Food Chemicals Codex: First Supplement to the Second Edition (1974)

Pages
113

Size
6 x 8

ISBN
0309338883

Subcommittee on Codex Specifications; Committee on Food Protection; Food and Nutrition Board; Division of Biological Sciences; Assembly of Life Sciences; National Research Council

FIRST SUPPLEMENT TO THE

FOOD CHEMICALS CODEX

SECOND EDITION

(First Supplement to F.C.C. II)

Effective June 15, 1974

Subcommittee on Codex Specifications
Committee on Food Protection
Food and Nutrition Board
Division of Biological Sciences
Assembly of Life Sciences
National Research Council

NATIONAL ACADEMY OF SCIENCES
Washington, D. C. 20418
1974

NOTICE

The project that is the subject of this report was approved by the Governing Board of the National Research Council, acting in behalf of the National Academy of Sciences. Such approval reflects the Board's judgment that the project is of national importance and appropriate with respect to both the purposes and resources of the National Research Council.

The members of the committee selected to undertake this project and prepare this report were chosen for recognized scholarly competence and with due consideration for the balance of disciplines appropriate to the project. Responsibility for the detailed aspects of this report rests with that committee.

Each report issuing from a study committee of the National Research Council is reviewed by an independent group of qualified individuals according to procedures established and monitored by the Report Review Committee of the National Academy of Sciences. Distribution of the report is approved, by the President of the Academy, upon satisfactory completion of the review process.

Acknowledgments. This study was supported in part by U.S. Food and Drug Administration Research Grant No. FD 00213, administered by the Department of Health, Education, and Welfare, U.S. Public Health Service.

Permission to adapt ASTM Methods D-2008-62, D-938-60, and D-721-56 T for use in this Codex has been granted by the American Society for Testing and Materials, 1961 Race Street, Philadelphia, Pa. 19103.

Compliance with Federal Statutes. The fact that an article appears in this Food Chemicals Codex does not exempt it from compliance with requirements of Acts of Congress or with regulations and rulings issued by agencies of the United States Government under authority of these Acts.

Revisions of the federal requirements that affect the Codex standards will be included in Codex Supplements as promptly as practicable.

ISBN 0-309-01949-4

Library of Congress Catalog Card No. 66-61563

Printed in the United States of America

Organization of the Food Chemicals Codex

National Academy of Sciences—National Research Council

PHILIP HANDLER, *President*

ASSEMBLY OF LIFE SCIENCES (1973–1974)

JAMES D. ELBERT, *Chairman* LEONARD LASTER, *Executive Director*

DIVISION OF BIOLOGICAL SCIENCES (1973–1974)

D. S. FARNER, *Chairman* R. B. STEVENS, *Executive Secretary*

FOOD AND NUTRITION BOARD (1973–1974)

L. J. FILER, JR., *Chairman* PAUL E. JOHNSON, *Executive Secretary*

COMMITTEE ON FOOD PROTECTION (1973–1974)

E. M. FOSTER, *Chairman* LORNE A. CAMPBELL, *Staff Officer* (until Oct. 1973)

FOOD CHEMICALS CODEX PROJECT (1966–1974)

DURWARD F. DODGEN, *Director* (1969–)
Consultant (1966–1969)

PAUL E. JOHNSON, *Director* (1966–1969) JUSTIN L. POWERS, *Consultant*

ADDITIONS, CHANGES, AND CORRECTIONS

Additions, changes, and corrections listed herein constitute revisions in the Food Chemicals Codex, Second Edition (F.C.C. II), effective June 15, 1974, unless otherwise indicated. Page numbers refer to F.C.C. II, unless indicated by a reference to pages in THIS SUPPLEMENT.

GENERAL PROVISIONS

Added Substances, page 8

Change part (e) of this paragraph to read:

(e) does not interfere with the assays and tests prescribed for determining compliance of the substances with the Food Chemicals Codex requirements, except that such interferences may be provided for in special cases that do not involve abrogation of the other requirements in this paragraph.

MONOGRAPHS

Alginic Acid, page 26

Change the paragraph entitled *Heavy Metals*, page 27, to read:

Heavy metals. Prepare and test a 500-mg. sample as directed in *Method II* under the *Heavy Metals Test*, page 920, but use nitric acid instead of sulfuric acid to wet the sample prior to ignition, and cautiously ignite in a platinum crucible. Any color does not exceed that produced in a control (*Solution A*) containing 20 mcg. of lead ion (Pb).

Change the paragraph entitled *Insoluble matter*, page 27, to read:

Insoluble matter. Disperse about 2 grams of the sample, accurately weighed, in 800 ml. of water in a 2000-ml. flask. Neutralize to pH 7 with sodium hydroxide T.S., and then add 3 ml. in excess. Cover the flask, heat to boiling, and boil for 1 hour with frequent stirring. Filter while hot through a tared Gooch crucible provided with a glass fiber filter (2.4-cm., No. 934AH, Reeve Angel & Co., Clif-

ton, N. J., or equivalent filter). If slow filtration is caused by high viscosity of the sample solution, boil until the viscosity is reduced enough to permit filtration. Wash the crucible thoroughly with hot water, dry the crucible and its contents at 105° for 1 hour, cool, and weigh.

Aluminum Ammonium Sulfate, page 34

Change the paragraph entitled *Selenium*, page 35, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Aluminum Potassium Sulfate, page 36

Change the paragraph entitled *Selenium*, page 37, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Aluminum Sodium Sulfate, page 37

Change the paragraph entitled *Selenium*, page 38, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Aluminum Sulfate, page 39

Insert the following SPECIFICATION (*Limits of Impurities*) for *Fluoride:*

Fluoride. Not more than 30 parts per million (0.003 percent).

Insert the following new paragraph to precede the paragraph entitled *Heavy metals*, page 40:

Fluoride. Determine as directed in the test for *Fluoride* under *Aluminum Ammonium Sulfate*, page 34.

Change the paragraph entitled *Selenium*, page 40, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Aluminum Sulfate Solution, page 40

Insert the following SPECIFICATION (*Limits of Impurities*) for *Fluoride:*

Fluoride. Not more than 30 parts per million (0.003 percent), calculated on the $Al_2(SO_4)_3.18H_2O$ determined in the *Assay*.

Insert the following new paragraph to precede the paragraph entitled *Heavy metals*, page 42:

Fluoride. Determine as directed in the test for *Fluoride* under *Aluminum Ammonium Sulfate*, page 34, using an accurately weighed amount of the solution equivalent to 1.67 grams of $Al_2(SO_4)_3.18H_2O$.

Change the paragraph entitled *Selenium*, page 42, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using an accurately weighed amount of the solution equivalent to 200 mg. of $Al_2(SO_4)_3.18H_2O$.

Ammonium Alginate, page 43

Change the paragraph entitled *Heavy metals*, page 44, to read:

Heavy metals. Determine as directed in the revised test for *Heavy metals* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Change the paragraph entitled *Insoluble matter*, page 44, to read:

Insoluble matter. Determine as directed in the revised test for *Insoluble matter* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Ammonium Bicarbonate, page 44

Change the SPECIFICATION (*Limits of Impurities*) for *Nonvolatile residue*, page 45, to read:

Nonvolatile residue. Not more than 0.05 percent (0.55 percent for products containing a suitable anticaking agent). [*Note:* This revision became effective on March 14, 1973.]

Ammonium Chloride, page 47

Change the SPECIFICATION for *Assay* to read:

Assay. Not less than 99.5 percent of NH_4Cl after drying.

Ammonium Saccharin, page 51

Change the last sentence of the *Description* to read:

It is intensely sweet.

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Toluenesulfonamides:*

Toluenesulfonamides. Not more than 100 parts per million (0.01 percent).

Change the paragraph entitled *Selenium*, page 52, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Insert the following new paragraph to precede the *Packaging and storage* statement, page 52:

Toluenesulfonamides. Determine as directed under *Sodium Saccharin*, page 56 of THIS SUPPLEMENT.

Ammonium Sulfate, page 52

Change the paragraph entitled *Selenium*, page 53, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

L-Asparagine

Insert the following new monograph to precede the monograph entitled DL-*Aspartic Acid*, page 69:

L-ASPARAGINE

L-α-Aminosuccinamic Acid

$H_2NOCCH_2CH(NH_2)COOH$

$C_4H_8N_2O_3.H_2O$ Mol. wt. 150.13

DESCRIPTION

White crystals or crystalline powder having a slightly sweet taste. It is soluble in water and practically insoluble in alcohol and in ether. Its solutions are acid to litmus. It melts at about 234°.

IDENTIFICATION

To 100 mg. of the sample add 5 ml. of sodium hydroxide T.S., heat on a water bath for 1 hour, adjust the pH to 5.0 with diluted hydrochloric acid T.S., and add 100 mg. of triketohydrindene hydrate. The vapor evolved changes to blue the color of acetaldehyde test paper (see page 105 of THIS SUPPLEMENT).

SPECIFICATIONS

Assay. Not less than 98.0 percent and not more than 101.0 percent of $C_4H_8N_2O_3$ after drying.

Loss on drying. Between 11.5 and 12.5 percent.

Specific rotation, $[\alpha]_D^{20°}$. Between +33.0° and +36.5° after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Residue on ignition. Not more than 0.1 percent.

TESTS

Assay. Dissolve about 130 mg. of the sample, previously dried at 105° for 4 hours and accurately weighed, in 3 ml. of formic acid and 50 ml. of glacial acetic acid, and titrate with 0.1 *N* perchloric acid, determining the end-point potentiometrically. Perform a blank determination (see page 2), and make any necessary correction. Each ml. of 0.1 *N* perchloric acid is equivalent to 13.21 mg. of $C_4H_8N_2O_3$.

Loss on drying, page 931. Dry at 105° for 4 hours.

Specific rotation, page 939. Determine in a solution containing 10 grams of a previously dried sample in sufficient 6 *N* hydrochloric acid to make 100 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Residue on ignition, page 945. Ignite 1 gram as directed in the general method.

Packaging and storage. Store in well-closed, light-resistant containers.

Functional use in foods. Nutrient; dietary supplement.

L-Aspartic Acid

Insert the following new monograph to precede the monograph entitled *Azodicarbonamide*, page 70:

L-ASPARTIC ACID

L-Aminosuccinic Acid

$HOOCCH_2CH(NH_2)COOH$

$C_4H_7NO_4$ Mol. wt. 133.10

DESCRIPTION

White, odorless crystals or crystalline powder having a slightly acid taste. It is slightly soluble in water but insoluble in alcohol and in ether. It melts at about 270°.

IDENTIFICATION

To 5 ml. of a 1 in 1000 solution add 1 ml. of triketohydrindene hydrate T.S. A bluish purple color appears.

SPECIFICATIONS

Assay. Not less than 98.5 percent of $C_4H_7NO_4$ after drying.

Specific rotation, $[\alpha]_D^{20°}$. Between +24.5° and +26.0° after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Loss on drying. Not more than 0.25 percent.

Residue on ignition. Not more than 0.1 percent.

TESTS

Assay. Dissolve about 250 mg., previously dried at 105° for 3 hours and accurately weighed, in 100 ml. of recently boiled and cooled water,

add phenolphthalein T.S., titrate with 0.1 *N* sodium hydroxide to the first appearance of a faint pink color that persists for at least 30 seconds. Each ml. of 0.1 *N* sodium hydroxide is equivalent to 13.31 mg. of $C_4H_7NO_4$.

Specific rotation, page 939. Determine in a solution containing 8 grams of a previously dried sample in sufficient 6 *N* hydrochloric acid to make 100 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Loss on drying, page 931. Dry at 105° for 3 hours.

Residue on ignition, page 945. Ignite 1 gram as directed in the general method.

Packaging and storage. Store in well-closed, light-resistant containers.

Functional use in foods. Nutrient; dietary supplement.

Azodicarbonamide, page 70

Change the paragraph entitled *Assay* to read:

Assay. Transfer about 225 mg. of the sample, previously dried in a vacuum oven at 50° for 2 hours and accurately weighed, into a 250-ml. glass-stoppered iodine flask. Add about 23 ml. of dimethyl sulfoxide to the flask, washing any adhered sample down with the solvent, then stopper the flask, and place about 2 ml. of the solvent in the cup or lip of the flask. Swirl occasionally until complete solution of the sample is effected, and then loosen the stopper to drain the remainder of solvent into the flask and to rinse down any dissolved sample into the solution. Add 5.0 grams of potassium iodide followed by 15 ml. of water, then immediately pipet 10 ml. of 0.5 *N* hydrochloric acid into the flask, and stopper rapidly. Swirl until the potassium iodide dissolves, and allow to stand for 20–25 minutes protected from light. Titrate the liberated iodine with 0.1 *N* sodium thiosulfate to the disappearance of the yellow color. Titrate with additional thiosulfate if any yellow color appears within 15 minutes. Perform a blank determination on a solution consisting of 25 ml. of dimethyl sulfoxide, 5.0 grams of potassium iodide, 15 ml. of water, and 5 ml. of 0.5 *N* hydrochloric acid, and make any necessary correction. Each ml. of 0.1 *N* sodium thiosulfate is equivalent to 5.804 mg. of $C_2H_4N_4O_2$.

Benzoic Acid, page 79

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Loss on drying:*

Loss on drying. Not more than 0.2 percent.

Insert the following new paragraph to precede the paragraph entitled *Readily carbonizable substances,* page 80:

Loss on drying, page 931. Dry over silica gel for 3 hours.

Benzyl Alcohol, page 84

Change the SPECIFICATION for *Refractive index* to read:

Refractive index. Between 1.539 and 1.541 at 20°.

Butyl Alcohol, page 107

Replace the last three sentences of the paragraph entitled *Butyl ether,* page 108, with the following:

The recorder should be operated in the 0–1 mv. range, with the detector voltage set at about 8, depending upon the particular instrument used. Under the conditions described, the butyl ether is eluted in about 6 minutes and the *n*-butyl alcohol in about 25 minutes, so that the relative retention of the butyl ether in butyl alcohol is about 0.24. A known mixture of about 1 percent butyl ether in butyl alcohol should be chromatographed to verify the retention time of the ether. The area of the butyl ether peak is not more than 0.2 percent of the total area of all peaks.

Caffeine, page 115

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Other alkaloids:*

Other alkaloids. Passes test.

Insert the following new paragraph to precede the paragraph entitled *Readily carbonizable substances,* page 116:

Other alkaloids. Add a few drops of mercuric-potassium iodide T.S. to 5 ml. of a 1 in 50 solution of the sample. No precipitate forms.

Calcium Acetate, page 116

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Fluoride:*

Fluoride. Not more than 50 parts per million (0.005 percent).

Insert the following new paragraph to precede the paragraph entitled *Heavy metals*, page 117:

Fluoride. Determine as directed in *Method III* under the *Fluoride Limit Test*, page 919, except in the *Procedure* use 10 ml. of 1 *N* hydrochloric acid to dissolve the sample.

Calcium Alginate, page 117

Replace *Identification tests A* and *B*, page 118, with the following:

A. Moisten about 5 mg. of the sample with water, and add 1 ml. of acid ferric sulfate T.S. Within five minutes a cherry-red color develops, finally becoming deep purple.

On page 118, designate *Identification test C* as *B*, and *D* as *C*.

Change the paragraph entitled *Heavy metals*, page 118, to read:

Heavy metals. Determine as directed in the revised test for *Heavy metals* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Change the paragraph entitled *Insoluble matter*, page 118, to read:

Insoluble matter. Determine as directed in the revised test for *Insoluble matter* under *Alginic Acid*, page 1 of THIS SUPPLEMENT, except add to the 2000-ml. flask, along with the sample, an amount of sodium hexametaphosphate equivalent to approximately twice the weight of the sample.

Calcium Carbonate, page 121

Change the SPECIFICATION (*Limits of Impurities*) for *Fluoride* to read:

Fluoride. Not more than 50 parts per million (0.005 percent). [*Note:* This revision became effective on March 14, 1973.]

Calcium Chloride Solution

Insert the following new monograph to precede the monograph entitled *Calcium Citrate*, page 126:

CALCIUM CHLORIDE SOLUTION

DESCRIPTION

Calcium chloride solution occurs as a clear to slightly turbid, colorless or slightly colored liquid at room temperature. It is nominally available in a concentration range of about 35% to 45% of $CaCl_2$. When diluted to a concentration of about 1 to 10 ($CaCl_2$ basis), it gives positive tests for *Calcium*, page 926, and for *Chloride*, page 926.

SPECIFICATIONS

Assay. Not less than 90.0 percent and not more than 110.0 percent, by weight, of the labeled amount of calcium chloride, expressed as $CaCl_2$.

Limits of Impurities

Alkalinity [as $Ca(OH)_2$]. Not more than 0.3 percent.

Arsenic (as As). Not more than 3 parts per million (0.0003 percent), calculated on the $CaCl_2$ determined in the *Assay*.

Fluoride. Not more than 40 parts per million (0.004 percent), calculated on the $CaCl_2$ determined in the *Assay*.

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent), calculated on the $CaCl_2$ determined in the *Assay*.

Lead. Not more than 10 parts per million (0.001 percent), calculated on the $CaCl_2$ determined in the *Assay*.

Magnesium and alkali salts. Not more than 5 percent, calculated on the $CaCl_2$ determined in the *Assay*.

TESTS

Assay. Transfer an accurately weighed amount of the solution, equivalent to about 1 gram of $CaCl_2$, into a 250-ml. volumetric flask, dissolve it in a mixture of 100 ml. water and 5 ml. of diluted hydrochloric acid T.S., dilute to volume with water, and mix. Transfer 50.0 ml. of this solution into a suitable container, and add 50 ml. of water. While stirring, preferably with a magnetic stirrer, add about 30 ml. of 0.05 *M* disodium ethylenediaminetetraacetate from a 50-ml. buret, then add 15 ml. of sodium hydroxide T.S. and 300 mg. of hydroxy naphthol blue indicator, and continue the titration to a blue end-point. Each ml. of 0.05 *M* disodium ethylenediaminetetraacetate is equivalent to 5.550 mg. of $CaCl_2$.

Alkalinity. Dilute an accurately weighed amount of the solution, equivalent to about 5 grams of $CaCl_2$, to 50 ml. with water, add phenolphthalein T.S., and titrate with 0.1 *N* hydrochloric acid. Each ml. of 0.1 *N* hydrochloric acid is equivalent to 3.71 mg. of $Ca(OH)_2$.

Arsenic. Dilute an accurately weighed amount of the solution, equivalent to 1 gram of $CaCl_2$, to 35 ml. with water. The resulting solution meets the requirements of the *Arsenic Test*, page 865.

Fluoride. Determine as directed in *Method III* under the *Fluoride Limit Test*, page 917, using as the sample an accurately weighed amount of the solution equivalent to 1 gram of $CaCl_2$.

Heavy metals. Dilute an accurately weighed amount of the solution, equivalent to 1 gram of $CaCl_2$, to 25 ml. with water. This solution meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. Dilute an accurately weighed amount of the solution, equivalent to 1 gram of $CaCl_2$, to 10 ml. with water. The resulting solution meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Magnesium and alkali salts. Dilute an accurately weighed amount of the solution, equivalent to 1 gram of $CaCl_2$, to 50 ml. with water, add 500 mg. of ammonium chloride, mix, and boil for about 1 minute. Rapidly add 40 ml. of oxalic acid T.S., and stir vigorously until precipitation is well established. Immediately add 2 drops of methyl red T.S., then add ammonia T.S., dropwise, until the mixture is just alkaline, and cool. Transfer the mixture into a 100-ml. cylinder, dilute with water to 100 ml., let it stand for 4 hours or overnight, and then decant the clear, supernatant liquid through a dry filter paper. To 50 ml. of the clear filtrate in a platinum dish add 0.5 ml. of sulfuric acid, and evaporate the mixture on a steam bath to a small volume. Carefully evaporate the remaining liquid to dryness over a free flame, and continue heating until the ammonium salts have been completely decomposed and volatilized. Finally, ignite the residue to constant weight. The weight of the residue does not exceed 25 mg.

Packaging and storage. Store in tight containers.

Functional use in foods. Miscellaneous and general purpose; sequestrant; firming agent.

Calcium Citrate, page 126

Change the paragraph entitled *Loss on drying* to read:

Loss on drying, page 931. Dry for 4 hours at 150°.

Calcium Disodium EDTA, page 127

Replace the paragraph entitled *Assay*, page 128, with the following:

Assay. Transfer about 1.2 grams of the sample, accurately weighed, into a 250-ml. beaker, and dissolve in 75 ml. of water. Add 25 ml. of diluted acetic acid T.S. and 1.0 ml. of diphenylcarbazone T.S., and titrate slowly with 0.1 *M* mercuric nitrate to the first appearance of a purplish color. Each ml. of 0.1 *M* mercuric nitrate is equivalent to 37.43 mg. of $C_{10}H_{12}CaN_2Na_2O_8$. [*Note:* See page 104 of THIS SUPPLEMENT for diphenylcarbazone T.S. and 0.1 *M* mercuric nitrate.]

Calcium Saccharin, page 154

Change the third sentence of the *Description* to read:

It is intensely sweet.

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Toluenesulfonamides:*

Toluenesulfonamides. Not more than 100 parts per million (0.01 percent).

Change the paragraph entitled *Selenium*, page 156, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Insert the following new paragraph to precede the *Packaging and storage* statement, page 156:

Toluenesulfonamides. Determine as directed under *Sodium Saccharin*, page 56 of THIS SUPPLEMENT.

Calcium Silicate, page 156

Change the first two lines of the paragraph entitled *Assay for calcium oxide*, page 157, to read:

Assay for calcium oxide. Using sodium hydroxide T.S., neutral-

ize to litmus the combined filtrate and washings retained in the *Assay for silicon dioxide*, add, while stirring, about 30 . . . .

Calcium Stearate, page 158

Change the first sentence of the *Description* to read:

Calcium stearate is a compound of calcium with a mixture of solid organic acids obtained from edible sources, and consists chiefly of variable proportions of calcium stearate and calcium palmitate.

Calcium Sulfate, page 163

Change the first sentence in the paragraph entitled *Assay* to read:

Assay. Dissolve about 350 mg., accurately weighed, in 100 ml. of water and 4 ml. of diluted hydrochloric acid T.S., boiling if necessary to effect solution, and cool.

Change the paragraph entitled *Selenium*, page 164, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Carnauba Wax, page 170

Change the SPECIFICATION for *Acid value* to read:

Acid value. Between 2 and 7.

Carrageenan, page 172

Change the SPECIFICATION for *Sulfate* to read:

Sulfate. Between 18 percent and 40 percent on a dry weight basis.

Insert the following new SPECIFICATION for *Viscosity:*

Viscosity of a 1.5 percent solution. Not less than 5 centipoises at 75°.

Insert the following new paragraph to precede the paragraph entitled *Sulfate*, page 173:

Viscosity of a 1.5 percent solution. Transfer 7.5 grams of the sample into a tared, 600-ml. tall-form (Berzelius) beaker, and disperse with agitation for 10–20 minutes in 450 ml. of deionized water. Add sufficient water to bring the final weight to 500 grams, and heat in a water bath, with continuous agitation, until a temperature of 80° is reached (20–30 minutes). Add water to adjust for loss by evaporation, cool to 76–77°, and place in a constant-temperature bath at 75°. Pre-heat the bob and guard of a Brookfield LVF viscometer to approximately 75° in water, then dry the bob and guard and attach them to the viscometer, which should be equipped with a No. 1 spindle (19 mm. in diameter, approx. 65 mm. in length) and UL (ultra low) adapter, and capable of rotating at 30 rpm. Adjust the height of the bob in the sample solution, start the viscometer rotating at 30 rpm, and, after six complete revolutions of the viscometer, take the viscometer reading on the 0–100 scale. Record the results in centipoises by multiplying the reading by 5. [*Note:* Some samples of carrageenan may be too viscous to be read on the 0–100 scale. Such samples obviously pass the specification, but if a viscosity reading is desired for other reasons, use a No. 2 spindle, take the reading on the 0–500 scale, and multiply the reading by 5 to obtain the viscosity in centipoises.]

Chlorine

Insert the following new monograph to precede the monograph entitled *Cholic Acid*, p. 188:

CHLORINE

Cl_2 Mol. wt. 70.91

DESCRIPTION

A greenish yellow gas, normally packaged as a liquid under pressure in containers approved by the U.S. Department of Transportation. At 60°F., it has a vapor pressure of 70.91 psig. Its vapor density is about 2.5 times that of air. About 0.8 pound (0.362 kg.) is soluble in 100 pounds (45.4 kg.) of water at 60°F. under atmospheric pressure.

Caution: Chlorine gas is a respiratory irritant. Large amounts cause coughing, labored breathing, and irritation of the eyes. In extreme cases, the difficulty in breathing may cause death due to suffocation. Liquid chlorine causes skin and eye burns on contact. (Safety precautions to be observed in handling the material are specified in the *Chlorine Manual*, available from The Chlorine Institute, 342 Madison Avenue, New York, NY 10017.)

SPECIFICATIONS

Assay. Not less than 99.5 percent, by volume.

Limits of Impurities

Moisture. Not more than 150 parts per million (0.015 percent), by weight.

Residue. Not more than 150 parts per million (0.015 percent), by weight, of non-volatile matter.

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 30 parts per million (0.003 percent).

Lead. Not more than 10 parts per million (0.001 percent).

Mercury. Not more than 1 part per million (0.0001 percent).

TESTS

Assay. Determine by A.S.T.M. Method E 412-70, "Assay of Liquid Chlorine (Zinc Amalgam Method)."

Moisture and **Residue.** Determine by A.S.T.M. Method E 410-70, "Moisture and Residue in Liquid Chlorine."

Sample Solution for the Determination of Arsenic, Heavy Metals, Lead, and Mercury. Dissolve the residue, obtained in the test for *Residue*, in 2.5 ml. of freshly prepared aqua regia, and dilute with water to a volume, in ml., equivalent to the weight, in grams, of the initial chlorine sample, so that 1 ml. of the final dilution is equivalent to 1 gram of chlorine.

Arsenic. A 1.0-ml. portion of the *Sample Solution*, diluted to 35 ml. with water, meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A 0.67-ml. portion of the *Sample Solution*, diluted to 25 ml. with water, meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A 1.0-ml. portion of the *Sample Solution*, mixed with 5 ml. of water and 11 ml. of diluted hydrochloric acid T.S., meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Mercury. Transfer 2.0 ml. of the *Sample Solution* into a 50-ml. beaker, add 10 ml. of water, 1 ml. of dilute sulfuric acid (1 in 5), and 1 ml. of potassium permanganate solution (1 in 25), cover with a watch glass, boil for a few seconds, and cool. Use the resulting solution as the *Sample Preparation* as directed under the *Mercury Limit Test*, page 934.

Packaging and storage. Store in suitable pressure containers, observing applicable federal regulations pertaining to shipping containers.

Functional use in foods. Antimicrobial; bleaching agent; oxidation agent.

L-Cysteine Monohydrochloride, page 226

Change the SPECIFICATION for *Specific rotation* to read:

Specific rotation, $[\alpha]_D^{20°}$. Between +5.0° and +8.0°.

Change the paragraph entitled *Specific rotation* to read:

Specific rotation, page 939. Determine in a solution containing 8 grams of undried sample in sufficient 1 *N* hydrochloric acid to make 100 ml.

1-Decanol (Natural), page 233

Change the SPECIFICATION for *Refractive index* to read:

Refractive index. Between 1.435 and 1.439 at 20°.

Dioctyl Sodium Sulfosuccinate, page 256

Change the SPECIFICATION for *Assay*, page 257, to read:

Assay. Not less than 98.5 percent of $C_{20}H_{37}NaO_7S$, calculated on the dried basis.

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Bis(2-ethylhexyl) maleate:*

Bis(2-ethylhexyl) maleate. Not more than 0.4 percent.

In the section entitled *Assay*, page 257, replace the paragraph entitled *Sample solution* with the following:

Sample solution. Transfer about 3.8 grams of the sample, accurately weighed, into a 500-ml. volumetric flask, dissolve in chloroform, dilute to volume with the same solvent, and mix.

