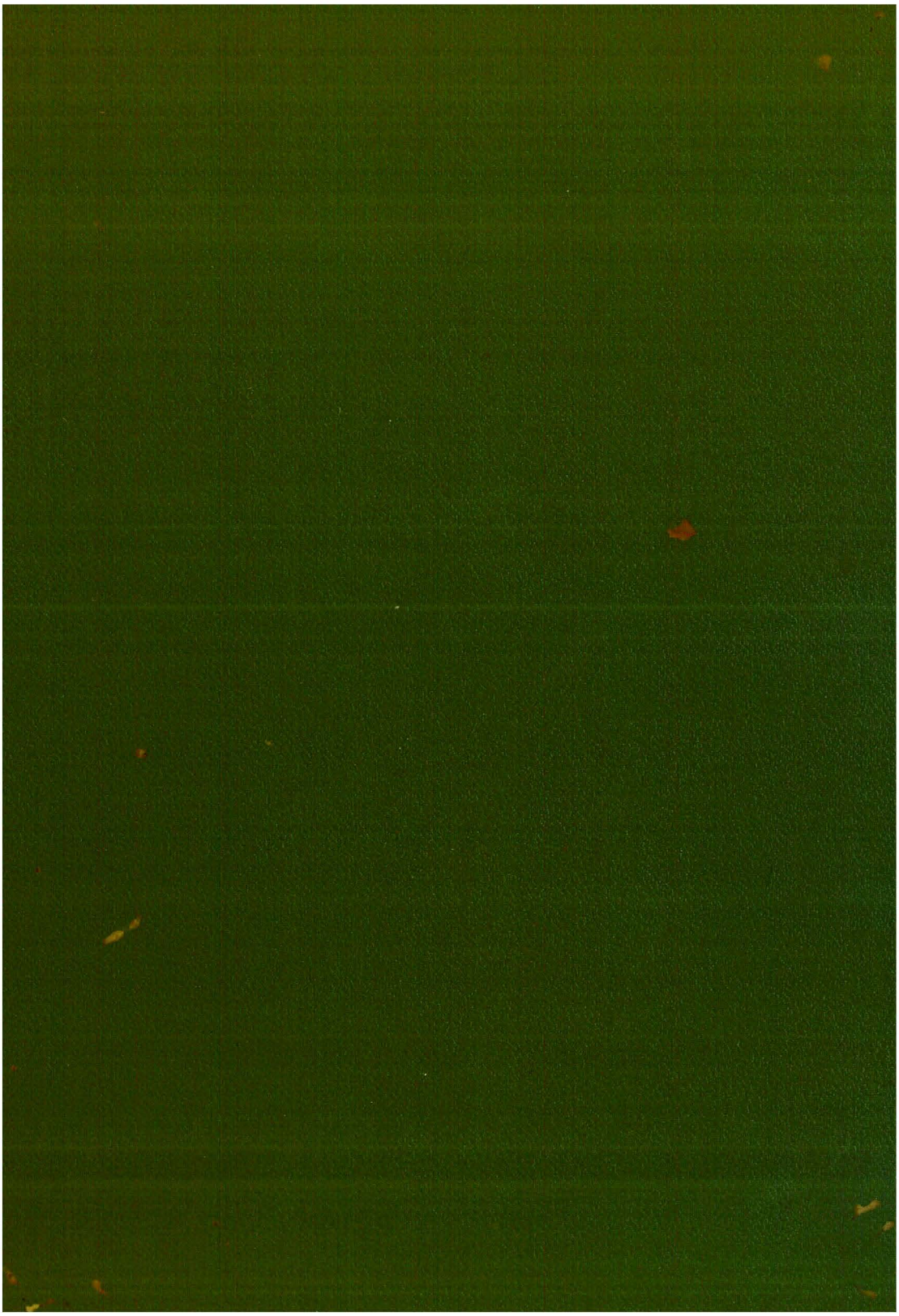

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TUESDAY—AFERNOON SESSION

PROCEEDINGS OF THE SIXTY-FOURTH ANNUAL MEETING OF THE ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS, 1950

The sixty-fourth annual meeting of the Association of Official Agricultural Chemists was held at the Shoreham Hotel, Washington, D. C., October 2, 3, and 4, 1950.

The meeting was called to order by the President, W. A. Queen, on the morning of October 2, at 10:00 o'clock.

OFFICERS, COMMITTEES, REFEREES, AND ASSOCIATE REFEREES OF THE ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS FOR THE YEAR ENDING NOVEMBER 1951

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F. H. WILEY (1952)
J. B. SMITH (1953)
A. H. ROBERTSON (1955)
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W. F. REINDOLLAR (Bureau of Chemistry, State Department of Health, Baltimore 18, Md.), *Chairman*

SUBJECTS, REFEREES, AND ASSOCIATE REFEREES†

SUBCOMMITTEE A:

E. L. GRIFFIN (1952) *Chairman;* J. B. SMITH (1954); and C. W. MARSHALL (1956).

FEEDING STUFFS:

Referee: M. P. Etheredge, Mississippi State College, State College, Miss.
MINERAL MIXED FEEDS (CALCIUM AND IODINE):
A. T. Perkins, Kansas State College, Manhattan, Kans.
LACTOSE IN MIXED FEEDS:
C. W. Sievert, American Dry Milk Institute, Chicago 1, Ill.
FAT IN FISH MEAL:
Maurice E. Stansby, Fish and Wildlife Service, Seattle 2, Wash.
CRUDE FAT OR ETHER EXTRACT:
H. H. Hoffman, Department of Agriculture, Tallahassee, Fla.
MICROSCOPIC EXAMINATION:
J. A. Schrader, Agricultural Experiment Sta., Lexington 29, Ky.
MINERAL CONSTITUENTS OF MIXED FEEDS:
J. C. Edwards, Department of Agriculture, Tallahassee, Fla.
PROTEIN EVALUATION IN FISH AND ANIMAL PRODUCTS:
Frank J. Kokoski, N. Y. Dept. Agriculture and Markets, Albany, N. Y.
TANKAGE (HIDE, HOOF, HORN, AND HAIR CONTENT):
A. T. Perkins
SULFA DRUGS IN FEEDS:
R. T. Merwin, Agricultural Experiment Station, New Haven, Conn.

