as oleic acid) is obtained by the following equation and it should not be more than 10%.

$$\text{Free fatty acid (as oleic acid)} = \frac{V \times N \times 28.2}{W}$$

V :

Consumed amount of 0.25 N sodium hydroxide solution (ml)

N : Normality of 0.25 N sodium hydroxide solution

W : Amount of sample (g)

(3) Saponification Value : 3 g of Rice Bran Wax is precisely weighted into a flask and dissolved in 25 ml of xylene by shaking until the solution becomes clear or slightly turbid, where 50 ml of ethyl alcohol and 25 ml of 0.5 N alcoholic solution of potassium hydroxide are added. Attach a reflux condenser. The solution is saponified for 2 hour in a water bath. Saponification value should be 70~160 under

Saponification Value in Oils Test.

- (4) Iodine Value : Approximately 1g of Rice Bran Wax is precisely weighted into a 500 ml Erlenmeyer flask, and 20 ml of carbon tetrachloride is added to dissolve the sample. Add 25 ml of Weiss solution, and shake with stopper. The flask is set aside for 30 minutes in a dark place. 20 ml of potassium iodide solution and 100 ml of water (previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution (indicator : 1 ml of starch solution). The content should not be more than 20. Separately, a blank test is carried out by the same procedure.

$$\text{Iodine Value} = \frac{(A - B) \times 1.269 \times f}{C}$$

A : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

B : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

f : Activity of 0.1 N sodium thiosulfate solution in this test

C : Amount of sample(g)

- (5) Arsenic : Place 0.25 g of Rice Bran Wax in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

- (6) Lead : When 5.0 g of Rice Bran Wax is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Residue on Ignition Residue on Ignition of Rice Bran Wax should not be more than 0.3%.

138. Arabino Galactan

Definition Arabino Galactan is a polysaccharide obtained by extracting roots or stems of *Larix occidentalis* NUTT. of pinaceae with water.

Compositional Specifications of Arabino Galactan

Description Arabino Galactan is white~pale yellowish brown powder with a slight characteristic scent.

Identification (1) When 6 g of Arabino Galactan is gently mixed and stirred in 10 ml of water, it readily dissolves and turns into a slightly viscous liquid.

(2) Add 5 ml of water to 5 ml of the solution from (1), and then add 5 ml of sodium borate solution (1→20). When Cetylpyridinium Chloride solution (1→20) is drop-wise added to the resulting solution, white precipitates are formed.

Purity (1) Arsenic : Place 0.25 g of Arabino Galactan in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more than 4 ppm.

(2) Lead : When 5.0 g of Arabino Galactan is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Total Viable Aerobic Count : When Arabino Galactan is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 per 1 g

(4) E. Coli : When Arabino Galactan is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Ash When Arabino Galactan tested by Ash and Acid-Insoluble Ash Limit, the amount of ash should be 4%.

Loss on Drying When Arabino Galactan is dried for 5 hours at 105°C, the loss should not be more than 12%.

139. Onion Color

Definition Onion Color is a pigment obtained by extracting bulbs of Onions (*Allium cepa* L.) of liliaceae with ethyl alcohol. Its major pigment component is Quercetin ($C_{15}H_{10}O_7=302.23$) of flavonoids. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Onion Color

Content Color value ($E_{1cm}^{10\%}$) of Onion Color should be more than the indicated value.

Description Onion Color is brown liquid, paste, powder, or paste with a slight characteristic scent.

Identification (1) Citrate buffer (pH 7.0) solution (1→100) of Onion Color is yellowish~reddish brown in color.

(2) When the solution in (1) is acidified with hydrochloric acid, the pigment becomes insoluble and brown precipitates are formed.

(3) When ferric chloride solution is added to the solution in (1), milky white precipitates are formed.

Purity (1) Arsenic : Place 0.25 g of Onion Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Onion Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay (Color Value) Appropriate amount of Onion Color is precisely weighted so that the measuring absorption of Onion Color is within 0.3~0.7 and dissolved in 5 ml of sodium carbonate (anhydrous) solution (1→200). Citrate buffer solution with pH 7.0 is added so that the total volume is 100 ml accurately. 5 ml of this solution is diluted to 100 ml with Citrate buffer solution with pH 7.0 (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using Citrate buffer solution with pH 7.0 as a reference solution, absorption A is measured at 500 nm wavelength with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1\text{cm}}^{10\%}) = \frac{A \times 200}{\text{Weight of sample (g)}}$$

◦ Citrate buffer solution (pH 7.0)

Solution 1 : 1 ℓ of solution containing 21 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$)

Solution 2 : 1 ℓ of solution containing 71.6 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)

Solution 1 and Solution 2 are mixed well (35 : 165) and its pH is adjusted to 7.0.

140. γ -Oryzanol

Definition γ -Oryzanol is obtained by the following procedure. Rice bran or embryo bud oil is distributed with hydrous ethyl alcohol and hexane or acetone at room temperature. It is then obtained from the fraction of hydrated ethyl alcohol or by treating with resin and purified. Its component is γ -Oryzanol.

Compositional Specifications of γ -Oryzanol

Content The content (mg) of Oryzanol A ($C_{40}H_{58}O_4$) of γ -Oryzanol should be more than the indicated amount.

Description γ -Oryzanol is pale yellow~yellow crystalline powder. It can be scentless or have a slight characteristic scent.

Identification (1) When 0.01 g of γ -Oryzanol is dissolved in 10 ml of alcoholic potassium hydroxide solution, this solution is yellow color.

(2) When 0.01 g of γ -Oryzanol is dissolved in 5 ml of chloroform, where 4 drops of sulfuric acid is added and mixed by shaking, the solution becomes yellow. When 10 drops of anhydrous acetic acid are added, the color of the solution changes to reddish violet then slowly to green.

(3) When 0.01 g of γ -Oryzanol is dissolved in 5 ml of chloroform, where 5 drops of sulfuric acid is added and mixed by shaking and then settled, chloroform layer is pale yellow and aqueous layer is orange in color.

(4) A solution of material in n-heptane (1→100,000) shows maximum absorptions at 229~233 nm, 289~293 nm, 313~317 nm.

Purity (1) Arsenic : Place 0.25 g of γ -Oryzanol in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of γ -Oryzanol is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Loss on Drying When γ -Oryzanol is dried for 3 hours at 105°C, the loss should not be more than 0.5%.

Residue on Ignition When Residue on Ignition is done with precisely weighted

material, the amount of residue should not be more than 0.1%.

Assay Dry γ -Oryzanol, weight precisely 0.05 g of γ -Oryzanol, and dissolve the sample in 70 ml of n-heptane by heating at 70~80°C. N-heptane is added to bring the total volume to 100 ml accurately. 2 ml of this solution is further diluted to 100 ml with n-heptane (Test Solution). Using n-heptane as a reference, absorption A of Test Solution is measured at the maximum absorption band near 315 nm with a path length of 1cm. The content of oryzanol A is obtained by the following equation.

$$\text{Content of Oryzanol A (mg)} = 1$$

141. Milt Protein

Definition Milt Protein is obtained by the following procedure. Hexane and alkaline proteins in testicles of salmon (*Oncorhynchus keta* WALBAUM) of salmonidae or skipjack tuna (*Katsuwonus pelamis* LINNAEUS) of scombridae are decomposed by acid, which is then neutralized. Its component is alkaline protein (protamine, histone).

Compositional Specifications of Milt Protein

Content If Milt Protein is converted to a dehydrated form, it should contain no less than 50% protamine.

Description Milt Protein is white~pale yellow powder with characteristic taste.

Identification (1) 1 mg of Milt Protein is dissolved in 2 ml of water. 5 drops of a solution containing 0.1 g of α -naphthol solution in 100 ml diluted ethyl alcohol (7→10) and 5 drops of sodium hypochlorite solution (4~6%) are added to this solution. When this solution is alkalinized with sodium hydroxide solution, it becomes clear red.

(2) 5 mg of Milt Protein is dissolved in 1 ml of water by heating. When 1 drop of sodium hydroxide solution (1→10) and 2 drops of copper sulfate solution (1→7) are added, it becomes reddish violet.

Purity (1) Clarity of Solution : When 0.5 g of Milt Protein is mixed with 50 ml of water for 5 minutes, its color is colorless~pale yellow and its turbidity should be low or better.

(2) Arsenic : Place 0.25 g of Milt Protein in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(3) Heavy Metals : Carbonize 1 g of Milt Protein by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a

Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. This solution is used as the test solution. The content should not be more than 20 ppm under Heavy Metal Limit Test. Standard color solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. Add 3 drops of hydrochloric acid to the residue, which is then transferred into another Nestler cylinder as described test solution. Then add 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water to make the total volume to 50 ml. This solution is used as the Standard color solution.

Loss on Drying When Milt Protein is dried for 3 hours at 100°C, the loss should not be more than 7.0%.

Ash When Milt Protein tested by Ash and Acid-Insoluble Ash Limit, the amount of ash should not be more than 12%.

Assay Approximately 150 mg of Milt Protein is precisely weighted and tested by Kjeldahl Method in Nitrogen Determination. The content is calculated by the following equation.

$$1 \text{ ml of } 0.1 \text{ N sulfuric acid} = 1.401 \text{ mg N}$$

$$\text{Content (\%)} = \frac{\text{Amount of nitrogen (mg)} \times 3.19}{\text{Weight of sample (mg)} \times \frac{100 - \text{loss on drying (\%)}}{100}} \times 100$$

142. Purple Sweet Potato Color

Definition Purple Sweet Potato Color is a pigment obtained by extracting tuberous roots of sweet potato (*Ipomoea batatas* POIR. and its variety) of convolvulaceae with water. Its major pigment component is anthocyanin. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Purple Sweet Potato Color

Content Color value ($E_{1cm}^{10\%}$) of Purple Sweet Potato Color should be more than the indicated value.

Description Purple Sweet Potato Color is dark red liquid, paste, powder, or paste with a slight characteristic scent.

Identification (1) A solution (1→100) of Purple Sweet Potato Color in citrate buffer solution (pH 3.0) is red color and has a maximum absorption band near 530 nm.
(2) When the solution in (1) is alkalized with sodium hydroxide solution (1→25), the color changes to dark green.

Purity (1) Arsenic : Place 0.25 g of Purple Sweet Potato Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.
(2) Lead : When 5.0 g of Purple Sweet Potato Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 8 ppm.

Assay (Color Value) Appropriate amount of Purple Sweet Potato Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in citrate buffer solution with pH 3.0 so that the total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citrate buffer solution with pH 3.0 as a reference solution, absorption A is measured at the maximum absorption near 530 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citrate buffer solution (pH 3.0)

Solution 1 : 1 ℓ of solution containing 121g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$)

Solution 2 : 1 ℓ of solution containing 71.6g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)

Solution 1 and Solution 2 are mixed well (159:41) and its pH is adjusted to 3.0.

143. Purple Yam Color

Definition Purple yam color is a pigment obtained by extracting tuberous roots of yam (*Dioscorea alata* Linné) of dioscoreaceae with water. Its major pigment component is cyanidin acylglucoside. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Purple Yam Color

Content Color value ($[E]_{1cm}^{10\%}$) of Purple yam color should be more than the indicated value.

Description Purple yam color is dark red liquid, paste, powder, or paste with a slight characteristic scent.

Identification (1) A solution (1→100) of Purple yam color in citrate buffer solution (pH 3.0) is red color and has a maximum absorption band near 530 nm..

(2) When the solution in (1) is alkalinized with sodium hydroxide solution (1→25), its color changes to dark green.

Purity (1) Arsenic : Place 0.25 g of Purple yam color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Purple Yam Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay (Color Value) Appropriate amount of Purple yam color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in citrate buffer solution (pH 3.0) so that the total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citrate buffer solution (pH 3.0) as a reference solution, absorption A is measured at the maximum absorption near 530 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citrate buffer solution (pH 3.0)

Solution 1 : 1 ℓ of solution containing 121g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 1 ℓ of solution containing 71.6g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (159 : 41) and its pH is adjusted to 3.0.

144. Perilla Color

Definition Perilla Color is a pigment obtained by extracting leaves of perilla (*Perilla frutescens* BRITT. Var. *acuta* KUDO.) of labiatae with ethyl alcohol. Its major pigment component is shisonin, malonyl shisonin. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Perilla Color

Content Color value ($E_{1cm}^{10\%}$) of Perilla Color should be more than the indicated value.

Description Perilla Color is dark red liquid, paste, powder, or paste with a slight characteristic scent.

Identification (1) A solution (1→100) of Perilla Color in citrate buffer solution (pH 3.0) is red color and has a maximum absorption band near 520 nm.

(2) When the solution in (1) is alkalinized with sodium hydroxide solution (1→25), its color changes to dark red.

Purity (1) Arsenic : Place 0.25 g of Perilla Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Perilla Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay (Color Value) Appropriate amount of Perilla Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in citrate buffer solution (pH 3.0) so that the total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citrate buffer solution (pH 3.0) as a reference solution, absorption A is measured at the maximum absorption near 520 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citrate buffer solution (pH 3.0)

Solution 1 : 1 ℓ of solution containing 121g of citric acid ($C_6H_8O_7 \cdot H_2O$).

Solution 2 : 1 ℓ of solution containing 71.6g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (159 : 41) and its pH is adjusted to 3.0.

145. Carotene

Definition Carotene is a collective name of sweet potato carotene, dunaliella carotene, carrot carotene, and palm oil carotene. Its major component is carotene. Sweet potato carotene is obtained by extracting tuberous roots of sweet potato (*Ipomoea batatas* POIR.) of convolvulaceae with organic solvents such as acetone, isopropyl alcohol, methyl alcohol, and hexane. Dunaliella carotene is obtained by extracting *Dunaliella salina* and *Dunaliella bardawil* with carbon dioxide, fats and related substances, or organic solvents such as acetone, isopropyl alcohol, methyl alcohol, and hexane. Carrot carotene is obtained by extracting dried roots of carrot (*Daucus carota* L., etc) of umbelliferae with fats and related substances, or organic solvents such as acetone, isopropyl alcohol, methyl alcohol, and hexane. Palm oil carotene is obtained by adsorptive separation of elaeis (*Elaeis guineensis* JACQ.) palm oil of palmae or extracting the separated unsaponifiable matter with organic solvents such as acetone, isopropyl alcohol, methyl alcohol, and hexane. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Carotene

Content Color value ($E_{1cm}^{10\%}$) of Carotene should be more than the indicated value.

Description Carotene is reddish brown~venetian red liquid, paste, powder, or paste with a slight characteristic scent.

Identification (1) A solution of Carotene in cyclohexane (1→400) is orange color and has a maximum absorption bands near 450 nm and 480 nm.

(2) When 1 ml of antimony trichloride solution is added to a solution of Carotene in cyclohexane (1→100), it becomes bluish green.

Purity (1) Arsenic : Place 0.25 g of Carotene in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Lead : When 5.0 g of Carotene is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Residual Solvents : When Carotene is tested by Purity (4) for Paprika Extract

Pigments, the content of residual solvents should be,

Acetone	Not more than 30ppm
Isopropyl alcohol	Not more than 50ppm
Methyl alcohol	Not more than 50ppm
Hexane	Not more than 25ppm

Assay (Color Value) Appropriate amount of Carotene is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in 10 ml of chloroform, where cyclohexane is added to bring the total volume to 100 ml. 5 ml of this solution is diluted to 100 ml with cyclohexane (if it is water soluble, water is used). 10 ml of this solution is further diluted to 100 ml with cyclohexane (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using cyclohexane as a reference solution, absorption A is measured at the maximum absorption near 455 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1cm}^{10\%}) = \frac{A \times 2,000}{\text{Weight of sample (g)}}$$

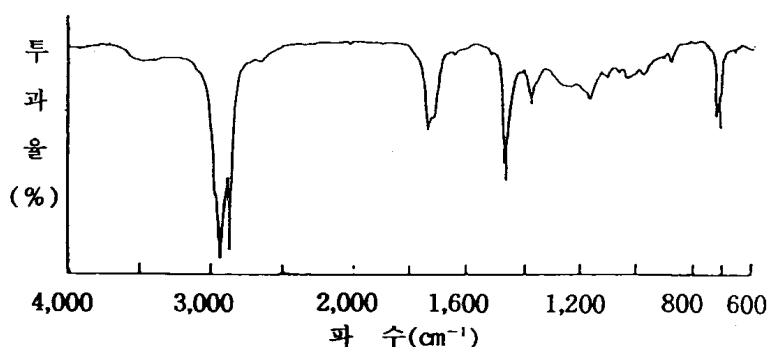
146. Candelilla Wax

Definition Candelilla Wax is obtained by sampling and purifying stems of candelilla (*Euphorbia Antisyphilitica* ZUCC.) of euphorbiaceae.

Compositional Specifications of Candelilla Wax

Description Candelilla Wax is almost tasteless pale yellow~yellowish brown solid with resinous odor.

Identification (1) 1~2 mg of Candelilla Wax is analyzed by Potassium Bromide Disk Method in Infrared Spectrophotometry (1). Its spectrum is shown below.



Purity (1) Melting Point : Melting point of Candelilla Wax should be in a temperature range of 68~73°C.

(2) Acid value : Approximately 3 g of Candelilla Wax is precisely weighted and dissolved in 80 ml mixture of xylan and ethyl alcohol (3 : 5) (Test Solution). When Test Solution is tested by Acid Value Test Methods in Flavoring Substances Test. 12.0~24.0 (titration should be carried out when it is warm.).

(3) Saponification Value : 1 g of Candelilla Wax is precisely weighted into a saponification flask, 50ml of mixture of xylan and ethyl alcohol (3→5) in ethyl alcohol and 25 ml of alcoholic solution of potassium hydroxide are added. After attaching a reflux condenser, the solution is heated for 1 hour while shaking occasionally. Make warm. A few drops of phenolphthalein TS are added to the solution, which is then titrated with 0.5 N hydrochloric acid. Saponification value is calculated using the following equation and should be 43~65.

(4) Arsenic : Place 0.25 g of Carotene in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the

residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(5) Lead : When 5.0 g of Candelilla Wax is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(6) Mercury : When Candelilla Wax is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

Residue on Ignition Residue on Ignition of Candelilla Wax should not be more than 0.3%.

147. Chlorophyll

Definition Chlorophyll is a pigment obtained by extracting chlorella (*Chlorella pyrenoides* CHIK, etc.) of chlorella, spinach (*Spinacia oleracea* L.) of chenopodiaceae, comfley (*Symphytum officinale* LEDEB) of borraginaceae, and Spirulina (*Spirulina platensis* NORD.), a blue-green algae (GEITLER, etc.) with ethyl alcohol or organic solvents such as acetone, isopropyl alcohol, methyl alcohol, and hexane. Its major pigment component is Chlorophylls. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Chlorophyll

Content Color value ($E_{1cm}^{10\%}$) of Chlorophyll should be more than the indicated value.

Description Chlorophyll is green~dark green liquid or paste with a slight characteristic scent.

Identification

- (1) A solution of Chlorophyll in n-hexane (1→100) is green color and has maximum absorption bands near 415 nm, 425 nm and 660 nm. Weigh 1 g which is converted to 600 of color value from indicated value of Chlorophyll. It is dissolved in 100 mL of n-hexane and this solution shows green color. When mixed and shaken with 0.5 mL of hydrochloric acid, the color of this solution is changed to yellow with green.
- (2) Weigh 1 g which is converted to 600 of color value from indicated value of Chlorophyll. It is dissolved in 100 mL of ethanol and this solution shows a red fluorescence.
- (3) A solution of Chlorophyll in n-hexane has maximum absorption bands near 410~430 nm and 660~670 nm.
- (4) Weigh 1 g which is converted to 600 of color value from indicated value of Chlorophyll and dissolve in 30 mL of n-hexane. 2 μ l of this solution drop-wise added on to a thin layer plate, which is prepared by using silica gel (activated by heating at 110°C for 1 hour) for thin layer chromatography. Using a mixture of n-hexane : acetone : tert-butylalcohol (10:1:1) as a developing solvent, each plate is developed up to 10 cm, and then dried in air. R_f value shows yellowish green color(chlorophyll b), green color(chlorophyll a) and gray color(feofitin) spots at near 0.3, 0.4 and 0.65. When these plates are observed under UV light (major wavelength at 366 nm) in a dark place, they show a red fluorescence. And R_f value shows yellow color(xanthophyll) and orange yellow color(β -carotene) spots at near 0.25 and 0.95. When these plates are observed under UV light (major wavelength at 366 nm) in a dark place, they don't show any fluorescence.

- Purity** (1) Arsenic : Place 0.25 g of Chlorophyll in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more than 4 ppm.
- (2) Lead : When 5.0 g of Chlorophyll is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
- (3) Cadmium : When 5.0 g of Chlorophyll is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.
- (4) Mercury : When Chlorophyll is tested by Mercury Limit Test, its content should not be more than 1.0ppm.
- (5) Residual Solvents : When Chlorophyll is tested by Purity (4)(5) for Paprika Extract Pigments, the content of residual solvents should be

Acetone	}	Not more than 50ppm (individual or total if combined)
Isopropyl alcohol		
Methyl alcohol		
Hexane		
Methylene chloride		Not more than 10ppm

Assay (Color Value) Appropriate amount of Chlorophyll is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in n-hexane so that the total volume is 100 ml (if it is water soluble, water is used). 5 ml of this solution is diluted to 100 ml with n-hexane (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using n-cyclohexane as a reference solution, absorption A is measured at the maximum absorption near 660 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1\text{cm}}^{10\%}) = \frac{A \times 200}{\text{Weight of sample (g)}}$$

148. Chitin

Definition Chitin is obtained by treating shells of crustacea with acids. Its component is *N*-Acetylglucosamine.

Compositional Specifications of Chitin

Description Chitin is white~pale yellow or red powder or scale with a slight characteristic scent.

Identification When 5 ml of anthrone solution and 1 ml of water are added to 0.2 g of Chitin, which is heated in a water bath, it becomes blue~green.

Purity (1) Arsenic : Place 0.25 g of Chitin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more than 4 ppm.

(2) Lead : When 5.0 g of Chitin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Loss on Drying

When Chitin is dried for 4 hours at 105°C, the loss should not be more than 15%.

Residue on Ignition

Residue on Ignition of Chitin (converted to dried form) should not be more than 5%.

149. Thaumatin

Definition Thaumatin is obtained by purifying the water extracts of seeds of *Thaumatococcus daniellii* Benth. Its component is Thaumatin.

Compositional Specifications of Thaumatin

Content When Thaumatin is quantitatively analyzed, it should contain more than the amount indicated as thaumatin.

Description Thaumatin is scentless milky yellowish brown~grayish brown powder, flakes, or solid with a cool and strong sweet taste.

Identification (1) Dissolve 0.1 g of Thaumatin in 10 ml of sodium hydroxide solution by heating and cooled. When 0.5 ml of cupper sulfate solution (1→100) is added to this solution, it becomes reddish violet~bluish green in color.

(2) 2 ml of buffer solution (ninhydrine/acetic acid) and 2 ml of hydrazine sulfate solution (0.26→500) are added to 2 ml aqueous solution of Thaumatin (1→100). Upon heating a water bath, this solution turns bluish violet.

Standard Solution

- Ninhydrine/Acetic acid buffer solution : Dissolve 2 g of ninhydrine in 50 ml of water, where 25 ml of acetic acid buffer and water are added to bring up the total volume to 100 ml.
- Acetic acid buffer : Dissolve 82 g of anhydrous sodium acetate in 140 ml of water. 25 ml of acetic acid and water are added to bring up the total volume to 250 ml. By adding acetic acid or sodium acetate solution (2→15), pH of this solution is adjusted to $\text{pH } 5.51 \pm 0.03$.

(3) The solution obtained in Assay has a maximum absorption band near 277 nm.

Purity (1) Arsenic : Place 0.25 g of Thaumatococcus in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 2 ppm.

(2) Lead : When 5.0 g of Thaumatin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 3.0 ppm.

(3) Aluminium : When 5.0 g of Thaumatin is tested by Atomic Absorption

Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 100 ppm.

- (4) Carbohydrate : 0.2 g of Thaumatococcus is precisely weighted, dissolved in water to make 100 ml, test solution. 0.2 ml of test solution is taken into a test tube made of glass, cooled in an ice water bath, 1.2 ml of Cysteine · sulfuric acid solution which is previously cooled in an ice water bath is added, with a stopper, shaken vigorously, and mixed. Test tube is set aside in an ice water bath for 2 minutes, at room temperature for 3 minutes, and heated for 3 minutes in boiling water. It is immediately immersed in an ice water bath and set aside for 5 minutes. The absorption is measured at 412 nm wavelength with 1cm path length. Then the concentration of carbohydrate(as glucose) in test solution is calculated from calibration curve. The content of carbohydrate should not be more than 3.0%.

Content of Carbohydrate(%)=

$$\frac{\text{concentration of carbohydrate in test solution(as glucose, } \mu\text{g/ml)}}{\text{Weight of sample(g)} \times 1 - (\text{loss on drying}(\%)/100)} \times \frac{100}{10^6} \times 100$$

Preparation of calibration curve : Dissolve standard of glucose in water to prepare the concentration to 10~100 $\mu\text{g/ml}$. Calibration curve is prepared from the absorption measured by same procedure of test solution.

Solution

L-Cysteine Solution : Dissolve 3 g of Cysteine hydrochloric acid hydrate in water to make 100 ml.

Cysteine · sulfuric acid : Mix 0.5 ml of L-Cysteine solution and 25 ml of 86% sulfuric acid. This is prepared freshly before use.

- (5) Total viable aerobic count : When Thaumatococcus proceed as directed under Total viable aerobic count for Coliform in General Testing Methods in 「Standards and Specifications for Foods」, it should not be more than 1,000 per 1 g.
- (6) E. coli : When Thaumatococcus proceed as directed under Microbiological Methods for E. coli in General Testing Methods in 「Standards and Specifications for Foods」, it should be negative (-).

Loss on Drying When Thaumatococcus is dried for 5 hours at 105°C, the loss should not be more than 9%.

Ash After carbonizing at 500°C, Thaumatococcus tested by Ash and Acid-Insoluble Ash Limit. The amount of ash should not be more than 1.0%.

Residue on Ignition When Residue on Ignition is done with precisely weighed 5 g of Thaumatococcus, the amount of residue should not be more than 0.05%.

Assay Approximately 1 g of Thaumatococcus is precisely weighted and dissolved in water (total volume = 100 ml), which is then filtered through a filter paper. 5 ml of this solution is diluted 100 ml with water (Test Solution). Absorption A of Test Solution is measured at the maximum absorption near 277 nm with 1cm path length using water as a reference. The content is obtained by the following equation.

$$\text{Content(\%)} = \frac{A \times 100}{0.567 \times S}$$

S : Weight of sample (g)

150. Furcelleran

Definition Furcelleran is a polysaccharide obtained by extracting leaves of *Furcellaria fastigata* HUD. (a red algae) with water.

Compositional Specifications of Furcelleran

Description Furcellerane is scentless white~pale yellow powder with a slightly salty taste.

Identification (1) 4 g of Furcelleran is stirred in 200 ml of water in approximately 80°C water bath until a viscous liquid is obtained. When this viscous liquid is set aside and cooled to room temperature, it forms a gel.

(2) 0.1 g of Furcelleran is dissolved in 20 ml water. To this solution, 3 ml of barium chloride solution and 5 ml of hydrochloric acid (1→4) are added to form precipitates, which is then filtered. When the filtrate is boiled for 5 minutes, white crystalline precipitates are formed.

Purity (1) Sulfate(SO₄) : Furcelleran is dried for 5 hours at 105°C. Approximately 1 g of the dried material is precisely weighted into a 100 ml round bottom flask, and 50 ml of diluted hydrochloric acid (1→4) is added. A reflux condenser is attached, and heated for 1 hour. 25 ml of hydrogen peroxide is added to the flask, which is then heated again for approximately 5 hours. If necessary, the decomposed solution is filtered. Transfer the filtrate into a beaker. While the filtrate is boiling, 10 ml of barium chloride solution is slowly added to the beaker, which is heated for 2 hours in a water bath. Cool the solution. It is filtered through a quantitative filter paper (5 type C). The residue is washed with warm water until the wash water doesn't show the reaction of chlorides. The residue is dried along with the filter paper, which is then heat treated in a porcelain crucible until the weight becomes constant. The remaining residue is weighted as barium sulfate (B). The content of sulfate (SO₄) is calculated by the following equation. It should be 8.0~40.0%

$$\text{Content of Sulfate (SO}_2\text{) (\%)} = \frac{\text{Weight of barium sulfate (g)} \times 0.4116}{\text{Weight of sample (g)}} \times 100 (\%)$$

(2) Arsenic : Place 0.25 g of Furcelleran in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution

proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

- (3) Lead : When 5.0 g of Furcelleran is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
- (4) Residual solvent : 2 g of Furcelleran is precisely weighed into a 300 ml round bottom distilling flask, 200 ml of water is added, boiling chips and 1 ml of silicone resin are added and mixed well. A fractionating column is connected to flask, 4 ml of internal standard solution is precisely weighed and added to it. While adjusting the heat so that the foam does not enter the column, distill the solution at the rate of 2~3 ml per 1 minute until the milky liquid becomes about 90 ml, and water is added to make 100 ml, test solution. However, tert-butyl alcohol (1→1,000) is used as internal standard solution. Separately, 0.5 g each of methyl alcohol and isopropyl alcohol is precisely measured and water is added to 500 ml. Again 2 ml of this solution and 4 ml of internal standard solution is weighed, water is added to make 100 ml, mixed standard solution. 2μl of test solution and mixed standard solution is taken respectively, and injected to gas chromatograph with the following operation condition. Then, Ratio of methyl alcohol and isopropyl alcohol peak area against tert-butyl alcohol peak area, Q_{T1} , Q_{T2} and Q_{S1} , Q_{S2} , is measured respectively, and measure the content of methyl alcohol and isopropyl alcohol under following equation, it should be not more than 0.1% as individual or sum if used together.

$$\text{Content of methyl alcohol(\%)} = \frac{\text{Weight of methyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_{T1}}{Q_{S1}} \times \frac{2 \times 100}{500 \times 100} \times 100$$

$$\text{Content of Isopropyl alcohol(\%)} = \frac{\text{Weight of isopropyl alcohol(g)}}{\text{Weight of sample(g)}} \times \frac{Q_{T2}}{Q_{S2}} \times \frac{2 \times 100}{500 \times 100} \times 100$$

Q_{T1} : Ratio of methyl alcohol peak area against tert-butyl alcohol peak area in Test Solution

Q_{T2} : Ratio of isopropyl alcohol peak area against tert-butyl alcohol peak area in Test Solution

Q_{S1} : Ratio of methyl alcohol peak area against tert-butyl alcohol peak area in mixed standard Solution

Q_{S2} : Ratio of isopropyl alcohol peak area against tert-butyl alcohol peak area in mixed standard Solution

Column : PLOT Q or its equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Injection Port Temperature : 200°C

Column Temperature : 120°C

Detector Temperature : 300°C

Carrier gas : Nitrogen or Helium

Loss on Drying When Furcelleran is dried for 5 hours at 105°C, the loss should not be more than 12%.

Ash When Furcelleran is tested by Ash and Acid-Insoluble Ash Limit, it should not be more than 40%.

151. Grape Juice Color

Definition Grape Juice Color is a pigment obtained after removing precipitates from juice extracts of grapes (*Vitis labrusca* Linné or *Vitis vinifera* Linné) of vitaceae. Its major pigment component is malvidin-3-glycoside. Dilutant, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Grape Juice Color

Content Color value ($E_{1\text{cm}}^{10\%}$) of Grape Juice Color should be more than the indicated value.

Description Grape Juice Color is dark red liquid, paste, powder, or paste with a slight characteristic scent.

Identification (1) A solution of Grape Juice Color in citrate buffer solution (pH 3.0, 1→100) is red in color and has a maximum absorption band near 525 nm.
(2) When the solution in (1) is alkalinized with sodium hydroxide solution (1→25), its color changes to dark green.

Purity (1) Arsenic : Place 0.25 g of Grape Juice Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.
(2) Heavy Metals : Carbonize 1 g of Grape Juice Color by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. This solution is used as the test solution. The content should not be more 20 ppm under Heavy Metal Limit Test. Standard color solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and

evaporated to dryness in a crucible that is made of the same material used for test solution preparation. Add 3 drops of hydrochloric acid to the residue, which is then transferred into another Nestler cylinder as described test solution. Then add 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water to make the total volume to 50 ml. This solution is used as the Standard color solution.

(2) Lead : When 5.0 g of Tomato Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay Appropriate amount of Grape Juice Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in citric acid buffer solution (pH 3.0) so that the total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citric acid buffer solution (pH 3.0) as a reference solution, absorption A is measured at the maximum absorption near 525 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1\text{cm}}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citric acid buffer solution (pH 3.0)

Solution 1 : 1 ℓ of solution containing 121g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 1 ℓ of solution containing 71.6 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (159 : 41) and its pH is adjusted to 3.0.

152. ϵ -Polylysine

Definition ϵ -Polylysine is obtained by adsorption (with an ion exchange resin), separation, and purification of culture solution of *Streptomyces albulus* (a kind of actinomycetes). Its component is ϵ -Polylysine.

Compositional Specifications of ϵ -Polylysine

Content Dried ϵ -Polylysine should contain no less than 87% of ϵ -Polylysine.

Description ϵ -Polylysine is highly hygroscopic pale yellow powder with a slightly bitter taste.

Identification (1) When 1 ml of Dragendorff solution is added to an aqueous solution of ϵ -Polylysine (0.1→100), reddish brown precipitates are formed.

(2) 0.1 g of ϵ -Polylysine is dissolved in 100 ml of phosphate buffer solution (pH 6.8). When 1 ml of methyl orange solution is added to 1 ml of this solution, reddish brown precipitates are formed.

Purity (1) Arsenic : Place 0.25 g of ϵ -Polylysine in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Heavy Metals : Carbonize 1 g of ϵ -Polylysine by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. This solution is used as the test solution. The content should not be more 20 ppm under Heavy Metal Limit Test. Standard color solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. Add

3 drops of hydrochloric acid to the residue, which is then transferred into another Nestler cylinder as described test solution. Then add 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water to make the total volume to 50 ml. This solution is used as the Standard color solution.

Loss on Drying When ϵ -Polylysine is dried for 3 hours at 105°C, the loss should not be more than 20%.

Residue on Ignition When thermogravimetric analysis is done with accurately weighted 1 g of ϵ -Polylysine, the amount of residue should not be more than 1.0%.

Assay (Color Value) Approximately 100 mg of dried ϵ -Polylysine is precisely weighted and tested by Kjeldahl Method in Nitrogen Determination. The content of ϵ -Polylysine is obtained by the following equation.

1 ml of 0.1 N sulfuric acid = 1.401 mg N

$$\text{Content (\%)} = \frac{\text{Amount of nitrogen (mg)} \times 5.24}{A \times \frac{100 - B}{100}} \times 100$$

A : Amount of sample (mg)

B : Loss on Drying (%)

153. Pullulan

Definition Pullulane is obtained by separation and purification of polysaccharides produced by black yeast (*Aureobasidium pullulans* (DE BARY) ARN.). Its major component is neutral polysaccharides.

Compositional Specifications of Pullulan

Description Pullulane is white~pale yellowish white powder. It may be scentless or may have a slight characteristic scent.

Identification (1) When 10 g of Pullulane is slowly mixed (in small portions at a time) into 100 ml while stirring, it becomes a viscous solution.

(2) When 0.1 ml of pullulanase solution is added to and mixed with 10 ml of the solution obtained in (1) and set aside, viscosity disappears.

(3) When 2 ml of polyethylene glycol 600 is added to 10 ml of an aqueous solution of Pullulane (1→50), white precipitates are formed immediately.

Purity (1) Viscosity : Approximately 10 g of dried Pullulane is precisely weighted and dissolved in water (total weight = 100 g). Viscosity of this solution is measured at $30 \pm 0.1^\circ\text{C}$ by 1. Capillary Viscosity Measurement in Viscosity Measurement. It should be 15~180cps.

(2) Arsenic : Place 0.5 g of Pullulane in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at $450\sim 550^\circ\text{C}$. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at $450\sim 550^\circ\text{C}$. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 2 ppm.

(3) Lead : When 5.0 g of Pullulane is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Protein : When approximately 3 g of Pullulane is precisely weighted and tested by semi-micro Kjeldahl Method in Nitrogen Determination. By multiplying the amount of nitrogen with a nitrogen coefficient 6.25, the amount of protein is obtained. The content should not be more than 0.3%. However, the amount of sulfuric acid used for decomposition is 12 ml and the amount of sodium hydroxide solution (2→5) is 40 ml.

$$1 \text{ ml of } 0.01 \text{ N sulfuric acid} = 0.140 \text{ mg N}$$

- (5) Monosaccharides, disaccharides, and oligosaccharide : 0.8 g of ϵ -Polylysine is precisely weighted and water is added to make 100 ml. Transfer 1 ml of this solution into a centrifuged tube, 0.1 ml of saturated solution of potassium chloride and 3 ml of methyl alcohol are added, vigorously shaken for 20 seconds, mixed, and centrifuged at 11,000rpm for 10 minutes. 5 ml of anthrone solution is added to 0.2 ml of supernatant, immediately mixed, heated for 15 minutes in a water bath, test solution. Absorption is measured at 620nm wavelength. Separately, 5 ml each of anthrone solution is added to 0.2 ml of standard solution and for blank test, 0.2 ml of water respectively, proceed under same procedure as test solution, and each absorption is measured. The content of monosaccharides, disaccharides, and oligosaccharide should not be more than 10% (as glucose) by the following equation.

$$\text{content of monosaccharides, disaccharides, and oligosaccharide(\%)} = \frac{(A_t - A_b) \times 0.41 \times G \times 100}{(A_s - A_b) \times W}$$

A_t : Absorption of test solution

A_b : Absorption of blank test solution

A_s : Absorption of standard solution

G : Weight of glucose

W : Weight of sample

Standard solution : 0.2g of glucose is precisely weighted and dissolved in water to make 1,000 ml.