In the section entitled *Assay*, page 257, replace the paragraph entitled *Procedure* with the following:

Procedure. Pipet 10.0 ml. of the *Sample solution* into a 250-ml. flask, and add 40 ml. of chloroform, 50 ml. of *Salt solution*, and 10 drops of bromophenol blue T.S. Titrate with *Tetra-n-butylammonium iodide solution* to the first appearance of a blue color in the chloroform layer after vigorous shaking. Calculate the percent of $C_{20}H_{37}NaO_7S$ by the formula $(V \times 1.250 \times 444.6 \times 10)/(W \times 369.4)$, in which V is the volume, in ml., of *Tetra-n-butylammonium iodide solution* required; 444.6 is the molecular weight of dioctyl sodium sulfosuccinate; W is

the weight, in grams, of the sample taken; and 369.4 is the molecular weight of tetra-*n*-butylammonium iodide.

Insert the following new paragraphs to precede the paragraph entitled *Heavy metals*, page 258:

Bis(2-ethylhexyl) maleate

Supporting electrolyte. Dissolve 21.2 grams of anhydrous lithium perchlorate ($LiClO_4$) in 175 ml. of water in a 250-ml. beaker. Adjust the pH of this solution to 3.0 by the dropwise addition of glacial acetic acid (usually 1 or 2 drops is sufficient), using a suitable pH meter. Quantitatively transfer the solution into a 200-ml. volumetric flask, dilute to volume with water, and mix.

Standard solution. Transfer 100–110 mg. of bis(2-ethylhexyl) maleate*, accurately weighed, into a 100-ml. volumetric flask. Record the exact weight, to the nearest 0.1 mg., as W_A. Add 60–70 ml. of isopropyl alcohol, swirl to dissolve, then dilute to volume with water, and mix.

Sample stock solution. Transfer 12.5 grams of the sample, accurately weighed, into a 150-ml. beaker. Record the exact weight, to the nearest 10 mg., as W_S. Add 80–90 ml. of isopropyl alcohol, and stir with a glass stirring rod until the sample is dissolved. Quantitatively transfer this solution, with the aid of isopropyl alcohol, into a 250-ml. volumetric flask, then dilute to volume with isopropyl alcohol, and mix.

Test solution A. Pipet 50.0 ml. of the *Sample stock solution* and 20.0 ml. of the *Supporting electrolyte* into a 100-ml. volumetric flask. Dilute to within 15 mm. of the graduated volume line with isopropyl alcohol, stopper, shake to facilitate solution, and set aside for 2 minutes. Dilute to volume with isopropyl alcohol, and mix. A completely clear solution should be obtained.

Test solution B. Pipet 50.0 ml. of the *Sample stock solution*, 10.0 ml. of the *Standard solution*, and 20.0 ml. of the *Supporting electrolyte* into a 100-ml. volumetric flask, and complete the preparation as described for *Test solution A*.

Blank. Pipet 20.0 ml. of the *Supporting electrolyte* into a 100-ml. volumetric flask, dilute to volume with isopropyl alcohol, and mix.

Procedure. Rinse a polarographic H-cell several times with small portions of *Test solution A*, then fill the cell half-full with the solution, place a paper tissue in the top of the sample side of the cell, and pass a moderate stream of nitrogen through the solution for 15 minutes. (*Note:* The nitrogen should first be saturated by passing it through a suitable scrubber containing isopropyl alcohol.) After 15 minutes, divert the nitrogen stream over the surface of the solution, and remove the tissue from the cell.

Set the polarizing voltage of a suitable, previously calibrated polaro-

* A suitable grade of bis(2-ethylhexyl) maleate is available as OT-35 from American Cyanamid Company, Fine Chemicals Department, Pearl River, New York 10965.

graph (Metrohm Polarocord E-261 or equivalent) at −1.3 volts, adjust the current sensitivity to the lowest range (most sensitive) at which the current oscillations will remain on scale, and record the polarogram, scanning a voltage range of −0.9 to −1.5 volts at this sensitivity and using a saturated calomel electrode as the reference electrode. Record the average oscillations, in mm., at −1.3 volts as *A*, and those at −1.0 volt as *B*. (*Note:* If a manual polarograph is used, record the average oscillations of the solutions at −1.3 volts and −1.0 volt, respectively.)

Repeat the entire procedure using *Test solution B*, recording the average oscillations at −1.3 volts as *D*, and those at −1.0 volt as *E*. Similarly, repeat the entire procedure using the *Blank*, recording the average oscillations at −1.3 volts as *G*, and those at −1.0 volt as *H*.

Calculation. Make the following preliminary calculations (in microamps) to obtain *C* (net diffusion current of *Test solution A*); *F* (net diffusion current of *Test solution B*); *I* (net current introduced by the *Blank*); *J* (diffusion current due to added maleate); and *K* (diffusion current due to originally present maleate): $C = (A - B) \times S_1$; $F = (D - E) \times S_2$; $I = (G - H) \times S_3$; $J = F - C$; and $K = C - I$; in which S_1, S_2, and S_3 represent the current sensitivities used for *Test solution A*, *Test solution B*, and the *Blank*, respectively.

Finally, calculate the percentage of bis(2-ethylhexyl) maleate in the original sample taken by the formula $(K \times 50W_A)/(J \times W_S)$.

Enzyme Preparations

Insert the following new monograph to precede the monograph entitled *Erythorbic Acid*, page 266:

ENZYME PREPARATIONS

DESCRIPTION

Enzyme preparations used in food processing are derived from animal, plant, or microbial sources (see *Classification* below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one or more active components as well as diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practice.

The individual preparations are usually named according to the substance to which they are applied, such as *Protease* or *Amylase;* such traditional names as *Malt*, *Pepsin*, and *Rennet* are also used, however.

The color of the preparations—which may be liquid, semi-liquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are

sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand.

The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for Food Chemicals Codex purposes) the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food in order to achieve the desired effect.

Additional information relating to the nomenclature and the sources from which the active components are derived is provided in the General Tests section under *Enzyme Preparations*, page 62 of THIS SUPPLEMENT.

CLASSIFICATION

ANIMAL-DERIVED PREPARATIONS

Catalase (bovine liver). Partially purified liquid or powdered extracts from bovine liver. Major active principle: *catalase.* Typical application: manufacture of certain cheeses.

Lipase, Animal. Obtained from two primary sources: (1) edible forestomach tissue of calves, kids, or lambs, and (2) animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts. Dispersible in water; insoluble in alcohol. Major active principle: *lipase.* Typical applications: manufacture of cheese; modification of lipids.

Pepsin. Obtained from the glandular layer of hog stomach. White to light tan water-soluble powders, amber pastes, or clear amber to brown aqueous liquids. Major active principle: *pepsin.* Typical applications: preparation of fish meal and other protein hydrolysates; clotting of milk in manufacture of cheese (in combination with rennet).

Rennet. Aqueous extracts made from the fourth stomach of calves, kids, or lambs. Clear amber to dark brown liquid preparations, or white to tan powders. Major active principle: *protease* (rennin). Typical application: manufacture of cheese.

Rennet, Bovine. Aqueous extracts made from the fourth stomach of bovine animals, sheep, and goats. Clear amber to dark brown liquids, or white to tan powders. Major active principle: *protease* (rennin). Typical application: manufacture of cheese.

Trypsin. Obtained from purified extracts of porcine or bovine pancreas. White to tan amorphous powders, which are soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *trypsin.* Typical applications: baking; meat tenderizing; production of protein hydrolysates.

PLANT-DERIVED PREPARATIONS

Bromelain. The purified proteolytic substance derived from the pineapples *Ananas comosus* and *Ananas bracteatus* L. White to light tan amorphous powder. Soluble in water (the solution being colorless to light yellow and somewhat opalescent) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *bromelain.* Typical applications: chillproofing of beer; meat tenderizing; preparation of precooked cereals; production of protein hydrolysates.

Ficin. The purified proteolytic substance derived from the latex of *Ficus* sp., which include a variety of tropical fig trees. White to off-white powders, which are completely soluble in water. (Liquid fig latex concentrates are light brown to dark brown in color.) Major active principle: *ficin.* Typical applications: chillproofing of beer; meat tenderizing; dough conditioner in baking.

Malt. The product of the controlled germination of barley. Clear amber to dark brown liquid preparations, or white to tan powders. Major active principles: (1) *α-amylase* and (2) *β-amylase.* Typical applications: baking; manufacture of alcoholic beverages; manufacture of syrups.

Papain. The purified proteolytic substance derived from the fruit of the papaya *Carica papaya* L. (Fam. Caricaceae). Produced as white to light tan amorphous powders, or as liquids. Soluble in water (the solution being colorless or light yellow and somewhat opalescent) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *papain* and (2) *chymopapain.* Typical applications: chillproofing of beer; meat tenderizing; preparation of precooked cereals; production of protein hydrolysates.

MICROBIALLY-DERIVED PREPARATIONS

Carbohydrase (*Aspergillus niger* var.). Produced by the controlled fermentation of *Aspergillus niger* var. as off-white to tan amorphous powders, or as tan to dark brown liquids. Practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *α-amylase;* (2) *pectinase* (usually a mixture of pectin methylesterase and polygalacturonase); (3) *cellulase;* (4) *glucoamylase* (amyloglucosidase); and (5) *lactase.* Typical applications: preparation of starch syrups, alcohol, beer, ale, fruit juices, chocolate syrup, bakery products, liquid coffee, wine, dextrose, and dairy products.

Carbohydrase (*Aspergillus oryzae* var.). Produced by the controlled fermentation of *Aspergillus oryzae* var. as off-white to tan amorphous powders, or as liquids. Soluble in water (the solutions being light yellow to dark brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *α-amylase* and (2) *glucoamylase* (amyloglucosidase). Typical applica-

tions: preparation of starch syrups, alcohol, beer, ale, bakery products, and dextrose.

Carbohydrase (*Rhizopus oryzae* var.). A group of enzyme preparations produced by the controlled fermentation of *Rhizopus oryzae* var. as powders or liquids. Major active principles: (1) *α-amylase;* (2) *pectinase;* and (3) *glucoamylase* (amyloglucosidase). Typical applications: preparation of starch syrups and fruit juices; manufacture of cheese.

Carbohydrase (*Saccharomyces* species). The purified enzyme produced by the controlled fermentation of a number of species of *Saccharomyces* traditionally used in the manufacture of food. White to tan amorphous powders. Soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *invertase* and (2) *lactase.* Typical applications: manufacture of candy and ice cream; modification of dairy products.

Carbohydrase and Protease, Mixed (*Bacillus subtilis*). Produced by the controlled fermentation of *Bacillus subtilis* var. as off-white to tan amorphous powders, or as liquids. Soluble in water (the solutions usually being light yellow to dark brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *α-amylase* and (2) *protease.* Typical applications: preparation of starch syrups, alcohol, beer, dextrose, bakery products, fish meal; meat tenderizing; preparation of protein hydrolysates.

Catalase (*Aspergillus niger* var.). Produced by the controlled fermentation of *Aspergillus niger* var. as off-white to tan amorphous powders, or as liquids. Soluble in water (the solutions usually being tan to brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase.* Typical applications: manufacture of cheese and egg products.

Catalase (*Micrococcus lysodeikticus*). Partially purified liquid or powdered extracts from submerged fermentations of *Micrococcus lysodeikticus.* Major active principle: *catalase.* Typical application: manufacture of cheese.

Glucose Oxidase (*Aspergillus niger* var.). Produced by the controlled fermentation of *Aspergillus niger* var. as off-white to tan amorphous powders. Soluble in water (the solutions usually being light tan to brown in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *glucose oxidase* and (2) *catalase.* Typical applications: removal of sugar from liquid eggs; deoxygenation of citrus beverages.

Lipase (*Aspergillus niger* var.). Produced by the controlled fermentation of *Aspergillus niger* var. as off-white to tan amorphous powders. Soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether.

Major active principle: *lipase.* Typical applications: hydrolysis of lipids (e.g., fish oil concentrates).

Lipase (*Aspergillus oryzae* var.). Produced by the controlled fermentation of *Aspergillus oryzae* var. as off-white to tan amorphous powders, or as liquids. Soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase.* Typical application: hydrolysis of lipids (e.g., fish oil concentrates).

Protease (*Aspergillus oryzae* var.). Produced by the controlled fermentation of species of *Aspergillus oryzae* var. The purified enzyme occurs as off-white to tan amorphous powders. Soluble in water (the solutions usually being light yellow in color) but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease.* Typical applications: chillproofing of beer; bakery products; meat tenderizing; production of protein hydrolysates.

Rennet, Microbial (*Endothia parasitica*). Produced by the controlled fermentation of non-pathogenic species of *Endothia parasitica* as an off-white to tan amorphous powder, or as a liquid. The powders are soluble in water (the solutions usually being tan to dark brown in color) but are practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease.* Typical application: manufacture of cheese.

Rennet, Microbial (*Mucor* species). Produced by the controlled fermentation of *Mucor miehei* or *M. pusillus* as white to tan amorphous powders. The powders are soluble in water (the solutions usually being light yellow in color) but are practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease.* Typical application: manufacture of cheese.

REACTIONS CATALYZED

[*Note:* The reactions catalyzed by any given active component are essentially the same, regardless of the source from which that component is derived.]

α-Amylase. Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides.

β-Amylase. Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding *beta* limit dextrins.

Bromelain. Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, or leucine or glycine), yielding peptides of lower molecular weight.

Catalase. $2H_2O_2 \rightarrow O_2 + 2H_2O$.

Cellulase. Hydrolysis of β-1,4-glucan bonds in such polysaccharides as cellulose, yielding β-dextrins.

Ficin. Hydrolysis of polypeptides, amides, and esters (especially at

bonds involving basic amino acids, or leucine or glycine), yielding peptides of lower molecular weight.

Glucoamylase (*Amyloglucosidase*). Hydrolysis of α-1,4- and α-1,6-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding glucose.

Glucose Oxidase. β-D-glucose + O_2 → D-glucono-δ-lactone + H_2O_2.

Invertase. Hydrolysis of sucrose to a mixture of glucose and fructose (invert sugar).

Lactase. Hydrolysis of lactose to a mixture of glucose and galactose.

Lipase. Hydrolysis of triglycerides of simple fatty acid esters, yielding mono- and diglycerides, glycerol, and free fatty acids.

Pectinase

Pectin Methylesterase. Demethylation of pectin.

Polygalacturonase. Hydrolysis of α-1,4-galacturonide bonds in pectin.

Pepsin. Hydrolysis of polypeptides, including those with bonds adjacent to aromatic or decarboxylic L-amino acid residues, yielding peptides of lower molecular weight.

Protease (general). Hydrolysis of polypeptides, yielding peptides of lower molecular weight.

Rennin. Hydrolysis of polypeptides; specificity may be similar to pepsin.

Trypsin. Hydrolysis of polypeptides, amides, and esters at bonds involving the carboxyl groups of L-arginine and L-lysine, yielding peptides of lower molecular weight.

SPECIFICATIONS

In addition to the *General Requirements* listed in the General Tests section under *Enzyme Preparations*, page 62 of THIS SUPPLEMENT, all enzyme preparations covered herein (see *Classification* above) must meet the following *Specifications:*

Assay. Not less than 85 percent and not more than 115 percent of the declared activity.

Limits of Impurities

Aflatoxin. Not more than 5 parts per billion (0.0000005 percent).

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Coliforms. Not more than 30 per gram.

Heavy metals (as Pb). Not more than 40 parts per million (0.004 percent).

Lead. Not more than 10 parts per million (0.001 percent).

Pseudomonas aeruginosa. Negative by test.

Salmonella sp. Negative by test.

TESTS

Assay. The following procedures, which are included in the General Tests section under *Enzyme Preparations*, page 62 of THIS SUPPLEMENT, are provided for application as necessary in determining compliance with the declared representations for enzyme activity*: Alpha-Amylase Activity (Non-Bacterial); Bacterial Alpha-Amylase Activity (BAU); Catalase Activity; Cellulase Activity; Diastase Activity (Diastatic Power, DP); Esterase Activity; Glucoamylase Activity (Amyloglucosidase Activity); Glucose Oxidase Activity; Invertase Activity; Lactase (β-Galactosidase) Activity; Lipase (*Aspergillus oryzae*) Activity; Milk-clotting Activity; Papain (Proteolytic Activity); Pepsin Activity; Protease Activity, Bacterial (PC); Proteolytic Activity (HUT); and Trypsin Activity.

Aflatoxin. Determine as directed in J.A.O.A.C. *49*, 544 (1966); Pons *et al.*, Determination of Aflatoxins in Agricultural Products: Use of Aqueous Acetone for Extraction.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Coliforms. Determine as directed in Section 41.009, Official Methods of Analysis of the A.O.A.C., 11th Edition (1970), page 841.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Pseudomonas aeruginosa. Determine as directed under *Microbial Limit Tests*, U.S.P. XVIII, page 850.

Salmonella sp. Determine as directed in Section 10, Bacteriological Analytical Manual, 2nd Edition (1969), U. S. Department of Health, Education, and Welfare.

Packaging and storage. Store in tight containers in a cool, dry place.

Functional use in foods. Enzyme (see discussion under *Classification* above).

* Because of the varied conditions under which pectinases are employed, and because laboratory hydrolysis of a purified pectin substrate does not correlate with results observed with the natural substrates under use conditions, it is recommended that pectinase suppliers and users develop their own assay procedures that would relate to the specific application under consideration.

Ethoxylated Mono- and Diglycerides, page 268

Change the statement on *Functional use in foods*, page 269, to read:

Functional use in foods. Dough conditioner; emulsifier.

Ethyl Alcohol, page 277

Change the SPECIFICATION (*Limits of Impurities*) for *Alkalinity* to read:

Alkalinity (as NH_3). Not more than 3 parts per million (0.0003 percent). [*Note:* The test procedure for *Alkalinity* is correct as shown on page 278.]

Ethyl Isovalerate, page 291

Change the synonym, appearing directly under the monograph title, to read:

Ethyl 3-Methylbutyrate

Ferric Ammonium Citrate, Brown and **Ferric Ammonium Citrate, Green**

Insert the following two new monographs to precede the monograph entitled *Ferric Phosphate*, page 309:

FERRIC AMMONIUM CITRATE, BROWN

Iron Ammonium Citrate

DESCRIPTION

A complex salt of undetermined structure, composed of iron, ammonia, and citric acid and occurring as thin, transparent brown, reddish brown, or garnet red scales or granules, or as a brownish yellow powder. It is odorless or has a slight ammoniacal odor and has a mild iron-metallic taste. It is very soluble in water but is insoluble in alcohol. The pH of a 1 in 20 solution is about 5.0–8.0. It is deliquescent in air and is affected by light.

IDENTIFICATION

A. A 500-mg. sample, when ignited, chars and leaves a residue of iron oxide.

B. To 5 ml. of a 1 in 10 solution of the sample add 0.3 ml. of potassium permanganate T.S. and 4 ml. of mercuric sulfate T.S., and then heat the mixture to boiling. A white precipitate forms.

C. Dissolve about 500 mg. of the sample in 5 ml. of water, and add 5 ml. of sodium hydroxide T.S. A reddish brown precipitate forms, and ammonia is evolved when the mixture is heated.

SPECIFICATIONS

Assay. Not less than 16.5 percent and not more than 18.5 percent of iron (Fe).

Limits of Impurities

Arsenic (as As). Not more than 1 part per million (0.0001 percent).

Ferric citrate. Passes test.

Lead. Not more than 10 parts per million (0.001 percent).

Mercury. Not more than 1 part per million (0.0001 percent).

Oxalate. Passes test.

Sulfate. Not more than 0.3 percent.

TESTS

Assay. Transfer about 1 gram, accurately weighed, into a 250-ml. glass-stoppered Erlenmeyer flask, and dissolve in 25 ml. of water and 5 ml. of hydrochloric acid. Add 4 grams of potassium iodide, stopper, and allow to stand protected from light for 15 minutes. Add 100 ml. of water, and titrate the liberated iodine with 0.1 *N* sodium thiosulfate, using starch T.S. as the indicator. Perform a blank determination (see page 2), and make any necessary correction. Each ml. of 0.1 *N* sodium thiosulfate is equivalent to 5.585 mg. of iron (Fe).

Arsenic. Determine as directed in the test for *Arsenic* under *Ferric Phosphate*, page 310.

Ferric citrate. Add potassium ferrocyanide T.S. to a 1 in 100 solution of the sample. No blue precipitate forms.

Lead [*Note:* The following method has been found to be satisfactory when the particular atomic absorption spectrophotometer specified is used. The method may be modified as necessary for use with other suitable atomic absorption spectrophotometers capable of determining lead in the sample at the limit specified.]

Standard preparation. Transfer 10.0 ml. of *Lead Nitrate Stock Solution* (see page 921) into a 500-ml. volumetric flask, dilute to volume with water, and mix. This solution should be prepared on the day of use. Each ml. contains the equivalent to 2 mcg. of lead ion (Pb).

Sample preparation. Transfer about 15 grams of the sample, accurately weighed, into a 100-ml. volumetric flask (previously rinsed with nitric acid and water), dissolve in a mixture of 50 ml. of water and 1 ml. of nitric acid, dilute to volume with water, and mix.

Procedure. Using a Perkin-Elmer 403 atomic absorption spectrophotometer equipped with a deuterium arc background corrector, digital readout device, and a burner head capable of handling 15 percent solids content, blank the instrument with water following the manufacturer's operating instructions. Aspirate a portion of the *Standard preparation*, and record the absorbance as A_S; then aspirate a portion of the *Sample preparation*, and record the absorbance as A_U. Calculate the lead content, in parts per million, of the sample taken by the formula $100 \times (C/W) \times (A_U/A_S)$, in which C is the concentration of Pb in the *Standard preparation*, in mcg. per ml., and W is the weight of the sample taken, in grams.

Mercury

Standard preparations. Prepare a solution containing 1 mcg. of mercury (Hg) per ml. as directed for *Standard preparation* under *Mercury Limit Test*, page 934. Pipet 0.25, 0.50, 1.0, and 3.5 ml. of this solution into each of four glass-stoppered bottles of about 300-ml. capacity, such as B.O.D. (biological oxygen demand) bottles. Dilute the contents of each bottle to 100 ml. with water, and mix. These solutions contain the equivalent to 0.25, 0.50, 1.0, and 3.5 parts per million of Hg, respectively.

Sample preparation. Transfer 1.000 gram of the sample into a 200-ml. screw-cap centrifuge bottle, and add 5 ml. of nitric acid and 5 ml. of hydrochloric acid. Close the bottle tightly with a Teflon-lined screw-cap, digest on a steam bath for 1 hour, and cool. Quantitatively transfer into a suitable glass-stoppered bottle (see *Standard preparations*), dilute to 100 ml. with water, and bubble air through the sample for 2 minutes. Prepare a reagent blank in the same manner.

Apparatus. Use a suitable atomic absorption spectrophotometer assembly designed for mercury analysis, such as the Coleman MAS-50 Mercury Analyzer. (*Note:* The *Apparatus* and *Procedure* described under *Mercury Limit Test*, page 934, may be suitably modified for this determination.)

Procedure. Add 5 ml. of a 10 percent stannous chloride solution (prepared fresh each week by dissolving 20 grams of $SnCl_2.2H_2O$ in 40 ml. of warm hydrochloric acid and diluting with 160 ml. of water) to the solution to be tested, and immediately insert the bubbler of the mercury analysis apparatus. Obtain the absorbance reading by following the instrument manufacturer's operating instructions. Correct the sample readings for the reagent blank, and determine the mercury concentration of the *Sample preparation* from a standard curve prepared by plotting the readings obtained with the *Standard preparations* against mercury concentration, in parts per million.

Oxalate. Transfer 1 gram of the sample into a 125-ml. separator, dissolve in 10 ml. of water, add 2 ml. of hydrochloric acid, and extract successively with one 50-ml. portion and one 20-ml. portion of ether. Transfer the combined ether extracts to a 150-ml. beaker, add 10 ml.

of water, and remove the ether by evaporation on a steam bath. Add 1 drop of glacial acetic acid and 1 ml. of calcium acetate solution (1 in 20) to the residual aqueous solution. No turbidity is produced within 5 minutes.

Sulfate, page 879. Any turbidity produced by a 100-mg. sample does not exceed that shown in a control containing 300 mcg. of sulfate (SO_4).

Packaging and storage. Store in tight, light-resistant containers in a cool place.

Functional use in foods. Nutrient; dietary supplement.

FERRIC AMMONIUM CITRATE, GREEN

Iron Ammonium Citrate

DESCRIPTION

A complex salt of undetermined structure, composed of iron, ammonia, and citric acid and occurring as thin, transparent green scales, as granules, as a powder, or as transparent green crystals. It is odorless and has a mild iron-metallic taste. It is very soluble in water but is insoluble in alcohol. Its solutions are acid to litmus. It may deliquesce in air and is affected by light.

IDENTIFICATION

It responds to the *Identification tests* under *Ferric Ammonium Citrate, Brown,* page 25 of THIS SUPPLEMENT.

SPECIFICATIONS

Assay. Not less than 14.5 percent and not more than 16.0 percent of iron (Fe).

Limits of Impurities

Arsenic (as As). Not more than 1 part per million (0.0001 percent).

Ferric citrate. Passes test.

Lead. Not more than 10 parts per million (0.001 percent).

Mercury. Not more than 1 part per million (0.0001 percent).

Oxalate. Passes test.

Sulfate. Not more than 0.3 percent.

TESTS

Determine as directed for the respective *Tests* under *Ferric Ammonium Citrate, Brown,* page 25 of THIS SUPPLEMENT.

Packaging and storage. Store in tight, light-resistant containers in a cool place.

Functional use in foods. Nutrient; dietary supplement; anticaking agent for sodium chloride.

Ferric Phosphate, page 309

Change the first sentence of the *Description* to read:

Ferric phosphate contains from one to four molecules of water of hydration.

Change the SPECIFICATION for *Assay* to read:

Assay. Not less than 26.0 percent and not more than 32.0 percent of Fe.

Replace the section entitled *Mercury*, page 312, with the following:

Mercury

Standard preparations. Dissolve 338.5 mg. of mercuric chloride, $HgCl_2$, in about 200 ml. of water in a 250-ml. volumetric flask, add 14 ml. of dilute sulfuric acid (1 in 2), dilute to volume with water, and mix. Pipet 10.0 ml. of this solution into a 1000-ml. volumetric flask containing about 800 ml. of water and 56 ml. of dilute sulfuric acid (1 in 2), dilute to volume with water, and mix. Pipet 10.0 ml. of the second solution into a second 1000-ml. volumetric flask containing 800 ml. of water and 56 ml. of dilute sulfuric acid (1 in 2), dilute to volume with water, and mix. Each ml. of this diluted stock solution contains 0.1 mcg. of Hg. Pipet 1.25, 2.50, 5.00, 7.50, and 10.00 of the last solution (equivalent to 0.125, 0.250, 0.500, 0.750, and 1.00 mcg. of Hg, respectively) into each of five 150-ml. beakers. To each add 25 ml. of aqua regia, cover with watch glasses, heat just to boiling, simmer for about 5 minutes, and cool to room temperature. Transfer the solutions into separate 250-ml. volumetric flasks, dilute to volume with water, and mix. Transfer a 50.0-ml. aliquot from each solution into respective 150-ml. beakers, and to each add 1.0 ml. of dilute sulfuric acid (1 in 5) and 1.0 ml. of a filtered solution of potassium permanganate (1 in 25). Heat the solutions just to boiling, simmer for about 5 minutes, and cool.

Sample preparation. Transfer 5.00 grams of the sample into a 150-ml. beaker, add 25 ml. of aqua regia, cover with a watch glass, and allow to stand at room temperature for about 5 minutes. Heat just to boiling, allow to simmer for about 5 minutes, and cool. Transfer the solution into a 250-ml. volumetric flask, dilute to volume with water, and mix. (*Note:* Disregard any undissolved material that may be present.) Transfer a 50.0-ml. aliquot of this solution into a 150-ml. beaker, and add 1.0 ml. of dilute sulfuric acid (1 in 5) and 1.0 ml. of a filtered solution of potassium permanganate (1 in 25). Heat the solution just to boiling, simmer for about 5 minutes, and cool. Prepare a reagent blank in the same manner.