FERTILIZERS:

Referee: F. W. Quackenbush, Agricultural Experiment Station, Lafayette, Ind.
SAMPLING AND PREPARATION OF SAMPLE:
H. R. Allen, Agricultural Experiment Station, Lexington 29, Ky.
PHOSPHORIC ACID:
K. D. Jacob, Plant Industry Station, Beltsville, Md.
FREE WATER:
W. L. Hill, Plant Industry Station, Beltsville, Md.
NITROGEN:
H. A. Davis, Agricultural Experiment Station, Durham, N. H.

† Referees appointed during the year for unassigned subjects will be announced in *The Journal.*

MAGNESIUM AND MANGANESE:
John B. Smith, Agricultural Experiment Station, Kingston, R. I.
ACID- AND BASE-FORMING QUALITY

POTASH:
O. W. Ford, Purdue University, Lafayette, Ind.
SULFUR:
Gordon Hart, Department of Agriculture, Tallahassee, Fla.
COPPER AND ZINC:
H. J. Webb, A. and M. College of South Carolina, Clemson, S. C.
BORON:

INERT MATERIALS:
K. G. Clarke, Division of Fertilizer and Agricultural Lime, Beltsville, Md.

ECONOMIC POISONS:

Referee: J. J. T. Graham, Production and Marketing Administration, Insecticide Division, Washington 25, D. C.
HERBICIDES:
A. B. Heagy, Md. Inspection and Regulatory Service, College Park, Md.
RODENTICIDES:
J. B. La Clair, Department of Agriculture, Sacramento 14, Calif.
BENZENEHEXACHLORIDE:
C. V. Bowen, Bur. Entomology and Plant Quarantine, Beltsville, Md.
ORGANIC THIOCYANATES:
H. A. Rooney, Department of Agriculture, Sacramento 14, Calif.
DIMETHYL DITHIO CARBAMATES:
J. D. Patterson, Department of Agriculture, Salem, Oreg.
DDT:
E. E. Fleck, Bur. Entomology and Plant Quarantine, Beltsville, Md.
PARATHION:
F. I. Edwards, Bur. Entomology and Plant Quarantine, Beltsville, Md.
CHLORDANE AND TOXAPHENE:
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PYRETHRINS:
D. Kelsey, Production and Marketing Adm., Insecticide Div., Beltsville, Md.
PIPERONYL BUTOXIDE:
B. L. Samuel, Div. Chemistry, Dept. of Agriculture, Richmond, Va.
ROTENONE:
R. Payfer, Plant Products Div., Dept. of Agriculture, Ottawa, Canada.
ALLETHRIN:

QUATERNARY AMMONIUM COMPOUNDS:
R. L. Caswell, Production and Marketing Adm., Insecticide Div., Beltsville, Md.
PHENOLIC DISINFECTANTS:
W. A. Affens, Production and Marketing Adm., Insecticide Div., Beltsville, Md.

DISINFECTANTS:

Referee: L. S. Stuart, Production and Marketing Adm., Insecticide Div., Beltsville, Md.

MEDIA FOR DISINFECTANT TESTING:

Michael J. Pelzar, Dept. of Bacteriology, Univ. of Md., College Park, Md.

PLANTS:

Referee: E. J. Miller, Agricultural Experiment Station, East Lansing, Mich.

SAMPLING:

E. J. Miller

IODINE AND BORON:

L. K. Wood, Agricultural Experiment Station, Lexington 29, Ky.

SUGAR:

Kenneth T. Williams, Western Regional Research Lab., Albany, Calif.

ZINC:

E. J. Benne, Agricultural Experiment Station, East Lansing, Mich.

COPPER AND COBALT:

Kenneth C. Beeson, U. S. Plant, Soil, and Nutritional Lab., Ithaca, N. Y.

CAROTENE:

E. J. Benne

SODIUM:

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STARCH:

Carroll L. Hoffpauir, Southern Regional Research Lab., New Orleans, La.

SOILS AND LIMING MATERIALS:

Referee: W. H. MacIntire, Agricultural Experiment Station, Knoxville 16, Tenn.

HYDROGEN-ION CONCENTRATION OF SOILS:

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BORON

L. K. Wood

FLUORINE:

L. J. Hardin, Agr. Expt. Station, Knoxville, Tenn.

ZINC AND COPPER:

W. L. Lott, U. S. Bur. Plant Industry, Soils, and Agr. Eng., Raleigh, N. C.

EXCHANGEABLE CALCIUM AND MAGNESIUM:

W. M. Shaw, Agricultural Experiment Station, Knoxville 16, Tenn.

EXCHANGEABLE HYDROGEN:

W. M. Shaw

EXCHANGEABLE POTASSIUM:

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PHOSPHORUS:

L. E. Ensminger, Alabama Experiment Station, Auburn, Ala.

MOLYBDENUM:

W. O. Robinson, Bur. Plant Industry, Beltsville, Md.

STANDARD SOLUTIONS:

Referee: H. G. Underwood, Food and Drug Administration, Washington, D.C.