Anthrone solution : 0.2 g of anthrone is dissolved in 100 g of 75%(v/v) sulfuric acid. This is prepared freshly before use.

- (6) Coliform Group : When Pullulane is tested by Microbiological Methods for Coliform Group in General Testing Methods in 「Standards and Specifications for Foods」, it should be negative (-).
- (7) Salmonella : When Pullulane is tested by Microbiological Methods for Salmonella in General Testing Methods 「Standards and Specifications for Foods」, it should be negative (-).
- (8) The number of Fungi : When Pullulane is tested by Microbiological Methods for The number of Fungi in General Testing Methods in 「Standards and Specifications for Foods」, it should not be more than 100 per 1 g.

Loss on Drying When Pullulane is vacuum dried for 6 hours at 90℃, the loss should not be more than 8.0%.

Residue on Ignition Residue on Ignition of Pullulane should not be more than 5.0%.

154. Pecan Nut Color

Definition Pecan Nut Color is a pigment obtained by extracting outer peel and inner peel of pecans (*Carya Pecan* ENGL. Et GRAEBN.) of Juglandacea with ethyl alcohol. Its major pigment component is flavonoid. Diluent, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Pecan Nut Color

Content The color value ($E_{1cm}^{10\%}$) of Pecan Nut Color should be more than the indicated value.

Description Pecan Nut Color is brown liquid or powder with a slight characteristic scent.

Identification (1) An aqueous solution (1→500) of Pecan Nut Color is brown in color.

(2) When the solution in (1) is acidified with 10 ml of hydrochloric acid, brown precipitates are formed.

(3) When ferric chloride solution (1→10) is added to the solution in (1), milky white precipitates are formed.

Purity (1) Arsenic : Place 0.25 g of Pecan Nut Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(2) Heavy Metals : Carbonize 1 g of Pecan Nut Color by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. This solution is used as the test solution. The content should not be more 20 ppm under Heavy Metal Limit Test. Standard color solution is prepared by the following procedure. 2 ml of nitric

acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. Add 3 drops of hydrochloric acid to the residue, which is then transferred into another Nestler cylinder as described test solution. Then add 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water to make the total volume to 50 ml. This solution is used as the Standard color solution

Assay (Color Value) Appropriate amount of Pecan Nut Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in citric acid buffer solution (pH 7.0) so that the total volume is 100 ml. 1 ml of this solution is diluted to 100 ml with citric acid buffer solution (pH 7.0) (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citric acid buffer solution (pH 7.0) as a reference solution, absorption A is measured at the maximum absorption near 500 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } (E_{1\text{cm}}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

◦ Citric acid buffer solution (pH 7.0)

Solution 1 : 1 ℓ of solution containing 21 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 1 ℓ of solution containing 71.6 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (35:165) and its pH is adjusted to 7.0.

155. Hesperidin

Definition Hesperidin is obtained by purifying the extracts of peels, juices, or seeds of tangerine (*Citrus paradisi* MACF.) of rutaceae with water, ethyl alcohol or organic solvents. Its component is hesperidin.

Compositional Specifications of Hesperidin

Content When Hesperidin is dried, it should contain no less than 95.0% of hesperidin ($C_{28}H_{34}O_{15} = 610.57$).

Description Hesperidin is almost scentless and tasteless white~pale yellow crystallite or crystalline powder.

Identification (1) When Hesperidin is dissolved in sodium hydroxide solution (1→20) or heated anhydrous sodium carbonate solution (1→100), it is orange yellow~reddish yellow in color.

(2) 5 ml of ethyl alcohol and 1 ml of sodium hydroxide solution (1→20) are added to 0.1 g of Hesperidin. When the mixture is boiled for 2~3 minutes and cooled, the filtrate is yellow in color.

(3) 5 ml of ethyl alcohol is added to 0.1 g of Hesperidin, which is heated, cooled, and filtered. When 1 ml of hydrochloric acid and 0.01g of magnesium powder are added to 4 ml of the filtrate, the liquid shows red in color.

(4) 10 ml of hydrochloric acid (1→9) is added to 0.1 g of Hesperidin, which is boiled for 5 minutes. Cool the solution, it is filtered. The filtrate is neutralized with sodium hydroxide solution (1→4). When 3 ml of Fehling solution is added to the resulting solution, red precipitates are formed.

Purity (1) Clarity of Solution : A solution of 1 g of Hesperidin in 10 ml of sodium hydroxide solution (4.3→100) should be orange yellow~yellowish brown and almost clear (or less).

(2) Arsenic : Place 0.5 g of Hesperidin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 4 ppm.

(3) Lead : When 5.0 g of Hesperidin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Loss on Drying When 1 g of Hesperidin is dried for 3 hours at 105°C, the loss should not be more than 5%.

Residue on Ignition When thermogravimetric analysis is done with 1 g of Hesperidin, the amount of residue should not be more than 0.3%.

Assay After drying for 3 hours at 105°C, 50 mg of Hesperidin is precisely weighted and dissolved in 0.01 N sodium hydroxide solution (total volume = 100 ml). 2 ml of this solution is diluted to 50 ml with 0.01 N potassium hydroxide solution (Test Solution). Absorption A of the Test Solution is measured at 286 nm and the content (%) of Hesperidin is obtained from the following equation.

$$\text{Content of hesperidin (C}_{28}\text{H}_{34}\text{O}_{15}) = \frac{A}{251.7} \times \frac{25,000}{\text{Weight of sample (mg)}} \times 100 (\%)$$

156. Enzymatically Modified Rutin

Definition Enzymatically Modified Rutin is obtained after removing rhamnose from rutin by treating with partially hydrolyzing enzyme. Or it is obtained by treating rutin with transaminase followed by reacting with glucose. Its component is α -glycorutin.

Compositional Specifications of Enzymatically Modified Rutin

Content Dried Enzymatically Modified Rutin should contain no less than 60.0% of enzymatically modified rutin

Description Enzymatically Modified Rutin is yellow~yellowish brown powder with a slight characteristic scent.

Identification (1) 5 mg of Enzymatically Modified Rutin dissolve in 10 ml of ethyl alcohol. When 1~2 drops of ferric chloride solution (1→50) are added to this solution, it becomes brown in color.

(2) When 5 ml of sodium hydroxide solution (1→1,000) is added to 5 mg of Enzymatically Modified Rutin, the solution shows orange~yellow color.

(3) 5 mg of Enzymatically Modified Rutin dissolve in water, where 2 ml of hydrochloric acid and 0.05 g of magnesium powder are added. The color of the solution slowly changes to orange.

(4) 0.1 g of Enzymatically Modified Rutin dissolve in 100 ml of 1 N sulfuric acid. When this solution is boiled for 2 hours and cooled, yellow precipitates are formed..

(5) A solution of 0.01 g of Enzymatically Modified Rutin in 500 ml of 0.085% phosphoric acid solution has a maximum absorption band near 258 nm and 351 nm.

Purity (1) Arsenic : Place 0.5 g of Enzymatically Modified Rutin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue. which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should not be more 2 ppm.

(2) Lead : When 5.0 g of Enzymatically Modified Rutin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay Enzymatically Modified Rutin is precisely weighted and dissolved in 50 ml of water so that the measured absorption lies within a range of 0.3~0.7. If necessary, it is filtered through a glass filter, which is washed with water. The filtrate and wash

water are combined so that the total volume is 100 ml. 1 ml of this solution is diluted to 100 ml with 0.085% phosphoric acid (Test Solution). Separately, rutin standard is dried for 2 hours at 135°C and approximately 0.2g of it is precisely weighted and dissolved in 80 ml of methyl alcohol by heating. Cool the solution, this solution is diluted to 100 ml with methyl alcohol. 1 ml of this solution is further diluted to 100 ml with 0.085% phosphoric acid (Standard Solution). Using 0.085% phosphoric acid as a reference, absorption near 351 nm is measured for Test (A1) and Standard (A2) Solutions. The content of enzymatically modified rutin (the amount converted as rutin) is obtained by the following equation. The amount converted as rutin is the content enzymatically modified rutin.

$$A = \frac{A1 \times R}{A2 \times S} \times 100$$

A : Content of enzymatically treated rutin (converted as rutin) (%)

A1 : Absorbance of Test Solution

A2 : Absorbance of Standard Solution

S : Amount of sample (mg)

R : Amount of rutin Standard (mg)

157. Natural Flavoring Substances

Definition These materials are obtained from the following origins by processes such as extraction and distillation. They are used to add or enhance aroma. There are refined oils, extracts, and Oleoresin (spice oleoresins whose specification is separately set is excluded). However, water, ethanol, vegetable oil can be added for preserving quality. Also, flavor ingredients obtained from each raw materials can be combined in a way which doesn't cause chemical change.

- (1) When natural flavoring substances are prepared or processed, appropriate solvents (ethyl alcohol, hexane, isopropyl alcohol) are used individually or together, and they are obtained by extraction from each raw material. Used solvents should be removed upon specification of residual solvents.

Compositional Specifications of natural flavoring substances

Purity Residual Solvents (limited to form of oleoresin) : When natural flavoring substances is tested by Purity (5) for Paprika Extract Pigments, the content of residual solvents should be,

Isopropyl alcohol	Not more than 50ppm
Hexane	Not more than 25ppm

No.	General Name	Origin Name
1	Alfalfa	<i>Medicago sativa</i> L.
2	Almond, bitter(free from prussicacid) (Bitter almond)	<i>Prunus amygdalus</i> Batsch. <i>Prunus armeniaca</i> L., or <i>Prunus persica</i> (L.) Batsch.
3	Ambergris	<i>Physeter macrocephalus</i> L.
4	Ambrette(seed)	<i>Hibiscus moschatus</i> Moench.
5	Amyris(West Indian sandalwood)	<i>Amyris balsamifera</i> L.
6	Angelica root	<i>Angelica archangelica</i> L.
7	Angelica seed	<i>Angelica archangelica</i> L.
8	Angelica stem	<i>Angelica archangelica</i> L.
9	Angola weed	<i>Rocella fuciformis</i> Ach
10	Angostura (Cusparia bark)	<i>Galipea officinalis</i> Hancock.
11	Anise	<i>Pimpinella anisum</i> L.
12	Apricot kernel (Pescic oil)	<i>Prunus armeniaca</i> L.
13	Arnica flowers	<i>Arnica montana</i> L., <i>A. fulgens</i> Pursh, <i>A. sororia</i> <i>A. fulgens</i> Pursh, <i>A. sororia</i> Greene, or <i>A. cordifolia</i> Hooker.
14	Artemisia (Wormwood)	<i>Artemisia</i> spp.
15	Artichoke leaves	<i>Cynara scolymus</i> L.
16	Asafetida	<i>Ferula</i> (<i>Ferula assa-foetida</i> L. and related spp. Of <i>Ferula</i> .)

No.	General Name	Origin Name
17	Balm(Lemon balm, Melissa)	Melissa officinalis L.
18	Balsam of Peru	Myroxylon pereirae Klotzsch.
19	Basil	Ocimum basilicum L.
20	Bay leaves	Laurus nobilis L.
21	Bay(Myrcia oil)	Pimenta racemosa (Mill.) J. W. Moore.
22	Benzoin resin	Styrax benzoin Dryander, S. paralleloneurus Pekins, S. tonkinensis(Pierre) Craib ex Hartwich, or other spp. Of the Section Anthostyrax of the genus Styrax.
23	Bergamot(Bergamot orange)	Citrus aurantium L. subsp. Beramia Wright et Arn.
24	Blackberry bark	Rubus, Section Eubatus.
25	Bois de rose	Aniba rosaeodora Ducke.
26	Boldus(Boldo) leaves	Peumus boldus Mol.
27	Boronia flowers	Boronia megastigma Nees.
28	Bryonia root	Bryonia alba L., or B. diocia Jacq
29	Buchu leaves	Barosma betulina Bartl. Et Wendl., B. crenulata(L.) Hook. Or B. serratifolia Willd.
30	Buckbean leaves	Menyanthes trifoliata L.
31	Cacao	Theobroma cacao L.
32	Cajeput	Melaleuca leucadendron L. and other Melaleuca spp.
33	Camomile(Chamomile) flowers, Hungarian	Matricaria chamomilla L.
34	Camomile(Chamomile) flowers, Roman or English	Anthemis nobilis L.
35	Camphor tree	Cinnamomum camphora (L.) Nees et Eberm

No.	General Name	Origin Name
36	Cananga	Cananga odorata Hook. F. and Thoms.
37	Capsicum	Capsicum frutescens L. and Capsicum annuum L.
38	Caraway	Carum carvi L.
39	Cardamomseed (Cardamon)	Elettaria cardamomum Maton.
40	Carrot	Daucus carota L.
41	Cascara sagrada	Rhamnus purshiana DC.
42	Cascarilla bark	Croton eluteria Benn.
43	Cassia bark, Chinese	Cinnamomum cassia Blume.
44	Cassia bark, Padang or Batavia	Cinnamomum burmanni Blume.
45	Cassia bark, Saigon	Cinnamomum loureirii Nees.
46	Cassie flowers	Acacia farnesiana(L.) Willd.
47	Castoreum	Castor fiber L. and C. canadensis Kuhl.
48	Catechu, black	Acacia catechu Willd.
49	Cedar, white(Aborvitae), leaves, and twigs	Thuja occidentalis L.
50	Celery seed	Apium graveolens L.
51	Centuary	Centaurium umbellatum Gilib
52	Cherry pits	Prunus avium L. or P. cerasus L.
53	Cherry laurel leaves	Prunus laurocerasus L.
54	Cherry, wild, bark	Prunus serotina Ehrh.
55	Chervil	Anthriscus cerefolium (L.) Hoffm.
56	Chest nut leaves	Castanea dentata (Marsh.) Borkh
57	Chicory	Cichorium intybus L.

No.	General Name	Origin Name
58	Chirata	Swertia chirata Buch. Ham
59	Cinchona, red, bark	Cinchona succirubra Pav. Or its hybrids
60	Cinchona, yellow, bark	Cinchona ledgeriana Moens, C. calisaya Wedd., or hybrids of these with other spp. Of Cinchona.
61	Cinnamon bark, Ceylon	Cinnamomum zeylanicum Nees.
62	Cinnamon bark, Chinese	Cinnamomum cassia Blume.
63	Cinnamon bark, Saigon	Cinnamomum loureirii Nees.
64	Cinnamon leaf, Ceylon	Cinnamomum zeylanicum Nees.
65	Cinnamon leaf, Chinese	Cinnamomum cassia Blume.
66	Cinnamon leaf, Saigon	Cinnamomum loureirii Nees.
67	Citronella	Cymbopogon nardus Rendle.
68	Citrus peels	Citrus spp.
69	Civet(Zibeth, Zibet, Zibetum)	Civet cats, Viverra civetta Schreber and Viverra zibetha Schreber.
70	Clary(Clary sage)	Salvia sclarea L.
71	Clover	Trifolium spp.
72	Coca(decocainized)	Erythroxylum coca Lam. And other spp. Of Erythroxylum.
73	Coffee	Coffea spp.
74	Cognac oil, white and green	Ethyl oenanthata, so called.
75	Cola nut(Cola nut)	Cola acuminata Schott and Endl., and other spp. of Cola.
76	Copaiba	South American spp.of Copaifera L.
77	Coriander	Coriandrum sativum L.
78	Cork, oak	Quercus suber L., Q.occidentalis F. Gay, Q.acutissima, Q.mongolica, Q.serrata

No.	General Name	Origin Name
79	Costmary	<i>Chrysanthemum balsamita</i> L.
80	Costus root	<i>Saussurea lappa</i> Clarke.
81	Cumin(Cummin)	<i>Cuminum cyminum</i> L.
82	Curacao orange peel (Orange, bitter peel)	<i>Citrus aurantium</i> L.
83	Currant, black, buds and leaves	<i>Ribes nigrum</i> L.
84	Cusparia bark	<i>Galipea officinalis</i> Hancock.
85	Damiana leaves	<i>Turnera diffusa</i> Willd.
86	Dandelion	<i>Taraxacum officinale</i> Weber and T. <i>laevigatum</i> DC.
87	Dandelion root	<i>Taraxacum officinale</i> Weber and T. <i>laevigatum</i> DC.
88	Davana	<i>Artemisia pallens</i> Wall.
89	Dill, Indian	<i>Anethum sowa</i> Roxb., (<i>Pucedanum</i> <i>graveolens</i> Benth et Hook., <i>Anethum</i> <i>graveolens</i> L.)
90	Dittany(Fraxinella) roots	<i>Dictamnus albus</i> L.
91	Dittany of Crete	<i>Origanum dictamnus</i> L.
92	Dog grass(Quackgrass, Triticum)	<i>Agropyron repens</i> (L.) Beauv.
93	Dragon's blood (Dracorubin)	<i>Daemonorops</i> spp.
94	Elder tree leaves	<i>Sambucus nigra</i> L.
95	Elder flowers	<i>Sambucus canadensis</i> L. and <i>S. nigra</i> I.
96	Elecampane rhizome and roots	<i>Inula helenium</i> L.
97	Elemi	<i>Canarium commune</i> L. or <i>C. luzonicum</i> Miq.
98	Erigeron	<i>Erigeron canadensis</i> L.

No.	General Name	Origin Name
99	Estragole(Estragon, Esdragol, Esdragon, Tarragon)	Artemisia dracunculus L.
100	Eucalyptus globulus leaves	Eucalyptus globulus Labill.
101	Fennel, sweet	Foeniculum vulgare Mill.
102	Fenugreek	Trigonella foenum graecum L.
103	Fir("pine")needles and twigs	Abies sibirica Ledeb., A. alba Mill., A. sachalinesis Masters or A. mayriana Miyabe et Kudo.
104	Fir, balsam, needles and twigs	Abies balsamea(L.) Mill.
105	Galanga(Galangal)	Alpinia officinarum Hance.
106	Galanga, greater	Alpinia galanga Willd.
107	Galbanum	Ferula galbaniflua Boiss. Et Buhse and other Ferula spp.
108	Gambir(Catechu, Pale)	Uncaria gambir Roxb.
109	Genet flowers	Spartium junceum L.
110	Gentian, stemless	Gentiana acaulis L.
111	Gentian rhizome or roots	Gentiana lutea L.
112	Geranium	Pelargonium spp.
113	Geranium, East Indian	Cymbopogon martini Stapf.
114	Geranium, rose	Pelargonium graveolens L'Her.
115	Germander, chamaedrys	Teucrium chamaedrys L.
116	Germander, golden	Teucrium polium L.
117	Ginger	Zingiber officinale Rosc.
118	Grapefruit	Citrus paradisi Macf.
119	Guaiac	Guaiacum officinale L., G. santum L., Bulnesia sarmienti Lor.

No.	General Name	Origin Name
120	Guarana	Paullinia cupana HBK.
121	Guava	Psidium spp.
122	Haw, black, bark	Viburnum dilatatum Thunb.
123	Hemlock needles 및 twigs	Tsuga canadensis(L.) Carr. Or T. heterophylla(Raf.) Sarg
124	Hickory bark	Carya spp.
125	Horehound(Hoarhound)	Marrubium vulgare L.
126	Hops	Humulus lupulus L.
127	Horsemint	Monarda punctata L.
128	Hyacinth flowers	Hyacinthus orientalis L.
129	Hyssop	Hyssopus officinalis L.
130	Iceland moss	Cetraria islandica Ach
131	Immortelle	Helichrysum augustifolium DC.
132	Imperatoria	Peucedanum ostruthium (L.) Koch(Imperatoria ostruthium L.)
133	Jasmine	Jasminum officinale L. and other spp. Of Jasminum.
134	Juniper(berries)	Juniperus communis L.
135	Labdanum	Cistus spp.
136	Laurel berries	Laurus nobilis L.
137	Laurel leaves	Laurel (Laurus spp.
138	Lavender	Lavandula officinalis Chaix.
139	Laverder, spike	Lavandula latifolia Vill.
140	Lavandin	Hybrids between Lavandula
141	Lemon	Citrus limon(L.) Burm. f.
142	Lemon grass	Cymbopogon citratus DC. and Cymbopogon lexiuosus Stapf.
143	Lemon peel	Citrus limon (L.) Burm. f.
144	Lime	Citrus aurantifolia Swingle.
145	Linaloe wood	Bursera delpechiana Poiss. and other Bursera spp.
146	Linden flowers	Tilia spp.

No.	General Name	Origin Name
147	Linden leaves	<i>Tilia</i> spp.
148	Locust bean(Carob bean)	<i>Ceratonia siliqua</i> L.
149	Lovage	<i>Levisticum officinale</i> Koch
150	Lungmoss (Lungwort)	<i>Sticta pulmonacea</i> Ach.
151	Lupulin	<i>Humulus lupulus</i> L.
152	Mace	<i>Myristica fragrans</i> Houtt.
153	Maidenhair fern	<i>Adiantum capillus-veneris</i> L.
154	Mandarin	<i>Citrus reticulata</i> Blanco.
155	Maple, mountain	<i>Acer spicatum</i> Lam.
156	Marjoram, sweet	<i>Majorana hortensis</i> Moench.
157	Mate	<i>Ilex paraguariensis</i> St. Hil.
158	Menthol	<i>Mentha</i> spp.
159	Menthyl acetate	<i>Mentha</i> spp.
160	Mimosa(Black wattle) flowers	<i>Acacia decurrens</i> Willd. var. <i>dealbata</i>
161	Molasses(extract)	<i>Saccarum officinarum</i> L.
162	Mullein flowers	<i>Verbascum phlomoides</i> L. or <i>V. thapsiforme</i> Schrad.
163	Musk(Tonquin musk)	Musk deer, <i>Moschus moschiferus</i> L.

No.	General Name	Origin Name
164	Mustard	Brassica spp.
165	Myrrh	Commiphora molmol Engl., C. abyssinica (Berg) Engl., or other Commiphora spp.
166	Myrtle leaves	Myrtus communis L.
167	Naringin	Citrus paradisi Macf.
168	Neroli, bigarade	Citrus aurantium L.
169	Nutmeg	Myristica fragrans Houtt.
170	Olibanu	Boswellia carteri Birdw. and other Boswellia spp.
171	Onion	Allium cepa L.
172	Opopanax(Bisabolmyrrh)	Opopanax chironium Koch (true opopanax) of Commiphora erythraea Engl. var. Llabrescens.
173	Orange, bitter, flowers	Citrus aurantium L.
174	Orange, bitter, peel	Citrus aurantium L.
175	Orange, sweet	Citrus sinensis (L.) Osbeck.
176	Orange, sweet, flowers	Citrus sinensis (L.) Osbeck.
177	Orange, sweet, peel	Citrus sinensis (L.) Osbeck.
178	Orange leaf	Citrus sinensis (L.) Osbeck.
179	Origanum	Origanum spp.
180	Orris root	Iris germanica L.(including its variety florentina Dykes) and I. pallida Lam.

No.	General Name	Origin Name
181	Palmarosa	<i>Cymbopogon martini</i> Stapf.
182	Paprika	<i>Capsicum annum</i> L.
183	Parsley	<i>Petroselinum crispum</i> (Mill.) Mansf.
184	Passion flower	<i>Passiflora incarnata</i> L.
185	Patchouly	<i>Pogostemoncablin</i> Benth. And <i>P. heyneanus</i> Benth.
186	Peach leaves	<i>Prunus persica</i> (L.) Batsch
187	Peach kernel (Persic oil)	<i>Prunus persica</i> Sieb. Et Zucc.
188	Peanut stearine	<i>Arachis hypogaea</i> L.
189	Pennyroyal, American	<i>Hedeoma pulegioides</i> (L.) Pers
190	Pennyroyal, European	<i>Mentha pulegium</i> L.
191	Pepper, black	<i>Piper nigrum</i> L.
192	Pepper, white	<i>Piper nigrum</i> L.
193	Peppermint	<i>Mentha piperita</i> L.
194	Peruvian balsam	<i>Myroxylon pereirae</i> klotzsch.
195	Petitgrain	<i>Citrus aurantium</i> L.
196	Petitgrain lemon	<i>Citrus limon</i> (L.) Burm .f.
197	Petitgrain mandarin or tangerine	<i>Citrus reticulata</i> Blanco.
198	Pimenta(Allspice)	<i>Pimenta officinalis</i> Lindl.
199	Pimenta leaf	<i>Pimenta officinalis</i> Lindl.
200	Pine, dwarf, needles, and twigs	<i>Pinus mugo</i> Turra var. <i>pumilio</i> (Haenke) Zenari

No.	General Name	Origin Name
201	Pine, Scotch, needles, and twigs	<i>Pinus sylvestris</i> L.
202	Pine, white, bark	<i>Pinus strobus</i> L.
203	Pine, white oil	<i>Pinus palustris</i> Mill., and other <i>Pinus</i> spp.
204	Pipsissewa leaves	<i>Chimaphila umbellata</i> Nutt.
205	Pomegranate	<i>Punica granatum</i> L.
206	Poplar buds	<i>Populus balsamifera</i> L.(<i>P.tacamahacca</i> Mill.), <i>P. candicans</i> Ait., or <i>P. nigra</i> L.
207	Prickly ash bark	<i>Xanthoxylum</i> (or <i>Zanthoxylum</i>) <i>Americanum</i> Mill. Or <i>Xanthoxylum clavaherculis</i> L.
208	Quassia	<i>Picrasma excelsa</i> (Sw.) Planch, or quassia (<i>Quassia amara</i> L.)
209	Quebracho bark	<i>Aspidosperma quebracho- blanco</i> Schlecht, or (<i>Quebrachia lorentzii</i> (Griseb)).
210	Quillaia (Soapbark)	<i>Quillaja saponaria</i> Mol
211	Quince seed	<i>Cydonia oblonga</i> Miller.
212	Red saunders (Red sandalwood)	<i>Pterocarpus san alinus</i> L.
213	Rhatany root	<i>Krameria triandra</i> Ruiz et Pav. Or <i>K. argentea</i> Mart.
214	Rhubarb, garden root	<i>Rheum rhaponticum</i> L.
215	Rhubarb root	<i>Rheum officinale</i> Baill., <i>R. palmatum</i> L., or other spp.(excepting <i>R. rhaponticum</i> L.) or hybrids of <i>Rheum</i> grown in China.
216	Rose absolute	<i>Rosa alba</i> L., <i>Rosa centifolia</i> L., <i>Rosa damascena</i> Mill., <i>Rosa gallica</i> L., and vars. of these spp.
217	Rose (otto of roses, attar of roses)	<i>Rosa alba</i> L., <i>Rosa centifolia</i> L., <i>Rosa damascena</i> Mill., <i>Rosa gallica</i> L., and vars. of these spp.

No.	General Name	Origin Name
218	Rose buds	Rosa alba L., Rosa centifolia L., Rosa damascena Mill., Rosa gallica L., and vars. of these spp.
219	Rose flowers	Rosa alba L., Rosa centifolia L., Rosa damascena Mill., Rosa gallica L., and vars. of these spp.
220	Rose fruits(hips)	Rosa alba L., Rosa centifolia L., Rosa damascena Mill., Rosa gallica L., and vars. of these spp.
221	Rose geranium	Pelargonium graveolens L'Hdr.
222	Rose leaves	Rosa spp.
223	Rosemary	Rosmarinus officinalis
224	Saffron	Crocus sativus L.
225	Sage	Salvia officinalis L.
226	Sage, Greek	Salvia triloba L.
227	Sage, Spanish	Salvia lavandulaefolia Vahl.
228	St. John's loaf bread	Ceratonia siliqua L.
229	St. Johnswort leaves, flowers, and caulis	Hypericum perforatum L.
230	Sandalwood, white(yellow, or East Indian)	Santalum album L.
231	Sandarac	Tetraclinis articulata(Vahl.), Mast
232	Sarsaparilla	Smilax aristolochiaefolia Mill., (Mexican sarsaparilla), S. regelii Killip et Morton(Honduras sarsaparilla), S. febrifuga Kunth (Ecuadorean sarsaparilla), or undetermined Smilax spp.(Ecuadorean or Central Americal sarsaparilla).

No.	General Name	Origin Name
233	Sassafras leaves	Sassafras albidum (Nutt.) Nees
234	Savory, summer	Satureia hortensis L.
235	Savory, winter	Satureia Montana L.
236	Schinus molle	Schinus molle L.
237	Senna, Alexandria	Cassia acutifolia Delile
238	Simaruba bark	Simaruba amara Aubl
239	Sloe berries (Blackthorn berries)	Prunus spinosa L.
240	Snakeroot, Canadian (Wild ginger)	Asarum canadense L.
241	Spearmint	Mentha spicata L.
242	Spike lavender	Lavandula latifolia Vill.
243	Spruce needles and twigs	Picea glauca(Moench) Voss or P. mariana(Mill.) BSP.
244	Storax(Styrax)	Liquidambar orientalis Mill. or L. styraciflua L.
245	Tamarind	Tamarindus indica L.
246	Tangerine	Citrus reticulata Blanco.
247	Tansy	Tanacetum vulgare L.
248	Tea	Thea sinensis L.
249	Thistle, blessed(Holy thistle)	Onicus benedictus L.
250	Thyme	Thymus vulgaris L. andThymus zygis var. gracils Boiss.
251	Thyme, white	Thymus vulgaris L. and Thymus zygis var. gracils Boiss.
252	Thyme, wild or creeping	Thymus serpyllum L.

No.	General Name	Origin Name
253	Tuberose	<i>Polianthes tuberosa</i> L.
254	Tolu	<i>Myroxylon balsamum</i> (L.) Harms
255	Turmeric	<i>Curcuma longa</i> L.
256	Valerian rhizome and roots	<i>Valeriana officinalis</i> L.
257	Vanilla	<i>Vanilla planifolia</i> andr. Or <i>Vanilla tahitensis</i> . J. W. Moore.
258	Veronica	<i>Veronica officinalis</i> L.
259	Vervain, European	<i>Verbena officinalis</i> Linne <i>Verbena officinalis</i>
260	Vetiver	<i>Vetiveria zizanioides</i> Stapf.
261	Violet, Swiss	<i>Viola calcarata</i> L.
262	Violet, flowers	<i>Viola odorata</i> L.
263	Violet, leaves	<i>Viola odorata</i> L.
264	Violet, leaves absolute	<i>Viola odorata</i> L.
265	Walnut husks (hulls), leaves and green nuts	<i>Juglans nigra</i> L. or <i>J. regia</i> L.
266	Wild cherry bark	<i>Prunus serotina</i> Ehrh.
267	Woodruff, sweet	<i>Asperula odorata</i> L.
268	Yucca, Joshua tree	<i>Youcca brevifolia</i> Engelm
269	Ylang ylang	<i>Cananga odorata</i> Hook. F. and Thoms.
270	Yucca, Mohave	<i>Yucca schidigera</i> Roezl ex Ortgies (Y. <i>Mohavensis</i> Sarg)
271	Zedoary bark	<i>Curcuma zedoaria</i> Rosc.
272	Mastic	<i>Pistacia lentiscus</i> LINNE
273	Other natrual flavorings : falvorings obtained by manufacturing/processing raw materials that are appropriate for 2. Requirements for Raw Materials. Common in Food Codes	

158. Dammar Gum

Danmmar Resin

Definition Dammar Gum is obtained by drying exudates of trees of *Agathis*, *Hopea*, or *Shorea* genus. It consists of polysaccharides and acidic and neutral terpenoid compounds.

Compositional Specifications of Dammar Gum

Description Dammar Gum is white, pale yellow~dark brown transparent or semitransparent granular or solid resin.

Identification 20 μ l of 10% solution of Dammar Gum in chloroform is spotted on a pre-activated 0.2 mm silica gel (Merck F₂₅₄ or its equivalent) for Thin Layer Chromatography. It is then developed using a solution of ethyl ether and hexane (30 : 25). Sulfuric acid is sprayed on the plate, which is then dried for 3 minutes at 180°C. Two dark spots at R_f values of 0.8 and 0.7 are observed.

Purity (1) Lead : When 5.0 g of Dammar Gum is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(2) Acid Value : Approximately 5 g of Dammar Gum Dissolve in 30 ml of toluene and 30 ml of neutralized ethyl alcohol. This is used as test solution. The test solution is proceeded as directed under Acid value in Fats Test. The Acid value of the solution should be 20~40.

(3) Iodine Value : Approximately 1 g of Dammar Gum is precisely weighted into a 500 ml Erlenmeyer flask with a stopper and 20 ml of carbon tetrachloride is added to dissolve the material. After adding 25 ml of Weiss solution, a stopper is placed, shaken and the flask is set aside for 30 minutes in a dark place. 20 ml of potassium iodide solution and 100 ml of water (previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution (indicator : 1 ml starch solution). The iodine Value should be 10~40. Separately, a blank test is carried out by the same procedure.

$$\text{Iodine Value} = \frac{(A - B) \times 1.269}{C}$$

A : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

B : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

C : Amount of sample(g)

(4) Melting Point : Melting point should be in a temperature range of 90~95°C.

(5) Softening Point : Softening point should be 86~90°C.

(6) *E. coli* : Pasteurized saline solution is added to 25 g of Dammar Gum (total volume = 250 ml). It is tested by the (2) Allowed Limit Test in Microbiological Methods for *E. coli* in General Testing Methods in Food Code. It should be negative.

(7) *Salmonella* : Pasteurized saline solution is added to 25 g of Dammar Gum (total volume = 250 ml). It is tested by the Microbiological Methods for *Salmonella* in General Testing Methods in Food Code. It should be negative.

Ash When Dammar Gum tested by Ash and Acid-Insoluble Ash Limit, the amount of ash should not be more than 0.5%.

Loss on Drying When Dammar Gum is dried for 18 hours at 105°C, the loss should not be more than 6%.

159. Chitosanase

Definition Chitosanase is an enzyme obtained from cultures of *Aeromonas* genus, *Bacillus* genus, or *Trichoderma viride*. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Chitosanase

Content Chitosanase contains 90~130% of the indicated activity as Chitosanase.

Description Chitosanase is white~pale yellow~brown powder or transparent~brown liquid.

Purity (1) Arsenic : Place 0.25 g of Chitosanase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Heavy Metals : Carbonize 0.5 g of Chitosanase by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid, it is heated until white smoke disappears, which is then reduced to ash by further heating at 450~550°C. Cool the solution, 2 ml of hydrochloric acid is added, which is then evaporated to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, 1 drop of phenolphthalein indicator solution is added, then ammonia solution is added until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water. 50 ml of test solution is prepared by adding 2 ml of diluted acetic acid (1→20) and water to make 50 ml. When this solution tested for Heavy Metals, the content should not be more than 40ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water are added to bring the total volume to 50 ml.

(3) Lead : 0.8 g of Chitosanase (if it is liquid, it is concentrated by evaporation in a

water bath) is mindly carbonized by heating, which is reduced ash by further heat treatment at a temperature below 500°C. Carefully 20 ml of dilute nitric acid is added to the ash. Boil the sample for 5 minutes and cool. It is then filtered (if necessary). The residue is washed with water, which is then added to the filtrate. Water is added so that total volume of this solution becomes 50 ml. This test solution is used as the test solution. The detected amount of lead should not be more than 10ppm.

- (4) Coliform Group : When Chitosanase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code. It should contain 30 or less per 1 g of this product.
- (5) Salmonella : When Chitosanase proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in Food Code. It should be negative (-).

Assay(activity)

- Application and Principle : This test is to measure the activity of Chitosanase in an enzyme that is obtained from cultures of *Aeromonas* genus, *Bacillus* genus, or *Trichoderma viride*. Activity test is based on hydrolysis of chitosan substrate (pH 5.0, 48°C). Glucosamine, a reducing sugar that is produced by hydrolysis, is reacted with alkaline ferricyanide solution and absorption of the reaction mixture is measured.
- Preparation of Test Solution : sample dissolve in 0.05 M acetate buffer solution so that the absorption difference between 1 ml of the final dilution and enzyme blank test solution is within 0.1~0.5 under the given test conditions.
- Test Procedure : 2 ml of substrate solution is placed in a test tube, where exactly 1 ml of Test Solution, which is previously isothermalized for 5 minutes in a 48°C water bath, is added, mixed by shaking, and set aside in the water bath. After exactly 5 minutes, the test tube is heated for 3 minutes in a boiling water bath to stop the enzyme reaction and cooled to room temperature. Separately, an enzyme blank test solution is prepared by using 1 ml of Test Solution which is previously deactivated by heating for 3 minutes in a water bath. Separately, 1.4 ml each of water is added to two test tubes. 0.1 ml of enzyme reaction solution and 0.1 ml of enzyme blank test solution is added to respective test tube. 2 ml of alkaline ferricyanide solution is added to each test tube, which is heated for 15 minutes in a boiling water bath and cooled to room temperature. Absorptions of enzyme Test Solution and enzyme blank test solution are measured at 420 nm with 1cm path length. Concentration of D-glucosamine (μmol/ml) is obtained from a standard curve.