Apparatus. Use a *Mercury Detection Instrument* and *Aeration Apparatus* as described under the *Mercury Limit Test*, page 934. For the purposes of the test described herein, the Techtron AA-1000 atomic absorption spectrophotometer, equipped with a 10-cm. silica absorption cell (Beckman Part No. 75144 or equivalent) and coupled with a strip chart recorder (Varian Series A-25 or equivalent), is satisfactory.

Procedure. Assemble the *Aerating Apparatus* as shown in Fig. 12, page 935. Use magnesium perchlorate as the absorbent in the absorption cell (*e*), fill gas washing bottle *c* with 60 ml. of water, and place stopcock *b* in the bypass position. Connect the assembly to the 10-cm. absorption cell (analogous to *f* in Fig. 12) of the spectrophotometer, and adjust the air or nitrogen flow rate so that, in the following procedure, maximum absorption and reproducibility are obtained without excessive foaming in the test solution. Obtain a baseline reading at 2537 Å by following the manufacturer's instructions for operating the particular *Mercury Detection Instrument* in use. Using the Techtron AA-1000 spectrophotometer, the following conditions are suitable: *slit width*, 2 Å; *lamp current*, 3 mA; and *scale expansion*, ×1. With the Varian A-25 recorder, set the *chart speed* at 25 inches/hour and the *span* at 2 mv. Precondition the apparatus by an appropriate modification of the procedures described below for treatment of the test solutions. (*Note*: The fritted bubbler in gas washing bottle *c* should be kept immersed in water between determinations. After each determination, wash the bubbler with a stream of water.)

Treat the blank, each of the *Standard preparations*, and the *Sample preparation* as follows: Transfer the solution to be tested to a 125-ml. gas washing bottle, using a few drops of hydroxylamine hydrochloride solution (1 in 10) to remove any manganese hydroxide from the beaker. Dilute to about 55 ml. with water, and add a magnetic stirring bar. Discharge the permanganate color by the dropwise addition of the hydroxylamine hydrochloride solution, swirling after each drop is added. Add 15.0 ml. of 10 percent stannous chloride solution (prepared by dissolving 20 grams of $SnCl_2.2H_2O$ in 40 ml. of warm hydrochloric acid and diluting with 160 ml. of water), and immediately connect the gas washing bottle to the *Aeration Assembly*. Switch on the magnetic stirrer, turn stopcock *b* from the bypass to the aerating position, and obtain the absorbance reading. Disconnect bottle *c* from the *Aerating Apparatus*, discard the solution just tested, wash bottle *c* with water, wash the fritted bubbler with water, and repeat the foregoing procedure with the remaining solutions. Correct the sample readings for the reagent blank, and determine the mercury concentration of the *Sample preparation* from a standard curve prepared by plotting the readings obtained with the *Standard preparations* against mercury concentration, in parts per million, suitable adjustments being made for dilution factors.

Ferric Pyrophosphate, page 312

Change the paragraph entitled *Mercury*, page 313, to read:

Mercury. Determine as directed in the revised test for *Mercury* under *Ferric Phosphate*, page 29 of THIS SUPPLEMENT.

Gibberellic Acid, page 343

Change the *Specification* for *Specific rotation* to read:

Specific rotation, $[\alpha]_D^{25}°$. Between +75.0° and +90.0°.

Change the paragraph entitled *Specific rotation*, page 344, to read:

Specific rotation, page 939. Determine in a solution in alcohol containing 100 mg. in each ml. Avoid the use of heat in preparing the solution.

L-Glutamine

Insert the following new monograph to precede the monograph entitled *Glycerin*, page 350:

L-GLUTAMINE

L-2-Aminoglutaramic Acid

$H_2NOCCH_2CH_2CH(NH_2)COOH$

$C_5H_{10}N_2O_3$ Mol. wt. 146.15

DESCRIPTION

White, odorless crystals or crystalline powder having a slightly sweet taste. It is soluble in water and practically insoluble in alcohol and in ether. Its solutions are acid to litmus. It melts with decomposition at about 185°.

IDENTIFICATION

To 5 ml. of a 1 in 1000 solution add 1 ml. of triketohydrindene hydrate T.S. A purple color appears.

SPECIFICATIONS

Assay. Not less than 98.0 percent and not more than 101.0 percent of $C_5H_{10}N_2O_3$ after drying.

Specific rotation, $[\alpha]_D^{20°}$. Between +6.3° and +7.3° after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Loss on drying. Not more than 0.2 percent.

Residue on ignition. Not more than 0.1 percent.

TESTS

Assay. Dissolve about 150 mg. of the sample, previously dried at 80° for 3 hours and accurately weighed, in 3 ml. of formic acid and 50 ml. of glacial acetic acid, and titrate with 0.1 *N* perchloric acid, determining the end-point potentiometrically. Perform a blank determination (see page 2), and make any necessary correction. Each ml. of 0.1 *N* perchloric acid is equivalent to 14.62 mg. of $C_5H_{10}N_2O_3$.

Specific rotation, page 939. Determine in solution containing 4 grams of a previously dried sample in sufficient water to make 100 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Loss on drying, page 931. Dry at 80° for 3 hours.

Residue on ignition, page 945. Ignite 1 gram as directed in the general method.

Packaging and storage. Store in well-closed, light-resistant containers.

Functional use in foods. Nutrient; dietary supplement.

Guar Gum, page 361

Change the first sentence of the paragraph entitled *Acid-insoluble matter*, page 362, to read:

Transfer 1.5 grams of the sample, accurately weighed, into a 250-ml. beaker containing 150 ml. of water and 1.5 ml. of concentrated sulfuric acid.

Change the last sentence of the paragraph entitled *Protein*, page 363, to read:

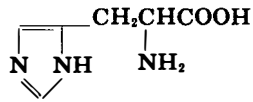
The percent of nitrogen determined, multiplied by 6.25, gives the percent of protein in the sample.

L-Histidine and L-Histidine Monohydrochloride

Insert the following two new monographs to precede the monograph entitled *Hops Oil*, page 374:

L-HISTIDINE

L-α-Amino-4(or 5)-imidazolepropionic Acid



$C_6H_9N_3O_2$ Mol. wt. 155.16

DESCRIPTION

White, odorless crystals or crystalline powder having a slightly bitter taste. It is soluble in water, very slightly soluble in alcohol, and insoluble in ether. It melts with decomposition between about 277° and 288°.

IDENTIFICATION

To 5 ml. of a 1 in 100 solution of the sample add 2 ml. of bromine T.S. A yellow color is produced. When the solution is heated gently, it first becomes colorless, then reddish brown, and finally it forms a dark gray precipitate.

SPECIFICATIONS

Assay. Not less than 98.0 percent and not more than 101.0 percent of $C_6H_9N_3O_2$ after drying.

Specific rotation, $[\alpha]_D^{20°}$. Between +11.5° and +13.0° after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Loss on drying. Not more than 0.2 percent.

Residue on ignition. Not more than 0.2 percent.

TESTS

Assay. Dissolve about 150 mg. of the sample, previously dried at 105° for 3 hours and accurately weighed, in 2 ml. of formic acid and 50 ml. of glacial acetic acid, and titrate with 0.1 *N* perchloric acid, deter-

mining the end-point potentiometrically. Perform a blank determination (see page 2), and make any necessary correction. Each ml. of 0.1 *N* perchloric acid is equivalent to 15.52 mg. of $C_6H_9N_3O_2$.

Specific rotation, page 939. Determine in a solution containing 11 grams of a previously dried sample in sufficient 6 *N* hydrochloric acid to make 100 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Loss on drying, page 931. Dry at 105° for 3 hours.

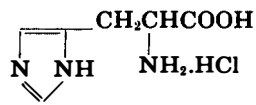
Residue on ignition, page 945. Ignite 1 gram as directed in the general method.

Packaging and storage. Store in well-closed, light-resistant containers.

Functional use in foods. Nutrient; dietary supplement.

L-HISTIDINE MONOHYDROCHLORIDE

L-α-Amino-4(or 5)-imidazolepropionic Acid Monohydrochloride



$C_6H_9N_3O_2.HCl.H_2O$ Mol. wt. 209.63.

DESCRIPTION

White, odorless crystals or crystalline powder having a slightly acid, bitter taste. It is soluble in water but insoluble in alcohol and in ether. It melts with decomposition at about 250° (after drying).

IDENTIFICATION

A. Heat 5 ml. of a 1 in 1000 solution with 1 ml. of triketohydrindene hydrate T.S. A reddish purple color is produced.

B. A 1 in 1000 solution gives positive tests for *Chloride*, page 926.

SPECIFICATIONS

Assay. Not less than 98.0 percent and not more than 101.0 percent of $C_6H_9N_3O_2.HCl.H_2O$ after drying.

Specific rotation, $[\alpha]_D^{20°}$. Between +8.5° and +10.5° after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Loss on drying. Not more than 0.3 percent.

Residue on ignition. Not more than 0.1 percent.

TESTS

Assay. Dissolve about 100 mg. of the sample, previously dried at 105° for 3 hours and accurately weighed, in 1 ml. of formic acid and 50 ml. of glacial acetic acid. Add 6 ml. of mercuric acetate T.S., and titrate with 0.1 *N* perchloric acid, determining the end-point potentiometrically. Perform a blank determination (see page 2), and make any necessary correction. Each ml. of 0.1 *N* perchloric acid is equivalent to 10.47 mg. of $C_6H_9N_3O_2.HCl.H_2O$.

Specific rotation, page 939. Determine in a solution containing 11 grams of a previously dried sample in sufficient 6 *N* hydrochloric acid to make 100 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Loss on drying, page 931. Dry at 105° for 3 hours.

Residue on ignition, page 945. Ignite 1 gram as directed in the general method.

Packaging and storage. Store in well-closed, light-resistant containers.

Functional use in foods. Nutrient; dietary supplement.

Hydroxypropyl Cellulose, page 385

On page 386, change "pH of a 2 percent solution" in the seventh and twenty-first lines to read:

pH of a 1 percent solution.

Hydroxypropyl Methylcellulose, page 387

Change the third sentence of the *Description* to read:

It occurs as a white to off-white, fibrous powder or as granules.

Change the SPECIFICATION (*Limits of Impurities*) for *Residue on ignition* to read:

Residue on ignition. Not more than 1.5 percent for products with viscosities greater than 50 centipoises; not more than 3.0 percent for products with viscosities of 50 centipoises or below.

Isoamyl Acetate, page 397

Replace the first sentence of the *Description* with the following:

A colorless liquid with a fruity, pear-like odor, composed of the acetates of mixed isomeric amyl alcohols, 2-methyl-1-butanol and 3-methyl-1-butanol predominating.

Isoamyl Butyrate, page 398

Replace the first two sentences of the *Description* with the following:

A colorless liquid with a strong, characteristic fruity odor, composed of the butyrates of mixed isomeric amyl alcohols, 2-methyl-1-butanol and 3-methyl-1-butanol predominating.

Isoamyl Formate, page 399

Replace the first two sentences of the *Description* with the following:

A colorless liquid with a plum-like odor, composed of the formates of mixed isomeric amyl alcohols, 2-methyl-1-butanol and 3-methyl-1-butanol predominating.

Isoamyl Hexanoate, page 400

Replace the first sentence of the *Description* with the following:

A colorless liquid with a characteristic fruity odor, composed of the hexanoates of mixed isomeric amyl alcohols, 2-methyl-1-butanol and 3-methyl-1-butanol predominating.

Isoamyl Isovalerate, page 401

Replace the first sentence of the *Description* with the following:

A clear, colorless liquid with a fruity odor which upon dilution resembles apples. It is composed of the isovalerates of mixed isomeric amyl alcohols, 2-methyl-1-butanol and 3-methyl-1-butanol predominating.

Isoamyl Salicylate, page 402

Replace the first sentence of the *Description* with the following:

A colorless liquid with a characteristic, pleasant odor, composed of the salicylates of mixed isomeric amyl alcohols, 2-methyl-1-butanol and 3-methyl-1-butanol predominating.

Lemongrass Oil, page 446

Change the paragraph entitled *Packaging and storage*, page 448, to read:

Packaging and storage. Store in a cool place in full, tight, preferably glass, aluminum, tin-lined, or other suitably lined containers, or in black iron unlined drums. If stored in glass containers, avoid exposure to light.

L-Leucine, page 451

Change the first phrase of the second sentence in the paragraph entitled *Assay*, page 452, to read:

Dissolve the sample in about 50 ml. of glacial acetic acid . . .

Locust Bean Gum, page 464

Replace the paragraph entitled *Galactomannans*, page 465, with the following:

Galactomannans. The remainder, after subtracting from 100 percent the sum of the percentages of *Acid-insoluble matter*, *Total ash*,

Loss on drying, and *Protein*, represents the percent of galactomannans in the sample.

Change the first sentence of the paragraph entitled *Acid-insoluble matter*, page 465, to read:

Transfer 1.5 grams of the sample, accurately weighed, into a 250-ml. beaker containing 150 ml. of water and 1.5 ml. of concentrated sulfuric acid.

Change the last sentence of the paragraph entitled *Protein*, page 466, to read:

The percent of nitrogen determined, multiplied by 6.25, gives the percent of protein in the sample.

L-Lysine Monohydrochloride, page 467

Change the SPECIFICATION for *Specific rotation* to read:

Specific rotation. $[\alpha]_D^{25°}$: Between +19.2° and +20.8° (equivalent to +24.0° to +26.0° as free lysine); $[\alpha]_D^{20°}$: between +19.0° and +21.5°

Magnesium Phosphate, Dibasic, page 475

Replace the paragraph entitled *Assay*, page 476, with the following:

Assay. Weigh accurately about 500 mg. of the residue obtained in the test for *Loss on ignition*, and dissolve it by heating in a mixture of 50 ml. of water and 2 ml. of hydrochloric acid. Cool, dilute to 100.0 ml. with water, and mix. Transfer 50.0 ml. of this solution into a 400-ml. beaker, add 100 ml. of water, and heat to 55–60°. From a buret add 15 ml. of 0.1 *M* disodium ethylenediaminetetraacetate, add a magnetic stirring bar, and adjust with sodium hydroxide T.S. to pH 10 while stirring. Add 10 ml. of ammonia-ammonium chloride buffer T.S. and 12 drops of eriochrome black T.S., and continue the titration with 0.1 *M* disodium ethylenediaminetetraacetate until the wine-red color changes to pure blue. Calculate the weight, in mg., of $Mg_2P_2O_7$ in the residue taken by the formula $2 \times 11.13 \times V$, in which V is the volume, in ml., of 0.1 *M* disodium ethylenediaminetetraacetate required in the titration of the 50.0 ml. aliquot.

Replace the first line of the paragraph entitled *Fluoride*, page 475, with the following:

Fluoride. Determine as directed in *Method III* under *Fluoride Limit Test*, page 919 (except in the *Procedure* use 10 ml. of 1 *N* hydrochloric acid to dissolve the sample), or use the following procedure: Transfer 5.0 grams of the sample into a 200-ml. distilling . . .

Magnesium Stearate, page 481

Change the first sentence of the *Description* to read:

Magnesium stearate is a compound of magnesium with a mixture of solid organic acids obtained from edible sources, and consists chiefly of variable proportions of magnesium stearate and magnesium palmitate.

Magnesium Sulfate, page 483

Change the paragraph entitled *Selenium*, page 484, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Manganese Sulfate, page 495

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Selenium:*

Selenium. Not more than 30 parts per million (0.003 percent).

Insert the following new paragraph to precede the *Packaging and storage* statement, page 496:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200-mg. of sample.

Methyl Formate, page 525

Change the paragraph entitled *Acidity*, page 526, to read:

Acidity. Transfer 50 ml. of the sample into a 300-ml. Erlenmeyer flask containing 1 ml. of bromocresol purple T.S. and 50 ml. of methanol that has been previously titrated with 0.1 *N* alcoholic potassium hydroxide to the first appearance of a bluish purple color, and titrate the sample with the alkali to the same color. Not more than 4.2 ml. is required.

Monosodium L-Glutamate, page 544

Change the SPECIFICATION (*Limits of Impurities*) for *Loss on drying* to read:

Loss on drying. Not more than 0.5 percent.

Change the paragraph entitled *Loss on drying*, page 545, to read:

Loss on drying, page 931. Dry at 100° for 5 hours.

Niacin, page 549

Change *Identification test C*, page 550, to read:

C. The infrared absorption spectrum of a mineral oil dispersion of the sample, previously dried at 105° for 1 hour, exhibits maxima only at the same wavelengths as that of a similar preparation of N.F. Niacin Reference Standard.

Papain, page 574

This monograph is subsumed by the new monograph for *Enzyme Preparations*, page 18 of THIS SUPPLEMENT.

Paraffin, Synthetic

Insert the following new monograph to precede the monograph entitled *Parsley Seed Oil*, page 576:

PARAFFIN, SYNTHETIC

Fischer-Tropsch Paraffin

DESCRIPTION

A very hard, white, practically tasteless and odorless wax. It is synthesized by the Fischer-Tropsch process from carbon monoxide and hydrogen, which are catalytically converted to a mixture of paraffin hydrocarbons; the lower molecular weight fractions are removed by distillation, and the residue is hydrogenated and further treated by percolation through activated charcoal. It is soluble in hot hydrocarbon solvents.

SPECIFICATIONS

Absorptivity. Less than 0.01 at 290 mμ, in decahydronaphthalene at 88° C. (190° F.).

Congealing point. Between 200° and 210° F. (93.3–98.9° C.).

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Lead. Not more than 3 parts per million (0.0003 percent).

Oil content. Not more than 0.50 percent.

TESTS

Absorptivity. Transfer about 100 mg. of the sample, accurately weighed, into a 100-ml. volumetric flask, dissolve in decahydronaphthalene at 88° C. (190° F.), dilute to volume at this temperature, and mix. Determine the absorbance of this solution in a 10-cm. cell at 290 mμ, with a suitable spectrophotometer, the cell holders of which shall maintain the temperature of the sample cell and the reference cell at 88° C. Use decahydronaphthalene at 88° C. in a matched cell as the blank. Cell lengths should be known to within ±0.5 percent or better of the nominal path length. Calculate the absorptivity (a) of the sample solution by the formula A/bc, in which A is the absorbance of the sample solution, corrected for the solvent blank; b is the exact path length of the sample cell, in cm.; and c is the exact concentration of the sample solution, in grams per liter. (*Note:* A suitable spectrophotometer, as applied in this test, is an accurately calibrated instrument capable of measuring absorbance with a repeatability of ±0.1 percent or better from an average of 0.4 absorbance level at 290 mμ; it has a spectral band width of 2 mμ or less, and wavelength measurements made with it shall be repeatable within ±0.2 mμ.)

Congealing point

Definition. The temperature at which the molten sample, when allowed to cool under the prescribed conditions, ceases to flow.

Thermometer jacket assembly. Use an A.S.T.M. Congealing Point Thermometer having a range of 68–213° F. and conforming to the requirements for an A.S.T.M. 54 F thermometer (see page 103 of THIS SUPPLEMENT). By means of a cork, fit the thermometer into a jacket consisting of a 1-ounce glass vial, 25 mm. in diameter and 55 mm. in height, and adjust the thermometer so that the bottom of the bulb is 10–15 mm. from the bottom of the vial.

Procedure. Place a sample, of sufficient size to represent exactly the material under test, in a casserole or other suitable dish, and heat slowly in a water bath to a temperature approximately 15° F. above the expected congealing point. Heat the *Thermometer jacket assembly* to approximately the same temperature as the prepared sample. When both the sample and the assembly have reached the required temperature, remove the assembly from the bath, then immediately remove the thermometer from its jacket, and immerse the thermometer bulb into the molten sample until the bulb is completely covered, taking care not to cover any part of the thermometer stem with the sample. As rapidly as possible, remove the thermometer and any adhering sample from the sample dish and place the thermometer in the jacket, holding both the thermometer and its jacket in a horizontal position during this operation. Rotate the thermometer in a horizontal position at the rate of approximately one revolution in 2 seconds, pausing momentarily at the completion of each revolution to inspect the drop of sample on the thermometer bulb. When the drop is observed to rotate with the bulb, read the thermometer and record the reading as the congealing point, reported to the nearest 0.5° F.

Arsenic. Prepare a *Sample Solution* as directed in the general method under *Chewing Gum Base*, page 877. This solution meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 1-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) as the control (*Solution A*).

Lead. Prepare a *Sample Solution* as directed in the general method under *Chewing Gum Base*, page 878. This solution meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Oil content. Determine as directed in the general method, page 97 of THIS SUPPLEMENT.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Masticatory substance in chewing gum base.

Pentaerythritol Ester of Partially Hydrogenated Wood Rosin, page 585

In the paragraph entitled *Arsenic*, change the page reference to *Chewing Gum Base* to read:

877

Pepsin, page 591

This monograph is subsumed by the new monograph for *Enzyme Preparations*, page 18 of THIS SUPPLEMENT.

Petroleum Wax and **Petroleum Wax, Synthetic**

Insert the following two new monographs to precede the monograph entitled *α-Phellandrene*, page 597:

PETROLEUM WAX

Refined Paraffin Wax; Microcrystalline Wax

DESCRIPTION

A refined mixture of solid hydrocarbons, paraffinic in nature, obtained from petroleum. It occurs as a translucent, tasteless and odorless wax. It may be prepared as "refined paraffin wax" or as "microcrystalline wax." The refined wax is usually obtained from a lower molecular weight fraction of petroleum and has lower viscosities when molten than the microcrystalline wax. The microcrystalline wax is usually higher in molecular weight, in flash point, and in melting point than the refined wax. These waxes are graded and sold according to melting point, which ranges from about 120° to 200° F. (48–93° C.), and color, which varies from amber to almost white. They exhibit a low order of solubility in organic solvents but are most soluble in aromatic hydrocarbons and least soluble in ketones, esters, and alcohols.

SPECIFICATIONS

Ultraviolet absorbance (polynuclear hydrocarbons). 280–289 mμ, not more than 0.15; 290–299 mμ, not more than 0.12; 300–359 mμ, not more than 0.08; 360–400 mμ, not more than 0.02.

The following additional specifications, where applicable, should conform to the representations of the vendor: **Color, Melting point,** and **Odor.**

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Lead. Not more than 3 parts per million (0.0003 percent).

TESTS

Ultraviolet absorbance. Determine as directed in the federal food additive regulation for Petroleum Wax (21 CFR 121.1156).

Color. Determine by any suitable procedure, such as A.S.T.M. D 1500–58 T.

Melting point. Determine by any suitable procedure, such as A.S.T.M. D 127–49.

Odor. Determine by any suitable procedure, such as A.S.T.M. D 1833.

Arsenic. Prepare a *Sample Solution* as directed in the general method under *Chewing Gum Base*, page 877. This solution meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 1-gram sample as directed in *Method I I* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) as the control (*Solution A*).

Lead. Prepare a *Sample Solution* as directed in the general method under *Chewing Gum Base*, page 878. This solution meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Masticatory substance in chewing gum base; protective coating; defoaming agent.

PETROLEUM WAX, SYNTHETIC

Synthetic Wax (Ethylene Polymer)

DESCRIPTION

A refined mixture of solid hydrocarbons, paraffinic in nature, prepared by the catalytic polymerization of ethylene. Synthetic petroleum wax ranges in melting point from about 185° to 240° F. (85–116° C.). The color of this wax varies from amber to almost white. It is

most soluble in aromatic hydrocarbons and least soluble in ketones, esters, and alcohols.

SPECIFICATIONS

Molecular weight (average). Between 500 and 1,200.

Ultraviolet absorbance (polynuclear hydrocarbons). 280–289 mμ, not more than 0.15; 290–299 mμ, not more than 0.12; 300–359 mμ, not more than 0.08; 360–400 mμ, not more than 0.02.

The following additional specifications, where applicable, should conform to the representations of the vendor: **Color, Melting point,** and **Odor.**

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Lead. Not more than 3 parts per million (0.0003 percent).

TESTS

Molecular weight

Apparatus. Use a suitable vapor pressure osmometer, such as the Hewlett-Packard Model 302A or equivalent, equipped with dual thermistor beads.

Calibration standards. Dissolve accurately weighed amounts of benzil ($C_6H_5COCOC_6H_5$) in *o*-dichlorobenzene to produce solutions containing approximately 3, 7, 10, and 15 mg. of benzil, respectively, per gram of solution, and heat to 100° on a steam bath.

Sample preparations. Dissolve accurately weighed amounts of the sample in *o*-dichlorobenzene to produce solutions containing approximately 10, 20, 35, and 50 mg. of sample, respectively, per gram of solution, and heat to 100° on a steam bath. (Other suitable concentrations that give ΔR readings between 5 and 25 may be used in the *Procedure* below.)

Procedure. Following the manufacturer's instructions, balance the osmometer to zero with *o*-dichlorobenzene on both thermistor beads, and establish the calibration constant K_S at 100°, using the four *Calibration standards.* When the temperature within the osmometer has re-equilibrated to 100°, place an aliquot of the most concentrated *Sample preparation* on the sample thermistor bead. After 4.0 minutes, balance the instrument to zero with the potentiometer, and record the ΔR value. Repeat this procedure with the same *Sample preparation* two or three times, and average the ΔR values for that concentration. In a similar manner, obtain the average ΔR values for each of the other three concentrations of the *Sample preparation.* Plot the four average ΔR values for the *Sample preparations* as a function of ΔR/concentration, and extrapolate the line to zero to obtain the constant K_U for

the sample. Divide K_S by K_U to obtain the molecular weight of the sample tested.

Ultraviolet absorbance. Determine as directed in the federal food additive regulation for Petroleum Wax (21 CFR 121.1156).

Color. Determine by any suitable procedure, such as A.S.T.M. D 1500–58 T.

Melting point. Determine by any suitable procedure, such as A.S.T.M. D 127–49.

Odor. Determine by any suitable procedure, such as A.S.T.M. D 1833.

Arsenic. Prepare a *Sample Solution* as directed in the general method under *Chewing Gum Base*, page 877. This solution meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 1-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) as the control (*Solution A*).

Lead. Prepare a *Sample Solution* as directed in the general method under *Chewing Gum Base*, page 878. This solution meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Masticatory substance in chewing gum base; protective coating; defoaming agent.

Potassium Alginate, page 641

Change the paragraph entitled *Heavy metals*, page 642, to read:

Heavy metals. Determine as directed in the revised test for *Heavy metals* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Change the paragraph entitled *Insoluble matter*, page 642, to read:

Insoluble matter. Determine as directed in the revised test for *Insoluble matter* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Potassium Metabisulfite, page 657

Change the paragraph entitled *Arsenic*, page 658, to read:

Arsenic. Dissolve 1 gram of the sample in 10 ml. of water in a 150-ml. beaker, cautiously add 10 ml. of nitric acid and 5 ml. of sul-

furic acid, and evaporate on a steam bath to a volume of about 5 ml. Place the beaker on a hot plate, and heat just to dense fumes of sulfur trioxide. Cool, cautiously wash down the side of the beaker with about 10 ml. of water, and again heat to dense fumes. Cool, repeat the washing and fuming procedure, and cool again. This solution meets the requirements of the *Arsenic Test*, page 865, omitting the addition of 20 ml. of dilute sulfuric acid (1 in 5).

Change the paragraph entitled *Selenium*, page 658, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 100 mg. of sample and 100 mg. of magnesium oxide.