VITAMINS:

Referee: Chester D. Tolle, Food and Drug Administration, Washington 25, D. C.

VITAMIN A IN OLEOMARGARINE:

J. B. Wilkie, Food and Drug Administration, Washington 25, D. C.

VITAMIN A IN FEEDS:

Maxwell L. Cooley, General Mills, Inc., Larrowe Div., Rossford, Ohio

VITAMIN B_6 (CHEMICAL):

W. L. Hall, Food and Drug Administration, Washington 25, D. C.

VITAMIN D—POULTRY FEED SUPPLEMENTS:

Leo Friedman, Food and Drug Administration, Washington 25, D. C.

NICOTINIC ACID (CHEMICAL):

J. P. Sweeney, Food and Drug Administration, Washington 25, D. C.

CAROTENE:

F. W. Quackenbush

PANTOTHENIC ACID (MICROBIOLOGICAL METHOD):

H. W. Loy, Jr.

FOLIC ACID (MICROBIOLOGICAL METHOD):

Laura Flynn, College of Agriculture, Univ. of Missouri, Columbia, Mo.

VITAMIN B_{12} (MICROBIOLOGICAL METHOD):

Carl H. Krieger, Wisconsin Alumni Research Foundation, Madison, Wis.

SUBCOMMITTEE B:

F. H. WILEY (1952), (Food and Drug Administration, Washington 25, D. C.), *Chairman;* HARRY J. FISHER (1954); and G. R. CLARK (1956)

RADIOACTIVITY:

Referee: A. Schwebel, National Bureau of Standards, Washington 25, D. C.

SPECTROGRAPHIC METHODS:

Referee: W. T. Mathis, Connecticut Agricultural Expt. Station, New Haven, Conn.

VEGETABLE DRUGS AND THEIR DERIVATIVES:

Referee: P. S. Jorgensen, Food and Drug Administration, San Francisco 2, Calif.

AMINOPYRINE, EPHEDRINE, AND PHENOBARBITAL:

H. C. Heim, School of Pharmacy, University of Colorado, Boulder, Colo.

QUININE AND STRYCHNINE:

D. J. Miller, Food and Drug Administration, Buffalo 3, N. Y.

RUTIN IN TABLETS:

A. Turner, Eastern Regional Research Lab., United States Department of Agriculture, Philadelphia, Pa.

SYNTHETIC DRUGS:

Referee: F. C. Sinton, Food and Drug Administration, New York 14, N. Y.

METHYLENE BLUE:

H. O. Moraw, Food and Drug Administration, Chicago 7, Ill.

SULFANILAMIDE DERIVATIVES:

H. W. Conroy, Food and Drug Administration, Kansas City 6, Mo.

PROPADRINE HYDROCHLORIDE:

A. W. Steers, Food and Drug Administration, Los Angeles 15, Calif.

SPECTROPHOTOMETRIC METHODS:

J. Carol, Food and Drug Administration, Washington 25, D. C.

PROPYLTHIOURACIL:

Gordon Smith, Food and Drug Administration, New York 14, N. Y.

DI- AND TRIPHENHYDRAMINE HYDROCHLORIDE (BENADRYL® AND PYRIBENZAMINE ®):

H. C. Heim

SYNTHETIC ESTROGENS:

Daniel Banes, Food and Drug Administration, Washington 25, D. C.

MISCELLANEOUS DRUGS:

Referee: Iman Schurman, Food and Drug Administration, Chicago 7, Ill.

MICROSCOPIC TESTS FOR ALKALOIDS AND SYNTHETICS:

W. V. Eisenberg, Food and Drug Administration, Washington 25, D. C.

MERCURY COMPOUNDS:

R. L. Herd, Food and Drug Administration, St. Louis 1, Mo.

ORGANIC IODIDES AND SEPARATION OF HALOGENS:

V. E. Stewart, State Department of Agriculture, Tallahassee, Fla.

ALKALI METALS:

H. F. O'Keefe, Food and Drug Administration, Chicago 7, Ill.

GLYCOLS AND RELATED COMPOUNDS:

Harry Isacoff, Food and Drug Administration, New York 14, N. Y.

PRESERVATIVES AND BACTERIOSTATIC AGENTS IN AMPUL SOLUTIONS:

C. N. Jones, Food and Drug Administration, New York 14, N. Y.

METHYL ALCOHOL:

J. F. Guymon, Agri. Expt. Sta., College of Agriculture, Davis, Calif.

COSMETICS:

Referee: G. R. Clark, Food and Drug Administration, Washington 25, D. C.

COSMETIC CREAMS:

C. F. Bruening, Food and Drug Administration, Chicago 7, Ill.

COSMETIC SKIN LOTIONS:

R. Hyatt, Food and Drug Administration, Cincinnati 2, Ohio

DEODORANTS AND ANTI-PERSPIRANTS:

HAIR DYES AND RINSES:

S. W. Newburger, Food and Drug Administration, Baltimore 2, Md.

MASCARA, EYEBROW PENCILS, AND EYE SHADOW:

Paul W. Jewel, Max Factor and Company, Hollywood, Calif.