Standard Curve

D-glucosamine hydrochloride 215.6 mg of pre-dried to a constant weight dissolve in water (total volume = 100 ml). Using this solution, glucosamine standard solutions are prepared so that 1 ml of each solution contains 2.0, 4.0, 6.0, 8.0, and 10.0 $\mu\text{mol/ml}$ of D-glucosamine, respectively. 1 ml of each standard solution and 2 ml of water are added to a test tube, which is boiled for 3 minutes in a boiling water bath and cooled to room temperature. The same procedure described below Separately, 1.4 ml each of water is added to two test tubes. in Test Procedure is followed. Separately, a reference solution is prepared using 1 ml of water instead of 1 ml of Standard Solution. Using this reference solution, absorptions of Standard Solutions are measured at 420 nm with 1cm path length. A calibration curve of absorption vs. concentration of glucosamine standard ($\mu\text{mol/ml}$) is prepared.

Enzyme activity is calculated by the following equation.

$$\text{CU/ml or g} = \frac{A}{5 \times W}$$

A : Concentration of D-glucosamine in Test Solution obtained from the standard curve ($\mu\text{mol/ml}$)

5 : Reaction time (minutes)

W : Amount of sample contained in 1 ml of Test Solution (ml or g)

Definition of Activity : 1 Chitosanase Unit(CU) corresponds to an amount of enzyme that frees reducing sugar (equivalent of 1 μmol of D-glucosamine) per minute under the test conditions above

Solutions

- 0.05 M Acetate Buffer Solution : 14.8 ml of 0.1 M acetic acid and 35.2 ml of 0.1 M sodium acetate solution are mixed and diluted to 100 ml with water. pH of this solution should be 5.0.
- Substrate Solution : 0.2 g of chitosan (Sigma-Aldrich Co. or its equivalent) is dispersed in 40 ml of water, which is then dissolved by adding 10 ml of 1.0 M acetic acid. pH of this solution is adjusted to 5.0 by adding 1.0 M sodium acetate solution. It is then diluted to 100 ml with 0.05 M acetate buffer solution. This solution is stored in a refrigerator.
- Alkaline Ferricyanide Solution : 0.5 g of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) dissolve in 1,000 ml of 0.5 M sodium carbonate solution. This

solution is sealed and stored in a brown bottle.

Storage standard of Chitosanase

Chitosanase is strongly hygroscopic, hence should be stored in a hermetic container in a cold dark place.

160. Smoke Flavours

Wood smoke flavours, Pyroligneous acid, Smoke condensate

Definition Smoke Flavors is a mixture obtained by thermally decomposing hard segments of unprocessed trees under the condition where the amount of air is limited or controlled, by distilling them in dry condition at 200~800°C, or by treating them with strong heating vapor at 300~500°C. The main compositions are carboxylic acid, compounds having the carboxyl functional, and phenol compounds. However, for quality conservation, water, plant oil, propylene glycol, and emulsifier can be added.

Compositional Specifications of Smoke Flavors

Description Smoke Flavors is black viscous semi solid~pale brown liquid with smoke-like pungent taste.

Purity (1) Acid value : 1 ml of Smoke Flavors is precisely weighted into a 250 ml beaker, where 100 ml of water is added. Stirred and filtered this solution . 0.1 N sodium hydroxide solution is added to the filtrate until the pH reaches 8.15 and the consumed amount is recorded. Acid value of smoke flavours is obtained by the following equation and it should be 2~20%.

$$\text{Acid value (\%)} = \frac{a \times F \times 6.005}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

a : consumed amount of 0.1 N sodium hydroxide solution (ml)

F : coefficient of 0.1 N sodium hydroxide solution

6.005 : Weight of acetic acid (mg) corresponding to 1 ml of 0.1 N sodium hydroxide solution

(2) Benzo(a)pyrene

Test Solution : Depending on the description of the sample, it is treated by the procedure in ① or ②.

① Liquid sample : 200 g of well mixed smoke Flavors is precisely weighted and transferred into a 1,000 ml separatory funnel with a stopcock using 100 ml of iso-octane, where 450 ml of 5.6% potassium hydroxide solution is added. It is well mixed and settled to separate phases. Iso-octane phase is collected. The aqueous layer is extracted twice with 100 ml each of iso-octane, which is added to the previous iso-octane phase. Again, the extracts are washed twice with 50 ml each of 5.6% potassium hydroxide solution, twice with 50 ml each of water, three times with 50 ml each of phosphoric acid, and three times with 100 ml each of water.

② Viscous liquid with solid precipitates or semi-solid : Approximately 25 g of well mixed smoke Flavors is precisely weighted into a 150 ml beaker. It dissolve by adding small amount of 20% potassium hydroxide solution. The resulting solution transfer into a 2,000 ml separatory funnel with a stopcock using 250 ml of 20% potassium hydroxide solution .The beaker is washed 4 times with 50 ml each of ethyl alcohol, which is added to the separatory funnel. 400 ml of ethyl alcohol is added to the funnel and mixed well. 250ml of iso-octane is added to the funnel and mixed. It is then set aside to separate the phases. The upper iso-octane phase is collected and the lower aqueous phase transfer into another separatory funnel with a stopcock. It is then extracted twice with 200 ml each of iso-octane, which is added to the previous iso-octane phase. The extracts are washed three times with 200 ml each of 5.6% potassium hydroxide solution, three times with 200 ml each of water, three times with 200 ml each of phosphoric acid, and three times with 200 ml each of water.

Test Procedure : Iso-octane solution in (① or ②) is passed through a column (230 × 38 mm ID, filled with 60 g of Florisil at the bottom and 50 g anhydrous sodium sulfate on top) that is pre-wetted with iso-octane. The separatory funnel is washed twice with 50 ml each of benzene, which is also passed through the column. The eluted solutions are collected. Additional 75 ml of benzene is eluted through the column and added to the previous solution. It is concentrated to approximately 5 ml to remove solvent by heating in a water bath under nitrogen atmosphere. The concentrate transfer to a 50 ml flask with a glass stopcock using benzene. It is then carefully concentrated to 0.2~0.3 ml in a water bath under nitrogen atmosphere. The residue transfer into a 125 ml beaker and washed 4 times with 5~10 ml each of hot methyl alcohol. It is then vacuum filtered into a 50 ml flask. The filtrate is concentrated to 3~5 ml at 40°C using a rotary evaporator. The concentrate transfer into a 15 ml test tube and washed 3 times with 1 ml of iso-octane. It is then evaporated to dryness under nitrogen atmosphere. The residue dissolve in a mixture of acetonitrile: methyl alcohol: water (2 : 2 : 1), where the total volume is 0.25 ml (Test Solution). Separately, a Standard Solution is prepared so that 1 ml of the solution contains 0.5~4.0 μg of benzo-pyrene. 20 μl each of Test and Standard Solutions are injected into a liquid chromatography under the following operation conditions and the content of benzo-pyrene is obtained by the following equation. The content should not be more than 0.002ppm.

$$\text{Amount of benzo-pyrene (ppm)} = \frac{\text{Concentration of Standard Solution}(\mu\text{g/ml})}{\text{Au} \times \text{dilution factor}} \times \frac{\text{Au} \times \text{dilution factor}}{\text{As} \times W_u}$$

Au : peak area of Test Solution

As : peak area of Standard Solution

Wu : Weight of sample(g)

Operation Conditions

-Detector : UV 289 nm

-Column : ODS (250 × 4.6 mm) or its equivalent

-Mobile Phase : Liquid A : water

Liquid B : methyl alcohol : acetonitrile (50 : 50)

-Concentration Gradient : After a linear concentration gradient between Solution A : Solution B (20 : 80 → 0 : 100) is carried out in 20 minutes, the column is maintained for 20 minutes with 100% of Solution B. After analysis, for the purpose of column stability, a concentration change of Solution A : Solution B (0 : 100 → 20 : 80) is applied in 5 minutes to the column and then the column is maintained for 20 minutes with 80% Solution B.

-Flow Rate : 1.0 ml/min

(3) Diethyl ether : Exactly 10 g of Smoke Flavors is extracted with 1 ml of toluene in a separatory funnel with a stopcock. Mixed by shaking and then settled. Toluene phase is collected and dehydrated by adding a small amount of anhydrous sodium sulfate (Test Solution). A solution of diethyl ether in toluene with a concentration of 250 µg/ml is prepared (Standard Solution). Same amount each of both solutions is injected into gas chromatography. The content of diethyl ether is obtained by the following equation and it should not be more than 20ppm.

$$\text{Amount of diethyl ether(ppm)} = \frac{\text{Concentration of Standard Solution}(\mu\text{g/ml})}{\text{Au} \times \text{Wu}} \times \frac{\text{Au} \times \text{dilution factor}}{\text{As}}$$

Au : peak area of Test Solution

As : peak area of Standard Solution

Wu : Weight of sample(g)

Operation Conditions

-Column : HP-FFAP (50 m × 320 µm × 0.5 µm) or its equivalent

-Detector : Flame Ionization Detector (FID)

-Temperature at injection hole : 150°C

-Column Temperature : 40°C

-Detector Temperature: 230°C

(4) Methyl Alcohol : 50 g of Smoke Flavours is tested by the Test Solution B of Purity (5) for 80. Paprika Extract Pigments in Food Additive Codes. The content of methyl alcohol should not be more than 50ppm.

(5) Phenol : Exactly 5 ml of 0.2% aqueous solution of Smoke Flavours is placed in a test tube. For a blank test, 5 ml of water is placed in another test tube. To each test tube, 1 ml of 0.05% copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) solution, 5 ml of sodium borate buffer solution, and 4 drops of 2,6-Dibromo-N-chloro-p-benzoquinoneimine solution are added. With a cap in place, each tube is vigorously shaken and set aside for exactly 10 minutes in a dark place for colorization. 10 ml each of n-butyl alcohol is added to each tube, which is then turned upside down 6~8 times without shaking. It is then centrifuged for 5 minutes at 700 rpm. Absorption of the supernatant is measured at 610 nm using the blank test solution as a reference. The content of phenol (as 2,6-dimethoxyphenol) is obtained from a standard curve and it should not be more than 16%.

Standard Curve

20 mg of 2,6-Dimethoxyphenol standard is precisely weighted and dissolved in water (total volume = 1,000 ml). Using this solution, a series of standard solutions are prepared so that each contain 1~20 µg/ml of the phenol. By following the same procedure as the Test Solution, absorptions at each concentration is measured at 610 nm and a standard curve is prepared.

Solutions

◦ Sodium Borate Buffer Solution : 24.8 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) dissolve in 900 ml of water, where pH is adjusted to 9.8 with sodium hydroxide solution. The total volume is brought up to 1,000 ml with water.

◦ 2,6-Dibromo-N-chloro-p-benzoquinoneimine Solution : 40 mg of 2,6-Dibromo-N-chloro-p-benzoquinoneimine dissolve in 10 ml of methanol. This solution is prepared just before use.

(6) Carbonyls : 1 ml of Smoke Flavours is diluted to 50 ml of carbonyl-removed alcohol. 5 ml of this solution is further diluted to 100 ml with a mixture of carbonyl-removed ethyl alcohol and toluene (1 : 9) (Test Solution). 1 ml each of Test Solution and toluene (for blank test) is placed in a flask, respectively, where 1

ml of toluene, 2 ml of saturated 2,4-DNPH solution, and 2 ml of TCA solution are added. Each test tube is covered with a glass stopper, heated for 30 minutes at 60°C, and cooled in an ice bath. 5 ml of potassium hydroxide solution and 25 ml of carbonyl-removed alcohol are added to each test tube, which is then colorized for exactly 10 minutes. Absorption is measured at 430 nm using the blank test solution as a reference. The content of carbonyl (as heptanal) is obtained from a standard curve and it should be 2~25 %.

Standard Curve

Benzene is added to precisely weighted 30 mg of heptanal standard so that the total volume is 1,000 ml. Using this solution, a series of standard solutions are prepared so that the concentrations lie within a range of 1~30 µg/ml (Standard Solutions). By following the same procedure as the Test Solution, absorptions at each concentration is measured at 430 nm and a standard curve is prepared.

Solutions

- Saturated 2,4-DNPH Solution : 0.05% 2,4-Dinitrophenylhydrazine solution in toluene is prepared. After shaking for 1 hour, it is set aside for over night. It is filtered prior to use. It should be used within 1 week.
 - TCA Solution : 4% (w/v) solution of trichloroacetic acid in toluene is prepared.
 - Potassium Hydroxide Solution : 4% (w/v) solution of potassium hydroxide solution in carbonyl-removed alcohol is prepared. This solution is freshly prepared before use.
- (7) Solid Content : 0.5 g (0.5 ml for liquid) of Smoke Flavors is precisely weighted and dried for 16 hours at 105°C. The total solid content should not be more than 18%.
- (8) Lead : 0.8 g of Smoke Flavors is taken and carbonized by quietly heating. Then at a temperature not above 500 , it is made ash, to which 20 ml of dilute nitric acid is then carefully added. The resulting solution is boiled for 5 minutes quietly and cooled, and filtered if necessary. The remnant is washed with water, and the washing is combined with the remaining solution, to which 50 ml of water is added. This is the test solution. When tested for lead, the amount should not be more than 2ppm.

161. Grape Seed Extract

Definition Grape Seed Extract is obtained from seeds of grapes (*Vitis labrusca* LINNE, *Vitis vinifera* LINNE) of vitaceae by extracting with hot water, heated ethyl alcohol or acetone at room temperature, fermenting the extracts using yeast, or hydrolyzing with tannase. Its major component is proanthocyanidin.

Compositional Specifications of Grape Seed Extract

Content Grape Seed Extract contains 90~130% of the indicated amount as proanthocyanidin.

Description Grape Seed Extract is pale brown~brown powder with a slightly puckery and sour taste.

Identification (1) 0.01 g of Grape Seed Extract dissolve in 10 ml of ethyl alcohol solution (10→100). When 1~2 drops of ferric chloride solution are added to this solution, it shows deep green~greenish brown color.

(2) 0.1 g of Grape Seed Extract dissolve in 10 ml of ethyl alcohol solution (10→100). When 1 ml of hydrochloric acid is added to this solution and heated in a water bath, it becomes red in color.

Purity (1) Arsenic : Place 0.25 g of Grape Seed Extract in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Heavy Metals : Carbonize 1 g of Grape Seed Extract by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid, it is heated until white smoke disappears, which is then reduced to ash by further heating at 450~550°C. Cool the solution, 2 ml of hydrochloric acid is added, which is then evaporated to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, 1 drop of phenolphthalein indicator solution is added, then ammonia solution is added until the color of the solution becomes pale red. The resulting solution transfer into a Nestler cylinder by rinsing with water. Test solution is prepared by adding 2 ml of diluted acetic acid (1→20) and water to make 50 ml. When this solution tested for Heavy Metals, the content

should not be more than 20ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water are added to bring the total volume to 50 ml.

(3) Coliform Group : Grape Seed Extract is tested by Microbiological Methods for Coliform Group in General Testing Methods in Food Code. It should be negative.

Loss on Drying When precisely weighted 3 g of Grape Seed Extract is dried for 3 hours at 105°C, the loss should not be more than 10%.

Assay Grape Seed Extract is precisely weighted (so that the concentration of proanthocyanidin is 10~50 mg) and dissolved in methyl alcohol (total volume = 100 ml, Test Solution). 0.5 ml of Test Solution is placed in a brown test tube, where 3.0 ml of vanillin solution in methanol (4→100) is added. It is stirred for 10 seconds. After adding exactly 1.5 ml of hydrochloric acid, it is capped immediately and set aside for 15 minutes at 18~22°C. Using water as a reference, absorption at 500 nm is measured. The content of (+) catechin equivalent is obtained from a standard curve. This is the content of proanthocyanidin. To correct for anthocyanidin present in the sample, the same procedure as Test Solution is followed with 3 ml of methanol instead of vanillin solution in methanol. Absorption is measured at 500 nm using water as a reference. This absorption value is subtracted from that of Test Solution. The content of (+) catechin equivalent in test solution is obtained from a standard curve. This is the content of proanthocyanidin.

Standard Curve

Methyl alcohol is added to precisely weighted 100 mg of (+) catechin standard (total volume = 100 ml). 1, 2, 3, 5 ml of this solution is diluted to 10 ml with methyl alcohol (Standard Solutions). 0.5 ml each of Standard Solution is placed in a brown test tube, where 3.0 ml of vanillin solution in methanol (4→100) is added. It is stirred for 10 seconds. After adding exactly 1.5 ml of hydrochloric acid, it is capped immediately and set aside for 15 minutes at 18~22°C. Using water as a reference, absorption of each Standard Solution at 500nm is measured. A standard curve of absorption vs. concentration of Standard Solution (mg/ml) is prepared. Separately, a blank test is carried out by following the same procedure as Test Solution with 0.5 ml of water instead of catechin standard solution.

The content of proanthocyanidin is obtained by the following equation.

$$\text{Content(\%)} = \frac{A}{\text{Weight of sample (mg)}} \times \text{dilution factor} \times 100$$

A : Amount of (+) catechin equivalent in Test Solution obtained from the standard curve (mg)

162. Phaffia Color

Definition Phaffia Color is a pigment obtained by extracting the cultures of an enzyme (Phaffia rhodozyma MILLER) with ethyl alcohol. Its major pigment component is Astaxanthin of carotinoids. Diluent, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Phaffia Color

Content Color value ($[E]_{1cm}^{10\%}$) of Phaffia Color should be more than the indicated value.

Description Phaffia Color is reddish brown~brown with a slight characteristic scent.

Identification A solution of Phaffia Color in petroleum ether (1→500) is orange in color and has a maximum absorption band near 474 nm.

Purity (1) Arsenic : Place 0.25 g of Phaffia Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Phaffia Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Residual Solvents : When Phaffia Color is tested by Purity (5) for Paprika Extract Pigments, the content of residual solvents should be,

Acetone	Not more than 30ppm
Hexane	Not more than 25ppm

Assay(Color Value) An appropriate amount of Phaffia Color is precisely weighted and mixed with 10ml of Dimethylsulfoxide (pre-heated to 55°C) so that the measured absorption lies within a range of 0.3~0.7. It is then reacted for 8 minutes in a 55°C water bath. To this reaction mixture, 3 ml of phosphate buffer solution (pH 7.0) and 30 ml of petroleum ether are added. It is well mixed and set aside to separate phases. Petroleum ether phase is collected. The lower aqueous phase is extracted twice with 30 ml each of petroleum ether, which is added to the previous petroleum ether phase. The total volume is brought up to 100 ml with petroleum ether (Test Solution). If necessary, the supernatant is centrifuged for use. Color value is obtained using the

following equation. Using petroleum ether as a reference, absorption A of the Test Solution is measured at a maximum absorption band near 474 nm with 1cm path length. Color value is obtained by the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Phosphate Buffer Solution (pH 7.0)

Solution 1 : 53.7 g of sodium phosphate, dibasic ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) dissolve in water (total volume = 1,000 ml).

Solution 2 : 20.4 g of potassium phosphate, monobasic(KH_2PO_4) dissolve in water(total volume = 1,000 ml).

Solutions 1 & 2 are well mixed in a volume ratio of 80 : 45 and the pH is adjusted to 7.0.

163. Tomato Color

Definition

Tomato Color is a pigment obtained from tomatoes (*Lycopersicon esculentum* MILLER) of solanaceae by the following processes. Tomatoes are extracted with oil/fat. Or, dehydrated tomatoes in room temperature or heating condition are extracted with hexane or acetone and solvents are removed. Or, tomato juice is partitioned. Its major pigment component is lycopene of carotinoids. Diluent, stabilizer, or solvent can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Tomato Color

Content Color value ($[E]_{1cm}^{10\%}$) of Tomato Color should be more than the indicated value.

Description Tomato Color is dark red powder or oily liquid with a slight characteristic scent.

Identification Test Solution obtained in Color Value section has a maximum absorption band near 472 nm.

Purity (1) Arsenic : Place 0.25 g of Tomato Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Tomato Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Cadmium : When 5.0 g of Tomato Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Mercury : When Tomato Color is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

(5) Residual Solvents : When Tomato Color is tested by Purity (5) for Paprika Extract Pigments, the content of residual solvents should be,

Acetone Not more than 30ppm

Hexane Not more than 25ppm

Residue on Ignition When Tomato Color is tested by the procedure in Residues on Ignition, its content should not be more than 0.1%.

Assay(color value) Appropriate amount of Tomato Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in 50 ml of dichloromethane. The total volume is brought up to 100 ml with petroleum ether. 1 ml of this solution is diluted to 100 ml with petroleum ether (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using petroleum ether as a reference solution, absorption A is measured at the maximum absorption near 472 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

164. Enzymatically Decomposed Apple Extract

Definition Enzymatically Decomposed Apple Extract is obtained by the following process. Pulps are removed from juices of apples (*Malus pumila* MILLER) of rosaceae. The clear supernatant is enzymatically treated, which is then purified. The effective components are chlorogenic acid and catechins. Diluent, stabilizer, or solvents can be added for the purpose of content adjustment and quality preservation.

Compositional Specifications of Enzymatically Decomposed Apple Extract

Content Enzymatically Decomposed Apple Extract contains 90~130% of the indicated amount as chlorogenic acid and catechins.

Description This is a yellowish brown powder with a hint of apple scent.

Identification 10 g of Enzymatically Decomposed Apple Extract dissolve in 100 ml of water. When 2 drops of ferric chloride solution are added to 5 ml of this solution, it turns blackish blue. Upon settling, blackish blue precipitates are formed.

Purity (1) Arsenic : 0.5 g of Enzymatically Decomposed Apple Extract is placed in a flask for decomposition. After adding 5 ml of sulfuric acid and 5 ml of nitric acid, the flask is slowly heated. 2~3 ml of nitric acid is added at a time and the flask is heated until the liquid becomes colorless~pale yellow. Cool the solution, 15 ml of saturated ammonium hydroxide is added to the flask, which is then heated and concentrated to 2~3 ml until thick white smoke is generated. The resulting concentrate is neutralized with ammonia water or ammonia solution. This test solution is tested for arsenic. Standard color reference is prepared with 1 ml of arsenic standard solution following the same procedure as the test solution. The detected amount of lead should not be more than 2ppm.

(2) Lead : When 5.0 g of Enzymatically Decomposed Apple Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Assay Precisely 20 mg of Enzymatically Decomposed Apple Extract is mixed with a mixture of 0.2M phosphate buffer solution (pH 3.0) : methyl alcohol : water (2 : 3 : 15) (total volume = 25 ml). It is filtered through a 0.45 μ m Millipore filter (Test Solution). Separately, a mixed standard solution is prepared with a mixture of 0.2 M phosphate buffer solution (pH 3.0) : methyl alcohol : water (2 : 3 : 15) so that the final concentrations are epigallocatechin 360ppm, chlorogenic acid 55ppm, epicatechin 100ppm, epigallocatechin gallate 80ppm, and epicatechin gallate 70ppm. 20 μ l each of mixed standard solution and Test Solution is injected into liquid chromatography under the following Operation Conditions. The contents of chlorogenic acid and

catechins are separately calculated by the following equations.

① chlorogenic acid

$$\text{Content (\%)} = \frac{\text{Concentration of chlorogenic acid}}{\text{Standard Solution. (ppm)}} \times \frac{S_a}{S_t} \times \frac{D}{W} \times \frac{100}{10^6}$$

S_a : Peak area of Test Solution

S_t : Peak area of Standard Solution

W : Weight of sample(g)

D : Dilution factor of Test Solution

② catechins : The content of catechins is a sum of the contents of epigallocatechin, epicatechin, epigallocatechin gallate, and epicatechin gallate.

$$\text{Content (\%)} = \frac{\text{concentration of corresponding Catechin Standard Solution. (ppm)}}{\text{Standard Solution. (ppm)}} \times \frac{S_a}{S_t} \times \frac{D}{W} \times \frac{100}{10^6}$$

S_a : Peak area corresponding catechin in Test Solution

S_t : Peak area corresponding catechin in Standard Solution

W : Weight of sample(g)

D : Dilution factor of Test Solution

Operation Conditions

-Detector : UV detector, 280 nm

-Column : μ Bondapak C₁₈ (3.9 \times 300 mm, 10 μ m) or its equivalent

-Column Temperature : 40°C

-Mobile Phase : acetonitrile : acetic acid : methyl alcohol : water
(113 : 5 : 20 : 862)

-Flow Rate : 1 ml/min

◦ 0.2 M Phosphate Buffer Solution (pH 3.0) : 0.2 M potassium phosphate, monobasic solution and 0.2 M phosphoric acid are well mixed. Its pH is adjusted to 3.0.

165. Sesame Seed Oil Unsaponified Matter

Definition Sesame Seed Oil Unsaponified Matter is obtained by extracting seeds (or residues after extracting oil) of sesame (*Sesamum indicum* LINNE) of pedaliaceae with alcohol. Its components are sesamin, sesamolin, and sesamol.

Compositional Specifications of Sesame Seed Oil Unsaponified Matter

Content Sesame Seed Oil Unsaponified Matter (as sesamin) should contain more than the indicated content.

Description Sesame Seed Oil Unsaponified Matter is white~yellow crystallite or crystalline powder.

Identification (1) When Sesame Seed Oil Unsaponified Matter is tested by Assay, a sesamin peak at 285 nm is observed.

(2) A solution of 5 mg of Sesame Seed Oil Unsaponified Matter in 10 ml of methyl alcohol has maximum absorption bands at 237 nm and 287 nm.

(3) 5 mg of Sesame Seed Oil Unsaponified Matter dissolve in 10 ml of methyl alcohol (Test Solution). Separately, 1 mg of sesamin standard dissolve in 10 ml of methyl alcohol (Standard Solution). 10 μ l each of Test and Standard Solutions is spotted on a silica 60F (60F254 Silica plate) for thin layer chromatography. It is developed using a mixture of chloroform : ethylether (9 : 1) as a developing solvent and blow-dried. When silica plates are observed under UV lamp, the distance from the starting line to the center of the spot (R_f) of Test Solution should be same as R_f of Standard Solution.

Purity (1) Arsenic : Place 0.25 g of Sesame Seed Oil Unsaponified Matter in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1 \rightarrow 50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Sesame Seed Oil Unsaponified Matter is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Loss on Drying : When 1 g of Sesame Seed Oil Unsaponified Matter is dried for 2 hours at 105°C, the loss should not be more than 1.0%.

Assay Approximately 5 mg of Sesame Seed Oil Unsaponified Matter is precisely

weighted and dissolved in a small amount of chloroform, which is diluted to 10ml with methyl alcohol. It is then filtered through a 0.45 µm Millipore filter (Test Solution). Separately, approximately 5 mg of sesamin standard is precisely weighted and dissolved in a small amount of chloroform, which is diluted to 10 ml with methyl alcohol. It is then filtered through a 0.45 µm Millipore filter (Standard Solution). 10 µl each of Standard and Test Solutions is injected into liquid chromatography under the following Operation Conditions. The content of sesamin is obtained by the following equation.

$$\text{Content(\%)} = \frac{Sa \times \text{Weight of sesamin standard (mg)}}{St \times \text{Weight of sample (mg)}} \times 100$$

Sa : peak area of Test Solution

St : peak area of Standard Solution

Operation Conditions

- Detector : UV detector, 285 nm
- Column : ODS Hypersil (4.6 × 200 mm, 5 µm) or its equivalent
- Column Temperature : room temperature
- Mobile Phase : methyl alcohol : water (80 : 20)
- Flow Rate : 0.7 ml/min

166. Transglutaminase

Definition Transglutaminase is an enzyme obtained by the following procedure. Cultures of *Streptovercillium mobaraense* are extracted with water. The extracts are treated with cold ethyl alcohol to obtain enzyme. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Transglutaminase

Content Transglutaminase contains 90~130% of the indicated activity as Transglutaminase.

Description Transglutaminase is white~pale yellow~deep brown powder, granule, solid, or transparent~deep brown liquid.

Purity (1) Arsenic : Place 0.25 g of Transglutaminase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbon substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Heavy Metals : Carbonize 0.5 g of Transglutaminase by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid, Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution, Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. This solution is used as the test solution. When this solution tested for Heavy Metals, the content should not be more than 40ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water are added to bring the total volume to 50 ml.

- (3) Lead : 0.8 g of Transglutaminase (if it is liquid, it is concentrated by evaporation in a water bath) is mildly carbonized by heating, which is reduced ash by further heat treatment at a temperature below 500°C. Carefully 20 ml of dilute nitric acid is added to the ash. Boil the sample for 5 minutes and cool. It is filtered (if necessary), the residue is washed with water, which is then added to the filtrate. Water is added so that total volume of this solution becomes 50 ml. This test solution is tested for lead. The detected amount of lead should not be more than 10ppm.
- (4) Coliform Group : Transglutaminase is tested by Microbiological Methods for Coliform Group in General Testing Methods in Food Code. It should contain 30 or less per 1 g of Transglutaminase.
- (5) Salmonella : Transglutaminase is tested by Microbiological Methods for Salmonella in General Testing Methods in Food Code. It should be negative (-).

Assay(activity) Application and Principle : This test is to measure the activity of Transglutaminase in an enzyme that is obtained by treating the aqueous extracts from cultures of *Streptovorticillium mobaraense* with cold ethyl alcohol. Activity test is based on generation of glutamate- γ -hydroxamate from the reaction between glutamic acid group and hydroxylamine.

- Preparation of Test Solution : An appropriate amount of sample is dissolved in approximately 45 ml of 0.2 M tris-hydrochloric acid buffer solution (pH 6.0) by stirring for 30 minutes at room temperature. The solution is diluted exactly to 50 ml with 0.2 M tris-hydrochloric acid buffer solution (pH 6.0). The concentration should be such that an absorption value (measured by the following Test Procedure) lies within a range of 0.3~0.7.
- Test Procedure : Exactly 0.2 ml of Test Solution is placed in a test tube, which is pre-heated for 1 minutes in a $37 \pm 1^\circ\text{C}$ water bath. 2 ml of substrate solution (previously isothermalized for 10 minutes at $37 \pm 1^\circ\text{C}$) is added to Test Solution and mixed by shaking immediately. This solution is set aside in the same water bath for exactly 10 minutes at $37 \pm 1^\circ\text{C}$, where 2 ml of colorizing solution is added. The reaction is stopped and the reaction mixture is centrifuged for 10 minutes at 3,000 rpm to separate the precipitates. Absorption of the supernatant is measured at 525 nm using water as a reference. Separately, enzyme blank test solution is prepared as follows. 0.2 ml of Test Solution and 2 ml of colorizing solution are mixed by shaking and set aside for 10 minutes at $37 \pm 1^\circ\text{C}$. After adding 2 ml of substrate solution to the resulting solution, it is centrifuged at 3,000 rpm. Absorption is measured by the same procedure as the enzyme test solution. Absorption of Test Solution is obtained

by subtracting the absorption of enzyme blank test solution from that of enzyme test solution.

◦ Standard Curve

64.8 mg of L-glutamate- γ -monohydroxamate is precisely weighted and dissolved in 10 ml of 0.2 M tris-hydrochloric acid buffer solution (pH 6.0). Standard Solutions are prepared so that 1 ml each contains 8.0, 16.0, 20.0, 24.0, and 32.0 μmol of L-glutamate- γ -monohydroxamate. 2 ml of substrate solution is added to 0.2 ml of each Standard Solution at $37 \pm 1^\circ\text{C}$ and set aside for 10 minutes. After adding 2 ml of colorizing solution, precipitates are removed by the same procedure as Test Solution. Absorptions at 525 nm are measured using water as a reference. A standard curve of absorption vs. concentration of L-glutamate- γ -monohydroxamate ($\mu\text{mol}/\text{ml}$) is prepared.

Enzyme activity is calculated by the following equation.

$$\text{U/g} = \frac{C \times D}{W \times 10}$$

C : Concentration of hydroxamate of Test Solution obtained from the standard curve ($\mu\text{mol}/\text{ml}$)

D : Dilution factor of Test Solution (ml)

W : Dmount of sample(g)

10 : Reaction time (minutes)

Definition of Activity : 1 Transglucosidase unit corresponds to an amount of enzyme that produces 1 μmol per 1 minute of hydroxamic acid from the substrate under the test conditions above.

Solutions

◦ Substrate Solution : 2.42 g of tris(hydroxymethyl)amino-methane, 0.7 g of hydroxylamine hydrochloride, 0.31 g of glutathione, 1.01 g of carbobenzyloxy glutaminylglycine are precisely weighted and dissolved in 80 ml of water. pH is adjusted to 6.0 with 6 N hydrochloric acid. The total volume is brought up to 100 ml with water.

◦ 0.2 M Tris-Hydrochloric Buffer Solution (pH 6.0) : 24.22 g of tris(hydroxymethyl)amino-methane dissolve in 800 ml of water and pH is adjusted to 6.0 with 2.8 N hydrochloric

acid. The total volume is brought up to 1,000 ml with water. The solution is stored at 5°C in a refrigerator.

◦ Colorizing Solution

Solution 1 : 3 N hydrochloric acid

Solution 2 : 12 g of trichloro acetic acid (Cl_3COOH) dissolve in water (total volume = 100 ml).

Solution 3 : 5 g ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) dissolve in 0.1 N hydrochloric acid (total volume = 100 ml).

Same amounts of Solution 1, 2, and 3 are well mixed before use.

167. Gold Leaf

Gold

Definition Gold Leaf is a thin sheet of gold.

Compositional Specifications of Gold Leaf

Content Gold Leaf should contain no less than 94.4% of gold (Au).

Description Gold Leaf is yellow extremely thin and soft sheet.

Identification (1) Gold Leaf is insoluble in hydrochloric acid, nitric acid, and sulfuric acid but soluble in aqua Regia.

(2) 0.01 g of Gold Leaf dissolve in 5 ml mixed solution of nitric acid : hydrochloric acid : water (1 : 4 : 5) by heating. Cool the solution, 2 ml of hydrochloric acid is added to this solution, which is then concentrated by heating in a water bath. This process is repeated 4 times to remove nitric acid. 20 ml of water is added to the resulting residue, where sodium hydroxide solution is added to adjust the acidity to slightly acid. When 1 ml of 5-(p-dimethylamino- benzyldine)rhodanine solution in ethyl alcohol (1→3,000) to the resulting solution, it shows reddish violet in color.

Purity (1) Arsenic : 0.2 g of Gold Leaf is placed a platinum, quartz, and porcelain crucible, where 5 ml of aqua regia is added. It is heated until white smoke is generated. Cool the solution, water is carefully added to bring the total volume to 5 ml (Test Solution). Test Solution is tested for arsenic and the content of gold should not be more than 5ppm.

Assay 20 ml of dilute nitric acid (1→2) is added to precisely weighted 0.5 g of Gold Leaf, which is heated for 10 minutes at 50°C or lower. It is then thermally decomposed with 30 ml of aqua Regina, where water is added to bring the total volume to exactly 100 ml. 3 ml of the resulting solution is diluted to 200 ml with water. 5 ml of this solution is further diluted to 100 ml (Test Solution). Separately, 5 ml of gold standard solution (1 ml = 1,000 µg Au) for atomic absorption spectrophotometer is diluted to 50 ml with water. 2, 4, 6, 8, and 10 ml each of this solution is further diluted to 100 ml with water (Standard Solutions). Test Solution and each Standard Solution are analyzed with atomic absorption spectrophotometer by the following Operation Conditions. The content of gold is obtained from a calibration curve prepared from Standard Solutions.

Operation Conditions

-Gas : flammable gas : acetylene or hydrogen

Retarding gas : air

- Lamp : Gold, hollow cathode lamp
- Wavelength : 242.8 nm

168. Crayfish Color

○ Designed Cancellation

The date of cancellation: 27.3.'12 (Notification No. 2012-10).

169. Glucosamine

Definition Glucosamine is obtained by one of the following processes. Chitin or chitosan are extracted from shells of crustacea (crabs, shrimps, etc.) or bones of mollusca (squid, cuttle fish, etc). Chitin or chitosan is hydrolyzed with hydrochloric acid. Or it Dissolve in hydrochloric acid then hydrolyzed with chitosanase. Hydrolyzed material is separated and purified.

Compositional Specifications of Glucosamine

Content Glucosamine should contain no less than 80.0% of glucosamine ($C_6H_{13}NO_5 = 179.17$).

Description Glucosamine is white powder.

Identification When 0.2 g of Glucosamine in a mixture of 5 ml of anthrone solution and 1 ml of water is heated in a water bath, it shows blue~green in color.

Purity (1) Arsenic : Place 0.25 g of Glucosamine in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Glucosamine is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Clarity and Color of Solution : When 1 g of Glucosamine is dissolved in 20 ml of water, the solution should be clear.

(4) Acidity : pH of aqueous solution (10→100) of Glucosamine should be 3.0~5.0.

(5) Chloride : 0.1 g of Glucosamine is dissolved in 50 ml of methanol. Add 5 drops of potassium chromate solution(1→20) and the end point is the point where the color turns from yellow to reddish brown. When it is titrated by 0.1 N silver nitrate solution, the content which is calculated on the dried basis should be 16~18%.