Potassium Sulfate, page 670

Change the paragraph entitled *Selenium* to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Potassium Sulfite, page 670

Change the paragraph entitled *Arsenic*, page 671, to read:

Arsenic. Dissolve 1 gram of the sample in 10 ml. of water in a 150-ml. beaker, cautiously add 10 ml. of nitric acid and 5 ml. of sulfuric acid, and evaporate on a steam bath to a volume of about 5 ml. Place the beaker on a hot plate, and heat just to dense fumes of sulfur trioxide. Cool, cautiously wash down the side of the beaker with about 10 ml. of water, and again heat to dense fumes. Cool, repeat the washing and fuming procedure, and cool again. This solution meets the requirements of the *Arsenic Test*, page 865, omitting the addition of 20 ml. of dilute sulfuric acid (1 in 5).

Change the paragraph entitled *Selenium*, page 671, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample and 100 mg. of magnesium oxide.

Propylene Glycol, page 678

Change the *Specification* (*Limits of Impurities*) for *Residue on ignition*, page 679, to read:

Residue on ignition. Not more than 70 parts per million (0.007 percent).

Propylene Glycol Alginate, page 680

Change the paragraph entitled *Heavy metals*, page 682, to read:

Heavy metals. Determine as directed in the revised test for *Heavy metals* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Change the paragraph entitled *Insoluble matter*, page 682, to read:

Insoluble matter. Determine as directed in the revised test for *Insoluble matter* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Riboflavin, page 697:

Change the first sentence in the second paragraph of the *Assay*, page 698, to read:

In the same manner, prepare a standard solution to contain, in each ml., a quantity of U.S.P. Riboflavin Reference Standard, accurately weighed, equivalent to that of the solution prepared as directed in the preceding paragraph, and measure the intensity of its fluorescence in a fluorometer at about 530 mμ, using an excitation wavelength of about 440 mμ.

Change the first sentence in the third paragraph of the *Assay*, page 698, to read:

Similarly, measure the intensity of the fluorescence of the final solution of the riboflavin being assayed, before and after the addition of sodium hydrosulfite.

Change the last sentence in the second paragraph of the test for *Lumiflavin*, page 698, to read:

The absorbance of the filtrate, determined in a 1-cm. cell at 440 mμ with a suitable spectrophotometer, using alcohol-free chloroform as the blank, does not exceed 0.025.

Riboflavin 5′-Phosphate Sodium, page 699

Replace the first sentence of the paragraph entitled *Procedure*, under the *Assay*, page 700, with the following:

Procedure. With a suitable fluorometer, determine the intensity of the fluorescence of each solution at about 530 mμ, using an excitation wavelength of about 440 mμ. Record the fluorescence of the *Assay preparation* as I_U, and that of the *Standard preparation* as I_S.

Replace the first sentence of the paragraph entitled *Procedure*, page 702, under the test for *Free riboflavin and riboflavin diphosphate* (beginning on page 701), with the following:

Procedure. With a suitable fluorometer, determine the intensity of fluorescence of each sample solution and of the *Standard preparation* at about 530 mμ, using an excitation wavelength of about 440 mμ.

Saccharin, page 706

Change the third sentence of the *Description* to read:

It is intensely sweet.

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Toluenesulfonamides:*

Toluenesulfonamides. Not more than 100 parts per million (0.01 percent).

Change the paragraph entitled *Selenium*, page 707, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Insert the following new paragraph to precede the *Packaging and storage* statement, page 707:

Toluenesulfonamides. Determine as directed under *Sodium Saccharin*, page 56 of THIS SUPPLEMENT, but use 8.0 ml. of 15% sodium bicarbonate solution to dissolve the sample for the *Test preparation.*

Sodium Acid Pyrophosphate, page 719

Change the SPECIFICATION (*Limits of Impurities*) for *Fluoride*, page 720, to read:

Fluoride. Not more than 25 parts per million (0.0025 percent).

Change the paragraph entitled *Fluoride*, page 720, to read:

Fluoride. Weigh accurately 2.0 grams, and proceed as directed in *Method I* under the *Fluoride Limit Test*, page 917.

[*Note:* These revisions became effective on March 14, 1973.]

Sodium Alginate, page 721

Change the paragraph entitled *Heavy metals*, page 722, to read:

Heavy metals. Determine as directed in the revised test for *Heavy metals* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Change the paragraph entitled *Insoluble matter*, page 722, to read:

Insoluble matter. Determine as directed in the revised test for *Insoluble matter* under *Alginic Acid*, page 1 of THIS SUPPLEMENT.

Sodium Aluminum Phosphate, Acidic, page 722

Insert the following synonymn immediately under the monograph title:

SALP

Replace the paragraph entitled *Neutralizing value*, page 723, with the following:

Neutralizing value. Transfer 840.0 mg. of the sample into a 250-ml. beaker, and add 20 grams of sodium chloride, 5 ml. of sodium citrate solution (1 in 10), and 25 ml. of water. Add 120.0 ml. of 0.1 N sodium hydroxide from a buret, swirling during the addition, then place on a magnetic hot plate, and begin stirring at slow to medium speed, avoiding splattering. Bring to a boil in 3–5 minutes, and boil for exactly 5 minutes. Remove from the hot plate, cool immediately to 25°, place on an unheated magnetic stirrer, and titrate immediately, while stirring, to pH 8.5 with 0.2 N hydrochloric acid, determining the pH with a meter previously standardized with pH 7.0 buffer. Stir for an additional 5 minutes, and then add 0.2 N hydrochloric acid as necessary to obtain pH 8.5. Calculate the neutralizing value by the formula $(V_1N_1 - V_2N_2)(0.84 \times 100)/W$, in which V_1 and N_1 are the volume, in ml., and the exact normality, respectively, of the sodium hydroxide solution; V_2 and N_2 are the volume, in ml., and the exact

normality, respectively, of the hydrochloric acid solution; and *W* is the weight of the sample taken, in grams.

Sodium Ascorbate, page 725

Change the paragraph entitled *Loss on drying*, page 726, to read:

Loss on drying, page 931. Dry in vacuum over phosphorus pentoxide at 60° for 4 hours.

Sodium Bisulfate, page 728

Change the paragraph entitled *Selenium*, page 729, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Sodium Bisulfite, page 729

Change the paragraph entitled *Arsenic*, page 730, to read:

Arsenic. Dissolve 1 gram of the sample in 10 ml. of water in a 150-ml. beaker, cautiously add 10 ml. of nitric acid and 5 ml. of sulfuric acid, and evaporate on a steam bath to a volume of about 5 ml. Place the beaker on a hot plate, and heat just to dense fumes of sulfur dioxide. Cool, cautiously wash down the side of the beaker with about 10 ml. of water, and again heat to dense fumes. Cool, repeat the washing and fuming procedure, and cool again. This solution meets the requirements of the *Arsenic Test*, page 865, omitting the addition of 20 ml. of dilute sulfuric acid (1 in 5).

Change the paragraph entitled *Selenium*, page 730, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Sodium Chloride

Replace the entire monograph for *Sodium Chloride*, page 734, with the following:

SODIUM CHLORIDE

Salt

NaCl Mol. wt. 58.44

DESCRIPTION

Salt is a generic term applied to commercially produced sodium chloride. It is available in various crystalline forms, referred to as evaporated salt, rock salt, solar salt, or simply salt. It may contain up to 2 percent (total) of suitable food-grade anticaking, free-flowing, or conditioning agents, either singly or in combination. It may contain not more than 13 parts per million of sodium ferrocyanide, or not more than 25 parts per million of green ferric ammonium citrate as crystal-modifying and anticaking agents. If labeled as iodized, it contains not less than 0.006 percent and not more than 0.010 percent of potassium iodide.

Sodium chloride is a transparent to opaque, white crystalline solid of variable particle size. (Rock salt may be white to off-white in color.) It remains dry in air at a relative humidity below 75 percent but becomes deliquescent at higher humidities. One gram is soluble in 2.8 ml. of water at 25°, in 2.7 ml. of boiling water, and in about 10 ml. of glycerin. Sodium chloride containing water-insoluble anticaking, free-flowing, and conditioning agents may produce cloudy solutions, or may dissolve incompletely. A 1 in 20 solution usually has a pH between 5.5 and 8.5 (the pH may be higher if alkaline conditioning agents have been added), and gives positive tests for *Sodium*, page 928, and for *Chloride*, page 926.

SPECIFICATIONS

Assay. Not less than 97.5 percent of NaCl after drying at 625° for 2 hours, the remainder consisting chiefly of up to 2 percent of suitable food-grade anticaking, free-flowing, or conditioning agents, with minor amounts of innocuous naturally occurring components such as alkaline and/or alkaline earth sulfates and chlorides.

Iodine. Not less than 0.006 percent and not more than 0.010 percent of potassium iodide. [*Note:* This specification applies only to iodized salt.]

Iron. Not more than 16 parts per million (0.0016 percent) of Fe. [*Note:* This specification applies only to products to which green ferric ammonium citrate has been added.]

Loss on drying. Not more than 0.5 percent.

Sodium ferrocyanide. Not more than 13 parts per million (0.0013 percent) of anhydrous $Na_4Fe(CN)_6$. [*Note:* This specification applies only to products to which sodium ferrocyanide has been added.]

Limits of Impurities

Arsenic (as As). Not more than 1 part per million (0.0001 percent).

Calcium and magnesium. Not more than 2 percent.

Heavy metals (as Pb). Not more than 4 parts per million (0.0004 percent).

TESTS [*Note:* In the following procedures, it may be necessary to filter the sample solutions to avoid interference by insoluble or suspended anticaking, free-flowing, or conditioning agents.]

Assay. Weigh accurately about 250 mg. of the sample, previously dried at 625° for 2 hours, and dissolve it in 50 ml. of water in a glass-stoppered flask. Add, while agitating, 3 ml. of nitric acid, 5 ml. of nitrobenzene, 50.0 ml. of 0.1 *N* silver nitrate, and 2 ml. of ferric ammonium sulfate T.S. Shake well, and titrate the excess silver nitrate with 0.1 *N* ammonium thiocyanate. Each ml. of 0.1 *N* silver nitrate is equivalent to 5.844 mg. of NaCl.

Iodine. Transfer about 20 grams of the sample, accurately weighed, into a 600-ml. beaker, and dissolve in about 300 ml. of water. Add a few drops of methyl orange T.S., neutralize the solution with phosphoric acid (85%), and then add 1 ml. excess of the acid. Add 25 ml. of bromine T.S. and a few glass beads, boil until the solution is clear, then boil for an additional five minutes. Add about 50 mg. of salicylic acid crystals, 1 ml. of phosphoric acid, and 10 ml. of potassium iodide solution (1 in 20), and titrate to a pale yellow color with 0.01 *N* sodium thiosulfate. Add 1 ml. of starch T.S., and continue the titration to the disappearance of the blue color. Each ml. of 0.01 *N* sodium thiosulfate is equivalent to 0.2767 mg. of KI.

Iron. Dissolve 625.0 mg. of the sample in 10 ml. of diluted hydrochloric acid T.S., and dilute to 50 ml. with water. Add about 40 mg. of ammonium persulfate crystals and 10 ml. of ammonium thiocyanate T.S. Any red or pink color does not exceed that produced by 1.0 ml. of *Iron Standard Solution* (10 mcg. Fe) in an equal volume of solution containing 2 ml. of hydrochloric acid and the quantities of ammonium persulfate and ammonium thiocyanate used in the test.

Loss on drying, page 931. Dry at 110° for 2 hours.

Sodium ferrocyanide. Dissolve 9.62 grams of the sample in 80 ml. of water in a 150-ml. glass-stoppered cylinder or flask. Prepare a standard solution containing 125 mcg. of $Na_4Fe(CN)_6$ in each ml. by dissolving 99.5 mg. of $Na_4Fe(CN)_6.10H_2O$ in 500.0 ml. of water, then transfer 1.0 ml. of this solution into a similar 150-ml. container for the control. To each container add 2 ml. of ferrous sulfate T.S. and 1 ml. of diluted sulfuric acid T.S., dilute to 100 ml. with water, and mix. Transfer 50-ml. portions of the respective solutions into matched color-comparison tubes. The sample solution shows no more blue color than the control.

Arsenic. A solution of 3 grams of the sample in 25 ml. of water meets the requirements of the *Arsenic Test*, page 865.

Calcium and magnesium

Standard EDTA solution. Dissolve 4.0 grams of disodium ethylenediaminetetraacetate, $C_{10}H_{14}N_2Na_2O_8.2H_2O$, in sufficient water to make 1000 ml.

Magnesium sulfate solution. Dissolve 2.6 grams of magnesium sulfate, $MgSO_4.7H_2O$, in sufficient water to make 1000 ml.

Buffer solution. (a) Initial preparation: Transfer 67.5 grams of ammonium chloride into a 1000-ml. volumetric flask, and dissolve in 570 ml. of concentrated ammonium hydroxide. Use 2 ml. of this solution as directed below under *Titer determination.* (b) Final preparation: Pipet 50.0 ml. of *Magnesium sulfate solution* into the flask, add exactly the volume *T*, in ml., of *Standard EDTA solution*, determined as directed below under *Titer determination*, then dilute to volume with water, and mix.

Titer determination. Pipet 50.0 ml. of *Magnesium sulfate solution* into a 400-ml. beaker, and add 200 ml. of water, 2 ml. of *Buffer solution* (initial preparation), 1.0 ml. of potassium cyanide solution (1 in 20), and 5 drops of eriochrome black T.S. or other suitable indicator. Titrate with the *Standard EDTA solution*, while stirring with a magnetic stirrer, to a true blue end-point. Record the volume *T*, in ml., of *Standard EDTA solution* equivalent to 50.0 ml. of the *Magnesium sulfate solution.*

Standardization of EDTA solution. Transfer about 1 gram, accurately weighed, of primary standard calcium carbonate, $CaCO_3$, into a 1000-ml. volumetric flask, dissolve in 800 ml. of water containing 5 ml. of concentrated hydrochloric acid, dilute to volume with water, and mix. Pipet 25.0 ml. of this solution into a 400-ml. beaker, and add 200 ml. of water, 2 ml. of *Buffer solution* (final preparation), 1.0 ml. of potassium cyanide solution (1 in 20), and 20 drops of eriochrome black T.S. or other suitable indicator. Titrate with the *Standard EDTA solution*, stirring with a magnetic stirrer, to a true blue end-point. Calculate the factor *F*, giving the number of mg. of Ca equivalent to 1.0 ml. of *Standard EDTA solution*, by the formula $10.011\ w/v$, in which w is the exact weight, in grams, of the primary standard calcium carbonate taken, and v is the volume, in ml., of the *Standard EDTA solution* required in the titration.

Sample preparation for rock and solar salt. Transfer 50.0 grams of the sample into a 500-ml. volumetric flask, dissolve in 400 ml. of water containing 2 ml. of concentrated hydrochloric acid, dilute to volume with water, and mix. Filter a 50-ml. aliquot, then pipet 10.0 ml. of the filtrate into a 400-ml. beaker, and add 190 ml. of water.

Sample preparation for evaporated salt. Transfer 10.0 grams of the sample into a 400-ml. beaker, and dissolve in 100 ml. of water. If free-flowing agents are present, filter and rinse quantitatively. Dilute the solution or filtrate to 200 ml. with water.

Procedure. To the *Sample preparation* add 5 ml. of *Buffer solution* (final preparation), 1 ml. of potassium cyanide solution (1 in 20), and 5 drops of eriochrome black T.S. or other suitable indicator. Begin stirring with a magnetic stirrer, and titrate with *Standard EDTA solution* to a true blue end-point, recording the volume, in ml., required as V. Calculate the parts per million of total calcium and magnesium (both expressed as Ca) in the sample by the formula $V \times F \times 1000/W$, in which W is the weight, in grams, of salt sample in the final solution titrated.

Heavy metals. A solution of 5 grams of the sample in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Packaging and storage. Store in well-closed containers.

Labeling. Label the product to indicate whether or not it is iodized.

Functional use in foods. Nutrient; preservative; flavoring agent and intensifier.

Sodium Citrate, page 736

Change the second sentence of the paragraph entitled *Assay* to read:

Add 100 ml. of glacial acetic acid, stir until completely dissolved, and titrate with 0.1 N perchloric acid, using crystal violet T.S. as the indicator.

Sodium Ferric Pyrophosphate, page 740

Change the paragraph entitled *Mercury*, page 741, to read:

Mercury. Determine as directed in the test for *Mercury* under *Ferric Phosphate*, page 29 of THIS SUPPLEMENT.

Sodium Metabisulfite, page 748

Change the paragraph entitled *Arsenic* to read:

Arsenic. Dissolve 1 gram of the sample in 10 ml. of water in a 150-ml. beaker, cautiously add 10 ml. of nitric acid and 5 ml. of sulfuric acid, and evaporate on a steam bath to a volume of about 5 ml. Place the beaker on a hot plate, and heat just to dense fumes of sulfur trioxide. Cool, cautiously wash down the side of the beaker with about 10 ml. of water, and again heat to dense fumes. Cool, repeat the

washing and fuming procedure, and cool again. This solution meets the requirements of the *Arsenic Test*, page 865, omitting the addition of 20 ml. of dilute sulfuric acid (1 in 5).

Change the paragraph entitled *Selenium*, page 749, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample and 100 mg. of magnesium oxide.

Sodium Metaphosphate, page 749

In the first line of the paragraph entitled *Assay*, page 750, change "about 250 mg." to read:

... about 200 mg.

Sodium Methylate, page 751

Change the SPECIFICATION (*Limits of Impurities*) for *Sodium hydroxide* to read:

Sodium hydroxide. Not more than 1.7 percent.

Sodium Saccharin, page 764

Delete the fourth sentence of the *Description.*

Insert the following new SPECIFICATION (*Limits of Impurities*) for *Toluenesulfonamides:*

Toluenesulfonamides. Not more than 100 parts per million (0.01 percent).

Change the paragraph entitled *Selenium*, page 765, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Insert the following paragraphs to precede the *Packaging and storage* statement, page 765:

Toluenesulfonamides

Methylene chloride. Use a suitable grade (such as that obtainable

from Burdick & Jackson Laboratories, Inc.), equivalent to the product obtained by distillation in all-glass apparatus.

Internal standard stock solution. Transfer 100.0 mg. of *n*-tricosane (95%, obtainable from Chemical Samples Co.) into a 10-ml. volumetric flask, dissolve in *n*-heptane, dilute to volume with the same solvent, and mix.

Stock standard preparation. Transfer 20.0 mg. each of reagent grade *o*-toluenesulfonamide and *p*-toluenesulfonamide into a 10-ml. volumetric flask, dissolve in methylene chloride, dilute to volume with the same solvent, and mix.

Diluted standard preparations. Pipet into five 10-ml. volumetric flasks 0.1, 0.25, 1.0, 2.5, and 5.0 ml., respectively, of the *Stock standard preparation.* Pipet 0.25 ml. of the *Internal standard stock solution* into each flask, dilute each to volume with methylene chloride, and mix. These solutions contain, respectively, 20, 50, 200, 500, and 1000 mcg. per ml. of each toluenesulfonamide, plus 250 mcg. of *n*-tricosane.

Test preparation. Dissolve 2.00 grams of the sample in 8.0 ml. of 5% sodium bicarbonate solution, and mix the solution thoroughly with 10.0 grams of chromatographic siliceous earth (Celite 545, Johns-Manville, or equivalent). Transfer the mix into a 25 × 250-mm. chromatographic tube having a fritted glass disk and a Teflon stopcock at the bottom, and a reservoir at the top. Pack the contents of the tube by tapping the column on a padded surface, and then by tamping firmly from the top. Place 100 ml. of methylene chloride in the reservoir, and adjust the stopcock so that 50 ml. of eluate is collected in 20–30 minutes. To the eluate add 25 µl. of *Internal standard stock solution*, mix, and then concentrate the solution to a volume of 1.0 ml. in a suitable concentrator tube fitted with a modified Snyder column, and using a Kontes tube heater maintained at 90°.

Procedure. Inject 2.5 µl. of the *Test preparation* into a suitable gas chromatograph equipped with a flame-ionization detector. The column is of glass, 10 ft. (approx. 3 m.) in length and 2 mm. in inside diameter, and it is packed with 3% phenyl methyl silicone (OV-17), Applied Science Laboratories, Inc., or equivalent) on 100- to 120-mesh silanized calcined diatomaceous silica (Gas-Chrom Q, Applied Science, or equivalent). [*Caution:* The glass column should extend into the injector for on-column injection and into the detector base to avoid contact with metal.] The carrier is helium, flowing at a rate of 30 ml. per minute. The injection port, column, and detector are maintained at 225°, 180°, and 250°, respectively. The instrument attenuation setting should be such that 2.5 µl. of the *Diluted standard preparation* containing 200 mcg. per ml. of each toluenesulfonamide gives a response of 40–80% of full-scale deflection. Record the chromatogram, note the peaks for *o*-toluenesulfonamide, *p*-toluenesulfonamide, and the *n*-tricosane internal standard, and calculate the areas for each peak by suitable means. The retention times for *o*-toluenesulfonamide, *p*-toluenesulfonamide, and *n*-tricosane are about 5, 6, and 15 minutes, respectively.

In a similar manner, obtain the chromatograms for 2.5-μl. portions of each of the five *Diluted standard preparations*, and for each solution determine the areas of the *o*-toluenesulfonamide, *p*-toluenesulfonamide, and *n*-tricosane peaks. From the values thus obtained, prepare standard curves by plotting concentration of each toluenesulfonamide, in. mcg. per ml., vs. the ratio of the respective toluenesulfonamide peak area to that of *n*-tricosane. From the standard curve determine the concentration, in mcg. per ml., of each toluenesulfonamide in the *Test preparation*. Divide each value by 2 to convert the result to parts per million of the toluenesulfonamide in the 2-gram sample taken for analysis. [*Note:* If the toluenesulfonamide content of the sample is greater than about 500 parts per million, the impurity may crystallize out of the methylene chloride concentrate (see *Test preparation*). Although this level of impurity exceeds that permitted by the specification, the analysis may be completed by diluting the concentrate (usually 1:10 is satisfactory) with methylene chloride containing 250 mcg. of *n*-tricosane per ml., and by applying appropriate dilution factors in the calculation. Care must be taken to redissolve completely any crystalline toluenesulfonamide to give a homogeneous solution.]

Sodium Silicoaluminate, page 767

Change the SPECIFICATION for *Assay*, *Sodium oxide*, to read:

Sodium oxide: Not less than 4.0 percent and not more than 7.0 percent of Na_2O after drying.

Replace the sections entitled *Silicon dioxide* and *Aluminum oxide*, under *Assay*, page 768, with the following:

Silicon dioxide. Transfer about 500 mg., previously dried at 105° for 2 hours and accurately weighed, into a 250-ml. beaker, wash the sides of the beaker with a few ml. of water, and then add 30 ml. of 72 percent perchloric acid and 15 ml. of hydrochloric acid. Heat on a hot plate in a hood until dense white fumes are evolved, cool, add 15 ml. of hydrochloric acid, and heat again to dense white fumes. Cool, add 70 ml. of water, and filter through Whatman No. 40 or equivalent filter paper. Wash the filter paper and precipitate with hot water until free from perchloric acid, collecting the filtrate in a 250-ml. Erlenmeyer flask. Retain the filtrate for the determination of *Aluminum oxide.*

Transfer the filter paper and precipitate into a tared platinum crucible, char, and ignite at 900° to constant weight. Moisten the residue with a few drops of water, then add 15 ml. of hydrofluoric acid and 8 drops of sulfuric acid, and heat on a hot plate until white fumes of sulfur trioxide are evolved. Cool, add 5 ml. of water, 10 ml. of hy-

drofluoric acid, and 3 drops of sulfuric acid, and evaporate to dryness on the hot plate. Heat cautiously over an open flame until sulfur trioxide fumes have ceased, and ignite at 900° to constant weight. The weight loss after the addition of hydrofluoric acid represents the weight of SiO_2 in the sample taken. Retain the residue for the determination of *Aluminum oxide*.

Aluminum oxide. Add 2 grams of potassium pyrosulfate to the residue obtained in the *Silicon dioxide* determination, heat over a Meker burner until a clear melt is obtained, and cool. Add a few ml. of water and a few drops of sulfuric acid, and heat until the residue is dissolved, adding more water if necessary. Transfer this solution into the Erlenmeyer flask containing the filtrate obtained in the *Silicon dioxide* determination, and add ammonia T.S. until the aluminum hydroxide precipitate formed goes back slowly into solution after the last drop of the T.S. is added. Add 50.0 ml. of 0.05 *M* disodium ethylenediaminetetraacetate, and boil gently for 5 minutes. Cool, and add in the order given and with continuous stirring 20 ml. of pH 4.5 buffer solution (77.1 grams of ammonium acetate and 57 ml. of glacial acetic acid in 1000 ml. of solution), 50 ml. of alcohol, and 2 ml. of dithizone T.S. Titrate with 0.05 *M* zinc sulfate until the color changes from green-violet to rose-pink, and perform a blank determination (see page 2). Each ml. of 0.05 *M* disodium ethylenediaminetetraacetate is equvalent to 2.549 mg. of Al_2O_3.

Sodium Sulfate, page 775

Change the paragraph entitled *Selenium*, page 776, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Sodium Sulfite, page 776

Change the paragraph entitled *Arsenic*, page 777, to read:

Arsenic. Dissolve 1 gram of the sample in 10 ml. of water in a 150-ml. beaker, cautiously add 10 ml. of nitric acid and 5 ml. of sulfuric acid, and evaporate on a steam bath to a volume of about 5 ml. Place the beaker on a hot plate, and heat just to dense fumes of sulfur trioxide. Cool, cautiously wash down the side of the beaker with about 10 ml. of water, and again heat to dense fumes. Cool, repeat the washing and fuming procedure, and cool again. This solution

meets the requirements of the *Arsenic Test*, page 865, omitting the addition of 20 ml. of dilute sulfuric acid (1 in 5).

Change the paragraph entitled *Selenium*, page 777, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample and 100 mg. of magnesium oxide.

Sodium Thiosulfate, page 778

Change the paragraph entitled *Selenium*, page 780, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Sorbitan Monostearate, page 784

Change the paragraph entitled *Standard Solution*, under *Identification test B*, to read:

Standard Solution. Transfer 50 mg. each of sorbitol, of N.F: 1,4-Sorbitan Reference Standard, and of N.F. Isosorbide Reference Standard into a 1-ml. volumetric flask, dissolve and dilute to volume with water, and mix.

Sulfuric Acid, page 802

Change the paragraph entitled *Selenium*, page 803, to read:

Selenium. Determine as directed in *Method II* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

Terpene Resin, Synthetic, page 811

Change the second sentence of the *Description* to read:

The polymer is prepared by a batch or continuous process and is usually purified by steam and water washings.

Tocopherols Concentrate, Mixed, page 821

Change the SPECIFICATION for *Assay* to read:

Assay. Not less than 34.0 percent of total tocopherols. Not less than 50.0 percent of the total tocopherols consists of *d*-alpha-tocopherol ($C_{29}H_{50}O_2$).

Undecanal, page 845

Change the first synonym, appearing immediately under the monograph title, to read:

Aldehyde C-11 Undecylic

Zinc Sulfate, page 859

Change the paragraph entitled *Selenium*, page 860, to read:

Selenium. Determine as directed in *Method I* under the *Selenium Limit Test*, page 102 of THIS SUPPLEMENT, using 200 mg. of sample.

GENERAL TESTS AND APPARATUS

Chewing Gum Base, page 873

Change the italicized sentence near the bottom of page 877, in the section entitled *Sample Solution for Arsenic Test*, to read:

Maintain oxidizing conditions at all times during the digestion by adding peroxide whenever the mixture turns brown or darkens.

Enzyme Preparations

Insert the following new General Tests chapter to precede the chapter entitled *Essential Oils and Related Substances*, page 892:

ENZYME PREPARATIONS

General Requirements

Enzyme preparations are produced in accordance with good manufacturing practices. Regardless of the source from which they are derived, they cause no increase in the total microbial count in the treated food over the level accepted for the respective food.

Animal tissues used for the production of enzymes must comply with the applicable federal meat inspection requirements and must be handled in accordance with good hygienic practices.

Plant material used in the production of enzymes, or culture media used for the growth of micro-organisms, consists of components that leave no residues harmful to health in the finished food under normal conditions of use.