SUN TAN PREPARATIONS:

COAL-TAR COLORS:

Referee: K. A. Freeman, Food and Drug Administration, Washington 25, D. C.
INTERMEDIATES IN TRIPHENYL-METHANE DYES:
A. T. Schram, National Aniline Division, P.O. Box 975, Buffalo 5, N. Y.
ETHER EXTRACT IN COAL-TAR COLORS:
S. S. Forrest, Food and Drug Administration, Washington 25, D. C.
HALOGENS IN HALOGENATED FLUORESCEINS:
J. H. Jones, Food and Drug Administration, Washington 25, D. C.
IDENTIFICATION OF COAL-TAR COLORS:
Rachel Selar, Food and Drug Administration, Washington 25, D. C.
VOLATILE AMINE INTERMEDIATES IN COAL-TAR COLORS:
J. O. Millham, Food and Drug Administration, Washington 25, D. C.
NON-VOLATILE UNSULFONATED AMINE INTERMEDIATES IN COAL-TAR COLORS:
K. S. Heine, Jr., Food and Drug Administration, Washington 25, D. C.
SULFONATED AMINE INTERMEDIATES IN COAL-TAR COLORS:
N. Ettlestein, Food and Drug Administration, Washington 25, D. C.
UNSULFONATED PHENOLIC INTERMEDIATES IN COAL-TAR COLORS:
H. Holtzman, Ansbacher-Siegle Corp., Rosebank, Staten Island, N. Y.
SULFONATED PHENOLIC INTERMEDIATES IN COAL-TAR COLORS:
John Walton, H. Kohnstamm & Company, Brooklyn 31, N. Y.
INTERMEDIATES DERIVED FROM PHTHALIC ACID:
C. Graichen, Food and Drug Administration, Washington 25, D. C.
LAKES AND PIGMENTS:
C. Graichen
SPECTROPHOTOMETRIC TESTING OF COAL-TAR COLORS:
J. H. Jones
SUBSIDIARY DYES IN D&C COLORS:
L. Koch, H. Kohnstamm and Company, Brooklyn 31, N. Y.
HEAVY METALS IN COAL-TAR COLORS:
C. Stein, Food and Drug Administration, Washington 25, D. C.
ARSENIC AND ANTIMONY IN COAL-TAR COLORS:
L. S. Harrow
SUBSIDIARY DYES IN FD&C COLORS:
M. Dolinsky, Food and Drug Administration, Washington 25, D. C.
BOILING RANGE OF AMINES DERIVED FROM COAL-TAR COLORS:
L. S. Harrow
PAPER CHROMATOGRAPHY OF COAL-TAR COLORS:
Doris Tilden, Food and Drug Administration, San Francisco, Calif.
INORGANIC SALTS IN COAL-TAR COLORS:
K. S. Heine, Jr.

SUBCOMMITTEE C:

P. A. CLIFFORD (1952), Food and Drug Administration, Washington 25, D. C., *Chairman;* A. H. ROBERTSON (1954); and S. ALFEND (1956)

PROCESSED VEGETABLE PRODUCTS:

Referee: L. M. Beacham, Food and Drug Administration, Washington 25, D. C.

QUALITY FACTORS:

R. D. Lovejoy, Food and Drug Administration, Washington 25, D. C.

MOISTURE IN DRIED VEGETABLES:

B. Makower, Western Regional Research Laboratory, Albany 6, Calif.

CATALASE IN FROZEN VEGETABLES:

B. M. Gutterman, Food and Drug Administration, Washington 25, D. C.

PEROXIDASE IN FROZEN VEGETABLES:

M. A. Joslyn, College of Agr., Univ. of Calif., Berkeley 4, Calif.

COFFEE AND TEA:

Referee: S. T. Colamaria, Food and Drug Administration, Boston, Mass.

CHLOROGENIC ACID IN COFFEE:

L. C. Weiss, Food and Drug Administration, Los Angeles, Calif.

MOISTURE IN COFFEE AND TEA:

DAIRY PRODUCTS:

Referee: W. Horwitz, Food and Drug Administration, Minneapolis 1, Minn.

PHOSPHATASE TEST IN DAIRY PRODUCTS:

L. H. Burgwald, Ohio State University, Columbus, Ohio

SAMPLING, FAT, AND MOISTURE IN HARD CHEESES:

W. Horwitz

PREPARATION OF BUTTER SAMPLES:

A. L. Weber, Food and Drug Administration, New York 14, N. Y.

TESTS FOR RECONSTITUTED MILK:

George F. Edwards, Mass. Dept. of Health, State House Boston, Mass.

FAT IN DAIRY PRODUCTS:

Ernest O. Herreid, Illinois Agricultural Expt. Sta., Urbana, Ill.

SAMPLING, AND PREPARATION OF SAMPLE, OF SOFT CHEESES:

Sam Perlmutter, Food and Drug Administration, Minneapolis, Minn.

FROZEN DESSERTS:

H. M. Boggs, Food and Drug Administration, Philadelphia, Pa.

EGGS AND EGG PRODUCTS:

Referee: F. J. McNall, Food and Drug Administration, Cincinnati 2, Ohio

ADDED GLYCEROL:

George E. Keppel, Food and Drug Administration, Minneapolis 1, Minn.

AMMONIA NITROGEN:

E. B. Boyce, State Dept. Public Health, Boston 33, Mass.

DECOMPOSITION AND FILTH IN FOODS (CHEMICAL INDICES):

Referee: W. I. Patterson, Food and Drug Administration, Washington 25, D. C.

FISH PRODUCTS (ACIDS):

Fred Hillig, Food and Drug Administration, Washington 25, D. C.

SHELLFISH:

George McClellan, Food and Drug Administration, New Orleans 16, La.

ANIMAL FECAL MATTER:

John Thomas, Food and Drug Administration, New Orleans, La.