1 ml of 0.1 N silver nitrate solution = 3.545mg Cl

(6) Total Viable Aerobic Count : When Glucosamine is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 per 1 g

(7) Coliform Group : When Glucosamine proceed as directed under Microbe Test Methods for Coliform Group in General Test Methods in Food Code, it should contain not more than 30 colonies per 1 g of this product.

Loss on Drying When Glucosamine is dried for 4 hours at 105°C, the loss should not be more than 1.0%.

Residue on Ignition Residue on Ignition should not be more than 1.0%.

pH pH of aqueous solution (1→100) should be 4.0~7.0 (measured by glass electrode).

Coliform Group Glucosamine is tested by Microbiological Methods for Coliform Group in General Testing Methods in Food Code. It should be negative.

Assay 0.02 g of Glucosamine is precisely weighted and dissolved in 20 ml of water. It is then diluted to 100 ml with water (Test Solution). 1 ml of Test Solution transfer into a test tube with a stopper, where 2 ml of acetyl acetone is added. It is mixed and heated for 1 hour at 96°C. It is cooled in running water. 20 ml of 96% alcohol is added to this solution, where 2 ml of p-Dimethylaminobenzaldehyde is added and mixed. The resulting solution is set aside for 1 hour at room temperature. Absorption (AT) at 535 nm is measured. Separately, a Standard Solution is prepared by dissolving D-glucosamine standard in water so that it contains 100~500 µg/ml. Absorption (As) of 1 ml of Standard Solution is measured by the same procedure as Test Solution..

$$\text{Contents (\%)} = \frac{C \times 100}{\text{Weight of sample (g)}} \times \frac{A_T}{A_S} \times \frac{100}{10^6}$$

C : Concentration of Standard Solution as glucosamine (µg/ml)

Solutions

- Acetyl acetone solution : 1.5 ml of acetyl acetone (purified by distillation, boiling point : 138~140°C) is mixed with 1.2 N sodium carbonate solution (total volume = 50 ml).
- p-Dimethylaminobenzaldehyde solution : 1.6 g of p-Dimethylaminobenzaldehyde dissolve in 30 ml of hydrochloric acid, where 30 ml of 96% alcohol is added.

170. Laver Color

Definition Laver Color is a pigment obtained by extracting fronds of laver (*Porphyra tenera* KJELLM.) of bangiaceae (a red algae) with water or faintly acidic aqueous solution at room temperature. It's major pigment component is phycoerythrin. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Laver Color

Content Color value ($[E]_{1cm}^{10\%}$) of Laver Color should be more than the indicated value.

Description Laver Color is orange~red paste or liquid with a characteristic scent.

Identification (1) A solution of Laver Color in citric acid buffer solution with pH 6.0 (1→100) is pink~red in color.

(2) When 4.0 g of ammonium sulfate is dissolved in 10 ml of the solution in (1) and set aside, red precipitates are formed.

(3) A solution of Laver Color in citric acid buffer solution with pH 6.0 has maximum absorption bands near 565 nm, 540 nm, and 490 nm.

Purity (1) Arsenic : Place 0.25 g of Laver Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Laver Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay(color value) Appropriate amount of Laver Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in citric acid buffer solution (pH 6.0) so that the total volume is 100 ml (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citric acid buffer solution (pH 6.0) as a reference solution, absorption A is measured at the maximum absorption near 565 nm with 1 cm path length. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 10}{\text{Weight of sample (g)}}$$

◦ Citric acid·dibasic sodium phosphate buffer solution (pH 6.0)

Solution 1 : 0.1 M citric acid solution : 1L of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2 M dibasic sodium phosphate solution : 1L of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (73.7:126.3) and its pH is adjusted to 6.0.

171. Garden Balsam Extract

Definition Garden Balsam Extract is obtained by extracting root cortex of garden balsam (*Impatiens balsamina* LINNE) of Balsaminaceae with hydrated ethyl alcohol at room temperature. Its major component is quercetin.

Compositional Specifications of Garden Balsam Extract

Content Garden Balsam Extract should contain more than the indicated amount of quercetin ($C_{15}H_{10}O_7$).

Description Garden Balsam Extract is yellowish brown liquid with characteristic scent and slightly bitter taste.

Identification 5 mg of Garden Balsam Extract dissolve in 10 ml of 50% alcohol (Test Solution). Separately, 5 mg of quercetin standard dissolve in 10 ml of 50% alcohol (Standard Solution). Spot aliquots 2 μ l of each solution to a thin layer plate by using silica gel (with phosphor) for thin layer chromatography. Using a mixture of n-butyl alcohol : water : acetic acid (7 : 2 : 1) as a developing solvent, each plate is developed and dried in air. When these plates are observed under UV light, the spot for Test Solution should have the same color tone and position as the spot for Standard Solution.

Purity (1) Arsenic : Place 0.25 g of Garden Balsam Extract in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Garden Balsam Extract is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Residue on Ignition when Residue on Ignition analysis is done with accurately weighted 1 g of Garden Balsam Extract, the amount of Residue on Ignition should not be more than 1.0%.

Assay Approximately 0.4 g of Garden Balsam Extract is precisely weighted and dissolved in methyl alcohol (total volume = 50 ml), which is filtered through a 0.5 μ m Millipore filter (Test Solution). Separately, 50 mg of quercetin standard is precisely weighted and dissolved in methyl alcohol (total volume = 50 ml), which is filtered

through a 0.5 μm Millipore filter (Standard Solution). 10 μl each of Test and Standard Solutions is injected into a high-performance liquid chromatography under the following Operation Conditions. The content of quercetin is obtained by the following equation.

$$\text{Content (\%)} = \frac{\text{Weight of standard (mg)}}{\text{Weight of sample (mg)}} \times \frac{\text{peak area of Test Solution}}{\text{peak area of Standard Solution}} \times 100$$

Operation Conditions

- Detector : UV 375 nm
- Column : μ -Bondapak C₁₈ (3.9 mm \times 300 mm) or its equivalent
- Column Temperature : room temperature
- Mobile Phase : methyl alcohol : water : acetic acid (15:3:1)
- Flow Rate : 1.0 ml/min

172. Krill Color

○ Designed Cancellation

The date of cancellation: 27.3.'12 (Notification No. 2012-10).

173. Red Radish Color

Definition Red Radish Color is a pigment obtained by extracting reddish violet roots of radish (*Raphanus sativus* LINNE) of cruciferae with water or hydrated ethyl alcohol at room temperature. Its major pigment component is Pelargonidin acylglucoside of anthocyanins. Diluent and stabilizer can be added for the purpose of color value adjustment and quality preservation.

Compositional Specifications of Red Radish Color

Content Color value ($[E]_{1cm}^{10\%}$) of Red Radish Color should be more than the indicated value.

Description Red Radish Color is dark red powder with a slight characteristic scent.

Identification (1) Test Solution obtained in Color Value section shows red color and a maximum absorption band near 515 nm.

Purity (1) Arsenic : Place 0.25 g of Red Radish Color in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Red Radish Color is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

Assay(Color Value) Appropriate amount of Red Radish Color is precisely weighted so that the absorption is within 0.3~0.7 and dissolved in citric acid·dibasic sodium phosphate buffer solution with pH 3.0 (total volume 100 ml). 1 ml of this solution is diluted to 100 ml with citric acid·dibasic sodium phosphate buffer solution with pH 3.0 (Test Solution). If necessary, the solution is centrifuged and the supernatant is used. Using citric acid·dibasic sodium phosphate buffer solution with pH 3.0 as a reference solution, absorption A is measured at the maximum absorption near 515 nm with 1cm path length. Color value is obtained using the following equation.

$$\text{Color Value } ([E]_{1cm}^{10\%}) = \frac{A \times 1,000}{\text{Weight of sample (g)}}$$

◦ Citric acid·dibasic sodium phosphate buffer solution (pH 3.0)

Solution 1 : 0.1 M citric acid solution : 1 L of solution containing 21.01 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$).

Solution 2 : 0.2 M dibasic sodium phosphate solution : 1 L of solution containing 71.63 g of dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$).

Solution 1 and Solution 2 are mixed well (159:41) and its pH is adjusted to 3.0.

174. Enzymatically Modified Stevia

Glucosyl Stevia

Definition Enzymatically Modified Stevia is obtained by addition of glucose to stevia extracts using α -glucosyltransferase. Its components are α -glucosylstevioside, etc.

Compositional Specifications of Enzymatically Modified Stevia

Content If Enzymatically Modified Stevia is converted to a dehydrated form, it should contain no less than 80.0 % of steviolglycoside and not more than 15.0 % of unreacted steviolglycoside.

Description Enzymatically Modified Stevia is white~pale yellow powder, flakes, or granule with a cool, sweet taste. It have a slight characteristic scent.

Identification (1) 1.2 g of Enzymatically Modified Stevia dissolve in 100 ml of water, where 100 ml of n-butyl alcohol is added and shaken. It is set aside to separate two phases. N-butyl alcohol phase is taken and filtered if necessary. 5 ml of anthrone solution is slowly added along the inner wall of a container with 5 ml of this solution. The boundary area becomes blue~green in color.

(2) 40 ml of sulfuric acid (1 \rightarrow 5) is added to 2.4 g of Enzymatically Modified Stevia in a flask. A reflux condenser is attached to the flask, which is heated for 2 hours in a water bath. Cool the solution, it is extracted twice with 50 ml each of ether. The extracts are washed twice with water, dehydrated with anhydrous sodium sulfate, and evaporated to dryness. The residue dissolve in 10 ml of methyl alcohol, where 10 ml of water is added. Formed precipitates are filtered. These precipitates are washed with a small amount of 50% methyl alcohol and dried for 2 hours at 105°. The melting point of residue should be 226~230°.

(3) 2.4 g of Enzymatically Modified Stevia dissolve in 100 ml of water. The solution is halved. One portion is tested by (3) Assay for Unreacted Steviolglycoside in Assay and a group of peaks should be observed after the peak for Rebaudioside A. 500 GUN of glucoamylase per 1g of Enzymatically Modified Stevia is added to the other portion of the solution, which is reacted for 24 hours at 55°. It is then filtered through a 0.45 μ m Millipore filter and the filtrate is analyzed by Assay for Unreacted Steviolglycoside. The group of peaks observed after the peak of Rebaudioside A should almost disappear and the area for the group of peaks before the peak of Rebaudioside A should increase.

Purity (1) Arsenic : Place 0.5 g of Enzymatically Modified Stevia in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1 \rightarrow 50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at

450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 2ppm.

- (2) Lead : When 5.0 g of Enzymatically Modified Stevia is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Loss on Drying When 1 g of Enzymatically Modified Stevia is dried for 4 hours at 105°C, the loss should not be more than 6%.

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 1 g of Enzymatically Modified Stevia, the amount of Residue on Ignition should not be more than 1%.

Assay (1) Steviolglycoside : The content of steviolglycoside is a sum of ① steviol content and ② sugar content in glycoside.

① Assay for Steviol : Approximately 100 mg of Enzymatically Modified Stevia is precisely weighted into a 30 ml Erlenmeyer flask, where 10 ml of 20% sulfuric acid is added and a reflux condenser is attached. It is heated for 2 hours in a water bath and cooled in a running water. The solution transfer into a separatory funnel using 10 ml of water. The flask is again washed three times with 30 ml each of ether, which is added to the funnel. The funnel is well shaken and set aside. The aqueous layer is discarded and the ether phase is washed twice with 20 ml each of water. The aqueous layer is completely removed. The ether phase transfer into a separate flask. The funnel is washed twice with 10 ml each of ether, which is added to the flask. 15g of anhydrous sodium sulfate is added and mixed well by shaking. The ether phase is decanted into another flask. The remaining anhydrous sodium sulfate washed twice 10 ml each of ether, which is added to the flask. After evaporate the ether, the residue dissolve in 10 ml of ethyl acetate, where 3 ml of 4% diazomethane ether solution is added and a cap is placed. It is then set aside for 20 minutes while stirring occasionally. 0.5 ml of acetic acid is well mixed with this solution, where 2 ml of an internal standard solution of squalene in n-butyl alcohol (12.5 mg/ml) (Test Solution). Separately, 50 mg of stevioside standard (previously dried for 2 hours at 105°C) is precisely weighted and treated by the same procedure as Test Solution (Standard Solution). Test and Standard Solutions are separately injected into gas chromatography under the following Operation Conditions. The content of steviol is calculated by the following equation.

$$\text{Steviol Content (\%)} = \frac{A \times \text{amount of stevioside standard (mg)}}{As \times \text{amount of sample as a dehydrated form (mg)}} \times 100 \times K$$

A : Peak area ratio of iso-steviol methyl ester in Test Solution vs. sualene

As : Peak area ratio of iso-steviol methyl ester in Standard Solution vs. sualene

K : Conversion factor to steviol $318.46/804.88 = 0.3957$

Operation Conditions

-Column : DB-17 (30 m \times 250 μm \times 0.25 mm) or its equivalent

-Detector : Flame Ionization Detector(FID)

-Temperature at injection hole : 260°

-Column Temperature : 235°

-Detector Temperature : 260°

-Carrier Gas and Flow Rate : Nitrogen or helium, flow rate and column temperature are adjusted so that the peak of iso-steviol methyl ester appears in 7~15 minutes.

② Assay for Sugar in Glycoside

- Preparation of Test Solutions : Approximately 1.0 g of Enzymatically Modified Stevia is precisely weighted and dissolved in 50 ml of water. The solution transfer into a 2.5 cm (diameter) resin column that is made using 50 ml of adsorption resin (Amberlite XAD-7) for enzymatically modified stevia. It is then drained out for 1 minute at a rate of 3 ml/min or lower. The column is washed with 250 ml of water. Adsorbed matter is eluted for 1 minute using 250 ml of 50% ethyl alcohol or 90% methyl alcohol at a flow rate of 3 ml/min or lower. The eluted solution is concentrated and dried with a vacuum evaporator. The residue dissolve in water (total volume = 500 ml). 1 ml of this solution is diluted to 50 ml with water (Test Solution)
- Test Procedure : 2 ml of Test Solution is placed in a test tube with a stopper. While cooling in an ice bath, exactly 6 ml of anthrone solution is added to the test tube and well mixed with Test Solution by shaking. It is then heated for exactly 16 minutes in heating water bath and cooled in an ice bath. Absorption of this solution is measured at 620 nm using water as a reference. Concentration of glucose ($\mu\text{g/ml}$) in Test Solution is obtained from a glucose standard curve. Standard Solutions are prepared using glucose (dried for 2 hours at 105°) so that each 1 ml solution contains 10 μg , 30 μg , and 50 μg of glucose. Glucose Standard Curve prepared by following the same procedure as Test Solution with Standard Solutions. The content of sugar in

steviolglycoside is obtained by the following equation.

$$\text{Content of sugar in steviolglycoside (\%)} = \frac{b \times 0.9 \times 50 \times 500}{Y \times 1000 \times 1000} \times 100 = \frac{2.25b}{Y}$$

b : Glucose concentration in Test Solution from Standard Curve ($\mu\text{g/ml}$)

Y : Amount of sample as a dehydrated form (g)

(2) Assay for Unreacted Steviolglycoside : Approximately 1.5 g of Enzymatically Modified Stevia is precisely weighted and dissolved in water (total volume = 100 ml) (Test Solution). Separately, Stevioside standard and Rebaudioside A standard are dried for 2 hours at 105° . 50 mg of each standard dissolve in mobile phase (total volume = 100 ml) (Standard Solutions). Test and Standard Solutions are separately injected into liquid chromatography under the following Operation Conditions and the content of unreacted steviolglycoside is obtained. Peak areas of stevioside, rebaudioside A, rebaudioside C, and dulcoside A in Test Solution are obtained. Also peak areas of stevioside and rebaudioside A are obtained. The contents of the 4 components are obtained by the following equations. The sum of these contents is the content of unreacted steviolglycoside. When the peak of rebaudioside A show up, the mobile phase is changed to 50 : 50 composition and the residuals in the column is washed out.

$$\begin{aligned} \text{Content of stevioside (\%)} &= \frac{\text{Amount of stevioside standard (mg)}}{\text{Weight of sample as a dehydrated form (mg)}} \times \\ &\quad \frac{\text{peak area of stevioside in Test Solution}}{\text{peak area of stevioside in Standard Solution}} \times 100 \end{aligned}$$

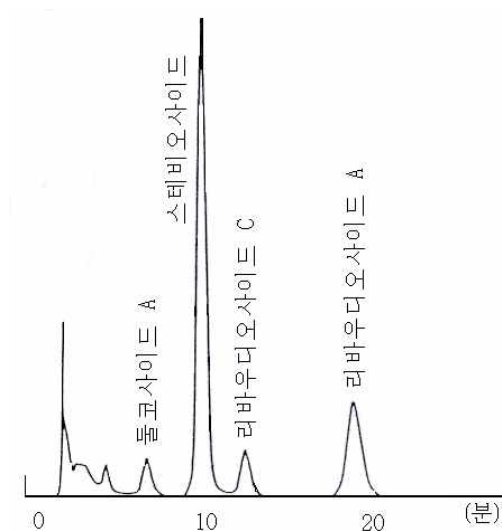
$$\begin{aligned} \text{Content of dulcoside A (\%)} &= \frac{\text{Amount of stevioside standard (mg)}}{\text{Weight of sample as a dehydrated form (mg)}} \times \\ &\quad \frac{\text{peak area of dulcoside in Test Solution}}{\text{peak area of dulcoside in Standard Solution}} \times 100 \end{aligned}$$

$$\begin{aligned} \text{Content of rebaudioside A (\%)} &= \frac{\text{Amount of rebaudioside A standard (mg)}}{\text{Weight of sample as a dehydrated form (mg)}} \times \\ &\quad \frac{\text{peak area of rebaudioside A in Test Solution}}{\text{peak area of rebaudioside A in Standard Solution}} \times 100 \end{aligned}$$

$$\text{Content of rebaudioside C (\%)} = \frac{\text{Amount of rebaudioside A standard (mg)}}{\text{Weight of sample as a dehydrated form (mg)}} \times$$

$$\frac{\text{peak area of rebaudioside C in Test Solution}}{\text{peak area of rebaudioside C in Standard Solution}} \times 100$$

When a test is carried out under the following Operation Conditions, the distribution of stevioside, rebaudioside A, rebaudioside C, and dulcoside A is as follows.

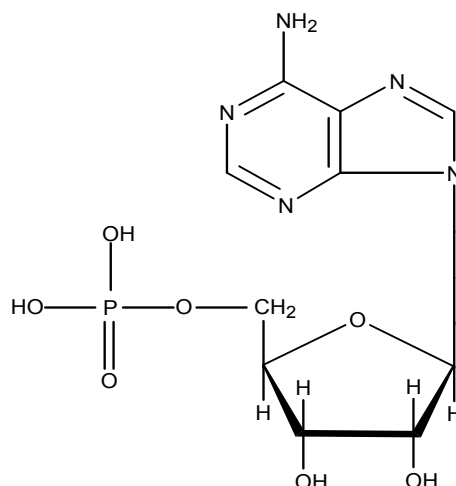


Operation Conditions

- Detector : UV 210 nm
- Column : μ -Bondapak C₁₈ or its equivalent
- Column Temperature : Room temperature
- Mobile Phase : Acetonitrile : Water (80 : 20)
- Flow Rate : 1.0 ml/min

◦ Anthrone solution : 200 mg of anthrone is precisely weighted and dissolved in 100 ml of sulfuric acid. While cooling, this solution is carefully added and mixed into 20 ml of water.

175. 5'-Adenylic acid

Chemical Formula $C_{10}H_{14}N_5O_7P$

Molecular Weight 347.22

Definition 5'-Adenylic acid is obtained by hydrolyzing (with enzyme) the hexane extracted from yeast (*Candida utilis*, *Kluyveromyces fragilis*) with hot water under the presence of salts..

Compositional Specifications of 5'-Adenylic acid

Content If 5'-Adenylic acid is converted to a dehydrated form, it should contain 98.0~102.0% 5'-adenylic acid ($C_{10}H_{14}N_5O_7P$).

Description 5'-Adenylic acid is white crystalline powder.

Identification (1) 0.2 g of 5'-Adenylic acid is precisely weighted and dissolved in 10 ml of 0.1 N sodium hydroxide solution, which is diluted to 200 ml with water. 2 ml of this solution is diluted to 100 ml with 0.01 N hydrochloric acid. The resulting solution has a maximum absorption band near 257 nm.

(2) 1 ml of hydrochloric acid and 1 ml of bromine solution are added to 3 ml aqueous solution of 5'-Adenylic acid (3→10,000), which is then heated for 30 minutes. Bromine is removed in a flowing air. 0.2 ml of orcin solution in alcohol (1→10) is added to this solution. To the resulting solution, 3 ml of ammonium ferric sulfate · hydrochloric acid solution (1→1,000) is added and heated for 20 minutes in a water bath. The final solution turns green.

(3) 0.25 g of 5'-Adenylic acid is precisely weighted and dissolved in 1 ml of sodium hydroxide solution (1→25), which is diluted to 5 ml of water. When 2 ml of magnesia solution is added to this solution, precipitates are not observed. To resulting solution, 7 ml of nitric acid is added and boiled for 10 minutes in a water bath. When the resulting solution is neutralized with sodium hydroxide solution (1→

25), it shows the reaction of (B) Phosphate Salts in Identification.

Purity (1) Arsenic : 0.25 g of 5'-Adenylic acid dissolve in 5 ml of diluted hydrochloric acid (Test Solution). When Test Solution is tested for arsenic, the content should not be more than 4ppm.

(2) Lead : When 5.0 g of 5'-Adenylic acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Solution: 0.5g of 5'-Cytidylic acid is weighted and dissolved in 2ml of sodium hydroxide solution, which is diluted to 10ml with water. the solution is nearly colorless and clear.

(4) Other nucleic acid hydrolysates: 0.1g of 5'-Cytidylic acid is weighted and dissolved in 0.5ml of sodium hydroxide solution, which is diluted to 20ml with water. When 1 $\mu\ell$ of the test solution is tested with the mixed solution of acetone·ammonia solution:n-propanol (2:5:6) by Thin Layer Chromatography, it should not show spots except for one original spot. Supporting material of thin layer plate must be used as dried one with silicagel(added fluorescent agent) for 1 hour at 110°C. When the solvent is developed up to 10cm from the base line, stop the developing. After drying with wind, the plates are observed under a UV light (about 250nm wavelength). The control solution is not used.

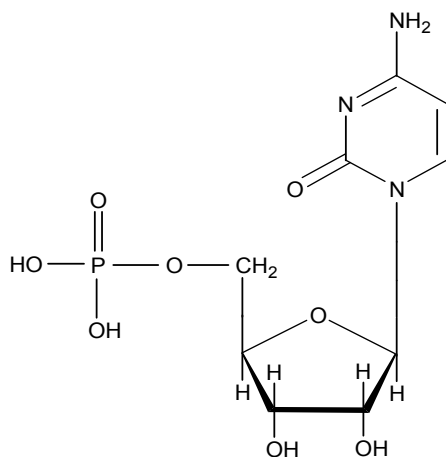
(5) Absorption Ratio : 20 mg of 5'-Adenylic acid is precisely weighted and dissolved in hydrochloric acid (1→1,000) (total volume = 1,000 ml). Absorptions A_1 , A_2 , and A_3 are measured at 250 nm, 260 nm, and 280 nm are measured. A_1/A_2 and A_3/A_2 should be 0.82~0.88 and 0.19~0.23.

Loss on Drying When 5'-Adenylic acid is dried for 4 hours at 120°C, the loss should not be more than 6.0%.

Assay 0.2 g of 5'-Adenylic acid is precisely weighted and dissolved in 10 ml of 0.1 N sodium hydroxide solution , which is diluted to 200 ml with water. 2 ml of this solution is further diluted to 200 ml with 0.01 N hydrochloric acid (Test Solution). Absorption A of Test Solution is measured at 257 nm with 1cm path length using 0.01 N hydrochloric acid as a reference. The content of 5'-adenylic acid is obtained by the following equation.

$$\text{Content (\%)} = \frac{0.2}{\text{Weight of sample (g)}} \times \frac{229.9 \times A}{100 - \text{loss on drying (\%)}} \times 100$$

176. 5'-Cytidylic acid



Chemical Formula $C_9H_{14}N_3O_8P$

Molecular Weight 323.20

Definition 5'-Cytidylic acid is obtained by enzymatically hydrolyzing and separating hexane that is obtained by extracting yeast (*Candida utilis*, *Kluyveromyces fragilis*) with hot water under a presence of salts. Its component is 5'-cytidylic acid.

Compositional Specifications of 5'-Cytidylic acid

Content If 5'-Cytidylic acid is converted to a dehydrated form, it should contain 98.0~102.0% 5'-cytidylic acid ($C_9H_{14}N_3O_8P$).

Description 5'-Cytidylic acid is white crystalline powder.

Identification (1) 0.2 g of 5'-Cytidylic acid is precisely weighted and dissolved in 10 ml of 0.1 N sodium hydroxide solution, which is diluted to 200 ml with water. 2 ml of this solution is diluted to 100 ml with 0.01 N hydrochloric acid. The resulting solution has a maximum absorption band near 280 nm.

(2) 1 ml of hydrochloric acid and 1 ml of bromine solution are added to 3 ml aqueous solution of 5'-Cytidylic acid (3→10,000), which is then heated for 30 minutes. Bromine is removed in a flowing air. 0.2 ml of orcin solution in alcohol (1→10) is added to this solution. To the resulting solution, 3 ml of ammonium ferric sulfate-hydrochloric acid solution (1→1,000) is added and heated for 20 minutes in a water bath. The final solution turns green.

(3) 0.25 g of 5'-Cytidylic acid is precisely weighted and dissolved in 1 ml of sodium hydroxide solution (1→25), which is diluted to 5 ml of water. When 2 ml of magnesia solution is added to this solution, precipitates are not observed. To resulting solution, 7 ml of nitric acid is added and boiled for 10 minutes in a water bath. When the resulting solution is neutralized with sodium hydroxide solution (1→

25), it shows the reaction of (B) Phosphate Salts in Identification.

Purity (1) Arsenic : 0.25 g of 5'-Cytidylic acid dissolve in 5 ml of diluted hydrochloric acid (Test Solution). When Test Solution is tested for arsenic, the content should not be more than 4ppm.

(2) Lead : When 5.0 g of 5'-Cytidylic acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Solution: 0.5g of 5'-Cytidylic acid is weighted and dissolved in 2ml of sodium hydroxide solution, which is diluted to 20ml with water. the solution is nearly colorless and clear.

(4) Other nucleic acid hydrolysates: 0.1g of 5'-Cytidylic acid is weighted and dissolved in 0.5ml of sodium hydroxide solution, which is diluted to 20ml with water. When 1 $\mu\ell$ of the test solution is tested with the mixed solution of acetone·ammonia solution:n-propanol (2:5:6) by Thin Layer Chromatography, it should not show spots except for one original spot. Supporting material of thin layer plate must be used as dried one with silicagel(added fluorescent agent) for 1 hour at 110°C. When the solvent is developed up to 10cm from the base line, stop the developing. After drying with wind, the plates are observed under a UV light (about 250nm wavelength). The control solution is not used.

(5) Absorption Ratio : 20 mg of 5'-Cytidylic acid is precisely weighted and dissolved in hydrochloric acid (1→1,000) (total volume = 1,000 ml). Absorptions A_1 , A_2 , and A_3 are measured at 250 nm, 260 nm, and 280 nm are measured. A_1/A_2 and A_3/A_2 should be 0.40~0.52 and 1.85~2.20, respectively.

Loss on Drying When 5'-Cytidylic acid is dried for 4 hours at 120°C, the loss should not be more than 6.0%.

Assay 0.2 g of 5'-Cytidylic acid is precisely weighted and dissolved in 10 ml of 0.1 N sodium hydroxide solution, which is diluted to 200 ml with water. 2 ml of this solution is further diluted to 100 ml with 0.01 N hydrochloric acid (Test Solution). Absorption A of Test Solution is measured at 280 nm with 1cm path length using 0.01 N hydrochloric acid as a reference. The content of 5'-adenylic acid is obtained by the following equation.

$$\text{Content (\%)} = \frac{0.2}{\text{Weight of sample (g)}} \times \frac{127.2 \times A}{100 - \text{loss on drying (\%)}} \times 100$$

177. Oxygen

Chemical Formula O_2
Molecular Weight 16.00

Compositional Specifications of Oxygen

Content Oxygen should contain no less than 99.0% of oxygen (O_2).

Description Oxygen is colorless scentless gas.

Identification Upon contact with a piece of wood of which the flame is extinguished, a violent flame is generated.

Purity When the volume of Oxygen is measured, it should be converted to the volume at 20° and under 760 mmHg.

- (1) Carbon Dioxide : Both ends of a carbon dioxide detecting tube are broken off. One end is connected to an oxygen cylinder and the other to an appropriate flow meter. When approximately 1050 ± 50 ml of oxygen is passed through at an appropriate flow rate for the tube, the content of carbon dioxide should not be more than 300 μ l/l.
- (2) Carbon Monoxide : Both ends of a carbon monoxide detecting tube are broken off. One end is connected to an oxygen cylinder and the other to an appropriate flow meter. When approximately 1050 ± 50 ml of oxygen is passed through at an appropriate flow rate for the tube, the content of carbon monoxide should not be more than 10 μ l/l.
- (3) Scent : When the valve of an oxygen cylinder is gently opened and the scent of oxygen is smelled (care must be taken to avoid direct facial contact), there should not be any noticeable scent.

Assay

Apparatus

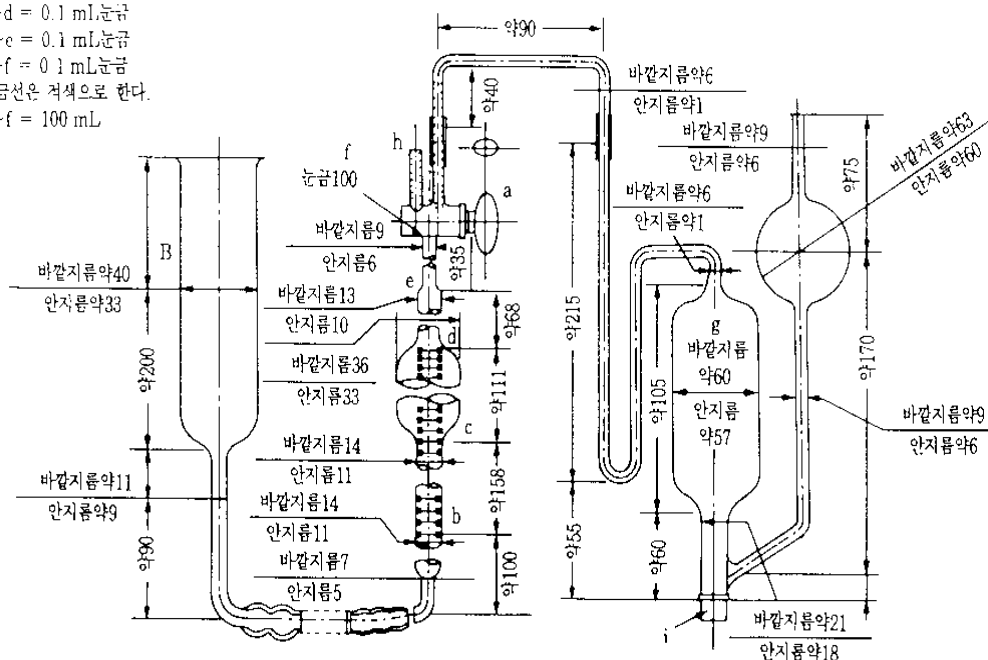
A is a 100 ml gas burette with a two-way stopcock. Scale marks for b~c, d~e, and e~f are at 0.1 ml, while those for c~d are at 2 ml. A is connected to a alidade B and a thick rubber hose. Approximately 50% volume of A and B is filled with ammonium chloride · ammonia solution. An inlet g of gas pipette C is filled (up to its top) with a finely coiled copper wire (diameter : Not more than 2 mm), filled with 125 ml of ammonium chloride · ammonia solution, plugged with a rubber stopper i, and connected to A with a think rubber tube.

Procedure : a is opened and B is lowered. The solution in g is sucked up to the stopcock of a and stopcock a is closed. sample inlet h of a is opened and B is raised

so that A and h are filled with ammonium chloride-ammonia solution. Then a is closed and the sample cylinder is connected to h. Again, a is opened and B is lowered and approximately 100 ml of sample is introduced. The opening to c in a is opened and B is raised so that the sample is flown into g then a is closed. c is gently shaken back and forth. Unadsorbed gas is flown back to A by opening a and lowering B and its volume is measured. This operation is repeated until the volume of unadsorbed gas becomes constant. The volume at this point is measured V (ml). The oxygen content is obtained by the following equation. When ammonium chloride-ammonia solution is freshly replaced, the above Procedure are repeated 4 times and the average is taken. V and the volume of the sample are converted to volumes at 20° and under 760 mmHg.

$$\text{Oxygen (O}_2\text{) content (\%)} = \frac{\text{converted volume of sample (mL)} - \text{converted } V(\text{mL})}{\text{converted volume of sample (mL)}}$$

b~c = 0.1 mL 눈금
c~d = 0.1 mL 눈금
d~e = 0.1 mL 눈금
e~f = 0.1 mL 눈금
눈금선은 적색으로 한다.
b~f = 100 mL



178. Erythritol

Erythrite

Chemical Formula $C_4H_{10}O_4$

Molecular Weight 122.12

Definition Erythritol is obtained by filtering, purifying, crystallizing, and washing the fermented liquid obtained from yeast such as *Moniliella pollinis*, *Trichosporonoides megachilensis* or *Candida lipolytica*(*Yarrowia lipolytica*). Its component is erythritol.

Compositional Specifications of Erythritol

Content Dried Erythritol should contain no less than 99.0% of erythritol ($C_4H_{10}O_4$).

Description Erythritol is scentless white crystalline powder with a sweet taste.

Identification (1) Erythritol is readily soluble in water, slightly soluble in alcohol, and insoluble in ether.

(2) Melting point should be in a temperature range of 119~123°C.

(3) When Erythritol is tested by Assay, the retention time of main peak of Test Solution is identical to that of erythritol standard solution.

Purity (1) Reducing Sugar (as glucose) : 500 mg of Erythritol is precisely weighted and dissolved in 2 ml of water and shaken, where 2 ml of Fehling solution is added. It is then heated to boil and cooled (Test Solution). Separately, 2 ml of Fehling solution is added to 2 ml of glucose solution (containing 0.75 mg of glucose per 1 ml), which is heated to boil and cooled (Standard Solution). When two solutions are compared, precipitates in Test Solution should be less than the reddish brown precipitates in Standard Solution (Not more than 0.3%).

(2) Ribitol and Glycerol : Erythritol is tested by Assay and the contents of ribitol and glycerol are obtained by the following equations. The sum of the contents should not be more than 0.1%. The relative retention times for erythritol, glycerol, and ribitol are 1.0, 1.10, and 0.93, respectively.

$$\text{Ribitol (\%)} = \frac{\text{peak area of ribitol}}{\text{sum of peak areas of erythritol, glycerol, and ribitol}} \times 100$$

$$\text{Glycerol (\%)} = \frac{\text{peak area of glycerol}}{\text{sum of peak areas of erythritol, glycerol, and ribitol}} \times 100$$

(3) Lead : Lead : When 5.0 g of Erythritol is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content

should not be more than 5.0 ppm.

Loss on Drying When Erythritol is dried for 4 hours at 105°C, the loss should not be more than 0.2%.

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 2 g of Erythritol, the amount of Residue on Ignition should not be more than 0.1%.

Assay Approximately 2 g of dried Erythritol is precisely weighted and dissolved in water (total volume = 50 ml) (Test Solution). Separately, 2 g of dried erythritol standard is precisely weighted and dissolved in water (total volume = 50 ml) (Standard Solution). 10 μ l each of Test and Standard Solutions are injected into liquid chromatography under the following Operation Conditions and the content of erythritol is obtained by the following equation.

$$\text{Content (\%)} = \frac{\text{Weight of standard (g)}}{\text{Weight of sample (g)}} \times \frac{\text{peak area of Test Solution}}{\text{peak area of Standard Solution}} \times 100$$

Operation Conditions

- Detector : RI detector
- Column : MCI-CKO8SH, Shodex KC811 or its equivalent
- Column Temperature : 60°C
- Mobile Phase : Water
- Flow Rate : 1.0 ml/min

179. Quercetin

Definition Quercetin is obtained by hydrolyzing rutin with acidic aqueous solution or enzyme. Its major component is quercetin.

Compositional Specifications of Quercetin

Content If Quercetin is converted to a dehydrated form, it should contain no less than 95.0% quercetin ($C_{15}H_{10}O_7$).

Description Quercetin is yellow crystalline powder with slight characteristic scent.

Identification (1) 5 mg of Quercetin dissolve in 10 ml of alcohol. When 1~2 drops of ferric chloride solution (1→50) are added to this solution, a greenish brown band appears.

(2) When 5 mg of Quercetin dissolve in 5ml of sodium hydroxide solution (1→100), it shows yellow~orange in color.

(3) 5 mg of Quercetin dissolve in 5 ml of alcohol. When 2 ml of hydrochloric acid and 0.05 g of magnesium are added, the solution slowly turns red.