Preparations derived from microbial sources are produced by methods and under culture conditions that ensure a controlled fermentation, thus preventing the introduction of micro-organisms that could be the source of toxic materials and other undesirable substances.

The carriers, diluents, and processing aids used in the production of the enzyme preparations shall be substances that are accepted for general use in foods, including water and substances that are insoluble in foods but removed from the foods after processing.

Although tolerances have not been established for mycotoxins other than aflatoxin (see *Limits of Impurities* in the general monograph), appropriate measures should be taken to ensure that the products do not contain such contaminants.

General Information

A list of the enzymes covered by the general monograph is shown in the accompanying table. Also incorporated in the table are the trivial names by which each is commonly known, as well as the systematic name in accordance with the International Union of Biochemistry (IUB) and the IUB number of the major component or of the enzyme for which the preparation is standardized.

General Test Procedures

The following procedures are provided for application as necessary in determining compliance with the vendor's declared representations for enzyme activity:

ALPHA-AMYLASE ACTIVITY

(Non-Bacterial)

Application and Principle. This procedure is for the determination of α-amylase activity of enzyme preparations derived from *Aspergillus niger*, var.; *Aspergillus oryzae*, var.; *Rhizopus oryzae*, var.; and barley malt. The assay is based on the time required to obtain

Enzyme Preparations Used in Food Processing

Trivial Name	Classification	Source	Systematic Name (IUB)*	IUB No*
α-Amylase	carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Rhizopus oryzae*, var. (4) *Bacillus subtilis*, var. (5) barley malt	α-1,4-glucan 4-glucanohydrolase	3.2.1.1
β-Amylase	carbohydrase	barley malt	α-1,4-glucan maltohydrolase	3.2.1.2
Bromelain	protease	pineapples: *Ananas comosus*, *Ananas bracteatus* (L)	None	3.4.4.24
Catalase	oxidoreductase	(1) *Aspergillus niger*, var. (2) bovine liver (3) *Micrococcus lysodeikticus*	Hydrogen peroxide: hydrogen peroxide oxidoreductase	1.11.1.6
Cellulase	carbohydrase	*Aspergillus niger*, var.	β-1,4-glucan glucanohydrolase	3.2.1.4
Ficin	protease	figs: *Ficus* sp.	None	3.4.4.12
Glucoamylase (Amyloglucosidase)	carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Rhizopus oryzae*, var.	α-1,4-glucan glucohydrolase	3.2.1.3
Glucose Oxidase	oxidoreductase	*Aspergillus niger*, var.	β-D-glucose:oxygen oxidoreductase	1.1.3.4
Invertase	carbohydrase	*Saccharomyces* sp.	β-D-fructofuranoside fructohydrolase	3.2.1.26
Lactase	carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Saccharomyces* sp.	β-D-galactoside galactohydrolase	3.2.1.23
Lipase	lipase	(1) edible forestomach tissue of calves, kids, and lambs (2) animal pancreatic tissues (3) *Aspergillus oryzae*, var. (4) *Aspergillus niger*, var.	glycerol-ester hydrolase	3.1.1.3
Papain	protease	papaya: *Carica papaya* (L)	None	3.4.4.10
Pectinase†	carbohydrase	(1) *Aspergillus niger*, var. (2) *Rhizopus oryzae*, var.	Poly-α-1,4-galacturonide glycanohydrolase Pectin pectyl-hydrolase	3.2.1.15 3.1.1.11
Pepsin	protease	porcine or other animal stomachs	None	3.4.4.1
Protease (General)	protease	(1) *Aspergillus oryzae*, var. (2) *Bacillus subtilis*, var.		
Rennet and other milk clotting enzymes	protease	(1) fourth stomach of ruminant animals (2) *Endothia parasitica* (3) *Mucor miehei*, *M. pusillus*	None	3.4.4.3
Trypsin	protease	animal pancreas	None	3.4.4.4

* Enzyme Nomenclature. Recommendations 1964 of International Union of Biochemistry, Elsevier, Amsterdam (1965).
† Usually a mixture of polygalacturonase and pectin methylesterase.

a standard degree of hydrolysis of a starch solution at 30° ± 0.1°. The degree of hydrolysis is determined by comparing the iodine color of the hydrolysate with that of a standard.

Apparatus

Reference Color Standard. Use a special Alpha-Amylase Color Disk available as Catalog No. 620-S5 from Hellige, Inc., 3718 Northern

Blvd., Long Island City, New York. Alternatively, a color standard may be prepared by dissolving 25.0 grams of cobaltous chloride ($CoCl_2.6H_2O$) and 3.84 grams of potassium dichromate in 100 ml. of 0.01 *N* hydrochloric acid. This standard is stable indefinitely when stored in a stoppered bottle or comparator tube.

Comparator. Use either the standard Hellige comparator (Catalog No. 607) or the pocket comparator (Catalog No. 605) with prism attachment (Catalog No. 605A). The comparator should be illuminated with a 100-watt frosted lamp placed six inches from the rear opal glass of the comparator and mounted so that direct rays from the lamp do not shine into the operator's eyes.

Comparator Tubes. Use the precision-bored square tubes with a 13-mm. viewing depth that are supplied with the Hellige comparator. Suitable tubes are also available from other apparatus suppliers (e.g., Coleman Universal Distributors).

Reagents and Solutions

Buffer Solution (pH 4.8). Dissolve 164 grams of anhydrous sodium acetate in about 500 ml. of water, add 120 ml. of glacial acetic acid, and adjust the pH to 4.8 with glacial acetic acid. Dilute to 1000.0 ml. with water, and mix.

β-Amylase Solution. Dissolve 250 mg. of β-amylase, free from α-amylase, in 5 ml. of water. The enzyme, which has been standardized to 2000° diastatic power, is distributed by the Wallerstein Company, 6301 Lincoln Avenue, Morton Grove, Ill. 60053. (*Note:* The enzyme should be stored in a refrigerator, and it should be allowed to warm to room temperature before opening, in order to prevent condensation of moisture.)

Special Starch. Use starch designated as "Starch (Lintner) Soluble," Baker Analyzed Reagent Catalog No. 4010. Before using new batches, test them in parallel with previous lots known to be satisfactory. Variations of more than ± 3° diastatic power in the averages of a series of parallel tests indicate an unsuitable batch.

Buffered Substrate Solution. Disperse 10.0 grams (dry-weight basis) of *Special Starch* in 100 ml. of cold water, and pour slowly into 300 ml. of boiling water. Boil with stirring for 1 to 2 minutes, then cool, and add 25 ml. of *Buffer Solution*, followed by all of the *β-Amylase Solution.* Quantitatively transfer the mixture into a 500-ml. volumetric flask with the aid of water saturated with toluene, dilute to volume with the same solvent, and mix. Store the solution at 30° ± 2° for not less than 18 nor more than 72 hours before use. (This solution is also known as "buffered limit dextrin substrate.")

Stock Iodine Solution. Dissolve 5.5 grams of iodine and 11.0 grams of potassium iodide in about 200 ml. of water, dilute to 250 ml. with water, and mix. Store in a dark bottle and make a fresh solution every 30 days.

Dilute Iodine Solution. Dissolve 20 grams of potassium iodide in

300 ml. of water, and add 2.0 ml. of *Stock Iodine Solution.* Quantitatively transfer into a 500-ml. volumetric flask, dilute to volume with water, and mix.

Sample Preparation. Prepare a solution of the sample so that 5 ml. of the final dilution will give an end-point between 10 and 30 minutes under the conditions of the assay.

For barley malt, finely grind 25 grams of the sample in a Miag-Seck mill, available from the Schock Gusmer Division of the Pfaudler Co., 1000 West Avenue, Rochester, New York 10003, or from Ludwig Baer Machinery, Inc., 270 Madison Avenue, New York, NY 10016. Quantitatively transfer the powder into a 1000-ml. Erlenmeyer flask, add 500 ml. of a 0.5% solution of sodium chloride, and allow the infusion to stand for 2.5 hours at 30° ± 0.2°, agitating the contents by gently rotating the flask at 20-minute intervals. (*Caution:* The infusion must not be mixed by inverting the flask, and the quantity of the grist left adhering to the inner walls of the flask as a result of agitation must be as small as possible.) Filter the infusion through a 32-cm. fluted filter of Whatman No. 1, or equivalent, paper on a 20-cm. funnel, returning the first 50 ml. of filtrate to the filter. Collect the filtrate until 3 hours have elapsed from the time the sodium chloride solution and the sample were first mixed. Pipet 20.0 ml. of the filtered infusion into a 100-ml. volumetric flask, dilute to volume with the 0.5% sodium chloride solution, and mix.

Procedure

Pipet 5.0 ml. of *Dilute Iodine Solution* into a series of 13 × 100-mm. test tubes, and place them in a water bath maintained at 30° ± 0.1°, allowing 20 tubes for each assay.

Pipet 20.0 ml. of the *Buffered Substrate Solution,* previously heated in the water bath for 20 minutes, into a 50-ml. Erlenmeyer flask, and add 5.0 ml. of 0.5% sodium chloride solution, also previously heated in the water bath for 20 minutes. Place the flask in the water bath.

At zero time, rapidly pipet 5.0 ml. of the *Sample Preparation* into the equilibrated substrate, mix immediately by swirling, and then stopper the flask, which should be placed back in the water bath. After 10 minutes, transfer 1.0 ml. of the reaction mixture from the 50-ml. flask into one of the test tubes containing the *Dilute Iodine Solution,* shake the tube, then pour its contents into a *Comparator Tube,* and immediately compare with the *Reference Color Standard* in the *Comparator,* using a tube of water behind the color disk. (*Note:* Be certain that the pipet tip does not touch the iodine solution; carry-back of iodine to the hydrolyzing mixture will interfere with enzyme action and will affect the results of the determination.) In the same manner, repeat the transfer and comparison procedure at accurately timed intervals until the α-amylase color is reached, at which time the elapsed time should be recorded. In cases where two comparisons 30 seconds apart show that one is darker and the other lighter than the

Reference Color Standard, the end-point is recorded to the nearest quarter-minute. The 13-mm. *Comparator Tube* should be shaken out between successive readings. Slight differences in color discrimination between operators may be minimized by the use of a prism attachment and by maintaining a 6- to 10-inch distance between the *Comparator* and the operator's eye.

Calculation

One α-amylase dextrinizing unit (DU) is defined as the quantity of α-amylase that will dextrinize soluble starch in the presence of an excess of β-amylase at the rate of 1 gram per hour at 30°.

Calculate the α-amylase dextrinizing units in the sample as follows:

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

$$\text{DU (dry basis)} = \frac{\text{DU (solution)} \times 100}{(100 - M)},$$

in which W is the weight, in grams, of the enzyme sample added to the incubation mixture in the 5-ml. aliquot of the *Sample Preparation* used; T is the elapsed dextrinizing time, in minutes; 24 is the product of the weight of the starch substrate (0.4 gram) and 60 minutes; and M is the percent moisture in the sample, determined by suitable means.

BACTERIAL ALPHA-AMYLASE ACTIVITY (BAU)

Application and Principle. This procedure is for the determination of α-amylase activity, expressed as Bacterial Amylase Units (BAU), of enzyme preparations derived from *Bacillus subtilis*, var. It is not applicable to products that contain β-amylase. The assay is based on the time required to obtain a standard degree of hydrolysis of a starch solution at 30° ± 0.1°. The degree of hydrolysis is determined by comparing the iodine color of the hydrolysate with that of a standard.

Apparatus. Use the *Reference Color Standard*, the *Comparator*, and the *Comparator Tubes* as described under *Alpha-Amylase Activity (Non-Bacterial)*, page 62 of THIS SUPPLEMENT, but use either daylight or daylight-type fluorescent lamps as the light source for the *Comparator*. (Incandescent lamps give slightly lower results.)

Reagents and Solutions

pH 6.6 Buffer. Solution A: Dissolve 9.1 grams of monobasic potassium phosphate (KH_2PO_4) in sufficient water to make 1000 ml. *Solution B:* Dissolve 9.5 grams of dibasic sodium phosphate (Na_2HPO_4) in sufficient water to make 1000 ml. Add 400 ml. of *Solution A* to 600 ml. of *Solution B*, mix, and adjust the pH to 6.6, if necessary, by the addition of *Solution A* or *Solution B* as required.

Dilute Iodine Solution. Prepare as directed under *Alpha-Amylase Activity (Non-Bacterial)*, page 62 of THIS SUPPLEMENT.

Special Starch. Use the material described under *Alpha-Amylase Activity* (*Non-Bacterial*), page 62 of THIS SUPPLEMENT.

Starch Substrate Solution. Disperse 10.0 grams (dry-weight basis) of *Special Starch* in 100 ml. of cold water, and pour slowly into 300 ml. of boiling water. Boil with stirring for 1 to 2 minutes, and then cool with continuous stirring. Quantitatively transfer the mixture into a 500-ml. volumetric flask with the aid of water, add 10 ml. of *pH 6.6 Buffer*, dilute to volume with water, and mix.

Sample Preparation. Prepare a solution of the sample so that 10 ml. of the final dilution will give an end-point between 15 and 35 minutes under the conditions of the assay.

Procedure

Pipet 5.0 ml. of *Dilute Iodine Solution* into a series of 13 × 100-mm. test tubes, and place them in a water bath maintained at 30° ± 0.1°, allowing 20 tubes for each assay.

Pipet 20.0 ml. of the *Starch Substrate Solution* into a 50-ml. Erlenmeyer flask, stopper, and allow to equilibrate for 20 minutes in the water bath at 30°.

At zero time, rapidly pipet 10.0 ml. of the *Sample Preparation* into the equilibrated mixture, and continue as directed in the *Procedure* under *Alpha-Amylase Activity* (*Non-Bacterial*), page 62 of THIS SUPPLEMENT, beginning with ". . . mix immediately by swirling, and then stopper the flask . . ."

Calculation

One Bacterial Amylase Unit (BAU) is defined as that quantity of enzyme that will dextrinize 1 mg. of starch per minute under the specified test conditions.

Calculate the α-amylase activity of the sample, expressed as BAU, by the formula:

$$\text{BAU per gram} = 40F/T,$$

in which 40 is a factor (400/10) derived from the 400 mg. of starch (20 ml. of a 2% solution) and the 10-ml. aliquot of *Sample Preparation* used; F is the dilution factor (total dilution volume/sample weight, in grams); and T is the dextrinizing time, in minutes.

CATALASE ACTIVITY

Application and Principle. This procedure is for the determination of catalase activity, expressed as Baker Units, of preparations derived from *Aspergillus niger*, var.; bovine liver; or *Micrococcus lysodeikticus*. The assay is an exhaustion method based on the breakdown of hydrogen peroxide by catalase, and the simultaneous breakdown of the catalase by the peroxide, under controlled conditions.

Reagents and Solutions

0.250 N Sodium Thiosulfate. Dissolve 62.5 grams of sodium thiosulfate, $Na_2S_2O_3.5H_2O$, in 750 ml. of recently boiled and cooled water, add 3.0 ml. of 0.2 *N* sodium hydroxide as a stabilizer, dilute to 1000 ml. with water, and mix. Standardize as directed for *0.1 N Sodium Thiosulfate*, page 1002, and adjust to exactly 0.250 *N* if necessary.

Peroxide Substrate Solution. Dissolve 25.0 grams of anhydrous dibasic sodium phosphate (Na_2HPO_4), or 70.8 grams of $Na_2HPO_4.12H_2O$, in about 1500 ml. of water, and adjust to pH 7.0 ± 0.1 with 85% phosphoric acid. Cautiously add 100 ml. of 30% hydrogen peroxide, dilute to 2000 ml. in a graduate, and mix. Store in a clean amber bottle, loosely stoppered. The solution is stable for more than one week if kept at 5° in a full container. (With freshly prepared substrate, the blank will require about 16 ml. of 0.250 *N* sodium thiosulfate. If the blank requires less than 14 ml., the substrate solution is unsuitable and should be prepared fresh again. It is essential that the sample titration is between 50% and 80% of that required for the blank.)

Procedure. Pipet an aliquot of not more than 1.0 ml. of the sample, previously diluted to contain approximately 3.5 Baker Units of catalase, into a 200-ml. beaker. Rapidly add 100 ml. of *Peroxide Substrate Solution*, previously adjusted to 25°, and stir immediately for 5 to 10 seconds. Cover the beaker, and incubate at 25° ± 1° until the reaction is completed. Stir vigorously for 5 seconds, and then pipet 4.0 ml. from the beaker into a 50-ml. Erlenmeyer flask. Add 5 ml. of 2 *N* sulfuric acid to the flask, mix, then add 5.0 ml. of 40% potassium iodide solution, freshly prepared, and 1 drop of 1% ammonium molybdate solution, and mix. While continuing to mix, titrate rapidly to a colorless end-point with *0.250 N Sodium Thiosulfate*, recording the volume, in ml., required as *S*. Perform a blank determination with 4.0 ml. of *Peroxide Substrate Solution*, and record the volume required, in ml., as *B*. (*Note:* When preparations derived from beef liver are tested, the reaction is complete within 30 minutes. Preparations derived from *Aspergillus* and other sources may require up to 1 hour. In assaying an enzyme of unknown origin, a titration should be run after 30 minutes and then at 10-minute intervals thereafter. The reaction is complete when two consecutive titrations are the same.)

Calculation. One Baker Unit is that amount of catalase that will decompose 266 mg. of hydrogen peroxide under the conditions of the assay.

Calculate the activity of the sample by the formula:

$$\text{Baker Units/gram or ml.} = 0.4(B - S) \times (1/C),$$

in which *C* is the ml. of aliquot of original enzyme preparation added to each 100 ml. of *Peroxide Substrate Solution*, or, when 1 ml. of diluted enzyme is used, *C* is the dilution factor.

CELLULASE ACTIVITY

Application and Principle. This procedure is for the determination of cellulase enzymes derived from *Aspergillus niger*, var., and *Aspergillus oryzae*, var. The assay is based on the time required to reduce the viscosity of a soluble cellulose from 400 centipoises to 300 centipoises at pH 5.0.

Apparatus

Viscometer. Use a Brookfield Model LVF or equivalent type viscometer, with a No. 1, Spindle, capable of rotating at 12 rpm and of being read in centipoises. A suitable viscometer is available from Brookfield Engineering Laboratories, Inc., 240 Cushing St., Stoughton, Mass. 02072.

Sample Container. Use a 250-ml. beaker, or equivalent container, designed for use with the Brookfield viscometer. Berzelius beakers, available as Corning Catalog No. 1140, are suitable for this purpose.

Beater. Use a wire whip hand beaker, such as the Ekco Presto-Whip with a spiral cone (available at hardware stores).

Reagents and Solutions

Sodium Acetate Buffer, pH 5.0. Dissolve 34 grams of sodium acetate, $NaC_2H_3O_2.3H_2O$, in about 800 ml. of water, and adjust the pH to 5.0 with glacial acetic acid. Quantitatively transfer the solution into a 1000-ml. volumetric flask, dilute to volume with water, and mix.

Standard Solution. Weigh accurately 1 gram of a standard cellulase preparation (available as Cellase 1000 Reference Standard, from Wallerstein Company, 6301 Lincoln Avenue, Morton Grove, Ill. 60053), and dissolve it in 100 ml. of water. Quantitatively transfer the solution into a 1000-ml. volumetric flask, dilute to volume with water, and mix. Each ml. of this solution contains 2.6 cellulase activity (CA) units.

Substrate Solution. Sift 132 grams of sodium carboxymethylcellulose (cellulose gum, Hercules Type 7-LF) through a household-type tea strainer or 40-mesh screen, and add with continuous stirring to approximately 2125 ml. of water. Add 375 ml. of *Sodium Acetate Buffer*, and continue stirring until most of the gum has gone into solution. Allow the mixture to stand at room temperature for 2–3 hours, stirring frequently to assure uniform and complete dispersion of the gum. (*Note:* Use only gentle mixing so as not to shear the polymer mechanically.)

Since the substrate may vary from lot to lot, each lot should be checked, by the *Procedure* below, before use in assaying the enzyme unknown. The viscosity of the *Substrate Solution* should be reduced from 400 cps to 300 cps in 277 $\pm$ 10 seconds by 5.0 ml. of the *Standard Solution.* If the viscosity-reduction time does not fall within this range, appropriate dilutions of the *Substrate Solutions* should be made.

Sample Preparation. Prepare a solution of the enzyme preparation

in water so that each 5 ml. of the final dilution contains between 2 and 10 cellulase activity (CA) units.

Procedure. Transfer 200 grams of the *Substrate Solution* into a *Sample Container*, and equilibrate for 15 minutes in a water bath maintained at 35° ± 0.1°. At zero time, rapidly pipet 5.0 ml. of the *Sample Preparation* into the equilibrated substrate, mix immediately for 15 seconds with the *Beater*, and then lower the Viscometer spindle as rapidly as possible into the mixture. *Do not remove the Sample Container from the water bath at any time during the determination.* Begin stirring at 12 rpm, and start timing with a stopwatch when the reading indicates a viscosity of 400 cps. Continue timing until the viscosity is reduced to 300 cps, and record the elapsed time, T_U, in seconds. (*Note:* The elapsed time should fall between 150 and 600 seconds; if longer times are required, use a higher concentration of enzyme in the *Sample Preparation.*)

In the same manner, treat 200 grams of the *Substrate Solution* with 5.0 ml. of the *Standard Solution*, and record the elapsed time.

Calculation

One cellulase activity (CA) unit is defined as that quantity of enzyme required to reduce the viscosity of 200 grams of a 5% solution of the specified sodium carboxymethyl cellulose substrate from 400 cps to 300 cps at 35° ± 0.1° and pH 5.0, in one hour.

Calculate the activity of the enzyme preparation taken for analysis by the formula:

$$\text{CA, Units/gram} = \frac{1000 \times 60 \times 60}{W \times T_U}$$

in which W is the weight, in mg., of cellulase contained in the 5-ml. aliquot of the *Sample Preparation used.*

DIASTASE ACTIVITY (Diastatic Power, DP)

Application and Principle. This procedure is for the determination of amylase activity of barley malt and other enzyme preparations. The assay is based on a 30-minute hydrolysis of a starch substrate at pH 4.6 and 20°. The reducing sugar groups produced on hydrolysis are measured in a titrimetric procedure using alkaline ferricyanide.

Apparatus

Mill. Use a laboratory mill of the type Miag-Seck, for fine grinding of malt, available from the Schock Gusmer Division of the Pfaudler Co., 1000 West Avenue, Rochester, N.Y. 10003, or from Ludwig Baer Machinery, Inc., 270 Madison Avenue, New York, N.Y. 10016.

Reagents and Solutions

Acetate Buffer Solution. Dissolve 68 grams of sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$, in 500 ml. of 1 *N* acetic acid in a 1000-ml. volumetric flask, dilute to volume with water, and mix.

Special Starch. Use the material described under *Alpha-Amylase Activity (Non-Bacterial)*, page 62 of THIS SUPPLEMENT.

Starch Substrate Solution. Disperse 20.0 grams (dry-weight basis) of *Special Starch* in 50 ml. of water, mix to a fine paste, and pour slowly into 750 ml. of boiling water. Boil with stirring for 2 minutes, cool, add 20 ml. of *Acetate Buffer Solution*, and mix. Quantitatively transfer into a 1000-ml. volumetric flask, dilute to volume with water, and mix.

Acetic Acid-Potassium Chloride-Zinc Sulfate Solution (A–P–Z). Dissolve 70 grams of potassium chloride and 20 grams of zinc sulfate ($ZnSO_4.7H_2O$) in 700 ml. of water in a 1000-ml. volumetric flask, add 200 ml. of glacial acetic acid, dilute to volume with water, and mix.

Alkaline Ferricyanide Solution, 0.05 N. Dissolve 16.5 grams of potassium ferricyanide, $K_3Fe(CN)_6$, and 22 grams of anhydrous sodium carbonate in 800 ml. of water in a 1000-ml. volumetric flask, dilute to volume with water, and mix.

Potassium Iodide Solution. Dissolve 50 grams of potassium iodide in 50 ml. of water in a 100-ml. volumetric flask, dilute to volume with water, and mix. Add 2 drops of 50% sodium hydroxide solution, and mix. The solution should be colorless.

Sample Preparation

Malt Samples. Grind 30 grams of the sample to a fine powder in a Maig-Seck mill. Accurately weigh 25 grams of the powder, and transfer it into a 1000-ml. Erlenmeyer flask. Add 500 ml. of a 0.5% sodium chloride solution, and allow the infusion to stand for 2.5 hours at 20° ± 0.2°, agitating the contents by gently rotating the flask at 20-minute intervals. (*Note:* The infusion must not be mixed by inverting the flask, and the quantity of grist left adhering to the inner walls of the flask as a result of agitation must be as small as possible. Gentle swirling of the contents of the flask without splashing against the walls will produce sufficient mixing.) Filter the infusion through a 32-cm. fluted filter of Whatman No. 1, or equivalent, paper on a 20-cm. funnel, returning the first 50 ml. of filtrate to the filter. Place a watch-glass over the funnel, and use a suitable cover around the stem and over the receiver to reduce evaporation losses during filtration. Collect the filtrate until 30 minutes of filtration time have elapsed. Pipet 20.0 ml. of the filtrate into a 100-ml. volumetric flask, dilute to volume with 0.5% sodium chloride solution, and mix.

Other Enzyme Preparations. Prepare a solution so that 10 ml. of the final dilution will give a diastatic power (DP) value between 2° and 150°.

Procedure

Pipet 10.0 ml. of the *Sample Preparation* into a 250-ml. volumetric flask, and at zero time, add 200 ml. of *Starch Substrate Solution*, previously equilibrated for 30 minutes in a water bath maintained at 20° ± 0.2°. Start the stopwatch at zero time.

Place the mixture in the water bath at 20°, and allow it to cool for exactly 30 minutes, then add 20.0 ml. of 0.5 *N* sodium hydroxide, dilute to volume with water, and mix.

Prepare a blank by adding 20.0 ml. of 0.5 *N* sodium hydroxide to a 250-ml. volumetric flask, followed by 10.0 ml. of the *Sample Preparation*. Swirl to mix, add 200 ml. of *Starch Substrate Solution*, dilute to volume with water, and mix.

Pipet 5.0 ml. of the sample digestion mixture into a 125-ml. Erlenmeyer flask, add 10.0 ml. of *Alkaline Ferricyanide Solution*, and swirl to mix. Heat the flask for exactly 20 minutes in a boiling water bath, and then cool to room temperature. Add 25 ml. of *A–P–Z Solution*, followed by 1 ml. of *Potassium Iodide Solution*, and swirl to mix. Titrate with 0.05 *N* sodium thiosulfate to the complete disappearance of the blue color, recording the volume, in ml., of 0.05 *N* sodium thiosulfate required as *S*.

Treat the blank solution in the same manner as described for the sample, recording the volume, in ml., of 0.05 *N* sodium thiosulfate required as *B*.

Calculation

One unit of diastase activity, expressed as degrees diastatic power (DP°), is defined as that amount of enzyme, contained in 0.1 ml. of a 5% solution of the sample enzyme preparation, that will produce sufficient reducing sugars to reduce 5 ml. of Fehling's solution when the sample is incubated with 100 ml. of the substrate for 1 hour at 20°. (*Note:* The definition of the unit does not correspond to the method of the determination.)

Calculate the diastase activity, expressed as DP°, of the sample by the formulas:

$$\text{DP°, as is basis} = (B - S) \times 23, \text{ and}$$

$$\text{DP°, dry basis} = (\text{DP°, as is basis} \times 100)/(100 - M),$$

in which 23 is a factor, determined by collaborative study, required to convert to the units of the definition; and *M* is the percent moisture of the sample, determined by suitable means.

ESTERASE ACTIVITY

Application and Principle. This procedure is primarily applicable to esterases from animal pregastric sources. The analysis is performed by potentiometric titration.

Apparatus. Use a suitable automatic recording titrator equipped with thermostatic control (Thermostatic Recording pH Stat, Sargent-Welch, or equivalent).