PINEAPPLE (DECOMPOSITION, CARBOHYDRATE):
J. F. Weeks, Jr., Food and Drug Administration, New Orleans, La.
APPLES (GALACTURONIC ACID) AND BLACKHEART IN PINEAPPLE:
W. O. Winkler, Food and Drug Administration, Washington 25, D. C.
STRAWBERRIES (PIGMENTS):
H. P. Bennett, Food and Drug Administration, New Orleans, La.
STRAWBERRIES (GALACTURONIC ACID):
P. A. Mills, Food and Drug Administration, San Francisco, Calif.
TOMATOES (SUCCINIC ACID):
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URIC ACID IN CEREAL PRODUCTS:
Helen Barry, Food and Drug Administration, New Orleans, La.
URIC ACID IN FRUIT PRODUCTS:
Doris Tilden, Food and Drug Administration, San Francisco, Calif.
URIC ACID IN NUTS:
H. M. Bollinger, Food and Drug Administration, Los Angeles, Calif.
FISH (INDOLE):
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LACTIC ACID:
L. C. Mitchell, Food and Drug Administration, Minneapolis, Minn.
SPINACH (GALACTURONIC AND SUCCINIC ACIDS):
H. D. Silverberg, Food and Drug Administration, St. Louis, Mo.
TOMATOES (GALACTURONIC ACID):
M. Dow, Food and Drug Administration, St. Louis, Mo.

GELATINE, DESSERT PREPARATIONS, AND MIXES:

Referee: Sumner C. Rowe, Food and Drug Administration, Washington 25, D. C.
GELATINE AND GELATINE DESSERTS (COMPOSITION):
Joseph H. Cohen, General Foods Corporation, Woburn, Mass.

FISH AND OTHER MARINE PRODUCTS:

Referee: Menno D. Voth, Food and Drug Administration, Boston 10, Mass.

GUMS IN FOODS:

Referee: F. Leslie Hart, Food and Drug Administration, Los Angeles 15, Calif.
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M. J. Gnagy, Food and Drug Administration, Los Angeles, Calif.
FROZEN DESSERTS:

CACAO PRODUCTS:
Flora G. Mendelsohn, Food and Drug Adm., Los Angeles 15, Calif.
CATSUP AND RELATED TOMATO PRODUCTS:
T. E. Strange, Food and Drug Administration, Portland, Ore.
STARCHY SALAD DRESSINGS:
M. J. Gnagy

MEAT AND MEAT PRODUCTS:

Referee: Roger M. Mehurin, Meat Inspection Div., Bur. of Animal Ind., Washington 25, D. C.

DEFATTED MILK SOLIDS IN MEAT PRODUCTS:

CREATIN IN MEAT PRODUCTS:

J. M. McCoy, Meat Inspection Division, Bureau of Animal Industry, Washington 25, D. C.

HORSEMEAT IN GROUND MEAT:

C. E. Hynds, State Food Laboratory, Albany, N. Y.

STARCH IN MEAT PRODUCTS:

R. A. Chapman, Dept. National Health and Welfare, Ottawa, Can.

METALS, OTHER ELEMENTS, AND RESIDUES IN FOODS:

Referee: H. J. Wichmann, Food and Drug Administration, Washington 25, D. C.

COPPER:

A. L. Brandon, Anheuser-Busch Company, Inc., St. Louis, Mo.

ZINC:

O. R. Alexander, American Can Company, Maywood, Ill.

MERCURY:

A. K. Klein, Food and Drug Administration, Washington, D. C.

DDT AS SPRAY RESIDUE ON FOODS:

R. H. Carter, Bureau of Entomology and Plant Quarantine, Beltsville, Md.

INSECTICIDES IN CANNED FOODS:

E. T. Abeling, Beechnut Packing Co., Canajoharie, N. Y.

PARATHION:

P. A. Clifford, Food and Drug Administration, Washington 25, D. C.

SODIUM FLUOROACETATE (1080):

L. L. Ramsey, Food and Drug Administration, Washington 25, D. C.

METHOXYCHLOR:

E. Laug, Food and Drug Administration, Washington 25, D. C.

MICROBIOLOGICAL METHODS:

Referee: G. G. Slocum, Food and Drug Administration, Washington 25, D. C.

CANNED MEATS:

M. L. Laing, Armour & Company, Chicago 9, Ill.

CANNED ACID FOODS:

A. P. Dunningan, Food and Drug Administration, Washington 25, D. C.

CANNED VEGETABLES:

C. W. Bohrer, Natl. Canners Assn., Washington, D. C.

EGGS AND EGG PRODUCTS:

M. T. Bartram, Food and Drug Administration, Washington 25, D. C.

NUTS AND NUT PRODUCTS:

William R. North, Food and Drug Administration, Washington 25, D. C.

FROZEN FRUITS AND VEGETABLES:

H. E. Goresline, Production and Marketing Administration, Poultry Division, Washington 25, D. C.

SUGAR:

E. J. Cameron, Natl. Canners Assn., Washington, D. C.

CANNED FISHERY PRODUCTS:

L. R. Shelton, Jr., Food and Drug Administration, Washington 25, D. C.

MICROCHEMICAL METHODS:

Referee: C. O. Willits, Eastern Regional Research Lab., Philadelphia, Pa.
ELEMENTAL ANALYSIS:
C. L. Ogg, Eastern Regional Research Laboratory, Philadelphia, Pa.
GROUP ANALYSIS:
A. Steyermark, Hoffman-La Roche, Nutley, N. J.

NUTS AND NUT PRODUCTS:

Referee: A. M. Henry, Food and Drug Administration, Atlanta 3, Ga.
SHREDDED COCONUT (GLYCOLS AND GLYCEROL):
A. J. Shingler, Food and Drug Administration, Atlanta, Ga.
FREE FATTY ACIDS:
W. B. Tarver, Food and Drug Administration, Atlanta, Ga.