(4) A solution of 10 mg of Quercetin in 500 ml alcohol has maximum absorption bands near 255 nm and 370 nm.

Purity (1) Arsenic : Place 0.25 g of Quercetin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Lead : When 5.0 g of Quercetin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Loss on Drying When Quercetin is dried for 2 hours at 135°C, the loss should not be more than 13.0%.

Assay Approximately 50 mg of Quercetin is precisely weighted and dissolved in methyl alcohol (total volume = 50 ml), which is filtered through a 0.5 μ m Millipore filter (Test Solution). Separately, quercetin ($C_{15}H_{10}O_7 \cdot 2H_2O$) standard is precisely weighted so that it contains 50 mg as quercetin and dissolved in methyl alcohol (total volume = 50 ml), which is filtered through a 0.5 μ m Millipore filter (Test Solution). 10 μ l each of Test and Standard Solutions are injected into a high speed liquid

chromatography under the following Operation Conditions and the content of quercetin is obtained by the following equation.

Content (%) =

$$\frac{\text{Weight of standard (as quercetin) (mg)}}{\text{Weight of sample (as dried form) (mg)}} \times \frac{\text{peak area of Test Solution}}{\text{peak area of Standard Solution}} \times 100$$

Operation Conditions

- Detector : UV 375 nm
- Column : μ -Bondapak C₁₈ (3.9 mm \times 300 mm) or its equivalent
- Column Temperature : room temperature
- Mobile Phase : methyl alcohol : water : acetic acid (15 : 3 : 1)
- Flow Rate : 1.0 ml/min

180. Lauric Acid

Decanoic acid

Chemical Formula : $C_{12}H_{24}O_2$

Molecular Weight : 200.32

Definition Lauric Acid is a solid fatty acid obtained from coconut oil and other vegetable oils. Its major component is lauric acid ($C_{12}H_{24}O_2$).

Compositional Specifications of Lauric Acid

Description Lauric Acid is white~pale yellow crystalline solid or powder.

Purity (1) Acid Value : When 0.5 g of Lauric Acid is precisely weighted, and proceeded as directed under Acid value in Fats Test, the Acid value should be 252~287.

(2) Solidification point : Solidification point of Lauric Acid should be 26.0~44.0.

(3) Lead :When 5.0 g of Lauric Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Arsenic : Place 0.25 g of Lauric Acid in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(5) Mercury : When Lauric Acid is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

(6) Iodine Value : Approximately 8.3 g of Lauric Acid is precisely weighted into a 500 ml Erlenmeyer flask with a stopper which contains 20 ml of 1 : 1 mixture of glacial acetic acid : cyclohexane and 25 ml of Weiss solution. A stopper is placed on the flask which is vigorously shaken and set aside for 1 hour in a dark place. 20 ml of potassium iodide solution and 100 ml of water(previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution. 0.1 N sodium thiosulfate solution is added drop wise until yellow color disappears. Starch solution is added and the titration is continued until the blue color disappears completely. Near the end point, the flask is vigorously shaken with

a stopper. Separately, a blank test is carried out by the same procedure. Iodine value is obtained by the following equation and it should not be more than 3.0.

$$\text{Iodine Value} = \frac{(B - S) \times 1.269}{\text{Weight of sample (g)}}$$

B : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

S : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

- (7) Saponification Value : 3 g of Lauric Acid is precisely weighted into a 250 ml flask, where 50 ml of 0.5 N alcoholic solution of potassium hydroxide is added. This solution is used as test solution. When test solution is proceeded as directed under Acid value in Fats Test, the Acid value should be 253~287.
- (8) Unsaponifiable matter : 5 g of Lauric Acid is precisely weighted into a 250 ml flask, where 2g of potassium hydroxide and 40 ml of alcohol are added and gently refluxed for 1 hour with a reflux condenser. The solution transfer into a separatory funnel (3.5 cm diameter x 30 cm length with 40 ml, 80 ml, and 130 ml scale marks) with a stopcock. The flask is washed with sufficient amount of alcohol, which is added to the funnel (total volume = 40 ml). The flask is washed with warm and cold water, which is added to the funnel (total volume = 80 ml). Finally, the flask is washed with a few ml of petroleum ether, which is added to the funnel. Cool the solution, 50 ml of petroleum ether is added to the funnel. The funnel is shaken vigorously for 1 minute and then settled to separate two phases completely. The supernatant ether layer is collected in a 500 ml separatory funnel with a stopcock. The aqueous layer is again extracted 6 times with 50 ml each of ether. These extracts are added to the first extract. The combined extracts are washed with 25 ml of 10% alcohol. This procedure is repeated until the aqueous layer doesn't get colored by phenolphthalein TS. When this is accomplished, aqueous phase is discarded and the ether extract transfer into a pre-weighted beaker. With 10 ml of ether, the funnel is washed, which is added to the beaker. Ether layer is evaporated to dryness in a water bath, which is then dried at 100°C for 30 minutes until the weight becomes constant. Then the residue is cooled in a desiccator and weighted. The residue dissolve in 50 ml of warm alcohol (neutralized with sodium hydroxide using phenolphthalein as an indicator). The resulting solution is titrated with 0.02 N sodium hydroxide solution until a pale red color persists. The amount of oleic acid is obtained by multiplying the consumed amount of sodium hydroxide solution with 5.659(mg). The exact amount of unsaponifiables is obtained by subtracting the

amount of fatty acid (as oleic acid) from the amount of residues. The content of unsaponifiable matter is calculated by the following equation and it should not be more than 0.3%.

Unsaponifiable matter(%) =

$$\frac{\text{Weight of residue (mg)} - \text{Weight of oleic acid (mg)}}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

Water Content Water content of Lauric Acid is determined by water determination (Karl-Fisher Titration) and should not be more than 0.2%

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 10 g of Lauric Acid, the amount of residue should not be more than 0.1%.

181. Stearic Acid

Octadecanoic acid

Chemical Formula : $C_{18}H_{36}O_2$

Molecular Weight : 284.18

Definition Stearic Acid is a solid fatty acid obtained from fats. It consists of a mixture of stearic acid ($C_{18}H_{36}O_2$) and palmitic acid ($C_{16}H_{32}O_2$). Its major component is stearic acid ($C_{18}H_{36}O_2$).

Compositional Specifications of Stearic Acid

Description Stearic Acid is white~pale yellow crystalline solid or powder.

Purity (1) Acid Value : When 0.5 g of Stearic Acid is precisely weighted, and proceeded as directed under Acid value in Fats Test, the Acid value should be 196~211.

(2) Solidification point : Solidification point of Stearic Acid should be 54.5~69.0.

(3) Lead : When 5.0 g of Stearic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Arsenic : Place 0.25 g of Stearic Acid in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(5) Mercury : When Stearic Acid is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

(6) Iodine Value : Approximately 3.6 g of Stearic Acid is precisely weighted into a 500 ml Erlenmeyer flask with a stopper which contains 20 ml of 1 : 1 mixture of glacial acetic acid : cyclohexane and 25 ml of Weiss solution. A stopper is placed on the flask which is vigorously shaken and set aside for 1 hour in a dark place. 20 ml of potassium iodide solution and 100 ml of water (previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution. Sodium thiosulfate solution is added drop wise until yellow color disappears. Starch solution is added and the titration is continued until the blue

color disappears completely. Near the end point, the flask is vigorously shaken with a stopper. Separately, a blank test is carried out by the same procedure. Iodine value is obtained by the following equation and it should not be more than 7.0.

$$\text{Iodine Value} = \frac{(B - S) \times 1.269}{\text{Weight of sample (g)}}$$

B : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

S : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

- (7) Saponification Value : 3 g of Stearic Acid is precisely weighted into a 250 ml flask, where 50 ml of 0.5 N alcoholic solution of potassium hydroxide is added. After attaching a reflux condenser, the solution is saponified for 30~60 minutes. Cool the solution, the condenser is washed with small amount of water and removed. 1 ml of phenolphthalein TS is added. The resulting solution is then titrated with 0.5 N hydrochloric acid. The solution is boiled (red color appears again) and titrated again until the red color disappears. Saponification value is calculated using the following equation and should be 197~212.
- (8) Unsaponifiable matter : 5 g of Stearic Acid is precisely weighted into a 250 ml flask, where 2 g of potassium hydroxide and 40 ml of alcohol are added and gently refluxed for 1 hour with a reflux condenser. The solution transfer into a separatory funnel (3.5 cm diameter x 30 cm length with 40 ml, 80 ml, and 130 ml scale marks) with a stopcock. The flask is washed with sufficient amount of alcohol, which is added to the funnel (total volume = 40 ml). The flask is washed with warm and cold water, which is added to the funnel (total volume = 80 ml). Finally, the flask is washed with a few ml of petroleum ether, which is added to the funnel. Cool the solution, 50 ml of petroleum ether is added to the funnel. The funnel is shaken vigorously for 1 minute and then settled to separate two phases completely. The supernatant ether layer is collected in a 500 ml separatory funnel with a stopcock. The aqueous layer is again extracted 6 times with 50 ml each of ether. These extracts are added to the first extract. The combined extracts are washed with 25 ml of 10% alcohol. This procedure is repeated until the aqueous layer doesn't get colored by phenolphthalein TS. When this is accomplished, aqueous phase is discarded and the ether extract transfer into a pre-weighted beaker. With 10 ml of ether, the funnel is washed, which is added to the beaker. Ether layer is evaporated to dryness in a water bath, which is then dried at 100°C for 30 minutes until the weight becomes constant. Then the residue is cooled in a desiccator and weighted. The residue dissolve in 50 ml of warm alcohol (neutralized with sodium hydroxide

using phenolphthalein as an indicator). The resulting solution is titrated with 0.02 N sodium hydroxide solution until a pale red color persists. The amount of oleic acid is obtained by multiplying the consumed amount of sodium hydroxide solution with 5.659(gm). The exact amount of unsaponifiables is obtained by subtracting the amount of fatty acid (as oleic acid) from the amount of residues. The content of unsaponifiable matter is calculated by the following equation and it should not be more than 1.5%.

Unsaponifiable matter(%) =

$$\frac{\text{Weight of residue (mg)} - \text{Weight of oleic acid (mg)}}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

Water Content Water content of Stearic Acid is determined by water determination (Karl-Fisher Titration) and should not be more than 0.2%

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 2 g of Stearic Acid, the amount of Residue on Ignition should not be more than 0.1%.

182. Palmitic Acid

Hexadecanoic acid

Chemical Formula : $C_{16}H_{32}O_2$

Molecular Weight : 256.43

Definition Palmitic Acid is a solid fatty acid obtained from fats. It consists of a mixture of palmitic acid ($C_{16}H_{32}O_2$) and stearic acid ($C_{18}H_{36}O_2$). Its major component is palmitic acid ($C_{16}H_{32}O_2$).

Compositional Specifications of Palmitic Acid

Description Palmitic Acid is white~pale yellow crystalline solid or powder.

Purity (1) Acid Value : When 0.5 g of Palmitic Acid is precisely weighted, and proceeded as directed under Acid value in Fats Test, the Acid value should be 204~220.

(2) Solidification point : Solidification point of Palmitic Acid should be 53.3~62.0.

(3) Lead : When 5.0 g of Palmitic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Arsenic : Place 0.25 g of Palmitic Acid in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(5) Mercury : When Palmitic Acid is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

(6) Iodine Value : Approximately 12.5 g of Palmitic Acid is precisely weighted into a 500 ml Erlenmeyer flask with a stopper which contains 20 ml of 1 : 1 mixture of glacial acetic acid : cyclohexane and 25 ml of Weiss solution. A stopper is placed on the flask which is vigorously shaken and set aside for 1 hour in a dark place. 20 ml of potassium iodide solution and 100 ml of water (previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution. 0.1 N sodium thiosulfate solution is added drop wise until yellow color disappears. Starch solution is added and the titration is continued until

the blue color disappears completely. Near the end point, the flask is vigorously shaken with a stopper. Separately, a blank test is carried out by the same procedure. Iodine value is obtained by the following equation and it should not be more than 2.0.

$$\text{Iodine Value} = \frac{(B - S) \times 1.269}{\text{Weight of sample (g)}}$$

B : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

S : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

(7) Saponification Value : 3 g of Palmitic Acid is precisely weighted into a 250 ml flask, where 50 ml of 0.5 N alcoholic solution of potassium hydroxide is added. After attaching a reflux condenser, the solution is saponified for 30~60 minutes. This solution is used as test solution, tested under Saponification value in Fats Test, boiled (red color appears again) and titrated again until the red color disappears. Saponification value should be 205~221.

(8) Unsaponifiable matter : 5 g of Palmitic Acid is precisely weighted into a 250 ml flask, where 2 g of potassium hydroxide and 40 ml of alcohol are added and gently refluxed for 1 hour with a reflux condenser. The solution transfer into a separatory funnel (3.5 cm diameter x 30 cm length with 40 ml, 80 ml, and 130 ml scale marks) with a stopcock. The flask is washed with sufficient amount of alcohol, which is added to the funnel (total volume = 40 ml). The flask is washed with warm and cold water, which is added to the funnel (total volume = 80 ml). Finally, the flask is washed with a few ml of petroleum ether, which is added to the funnel. Cool the solution, 50 ml of petroleum ether is added to the funnel. The funnel is shaken vigorously for 1 minute and then settled to separate two phases completely. The supernatant ether layer is collected in a 500 ml separatory funnel with a stopcock. The aqueous layer is again extracted 6 times with 50 ml each of ether. These extracts are added to the first extract. The combined extracts are washed with 25 ml of 10% alcohol. This procedure is repeated until the aqueous layer doesn't get colored by phenolphthalein TS. When this is accomplished, aqueous phase is discarded and the ether extract Transfer into a pre-weighted beaker. With 10 ml of ether, the funnel is washed, which is added to the beaker. Ether layer is evaporated to dryness in a water bath, which is then dried at 100°C for 30 minutes until the weight becomes constant. Then the residue is cooled in a desiccator and weighted. The residue dissolve in 50 ml of warm alcohol (neutralized with sodium hydroxide using phenolphthalein as an indicator). The resulting solution is titrated with 0.02N sodium hydroxide solution until a pale red color persists. The amount of oleic acid

is obtained by multiplying the consumed amount of sodium hydroxide solution with 5.659 mg. The exact amount of unsaponifiables is obtained by subtracting the amount of fatty acid (as oleic acid) from the amount of residues. The content of unsaponifiable matter is calculated by the following equation and it should not be more than 1.5%.

Unsaponifiable matter(%) =

$$\frac{\text{Weight of residue (mg)} - \text{Weight of oleic acid (mg)}}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

Water Content Water content of Palmitic Acid is determined by water determination (Karl-Fisher Titration) and should not be more than 0.2%.

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 2 g of Palmitic Acid, the amount of residue should not be more than 0.1%.

183. Pancreatin

Definition Pancreatin is obtained by extracting pancreas of cows or pigs. It is an enzyme that can decompose starches, fats, and proteins. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Pancreatin

Content Pancreatin contains 90~130% of each indicated activity of amylase, lipase, and protease.

Description Pancreatin is white~pale yellow powder with a characteristic scent.

Purity (1) Heavy Metals : Carbonize 0.667 g of Pancreatin by heating mildly in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. When this solution tested for Heavy Metals, the content should not be more than 30ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water are added to bring the total volume to 20 ml.

(2) Lead : 1.6 g of Pancreatin is mildly carbonized by heating, which is reduced ash by further heat treatment at a temperature below 500°C. Carefully 20 ml of dilute nitric acid is added to the ash. Boil the sample for 5 minutes and cool. It is filtered (if necessary). The residue is washed with water, which is then added to the filtrate. Water is added so that total volume of this solution becomes 50 ml. This test solution is tested for lead. The detected amount of lead should not be more than 5ppm.

(3) Coliform Group : Pancreatin is tested by Microbiological Methods for Coliform Group in General Testing Methods in Food Code. It should contain 30 or less per

1g of this product.

- (4) Salmonella : Pancreatin is tested by Microbiological Methods for Salmonella in General Testing Methods in Food Code. It should be negative (-).

Assay (Activity)

(1) Activity of Amylase

Application and Principle : This test is to measure the activity of amylase in an enzyme that is obtained from pancreas of cows and pigs.

- Preparation of Test Solution : When the amylase activity of Pancreatin is same as that of USP pancreatin standard, 40 mg of this additive is precisely weighted into a mortar, where 3 ml of phosphate buffer solution (pH 6.8) is added. It is then ground for 5~10 minutes and diluted to 100 ml with phosphate buffer solution (pH 6.8) (Test Solution). When the amylase activities are different, Test Solution is prepared (following the same procedure) so that 1 ml of the final dilution contains the same activity as 1 ml of Standard Solution.
- Test Procedure : Four 250 ml round bottom flasks with stoppers are labeled as S, U, BS, and BU, respectively. To each flask, 25 ml of substrate solution, 10 ml of phosphate buffer solution (pH 6.8), and 1 ml of sodium chloride solution (11.7→1,000) are added. A stopper is placed on each flask, which is then mixed and set aside in a $25 \pm 0.1^\circ\text{C}$ until it is isothermalized. 2 ml each of 1 N hydrochloric acid is added to BU & BS, which are well mixed and placed in the water bath. 1 ml each of Test Solution is added to U & BU and 1 ml each of Standard Solution is added to S & BS, which are well mixed and placed in the water bath. After exactly 10 minutes, 2 ml each of 1N hydrochloric acid is added to S & U. While stirring continuously, 10ml each of 1N iodine solution is added to each flask, where 45 ml each of 0.1 N sodium hydroxide solution is immediately added. The flasks are set aside for 15 minutes at $15 \sim 25^\circ\text{C}$ in a dark place. After adding 4 ml each of 2 N sulfuric acid to each flask, it is titrated with 0.1 N sodium thiosulfate solution until blue color disappears. The consumed amounts of 0.1 N sodium thiosulfate solution for U, S, BU, and BS are V_U , V_S , V_{BU} , and V_{BS} , respectively.

Amylase activity of an enzyme is obtained by the following equation.

$$\text{Amylase activity(USP Units/mg)} = \frac{C_s}{W_u} \times \frac{(V_{BU} - V_U U)}{(V_{BS} - V_S)} \times 100$$

C_s : Amylase activity of Standard Solution (USP units/ml)

W_u : Amount of sample (mg)

Solutions

- Standard Solution : 20 mg of USP pancreatin standard is precisely weighted into a mortar, where 30 ml of phosphate buffer solution (pH 6.8) is added. It is ground for 5~10 minutes and diluted to 50 ml with phosphate buffer solution (pH 6.8) (Standard Solution). USP Units of amylase per 1 ml of this solution is calculated.
- Phosphate Buffer Solution (pH 6.8)
Solution 1 : 13.6 g of potassium phosphate, monobasic dissolve in 500 ml of water.
Solution 2 : 14.2 g of sodium phosphate, dibasic dissolve in 500 ml of water. 51 ml of Solution 1 and 49 ml of Solution 2 are well mixed. pH is adjusted to 6.8, if necessary.
- Substrate Solution : 10 ml of water is added to purified soluble starch (2.0 g as a dried form) and stirred. 160 ml of water is added to this mixture, which is continuously stirred and heated to boil. Cool the solution, water is added to bring the volume to 200 ml. This solution is freshly prepared before use.

(2) Activity of Lipase

Application and Principle : This test is to measure the activity of lipase in an enzyme that is obtained from pancreas of cows and pigs.

- Preparation of Test Solution : 200 mg of Pancreatin is precisely weighted into a mortar. It is ground for 10 minutes with 3 ml of water. It is diluted so that 1 ml of the final dilution contains 8~16 USP units of lipase (Test Solution).
- Test Procedure : 10.0 ml of substrate solution, 8.0 ml of tris buffer solution, 2.0 ml of sodium tauro-cholate solution, and 9 ml of water are added to a 50 ml receiving container with a cap and mixed. It is capped and continuously stirred in a water bath with a temperature controller. The mixture is isothermalized at $37 \pm 0.1^{\circ}\text{C}$ in the water bath. pH of the mixture is adjusted to 9.2 with 0.1 N sodium hydroxide solution. 1.0 ml of Test Solution is added to the mixture, which is titrated with 0.1 N sodium-hydroxide solution for 5 minutes until pH reaches 9.0 and consumed amount of 0.1 N sodium hydroxide solution (ml) per minute is recorded. Separately, the same procedure is carried out with Standard Solution and a titration curve of 0.1 N sodium hydroxide solution consumption (ml) vs. time (minute) is prepared. Using only the straight line region of the titration curve, 0.1N sodium hydroxide solution consumption rate (ml/min) for Test Solution is obtained.

Activity of lipase is obtained by the following equation.

$$\text{Activity of lipase (USP Units/mg)} = \frac{A \times V}{W}$$

A : Activity of lipase per 1 ml of Standard Solution (USP Units/ml)

V : Consumption rate for Test Solution corresponding to straight line region of standard titration curve (ml/min)

W : Amount of sample contained in 1 ml of 1 ml Test Solution (mg)

Solutions

- Standard Solution : 200 mg of USP pancreatin standard is precisely weighted into a mortar. It is ground for 10 minutes with 3 ml of water. It is diluted with cold water so that 1 ml of the final dilution contains 8~16 UPS units of lipase (Standard Solution). This suspension is maintained at 4°C and mixed prior use. 5~15 ml of this cold suspension is warmed to 20°C just before use to for accurate measurement of volume.
- Arabic gum Solution : Arabic gum solution (1→10) is centrifuged until it becomes clear. Only the clear solution is used.
- Substrate Solution : 165 ml of Arabic gum solution, 20 ml of olive oil, and 15 g of ice are mixed using a homogenizer. The mixture is cooled to 5°C in an ice bath. It is then homogenized for 15 minutes.
- Tris Buffer Solution : 60 mg of tris(hydroxymethyl)aminomethane and 234 mg of sodium chloride dissolve in water (total volume = 100ml).
- Sodium tauro-cholate Solution : A solution is prepared so that it contains 80.0 mg of USP sodium tauro-cholate standard per 1 ml.

(3) Activity of Protease

- Application and Principle : This test is to measure the activity of protease in an enzyme that is obtained from pancreas of cows and pigs.
- Preparation of Test Solution : 100 mg of Pancreatin is precisely weighted into a mortar. It is ground for 5~10 minutes with 3 ml of phosphate buffer solution (pH 7.5). It is diluted so that 1 ml of the final dilution contains 2.5 UPS units of lipase (Test Solution).
- Test Procedure : Test tubes are labeled as S₁, S₂, and S₃ for standards and U for enzyme test. 2.0, 1.5, 1.5, 1.0 ml of phosphate buffer solution (pH 7.5) are added to S₁, S₂, U, and S₃, respectively. 5.0 ml each of trichloro acetic acid solutions added to each test tube, which is labeled as S_{1B}, S_{2B}, S_{3B} and U_B, respectively. Separately, for a blank test, 5 ml of trichloro acetic acid solution and 3ml of phosphate buffer solution (pH 7.5) are mixed in a test tube (labeled as B). All the test tubes (with stirring

glass rods) are isothermalized in a 40°C water bath. 2.0 ml each of substrate solution (isothermalized at 40°C) is added to each test tube at a regular time interval. After 60 minutes, the reaction is stopped by adding 5.0 ml each of trichloro acetic acid solution to S₁, S₂, S₃, and U. All the test tubes are removed from the water bath. They are set aside for 10 minutes at room temperature until proteins are settled down and filtered. Absorption of the completely clear supernatant of each solution is measured at 280 nm with 1cm path length using blank test solution B as a reference. Absorption values are corrected by respectively subtracting absorptions of S_{1B}, S_{2B}, and S_{3B} filtrates from those of S₁, S₂, and S₃ filtrates. A standard curve is prepared for the corrected absorption of each standard solution vs. its concentration. A concentration of corrected Test Solution (U-U_B) is obtained from corrected standard curve.

Enzyme activity of protease is obtained by the following equation.

$$\text{Activity of Protease (USP units/mg)} = A \times C \times \frac{10}{W}$$

A : Activity of protease of standard material (USP Units/mg)

C : Concentration of standard material corresponding to enzyme Test Solution obtained from the standard curve (mg/ml)

W : Amount of sample in 1.5 ml of Test Solution (mg)

Solutions

- Standard Solution : 100 mg of USP pancreatin standard is precisely weighted and dissolved in phosphate buffer solution (pH 7.5) so that the total volume is 100ml, which is set aside for 25 minutes at room temperature. This solution is diluted with phosphate buffer solution (pH 7.5) so that 1 ml of the final dilution contains 2.5 UPS Units of protease activity (Standard Solution).
- Phosphate Buffer Solution (pH 7.5) : 6.8 g of potassium phosphate monobasic, and 1.8g of sodium hydroxide are dissolved in 950 ml of water. pH of this solution is adjusted to pH 7.5 ± 0.2 with 0.2 N sodium hydroxide solution. It is then further diluted to 1,000 ml. This solution is stored in a refrigerator.
- Substrate Solution : 1.25 g of casein is well dispersed in 5 ml of water, where 10 ml of 0.1 N sodium hydroxide solution is added. After shaking for 1 minute, 50 ml of water is added and the resulting solution is shaken for approximately 1 hour. pH of this solution is adjusted

to 8.0 ± 0.1 using 1 N sodium hydroxide solution or 1 N hydrochloric acid. It is then diluted to 100 ml with water. This solution is freshly prepared before use.

◦ Trichloroacetic acid solution : 50 g of trichloroacetic acid dissolve in water to make total volume = 1,000 ml. This solution is stored at room temperature.

-Filter Paper : 5 ml of trichloroacetic acid solution is filtered through a filter paper. Absorption of the filtrate is measured at 280 nm with 1cm path length using unfiltered trichloroacetic acid solution as a reference. The absorption should not be more not more than 0.04. If it is higher, the filter paper is washed with trichloroacetic acid solution until it becomes should not be more than 0.04.

Stotage standard of Pancreatin

Pancreatin should be stored in a hermetic container in a cold dark place.

184. Ferulic Acid

Chemical Formula $C_{10}H_{10}O_4$

Molecular Weight 194.18

Definition Ferulic acid is obtained by the following procedure. Rice bran oil from rice (*Oryza sativa* LINNE) of gramineae is fractioned with hydrated ethyl alcohol and hexane at room temperature. γ -Oryzanol, which is obtained from the fraction of ethyl alcohol, is hydrolyzed with hot sulfuric acid and purified. Its major component is ferulic acid.

Compositional Specifications of Ferulic Acid

Content If Ferulic acid is converted to a dehydrated form, it should contain not less than 98.0% ferulic acid ($C_{10}H_{10}O_4 = 194.18$).

Description Ferulic acid is white~pale yellowish brown crystalline powder with slight characteristic or no scent.

Identification (1) A solution of Ferulic acid in ethyl alcohol (1→100,000) has maximum absorption bands at 234~238 nm and 320~324 nm.

(2) When 0.01 g of Ferulic acid dissolve in 10 ml of 10% alcoholic solution of potassium hydroxide by heating, it becomes yellow in color.

(3) 0.01 g of Ferulic acid dissolve in 2 ml of acetone. When 0.1 ml of a solution of ferric chloride in ethyl alcohol (1→50) is added to this solution, it becomes reddish brown in color.

Purity (1) Arsenic : Place 0.25 g of Ferulic acid in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Ferulic acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

Loss on Drying When 1 g of Ferulic acid is dried for 3 hours at 105°C, the loss weight should not be more than 0.5%.

Residue on Ignition When Residue on Ignition analysis is done with accurately

weighted 5 g of Ferulic acid, the amount of Residue on Ignition should not be more than 0.1%.

Assay Approximately 0.5 g of Ferulic acid is precisely weighted and dissolved in 50 ml of 50 v/v% ethyl alcohol solution by heating in a water bath. Cool the solution, it is titrated with 0.1 N sodium hydroxide solution. Separately, a blank test is carried out by the same method.

0.1 N sodium hydroxide solution 1 ml = 19.418 mg $C_{10}H_{10}O_4$

185. Castor oil

Definition Castor oil is nonvolatile oil obtained from seeds of castor-oil plant (*Ricinus communis* L.) of euphorbiaceae. It is a triglyceride mainly consisting ricinoleic acid.

Compositional Specifications of Castor oil

Description Castor oil is almost colorless or pale yellow viscous liquid.

Identification (1) Castor oil is soluble in 95% alcohol, miscible in anhydrous alcohol, and slightly soluble in petroleum ether.

(2) Specific gravity should be 0.952~0.966.

(3) Refractive Index $[n]_D^{20}$ should be 1.477~1.481.

Purity (1) Arsenic : Place 0.25 g of Castor oil in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Castor oil is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(3) Acid Value : 5.0 g of Castor oil is precisely weighted and dissolved in approximately 50 ml of alcohol (neutralized with 0.1 N potassium hydroxide solution using phenolphthalein TS) or a mixture of alcohol and ether (1 : 1) (heated if necessary). When this test solution is proceeded as directed under Acid value in Fats Test, the value should not be more than 2.

(4) Hydroxyl Value : 1.5 g of Castor oil is precisely weighted into a 250 ml flask and dissolved by adding 5 ml mixture of pyridine·anhydrous acetic acid (3:1 mixture of freshly distilled pyridine and anhydrous acetic acid). Separately, 5 ml mixture of pyridine·anhydrous acetic acid is added to a 250 ml flask as a blank test. A reflux condenser is attached to each flask. It is then heated for 1 hour in a water bath. 10 ml of water is added through the condenser and it is heated again for 10 minutes. After cooling to room temperature, 15 ml of n-butyl alcohol (neutralized with 0.5 N alcoholic potassium hydroxide solution using phenolphthalein TS) is added through the condenser, the condenser is removed, and inner wall of each flask is washed with 10 ml of n-butyl alcohol. 1 ml of phenolphthalein TS is added to each flask

and each solution is titrated with 0.5 N alcoholic solution of potassium hydroxide until it becomes pale red. The consumed amount (ml) of alcoholic solution is S and B for sample and blank test, respectively. Separately, free acid is corrected by the following procedure. 10 g of sample is precisely weighted and dissolved in 10 ml of pyridine (neutralized using phenolphthalein TS and freshly distilled). 1 ml of phenolphthalein TS is added to this solution, which is titrated with 0.5 N alcoholic solution of potassium hydroxide until it becomes red. The consumed amount (ml) of alcoholic solution is A. Hydroxyl value, that is calculated by the following equation, should be 160~168.

$$\text{Hydroxyl Value} = [B + \left(\frac{WA}{C}\right) - S] \times \frac{28.05}{W}$$

W : Weight of sample for acetylation (g)

C : Weight of sample for free acid measurement (g)

- (5) Saponification Value : 3 g is precisely weighted into a 250 ml flask. After adding 50 ml of 0.5 N alcoholic solution of potassium hydroxide, a reflux condenser is attached and quietly saponified for 30 minutes~1 hour. This solution is used as test solution, tested under Saponification value in Fats Test, boiled (red color appears again) and titrated again until the red color disappears. Saponification value should be 176~185.
- (6) Iodine Value : Approximately 300 mg of Castor oil is precisely weighted into a 500 ml Erlenmeyer flask with a stopper which contains 20 ml of 1 : 1 mixture of glacial acetic acid : cyclohexane and 25 ml of Weiss solution. A stopper is placed on the flask which is vigorously shaken and set aside for 1 hour in a dark place. 20 ml of potassium iodide solution and 100 ml of water (previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution. Sodium thiosulfate solution is added drop wise until yellow color disappears. Starch solution is added and the titration is continued until the blue color disappears completely. Near the end point, the flask is vigorously shaken with a stopper. Separately, a blank test is carried out by the same procedure. Iodine value is obtained by the following equation and it should be 83~88.

$$\text{Iodine Value} = \frac{(B - S) \times 1.269}{\text{Weight of sample (g)}}$$

B : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

S : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

186. Myristic Acid

Tetradecanoic acid

Chemical Formula $C_{14}H_{28}O_2$

Molecular Weight 228.38

Definition Myristic Acid is a solid fatty acid obtained from coconut oil and other fats. Its major component is myristic acid ($C_{14}H_{28}O_2$).

Compositional Specifications of Myristic Acid

Description Myristic Acid is white~pale yellow crystalline solid or powder.

- Purity**
- (1) Acid Value : When 0.5 g of Myristic Acid is precisely weighted, and proceeded as directed under Acid value in Fats Test, the Acid value should be 242~249.
 - (2) Solidification point : Solidification point of Myristic Acid should be 48.0~55.5.
 - (3) Lead : When 5.0 g of Myristic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.
 - (4) Arsenic : Place 0.25 g of Myristic Acid in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
 - (5) Mercury : When Myristic Acid is tested by Mercury Limit Test, its content should not be more than 1.0ppm.
 - (6) Iodine Value : Approximately 25 g of Myristic Acid is precisely weighted into a 500 ml Erlenmeyer flask with a stopper which contains 20 ml of 1 : 1 mixture of glacial acetic acid : cyclohexane and 25 ml of Weiss solution. A stopper is placed on the flask which is vigorously shaken and set aside for 1 hour in a dark place. 20 ml of potassium iodide solution and 100 ml of water(previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution. 0.1 N sodium thiosulfate solution is added drop wise until yellow color disappears. Starch solution is added and the titration is continued until the blue color disappears completely. Near the end point, the flask is vigorously

shaken with a stopper. Separately, a blank test is carried out by the same procedure. Iodine value is obtained by the following equation and it should not be more than 1.0.

$$\text{Iodine Value} = \frac{(B - S) \times 1.269}{\text{Weight of sample (g)}}$$

B : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

S : Consumed amount of 0. 1N sodium thiosulfate solution in the test for sample (ml)

- (7) Saponification Value : 3 g of Myristic Acid is precisely weighted into a 250 ml flask. After adding 50 ml of 0.5 N alcoholic solution of potassium hydroxide, a reflux condenser is attached and quietly saponified for 30 minutes~1 hour. This solution is used as test solution, tested under Saponification value in Fats Test, boiled (red color appears again) and titrated again until the red color disappears. Saponification value should be 242~251.
- (8) Unsaponifiable matter : 5 g of Myristic Acid is precisely weighted into a 250 ml flask, where 2 g of potassium hydroxide and 40 ml of alcohol are added. After attaching a reflux condenser, gently refluxed for 1 hour. The solution transfer into a separatory funnel (3.5 cm diameter x 30 cm length with 40 ml, 80 ml, and 130 ml scale marks) with a stopcock. The flask is washed with sufficient amount of alcohol, which is added to the funnel (total volume= 40 ml). The flask is washed with warm and cold water, which is added to the funnel (total volume = 80 ml). Finally, the flask is washed with a few ml of petroleum ether, which is added to the funnel. Cool the solution, 50 ml of petroleum ether is added to the funnel. The funnel is shaken vigorously for 1 minute and then settled to separate two phases completely. The supernatant ether layer is collected in a 500 ml separatory funnel with a stopcock. The aqueous layer is again extracted 6 times with 50 ml each of ether. These extracts are added to the first extract. The combined extracts are washed with 25 ml of 10% alcohol. This procedure is repeated until the aqueous layer doesn't get colorized by phenolphthalein TS. When this is accomplished, aqueous phase is discarded and the ether extract transfer into a pre-weighted beaker. With 10 ml of ether, the funnel is washed, which is added to the beaker. Ether layer is evaporated to dryness in a water bath, which is then dried at 100°C for 30 minutes until the weight becomes constant. Then the residue is cooled in a desiccator and weighted. The residue dissolve in 50 ml of warm alcohol (neutralized with sodium hydroxide using phenolphthalein as an indicator). The resulting solution

is titrated with 0.02 N sodium hydroxide solution until a pale red color persists. The amount of oleic acid is obtained by multiplying the consumed amount of sodium hydroxide solution with 5.659 (mg). The exact amount of unsaponifiables is obtained by subtracting the amount of fatty acid (as oleic acid) from the amount of residues. The content of unsaponifiable matter is calculated by the following equation and it should be 1%.

Unsaponifiable matter(%) =

$$\frac{\text{Weight of residue (mg)} - \text{Weight of oleic acid (mg)}}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

Water Content Water content of Myristic Acid proceed as directed under water determination (Karl-Fisher Titration) and the content should not be more than 0.2%.

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 2 g of Myristic Acid, the amount of Residue on Ignition should not be more than 0.1%.

187. Oleic Acid

(z)-9-Octadecanoic acid

Chemical Formula $C_{18}H_{34}O_2$

Molecular Weight 282.47

Definition Oleic Acid is a unsaturated fatty acid obtained from fats. Its major component is oleic acid ($C_{18}H_{34}O_2$).

Compositional Specifications of Oleic Acid

Description Oleic Acid is colorless~pale yellow oily liquid.