Reagents and Solutions

Sodium Caseinate. Use the hydrophile powder, soluble form, available from Sheffield Chemical (Div. Kraftco), 2400 Morris Ave., Union, N.J. 07083.

Hydroxylated Lecithin Solution. Use the material available from Food Technology, Inc., 5903 Northwest Highway, Chicago, Ill. 60600, and prepare a 10% solution in light mineral oil (FCC or USP grade).

Tri-n-butyrin. Use the material available as #726, Eastman Organic Chemicals, or equivalent.

Substrate Preparation. Disperse an amount of *Sodium Caseinate*, equivalent to 600 mg. of casein, in 95 ml. of water contained in a one-half pint freezer jar (Ball Mason, or equivalent) that fits the head of a suitable high-speed blender. Add 0.5 ml. of *Hydroxylated Lecithin Solution* and 5.0 ml. of *Tri-n-butyrin*, and mix for 60 seconds at low speed. Adjust the temperature of the mixture to 42°, and use within 4 hours.

Sample Preparation. Suspend or dissolve an accurately weighed amount of the enzyme preparation in water, and dilute to obtain an enzyme activity of 10–20 esterase units per ml.

Procedure. Fill the buret of the titrator with 0.025 *N* sodium hydroxide, and calibrate the instrument following the manufacturer's instructions, setting the temperature at 42° and the pH at 6.20. Mix the *Substrate Preparation* for about 15 seconds with a magnetic stirrer, then pipet 10.0 ml. into the reaction vessel of the titrator, and add a small stirring bar. Place the vessel on the titrator, add 1.0 ml. of the *Sample Preparation*, and equilibrate the mixture for 15 minutes. Actuate the recorder, and record the titration curve for 15 minutes. (*Note:* The recorder trace reflecting delivery of the titrant must be linear.) Determine the rate, in ml. per minute, at which the titrant was delivered during the titration, and record this value as *R*.

Calculation. One esterase unit is the activity that releases 1.25 micromoles of butyric acid per minute under the conditions of the test.

Calculate the activity of the enzyme preparation by the formula:

$$\text{Esterase units/gram} = \frac{R \times (0.025)(10^3)}{W \times 1.25}$$

in which *W* is the weight, in grams, of the enzyme preparation contained in the 1.0 ml. of *Sample Preparation* taken for analysis.

GLUCOAMYLASE ACTIVITY

(Amyloglucosidase Activity)

Application and Principle. This procedure is designed for the determination of the glucoamylase activity of preparations derived

from *Aspergillus niger*, var., but it may be modified for the determination of preparations derived from *Aspergillus oryzae*, var., and *Rhizopus oryzae*, var. (as indicated by the variations in the text below). The sample is allowed to convert a corn starch hydrolyzate solution, under carefully controlled conditions of time, temperature, pH, and concentration. The resulting reducing sugars are determined, and the activity is calculated as the weight, in grams, of reducing sugars produced by a unit quantity of sample in 1 hour under the specified conditions.

Reagents and Solutions

Starch Hydrolyzate Solution (4%). Weigh accurately an amount of 15–20 dextrose equivalent (DE) corn syrup solids corresponding to 40.0 grams of the dry substance. (If necessary, an equivalent amount of neutralized and filtered corn starch hydrolyzate having a DE of 15–20 may be substituted for the corn syrup solids. Suitable dried commercial products in this DE range may be obtained from A. E. Staley Manufacturing Company, Decatur, Ill. 62525; CPC International, Argo, Ill. 60501; and Grain Processing Corp., Muscatine, Iowa 52761.) Transfer quantitatively into a 1000-ml. volumetric flask, dilute to volume with water, and mix thoroughly. Prepare this solution fresh daily.

Acetate Buffer. Transfer 60 grams of glacial acetic acid into a 1000-ml. volumetric flask, dilute to volume with water, and mix. With the aid of a suitable pH meter, adjust the pH of this solution to 4.2 by the addition of a sodium acetate solution prepared by dissolving 136 grams of $NaC_2H_3O_2.3H_2O$ in sufficient water to make 1000 ml. (*Note:* A pH of 5.0 should be used when testing preparations derived from *Aspergillus oryzae* or *Rhizopus oryzae*.)

Fehling's Solution A. Prepare the *Copper Solution* (*A*) as directed under *Cupric Tartrate T.S., Alkaline*, page 989. [This solution is quite commonly carefully standardized for use in determination of dextrose equivalent in the Lane and Eynon method. However, the concentration of the solution is not highly critical in the Schoorl method (specified later under *Determination of Reducing Sugars*), and adjustment of its concentration is usually unnecessary. The theoretical blank titer is 27.8 ml., but the solution may be considered to be satisfactory if the titer is between 27.5 and 29.5 ml.]

Fehling's Solution B. Prepare the *Alkaline Tartrate Solution* (*B*) as directed under *Cupric Tartrate T.S., Alkaline*, page 989.

Sample Preparation. The *Procedure* below is based on the use of a sample containing 0.1–0.2 unit of glucoamylase activity. This sample size will produce 0.2–0.4 gram of reducing sugars under the conditions specified, and maximum accuracy is obtained in this range. For slightly less accurate results, an enzyme dosage range of 0.05–3.0 units may be used if necessary.

Liquid samples, solid samples, and liquid concentrates should be

prepared as directed in the following tables and the aliquot size indicated should be used in the *Procedure* (*Production of Reducing Sugars*):

Liquid Samples

Enzyme in Sample, Units/ml.	Dilute	Aliquot Size, ml.	Dilution Factor (F)
0.05 or less	...	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 ml. → 100 ml.	1.00	20
4.1–5.0	4.0 ml. → 100 ml.	1.00	25
5.1–7.0	3.0 ml. → 100 ml.	1.00	33.3
7.1–10.0	2.0 ml. → 100 ml.	1.00	50

Solid Samples and Liquid Concentrates

Enzyme in Sample, Units/gram	Sample Wt., grams*	Dilute to (ml.)	Aliquot Size, ml.
4 or less	10	1000	5.0
5–10	4	1000	5.0
11–25	1.6	1000	5.0
26–50	1.4	1000	3.0
51–75	1.25	1000	2.0
76–100	1.00	1000	2.0
101–150	1.25	1000	1.0
151–200	1.00	1000	1.0
201–250	1.50	2000	1.0
251–300	1.00	2000	1.0

* Accurately weigh the sample into a volumetric flask, fill the flask two-thirds full of water, and allow the stoppered flask to stand at room temperature for at least 30 minutes, shaking vigorously at least 5 times during that period. Dilute to volume with water, and mix well. Take the indicated aliquot from a portion of the sample solution that has been filtered through Whatman No. 12 or equivalent filter paper.

Procedure

Production of Reducing Sugars. Pipet 50.0 ml. of the *Starch Hydrolyzate Solution* and 5.0 ml. of *Acetate Buffer* into a 100-ml. volumetric flask. Prepare a second flask in the same manner for use as the control, and carry this flask through the same procedure concurrently, but use water in place of the *Sample Preparation.* Place the flask in a water bath maintained at 60°, and allow to stand for at least 10 minutes. (*Note:* Use 55° when testing preparations derived from *Aspergillus oryzae* and *Rhizopus oryzae.*) Pipet an appropriately sized aliquot of the *Sample Preparation* into the flask, and simultaneously begin timing the reaction. (*Note:* If a series of samples is being analyzed, pipet aliquots at timed intervals, so spaced as to permit neutralization of each after 120 minutes of reaction time.) Swirl the contents of the flask to mix thoroughly, and allow to stand in the

water bath for 120 minutes. When 115–118 minutes of the reaction period has passed, add 3 drops of phenolphthalein T.S., then when exactly 120 minutes has elapsed, remove the flask from the bath and immediately neutralize the contents by the addition of 2% sodium hydroxide solution, preferably added with a fast-flowing buret (about 3–7 ml. is usually required). Cool to room temperature in a running-water bath, dilute to volume with water, and mix thoroughly. Determine the reducing sugars content on a 10.0-ml. aliquot of this solution, and on a 10.0-ml. aliquot of the control, as directed below.

Determination of Reducing Sugars (Schoorl Method). (*Note:* This method is suitable for determining reducing sugars in soluble materials that are substantially free of protein. Samples containing significant amounts of protein can be analyzed, however, after treatment with a protein precipitant.) Pipet 10.0 ml. each of *Fehling's Solution A* and *B* into a 250-ml. Erlenmeyer flask, and then add 10.0 ml. of the sample solution obtained under *Production of Reducing Sugars* above. Prepare a second flask in the same manner for use as the control, using 10.0 ml. of the control solution, instead of the sample solution, obtained under *Production of Reducing Sugars*, and carry this flask concurrently through the same procedure described for the sample. (*Note:* If large numbers of samples are to be analyzed, the sample solutions may be pipetted into a series of flasks first. Each sample may be diluted to 30 ml. with water, and the *Fehling's Solution A* may be added at any time; however, *Fehling's Solution B* must not be added until just before heating begins, since the reaction is initiated at room temperature as soon as the solution is added.) Pipet water into the flask to make a total volume of 50 ml., and mix the contents of the flask by gentle swirling. Add two small glass beads, and close the mouth of the flask with a small funnel or glass bulb. Heat the solution, preferably with a hot plate, at such a rate that the solution is brought to boiling in just 3 minutes, and then continue boiling for exactly 2 minutes (total heating time, 5 minutes). Cool quickly to room temperature in an ice bath or in cold running water, and then rinse down the funnel (or bulb) and the walls of the flask with a few ml. of water. Add 10 ml. each of 30% potassium iodide solution and of 28% sulfuric acid, and titrate rapidly with 0.100 *N* sodium thiosulfate until the iodine color almost disappears. Add 1 ml. of starch T.S., and titrate dropwise, with continuous agitation, to the disappearance of the blue color. Record the volume, in ml., of 0.100 *N* sodium thiosulfate required for the sample solution as *S*, and that required for the control solution as *C*. Conduct two reagent blank determinations, substituting 30 ml. of water for the sample, and record the average volume, in ml., of the blanks as *B*. Obtain the *Titer Difference*, expressed as ml. of 0.100 *N* sodium thiosulfate, for the sample by subtracting *S* from *B*, recording the value thus obtained as T_S. Subtract *C* from *B* to obtain the *Titer Difference* for the control, and record this value as T_C. (See footnote to the table that follows.)

Conversion of Titer Difference to Reducing Sugars Content*

Titer Difference, ml.	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
	Reducing Sugar (as Dextrose), mg.									
0.0	0.0	0.3	0.7	1.0	1.3	1.6	1.9	2.2	2.5	2.8
1.0	3.2	3.5	3.8	4.1	4.4	4.7	5.0	5.3	5.6	5.9
2.0	6.4	6.6	6.9	7.2	7.5	7.8	8.1	8.5	8.8	9.1
3.0	9.4	9.8	10.1	10.4	10.7	11.0	11.4	11.7	12.0	12.3
4.0	12.6	13.0	13.3	13.6	14.0	14.3	14.6	15.0	15.3	15.6
5.0	15.9	16.3	16.6	16.9	17.2	17.6	17.9	18.2	18.5	18.9
6.0	19.2	19.5	19.8	20.1	20.5	20.8	21.1	21.4	21.8	22.1
7.0	22.4	22.7	23.0	23.3	23.7	24.0	24.3	24.6	24.9	25.2
8.0	25.6	25.9	26.2	26.6	26.9	27.3	27.6	28.0	28.3	28.6
9.0	28.9	29.3	29.6	30.0	30.3	30.6	31.0	31.3	31.6	31.9
10.0	32.3	32.7	33.0	33.3	33.7	34.0	34.3	34.6	35.0	35.3
11.0	35.7	36.0	36.3	36.7	37.0	37.3	37.6	38.0	38.3	38.7
12.0	39.0	39.3	39.6	40.0	40.3	40.6	41.0	41.3	41.7	42.0
13.0	42.4	42.8	43.1	43.4	43.7	44.1	44.4	44.8	45.2	45.5
14.0	45.8	46.2	46.5	46.9	47.2	47.6	47.9	48.3	48.6	48.9
15.0	49.3	49.6	49.9	50.3	50.7	51.1	51.4	51.7	52.1	52.4
16.0	52.8	53.2	53.5	53.9	54.2	54.5	54.9	55.3	55.6	56.0
17.0	56.3	56.7	57.0	57.3	57.7	58.1	58.4	58.8	59.1	59.5
18.0	59.8	60.1	60.5	60.9	61.2	61.5	61.9	62.3	62.6	63.0
19.0	63.3	63.6	64.0	64.3	64.7	65.0	65.4	65.8	66.1	66.5
20.0	66.9	67.2	67.6	68.0	68.4	68.8	69.1	69.5	69.9	70.3
21.0	70.7	71.1	71.5	71.9	72.2	72.6	73.0	73.4	73.7	74.1
22.0	74.5	74.9	75.3	75.7	76.1	76.5	76.9	77.3	77.7	78.1
23.0	78.5	78.9	79.3	79.7	80.1	80.5	80.9	81.3	81.7	82.1
24.0	82.6	83.0	83.4	83.8	84.2	84.6	85.0	85.4	85.8	86.2
25.0	86.6	87.0	87.4	87.8	88.2	88.6	89.0	89.4	89.8	90.2
26.0	90.7	91.1	91.5	91.9	92.3	92.7	93.1	93.5	93.9	94.3
27.0	94.8									

* Use of this table presumes the ability of the analyst to duplicate exactly the conditions under which the data were developed. The risk of error can be avoided by careful duplicate standardization with known quantities of pure dextrose (5 samples, ranging from 10 to 70 mg.). A plot of *Titer Difference* vs. mg. of dextrose is slightly curvilinear, passing through the origin. If use of a standardization curve is adopted, the thiosulfate solution need not be standardized. Some additional increase in accuracy results from use of a 0.065 *N* sodium thiosulfate solution, which increases the blank titer to about 44–45 ml.

Calculation

Reducing Sugars Content. By reference to the accompanying table, entitled *Conversion of Titer Difference to Reducing Sugars Content*, determine the weight, in mg., of reducing sugars equivalent to the volume T_S, and record the value thus obtained as W_S. In a similar manner, determine the weight of reducing sugars equivalent to the volume T_C, and record this value as W_C.

Calculate the total reducing sugars (as dextrose) produced by the aliquot of *Sample Preparation* taken by the formula:

$$D_S, \text{ grams} = (W_S \times 100)/(1000 \times 10).$$

Calculate the total reducing sugars (as dextrose) produced by the control by the formula:

$$D_C, \text{ grams} = (W_C \times 100)/(1000 \times 10).$$

Enzyme Activity, Liquid Samples. Calculate the glucoamylase activity of the liquid enzyme preparation taken for analysis by the formula:

$$\text{Glucoamylase, units/ml.} = (D_S - D_C) \times (F/2 \text{ hr.}),$$

in which F is the dilution factor appropriate for the enzyme preparation analyzed (see table on *Liquid Samples* under *Sample Preparation*), or F is a factor appropriate to any adaptations used.

Enzyme Activity, Solid Samples and Liquid Concentrates. Calculate the glucoamylase activity of solid samples or liquid concentrates taken for analysis by the formula:

$$\text{Glucoamylase, units/gram} = (D_S - D_C) \times V/(G \times A \times 2 \text{ hr.}),$$

in which V is the dilution volume, in ml., and A is the aliquot size, in ml., appropriate for the enzyme preparation analyzed (see table on *Solid Samples and Liquid Concentrates* under *Sample Preparation*), and G is the weight, in grams, of the enzyme preparation taken for analysis.

GLUCOSE OXIDASE ACTIVITY

Application and Principle. This procedure is for the determination of glucose oxidase activity of preparations derived from *Aspergillus niger*, var. The assay is based upon oxygen uptake in the presence of excess substrate, excess air, and excess catalase.

Apparatus

Warburg Apparatus. Use the apparatus, or its equivalent, supplied as Catalog No. 666900, Precision Scientific Co., 3737 W. Cortland St., Chicago, Ill. 60647.

Manometer. Use the manometer, or its equivalent, supplied as Catalog No. 66665, Precision Scientific Co.

Reaction Flasks. Use the 15-ml. flasks, or their equivalent, supplied as Catalog No. 66703, Precision Scientific Co.

Buffered Dextrose Substrate. Dissolve 14.2 grams of anhydrous dibasic sodium phosphate in about 750 ml. of water. Dissolve 4.0 grams of sodium dehydroacetate (Ganes Chemical Works or equivalent) in this solution, and adjust the pH to 5.9 ± 0.05 with 85% phosphoric acid. Finally, dissolve 33.0 grams of dextrose monohydrate in the solution, dilute to 1000.0 ml. with water, and mix. To ensure mutarotation to equilibrium, hold overnight at room temperature.

Sample Solution. Weigh accurately a suitable amount of the enzyme preparation, and dilute it with water to a known volume to

obtain a solution containing between 10 and 20 glucose oxidase units per ml. [*Note:* If the enzyme preparation contains less than one Baker unit of catalase (see Scott, D., and Hammer, F., *Enzymologia* 22, 194, 1960), catalase must be added to meet or exceed the minimum ratio.] Transfer a 1.0-ml. aliquot into a 100-ml. volumetric flask, dilute rapidly to volume with the *Buffered Dextrose Substrate* (previously adjusted to a temperature of 25°), and mix. This solution may be unstable and should be used as soon as possible.

Procedure. Pipet 2.0-ml. portions of the *Sample Solution* into four calibrated *Reaction Flasks*, taking care that none of the solution is pipetted into the wells of the flasks. Using rubber bands or springs, secure each flask to a calibrated *Manometer*, and place the assembly in the water bath, maintained at 30° ± 0.01°, of the *Warburg Apparatus*. Open the manometer stopcocks leading to the flasks, and allow the manometers to oscillate, using a mechanical shaker, at a rate of 120 times per minutes, with a stroke of 4 cm., for 10 minutes in order to equilibrate the temperature of the flasks. After temperature equilibration has been reached, adjust the manometers to the initial volume for the respective reaction flasks. Close the stopcocks, and shake again for 30 minutes. Readjust the manometers to their original volumes, and note the change in pressure (P), in mm., for each flask.

Calculation. One unit of glucose oxidase activity is defined as that quantity of enzyme that will cause the uptake of 10 $mm.^3$ of oxygen per minute in a Warburg manometer at 30° in the presence of excess air and excess catalase, and with a substrate containing 3.3% glucose monohydrate and 0.1 M phosphate buffer at pH 5.9, with 0.4% sodium dehydroacetate [see Scott, D., *J. Agric. Food Chem.* 1, 727 (1953)]. Calculate the activity of the enzyme preparation by the formula:

$$\text{Glucose Oxidase Units, per gram or per ml.} = (P \times C \times D)/(30 \text{ min.} \times 10 \text{ mm}^3. \times V),$$

in which P is the pressure drop, in mm., observed in the reaction flask, corrected for thermobarometer change*; C is the reaction flask constant†; D is the dilution factor of the enzyme solution; and V is the volume, in ml., of *Sample Solution* used in the *Procedure*. Average the four values thus calculated to obtain the activity of the enzyme preparation taken for analysis.

INVERTASE ACTIVITY

Application and Principle. This procedure is for the determination of invertase activity of yeast enzymes. The assay is based on a 30-minute hydrolysis of sucrose at 20° and pH 4.5. The degree of

* Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques*, Third Edition, pp. 6–7, Burgess Publishing Co., Minneapolis, Minn. (1957).
† Umbreit *et al.*, *Ibid.*, pp. 61–63.

hydrolysis is determined by measuring the optical rotation of the solution with a polarimeter.

Reagents and Solutions

Phosphate Buffer Solution. Dissolve 115 grams of monobasic sodium phosphate ($NaH_2PO_4.H_2O$) in sufficient water to make 500.0 ml.

Sucrose Substrate Solution. Dissolve 100 grams of sucrose (such as Domino Superfine cane sugar, available in supermarkets) in 300 ml. of water, add 20.0 ml. of *Phosphate Buffer Solution*, dilute to volume in a 1000-ml. volumetric flask, and mix.

Neutral Lead Acetate Solution. Dissolve 31 grams of lead acetate ($C_4H_6PbO_4.3H_2O$) in 50 ml. of water, adjust to pH 7.0 with sodium hydroxide T.S., and dilute to 80 ml. in a graduate. Filter through Whatman No. 1, or equivalent, filter paper, and store the filtrate in a glass-stoppered bottle.

Sample Preparation. Prepare a solution of the sample so that 10 ml. of the final dilution will give a polarimeter reading, in a 2-dm. tube, between 0 and +20. For solid preparations, transfer an accurately weighed portion into a mortar, triturate with at least 5 times the sample weight of water, and dilute quantitatively and stepwise to the desired concentration. Liquid sample should be pipetted directly into a volumetric flask and diluted to volume with water.

Procedure

Transfer 100.0 ml. of the *Sucrose Substrate Solution* into a 100/110-ml. sugar flask, and equilibrate to 20° for 15 minutes in a water bath maintained at 20° ± 0.1°. At zero time, rapidly pipet 10.0 ml. of the *Sample Preparation* into the flask, and invert the flask 5 or 6 times to mix. Start the stopwatch at zero time. Return the flask to the water bath, and allow it to stand for 30.0 minutes. If a large amount of insoluble matter is present in the *Sample Preparation*, invert the incubation mixture in the flask every 10 minutes.

At the end of the incubation period, add about 2 grams of sodium carbonate ($Na_2CO_3.H_2O$), and swirl to dissolve. Check the pH of the solution with a pH meter, and make the solution alkaline if necessary by the addition of sodium carbonate.

Pipet 50.0 ml. of the enzyme digest into a 100-ml. volumetric flask, add 6 drops of the *Neutral Lead Acetate Solution*, dilute to volume with water, and mix. To the solution add 3 grams of a suitable filter aid, such as a cellulose-type flocculent, and filter through Whatman No. 1, or equivalent, filter paper, discarding the first 3 ml. of filtrate. The subsequent filtrate must be perfectly clear in order to be read on the polarimeter.

Prepare enzyme blanks containing 10 ml. of the *Sample Preparation* in 100 ml. of water, and treat the blanks in the same manner as the enzyme digest.

Rinse a 2-dm. polarimeter tube three times with the solution to be polarized, discard the washes, and fill the tube well up into the tubula-

ture. Place the filled tube in the polarimeter, insert a thermometer (with a range of 10–30° C., graduated in 0.1°), and allow the solution to achieve equilibration to 20°. For each solution, determine the reading five times, and then average the readings for each. Subtract the average of the blanks from the average of the sample to obtain the net reading for the sample.

Calculation

One invertase unit is defined as that quantity of enzyme that will hydrolyze 77% of the sucrose applied under the conditions of the assay.

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

By interpolation from the standard curve, determine the activity (A) of the *Sample Preparation*. For every degree above 20° C. at which the sample is read, subtract 0.004 from the activity, or add 0.004 per degree below 20° C.

Calculate the invertase activity (IA) of the enzyme preparation as follows:

$$\text{IA, units/gram} = (A \times 2 \times 1000)/W,$$

in which 2 is a dilution factor; 1000 converts mg. to grams; and W is the weight, in mg., of the enzyme sample added to the incubation mixture in the 10-ml. aliquot of *Sample Preparation* used. (*Note:* The dilution factor, 2, is not exact, but it is satisfactory for this determination.)

LACTASE (β-Galactosidase) ACTIVITY

Application and Principle. This procedure is for the determination of lactase activity of enzyme preparations derived from *Aspergillus niger*, var., and *Saccharomyces* sp. The assay is based on a 15-minute hydrolysis of an *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate at 37° and at the specified pH (4.5 for *Aspergillus niger*, var., and 6.5 for *Saccharomyces* sp.).

Reagents and Solutions

Acetate Buffer (for *Aspergillus niger*, var.). Pipet 50 ml. of 2 N acetic acid into about 800 ml. of water, and add 2 N sodium hydroxide

until the pH is 4.5 ± 0.05, determined by pH meter. Transfer the solution into a 1000-ml. volumetric flask, dilute to volume with water, and mix.

P–E–M Buffer (for *Saccharomyces* sp.). Dissolve 27.2 grams of anhydrous monobasic potassium phosphate, 37.2 mg. of disodium ethylenediaminetetraacetate dihydrate, and 20.3 mg. of manganese chloride hexahydrate in about 800 ml. of water, and add 2 *N* sodium hydroxide until the pH is 6.5 ± 0.05, determined by pH meter. Transfer the solution into a 1000-ml. volumetric flask, dilute to volume with water, and mix.

Standard o-Nitrophenol Solution. Transfer 139.0 mg. of *o*-nitrophenol into a 1000-ml. volumetric flask, dissolve in 10 ml. of 95% alcohol, dilute to volume with water, and mix. Pipet 2-, 4-, 6-, 8-, 10-, 12-, and 14-ml. portions of this solution into a series of 100-ml. volumetric flasks, dilute each to volume with 1% sodium bicarbonate solution, and mix. The dilutions contain, respectively, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, and 0.14 μmoles of *o*-nitrophenol per ml. Determine the absorbance of each dilution at 420 mμ in a 1-cm. cell, with a suitable spectrophotometer, using water as the blank, and for each dilution plot absorbance against μmoles of *o*-nitrophenol: a straight line through the origin must be obtained. Divide the absorbance of each dilution by μmoles of *o*-nitrophenol to obtain the extinction coefficient (ϵ) at that dilution, and then average the seven values thus calculated: a value close to 4.65 should be obtained.

Aspergillus Substrate. Transfer 370.0 mg. of *o*-nitrophenyl-β-D-galactopyranoside into a 100-ml. volumetric flask, dissolve in about 75 ml. of *Acetate Buffer*, dilute to volume with this same solvent, and mix.

Saccharomyces Substrate. Transfer 250.0 mg. of *o*-nitrophenyl-β-D-galactopyranoside into a 100-ml. volumetric flask, dissolve in about 75 ml. of *P–E–M Buffer*, dilute to volume with this same solvent, and mix.

Test Preparation. Prepare a solution from the enzyme preparation so that 1 ml. of the final dilution will contain between 0.15 and 0.65 Lactase Unit. Weigh the enzyme, transfer it into a glass mortar, and triturate with the appropriate *Buffer* solution. Quantitatively transfer the mixture into a volumetric flask of appropriate size, dilute to volume with the *Buffer* solution, and mix.

Procedure. Pipet 4-ml. portions of the appropriate *Substrate* into a suitable series of 25 × 150-mm. test tubes, stopper, and equilibrate them in a water bath maintained at 37° ± 0.1°. At zero time, rapidly pipet 1 ml. of the *Test Preparation* into the equilibrated substrate, and mix by swirling, starting the stopwatch at zero time. After 15.0 minutes' incubation time, pipet 1 ml. from each incubation mixture into separate test tubes containing 1 ml. of 10% sodium carbonate solution, then mix by swirling, dilute to 10 ml. with water, and mix again. Determine the absorbance of each solution at 420 mμ in a 1-cm. cell, with a suitable spectrophotometer, using as the blank a

solution prepared in the same manner as for the sample, substituting 1 ml. of water for the *Test Preparation.*

Calculation

One Lactase Unit (LU) is defined as that quantity of enzyme that will liberate 1 μmole of *o*-nitrophenol per minute under the conditions of the assay.

Calculate the activity of the enzyme preparation taken for analysis as follows:

$$\text{LU/gram} = (A \times 5 \times 10)/(\epsilon \times 15 \times W),$$

in which A is the average of the absorbance readings for the sample; 5 is the volume, in ml., of the incubation mixture; 10 is the final volume, in ml., of the diluted incubation mixture; ϵ is the extinction coefficient, determined as directed under *Standard o-Nitrophenol Solution;* 15 is the incubation time, in minutes; and W is the weight, in grams, of original enzyme preparation contained in the 1-ml. aliquot of *Test Preparation* used.

LIPASE (*Aspergillus oryzae*) ACTIVITY

Application and Principle. This procedure is for the determination of lipase activity of preparations derived from *Aspergillus oryzae*, var. The assay is based on a 5-minute hydrolysis of an olive oil substrate at pH 6.5 and 30°. The fatty acids released on hydrolysis of the glycerol esters are determined by titration with sodium hydroxide.