OILS, FATS, AND WAXES:

Referee: J. Fitelson, Food and Drug Administration, New York 14, N. Y.
SPECTROPHOTOMETRIC METHODS:
Gardner Kirsten, Food and Drug Administration, New York 14, N. Y.
PEANUT OIL:
A. B. Karasz, Dept. Agriculture and Markets, Albany 1, N. Y.
ANTIOXIDANTS:
S. Kahan, Food and Drug Administration, New York 14, N. Y.
COAL-TAR COLORS IN OILS:
Marie Offutt, Food and Drug Administration, New York, N. Y.

SPICES AND OTHER CONDIMENTS:

Referee: E. C. Deal, Food and Drug Administration, New Orleans, La.
VINEGAR:
G. A. Michael, Dept. Public Health, State House, Boston 33, Mass.
VOLATILE OIL IN SPICES:

SUGAR, ASH, ETHER EXTRACT, AND PUNGENT PRINCIPLES IN MUSTARDS:
Jesse E. Roe, Food and Drug Administration, Denver, Colo.
PREPARATION OF SAMPLE, AND FAT IN MAYONNAISE AND SALAD DRESSING:
Juanita E. Breit, Food and Drug Administration, Minneapolis, Minn.
PREPARATION OF SAMPLE OF FRENCH DRESSING:
A. Kramer, Baltimore, Md.
SEEDS AND STEMS IN GROUND CHILI:
A. N. Prater, Gentry, Inc., Los Angeles 54, Calif.

ENZYMES:

Referee: J. W. Cook, Food and Drug Administration, San Francisco, Calif.
HYDROCYANIC ACID GLUCOSIDES:
W. O. Winkler, Food and Drug Administration, Washington 25, D. C.

SUBCOMMITTEE D:

J. WALTER SALE (1952) (Food and Drug Administration, Washington, D. C.), *Chairman;* C. S. FERGUSON (1954); and FLOYD ROBERTS (1956)

ALCOHOLIC BEVERAGES:

Referee: J. Walter Sale, Food and Drug Administration, Washington 25, D. C.

MALT BEVERAGES, SIRUPS, EXTRACTS, AND BREWING MATERIALS:
Robert I. Tenney, Wahl-Henius Inst., 64 E. Lake St., Chicago, Ill.

HOPS:
F. E. Connory, P. Ballentine & Sons, 57 Freeman St., Newark, N. J.

INORGANIC ELEMENTS IN BEER:
A. L. Brandon, Anheuser-Busch, Inc., St. Louis, Mo.

COLOR AND TURBIDITY IN BEER:
B. H. Nissen, Anheuser-Busch, Inc., St. Louis, Mo.

DISTILLED SPIRITS:
A. D. Etienne, Bureau of Internal Revenue, Washington 25, D. C.

CHROMATOGRAPHIC ABSORPTION:
Alex P. Mathers, Bur. Internal Revenue, Washington 25, D. C.

CARAMEL IN WINES:
Peter Valaer

CORDIALS AND LIQUEURS:
John B. Wilson, Food and Drug Administration, Washington 25, D. C.

METHANOL:
J. F. Guymon, Agr. Expt. Station, College of Agriculture, Davis, Calif.

CACAO PRODUCTS:

Referee: W. O. Winkler, Food and Drug Administration, Washington 25, D. C.

LECITHIN:
J. H. Bornmann, Food and Drug Administration, Chicago 7, Ill.

MALT SOLIDS:
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PECTIC ACID:
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CACAO INGREDIENTS:
W. O. WINKLER

LACTOSE:
Donald G. Mitchell, Walter Baker Co., Dorchester 24, Mass.

CEREAL FOODS:

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STARCH IN RAW AND COOKED CEREALS:
E. Steagall, Food and Drug Administration, Washington, D. C.

MILK SOLIDS AND BUTTERFAT IN BREAD:
V. E. Munsey

PROTEOLYTIC ACTIVITY OF FLOUR:
Byron S. Miller, Federal Hard Wheat Quality Lab., Manhattan, Kans.

SOYBEAN FLOUR:
T. C. Law, 16 Baker St., Atlanta, Ga.

BAKED PRODUCTS (SUGARS)
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UNSAPONIFIABLE MATTER AND STEROLS IN NOODLES AND BAKERY PRODUCTS:
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W. V. Eisenberg, Food and Drug Administration, Washington 25, D. C.
DAIRY AND EGG PRODUCTS:
Dorothy Scott, Food and Drug Administration, Washington, D. C.
NUT PRODUCTS:
Maryvee G. Yakowitz, Food and Drug Administration, Washington 25, D. C.
BAKED PRODUCTS, CEREALS, AND CONFECTIONERY:
J. F. Nicholson, Food and Drug Administration, Washington 25, D. C.
BEVERAGE MATERIALS:
F. A. Hodges, Food and Drug Administration, Washington 25, D. C.
FRUIT PRODUCTS:
W. G. Helsel, Food and Drug Administration, Washington 25, D. C.
VEGETABLE PRODUCTS:
F. R. Smith, Food and Drug Administration, Washington 25, D. C.
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C. R. Joiner, Food and Drug Administration, St. Louis 1, Mo.
MISCELLANEOUS MATERIALS:
A. H. Tillson, Food and Drug Administration, Washington, D. C.