- Purity**
- (1) Acid Value : When 0.5 g of Oleic Acid is precisely weighted, and proceeded as directed under Acid value in Fats Test, the Acid value should be 196~204.
 - (2) Solidification point : Solidification point of Oleic Acid should not be more than 10°C.
 - (3) Lead : When 5.0 g of Oleic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.
 - (4) Arsenic : Place 0.25 g of Oleic Acid in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
 - (5) Mercury : When Oleic Acid is tested by Mercury Limit Test, its content should not be more than 1.0ppm.
 - (6) Iodine Value : Approximately 0.3 g of Oleic Acid is precisely weighted into a 500 ml Erlenmeyer flask with a stopper which contains 20 ml of 1 : 1 mixture of glacial acetic acid : cyclohexane and 25 ml of Weiss solution. A stopper is placed on the flask which is vigorously shaken and set aside for 1 hour in a dark place. 20 ml of potassium iodide solution and 100 ml of water(previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution. 0.1 N sodium thiosulfate solution is added drop wise until yellow color disappears. Starch solution is added and the titration is continued until the blue color disappears completely. Near the end point, the flask is vigorously shaken with

a stopper. Separately, a blank test is carried out by the same procedure. Iodine value is obtained by the following equation and it should be 83~103.

$$\text{Iodine Value} = \frac{(B - S) \times 1.269}{\text{Weight of sample (g)}}$$

B : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

S : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

- (7) Saponification Value : 3 g of Oleic Acid is precisely weighted into a 250 ml flask, where 50 ml of 0.5 N alcoholic solution of potassium hydroxide is added. After attaching a reflux condenser, the solution is saponified for 30~60 minutes. This solution is used as test solution, tested under Saponification value in Fats Test, boiled (red color appears again) and titrated again until the red color disappears. Saponification value should be 196~206.
- (8) Unsaponifiable matter : 5 g of Oleic Acid is precisely weighted into a 250 ml flask, where 2 g of potassium hydroxide and 40 ml of alcohol are added and gently refluxed for 1 hour with a reflux condenser. The solution transfer into a separatory funnel (3.5 cm diameter \times 30 cm length with 40 ml, 80 ml, and 130 ml scale marks) with a stopcock. The flask is washed with sufficient amount of alcohol, which is added to the funnel (total volume = 40 ml). The flask is washed with warm and cold water, which is added to the funnel (total volume = 80 ml). Finally, the flask is washed with a few ml of petroleum ether, which is added to the funnel. Cool the solution, 50 ml of petroleum ether is added to the funnel. The funnel is shaken vigorously for 1 minute and then settled to separate two phases completely. The supernatant ether layer is collected in a 500 ml separatory funnel with a stopcock. The aqueous layer is again extracted 6 times with 50 ml each of ether. These extracts are added to the first extract. The combined extracts are washed with 25 ml of 10% alcohol. This procedure is repeated until the aqueous layer doesn't get colorized by phenolphthalein TS. When this is accomplished, aqueous phase is discarded and the ether extract transfer into a pre-weighted beaker. With 10 ml of ether, the funnel is washed, which is added to the beaker. Ether layer is evaporated to dryness in a water bath, which is then dried at 100°C for 30 minutes until the weight becomes constant. Then the residue is cooled in a desiccator and weighted. The residue dissolve in 50 ml of warm alcohol (neutralized with sodium hydroxide using phenolphthalein as an indicator). The resulting solution is titrated with 0.02 N sodium hydroxide solution until a pale red color persists. The amount

of oleic acid is obtained by multiplying the consumed amount of sodium hydroxide solution with 5.659 (mg). The exact amount of unsaponifiables is obtained by subtracting the amount of fatty acid (as oleic acid) from the amount of residues. The content of unsaponifiable matter is calculated by the following equation and it should not be more than 2.0%.

Unsaponifiable matter(%) =

$$\frac{\text{Weight of residue (mg)} - \text{Weight of oleic acid (mg)}}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

Water Content Water content of Oleic Acid proceed as directed under water determination (Karl-Fisher Titration) and should not be more than 0.2%..

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 10 g of Oleic Acid, the amount of residue on Ignition should not be more than 0.01%.

188. Capric Acid

Chemical Formula : $C_{10}H_{20}O_2$

Molecular Weight : 172.27

Definition Capric acid is a saturated fatty acid obtained from fat and its main ingredient is capric acid ($C_{10}H_{20}O_2$).

Composition Specifications of Capric Acid

Description Capric acid is a white crystal with characteristic smell.

Purity (1) Acid Value : When 0.5 g of Capric acid is precisely weighted, and proceeded as directed under Acid value in Fats Test, the Acid value should be 320~329.

(2) Solidification point : The Solidification point of Capric acid is $27.0 \sim 32.0^{\circ}\text{C}$

(3) Lead : When 5.0 g of Capric acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Arsenic : Place 0.25 g of Capric acid in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at $450 \sim 550^{\circ}\text{C}$. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at $450 \sim 550^{\circ}\text{C}$. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4 ppm.

(5) Mercury : When Capric acid is tested by Mercury Limit Test, its content should not be more than 1.0 ppm.

(6) Iodine Value : Approximately 5.9 g of Capric acid is precisely weighted into a 500 ml Erlenmeyer flask with a stopper which contains 20 ml of 1 : 1 mixture of glacial acetic acid : cyclohexane and 25 ml of Weiss solution. A stopper is placed on the flask which is vigorously shaken and set aside for 1 hour in a dark place. 20 ml of potassium iodide solution and 100 ml of water (previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution. 0.1 N sodium thiosulfate solution is added drop wise until yellow color disappears. Starch solution is added and the titration is continued until the blue color disappears completely. Near the end point, the flask is vigorously shaken with a stopper. Separately, a blank test is carried out by the same

procedure. Iodine value is obtained by the following equation and it should not be more than 0.6.

$$\text{Iodine Value} = \frac{(B - S) \times 1.269}{\text{Weight of Sample (g)}}$$

B : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

S : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

- (7) Saponification Value : 3 g of Capric acid is precisely weighted into a 250 ml flask, where 50 ml of 0.5 N alcoholic solution of potassium hydroxide is added. After attaching a reflux condenser, the solution is saponified for 30~60 minutes. This solution is used as test solution, tested under Saponification value in Fats Test, boiled (red color appears again) and titrated again until the red color disappears. Saponification value should be 320~331.
- (8) Unsaponifiable Matter : 5 g of Capric acid is precisely weighted into a 250 ml flask, where 2 g of potassium hydroxide and 40 ml of alcohol are added and gently refluxed for 1 hour with a reflux condenser. The solution transfer into a separatory funnel (3.5 cm diameter \times 30 cm length with 40 ml, 80 ml, and 130 ml scale marks) with a stopcock. The flask is washed with sufficient amount of alcohol, which is added to the funnel (total volume = 40 ml). The flask is washed with warm and cold water, which is added to the funnel (total volume = 80 ml). Finally, the flask is washed with a few ml of petroleum ether, which is added to the funnel. Cool the solution, 50 ml of petroleum ether is added to the funnel. The funnel is shaken vigorously for 1 minute and then settled to separate two phases completely. The supernatant ether layer is collected in a 500 ml separatory funnel with a stopcock. The aqueous layer is again extracted 6 times with 50 ml each of ether. These extracts are added to the first extract. The combined extracts are washed with 25 ml of 10% alcohol. This procedure is repeated until the aqueous layer doesn't get colorized by phenolphthalein TS. When this is accomplished, aqueous phase is discarded and the ether extract transfer into a pre-weighted beaker. With 10 ml of ether, the funnel is washed, which is added to the beaker. Ether layer is evaporated to dryness in a water bath, which is then dried at 100°C for 30 minutes until the weight becomes constant. Then the residue is cooled in a desiccator and weighted. The residue dissolve in 50 ml of warm alcohol (neutralized with sodium hydroxide using phenolphthalein as an indicator). The resulting solution is titrated with 0.02 N sodium hydroxide solution until a pale red color persists. The amount

of oleic acid is obtained by multiplying the consumed amount of sodium hydroxide solution with 5.659 (mg). The exact amount of unsaponifiables is obtained by subtracting the amount of fatty acid (as oleic acid) from the amount of residues. The content of unsaponifiable matter is calculated by the following equation and it should not be more than 0.2%.

Unsaponifiable matter(%) =

$$\frac{\text{Weight of residue (mg)} - \text{Weight of oleic acid (mg)}}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

Water Content Water content of Capric acid proceed as directed under water determination (Karl-Fisher Titration) and should not be more than 0.2%..

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 10 g of Capric acid, the amount of residue on Ignition should not be more than 0.1%.

189. Dextran

Definition Dextran is obtained by separating from culture medium of gram positive bacteria (*Leuconostoc mesenteroides*, *Streptococcus bovis* ORLA-JENSEN) and its main ingredient is dextran.

Composition Specifications of Dextran

Description Dextran is a white ~ pale yellow powder or solid without smell.

Identification When 2 ml of anthrone is added to 1 ml of the aqueous solution (1→3000) of Dextran, greenish blue appears, which becomes slowly to dark blue. Again 1 ml of sulfuric acid (1→2) or 1 ml of acetic acid is added, and then the color does not change.

Purity (1) Arsenic : Place 0.5 g of Dextran in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Dextran is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Total Viable Aerobic Count : When Dextran is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 5,000 per 1 g

(4) E. Coli : When 25 g of Dextran is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

(5) Total nitrogen : When 0.5 g of Dextran is precisely weighed, proceed as directed under Kjeldahl Method in Nitrogen Determination, the amount should not be more than 1.0%.

Residue on Ignition When Dextran proceed as directed under Residue on Ignition, the amount should not be more than 2.0%.

Loss on Drying When Dextran is dried at 105°C for 5 hours, the loss of Dextran should not be more than 10.0%.

Total viable aerobic count When Dextran proceed as directed under Total viable aerobic count Test of the General Testing Methods, Food Code, the amount of Dextran should not be more than 10,000 per 1 g.

190. Tea Catechin

Definition Tea Catechin is obtained by extracting from leaves or stems of *Camellia sinensis* O. KZE with water or ethyl alcohol and then purifying, or by extracting them with hot water and then separating with methanol or ethyl acetate, and its main ingredient is catechin.

Composition Specifications of Tea Catechin

Content Tea Catechin, when calculated on the anhydrous basis, should be 70~110% as catechin.

Description Tea Catechin is a white, pale yellow~dark brown powder, paste, or liquid with characteristic smell.

Identification (1) When 0.1 g of Tea Catechin dissolve in 10 ml of 50% ethyl alcohol and 2~3 drops of ferric chloride (1→50) are added, the solution becomes greenish purple~dark purple.

(2) The aqueous solution of Tea Catechin exhibits absorption maximum at a wavelength of 265~280 nm.

Purity (1) Arsenic : Place 0.25 g of Tea Catechin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Tea Catechin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Residual Solvent : 1g of Tea Catechin is precisely weighed into a sample vial, 5μl of water is added, and seal it quickly with a septum, test solution. Proceed headspace-gas chromatography under operation conditions below and measure each amount of acetone and ethyl acetate from each calibration curve, it should be not more than 25ppm as individual or sum if used together

Operation Condition

Column : HP-1 or its equivalent

Detector : Hydrogen Flame Ionization Detector (FID)

Injection Port Temperature : 110°C

Column Temperature : 40°C

Detector Temperature : 110°C

Carrier gas : Nitrogen or helium

Head space sampler

Heating temperature : 80°C

Heating time : 40 minutes

Sample gas injection : 0.4ml

Mixed standard solution : 1 g of acetone and 1 g of ethyl acetate is precisely weighed into each flask, water is added to make 100 ml. 2, 20, 40 ml each of this solution is taken, water is added to make 100 ml, each mixed standard solution. (1 ml of each mixed standard solution contains 200, 2,000, 4,000µg of acetone and ethyl acetate, respectively).

Preparation of calibration curve : 1 g of tannic acid, free of acetone and ethyl acetate, is precisely weighed into a vial, 5µl each of 200, 2,000, 4,000ppm of mixed standard solution is added respectively, and seal it quickly with a septum. Proceed headspace-gas chromatography under operation conditions below and measure the peak area of acetone and ethyl acetate. From the peak area, prepare each calibration curve.

Loss on Drying When Tea Catechin is dried at 100°C for 2 hours, the loss should not be more than 5%. (However, this applies only to powder products).

Assay 0.5 g of sample is precisely weighted and water content(W%) is measured. The amount that corresponds to about 30 mg of catechin of Tea Catechin is weighted, to which water is added. If necessary, it is heated for dissolution. The volume is made precisely 100 ml by adding water. To 5 ml of this solution, 5 ml of ferrous tartarate solution is added and then phosphate buffer (pH 7.5) is added to make precisely 25 ml for the test solution. With water as reference solution, absorbance is measure at 540 nm. Separately, the standard solution containing 5, 10, 15, 20, 25 mg of ethyl gallate (standard) are made. Using 5 ml of each of these standard solution and water, the same procedure as the test solution is performed to generate color. Then at 540 nm, absorbance is measured to determine the standard curve. From the absorbance of the test solution and the standard curve, the content (mg) of catechin in 100 ml of the test solution is determined, according to the following formula.

$$\text{Catechin Content (\%)} = \frac{C \times 1.5 \times 100}{\text{Weight of sample (mg)} \times (100 - W)} \times 100$$

C : Concentration (mg/100 ml) of ethyl gallate in the test solution obtained from the standard solution

1.5 : Absorption of Ethyl Gallate 1mg corresponds to the absorption of tea catechin 1.5 mg.

W : Water content (%)

191. Nitrogen

Chemical Formula N_2
Molecular Weight 28.00

Composition Specifications of Nitrogen

Content Nitrogen should not contain less than 99.0% of nitrogen.

Description Nitrogen is a colorless, odorless gas or liquid.

Identification The flame of burning wood splinter is extinguished in an atmosphere of nitrogen.

Purity (1) Oxygen : The oxygen detector whose scale is in the range of 0~100 μL and which is attached with electrochemical cell is used. The oxygen in a sample generates electronic signals in the detector, proportional to the oxygen content. With an appropriate pressure adjustor, a metal pipe that does not pass air through, and a prescribed flow speed, the detector is controlled according to the instruction of the manufacturer to pass the gas through until a fixed value is measured. When nitrogen passes the cell containing potassium hydroxide, the content of oxygen should not be more than 1%(v/v).

(2) Carbon Monoxide : Both ends of the carbon monoxide detection pipe, and one end is connected to the container of Nitrogen and the other end to an appropriate flow meter. When about 1050 ± 50 ml of Nitrogen is passed through the detection pipe in a proper flow speed, the amount should not be more than 10 μL .

Assay Gas chromatography is performed with the following operating conditions. The amount of the control gas (a) injected and the operation conditions are controlled so that the height of the nitrogen peak in the chromatogram obtained by injecting the control gas is 35% of the recorder of full scale. Also, the oxygen and nitrogen peaks should be distinctively separated in the chromatogram. The peak area obtained in the chromatogram of the sample gas should not be less than 99.0% of the peak area of the control gas (b), by injecting the control gas (b) and the sample gas that is to be tested.

Operation Condition

-Column : Stainless Steel 2 mm \times 2 m

-Packing material : Adsorption materials that can distinguish molecules of diameters 0.5 μm or less or those whose separation power is equivalent to them

- Carrier Gas : Helium 99.995% (v/v)
- Flow Speed : 40 ml/min
- Detector : Thermal Conductivity Detector
- Injector : Loop injector
- Column Temperature : 50°C
- Detector Temperature : 130°C
- Control Gas (a) : air
- Control Gas (b) : Nitrogen N₂ 99.999% (v/v) or more, CO 1ppm or less, O₂ 5ppm or less

192. Caprylic Acid (Octanoic Acid)

Chemical Formula : $C_8H_{16}O_2$

Molecular Weight : 144.21

Definition Caprylic Acid is a saturated fatty acid obtained from fat, whose main ingredient is caprylic acid.

Composition Specifications of Caprylic Acid

Description Caprylic Acid is a colorless oil with slightly unpleasant odor.

Purity (1) Acid Value : When 0.5 g of Caprylic Acid is precisely weighted, and proceeded as directed under Acid value in Fats Test, the Acid value should be 366~396.

(2) Solidification point : The solidification point of Caprylic Acid is 8~15 °C

(3) Lead : When 5.0 g of Caprylic Acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(4) Arsenic : Place 0.25 g of Caprylic Acid in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(5) Mercury : When Caprylic Acid is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

(6) Iodine Value : Approximately 12.5 g of Caprylic Acid is precisely weighted into a 500 ml Erlenmeyer flask with a stopper which contains 20 ml of 1 : 1 mixture of glacial acetic acid : cyclohexane and 25 ml of Weiss solution. A stopper is placed on the flask which is vigorously shaken and set aside for 1 hour in a dark place. 20 ml of potassium iodide solution and 100 ml of water (previously boiled and cooled) are added to the flask. The excess iodine is titrated with 0.1 N sodium thiosulfate solution. 0.1 N sodium thiosulfate solution is added drop wise until yellow color disappears. Starch solution is added and the titration is continued until the blue color disappears completely. Near the end point, the flask is vigorously shaken with a stopper. Separately, a blank test is carried out by the same

procedure. Iodine value is obtained by the following equation and it should not be more than 2.0.

$$\text{Iodine Value} = \frac{(B - S) \times 1.269}{\text{Weight of sample (g)}}$$

B : Consumed amount of 0.1 N sodium thiosulfate solution in the blank test (ml)

S : Consumed amount of 0.1 N sodium thiosulfate solution in the test for sample (ml)

- (7) Saponification Value : 2 g of Caprylic Acid is precisely weighted into a 250 ml flask, where 50 ml of 0.5 N alcoholic solution of potassium hydroxide is added. After attaching a reflux condenser, the solution is saponified for 30~60 minutes. This solution is used as test solution, tested under Saponification value in Fats Test, boiled (red color appears again) and titrated again until the red color disappears. Saponification value should be 366~398.
- (8) Unsaponifiable Matter : 5 g of Caprylic Acid is precisely weighted into a 250 ml flask, where 2 g of potassium hydroxide and 40 ml of alcohol are added and gently refluxed for 1 hour with a reflux condenser. The solution transfer into a separatory funnel (3.5 cm diameter \times 30 cm length with 40 ml, 80 ml, and 130 ml scale marks) with a stopcock. The flask is washed with sufficient amount of alcohol, which is added to the funnel (total volume = 40 ml). The flask is washed with warm and cold water, which is added to the funnel (total volume = 80 ml). Finally, the flask is washed with a few ml of petroleum ether, which is added to the funnel. Cool the solution, 50 ml of petroleum ether is added to the funnel. The funnel is shaken vigorously for 1 minute and then settled to separate two phases completely. The supernatant ether layer is collected in a 500 ml separatory funnel with a stopcock. The aqueous layer is again extracted 6 times with 50 ml each of ether. These extracts are added to the first extract. The combined extracts are washed with 25 ml of 10% alcohol. This procedure is repeated until the aqueous layer doesn't get colorized by phenolphthalein TS. When this is accomplished, aqueous phase is discarded and the ether extract transfer into a pre-weighted beaker. With 10 ml of ether, the funnel is washed, which is added to the beaker. Ether layer is evaporated to dryness in a water bath, which is then dried at 100°C for 30 minutes until the weight becomes constant. Then the residue is cooled in a desiccator and weighted. The residue dissolve in 50 ml of warm alcohol (neutralized with sodium hydroxide using phenolphthalein as an indicator). The resulting solution is titrated with 0.02 N sodium hydroxide solution until a pale red color persists. The amount

of oleic acid is obtained by multiplying the consumed amount of sodium hydroxide solution with 5.659 (mg). The exact amount of unsaponifiables is obtained by subtracting the amount of fatty acid (as oleic acid) from the amount of residues. The content of unsaponifiable matter is calculated by the following equation and it should not be more than 0.2%.

Unsaponifiable matter(%) =

$$\frac{\text{Weight of residue (mg)} - \text{Weight of oleic acid (mg)}}{\text{Weight of sample (g)}} \times \frac{100}{1,000}$$

Water Content Water content of Caprylic Acid proceed as directed under water determination (Karl-Fisher Titration) and should not be more than 0.4%.

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 10 g of Caprylic Acid, the amount of residue should not be more than 0.1%.

193. Naringin

Chemical Formula $C_{28}H_{32}O_{14}$

Molecular Weight MW 580.53

Definition Naringin is obtained by purifying the extracts of peels, juices, or seeds of tangerine (*Citrus paradisi* MACF.) of rutaceae with water, ethyl alcohol or methyl alcohol at room temperature. Its component is naringin

Compositional Specifications of Naringin

Content When Naringin is dried, it should contain 90~110% of the Naringin ($C_{28}H_{32}O_{14}$ = 580.53).

Description Naringin occurs as colorless~pale yellow crystal with strong bitter taste.

Identification (1) 5 mg of Naringin dissolve in 10ml of 50% ethyl alcohol. When 1~2 drops of ferric chloride solution (1→500) are added to this solution, it becomes brown in color.

(2) When 5mg of Naringin dissolve in 5ml of Sodium hydroxide solution, the solution shows orange yellow~yellow color.

(3) A solution of 10 mg of Naringin in 500ml of water has a maximum absorption band in the wavelength range of 280~285nm.

Purity (1) Arsenic: Place 0.5 g of Naringin in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 2 ppm.

(2) Lead : When 5.0 g of Naringin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Residual Solvents : When Naringin is tested by Purity (5) for Oleoresin Paprika, the content of methyl alcohol should not be more than 50 ppm.

Assay After drying for 3 hours at 105°C, approximately 0.2 g of Naringin is precisely weighted and dissolved in 50% ethyl alcohol (total volume = 100 ml). This solution is filtered through 0.45 µm filter. 1 ml of this solution is diluted to 100 ml with water (Test Solution). Using water as a reference, the absorbance is measured at 280 nm and the content of Naringin is obtained from the following equation.

$$\text{Content of Naringin (\%)} = \frac{A}{28.0} \times \frac{10,000}{\text{Weight of sample (mg)}} \times 100$$

A: Absorbance of the test solution

194. Enzymatically Modified Hesperidine

Definition Enzymatically Modified Hesperidine is obtained by adding glucose to hesperidine using α -glucosyltransferase. Its component is α -glucosylhesperidine.

Compositional Specifications of Enzymatically Modified Hesperidine

Content Dried the mixture form should contain no less than 27% of Enzymatically Modified Hesperidine as hesperetin glycoside and the mono form should contain no less than 70% of Enzymatically Modified Hesperidine as α -monoglucosylhesperidine.

Description Enzymatically Modified Hesperidine occurs as a light yellow~yellowish brown powder or crystalline powder with a slightly characteristic odor.

Identification (1) 5 mg of Enzymatically Modified Hesperidine dissolve in 10 ml of 50% ethyl alcohol. When 1~2 drops of ferric chloride solution (1→500) are added to this solution, it becomes brown in color.

(2) A solution of 10 mg of Enzymatically Modified Hesperidine in 500ml of water has a maximum absorption band in the wavelength range of 280–286nm.

(3) Approximately 0.5g of Enzymatically Modified Hesperidine is completely dissolved in 100ml of water. Use this solution as the test solution. Aside from that, approximately 0.2g of standard Hesperidine dissolve in 50 ml of sodium hydroxide solution (1→500). Prepare 40 ml of the standard solution through applying the mobile phase of the high performance liquid chromatography to 10 ml of the previous solution. When performed the high performance liquid chromatography for the test solution and the standard solution following the conditions listed below, peak of Enzymatically Modified Hesperidine should be at the location with earlier retention time compared to that of the Hesperidine, but it should display relatively similar UV absorption spectrum.

Operating Conditions

Detector: Photodiode array detector (Measured wavelength 280nm, 200–400nm)

Packing material: chemically bonded Octadecylsilane

Column: inner diameter 3.9–4.6mm, length 150–300mm

Column temperature: 40℃

Mobile phase: water : acetonitrile (4:1)

Flow rate: 0.5ml/min

Injection volume: 10ul

Purity (1) Arsenic: Place 0.5 g of Enzymatically Modified Hesperidine in a platinum,

quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 2ppm.

(2) Lead : When 5.0 g of Enzymatically Modified Hesperidine is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 10 ppm.

(3) Clarity and Color of Solution : When 0.5 g of Enzymatically Modified Hesperidine is dissolved in 100 ml of water, the solution should be clear.

Loss on Drying When 1.0 g of Enzymatically Modified Hesperidine is dried for 3 hours at 105°C, the loss should not be more than 6%.

Assay 1.0g of Enzymatically Modified Hesperidine is precisely weighted and dissolved in water to make 100 ml solution. 1 ml of this solution dissolve with 100 ml of distilled water and use this solution as the test solution. Aside from this, dry the standard Hesperidine compound in 135°C for 2 hours and then dissolve 1.0g of dried standard Hesperidine with 0.5N Sodium hydroxide solution and mark it up to 100ml. Dissolve and mark up 1.0ml of this solution to 100ml with water which is considered as the standard solution. Calculate the hesperetin glycoside (C) under the operation conditions shown below based on the high performance liquid chromatography through the sum of the monoglycosylhesperidine (A_1), diglycosylhesperidine (A_2), triglycosylhesperidine (A_3), tetraglycosylhesperidine (A_4), pentaglycosylhesperidine (A_5), and the quantity of hesperidine (B). However, calculate only the monoglycosylhesperidine (A_1) for the mono formation.

$$A_n = \frac{A_s \times a_t}{A_t \times a_s} \times F \times 100$$

$$B = \frac{B_s \times a_t}{A_t \times b_s} \times 100$$

$$C = A_1 + A_2 + A_3 + A_4 + A_5 + B$$

A_n : The specific quantity of glycosylhesperidine for selected from $A_1 \sim A_5$ during the examination

A_s : Each specific glycosylhesperidine peak area of the test solution

A_t : The hesperidine peak area of the standard hesperidine solution

a_s : The amount (g) collected in respect to the examination

a_t : The collected amount of the standard (purity correction) hesperidine (g)

F: The coefficient for the hesperidine conversion = $M/610$, M: molar mass of each glycosylhesperidine type (M: 772 for monoglycosylhesperidine, 934 for diglycosylhesperidine, 1096 for triglycosylhesperidine, 1258 for tetraglycosylhesperidine, 1420 for pentaglycosylhesperidine)

B: The amount of hesperidine (%) during the examination

B_s : The hesperidine peak area of the test solution

b_s : The amount collected (g) in respect to the examination

C: The amount of hesperetin glycoside during the examination (%)

Operating Condition

Detector: UV (Measured wavelength 280nm)

Column: Capcell pack C_{18} or its equivalent

Column temperature: 45°C

Mobile phase: Acetonitrile : water (20:80)

Flow rate: 0.5ml/min

Injection volume: 10 μ l

195. Maltotriohydrolase

G3 Producing Enzyme

Definition Maltotriohydrolase is an enzyme obtained from a culture of *Microbacterium imperiale*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Maltotriohydrolase

Content Maltotriohydrolase contains 90~130% of the indicated activity as G3 Maltotriohydrolase.

Description Maltotriohydrolase is white~pale yellow~brown powder or transparent brown liquid.

Purity (1) Arsenic: Place 0.25 g of Maltotriohydrolase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(2) Heavy Metals: Carbonize 1 g of Maltotriohydrolase by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. When this solution tested for Heavy Metals, the content should not be more than 40ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution,

2 ml of diluted acetic acid (1→20), and water are added to bring the total volume to 50 ml.

- (3) Lead: 0.8 g of Maltotriohydrolase (if it is liquid, it is concentrated by evaporation in a water bath) is mindly carbonized by heating, which is reduced ash by further heat treatment at a temperature below 500°C. Carefully 20 ml of dilute nitric acid is added to the ash. Boil the sample for 5 minutes and cool. It is filtered (if necessary). The residue is washed with water, which is then added to the filtrate. Water is added so that total volume of this solution becomes 50 ml. This test solution is tested for lead. The detected amount of lead should not be more than 10ppm.
- (4) Coliform group: Maltotriohydrolase is tested by Microbiological Methods for Coliform Group in section General Testing Methods in Food Code. It should contain no more than 30 per 1g of this product.
- (5) Salmonella: Maltotriohydrolase is tested by Microbiological Methods for Salmonella in section General Testing Methods in Food Code. It should be negative (-).

Assay(activity) Application and principle: This test is to measure the activity of G3 Maltotriohydrolase in an enzyme that is obtained from *Microbacterium imperiale*.

Preparation of Test Solution : Sample is diluted with calcium chloride · acetic acid buffer solution (pH 6.0) so that 1 ml of the solution contains 0.2~0.85 Unit.

Test Procedure : 0.5 ml of substrate solution and 0.4 ml of calcium chloride · acetic acid buffer solution (pH 6.0) are placed in a 50 ml volumetric flask, which is isothermalized in a $40 \pm 0.5^{\circ}\text{C}$ water bath for 10 minutes. Exactly 0.1 ml of Test Solution is added to the solution, mixed well by shaking, and set aside in a water bath. After exactly 15 minutes, 1 ml of alkaline copper solution is added to the solution, which is sealed and heated for exactly 20 minutes in a boiling water bath. Cool the solution, 1 ml of arsenic·ammonium molybdate solution is added and well mixed until red precipitates of cuprous oxide are completely dissolved. After setting aside for 20 minutes at room temperature, water is added to bring the total volume to 25 ml. Using water as a reference, the absorption (A_S) is measured at 520 nm with 1cm path length. Separately, as an enzyme blank test, 0.5 ml of substrate solution, 0.4 ml of calcium chloride acetate buffer solution (pH 6.0), 1 ml of alkaline copper solution, and 0.1 ml of Test Solution are well mixed and its absorption (A_B) is measured following the same procedure as the Test Solution.

Standard curve: Glucose is dried for 6 hours at 105°C. 1.0 g of dried glucose is precisely weighted and dissolved in water (total volume = 100 ml). 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, and 5.0 ml each of this solution is diluted to 100 ml with water. 1 ml of

the each resulting solution contains 0.1 mg, 0.2 mg, 0.3 mg, 0.4 mg, and 0.5 mg each of glucose. 1 ml of each glucose standard solution is placed in a 50 ml Nestler cylinder, where 1 ml of alkaline copper solution is added and well mixed. It is sealed, heated in a boiling water bath for exactly 20 minutes, and cooled immediately. 1 ml of arsenic·ammonium molybdate solution is added and well mixed until red precipitates of copper suboxide are completely dissolved. After setting aside for 20 minutes at room temperature, water is added to bring the total volume to 25 ml. Using water as a reference, the absorbance of each standard solution is measured at 520 nm with 1cm path length. A standard curve of absorbance vs. the amount of glucose (mg) is prepared.

Enzyme activity is calculated by the following equation.

$$\text{Maltotriohydrolase (unit/g)} = (A_S - A_B) \times F \times \frac{1}{15} \times \frac{1.0}{0.1} \times \frac{1}{0.180} \times \frac{N}{W}$$

F : Amount of glucose (mg) when the difference in absorption is 1.0 (obtained from the standard curve).

15 : Reaction time (minutes)

0.1: Volume of Test Solution (ml)

0.180 : Coefficient of glucose/1 μ mole (glucose 1 μ mole = 0.180 mg)

N : Dilution volume of Test Solution

W : Weight of sample(g)

Definition of Activity: Maltotriohydrolase unit is an amount of enzyme that produces reducing sugar that corresponds to 1 μ mol of glucose per minute under the conditions above.

Solutions

Substrate Solution : 1.0 g of soluble starch is dispersed in 10 ml of water, where 50 ml of boiling water is slowly added while stirring. It is then boiled for 5 minutes. After cooling water is added to bring the total volume to 100 ml.

Calcium Chloride · Acetic acid Buffer Solution (pH 6.0) : Two solutions of 5 mM anhydrous calcium chloride in 0.1 M acetic acid and 0.1 M sodium acetate solution are prepared. These solutions are mixed and adjusted pH 6.0.

Alkaline Solution : 200 g of anhydrous sodium sulfate, 25 g of anhydrous sodium carbonate, 20 g of sodium bicarbonate, and 25 g of potassium sodium tartrate are dissolved in water to make 1,000 ml solution.

Copper Solution: 30 g of copper sulfate dissolve in 150 ml of water and apply 4 drops of sulfuric acid. Mark up the volume of the content to 200 ml with water.

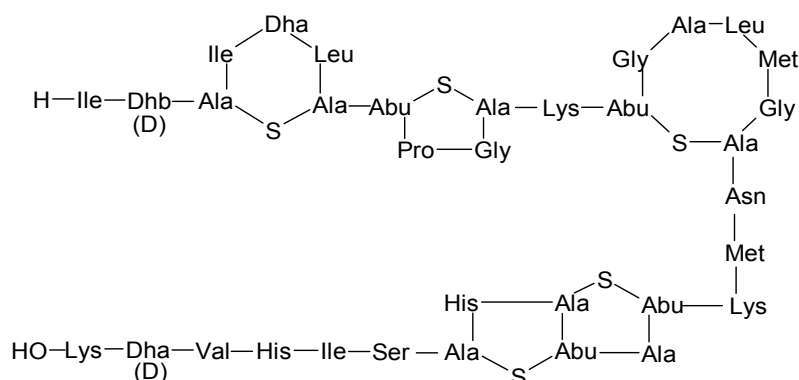
Alkaline Copper Solution: 25 ml of alkaline solution is mixed with 1 ml of copper solution. The solution is prepared before use.

Arsenic · Ammonium Molybdate Solution : 3 g of sodium arsenate, dibasic (7 hydrate) dissolve in 25 ml of water. 25 g of ammonium molybdate (4 hydrate) dissolve in 450 ml of water, where 21 ml of sulfuric acid is added. Sodium arsenate, dibasic solution is slowly added to ammonium molybdate solution while stirring. It is set aside for 24 hours at 37°C. It is stored in a brown bottle.

Storage standards of Maltotriohydrolase

Maltotriohydrolase is strongly hygroscopic, hence should be stored in a hermetic container in a cold dark place.

196. Nisin



Abu = alpha-aminobutylic acid

Dha = dehydroalanine

Dhb = dehydrobutyryne

Chemical Formula $C_{143}H_{230}N_{42}O_{37}S_7$

Molecular Weight 3354.12

Definition Nisin is a mixture of polypeptide produced by *Lactococcus lactis* (*Streptococcus lactis*), Lancefield group N and sodium chloride.

Compositional Specifications of Nisin

Content Nisin contains no less than 900 IU/mg of nisin ($C_{143}H_{230}N_{42}O_{37}S_7$).

Description Nisin occurs as a white, micronized powder.

Identification (1) Stability to acid : Suspend 100mg of Nisin in 0.02N hydrochloric acid as described in the preparation of the Nisin standard solutions under the Assay. After boiling this solution for 5 min, determine the Nisin concentration using test method under the Assay. No significant loss of activity is noted following this heat treatment. The Nisin concentration of the boiled Nisin is $100 \pm 5\%$. After adjusting the pH of the Nisin solution to 11 by adding 5N sodium hydroxide, heat the solution at 65°C for 30 min, and then cool. After adjusting the pH to 2.0 by adding hydrochloric acid, determine the Nisin concentration using Assay. Confirm complete loss of antimicrobial activity of Nisin following this treatment.

(2) Tolerance of *Lactococcus lactis* to high concentrations of Nisin: Prepare cultures of *Lactococcus lactis* (ATCC 11454, NCIMB 8586) in sterile skim milk by incubating for 18 hr at 30°C . Prepare one or more flasks containing 100 ml of litmus milk, and sterilize at 121°C for 15 min. Suspend 0.1 g of sample in the sterilized litmus milk, and allow to stand at room temperature for 2 hr. Add 0.1 ml of the *L. lactis* culture, and incubate at 30°C for 24 hr. *L. lactis* will grow in this concentration of

sample (about 1000 IU/ml).

Purity (1) Lead : When 5.0 g of Nisin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 1.0 ppm.

(2) Arsenic : Place 0.77g of Nisin in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 1.3ppm.

(3) Mercury : When Nisin is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

(4) Total viable aerobic count : When Nisin is tested by Microbiological Methods for Total viable aerobic count in General Testing Methods in Food Code, it should not be more than 10 CFU/g.

(5) E. coli: When Nisin is tested by Microbiological Methods for E. coli in General Testing Methods in Food Code, it should be negative in 25 g.

(6) Salmonella : When Nisin is tested by Microbiological Methods for Salmonella in General Testing Methods in Food Code, it should be negative in 25 g.

(7) Coliform Group : Taurine is tested by Microbiological Methods for Coliform Group in General Testing Methods in Food Code. It should be contain not more than 30 per 1 g of this product.

Sodium Chloride Approximately 20mg of Nisin, precisely weighted, dissolve in 50 ml of water contained in a glass-stoppered flask. Add, while agitating, 3 ml of nitric acid, 5 ml of nitrobenzene, 50 ml of 0.1N silver nitrate, and 2 ml of ferric ammonium sulfate. Shake well, and titrate the excess silver nitrate with 0.2N ammonium thiocyanate. The titration endpoint is indicated by the appearance of a red color. Perform a blank test using water, and calculate the content of sodium chloride in the sample by the formula. The content of sodium chloride should not be less than 50.0%.

$$\text{The content of sodium chloride (\%)} = (2 \times 5.844)(A - B) / S \times 100$$

A : The volume of 0.2N ammonium thiocyanate consumed by the blank test(ml)

B : The volume of 0.2N ammonium thiocyanate consumed by the sample test (ml)

S : Weight of sample (mg)

Assay

Medium : Dissolve 10 g of Bacteriological Peptone, 3 g of Beef Extract, 3 g of Sodium Chloride, 1.5 g of Autolyzed Yeast (Yeast Extract), 1 g of Brown Sugar, and 15 g of Agar in distilled water to a final volume of 1000 ml. Sterilize in an autoclave at 121°C for 15 min. The medium can be stored in a covered container at room temperature until use. At the time of use, melt the medium, and cool to approximately 50°C. Add 2% of a 1:1 mixture of Tween 20 (polyoxyethylene sorbitan monolaurate) and distilled water, previously held for 20~30 min at 48°C.