Reagents and Solutions

Sterol Extract. Use a suitable grade of multisterol extract containing sterols and higher alcohols in the free form only, such as Amerchol L-101, available from American Cholesterol Products, Edison, N.J. 08817.

Stock Buffer Solution. Dissolve 9.7 grams of sodium acetate ($NaC_2H_3O_2.3H_2O$) and 14.7 grams of U.S.P. sodium barbital in sufficient water to make 500 ml. Store this solution, which has a pH of 9.9, in a refrigerator.

Diluted Buffer Solution. Mix 40 ml. of *Stock Buffer Solution* with 16 ml. of 8.5% sodium chloride solution and 53 ml. of 0.1 *N* hydrochloric acid, dilute to 200 ml. with water, and mix. Adjust the pH of the final solution to 6.5, if necessary, with 0.1 *N* hydrochloric acid or 0.1 *N* sodium hydroxide. Store in a refrigerator until used, and discard if crystals appear during storage.

Substrate Emulsion. Stir 30 grams of gum arabic (acacia) and 270 ml. of water with a magnetic stirrer for 30 minutes at room temperature, breaking any lumps with a glass rod, and then cool to 5°. Transfer 6 grams of *Sterol Extract* into a 400-ml. beaker, add 72 grams of U.S.P. olive oil and 222 grams of the chilled gum arabic solution, mix with a glass rod, then pour into a homogenizing blender, and blend for

5 minutes. Adjust the pH to 6.3 with 0.5 *N* sodium hydroxide, cool to 5°, and blend again for 7 minutes. The temperature may have increased to 47°, and the pH to 6.5; if necessary, adjust the pH to 6.5 with 0.5 *N* sodium hydroxide. The emulsion is stable for at least 8 days when stored in a refrigerator. (*Caution:* Do not allow the suspension to freeze.)

Sample Preparation. Prepare a solution of the enzyme preparation so that 2 ml. of the final dilution will contain between 2 and 7 lipase units. Weigh or pipet the sample quantitatively, and use within 5 minutes of dilution.

Procedure

Mix 3 parts of the *Substrate Emulsion* with 1 part of the *Diluted Buffer Solution* (w/w), and transfer 8 ml. of the mixture into a 100-ml. beaker. Equilibrate to 30° by heating for 10 minutes in a water bath maintained at 30° ± 0.1°. At zero time, rapidly pipet 2.0 ml. of the *Sample Preparation* into the equilibrated substrate, starting the stopwatch at zero time. Mix with a glass rod, incubate for 5.0 minutes in the water bath, then add 40 ml. of alcohol (23A denatured alcohol is suitable), and immediately mix with the glass rod. Remove from the water bath, and titrate with 0.02 *N* sodium hydroxide from a 10-ml. microburet to a pH of 8.0, using a pH meter. Record the volume, in ml., of 0.02 *N* sodium hydroxide required as *S*.

Prepare a blank by mixing 8 ml. of the *Substrate Emulsion* with 40 ml. of alcohol and 2 ml. of the *Sample Preparation*, and titrate to pH 8.0 as directed above, recording the volume of 0.02 *N* sodium hydroxide required as *B*.

Calculation

One lipase unit (LU) is defined as that quantity of enzyme that will liberate the equivalent of 1 micromole of acid (H^+) per minute from the substrate, under the conditions of the assay.

Calculate the lipase activity (LA) of the sample, as the number of LU per gram of original preparation, as follows:

$$\text{LU/gram} = \frac{(K \times N \times 1000)}{(W \times 0.001 \times 5)},$$

in which *K* is the net titration value ($S - B$); *N* is the exact normality of the sodium hydroxide solution; 1000 converts millimoles of acid to micromoles; *W* is the weight, in mg., of the enzyme sample added to the incubation mixture in the 2-ml. aliquot of *Sample Preparation* used; 0.001 converts mg. to grams; and 5 is the reaction time, in minutes.

MILK-CLOTTING ACTIVITY

Application. This procedure is to be applied to enzyme preparations derived from either animal or microbial sources.

Apparatus

Bottle-rotating Apparatus. Use a suitable assembly, designed to rotate at a rate of 16 to 18 rpm, such as that available from Dries-Jacques Associates, 1804 East North Avenue, Milwaukee, Wisc. 53202.

Sample Bottles. Use 125-ml. squat, round, wide-mouth bottles such as those available as Catalog No. 2-903 from Fisher Scientific Co.

Substrate Solution. Dissolve 60 grams of low-heat, nonfat dry milk (such as Peake Grade A, available from Galloway West, Fond du Lac, Wisc. 54935) in 500 ml. of a solution, adjusted to pH 6.3 if necessary, containing in each ml. 2.05 mg. of sodium acetate ($NaC_2H_3O_2$) and 1.11 mg. of calcium chloride ($CaCl_2$).

Standard Preparation. Use a standard-strength rennet; bovine rennet; milk-clotting enzyme, microbial (*E. parasitica*); or milk-clotting enzyme, microbial (*Mucor* species) as appropriate for the preparation to be assayed. Such standards, which are available from commercial coagulant manufacturers, should be of known activity. Dilute the standard-strength material 1 to 200 with water, and mix. Equilibrate to 30° before use, and prepare no more than 2 hours prior to use.

Sample Preparation. Prepare aqueous solutions or dilutions of the sample to produce a final concentration such that the clotting time, as determined in the *Procedure* below, will be within 1 minute of that of the *Standard Preparation.* Prepare no more than 1 hour prior to use.

Procedure. Transfer 50.0 ml. of the *Substrate Solution* into each of four 125-ml. *Sample Bottles.* Place the bottles on the *Bottle-rotating Apparatus,* and suspend the apparatus in a water bath, maintained at 30° ± 0.5°, so that the bottles are at an angle of approximately 20–30° to the horizontal. Immerse the bottles so that the water level in the bath is about equal to the substrate level in the bottles. Begin rotating the apparatus at 16–18 rpm, then add 1.0 ml. of the *Sample Preparation* to each of two bottles, and record the exact time of addition. Add 1.0 ml. of the *Standard Preparation* to each of the other two bottles, recording the exact time. Observe the rotating bottles, and record the exact time of the first evidence of clotting (i.e., when fine granules or flecks adhere to the sides of the bottle). Variations in the response of different lots of the substrate may cause variations in clotting time; therefore, the test samples and standards should be measured simultaneously on the same substrate. Average the clotting time, in seconds, of the duplicate samples, recording the time for the *Standard Preparation* as T_S and that for the *Sample Preparation* as T_U.

Calculation. Calculate the activity of the enzyme preparation by the formula:

$$\text{Milk-clotting Units/ml.} = 100 \times (T_S/T_U) \times (D_S/D_U),$$

in which 100 is the activity assigned to the *Standard Preparation,* D_S is the dilution factor for the *Standard Preparation,* and D_U is the dilu-

tion factor for the *Sample Preparation.* (*Note:* The dilution factors should be expressed as fractions; e.g., a dilution of 1 to 200 would be expressed as 1/200).

PAPAIN

(Proteolytic Activity)

Application and Principle. This procedure is for the determination of the proteolytic activity of papain, ficin, and bromelain. The assay is based on a 60-minute proteolytic hydrolysis of a casein substrate at pH 6.0 and 40°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration; solubilized casein is then measured spectrophotometrically.

Reagents and Solutions

Sodium Phosphate Solution (0.05 *M*). Transfer 7.1 grams of anhydrous dibasic sodium phosphate into a 1000-ml. volumetric flask, dissolve in about 500 ml. of water, dilute to volume with water, and mix. Add 1 drop of toluene as preservative.

Citric Acid Solution (0.05 *M*). Transfer 10.5 grams of citric acid monohydrate into a 1000-ml. volumetric flask, dissolve in about 500 ml. of water, dilute to volume with water, and mix. Add 1 drop of toluene as preservative.

Phosphate-Cysteine-EDTA Buffer Solution. Dissolve 7.1 grams of anhydrous dibasic sodium phosphate in about 800 ml. of water, and then dissolve in this solution 14.0 grams of disodium ethylenediaminetetraacetate dihydrate and 6.1 grams of cysteine hydrochloride monohydrate. Adjust to pH 6.0 ± 0.1 with 1 *N* hydrochloric acid or 1 *N* sodium hydroxide, then transfer into a 1000-ml. volumetric flask, dilute to volume with water, and mix.

Trichloroacetic Acid Solution. Dissolve 30 grams of trichloroacetic acid in 100 ml. of water.

Casein Substrate Solution. Disperse 1 gram (moisture-free basis) of Hammersten casein in 50 ml. of *Sodium Phosphate Solution*, and heat for 30 minutes in a boiling water bath, with occasional agitation. Cool to room temperature, and, with rapid and continuous agitation, adjust to pH 6.0 ± 0.1 by the addition of *Citric Acid Solution.* (*Note:* Rapid and continuous agitation during the addition prevents casein precipitation.) Quantitatively transfer the mixture into a 100-ml. volumetric flask, dilute to volume with water, and mix.

Stock Standard Solution. Transfer 100.0 mg. of N.F. Papain Reference Standard into a 100-ml. volumetric flask, dissolve and dilute to volume with *Phosphate–Cysteine–EDTA Buffer Solution*, and mix.

Diluted Standard Solutions. Pipet 2, 3, 4, 5, 6, and 7 ml. of *Stock Standard Solution* into a series of 100-ml. volumetric flasks, dilute each to volume with *Phosphate-Cysteine-EDTA Buffer Solution*, and mix by inversion.

Test Solution. Prepare a solution from the enzyme preparation so that 2 ml. of the final dilution will give a ΔA in the *Procedure* between 0.2 and 0.5. Weigh the sample accurately, transfer it quantitatively to a glass mortar, and triturate with *Phosphate-Cysteine-EDTA Buffer Solution*. Transfer the mixture quantitatively into a volumetric flask of appropriate size, dilute to volume with *Phosphate-Cysteine-EDTA Buffer Solution*, and mix.

Procedure

Pipet 5 ml. of *Casein Substrate Solution* into each of a series of 25 × 150-mm. test tubes, allowing 3 tubes for the enzyme unknown, 6 for a papain standard curve, and 9 for enzyme blanks. Equilibrate the tubes for 15 minutes in a water bath maintained at 40° ± 0.1°. At zero time, rapidly pipet 2 ml. of each of the *Diluted Standard Solutions*, and 2-ml. portions of the *Test Solution*, into the equilibrated substrate, starting the stopwatch at zero time. Mix each by swirling, stopper, and place the tubes back in the water bath. After 60.0 minutes, add 3 ml. of *Trichloroacetic Acid Solution* to each tube. (*Caution:* Do not use mouth suction.) Mix each tube immediately by swirling.

Prepare enzyme blanks containing 5.0 ml. of *Casein Substrate Solution*, 3.0 ml. of *Trichloroacetic Acid Solution*, and 2.0 ml. of one of the appropriate *Diluted Standard Solution* or the *Test Solution*.

Return all tubes to the water bath, and heat for 30.0 minutes, allowing the precipitated protein to coagulate completely. Filter each mixture through Whatman No. 42, or equivalent, filter paper, discarding the first 3 ml. of filtrate. The subsequent filtrate must be perfectly clear. Determine the absorbance of each filtrate in a 1-cm. cell at 280 mμ, with a suitable spectrophotometer, against its respective blank.

Calculation

One N.F. papain unit (PU) is defined in this assay as that quantity of enzyme that liberates the equivalent of 1 mcg. of tyrosine per hour under the conditions of the assay.

Prepare a standard curve by plotting the absorbances of filtrates from the *Diluted Standard Solutions* against the corresponding enzyme concentrations, in mg./ml. By interpolation from the standard curve, obtain the equivalent concentration of the filtrate from the *Test Solution*.

Calculate the activity of the enzyme preparation taken for analysis as follows:

$$\text{N.F. PU/mg.} = (A \times C \times 10)/W,$$

in which A is the activity of N.F. Papain Reference Standard, in PU per mg.; C is the concentration, in mg. per ml., of Reference Standard from the standard curve, equivalent to the enzyme unknown; 10 is the total volume, in ml., of the final incubation mixture; and W is the weight, in mg., of original enzyme preparation in the 2-ml. aliquot of *Test Solution* added to the incubation mixture.

PEPSIN ACTIVITY

Application. This procedure is to be applied to preparations derived from porcine or other animal stomachs.

Measuring Vessels. Use 100-ml. conically-shaped measuring vessels complying with the following descriptions: (a) diameters not exceeding 1 cm. at the bottom; (b) comply in other respects with the water and sediment tube A.S.T.M. Standard Method D96-68; (c) graduated from 0 to 0.5 ml. in 0.05-ml. graduations, from 2 to 3 ml. in 0.1-ml. graduations, from 3 to 5 ml. in 0.2-ml. graduations, from 5 to 10 ml. in 1-ml. graduations, from 10 to 25 ml. in 5-ml. graduations, and with graduation marks at 50, 75, and 100 ml. (*Note:* Measuring vessels other than the type described herein may be used if they are of such design and graduation to permit measurement of the residue with equivalent accuracy.)

Reagents and Solutions

Hydrochloric Acid Solution. Mix 35 ml. of 1.0 *N* hydrochloric acid with 385 ml. of water.

Substrate. Boil one or more hen eggs for 15 minutes to provide coagulated albumen, and cool rapidly by immersion in cold water. Remove the shell and pellicle and all of the yolk, and at once rub the albumen through a clean, dry No. 40 sieve, rejecting the first portion that passes through the sieve.

Substrate Preparation. Place 10 grams of the *Substrate* in each of as many 100-ml. wide-mouth bottles as needed for the test, and immediately add 35 ml. of *Hydrochloric Acid Solution* (all at one time or in portions). By suitable means, thoroughly disintegrate the particles of albumen. Equilibrate to 52° before use in the *Procedure* below.

Standard Preparation. Dissolve 100 mg. of N.F. Pepsin Reference Standard in 150 ml. of *Hydrochloric Acid Solution.* Use this solution within 1 hour.

Sample Preparation. Dissolve 100 mg. of the sample pepsin, or an amount of the enzyme preparation that will provide a solution similar to or slightly stronger than the *Standard Preparation*, in 150 ml. of *Hydrochloric Acid Solution.* Use this solution within 1 hour.

Procedure. Pipet 5.0 ml. of the *Standard Preparation* into each of two bottles containing the *Substrate Preparation.* To two or more additional substrate bottles add graduated aliquots of the *Sample Preparation* so that one bottle will contain approximately the same amount, and the others will contain successively lesser amounts, of pepsin as is contained in the 5.0 ml. of the *Standard Preparation*, using, for example, 5.0, 4.9, and 4.8 ml. When less than 5.0 ml. of the *Sample Preparation* is used, add sufficient *Hydrochloric Acid Solution* to make 5.0 ml. of combined *Sample Preparation* plus acid added. At once stopper the bottles securely, invert them 3 times, and heat in a water bath, maintained at 52° ± 0.5°, for 2.5 hours, agitating the contents equally every 10 minutes by inverting the bottles once. Re-

move the bottles from the bath, and pour the contents of each into separate *Measuring Vessels.* Transfer the undigested albumen that adheres to the sides of the bottles into the respective *Measuring Vessel* with the aid of small portions of water until 50 ml. has been used for each. Mix the contents of each vessel, allow them to stand for 30 minutes, and then read for each the volume of undigested albumen. Average the sediment volumes in the two standard vessels, and note which of the sample vessels contains undigested albumen closest to the average for the standards. Finally, record as v the volume, in ml., of *Sample Preparation* that produced the undigested albumen closest to the average produced by the *Standard Preparations.*

Calculation. One Pepsin Unit is defined as that quantity of enzyme that digests 3,000 times its weight of coagulated egg albumen under the conditions of the assay.

Calculate the activity of the enzyme preparation by the formula:

$$\text{Pepsin Units/mg.} = 3000 \times (S/u) \times (5.0/v),$$

in which S is the weight, in mg., of N.F. Pepsin Reference Standard used to make the *Standard Preparation;* u is the weight, in mg., of enzyme preparation taken for analysis; and v is as defined in the *Procedure.*

PROTEASE ACTIVITY, BACTERIAL (PC)

Application and Principle. This procedure is for the determination of protease activity, expressed as PC units, of preparations derived from *Bacillus subtilis*, var. The assay is based on a 30-minute proteolytic hydrolysis of casein at 37° and pH 7.0. Unhydrolyzed casein is removed by filtration, and the solubilized casein is determined spectrophotometrically.

Reagents and Solutions

Casein. Use Hammersten grade casein, available from Nutritional Biochemical Corp., 21010 Miles Avenue, Cleveland, Ohio 44128.

Tris Buffer (pH 7.0). Dissolve 12.1 grams of enzyme-grade (or equivalent) tris(hydroxymethyl)aminomethane in 800 ml. of water, and titrate with 1 *N* hydrochloric acid to pH 7.0. Transfer into a 1000-ml. volumetric flask, dilute to volume with water, and mix.

TCA Solution. Dissolve 18 grams of trichloroacetic acid and 19 grams of sodium acetate trihydrate in 800 ml. of water in a 1000-ml. volumetric flask, add 20 ml. of glacial acetic acid, dilute to volume with water, and mix.

Substrate Solution. Dissolve 6.05 grams of enzyme-grade tris(hydroxymethyl)aminomethane in 500 ml. of water, add 8 ml. of 1 *N* hydrochloric acid, and mix. Dissolve 7 grams of *Casein* in this solution, and heat for 30 minutes in a boiling water bath, stirring occasionally.

Cool to room temperature, and adjust to pH 7.0 with 0.2 *N* hydrochloric acid, adding the acid slowly, with vigorous stirring, to prevent precipitation of the casein. Transfer the mixture into a 1000-ml. volumetric flask, dilute to volume with water, and mix.

Sample Preparation. Using *Tris Buffer*, prepare a solution of the sample enzyme preparation so that 2 ml. of the final dilution will contain between 10 and 44 PC units.

Procedure

Pipet 10.0 ml. of the *Substrate Solution* into each of a series of 25 × 150-mm. test tubes, allowing 1 tube for each enzyme test, 1 tube for each enzyme blank, and 1 tube for a substrate blank. Equilibrate the tubes for 15 minutes in a water bath maintained at 37° ± 0.1°.

At zero time, rapidly pipet 2.0 ml. of the *Sample Preparation* into the equilibrated substrate, starting the stopwatch at zero time. Mix, and replace the tubes in the water bath. Add 2 ml. of *Tris Buffer* (instead of the *Sample Preparation*) to the substrate blank. After exactly 30 minutes, add 10 ml. of *TCA Solution* to each enzyme incubation and to the substrate blank to stop the reaction. (*Caution:* Do not use mouth suction for the *TCA Solution.*) Heat the tubes in the water bath for an additional 30 minutes to allow the protein to coagulate completely.

At the end of the second heating period, shake each tube vigorously, and filter through 11-cm. Whatman No. 42, or equivalent, filter paper, discarding the first 3 ml. of filtrate. (*Note:* The filtrate must be perfectly clear.) Determine the absorbance of each sample filtrate in a 1-cm. cell, at 275 mμ, with a suitable spectrophotometer, using the filtrate from the substrate blank to set the instrument at zero. Correct each reading by subtracting the appropriate enzyme blank reading, and record the value so obtained as A_U.

Standard Curve. Transfer 100.0 mg. of L-tyrosine, chromatographic grade or equivalent (Calbiochem, La Jolla, Calif. 92037), previously dried to constant weight, to a 1000-ml. volumetric flask. Dissolve in 60 ml. of 0.1 *N* hydrochloric acid. When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 100 mcg. of tyrosine in 1.0 ml. Prepare 3 more dilutions from this stock solution to contain 75.0, 50.0 and 25.0 mcg. of tyrosine per ml. Determine the absorbance of the four solutions at 275 mμ in a 1-cm. cell on a suitable spectrophotometer vs. 0.006 *N* hydrochloric acid. Prepare a plot of absorbance vs. tyrosine concentration. Determine the slope of the curve in terms of absorbance per mcg. of tyrosine. Multiply this value by 1.10 and record it as A_S. A value of approximately 0.0084 should be obtained.

Calculation

One bacterial protease unit (PC) is defined as that quantity of enzyme that produces the equivalent of 1.5 mcg. per ml. of L-tyrosine per minute under the conditions of the assay.

From the *Standard Curve*, and by interpolation, determine the absorbance of a solution having a tyrosine concentration of 60 mcg. per ml. A figure close to 0.0115 should be obtained. Divide the interpolated value by 40 to obtain the absorbance equivalent to that of a solution having a tyrosine concentration of 1.5 mcg. per ml., and record the value thus derived as A_S.

Calculate the activity of the sample enzyme preparation by the formula:

$$\text{PC per gram} = (A_U/A_S) \times (22/30W),$$

in which 22 is the final volume, in ml., of the reaction mixture; 30 is the time of the reaction, in minutes; and W is the weight of the original sample taken, in grams.

PROTEOLYTIC ACTIVITY (HUT)

Application and Principle. This procedure is for the determination of the proteolytic activity, expressed as Hemoglobin Units on the Tyrosine Basis (HUT), of preparations derived from *Aspergillus oryzae*, var., and it may be used to determine the activity of other proteases at pH 4.7. The test is based on the 30-minute enzymatic hydrolysis of a hemoglobin substrate at pH 4.7 and 40°. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized hemoglobin in the filtrate is determined spectrophotometrically.

Reagents and Solutions

Hemoglobin. Use Hemoglobin Substrate Powder (Worthington Biochemical Corp., Freehold, N.J. 07728) or a similar, high-grade material that is completely soluble in water.

Acetate Buffer Solution. Dissolve 136 grams of sodium acetate ($NaC_2H_3O_2.3H_2O$) in sufficient water to make 500 ml. Mix 25.0 ml. of this solution with 50.0 ml. of 1 *M* acetic acid, dilute to 1000 ml. with water, and mix. The pH of this solution should be 4.7 ± 0.02.

Substrate Solution. Transfer 4.0 grams of the *Hemoglobin* into a 250-ml. beaker, add 100 ml. of water, and stir for 10 minutes to dissolve. Immerse the electrodes of a pH meter in the solution and adjust the pH to 1.7, stirring continuously, by the addition of 0.3 *N* HCl solution. After 10 minutes, adjust the pH to 4.7 by the addition of 0.5 *M* sodium acetate. Transfer the solution into a 200-ml. volumetric flask, dilute to volume with water, and mix. This solution is stable for about 5 days when refrigerated.

Trichloroacetic Acid Solution. Dissolve 140 grams of trichloroacetic acid in about 75 ml. of water. Transfer the solution to a 100-ml. volumetric flask, dilute to volume with water, and mix thoroughly.

Sample Preparation. Dissolve an amount of the sample in the *Acetate Buffer Solution* to produce a solution containing, in each ml.,

between 9 and 22 HUT. (Such a concentration will produce an absorbance reading, in the procedure below, within the preferred range of 0.2 to 0.5.)

Procedure. Pipet 10.0 ml. of the *Substrate Solution* into each of a series of 25 × 155-mm. test tubes: one for each enzyme test and one for the substrate blank. Heat the tubes in a water bath at 40° for about 5 minutes. To each tube except the substrate blank add 2.0 ml. of the *Sample Preparation*, and begin timing the reaction at the moment the solution is added; add 2.0 ml. of the *Acetate Buffer Solution* to the substrate blank tube. Close the tubes with No. 4 rubber stoppers, and tap each tube gently for 30 seconds against the palm of the hand to mix. Heat each tube in a water bath at 40° for exactly 30 minutes, and then pipet rapidly 10.0 ml. of the *Trichloroacetic Acid Solution* into each tube. (*Caution:* Do not use mouth suction on the pipet.) Shake each tube vigorously against the stopper for about 40 seconds, and then allow to cool to room temperature for 1 hour, shaking each tube against the stopper at 10- to 12-minute intervals during this period. Prepare enzyme blanks as follows: heat, in separate tubes, 10.0 ml. of the *Substrate Solution* and about 5 ml. of the *Sample Preparation* in the water bath for 30 minutes, then add 10.0 ml. of the *Trichloroacetic Acid Solution* to the *Substrate Solution*, shake well for 40 seconds, and to this mixture add 2.0 ml. of the pre-heated *Sample Preparation*. Shake again and cool at room temperature for 1 hour, shaking at 10- to 12-minute intervals.

At the end of 1 hour, shake each tube vigorously and filter through 11-cm. Whatman No. 42 or equivalent filter paper, refiltering the first half of the filtrate through the same paper. Determine the absorbance of each filtrate in a 1-cm. cell, at 275 mμ, with a suitable spectrophotometer, using the filtrate from the substrate blank to set the instrument to zero. Correct each reading by subtracting the appropriate enzyme blank reading, and record the value so obtained as A_U. (*Note:* If a corrected absorbance reading between 0.2 and 0.5 is not obtained, repeat the test using more or less of the enzyme preparation as necessary).

Standard Curve. Transfer 100.0 mg. of L-tyrosine, chromatographic grade or equivalent (Calbiochem, La Jolla, Calif. 92037), previously dried to constant weight, to a 1000-ml. volumetric flask. Dissolve in 60 ml. of 0.1 *N* hydrochloric acid. When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 100 mcg. of tyrosine in 1.0 ml. Prepare 3 more dilutions from this stock solution to contain 75.0, 50.0 and 25.0 mcg. of tyrosine per ml. Determine the absorbance of the four solutions at 275 mμ in a 1-cm. cell on a suitable spectrophotometer vs. 0.006 *N* hydrochloric acid. Prepare a plot of absorbance vs. tyrosine concentration. Determine the slope of the curve in terms of absorbance per mcg. of tyrosine. Multiply this value by 1.10 and record it as A_S. A value of approximately 0.0084 should be obtained.

Calculation. One HUT unit of proteolytic (protease) activity is defined as that amount of enzyme that produces, in 1 minute under the specified conditions, a hydrolysate whose absorbance at 275 mμ is the same as that of a solution containing 1.10 mcg. per ml. of tyrosine in 0.006 *N* hydrochloric acid.

Calculate the HUT per gram of the original enzyme preparation by the formula:

$$\text{HUT/gram} = (A_U/A_S) \times (22/30W),$$

in which 22 is the final volume of the test solution, 30 is the reaction time in minutes, and *W* is the weight of the original sample taken, in grams. (*Note:* The value for A_S, under carefully controlled and standardized conditions, is 0.0084; this value may be used for routine work in lieu of the value obtained from the standard curve, but the exact value calculated from the standard curve should be used for more accurate results and in cases of doubt.)

TRYPSIN ACTIVITY

Application. This procedure is for the determination of the trypsin activity of trypsin preparations derived from purified extracts of porcine or bovine pancreas.

Reagents and Solutions

Fifteenth molar phosphate buffer pH 7.6. Dissolve 4.54 grams of monobasic potassium phosphate in sufficient water to make 500 ml. of solution. Dissolve 4.73 grams of anhydrous dibasic sodium phosphate in sufficient water to make 500 ml. of solution. Mix 13 ml. of the monobasic potassium phosphate solution with 87 ml. of the anhydrous dibasic sodium phosphate solution.

Substrate Solution. Dissolve 85 7 mg. of *N*-benzoyl-L-arginine ethyl ester hydrochloride, suitable for use in assaying trypsin, in sufficient water to make 100 ml. (*Note:* Determine the suitability of the substrate and check the adjustment of the spectrophotometer by performing the assay using N.F. Trypsin Reference Standard.) Dilute 10.0 ml. of this solution to 100.0 ml. with *Fifteenth Molar Phosphate Buffer, pH 7.6.* Determine the absorbance of this solution at 253 mμ in a 1-cm. cell, with a suitable spectrophotometer, using water as the blank and maintaining the cell temperature at 25° ± 0.1°. Adjust the absorbance of the solution, if necessary, by the addition of *Fifteenth Molar Phosphate Buffer, pH 7.6* so that it measures not less than 0.575 and not more than 0.585. Use this solution within a period of 2 hours.