FLAVORS AND NON-ALCOHOLIC BEVERAGES:

Referee: John B. Wilson, Food and Drug Administration, Washington 25, D. C.
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PEEL OILS IN CITRUS JUICES:
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PRESIDENT'S ADDRESS*

THE A.O.A.C. AND FOOD STANDARDS

W. A. QUEEN (Food and Drug Administration, Washington, D. C.)

An organization which endures beyond a half century inevitably acquires a body of cherished traditions. This year marks the 66th anniversary of the Association of Official Agricultural Chemists. In the passing of these years perhaps one of the more questionable traditions has grown up that the President of the A.O.A.C. shall address the annual meeting. I am happy, nevertheless, to conform to this tradition and I welcome the opportunity which the occasion affords to express to you my appreciation for the honor of election as President of this Association. It is also within the A.O.A.C. tradition that the President is permitted to choose the subject matter for his remarks. I have accordingly elected to discuss the record of participation of the A.O.A.C. in the development of standards for foods and to suggest the importance of future participation by the Association in the food standardization program.

More than 60 years ago Professor John A. Myers was the President of our Association. In the Presidential address which he delivered in 1889 he said, concerning the A.O.A.C., "It is aiming to lay a foundation so solid that every court in this land must respect its conclusions, and every analytical chemist, whether he lives in this country or elsewhere, must be forced either to practice or admit the advantages and correctness of our system of analyses." The goal which was set for this Association by Professor Myers was high—laudably high! Of course, viewed strictly and literally, this goal has not yet been fully achieved. But there are many instances where we can now say, even in a quite literal sense, that every Federal court and many of the State courts in this land must, as matters of law, respect conclusions founded essentially upon A.O.A.C. work and must accept analytical methods as they are prescribed in our *Book of Methods*. The instances which I have in mind are the numerous ones where data resulting from A.O.A.C. work was used in the development of food standards and A.O.A.C. methods are cited in the standards. The status in the courts of these conclusions and analytical methods follows as a consequence of the legal effect given to food standards by the Federal Food, Drug, and Cosmetic Act.

The development of food standards, in the sense in which we use the term, goes back about 60 years and has its origin in work initiated by the A.O.A.C. This development can be traced through the evolution of the Committee on Food Standards, of which I have been privileged to serve

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as chairman for a number of years. I think it will be worth while to outline in a brief way the history of the Food Standards Committee. To those among you who are interested in a more detailed treatment of this history I recommend the Presidential address which was delivered by the late Walter S. Frisbie, my esteemed friend and predecessor, at our 1939 meeting. You will find this address published in the February 1940 issue of the *Journal of the A.O.A.C.*, and it is well worthy of review.

Within two or three years after the Association of Official Agricultural Chemists was permanently organized at the 1884 Convention of Agricultural Chemists, investigations were expanded from fertilizers to include feeds and dairy products and not long thereafter to many other foods. A review shows that between 1880 and 1890 ten states passed laws in regulation of food adulteration. In prompt response to the enactment of these food laws the Association's work in the investigation and adoption of analytical methods for various foods progressed apace, but official chemists soon realized that they had need for standards against which they could measure their analytical data concerning foods suspected of being adulterated. To conclude whether to charge adulteration it was not enough for an official to know that for an article represented as cheddar cheese, the A.O.A.C. methods of analysis proved a representative sample of the cheese to contain 42% water, and 45% milk fat on a moisture free basis. There was need for a food standard for cheddar cheese—a recognized, trustworthy definition provided with specifications as to the proper limits for moisture and fat. In his Presidential address in 1897 Professor William Frear discussed the serious problem of adulteration of food for man and he recommended that the A.O.A.C. take steps "to secure the establishment of standards of composition for pure food substances. . . . " He went on to say "In the absence of such an accepted set of standards each food chemist is compelled to formulate his own. The result is that the standards used by different chemists are based upon insufficient data and are formulated according to different canons, with the consequence that legal actions are instituted in good faith by control officers only to be lost by the failure of the several experts to agree upon the essential bases of comparison." Responding to the recommendation made by Professor Frear the Association appointed a Committee with Dr. Harvey W. Wiley as Chairman and Professor William Frear of Pennsylvania, E. H. Jenkins of Connecticut, M. A. Scovell of Kentucky and H. A. Weber of Ohio as co-members. This Committee published a report which is dated July 20, 1898. Copies of that report are now very rare but my office is fortunate enough to have one of them. It is captioned "Association of Official Agricultural Chemists of the United States Committee on Food Standards." This is the earliest record I have been able to find which is designated as a report of a "Committee on Food Standards." The rare foresight manifested in this first publication by the

Committee on Food Standards would warrant my giving you the complete 8-page report if time would permit. Being in position to observe some of the outward evidences of your inward reactions to this suggestion, however, I shall not attempt to give you the full report, but I at least want to bring parts of it to your attention. The report opens with a statement that "in the recognition of the growing need on the part of many of its members who are called upon to serve in their respective States as official food control chemists, for a compilation of trustworthy chemical and physical data representing the composition of foods" . . . the Association of Official Agricultural Chemists, at its late annual meeting, " . . . appointed a Committee on Food Standards to consider what course in reference to this subject should now be taken, and empowered [it] to proceed at once to the prosecution of any plans it might adopt relative to the subject and to call to its aid such expert assistance as it might select and enlist." In the report there is a tabulation of food categories arranged to facilitate the selection and compilation of data and the formulation of standards. In the tabulation each category is divided into classes of food, and for each class there is assigned a leading authority in the field of food chemistry to serve as referee. The classes are: meat and various other animal food products; milk and milk products; grains and grain products; fruits and vegetables; sugar and related substances; spices and other condiments; drinks; and a class designated "Substances artificially introduced with possible injury to health." In this last mentioned class the Committee listed such things as "preservatives, coloring matters, metals and metallic salts and other injurious substances." The current investigations on the use of chemicals in foods by the Delaney Committee of the House makes this last class of more than passing interest. Those "charter members" of the original Food Standards Committee were indeed possessed of remarkable foresight. Quoting further from the report we find "The committee . . . formulated certain general principles for guidance in the selection of chemical data, for the purpose of securing a satisfactory basis for the adoption of a set of American Food Standards." We have evidence that this original Committee on Food Standards actively prosecuted the program of work which it had outlined for itself. In 1900 the Committee tentatively adopted definitions and standards of identity for meat, lard, milk and certain milk products, including condensed milk and ice cream, jams and jellies, syrups, and for the flavoring extracts of cinnamon, lemon, orange and peppermint. So we see that this year we are celebrating the golden anniversary of this historic initial achievement in the promulgation of formal American Food Standards.