Assay Organism: Maintain *Micrococcus luteus* (ATCC 10240, NCIMB 8166) by subculturing on agar slants of the medium and incubating at 30°C for 48 hours. Prepared slants may be stored for a maximum of 14 days at 4°C until required. When required for use, the growth on the slant cultures is suspended in 7 ml of sterilized normal saline solution.

Nisin Standard Stock Solution: Suspend 100 mg of Nisin Standard (1,000 IU/mg), precisely weighted, in 80 ml of 0.02N hydrochloric acid. Set aside at room temperature for 2 hr. Dilute the suspension to a final volume of 100 ml with 0.02N hydrochloric acid (1,000 IU/ml). The standard stock solution can be stored for up to 7 days at 4°C.

Nisin Standard Solution: Weigh 0.5, 1.0, 2.5, 5.0 and 10.0 ml of Standard Stock Solution accurately into separate 1000 ml volumetric flasks. Dilute each flask to volume with 0.02N hydrochloric acid to make 1000 ml. (0.5, 1.0, 2.5, 5.0, 10.0 IU/ml). Each standard solution is prepared freshly on the day of use.

Preparation of the Standard Curve: Dilute the suspension of the assay organism to 1:10 using normal saline solution, and then mix thoroughly. Add 2 ml of this solution to each 100 ml of melted medium held at 48°C. Pour the inoculated medium to a depth of 3~4 mm (approximately 15 ml) into five Petri dishes, and allow to solidify. Invert the plates, and store at 4°C for 1 hr. Bore four 8~9 mm (in diameter) holes on 30 mm centers in each plate of the agar medium. Besides, absorbing disc can be used. Transfer 0.2 ml each of Nisin standard solutions of 0.5, 1.0, 2.5, 5.0, and 10.0 IU/ml into the holes, one concentration to a plate. Cover the plates, and incubate them overnight at 30°C. Measure the zones of inhibition to the nearest 0.1 mm by means of calipers or other appropriate devices. Plot the Nisin concentration against the zone diameters, and draw the best straight line through the plotted points.

Procedure: Suspend 100 mg of sample in 80 ml of 0.02N hydrochloric acid in a 100 ml volumetric flask, and set aside at room temperature for 2 hr. Dilute the solution to volume by adding 0.02N hydrochloric acid. Dilute to a 1:200 solution with 0.02N

hydrochloric acid. Proceed as described above for the standard curve, transferring in quadruplicate a measured volume of this solution(0.2ml) into the holes of four agar discs. Cover the plates, and incubate them overnight at 30°C. After incubation, measure the zones of inhibition. From the standard curve, determine the Nisin concentrations, and average the results.

Loss on Drying When 2 g of Nisin is dried for 2 hr at 105°C, the loss should not be more than 3.0%.

Storage Standards of Nisin

Nisin should be stored in well-closed containers at temperatures not exceeding 22°C

197. Tannase

Definition Tannase is the enzyme, which is obtained from the culture of *Aspergillus oryzae*. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Tannase

Content Tannase contains no less than 90 ~ 130% of indicated activity as a Tannase.

Description Tannase is a white ~ pale brown powder, granule, paste or colorless ~ pale brown liquid with a characteristic scentless or a characteristic scent.

Purity (1) Arsenic : Place 0.25g of Tannase in a platinum, quartz, or porcelain crucible.

Add 10ml of magnesium nitrate in ethyl alcohol(1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Heavy Metals : Carbonize 0.5 g of Tannase by heating gently in a quartz or porcelain crucible. Cool the solution, add 2 ml of nitric acid and 5 drops of sulfuric acid. Heat the crucible until fuming, and strongly heat the crucible to ash at 450~550°C. Cool the solution, add 2 ml of hydrochloric acid and evaporate to dryness in a water bath. 3 drops of hydrochloric acid and 10 ml of hot water are added to the resulting residue, which is then heated for 2 minutes. Cool the resulting residue, and add 1 drop of phenolphthalein indicator solution. Then add ammonia solution until the color of the solution becomes pale red. Transfer the resulting solution into a Nestler cylinder by rinsing with water, and then add 2 ml of diluted acetic acid (1→20) and water to make 50 ml. When this solution proceed as directed under Heavy Metals, the content should not be more 40ppm. Color standard solution is prepared by the following procedure. 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid are added and evaporated to dryness in a crucible that is made of the same material used for test solution preparation. 3 drops of hydrochloric acid are added to the residue, which is then transferred into another Nestler cylinder as described above. Finally, 2 ml of lead standard solution, 2 ml of diluted acetic acid (1→20), and water are added to make the total volume to 50 ml.

(3) Lead: 0.8g of Tannase is mindly carbonized by heating, which is reduced ash by further heat treatment at a temperature below 500°C. Carefully 20ml of dilute nitric

acid is added to the ash. Boil the sample for 5 minutes and cool. It is filtered (if necessary). The residue is washed with water, which is then added to the filtrate. Water is added so that the total volume of the solution becomes 50ml. This test solution is tested for Lead. The detected amount of Lead should not be more than 10ppm.

(4) Coliform Group: Tannase is tested by Microbiological Method for [Coliform Group] in General Testing Methods in Food Code. It should contain not more than 30 per 1g of this product.

(5) Salmonella : Tannase is tested by Microbiological Method for Salmonella] in General Testing Methods in Food Code. It should be negative(-).

Assay(activity) Application and Principle: This test is to measure the activity of Tannase in the enzyme which is obtained from the culture of *Aspergillus oryzae*. The activity test is based on the hydrolysis of depside bond of tannin acid substrate at 30°C. Absorbance difference is measured by using spectrophotometer at 310nm.

The preparation of Test Solution : When Tannase is weighted, 1 ml of the final diluent solution contains 1 Tannase unit. 50mM of citric acid buffer solution(pH 5.5) of the low temperature(5±3°C) is added to prepare Test solution.

Test Procedure : 4ml of substrate solution is added to a 25 × 150mm test tube and isothermalized for 10 minutes in a 30°C water bath. Precisely 1ml of Test solution is added to the test tube, and mixed well, and the reaction of the solution is conducted in a water bath(Reaction start). The test tube is separated with reaction solution A and B. After 10minutes from reaction start, 1ml of the reaction solution is taken in test tube A, and 9ml of 80% ethyl alcohol solution is added in the solution. Next, shake strongly and stop the reaction. This reaction solution is called as Solution A. After 20minutes from reaction start, 1ml of the reaction solution is taken in test tube B, and 9ml of 80% ethyl alcohol solution is added in the solution. Next, mix and stop the reaction. This reaction solution is called as Solution B. Solution A and B is diluted 10times by 80% ethyl alcohol, and these solutions are called as Enzyme test solution A and B. As control solution is 80% ethyl alcohol, each 1cm liquid layer of Enzyme test solution A and B, absorbance a and b, is measured at 310nm. The activity of the enzyme is calculated following the formula.

$$\text{Tannase unit/g} = \frac{(a - b) \times 20.3 \times 4}{10 \times 0.71 \times C}$$

20.3 : μmol of tannic acid contained 1.0ml of substrate solution

4: Substrate solution for reaction(ml)

10: The difference between final and initial reaction time(min)

0.71: Absorbance change in the completed hydrolysis of tannic acid 20.3μmol under

above condition.

C: Sample amount containing in 1ml of the Test Solution(g)

a: Absorbance of Enzyme test solution A

b: Absorbance of Enzyme test solution B

Only, the value of (a-b) should be 0.09~0.11

Definition of Activity : 1 Tannase unit corresponds to the amount of enzyme, which hydrolyze 1 μ mol of tannic acid per minutes under the above test conditions

Solutions

50mM citric acid buffer solution(pH 5.5)

Solution A : 10.5g of citric acid dissolve in 1000ml water.

Solution B : 14.7g of sodium citrate(2 hydrate) dissolve in 1000ml water.

A solution and B solution are mixed (138ml :500ml) and adjust pH to 5.5 with using both solutions.

Substrate solution : 0.32g of tannic acid (Sigma USP Grade) is weighted, and added in 10ml of 50mM citric acid(pH 5.5). Dissolve with warming and shaking. Add 50mM citric acid(pH 5.5) to make 100ml volume.

Storage standard of Tannase

Tannase should be stored in a hermetic container in a cold dark place.

198. Poly - γ -glutamic acid

Definition Poly - γ -glutamic acid is obtained by separating and refining the cultured residue solution after culturing *Bacillus subtilis* and *Bacillus subtilis chungkookjang*. Its compound is Poly - γ -glutamic acid.

Compositional Specifications of Poly- γ -glutamic acid

Content When Poly- γ -glutamic acid is weighted Dried it should contain no less than 95.0% as a Poly- γ -glutamic acid.

Description Poly- γ -glutamic acid is strong hygroscopic property, white powder, scentless and tasteless.

Identification (1) When Thin Layer Chromatography is tested after taking 0.1g of Poly - γ -glutamic acid, red spot should be identified at the same place as L-Glutamic acid. Test solution is made like that 0.1g of Poly - γ -glutamic acid is taken, and dissolve in 9.5ml of water, and then 0.5ml of 6N hydrochloric acid is added in the solution, and is hydrolyzed at 110°C for 24 hours. It can be filtered if it is necessary. Prepared test solution is conducted under the following condition.

Condition of Thin Layer Chromatography

Developing solvent : n-butyl alcohol : glacial acetic acid : water(2 : 1 : 1)

Thin layer plate: Silicagel

Developing distance: 10 ~15cm

Color reagent : 0.2g of Ninhydrin dissolve in unsaturated n-butyl alcohol to make 100ml.

(2) When 1g of Poly - γ -glutamic acid is taken, and measured by Potassium Bromide Disk Method of Infrared Spectrophotometry, Carboxyl group($1,735\text{cm}^{-1}$), amin group(1554cm^{-1}) and carboxyl group connected amin group(1650cm^{-1}) should be identified.

Purity (1) Arsenic : Place 0.5g of Poly - γ -glutamic acid in a platinum, quartz, or porcelain crucible. Add 10ml of magnesium nitrate in ethyl alcohol(1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Poly- γ -glutamic acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content

should not be more than 10 ppm.

(3) Total Viable Aerobic Count : When Poly-γ-glutamic acid is tested by Microbe Test Methods for Total Viable Aerobic Count (Number of General Germs) in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 10,000 per 1 g

(4) E. Coli : When Poly-γ-glutamic acid is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Loss on Drying When 3 g of Poly-γ-glutamic acid is dried for 3 hours at 105°C, the loss should not be more than 4%.

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 1 g of Poly-γ-glutamic acid, the amount of residue should not be more than 1.0%.

Assay Poly-γ-glutamic acid is dried and 100mg of Poly-γ-glutamic acid is weighted, and dissolved in 10ml water. It is called A solution. 0.5ml of A solution is taken. Add 10ml of 6N hydrochloric acid. Hydrolyze for 24 hours at 110°C to make Test Solution. Calculate the content of glutamic acid by using Amino Acid Analyzer following below condition after taking the appropriate amount of Test Solution. Separately, the appropriate amount of A solution, which is not hydrolyzed, is taken. Calculate the content of free glutamic acid by using Amino Acid Analyzer. Calculate the content of Poly-γ-glutamic acid following the formula.

$$\text{The content of free glutamic acid (\%)} = \frac{\text{The weight of glutamic acid(g)}}{\text{Weight of sample(g)}} \times 100$$

$$\text{The content of Poly-}\gamma\text{-glutamic acid(\%)} = \frac{\text{The weight of glutamic acid(g)}}{\text{Weight of sample(g)}} \times 0.88 \times 100 - \text{the content of free glutamic acid}$$

$$0.88 = \frac{129(\text{the molecular weight of glutamic acid residue in the Poly-}\gamma\text{-glutamic acid})}{147(\text{the molecular weight of glutamic acid})}$$

Operation condition of Amino Acid Analyzer

Column : HR Na column(4.6mm × 200mm) or equivalent

Column Temperature : 78°C

Detector and wave length : Spectrophotometer(570nm)

Mobile phase and mobile flow rate

- Buffer solution : Flow Lithium citrate buffer(pH 2.8) with flowing speed of 20ml/h.
- Reaction solution : Flow Ninhydrin solution with flowing speed of 25ml/h.
- Reaction Temperature : 135°C
- The amount of injection : 40 μ l

199. α -Galactosidase

Definition α -Galactosidase is the enzyme, which is obtained from the culture of *Aspergillus niger*. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of α -Galactosidase

Content α -Galactosidase contains 90 ~ 130% of indicated activity as α -Galactosidase..

Description Tannase is a white ~ pale yellow powder.

Purity (1) Arsenic : Place 0.25g of α -Galactosidase in a platinum, quartz, or porcelain crucible. Add 10ml of magnesium nitrate in ethyl alcohol(1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of α -Galactosidase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Coliform Group: α -Galactosidase is tested by Microbiological Method for (Coliform Group] in General Testing Methods in Food Code. It should contain not more than 30 per 1g of this product.

(4) Salmonella : α -Galactosidase is tested by Microbiological Method for [Salmonella] in General Testing Methods in Food Code. It should be negative(-).

(5) E. Coli : When α -Galactosidase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity) Application and Principle: This test is to measure the activity of α -Galactosidase in the enzyme which is obtained from the culture of *Aspergillus niger*. The activity test is based on the hydrolysis of p-nitrophenyl- α -D-galactopyranoside substrate for 15minutes at the temperature of 37°C, and pH 5.5.

Enzyme Test Solution : Sample dissolve in water, and 1ml of final diluent solution should be contained 0.001~ 0.003 galactosidase unit to prepare Test Solution.

Test Procedure : 2.0 ml of substrate solution is added to a 20 × 150mm test tube and isothermalized for 15 minutes in a 37 ± 0.2°C water bath. In the same condition with Substrate Solution, 1.0 ml of Enzyme Test Solution is precisely added to the test

tube, and mixed well. After 15minutes, 5.0 ml of sodium boric acid buffer(pH 9.7) is added in each test tube, and mixed in each test tube. Control is water. Measure the absorbance(A_S) at 405 nm. Separately, for Enzyme blank test, 1 ml of Enzyme Test Solution is taken. Add 5 ml of sodium boric acid buffer, and mix. 2 ml of Substrate Solution is placed in each test tube, and mixed, and conducted the same procedure as Enzyme Test solution by measuring absorbance(A_B) at 405nm. The activity of the enzyme is calculated following the formula.

$$\alpha\text{-Galactosidase(Gal U/g)} = \frac{(A_S - A_B) \times F}{\epsilon \times T \times M}$$

A_S : Absorbance of Test Solution

A_B : Absorbance of Control Solution

F : Dilution factor of Test Solution

T : Reaction Time(min)

M : Weight of sample(g) contained 1ml of Test Solution.

ϵ : Absorbance coefficient measured with standard 4-nitrophenol solution

Definition of Activity : 1 α -Galactosidase unit corresponds to the amount of enzyme, which isolated from 1 μ mol of p-nitrophenol per minutes under the above test conditions

Solutions

Acetic acid buffer solution(pH 5.5)

A Solution : 11.55ml of glacial acetic acid dissolve in 1,000ml water.

B Solution : 16.4g of sodium acetate dissolve in 1,000ml water.

7.5ml of A Solution and 42.5ml of B Solution are mixed, and adjusted to pH 5.5 by using A Solution and B Solution. Add water to make 1,000ml volume.

Substrate solution : 0.0383g of p-nitrophenyl- α -galactopyranoside is mixed to Acetic acid buffer solution, and is diluted to make 100ml volume.

Sodium boric acid buffer solution : 47.63g of Sodium boric acid dissolve in warm water, and cooled into room temperature. Add 20ml of 4N sodium hydroxide. After adjusting until pH 9.7 by using 4N sodium hydroxide, the solution is diluted to 2,000ml.

4-nitrophenol Standard Stock Solution : 4-nitrophenol is dried advance. 68.83mg of 4-nitrophenol is precisely weighted, and dissolved in water to make 1,000ml. 1ml of this solution should contain 0.5 μ mol of nitrophenol.

Standard 4-nitrophenol Solution : Pipet 4ml, 8ml and 16ml of 4-nitrophenol Standard Stock Solution into each test tube, and add water to make 50ml volume. The content of 4-nitrophenol in th diluted solution should be contained each 0.04, 0.08 and 0.16 μ mol per 1ml. After each solution is grouped with five test tubes, and 2.0ml of Substrate Solution is placed in each five test tubes. 1ml of Standard 4-nitrophenol Solution is added in each four test tubes, and 1.0ml of water is added to each fifth test tube instead of Standard 4-nitrophenol Solution. 5.0ml of Sodium boric acid buffer solution is added into each test tube, and mixed. Control solution is water. Absorbance is measured by 1cm of the liquid layer at 405nm, and the curve based on the amount of 4-nitrophenol is prepared. The average absorbance coefficient of Standard 4-nitrophenol Solution is calculated to divide the absorbance of each diluted solution into the concentrate of 4-nitrophenol(μ mol/ml)

$$\varepsilon = \frac{A_N}{C}$$

A_N : The absorbance of Standard 4-nitrophenol Solution

C : The concentrate of 4-nitrophenol

The value of absorbance coefficient should be obtained as an approximation value, 18.3.

Stotage standard of α -Galactosidase

α -Galactosidase should be stored in a hermetic container in a cold dark place.

200. β -Glycosidase

Definition β -Glycosidase is the enzyme, which is obtained from the culture of *Penicillium multicolor*. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of β -Glycosidase

Content β -Glycosidase contains no less than 90 ~ 130% of indicated activity as β -Glycosidase.

Description Tannase is a white ~ pale yellow powder.

Purity (1) Arsenic : Place 0.25g of β -Glycosidase in a platinum, quartz, or porcelain crucible. Add 10ml of magnesium nitrate in ethyl alcohol(1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of β -Glycosidase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Coliform Group: β -Glycosidase is tested by Microbiological Method for [Coliform Group] in General Testing Methods in Food Code. It should contain not more than 30 per 1g of this product.

(4) Salmonella : β -Glycosidase is tested by Microbiological Method for [Salmonella] in General Testing Methods in Food Code. It should be negative(-).

(5) E. Coli : When β -Glycosidase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity) Application and Principle: This test is to measure the activity of β -Glycosidase in the enzyme which is obtained from the culture of *Penicillium multicolor*. The activity test is based on the hydrolysis of 4-Nitrophenyl-primeveroside substrate for 10minutes at 40°C(pH 5.5).

Preparation of Test Solution : Sample dissolve in 20mM of acetic acid buffer solution. Following below method, Test Solution should be prepared in order that the range of absorbance is 0.3 ~ 0.7.

Test Procedure : 2.0 ml of substrate solution is added to a 25 × 150mm test tube(for

enzyme test) and isothermalized for 5 minutes in a 40°C water bath. 0.45ml of Test Solution is added in the test tube, and shaken. Isothermalize again for 10 minutes in a 40°C water bath, and add 2.5ml of 0.5M sodium carbonate. Separately, 2ml of Substrate Solution is added in the test tube for Enzyme Blank Test, and isothermalized for 5 minutes in a 40°C water bath. Add 2.5ml of 0.5M sodium carbonate. Cover with appropriate stopper. Up side down several times and shake. Add 0.45ml of Test Solution and isothermalize again for 10 minutes in a 40°C water bath. Control Solution is Enzyme Blank Test Solution and the absorbance is measured with 1cm of its liquid layer at 412nm. The enzyme activity is calculated following the formula.

$$\beta\text{-Glycosidase(U/g)} = \frac{(S_A - B_A) \times V}{f \times 10} \times \frac{1}{w}$$

S_A : Absorbance of Enzyme Test Solution

B_A : Absorbance of Enzyme Blank Test Solution

V : The amount of final reaction solution(ml)

f : Absorbance coefficient measured with standard 4-nitrophenol solution

10: Reaction time(minutes)

w : Weight of sample(g) contained final reaction solution

Definition of Activity : 1 β-Glycosidase unit corresponds to the amount of enzyme, which isolated from 1μmol of p-nitrophenol per minute under the above test conditions

Solutions

20mM acetic acid solution : 1.6g of sodium acetic acid(anhydrous) dissolve in water to make 1,000 ml volume

20mM acetic acid solution : 1.2 g of actic acid solution dissolvee in water to make 1,000 ml volume

20mM acetic acid buffer solution(pH 5.5) : Stir continually 20mM acetic acid solution and add 20mM acetic acid solution to adjust to pH 5.5

20mM substrate solution: 43.34mg of 4-Nitrophenyl-primeveroside(MW=433.4) dissolve in 20mM acetic acid buffer solution, and make 50ml volume..

0.5M sodium carbonate solution : 5.3g of sodium carbonate dissolve and made up 100ml.

Standard 4-Nitrophenol solution : 4-nitrophenol is dried advance. 139.0 mg of 4-nitrophenol is precisely weighted, and dissolved in 20mM acetic acid buffer solution to make 100ml. 1ml of this solution should contain 100 μ mol of nitrophenol. Dilute with 20mM acetic acid buffer solution to contain each 40, 80, 120, 160 and 200 μ mol per 1ml of this solution. 8 ml of 4-Nitrophenol solution of each concentrate is added to each test tube(five test tubes). Isothermalize for 5 minutes in a 40°C water bath. Add 10ml of 0.5M sodium carbonate solution, and mix. Water is used as reference solution. Absorbance is measured by 1cm of the liquid layer at 412nm, and the curve based on the amount of p-nitrophenol is prepared. the curve should be passed zero point and straight line. The average absorbance coefficient(f) of diluted solution is calculated to divide the absorbance of each diluted solution into the concentrate of p-nitrophenol(μ mol/ml), and the absorbance is measured.

Storage standard of β -Glycosidase

β -Glycosidase should be stored in a hermetic container in a cold dark place.

201. Glutaminase

Definition Glutaminase is the enzyme, which is obtained from the culture of *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Aspergillus* sp and *Candida* sp. Diluent or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of β -Glycosidase-Glutaminase

Content Glutaminase contains no less than 90 ~ 130% of indicated activity as β -Glycosidase..

Description Tannase is a white ~ pale yellow powder.

Purity (1) Arsenic : Place 0.25g of Glutaminase in a platinum, quartz, or porcelain crucible. Add 10ml of magnesium nitrate in ethyl alcohol(1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic Limit Test, it should be appropriate and should not be more 4ppm.

(2) Lead : When 5.0 g of Glutaminase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.

(3) Coliform Group: Glutaminase is tested by Microbiological Method for [Coliform Group] in General Testing Methods in Food Code. It should contain not more than 30 per 1g of this product.

(4) Salmonella : Glutaminase is tested by Microbiological Method for [Salmonella] in General Testing Methods in Food Code. It should be negative(-).

(5) E. Coli : When Glutaminase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity) Application and Principle: This test is to measure the activity of Glutaminase in the enzyme which is obtained from *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Aspergillus* sp and *Candida* sp. The activity test is based on the hydrolysis of L- γ -glutamyl-p-nitroanilide substrate for 10minutes at 37°C(pH 6.0). Produced p-nitroaniline is measured by the Absorbance Test.

Preparation of Test Solution : Sample dissolve in diluted solution. Following below method, Test Solution should be prepared in order that the difference of Absorbance($A_s - A_b$) is 0.3 ~ 1.0.

Standard Curve : Add 0.0414g of p-nitroaniline, 6g of acetic acid and 10ml of 1M acetate · sodium acetate buffer solution. Make up 100ml with water. 3 μ mol of p-Nitroaniline per 1ml should be contained in the solution. Dilute to contain 0.025, 0.05, 0.075, 0.1 and 0.125 μ mol by using 1.1M acetate · sodium acetate buffer solution. Control Solution is 1.1M acetate · sodium acetate buffer solution. The absorbance is measured with 1cm of its liquid layer at 410nm. The concentrate of p-Nitroaniline(F, μ mol/ml) is calculated by the difference 1.000.

Test Procedure : 4 ml of substrate solution is added to a 25 × 150mm test tube(for enzyme test) and isothermalized for 5 minutes in a 37 ± 0.2°C water bath. 0.5ml of Test Solution is promptly taken in the test tube, and shaken. Set aside again the water bath. After 10minutes, the reaction of the enzyme is stopped by 0.5ml of 10M acetic acid. 0.5ml of 10M acetic acid is added in the test tube for Enzyme Blank Test. Shake and add 0.5ml of Test Solution. Stir and isothermalize for 10minutes in the water bath. Control Solution is Enzyme Blank Test Solution and the absorbance is measured with 1cm of its liquid layer at 410nm. The enzyme activity is calculated following the formula.

$$\text{Glutaminase(GSU/g)} = (A_s - A_b) \times F \times D$$

F : The concentrate of p-nitroaniline(μ mol/ml), as the difference of absorbance obtained from Standard curve is 1.000

D : The dilution factor of sample

Definition of Activity : 1 Glutaminase unit corresponds to the amount of enzyme, which isolated from 1 μ mol of p-nitrophenol per minutes under the above test conditions

Solution

Substrate Solution : 0.0285g of L- γ -glutamyl-p-nitroanilide is weighted and dissolved in 0.2ml of 2N hydrochloric acid. Add approximately 70ml of water. 10ml of 1M acetate · sodium acetate buffer solution(pH 6.0) and 0.2ml of 2N sodium hydroxide are added in the solution. Make up 100ml with water. This solution is prepared freshly before use.

2N hydrochloric acid : 16.7 ml of hydrochloric acid dissolve with water to make up

100ml.

2N sodium hydroxide : 8g of sodium hydroxide dissolve with water to make up 100ml.

1M acetic acid : 60g of acetic acid dissolve in water to make 1,000 ml volume

1M sodium acetate solution : 136g of sodium acetate(three hydrous) dissolve in water to make 1,000 ml volume

1M acetate · sodium acetate buffer solution(pH 6.0) : 1M acetate is added in 1M sodium acetate. Adjust to pH 6.0

1M acetate · sodium acetate buffer solution : 60g of acetate and 100ml of 1M acetate · sodium acetate buffer solution(pH 6.0) are added in water to make 1,000 ml volume.

10% TRITON X-100 solution : 10g of TRITON X-100 is added in 70ml of water. dissolve to heat. Cool the solution, add water to make 100ml volume.

Diluted solution : 5.84g of sodium chloride dissolve in water. Add 100ml of 1M acetate · sodium acetate buffer solution(pH 6.0) and 1ml of 10% TRITON X-100 solution. Make 1000ml volume with water.

Storage standard of Glutaminase

Glutaminase should be stored in a hermetic container in a cold dark place.

202. Hydrogen

Chemical Formula H_2

Molecular Weight 2.00

Compositional Specifications of Hydrogen

Content Hydrogen contains no less than 99.9 % (V/V) of indicated activity as hydrogen (H_2)

Description Hydrogen is a colorless, tasteless and scentless gas.

Purity (1) Oxygen : Oxygen analysis of electric chemical type (Galvanic cell) which has the range of detector, 0~100 $\mu\text{l}/\text{ml}$ is used in this test. The amount of oxygen in hydrogen gas should be less 50 ppm when hydrogen gas is passed by operating the oxygen analysis (Galvanic cell).

(2) Carbon monoxide (CO), carbon oxide (CO_2) and methane (CH_4) : Carbon monoxide (CO), carbon oxide (CO_2) and methane (CH_4) are purged by using nitrogen gas. The injection amount for appropriate height of the standard gases is adjusted at the chromatograph which is obtained by inserting standard gases (CO , CO_2 and CH_4) verified correct concentration value. Next, standard gases (CO , CO_2 and CH_4) and sample are inserted into gas chromatograph. Then, each peak areas should be less than 50 ppm when the peak areas of the sample and standard gases are compared each other.

Operation Condition

Column : Porapak Q or its equivalent

Detector : Flame Ionization Detector (FID)

Thermal Conductivity Detector (TCD)

The amount of injection : Loop injection (1 ~ 2 ml)

Temperature of injection inlet : 120°C

Temperature of detector : 250°C

Temperature of column : Held at 35°C for 3 minutes and temperature is raised to 250°C at a rate of 35°C per minute.

Temperature of methanizer : 375°C

Carrier gas and flow rate : helium of more than 99.9995% and 25 ~ 30 ml per minute

Assay The content of hydrogen gas is calculated by one point standard quantity of

the peak area(or height) obtained from chromatogram by inserting sample and standard gas verified(99.9% and more than) to gas chromatograph with the following the operation condition.

Operating Condition

Column : Molecular sieve or its equivalent

Detector : Thermal Conductivity Detector(TCD)

The amount of injection : Loop injection (1 ~ 2 ml)

Temperature of injection inlet : 120℃

Temperature of detector : 250℃

Temperature of column : Temperature is raised from 50℃ to 250℃ at a rate of 50℃ per minute.

Carrier gas and flow rate : Argon and nitrogen and 25 ~ 30 ml per minute

203. Hyaluronic Acid

Definition Hyaluronic Acid is obtained by culturing and refining a cockscomb or *Streptococcus zooepidemicus*, its component is Hyaluronic acid which has bonding structure of N- acetylglucosamine and D-glucuronic acid.

Compositional Specifications of Hyaluronic Acid

Content When hyaluronic acid is converted to a dehydrated form, it contains more than 90 % of hyaluronic acid.

Description Hyaluronic acid is hygroscopic white~pale yellow powder or granules with slightly characteristic odor.

Identification (1) 0.1 g of hyaluronic acid dissolve in 100 ml of water, 10 ml of the solution is accurately taken into a test tube, and 2~3 drops of cetylpyridinium chloride solution(1→20) are added, then white suspension or precipitates are formed.
(2) 0.1 g of hyaluronic acid dissolve in 100 ml of water, 1 ml of the solution is accurately taken into a test tube, and 6 ml of sulfuric acid is added. It is heated in a water bath for 10 minutes, cooled, 0.2 ml of carbazole-ethanol TS is added, and allow to stand, the solution becomes red~reddish violet.

Purity (1) Acidity : 100 ml of water is added to 0.1 g of hyaluronic acid, shaken well and dissolved, then the pH of the solution should be 2.5~3.5.
(2) Arsenic : Place 0.25 g of hyaluronic acid in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 2ppm.
(3) Lead : When 5.0 g of hyaluronic acid is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.
(4) Other acidic mucopolysaccharides : 20 mg of hyaluronic acid is taken, 20 ml of 1N hydrochloric acid is added, and boiled for 30 minutes in a water bath and cooled. 5 ml of this solution is pipetted, 1.0 ml of 1N barium chloride solution is added, and allow to stand for 15 minutes, then the turbidity should increase not more than reference solution. Here, 1 ml of water is added and used as reference solution instead of 1N barium chloride solution.

Loss on Drying When 1 g of hyaluronic acid is dried for 4 hours at 105°C, the loss should not be more than 10%.

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 1 g of hyaluronic acid, the content of residue should not be more than 5%.

Assay 0.1 g of hyaluronic acid precisely dried, is accurately weighted, sodium chloride TS is added, shaken well for 3 hours, and mixed to make exactly 100 ml. 5 ml of this solution is accurately taken and sodium chloride TS is added to make exactly 100 ml, test solution. Separately, about 0.1 g of glucuronolactone is accurately weighted, sodium chloride TS is added, and dissolved to make exactly 100 ml. 3 ml of this solution is taken, sodium chloride TS is added to make exactly 100 ml, glucuronolactone standard solution. Pipette 5 ml of borax · sulfuric acid solution(0.95→100) into the test tube with stopper and cool down iced water, 1 ml each of test solution and glucuronolactone standard solution is added, maintain the temperature of these solutions become not more than room temperature, first slowly shaken and mixed, and next, vigorously shaken and mixed. Then it is boiled in a boiling water bath for 10 minutes, immediately cool down with ice water to room temperature, 0.2 ml of carbazole-ethanol solution(0.125→100) is added, shaken well and mixed. Again, it is boiled in a boiling water bath for 15 minutes and immediately cool down with ice water to room temperature. The solution, which is prepared with 1 ml of sodium chloride solution in the same manner above, is used as reference solution. The absorbance A_T and A_S of test solution and glucuronolactone standard solution at a 530nm wavelength determine and calculate the content of hyaluronic acid with following equation.

$$\text{Content(\%)} = \frac{\text{Content of glucuronolactone(mg)} \times A_T}{\text{Weight of sample(mg)} \times A_S} \times \frac{3}{5} \times 2.148 \times 100$$

$$2.148 = \frac{\text{Molecular weight of hyaluronic acid 1 unit (378.3)}}{\text{Molecular weight of glucuronolactone (176.12)}}$$

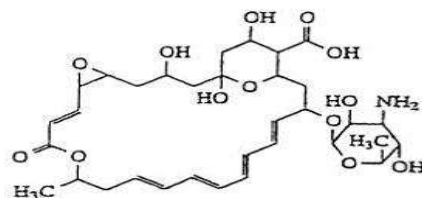
A_T : Absorbance of test solution

A_S : Absorbance of glucuronolactone standard solution

3/5 : dilution factor

204. Natamycin

Pimaricin

 $C_{33}H_{47}NO_{13}$

Molecular Weight : 665.73

Content The content should be more than 95.0% of Natamycin ($C_{33}H_{47}NO_{13}$, calculated on the anhydrous basis).

Description Natamycin is white to creamy white, crystalline powder.

Identification (1) On adding 1mg of Natamycin, on a spot plate, to 1ml of concentrated hydrochloric acid, a blue colour develops.

(2) A solution of 5mg of Natamycin in 0.1% glacial acetic acid in methanol has absorption maxima at about 290 nm, 303 nm and 318 nm.

Purity (1) Specific Rotation : 1 g of Natamycin(converted to a dehydrated form) dissolve in 100 ml of glacial acetic acid, measure the specific rotation, it should be $\alpha_D^{20} = +250 \sim +295^\circ$.

(2) Acidity : pH of suspension (1→100) should be 5.0~7.5. (measured by glass electrode).

(3) Lead : When 5.0 g of Natamycin is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 2.0 ppm.

(4) Arsenic : Place 0.25 g of Natamycin into a platinum, quartz, or porcelain crucible, add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.

(5) Mercury : When Natamycin is tested by Mercury Limit Test, its content should not be more than 1.0ppm.

(6) Total viable aerobic count : When Natamycin is tested by Microbiological Methods

for Total viable aerobic count in General Test Method in 「Standards and Specifications for Foods」, it should not be more than 100 per 1 g

Water Content Water content of accurately weighted 0.03 g of Natamycin is determined by direct titration method in water determination (Karl-Fisher Method) and should not be more than 9.0%.

Residue on Ignition When Residue on Ignition analysis is done with accurately weighted 2 g of Natamycin, Residue on Ignition should not be more than 0.5%.

Assay Transfer about 0.02 g of Natamycin Reference and the sample, accurately weighted, to a 100-ml volumetric flask. Add 5.0 ml of tetrahydrofuran, and sonicate for 10 min. Add 60 ml of methanol, and swirl to dissolve. Add 25 ml of water, and mix. Allow to cool to room temperature. Dilute with water to volume, mix, and filter through a membrane filter of 5- μ m or finer porosity. Separately inject about 20 μ l for each of the "standard" and "the sample" into the chromatograph, and record the peak areas of the major peaks. Calculate the content of Natamycin following equation with obtained height or area of peak. Preparation is done with using a light resistant container to block out direct sunlight.

Content of Natamycin($C_{33}H_{47}NO_{13}$) (%) =

$$\frac{\text{Weight of Natamycin reference standard converted into an anhydride (g)}}{\text{Weight of the sample converted into an anhydride (g)}} \times \frac{\text{Peak area of the sample solution}}{\text{Peak area of reference standard solution}} \times 100$$

ration Condition

Detector : UV 303nm

Column packing materials : 5~10 μ m octadecylsilanized silica for liquid chromatography

Column : stainless steel tube 4~6 mm \times 25 cm

Column temperature : room temperature

Mobile Phase : Dissolve 3.0 g of ammonium acetate and 1.0 g of ammonium chloride in 760 ml of water, and mix, and filter through a 0.5- μ m or finer porosity filter.

Flow rate : 2ml/min

Storage Standard of Natamycin

Natamycin should be stored in a light resistant container in a cold place.

205. Crude Magnesium Chloride(Sea Water)

Definition Crude magnesium chloride (Sea Water) is obtained by extracting and separating potassium chloride and sodium chloride from sea water, and its main ingredient is magnesium chloride.

Compositional Specifications of Crude Magnesium Chloride (Sea Water)

Content The sample contains 12.0~30.0% as crude magnesium chloride ($\text{MgCl}_2=95.21$).

Description Crude magnesium chloride (Sea Water) is colorless~pale yellow liquid with bitter taste.

Identification (1) When sodium hydroxide solution is added to Crude Magnesium Chloride (Sea Water), white gel phase precipitate is formed. Iodine TS is added to some of this solution, the precipitate turns dark brown. Also, although excess sodium hydroxide TS is added to some of the residue in the solution, precipitate doesn't dissolve.

(2) Crude Magnesium Chloride (Sea Water) shows the reaction (A) for Chlorides in Identification Tests.

Purity (1) Sulfate : Accurately weighted 0.25 g of Crude Magnesium Chloride (Sea Water) and dissolve in water to make 100 ml. When 2 ml of this solution is tested for sulfates, the content should not be more than the amount that corresponds to 0.5 ml of 0.01 N sulfuric acid.