Sample Preparations. Dissolve a sufficient amount of sample, accurately weighed, in 0.001 *N* hydrochloric acid to produce a solution containing about 3000 N.F. Trypsin units in each ml. Prepare 3 dilutions using 0.001 *N* hydrochloric acid so that the final solutions

will contain 12, 18 and 24 N.F. Trypsin units in each 0.2 ml. Use these concentrations in the *Procedure* below.

Procedure. Conduct the test in a spectrophotometer equipped to maintain a temperature of 25° ± 0.1° in the cell compartment. The temperature in the reaction cell should be determined before and after the measurement of absorbance to assure that the temperature does not change by more than 0.5°.

Pipet 0.2 ml. of 0.001 *N* hydrochloric acid and 3.0 ml. of *Substrate Solution* into a 1-cm. cell. Place this cell in the spectrophotometer and adjust the instrument so that the absorbance will read 0.050 at 253 mμ. Pipet 0.2 ml. of the *Sample Preparation* containing 12 N.F. units into another 1-cm. cell. Add 3.0 ml. of *Substrate Solution*, and place the cell in the spectrophotometer. At the same time the *Substrate Solution* is added, start a stopwatch, and read the absorbance at 30-second intervals for 5 minutes. Repeat the procedure with each of the *Sample Preparations* containing 18 and 24 N.F. units. Plot curves of absorbance vs. time for each concentration, and use only those values that form a straight line to determine the activity of the trypsin. Discard the values on the plateau, and take the average of the results from the 3 concentration levels as the actual activity of the trypsin.

Calculations. One N.F. Trypsin unit is the activity causing a change in the absorbance of 0.003 per minute under the conditions specified in this assay.

Calculate the number of N.F. Trypsin units per mg. at each level by the formula $(A_1 - A_2)/(T \times W \times 0.003)$, in which A_1 is the absorbance straight line final reading; A_2 is the absorbance straight line initial reading; T is the elapsed time, in minutes, between the initial and final readings; and W is the weight, in mg., of trypsin in the volume of solution used in determining the absorbance.

Essential Oils and Related Substances, page 892

Add the following *Note* at the end of the paragraph entitled *Hydroxylamine Hydrochloride Solution*, in the *Aldehydes* test on page 894:

[*Note:* When determining aldehydes in citrus oils (Lemon Oil; Lime Oil, Distilled; Mandarin Oil; Orange Oil; Orange Oil, Bitter; Tangerine Oil, etc.), use the following solution: Dissolve 45 grams of hydroxylamine hydrochloride in 130 ml. of water, add 850 ml. of *tert*-butyl alcohol, mix, and adjust to a pH of 3.4 with 0.5 *N* aqueous potassium hydroxide.]

Fluoride Limit Test, page 917

In the seventh line of the paragraph entitled *Procedure* (*Method III*), page 920, change "1 *M* sodium citrate" to read:

. . . 1 *M* sodium hydroxide . . .

Heavy Metals Test, page 920

Insert the following at the end of the *Procedure* section on page 921:

Method III. [reserved]

Method IV

Solution A (*Control*). Transfer a mixture of 8 ml. of sulfuric acid and 10 ml. of nitric acid into a 100-ml. Kjeldahl flask, clamp the flask at an angle of 45°, and then add in small increments an additional volume of nitric acid equal to that added in the preparation of *Solution B* below. Heat the solution to dense, white fumes, cool, and cautiously add 10 ml. of water. Add a volume of 30 percent hydrogen peroxide equal to that added in the preparation of *Solution B* below, then boil gently to dense, white fumes, and cool. Cautiously add 5 ml. of water, mix, and boil gently to dense, white fumes. Continue boiling until the volume is reduced to about 2 or 3 ml., then cool, and dilute cautiously with a few ml. of water. Into this solution pipet a volume of *Standard Lead Solution* containing the amount of lead ion (Pb) specified in the individual monograph, and mix. Transfer into a 50-ml. Nessler tube, rinse the flask with water, adding the rinsings to the tube until the volume is 25 ml., and mix. Adjust the pH to between 3.0 and 4.0 with stronger ammonia T.S. initially, and with ammonia T.S. as the desired range is approached, then dilute to 40 ml. with water, and mix.

Solution B (*Test preparation*). Transfer into a 100-ml. Kjeldahl flask (or a 300-ml. flask if the reaction foams excessively) the amount of the sample specified in the individual monograph, clamp the flask at an angle of 45°, and then add sufficient of a mixture of 8 ml. of sulfuric acid and 10 ml. of nitric acid to moisten the sample thoroughly. (*Note:* For liquid samples use 3 ml. of the acid mixture.) Warm gently until the reaction commences, allow the reaction to subside, and then add additional portions of the acid mixture, heating after each addition, until all of the 18 ml. of acid mixture has been added. Increase the heat, and boil gently until the reaction mixture darkens. Remove the flask from the heat, add 2 ml. of nitric acid, and heat to boiling again. Continue the intermittent heating and addition of 2-ml. portions of nitric acid until no further darkening occurs, then heat strongly to dense, white fumes, and cool. Cautiously add 5 ml. of water, mix, boil

gently to dense, white fumes, and continue heating until the volume is reduced to about 2 or 3 ml. Cool, cautiously add 5 ml. of water, and examine. If the solution is colored yellow, cautiously add 1 ml. of 30 percent hydrogen peroxide and again evaporate to dense, white fumes and to a volume of about 2 or 3 ml. Cool, dilute cautiously with a few ml. of water, and mix. Transfer into a 50-ml. Nessler tube, rinse the flask with water, adding the rinsings to the tube until the volume is 25 ml., and mix. Adjust the pH to between 3.0 and 4.0 with stronger ammonia T.S. initially, and with ammonia T.S. as the desired range is approached, then dilute to 40 ml. with water, and mix.

Procedure. To each tube add 10 ml. of freshly prepared hydrogen sulfide T.S., mix, allow to stand for 5 minutes, and view downward over a white surface. The color of *Solution B* is no darker than that of *Solution A*.

[*Note:* As indicated in the discussion of *Test Procedures* on page xi of the *Preface to the Second Edition*, a continuing effort is being made by the Food Chemicals Codex to improve the *Heavy Metals* test procedure and the methods of sample preparation. *Method IV*, shown above, was developed through a collaborative study sponsored jointly by the Food Chemicals Codex and the Committee on Analytical Reagents, American Chemical Society. The major difference between *Method IV* and *Method II* (page 921) is the manner in which the sample is prepared for the test. While *Method IV* is believed to be an improvement over *Method II* for certain substances, not enough experience has been gained with the new method for it to be specified in Food Chemicals Codex monographs at this time. It is being included in THIS SUPPLEMENT, however, for those users of the Codex who might wish to determine its suitability as an alternate for *Method II*. Reports of experience with *Method IV* would be welcome and should be sent to: Food Chemicals Codex, National Academy of Sciences, 2101 Constitution Avenue, N.W., Washington, D.C. 20418.]

Melting Range, page 931

Change the second paragraph under the section entitled *Apparatus*, page 932, to read:

The thermometer is preferably one that conforms to the specifications provided under *Thermometers*, page 966, selected for the desired accuracy and range of temperature.

Oil Content of Synthetic Paraffin

Insert the following new General Tests chapter to precede the chapter entitled *Optical Rotation*, page 939:

OIL CONTENT OF SYNTHETIC PARAFFIN

Apparatus

Filter Stick. Use either a 10-mm. diameter sintered-glass filter stick of 10 to 15 μ maximum pore diameter, or a filter stick made of stainless steel and having a 0.5-inch disk of 10 to 15 μ maximum pore diameter. Determine conformance with the pore diameter specified as follows: Clean sintered-glass filter sticks by soaking in hydrochloric acid, or stainless steel sticks by soaking in nitric acid, wash with water, rinse with acetone, and dry in air followed by drying in an oven at 105° for 30 minutes.

Thoroughly wet the clean filter stick by soaking in water, and then connect it with an apparatus (see Fig. 25) consisting of a mercury-filled manometer, readable to 0.5 mm.; a clean and filtered air supply; a drying bulb filled with silica gel; and a needle-valve type air pressure regulator. Apply pressure slowly from the air source, and immerse the filter just below the surface of water contained in a beaker.

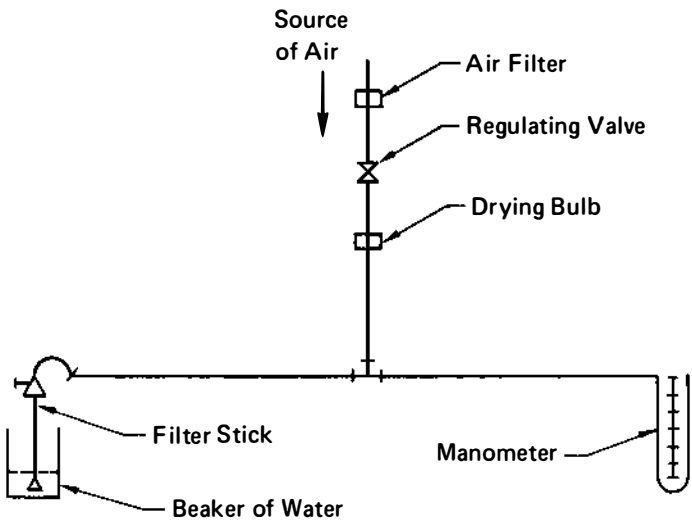


Fig. 25—Assembly for Checking Pore Diameter of Filter Sticks

(*Note:* If a head of liquid is noted above the surface of the filter after it is inserted into the water, the back-pressure thus produced should be subtracted from the observed pressure when the pore diameter is calculated as directed below.) Increase the air pressure to 10 mm. below the acceptable pressure limit, and then increase the pressure at a slow, uniform rate of about 3 mm. of mercury per minute until the first bubble passes through the filter. This can be conveniently observed by placing the beaker over a mirror. Read the manometer

when the first bubble passes off the underside of the filter. Calculate the pore diameter, in μ, by the formula $2180/p$, in which p is the observed pressure, in mm., corrected for any back-pressure as mentioned above.

Filtration Assembly. Connect the *Filter Stick* with an air pressure inlet tube and delivery nozzle and ground-glass joint to fit a 25 × 170-mm. test tube as shown in Fig. 26. If a stainless steel *Filter Stick* is used, make the connection to the test tube by means of a cork.

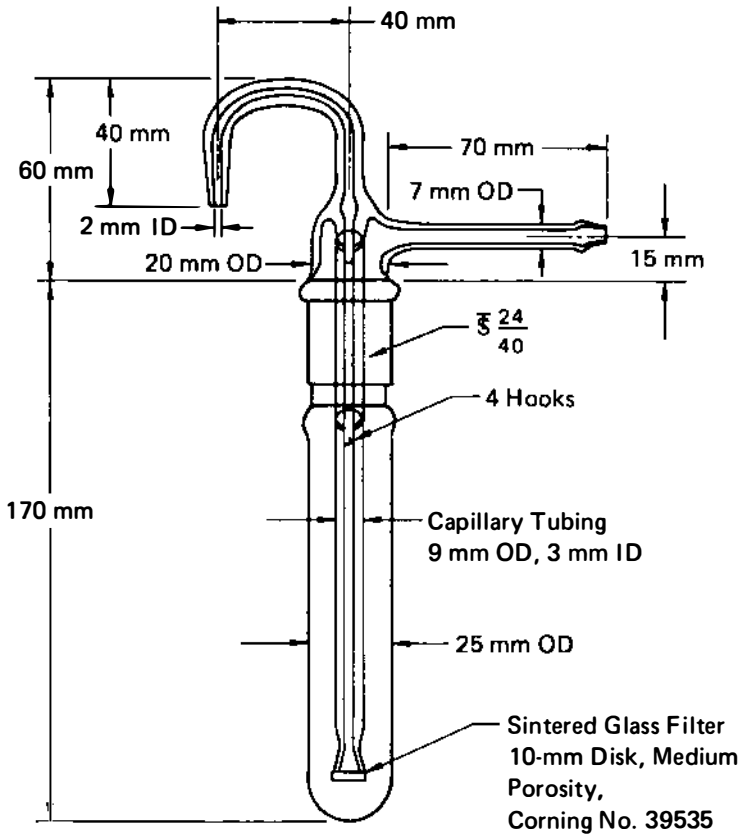


Fig. 26—Filtration Apparatus for Oil Content Determination

Cooling Bath. Use a suitable insulated box having 1-inch holes in the center to accommodate any desired number of test tubes. The bath may be filled with a suitable medium such as kerosine and may be cooled by circulating a refrigerant through coils, or by using solid carbon dioxide, to produce a temperature of $-30° \pm 2°$ F.

Air Pressure Regulator. Use a suitable pressure-reduction valve, or other suitable regulator, that will supply air to the *Filtration Assembly*

at the volume and pressure required to give an even flow of filtrate (see *Procedure*). Connect the regulator with rubber tubing to the end of the *Filter Stick* in the *Filtration Assembly*.

Thermometer. Use an A.S.T.M. Oil in Wax Thermometer having the range of −35° to +70° F. and conforming to the requirements for an A.S.T.M. 71F Thermometer (see page 103 of THIS SUPPLEMENT).

Weighing Bottles. Use glass-stoppered conical bottles having a capacity of 15 ml. The bottles are used as evaporating flasks in the *Procedure.*

Evaporation Assembly. The assembly consists of an evaporating cabinet capable of maintaining a temperature of 95° ± 2° F. around the evaporation flasks, and air jets (4 ± 0.2 mm. I.D.) for delivering a stream of clean, dry air vertically downward into the flasks. In the *Procedure* below, support each jet so that the tip is 15 ± 5 mm. above the surface of the liquid at the start of the evaporation. Supply the air (purified by passage through a tube of 1-cm. bore packed loosely to a height of 20 cm. with absorbent cotton) at the rate of 2 to 3 liters per minute per jet. The cleanliness of the air should be checked periodically to ensure that not more than 0.1 mg. of residue is obtained when 4 ml. of methyl ethyl ketone is evaporated as directed in the *Procedure.*

Wire Stirrer. Use a 250-mm. length of stiff iron or nichrome wire of about No. 20 B & S gauge. Form a 10-mm. diameter loop at each end, and bend the loop at the bottom end so that the plane of the loop is perpendicular to the length of the wire.

Sample Selection. If the sample is about 1 kg. or less, obtain a representative portion by melting the entire sample and stirring thoroughly. For samples greater than about 1 kg., exercise special care to ensure that a truly representative portion is obtained, noting that the oil may not be distributed uniformly throughout the sample and that mechanical operations may have expressed some of the oil.

Procedure. Melt a representative portion of the sample in a beaker, using a water bath or oven maintained at 160–210° F. As soon as the sample is completely melted, thoroughly mix it by stirring. Preheat a dropper pipet, provided with a rubber bulb and calibrated to deliver 1 ± 0.05 gram of molten sample, and withdraw a 1-gram portion of the sample as soon as possible after it has melted. Hold the pipet in a vertical position, and carefully transfer its contents into a clean, dry test tube previously weighed to the nearest mg. Evenly coat the bottom of the tube by swirling, allow the tube to cool, and weigh to the nearest mg. Calculate the sample weight, in grams, and record it as *B* (see *Calculation*). Pipet 15 ml. of methyl ethyl ketone (A.S.T.M. Specification D 740, or equivalent) into the tube, and immerse the tube up to the top of the liquid in a hot water or steam bath. Stir with an up-and-down motion with the *Wire Stirrer*, and continue heating and stirring until a homogeneous solution is ob-

tained, exercising care to avoid loss of solvent by prolonged boiling. (*Note:* If it appears that a clear solution will not be obtained, stir until any undissolved material is well dispersed so as to produce a slightly cloudy solution.)

After the sample solution is prepared, plunge the test tube into an 800-ml. beaker of ice water, and continue to stir until the contents are cold. Remove the stirrer, then remove the test tube from the bath, dry the outside of the tube with a cloth, and weigh to the nearest 100 mg. Calculate the weight, in grams, of solvent in the test tube, and record it as *C* (see *Calculation*). Place the tube in the *Cooling Bath*, maintained at −30° ± 2° F., and stir continuously with the *Thermometer* until the temperature reaches −25° ± 0.5° F., maintaining the slurry at a uniform consistency and taking precautions to prevent the sample from setting up on the walls of the tube or forming crystals.

Place the *Filter Stick* in a test tube and cool at −30° ± 2° F. in the *Cooling Bath* for a minimum of 10 minutes. Immerse the cooled *Filter Stick* in the sample, then connect the *Filtration Assembly*, seating the ground-glass joint of the filter so as to make an air-tight seal. Place an unstoppered *Weighing Bottle*, previously weighed together with the glass stopper to the nearest 0.1 mg., under the delivery nozzle of the *Filtration Assembly*. (*Note:* Suitable precautions and proper analytical technique should be applied to ensure the accuracy of the weight of the bottle. Prior to determining its weight, the bottle and its stopper should have been cleaned and dried, then rinsed with methyl ethyl ketone, wiped dry on the outside, dried in the *Evaporation Assembly* for about 5 minutes, and cooled. Then allow it to stand for about 10 minutes near the balance before weighing.)

Apply air pressure to the *Filtration Assembly*, immediately collect about 4 ml. of filtrate in the *Weighing Bottle*, and release the air pressure to permit the liquid to drain back slowly from the delivery nozzle. Stopper the bottle, and weigh it to the nearest 10 mg. without waiting for it to come to room temperature. Remove the stopper, transfer the bottle to the *Evaporation Assembly* maintained at 95° ± 2° F., and place it under an air jet centered inside the neck, with the tip 15 ± 5 mm. above the surface of the liquid. After the solvent has evaporated (usually less than 30 minutes time), stopper the bottle, and allow it to stand near the balance for about 10 minutes before it is weighed to the nearest 0.1 mg. Repeat the evaporation procedure for 5-minute periods until the loss between successive weighings is not more than 0.2 mg. Determine the weight of solvent evaporated, in grams, by subtracting the weight of the bottle plus oil residue from the weight of the bottle plus filtrate, and record the result as *D* (see *Calculation*).

Calculation. Calculate the percent, by weight, of oil in the sample by the formula $(100AC/BD) - 0.15$, in which *A*, *B*, *C*, and *D* are as indicated in the *Procedure*, and 0.15 is a factor to correct for solubility of the sample in the solvent at −25° F.

Oxygen Flask Combustion

Insert the following new General Tests chapter to precede the chapter entitled *pH Determination*, page 941:

OXYGEN FLASK COMBUSTION

Apparatus. The apparatus consists of a heavy-walled, deeply lipped or cupped, conical flask of a volume suitable for the complete combustion of the sample in which the particular element is being determined (e.g., see *Selenium Limit Test, Method I*, page 102 of THIS SUPPLEMENT). The flask is fitted with a round-glass stopper to which is fused a sample carrier consisting of heavy-gauge platinum wire and a piece of welded platinum gauze measuring about 1.5 × 2 cm. A suitable apparatus may be obtained as Catalog Nos. 6513-C20 (500-ml. capacity) and 6513-C30 (1000-ml. capacity) from Arthur H. Thomas Co., P.O. Box 779, Philadelphia, Pa. 19105. Equivalent apparatus available from other sources, or other suitable apparatus embodying the principles described herein, may also be used.

Procedure. (*Caution:* The analyst should wear safety glasses and should use a suitable safety shield between himself and the apparatus. Further safety measures should be observed as necessary to ensure maximum protection of the analyst. Furthermore, the flask must be scrupulously clean and free from even traces of organic solvents. Samples containing water of hydration or more than 1 percent of moisture should be dried at 140° for 2 hours before combustion.)

Accurately weigh the amount of sample specified in the monograph or general test. Solids should be weighed on a 4-cm. square piece of halide-free filter paper, which should be folded around the sample. Liquid samples not exceeding 0.2 ml. in volume should be weighed in tared cellulose acetate capsules [available as Catalog Nos. 6513-C80 (100 capsules) and 6513-C82 (1000 capsules) from the Arthur H. Thomas Co.]; gelatin capsules are satisfactory for liquid samples exceeding 0.2 ml. in volume. (*Note:* Gelatin capsules may contain significant amounts of combined halide or sulfur, in which case a blank determination should be made as necessary.) Place the sample, together with a filter paper fuse-strip, in the platinum gauze sample holder. Place the absorbing liquid, as specified in the individual monograph or general test, in the flask, moisten the joint of the stopper with water, and flush the air from the flask with a stream of rapidly flowing oxygen, swirling the liquid to facilitate its taking up oxygen. (*Note:* Saturation of the liquid with oxygen is essential for successful performance of this procedure.) Ignite the fuse-strip by suitable means. If the strip is ignited outside the flask, immediately plunge

the sample holder into the flask, invert the flask so that the absorption solution makes a seal around the stopper, and hold the stopper firmly in place. If the ignition is carried out in a closed system, the inversion of the flask may be omitted. After combustion is complete, shake the flask vigorously, and allow to stand for not less than 10 minutes with intermittent shaking. Then continue as directed in the individual monograph or general test chapter.

Selenium Limit Test, page 953

Replace the entire chapter with the following:

SELENIUM LIMIT TEST

2,3-Diaminonaphthalene Solution. On the day of use, dissolve 100 mg. of 2,3-diaminonaphthalene ($C_{10}H_{10}N_2$) and 500 mg. of hydroxylamine hydrochloride ($NH_2OH.HCl$) in sufficient 0.1 *N* hydrochloric acid to make 100 ml.

Selenium Stock Solution. Transfer 120.0 mg. of powdered metallic selenium into a 1000-ml. volumetric flask, and dissolve in 100 ml. of dilute nitric acid (1 in 2), warming gently on a steam bath to effect solution. Cool, dilute to volume with water, and mix. Transfer 5.0 ml. of this solution into a 200-ml. volumetric flask, dilute to volume with water, and mix. Each ml. of this solution contains 3 mcg. of selenium (Se).

Method I

Standard Preparation. Transfer 2.0 ml. of the *Selenium Stock Solution* into a 150-ml. beaker, add 50 ml. of 0.25 *N* nitric acid, and mix.

Sample Preparation. Transfer into a 1000-ml. combustion flask the amount of sample specified in the individual monograph (and the magnesium oxide, where required), and proceed as directed under *Oxygen Flask Combustion,* page 101 of THIS SUPPLEMENT, using 0.5 *N* nitric acid as the absorbing liquid. (*Note:* If the sample contains water of hydration or more than 1 percent of moisture, dry it at 140° for 2 hours before combustion.) Upon completion of combustion, place a few ml. of water in the cup or lip of the combustion flask, loosen the stopper of the flask, and rinse the stopper, sample holder, and sides of the flask with about 25 ml. of water. Transfer the solution into a 150-ml. beaker, heat gently to boiling, boil for 10 minutes, and cool.

Procedure. Using dilute ammonium hydroxide (1 in 2), adjust the pH of the *Standard Preparation,* of the *Sample Preparation,* and of 50 ml. of 0.25 *N* nitric acid, to serve as the blank, to 2 ± 0.2. Add 200

mg. of hydroxylamine hydrochloride to each beaker, swirl gently to dissolve, then without delay add 5 ml. of 2,3-*Diaminonaphthalene Solution* to each solution, and mix. Cover each beaker with a watch glass, and allow to stand at room temperature for 100 minutes. Transfer the solutions into separate separators with the aid of about 10 ml. of water, extract each solution with 5.0 ml. of cyclohexane, shaking each separator vigorously for 2 minutes, and allow the layers to separate. Discard the aqueous phases, and centrifuge the cyclohexane extracts to remove any traces of water. Determine the absorbance of each extract in a 1-cm. cell at the maximum at about 380 mμ, with a suitable spectrophotometer, using the blank to set the instrument. The absorbance of the extract from the *Sample Preparation* is not greater than that from the *Standard Preparation* when a 200-mg. sample is tested, or not greater than one-half the absorbance of the extract from the *Standard Preparation* when a 100-mg. sample is tested.

Method II

Standard Preparation. Transfer 2.0 ml. of the *Selenium Stock Solution* into a 150-ml. beaker, add 50 ml. of 2 *N* hydrochloric acid, and mix.

Sample Preparation. Transfer into a 150-ml. beaker the amount of sample specified in the individual monograph, dissolve in 25 ml. of 4 *N* hydrochloric acid, swirling if necessary to effect solution, heat gently to boiling, and digest on a steam bath for 15 minutes. Remove from heat, add 25 ml. of water, and allow to cool to room temperature.

Procedure. Place the beakers containing the *Standard Preparation* and the *Sample Preparation* in a fume hood. Cautiously add 5 ml. of ammonium hydroxide to each beaker and to a third beaker containing 2 *N* hydrochloric acid to serve as the blank. Allow the solutions to cool, and then adjust the pH of each solution to 2 ± 0.2 with dilute ammonium hydroxide (1 in 2). Continue as directed under *Procedure* in *Method I*, beginning with "Add 200 mg. of hydroxylamine hydrochloride . . . ."

Thermometers, page 966

In the table entitled *Thermometer Specifications*, add the following thermometers *For Special Use*, page 967:

A.S.T.M. No. E-	Range °C.	Range °F.	Subdivisions °C.	Subdivisions °F.	Immersion, mm.
54F[5]	. . . .	68 to 213	. . . .	0.5	Total
71F[6]	. . . .	−35 to +70	. . . .	1	76

[5] For determination of congealing point.
[6] For determination of oil in wax.

TEST SOLUTIONS

Insert the following new test solution to precede *Crystal Violet T.S.*, page 989:

Cupric Nitrate T.S. Dissolve 2.4 grams of cupric nitrate, $Cu(NO_3)_2.3H_2O$, in sufficient water to make 100 ml.

Insert the following new test solution to precede *α,α'-Dipyridyl T.S.*, page 989:

Diphenylcarbazone T.S. Dissolve about 1 gram of diphenylcarbazone ($C_{13}H_{12}N_4O$) in sufficient alcohol to make 100 ml. Store this solution in a brown bottle.

Insert the following new test solution to precede *Ferrous Sulfate T.S.*, page 990:

Ferric Sulfate T.S., Acid. Add 7.5 ml. of sulfuric acid to 100 ml. of water, and dissolve 80 grams of ferrous sulfate in the mixture with the aid of heat. Mix 7.5 ml. of nitric acid and 20 ml. of water, warm, and add to this the ferrous sulfate solution. Concentrate the mixture until, upon the sudden disengagement of ruddy vapors, the black color of the liquid changes to red. Test for the absence of ferrous iron and, if necessary, add a few drops of nitric acid and boil again. When the solution is cold, add sufficient water to make 110 ml.

VOLUMETRIC SOLUTIONS

Insert the following new volumetric solution to precede *Oxalic Acid, 0.1 N*, page 999:

Mercuric Nitrate, 0.1 M [32.46 grams $Hg(NO_3)_2$ per liter]. Dissolve about 35 grams of mercuric nitrate, $Hg(NO_3)_2.H_2O$, in a mixture of 5 ml. of nitric acid and 500 ml. of water, and dilute with water to 1000 ml. Standardize the solution as follows: Transfer an accurately measured volume of about 20 ml. of the solution into an Erlenmeyer flask, and add 2 ml. of nitric acid and 2 ml. of ferric ammonium sulfate T.S. Cool to below 20°, and titrate with 0.1 *N* ammonium thiocyanate to the first appearance of a permanent brownish color. Calculate the molarity.

INDICATOR PAPERS AND TEST PAPERS

Insert the following new test paper to precede *Cupric Sulfate Test Paper*, page 1007:

Acetaldehyde Test Paper. Use a solution prepared by mixing equal volumes of a 20 percent solution of morpholine and a 5 percent solution of sodium nitroferricyanide. Saturate the prepared filter paper in the mixture, and use the moistened paper without drying.

* * * * * * *

Individuals who wish to cut apart the corrections and additions in this First Supplement and paste them in the main volume of the *Food Chemicals Codex Second Edition* will need both sides of each page. Additional copies of the First Supplement are available at no charge and may be secured on request to:

Printing and Publishing Office
National Academy of Sciences
2101 Constitution Avenue, N.W.
Washington, D.C. 20418