(2) Bromide : Accurately weigh 1.0 g of Crude Magnesium Chloride (Sea Water), and dissolve in water to make 500 ml. Take 10 ml of this solution, make 100 ml with water. Again, 2 ml of this solution is taken, 3 ml of water, 2 ml of diluted phenol red TS and 1 ml of chloramine T solution(1→10,000) are added, and immediately mixed. Then it is allow to stand for 2 minutes, 0.15 ml of 0.1N sodium thiosulfate is added, mixed, and water is added to make 10 ml, test solution. Separately, potassium bromide is dried at 110°C for 4 hours, 2.979g of the solid is accurately weighted, water is added to make 1,000 ml, again 2 ml of this solution is accurately taken, and water is added to make 1,000 ml. 5 ml of this solution is taken, 2 ml of diluted phenol red TS and 1 ml of chloramine T solution(1→10,000) are added, immediately shaken and mixed. It is proceeded in the same manner as the test solution, reference solution. Using water as a reference, absorbance of the test solution and reference solution is measured at 590 nm, then the absorbance of test solution should not be higher than that of reference solution.

Diluted phenol red TS

Solution 1 : To 0.033g of phenol red, 1.5 ml of sodium hydroxide solution(2→25) and water are added and dissolved to make 100 ml.

Solution 2 : To 0.025g of ammonium sulfate, 235 ml of water is added, dissolved, 105 ml of sodium hydroxide solution(2→25) and 135 ml of acetic acid(3→25) are added and mixed well.

10 ml of solution 1 and 190 ml of solution 2 are mixed well. If needed, sodium hydroxide solution(2→25) or acetic acid (3→25) is added to adjust pH 4.7.

(3) Zinc: 4 g of Crude Magnesium Chloride (Sea Water) is accurately weighted, water is added to make 40ml, test solution. 30 ml of test solution is taken, 5 drops of acetic acid and 2 ml of potassium ferrocyanide solution(1→20) are added, shaken, mixed, and allow to stand for 10 minutes. The solution should not be more turbid than the following reference solution. To prepare reference solution, pipette 14ml of zinc standard solution, and add 10 ml of test solution and water to make 30ml. Add 5 drops of acetic acid and 2 ml of potassium ferrocyanide solution(1→20) to this solution, shake and mix, and allow to stand for 10 minutes. (not more than 70 ppm as Zinc)

Zinc standard solution : Accurately weigh 4.4g of zinc sulfate, dissolve with 1,000 ml water. Pipette 10 ml of this solution into a 1000 ml-volumetric flask with water to volume. 1ml of this solution contain 0.01 mg of zinc.

(4) Calcium : Accurately weigh 20 ml of test solution for assay, add water to make 100 ml. Add 0.2 ml of tartaric acid solution(1→5), then 10 ml of triethanol amine solution(3→10) and 10 ml of potassium hydroxide solution (1→10). Allow to stand for 5 minute, immediately titrate with 0.01 M EDTA determining endpoint indicator : 0.1 g of 2-oxy-1-(2'-oxy-4'-sulfo-1'-naphthylazo)-3-naphthoesan, and express the consumed volume as b (ml). At that time, the red-purple color of the solution completely disappears and the solution becomes blue. When the content of calcium calculate, it should not be more than 4.0% as calcium.

$$\text{Content of calcium(Ca)(\%)} = \frac{b \times 0.4008}{\text{Weight of sample(g)}}$$

(5) Sodium : 1.0 g of Crude Magnesium Chloride (Sea Water) is accurately weighted, water is added, and dissolved to make 1,000 ml. Again, 10 ml of this solution is taken and water is added to make 200 ml, test solution. Separately, sodium chloride is dried at 130°C for 2 hours, 2.542 g of the solid is accurately weighted, water is

added to make 1,000 ml. 2 ml of this solution is accurately taken and water is added to make 1,000 ml, reference solution. When test solution and reference solution are tested by Flame Atomic Absorption Spectrophotometry under following operation condition, the absorbance of test solution should not be higher than that of reference solution (not more than 4.0% as sodium).

Operation Condition

Light source lamp : Hollow cathode sodium lamp

Wavelength : 589.0nm

Combustible support gas : air

Combustible gas : acetylene

- (6) Potassium : Proceed the test by using test solution in Purity(5). Separately, potassium chloride is dried at 105°C for 2 hours, 1.907g of this solid is accurately weighted, and water is added to make 1,000 ml. 3 ml of this solution is taken, and water is added to make exactly 1,000 ml, reference solution. When test solution and reference solution are tested by Flame Atomic Absorption Spectrophotometry under following operation condition, the absorbance of test solution should not be higher than that of reference solution (not more than 6.0% as potassium).

Operation Condition

Light source lamp : Hollow cathode potassium lamp

Wavelength : 766.5nm

Combustible support gas : air

Combustible gas : acetylene

- (7) Arsenic : Place 0.25 g of Crude Magnesium Chloride (Sea Water) in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to it and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
- (8) Lead : Crude Magnesium Chloride is tested by Purity (2) for Sodium Metaphosphate(not more than 4.0 ppm).

Assay Accurately weigh 2 g of Crude Magnesium Chloride (Sea Water) and add water to make 200ml, test solution. To 5 ml of this solution, 50 ml of water and 5 ml of ammonia-ammonium chloride buffer (pH 10.7), titrate with 0.01 M EDTA solution determining endpoint (indicator : 2 drops of Eriochrome black T solution), and measure the consumed volume as a (ml). At that time the red color of the solution becomes blue. Calculate the content under following equation with using the consumed volume b (ml) obtained by Purity(4).

$$\text{Content of magnesium chloride(MgCl}_2\text{) (\%)} = \frac{(a - 0.25b) \times 0.952 \times 200}{\text{Weight of sample(g)} \times 5 \times 1,000} \times 100$$

$$1 \text{ ml of } 0.01\text{M E.D.T.A solution} = 0.952\text{mg MgCl}_2$$

206. Asparaginase

Definition Asparaginase is an enzyme obtained from a culture of *Aspergillus oryzae* and *Aspergillus niger*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Asparaginase

Content Asparaginase contains 90~130% of the indicated activity as Asparaginase.

Description Asparaginase is white~dark brown powder, granular, paste or colorless~dark brown liquid.

- Purity**
- (1) Arsenic : Place 0.25 g of Asparaginase in a platinum, quartz, or porcelain crucible. Add 10 ml solution of magnesium nitrate in ethyl alcohol (1→50) to it and then alcohol is ignited. It is then reduced to ash by heating at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath. When test for arsenic is carried out with this test solution, it should not be more than 4ppm.
 - (2) Lead : When 5.0 g of Asparaginase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5.0 ppm.
 - (3) Coliform Group : When Asparaginase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods in Food Code, it should not contain more than 30 per 1 g .
 - (4) Salmonella : When Asparaginase proceed as directed under Microbiological Methods for Salmonella in General Testing Methods in Food Code, it should be negative (-).
 - (5) E. coli : When 25 g of Asparaginase is tested by Microbiological Methods for E. coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay (activity)

Principle

The method is to measure ammonia which is generated from the hydrolysis of L-asparagine at 37°C(pH 7.0). Ammonia is subsequently combined with α-ketoglutarate to form L-glutamic acid, and it is measured by the consumed amount of NADH(Nicotineamide adenine dinucleotide, reduced).

Preparation of test solution : A certain amount of sample is taken and diluted with MOPS buffer solution so that 1 ml of the final diluted solution contains 0.4~1.0 ASNU. This solution is used as test solution. (Adjust absorption to be in the range of about 0.10~0.25).

Procedure : Equilibrate 2.4 ml of substrate solution in the $37 \pm 0.1^\circ\text{C}$ water bath for 10 minutes. Add 0.1 ml of test or control solution, immediately shaken, mixed and 1 ml of the solution transfer 1cm quartz cuvette. 0.1M MOPS buffer solution (pH 7.0) which contains Triton X-100, previously isothermalized, is used for blank test, and absorbance is rapidly measured at a 340 nm. Read the absorbance every 10 sec between 3 and 5 min from the start of the reaction for 2 min. (The absorbance at the reaction starting point should be 2.3~2.8. If the absorbance is below 2.3, prepare a new substrate solution). Carry out the test procedure in the same manner as above, at least twice for each test or control sample solution, and measure slope of absorbance curve per minute ($\Delta A/\text{minute}$). This slope should agree within 15 %.

Calculate the activity(ASNU/g) of test or control sample as follows :

$$\text{ASNU/g} = \frac{\Delta A/\text{minute} \times 2.5 \times D}{0.1 \times 6.3 \times 1 \times W}$$

$\Delta A/\text{minute}$: The absolute value of the decrease of absorbance per min for the test or control sample solution

2.5 : Volume of the final reaction solution (ml)

D : Dilution factor

0.1 : Volume of test solution used(ml)

6.3 : Extinction coefficient of NADH ($\text{ml} \cdot \mu\text{mol}^{-1} \cdot \text{cm}^{-1}$)

1.0 : Length of extinction cell (cm)

W : Weight of sample (g)

Definition of Activity :

One asparaginase unit(ANSU) is the amount of enzyme that produces $1\mu\text{mol}$ ammonia per minute under the above test operation condition.

Solutions

4M Sodium Hydroxide solution : Weigh 16 g of sodium hydroxide. Dissolve in water in a 100-ml volumetric flask. Add water to volume and mix until fully dissolved.

0.1M MOPS buffer solution (pH 7.0) : 20.9g of MOPS(Sigma M1254 or equivalent) dissolve in approximately 950ml of water in a 100 ml

volumetric flask. pH is adjusted to 7.0 with 4M sodium hydroxide solution, 1ml of Triton X-100 (Sigma T9284 or equivalent) is added, and water is added to volume and mix. The solution must be used on the day of preparation.

Substrate solution : 0.25g of L-asparagine (Sigma A7094 or equivalent) is weighted into a 25-ml volumetric flask, 20 ml of MOPS buffer solution (pH 7.0) is added, completely dissolved, and 0.011g of NADH(Roche 107735 or equivalent) is added. Again, to this, 0.063g of α -ketoglutarate(Sigma K3752 or equivalent) and at least 2,000 units of glutamate dehydrogenase (Fluka 49392 or equivalent) are added, and MOPS buffer solution (pH 7.0) is added to make 25 ml. The composition of the solution : 10mg/ml L-asparagine, 2.5mg/ml α -ketoglutarate, 0.44mg/ml NADH, Glutamate dehydrogenase, > 80 Unit/ml. This solution is stable for about 2 hours at room temperature.

Storage Standards of Asparaginase

Asparaginase should be stored in a hermetic container in a cold dark place.

207. α -Acetolactate decarboxylase

Definition It is an enzyme obtained from a culture of *Bacillus subtilis* that contains the gene for α -acetolactate decarboxylase from *Bacillus brevis*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of α -acetolactate decarboxylase

Content It contains 90~130% of the indicated activity as α -acetolactate decarboxylase.

Description It is white~dark brown powder or transparent~dark brown liquid.

- Purity** (1) Arsenic : Place 0.25 g of α -acetolactate decarboxylase in a platinum, quartz, or porcelain crucible. Add 10 ml of magnesium nitrate in ethyl alcohol (1→50) to the crucible, and ignite the sample with ethyl alcohol. Heat gently to ash at 450~550°C. If carbonaceous substance persists, wet with a suitable amount of nitric acid, and reheat severely to ash at 450~550°C. Cool the solution, and add 3 ml of hydrochloric acid to the residue, which is then dissolved by heating in a water bath, test solution. When test for arsenic is carried out with this test solution under IV. General Test Method in Korea Food Additives Code, it should not be more than 4ppm.
- (2) Lead : When 5.0 g of α -acetolactate decarboxylase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5ppm.
- (3) Coliform Group : α -acetolactate decarboxylase proceed as directed under Microbiological Methods for Coliform Group in General Testing Methods 「Standards and Specification for Foods (notified by KFDA)」. It should not be more than 30 per 1 g.
- (4) Salmonella : α -acetolactate decarboxylase is tested by Microbiological Methods for Salmonella in General Testing Methods 「Standards and Specification for Foods (notified by KFDA)」. It should be negative (-).
- (5) E. coli : α -acetolactate decarboxylase proceed as directed under Microbiological Methods for E. coli in General Testing Methods 「Standards and Specification for Foods (notified by KFDA)」. It should be negative (-).

Assay (Activity)

- Application and Principle : This test is to measure the activity of α -acetolactate decarboxylase in an enzyme that is obtained from a culture of *Bacillus subtilis* that contains the gene for α -acetolactate decarboxylase from *Bacillus brevis*. Activity test is based on measuring the absorbance of the mixture in the following manner: react the enzyme with α -acetolactate to produce acetoin, react the resultant acetoin with

mixture of 1-naphthol and creatine, measure absorbance.

- Preparation of test Solution : Dissolve the sample in the mixture of MES/Brij 35/NaCL solution and prepare final diluted solution containing 0.025 ~ 0.075 ADU per 1 ml.
- Test Procedure : Warm the enzyme solutions, MES buffer, and the substrate in water bath at 30°C for approximately 10minutes. Enzyme blank(B1). Pipette 0.2 ml of enzyme solution and 0.2 ml of MES buffer into a test tube. Mix, and immediately place the test tube back into the water bath at 30°C for 20min. Sample value(H1). Pipette 0.2 ml of enzyme solution and 0.2 ml of substrate into a test tube. Mix, and immediately place the test tube into the water bath at 30°C for 20min. Buffer blank(B2). Pipette 0.2 ml of MES buffer and 0.2 ml of MES/Brij 35/NaCL solution into a test tube. Mix, and immediately place the test tube into the water bath at 30°C for 20min. Buffer value(H2). Pipette 0.2 ml of MES/Brij 35/NaCL solution and 0.2 ml of substrate into a test tube. Mix, and immediately place the test tube into the water bath at 30°C for 20min. Exactly 20 min after mixing of each of solution B1, H1, B2, and H2, remove from water bath, add 4.6 ml of colour reagent, mix and leave at room temperature for exactly 40 min. At the end of this 40 min period measure the absorbance of the solution at 522 nm on a spectrophotometer or equivalent.
- Preparation of standard curve : Dissolve 0.1 g of acetoin in water in a 100-ml volumetric flask. Make to volume with water. Dilute 1, 2, 4, 6, and 8 ml of stock acetoin solution to volume with water in 100 ml volumetric flasks. Pipette 0.4 ml of the acetoin standard solutions into test tubes. Add 4.6 ml of colour reagent to each tube, mix, and let the test tube stand at room temperature for exactly 40 min. At the end of this 40 min period, measure absorbance at 522 nm. Plot optical density values at 522 nm for the acetoin standards against acetoin concentration($\mu\text{g/ml}$) of standard and generate a standard curve.

Activity of an enzyme is calculated by the following equation.

$$\text{Activity of an enzyme(ADU/g)} = \frac{\Delta A \times F}{88.1} \times 5.0 \times \frac{1}{20} \times \frac{1}{0.2} \times \frac{1}{w}$$

ΔA : $(H_1 - B_1) - (H_2 - B_2)$

F : The concentration($\mu\text{g/ml}$) of acetoin against one of absorbance obtained from standard curve

88.1 : Molecular weight of acetoin

5.0 : The volume of final enzyme solution

20 : reaction time(min)

0.2 : Taken volume of enzyme solution

W : Weight of enzyme in 1 ml of enzyme solution

Definition of activity : One α -acetolactate decarboxylase is the amount of enzyme which, by decarboxylation of α -acetolactate produces 1 μ mol of acetoin per min under above the test reaction conditions.

Solutions

- MES buffer (0.05 M, pH 6.0) : Dissolve 9.76 g of MES2-(N-morpholino)ethanesulphonic acid in approximately 900 ml of water. Adjust pH to 6.0 with 1N NaOH. Transfer to a 1,000 ml volumetric flask and make to volume with water. This solution may be kept for two weeks at room temperature.
- Brij 35 solution, 15% w/v : Dissolve 15.0 g of Brij 35(polyoxyethylene lauryl ether) in approximately 70 ml of water, heating to 60°C to aid dissolution. Cool the solution, transfer to a 100 ml volumetric flask and make to volume with water. This solution should be stored in a refrigerator, and can be kept for up to two months.
- MES / Brij 35 / NaCl solution : Dissolve 48.8 g of MES and 175.32 g of NaCl in approximately 4,500 ml of water. Add 17 ml of 15% Brij 35 solution. Adjust pH to 6.0 with 1 N NaOH. Transfer to a 5,000 ml volumetric flask and make to volume with water. This solution may be kept for two weeks at room temperature.
- α -Acetolactate substrate : Pipette 100 μ l of ethyl-2-acetoxy-2-methylacetolactate into a 50 ml volumetric flask. Add 6.0 ml of 0.5 N NaOH to the flask and stir for 20 min. Add MES buffer to bring the volume to approximately 40 ml. Adjust pH to 6.0 with 0.5 N HCl. Make to volume with MES buffer. This substrate should be made just before use.
- Colour reagent : Dissolve 5.0 g of 1-naphthol and 0.5 g of creatine in 1 N NaOH, make to volume with 1 N NaOH in a 500 ml volumetric flask. This

colour reagent should be made fresh just before use

Storage Standards of α -acetolactate decarboxylase

α -acetolactate decarboxylase should be stored in a hermetic container in a cold dark place.

208. Xylanase

Definition Xylanase is an enzyme obtained from cultures of *Thermomyces lanuginosus* and *Aspergillus oryzae* inserted the genes of xylanase. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Xylanase

Content When Xylanase is analyzed quantitatively, it contains 90~130% of the indicated activity as Xylanase.

Description Xylanase is white ~ dark brown power, granular, pasty substances or transparent ~ brown liquid.

- Purity**
- (1) Arsenic : 0.25 g of Xylanase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by gradually heating at 450~550°. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate (not more than 4ppm).
 - (2) Lead : When 5.0 g of Xylanase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm
 - (3) Coliform Group : Xylanase proceed as directed under Microbe Test Methods in Coliform Group in General Test Methods in Food Code. It should contain not more than 30 colonies per 1 g of this product.
 - (4) Salmonella : When Xylanase is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
 - (5) E. Coli : When 25 g of Xylanase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

Application and principle : Azo-wheat arabinoxylan substrate(colored with remazol) by treating with Xylanase for 15 minutes at pH 6.0, temperature 50℃ is precipitated with ethanol and activity test is based on colorimetry of the blue color of decomposition supernatant of remazol colored substrate which is not precipitated.

Preparation of Test Solution : When Xylanase is weighted, use water or phosphate

buffer solution so that 1 ml of the final diluent solution contains 0.4~1.4 Xylanase unit.

Procedure : Take precisely 0.1 ml of test solution into tube, keep it at $50\pm0.5^{\circ}\text{C}$ for 10 minutes. Then add precisely 0.9 ml of substrate solution and immediately shake it to mix. Keep this solution at $50\pm0.5^{\circ}\text{C}$ precisely for 30 minutes. Then add 5 ml of stop solution and immediately shake it to mix. Keep this solution for 30 minutes and centrifuge it by 4,000rpm for 15 minutes. Measure absorbance of supernatant at wavelength 585nm within 20 minutes.

Preparation of standard curve : Weigh precisely Xylanase(Novozyme co. or its equivalent) which contains 4000 Xylanase unit. After dissolving it in 0.1M phosphate buffer solution(pH 6.0), make to volume to 200 ml. Take precisely 2 ml, 3 ml, 4 ml, 5 ml, 6 ml and 7 ml of this solution. Then add 0.1M phosphate buffer solution(pH 6.0) to each solution to make to 100 ml. This solution is used as each standard solution. 1 ml of each solution contains Xylanase of 0.4, 0.6, 0.8, 1.2 and 1.4 unit. Take precisely 0.1 ml of standard solution into each tube, keep it at $50\pm0.5^{\circ}\text{C}$ for 10 minutes. Then add precisely 0.9 ml of substrate solution and immediately shake it to mix. Keep this solution at $50\pm0.5^{\circ}\text{C}$ precisely for 30 minutes. Then add 5 ml of stop solution and immediately shake it to mix. Keep this solution for 30 minutes and centrifuge it by 4,000rpm for 15 minutes. Measure absorbance of supernatant at wavelength 585nm within 20 minutes. The factor of enzyme(unit/ml) is plotted along the X axis and the absorbance is plotted along the Y axis. Prepare standard curve of enzyme activity.

Activity of an enzyme is calculated by the following equation.

$$\text{Xylanase (units/g)} = \frac{C}{W}$$

C : Activity of test solution is obtained from standard curve

W : Weight of sample in 1 ml of test solution(g)

Reagent

Substrate solution : Weigh precisely 0.5 g of azo-wheat arabinoxylan(Megazyme co. or its equivalent) and dissolve it in 0.1M phosphate buffer solution(pH 6.0). Add 0.1M phosphate buffer solution(pH 6.0) to make to 100ml.

Stop solution : Add 99.9% ethanol to 6.65 ml of 2N hydrochloric acid to make the total volume to 1,000 ml.

Phosphate buffer solution(pH 6.0) : Weigh 60.5 g of sodium phosphate, monobasic($\text{Na}_2\text{H}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and 10.945 g of sodium phosphate, dibasic($\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$) and dissolve them in 400 ml of water. Add 2 ml of 4N sodium hydroxide solution and

then add water to make to 500 ml. After taking 200 ml of this solution, add 1,600 ml of water to mix. Adjust pH to 6.0 with 4N sodium hydroxide solution or 2N hydrochloric acid and add water to make to 2,000 ml.

Storage Standard of Xylanase

Xylanase should be stored in a hermetic container in a cold dark place.

209. 5'-Deaminase

Definition 5'-Deaminase is an enzyme obtained from cultures of *Aspergillus melleus*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of 5'-Deaminase

Content When 5'-Deaminase is analyzed quantitatively, it contains 90~130% of the indicated activity as 5'-Deaminase.

Description 5'-Deaminase is white ~ dark brown power, granular, pasty substances or transparent ~ dark brown liquid.

- Purity**
- (1) Arsenic : 0.25 g of 5'-Deaminase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by gradually heating at 450~550°. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate (not more than 4ppm).
 - (2) Lead : When 5.0 g of 5'-Deaminase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm
 - (3) Coliform Group : 5'-Deaminase proceed as directed under Microbe Test Methods in Coliform Group in General Test Methods in Food Code. It should contain not more than 30 colonies per 1 g of this product.
 - (4) Salmonella : When 5'-Deaminase is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
 - (5) E. Coli : When 25 g of 5'-Deaminase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

Application and principle : Activity test is based on measuring the absorbance of the mixture in the following manner: react the 5'-Deaminase with adenosine 5'-monophosphate disodium substrate to produce inosine 5'-monophosphate sodium, measure absorbance of the rest of adenosine 5'-monophosphate disodium and inosine 5'-monophosphate sodium at the most great difference of absorbance wavelength

265nm.

Preparation of Test Solution : When 5'-Deaminase is weighted, use water or phosphate buffer solution so that 1 $\mu\ell$ of the final diluent solution contains 5~30 5'-Deaminase unit.

Procedure : Take 3 ml of substrate solution into tube, keep it at $37\pm0.5^\circ\text{C}$ precisely for 5 minutes. Then add precisely 1 ml of test solution and immediately shake it to mix. Keep this solution at $37\pm0.5^\circ\text{C}$ precisely for 15 minutes. Then add 4 ml of diluent perchloric acid(1 \rightarrow 30) and immediately shake it to mix. Take precisely 2 ml of this solution and add water to make to 100 ml. Using water as a reference solution, absorbance(A_T) is measured at wavelength 265 nm. Separately, take 3 ml of substrate solution and 4 ml of diluent perchloric acid(1 \rightarrow 30) into tube and immediately shake it to mix. And then add 1 ml of test solution and immediately shake it to mix. Take precisely 2 ml of this solution and add water to make to 100 ml. Using water as a reference solution, absorbance(A_B) of blank enzyme test solution is measured at wavelength 265 nm.

Activity of an enzyme is calculated by the following equation.

$$\begin{array}{l} \text{5'-Deaminase} \\ \text{(units/g)} \end{array} = (A_B - A_T) \times \frac{10}{0.001} \times \frac{8}{2} \times \frac{60}{15} \times \frac{1}{W}$$

A_B : Absorbance of blank enzyme test solution

A_T : Absorbance of test solution

10/0.001 : Unit Conversion Factor(When difference in absorption is 0.001, it corresponds to 10 unit)

W : Weight of sample in 1 ml of test solution(g)

Definition of Activity : 1 5'-Deaminase unit corresponds to the amount of enzyme which decreases 0.001 of difference in absorption for 60 minutes under the conditions above.

Reagent

Substrate solution : Dry adenosine 5'-monophosphate disodium in advance at 105°C for 4 hours. And calculate loss on drying. Weigh precisely 330.2 mg as the dried basis and dissolve it in about 25 ml of water. Adjust pH to 6.0 with 4N sodium hydroxide solution or 2N hydrochloric acid and add water to make to 100 ml. When using it, it is used as the mixture solution. The mixing ratio of this solution and 1/15M Phosphate buffer solution(pH 5.6) is 1:2.

1/15M phosphate buffer solution(pH 5.6) : Dissolve 9.07 g of potassium dihydrogen phosphate in water to make to 1,000 ml(A solution) and dissolve 9.46 g of anhydrate disodium hydrogen phosphate in water to make to 1,000 ml(B solution). The mixing

ratio of A solution and B solution is 14:1. Adjust pH to 5.6.

Storage Standard of 5'-Deaminase

5'-Deaminase should be stored in a hermetic container in a cold dark place.

210. α -Glucosidase

Definition α -Glucosidase is an enzyme obtained from cultures of *Aspergillus niger*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of α -Glucosidase

Content When α -Glucosidase is analyzed quantitatively, it contains 90~130% of the indicated activity as α -Glucosidase.

Description α -Glucosidase is white ~ dark brown power, granular, pasty substances or transparent ~ brown liquid.

- Purity**
- (1) Arsenic : 0.25 g of α -Glucosidase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by gradually heating at 450~550°. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate (not more than 4ppm).
 - (2) Lead : When 5.0 g of α -Glucosidase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm
 - (3) Coliform Group : α -Glucosidase proceed as directed under Microbe Test Methods in Coliform Group in General Test Methods in Food Code. It should contain not more than 30 colonies per 1 g of this product.
 - (4) Salmonella : When α -Glucosidase is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
 - (5) E. Coli : When 25 g of α -Glucosidase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

Application and principle : 4-Nitrophenyl- α -D-glucopyranoside substrate to produce p-nitrophenol by treating with α -Glucosidase at 40°C. is precipitated with ethanol and activity test is based on measuring absorbance of p-nitrophenol.

Preparation of Test Solution : Take the sample and add water to 10 ml of 1N diluted acetic acid • sodium acetate buffer solution(pH 5.0) to make to 1,000ml. Make the

final diluent solution with using 1N diluted acetic acid • sodium acetate buffer solution to 0.5 ml. Test Solution is prepared so that the absorbance to be measured will be within a range of 0.25~0.95 under the following test method. The solution is prepared before use.

Procedure : Take 2 ml of substrate solution into tube, keep it at $40 \pm 0.5^\circ\text{C}$ precisely for 5 minutes. Then add 0.5 ml of test solution and shake it to mix. Keep this solution at the constant temperature $40 \pm 0.5^\circ\text{C}$ precisely for 15 minutes. Add 0.5 ml of 10% sodium carbonate solution to this solution and mix it. Cool it in ice bath for 5 minutes and keep it in running water until measure the absorbance. Using water as a reference solution, absorbance(A_1) is measured at wavelength 420 nm. Separately, take 0.5 ml of test solution for blank enzyme test into tube. After adding 0.5 ml of 10% sodium carbonate solution to this solution, mix it. Then add 2 ml of substrate solution and cool it in ice bath for 5 minutes and keep it in running water until measure the absorbance. Using water as a reference solution, absorbance(A_2) of blank enzyme test solution is measured at wavelength 420 nm.

Activity of an enzyme is calculated by the following equation.

$$\alpha\text{-Glucosidase (units/g)} = (A_1 - A_2) \times F \times \frac{3}{0.5} \times \frac{1}{10} \times \frac{1}{W}$$

A_1 : Absorbance of enzyme reaction solution

A_2 : Absorbance of blank enzyme test solution

F : Amount of p-nitrophenol(When difference in absorption is 1.0 from standard curve)

10 : Reaction time(min)

W : Weight of sample in 1 ml of test solution(g)

Preparation of standard curve : Weigh 0.1391 g of p-nitrophenol and dissolve it in p-nitrophenol diluent solution, make to volume to 500 ml. Take precisely 0.5 ml, 1 ml, 1.5 ml, 2 ml, 2.5 ml of this solution. Then add p-nitrophenol diluent solution to each solution to make to 100 ml. This solution is used as each standard solution. 1 ml of each solution contains p-nitrophenol of 0.01, 0.02, 0.03, 0.04 and 0.05 $\mu\text{mol/ml}$. Using water as a reference solution, absorbance is measured at wavelength 420 nm. The concentration of p-nitrophenol($\mu\text{mol/ml}$) is plotted along the X axis and the absorbance is plotted along the Y axis. Prepare standard curve of enzyme activity.

Definition of Activity : 1 α -Glucosidase unit corresponds to the amount of enzyme which separates 1 μmol of p-nitrophenol from the substrate under the conditions above.

Reagent

Substrate solution : Dissolve 0.113 g of 4-Nitrophenyl- α -D-glucopyranoside in 35 ml of water. And add 5 ml of 1N acetic acid • sodium acetate buffer solution(pH 5.0) to this solution. Then add water to make to 50 ml. The solution is prepared before use.

1N acetic acid • sodium acetate buffer solution(pH 5.0) : After mixing 600 ml of 1N sodium acetate solution and 300ml of 1N acetic acid, adjust pH to 5.0 with 1N acetic acid.

p-nitrophenol diluent solution : Add water to 82 ml of 1N acetic acid • sodium acetate buffer solution(pH 5.0) to make to 1,000ml. Then add 200 ml of 10% sodium carbonate solution to this solution. The solution is prepared before use.

Storage Standard of α -Glucosidase

α -Glucosidase should be stored in a hermetic container in a cold dark place.

211. Phosphodiesterase

Definition Phosphodiesterase is an enzyme obtained from cultures of *Penicillium citrinum*. Dilutant or stabilizer can be added for the purpose of activity adjustment and quality preservation.

Compositional Specifications of Phosphodiesterase

Content When Phosphodiesterase is analyzed quantitatively, it contains 90~130% of the indicated activity as Phosphodiesterase.

Description α-Glucosidase is white ~ dark brown power, granular, pasty substances or transparent ~ brown liquid.

- Purity**
- (1) Arsenic : 0.25 g of Phosphodiesterase is placed in a platinum, quartz, or porcelain crucible. 10 ml of magnesium nitrate in ethyl alcohol (1→50) is added to the crucible and then alcohol is ignited. It is then reduced to ash by gradually heating at 450~550°. If carbonaceous substance persists, it is wetted with minute amount of nitric acid, which is further heat treated at 450~550°. After cooling, 3 ml of hydrochloric acid is added to the residue, which is then dissolved by heating in a water bath. When this test solution proceed as directed under Arsenic, it should be appropriate (not more than 4ppm).
 - (2) Lead : When 5.0 g of Phosphodiesterase is tested by Atomic Absorption Spectrophotometry or Inductively Coupled Plasma Emission Spectroscopy, its content should not be more than 5 ppm
 - (3) Coliform Group : Phosphodiesterase proceed as directed under Microbe Test Methods in Coliform Group in General Test Methods in Food Code. It should contain not more than 30 colonies per 1 g of this product.
 - (4) Salmonella : When Phosphodiesterase is tested by Microbe Test Methods for Salmonella in General Test Method 「Standards and Specifications for Foods」, it should be negative (-).
 - (5) E. Coli : When 25 g of Phosphodiesterase is tested by Microbe Test Methods for E. Coli in General Test Method 「Standards and Specifications for Foods」 (noticed by KFDA), it should be negative (-).

Assay(activity)

Application and principle : Adenosine 3'-monophosphate sodium salt substrate by treating with Phosphodiesterase produce phosphoric acid Change it to phosphomolybdic acid under acidic condition of perchloric acid. Reduce it with amidol solution and it produces molybdenum blue. Activity test is based on colorimetry of the blue color of molybdenum blue.

Preparation of Test Solution : When Phosphodiesterase is weighted, use water so that 1 ml of the final diluent solution contains 0.09~0.43 Phosphodiesterase unit.

Preparation of standard curve : Weigh 0.142 g of disodium hydrogen phosphate(anhydrous form) and make to volume to 100 ml($10\mu\text{mol/ml}$). Take precisely 1 ml, 5 ml, 10 ml, 15 ml, 20 ml of this solution. Then add water to each solution to make to 100 ml. This solution is used as each standard solution. Take 0.5 ml of this solution and add 4 ml of 6% perchloric acid solution to each solution. Then immediately shake it to mix. Add 0.4 ml of amidol solution and shake it to mix. Add precisely 0.2 ml of ammonium molybdate solution(8.3→100) and shake it to mix. Using water as a reference solution, absorbance(A_1, A_2, A_3, A_4 and A_5) of each solution is measured at wavelength 750 nm. Separately, take 0.5 ml of water and add 4 ml of 6% perchloric acid solution and shake it to mix. And then add 0.4 ml of amidol solution and shake it to mix. Add precisely 0.2 ml of ammonium molybdate solution(8.3→100) and shake it to mix. Using water as a reference solution, absorbance(A_0) is measured at wavelength 750 nm. The concentration($\mu\text{mol/ml}$) of phosphoric acid of each solution is plotted along the X axis and the absorbance($A_n - A_0$) is plotted along the Y axis. Prepare standard curve of phosphoric acid. And extinction coefficient of phosphoric acid is calculated by standard curve.

Procedure : Take 0.4 ml of substrate solution into tube, keep it at $70\pm 0.5^\circ\text{C}$ precisely for 5 minutes. Then add 0.1 ml of test solution and shake it to mix. Keep this solution at $70\pm 0.5^\circ\text{C}$ precisely for 15 minutes to react. Add 4 ml of 6% perchloric acid solution and shake it to mix. And then add 0.4 ml of amidol solution and shake it to mix. Add precisely 0.2 ml of ammonium molybdate solution(8.3→100) and shake it to mix. Keep it in running water for 15 minutes and using water as a reference solution. Absorbance(A_T) of enzyme reaction solution is measured at wavelength 750 nm. Separately, take 0.4 ml of substrate solution and add 4 ml of 6% perchloric acid solution and shake it to mix. And then add 0.1 ml of test solution and shake it to mix. And then add 0.4 ml of amidol solution and shake it to mix. Add 0.2 ml of ammonium molybdate solution(8.3→100) and shake it to mix. Keep it in running water for 15 minutes and using water as a reference solution. Absorbance(A_B) of blank enzyme test solution is measured at wavelength 750 nm.

Activity of an enzyme is calculated by the following equation.

$$\text{Phosphodiesterase (units/g)} = (A_T - A_B) \times \frac{1}{E} \times \frac{5.1}{0.1} \times \frac{1}{15} \times \frac{1}{W}$$

A_T : Absorbance of enzyme reaction solution

A_B : Absorbance of blank enzyme test solution

E : Extinction coefficient(absorbance of concentration($\mu\text{mol/ml}$) of phosphoric acid in

5.1 ml of amount of the total reaction solution)

15 : Reaction time(min)

W : Weight of sample in 1 ml of test solution(g)

Definition of Activity : 1 Phosphodiesterase unit corresponds to the amount of enzyme which separates 1 μmol of phosphoric acid per minute from the substrate under the conditions above.

Reagent

Substrate solution : Dissolve 0.113 g of 4-Nitrophenyl- α -D-glucopyranoside in 35 ml of water. And add 5 ml of 1N acetic acid • sodium acetate buffer solution(pH 5.0) to this solution. Then add water to make to 50 ml. The solution is prepared before use.

Substrate solution : Dry 0.1 g of adenosine 3'-monophosphate sodium salt in advance at 105°C for 4 hours. And calculate loss on drying. Weigh precisely adenosine 3'-monophosphate sodium salt as the dried basis corresponding to 0.0183 g. And dissolve it in 10ml of barbital sodium • hydrochloric acid buffer solution(pH 5.0). Then filter it with membrane filter(0.45 μm). The solution is prepared before use.

Amidol solution : Weigh 0.5 g of amidol and 10 g of sodium sulfite. Dissolve them in water to make to 50 ml and filter this solution. The solution is prepared before use.

6% perchloric acid solution : Dilute 20 ml of 60% perchloric acid with water to make to 200 ml.

Barbital sodium • hydrochloric acid buffer solution(pH 5.0) : Take 100 ml of barbital sodium • sodium acetate buffer solution(1/7mol/L) and 40 ml of sodium chloride solution(8.5→100). Add 100 ml of water to this solution and adjust pH to 5.0 with 1N hydrochloric acid. Then add water to make to 500 ml.

Barbital sodium • sodium acetate buffer solution(1/7mol/L) : Weigh 5.88g of barbital sodium and 2.34g of sodium acetic anhydride. And add water to make to 200 ml.

Storage Standard of Phosphodiesterase

Phosphodiesterase should be stored in a hermetic container in a cold dark place.