The year 1902 marked a significant step in the evolution of food standards toward their ultimate effective status as rules clothed with the force and effect of law. We find in the Appropriations Act for the Department

of Agriculture for the fiscal year beginning July 1, 1902, that general funds were appropriated "to enable the Secretary of Agriculture in collaboration with the Association of Official Agricultural Chemists, and such other experts as he may deem necessary, to establish standards of purity for food products and to determine what are regarded as adulterations therein, for the guidance of the officials of the various states and of the courts of Justice. . . . " A review of the statutes at large shows this authorization as being carried in the Agricultural Appropriation Acts for the next four successive years. These were the years immediately preceding the passage of the Federal Food and Drugs Act of 1906. During this five-year period the A.O.A.C. Committee on Food Standards made recommendations to the Secretary of Agriculture who promulgated them in publications called "Office of the Secretary Circulars." Standards were published in Circular No. 10 issued November 21, 1903, in Circular No. 13 issued December 20, 1904, and in Circular No. 19 issued June 26, 1906.

The legal status accorded the standards in these circulars by different courts is somewhat contradictory. A Federal District Court in St. Louis dismissed an information charging adulteration of a product represented as "Vanilla Extract" which failed to meet the standard for vanilla extract as published in Circular No. 19. The Judge said that Circular No. 19 could *not* be considered in determining the question of the guilt or innocence of the defendant. Under somewhat different circumstances, a Federal District Court in Cincinnati gave consideration to an adulteration charge which alleged that a product represented as "Lemon Extract" failed to meet the standard for lemon extract as published in Circular No. 19. The Cincinnati Judge was of the opinion that the Secretary of Agriculture had the power under the Appropriation Act to establish standards for food products and he said " . . . there seems to be no room for doubt that upon proof that the article did not conform to the requirements of the standard of purity established by the Secretary then an offense was charged under the laws of the United States." The charge of adulteration and the fine imposed on the defendant was sustained. So far as can be determined the question was not carried to appellate courts. Perhaps there was no appeal for the reason that these decisions came after Congress discontinued the inclusion of authorization to establish food standards in annual Agricultural Appropriation Acts.

Up to 1905 the only Committee on Food Standards was the one representing the A.O.A.C. but in the summer of 1905 the Association of State and National Food and Dairy Departments appointed a Committee on Standards. For some years following 1905 the two committees existed separately but with more or less collaboration between them. Although the Food and Drugs Act of June 30, 1906, made no specific provision for the promulgation of food standards, the Secretary of Agriculture appointed a committee of eight with the name "The United States

Commission on the Purity of Foods." He divided the membership of the Commission evenly by appointing four members from the Committee on Food Standards of the A.O.A.C. and four from the Committee on Standards of the Association of State and National Food and Dairy Departments. For the years from 1906 to 1913 the history we are following is somewhat confused. For example, Professors Jenkins and Scovell were members of the Committees of both Associations and were also members of the Secretary's Commission. To complicate the situation further the food standards committees of the two Associations also met as a joint committee. The United States Commission on the Purity of Foods was short-lived, apparently going out of existence when the Secretary called off its meeting for 1907.

In 1913 upon the recommendation of both Associations the Secretary of Agriculture appointed a committee which was called "The Joint Committee on Definitions and Standards." This committee had nine members, three representing our Association, three the Association of Dairy, Food and Drug Officials (successor of the Association of State and National Food and Dairy Departments), and three representing the Department of Agriculture. From 1913, forward the history of the Food Standards Committee is not difficult to follow. The original A.O.A.C. Committee on Food Standards was dissolved and the Joint Committee as appointed by the Secretary worked along the lines which the original committee had started. Sometime in the 1920's we find the gradual development of a shorter designation for the Joint Committee on Definitions and Standards, and around 1927 we find this group being called the "Food Standards Committee."

Following the passage of the Food and Drugs Act of 1906 the Agricultural Appropriation Acts did not authorize the Secretary to establish standards for foods, and unfortunately the Food and Drugs Act carried no such authorization. The courts held that the standards which were announced by the Secretary pursuant to the recommendation of the Joint Committee on Definitions and Standards did not have the force and effect of law. This was quite early recognized as a weakness in the 1906 Act. In his annual report for 1917 the Chief of the Bureau of Chemistry said "While the accomplishments of the Food and Drugs Act have been considerable, it must be admitted that it has its serious limitations. Especially conspicuous . . . [is] the lack of legal standards for foods, ... " Legislation was recommended to correct this deficiency in the Food and Drugs Act. A short but well directed step toward the goal of legal standards was achieved in 1930 when the so-called McNary-Mapes Amendment was enacted. This amendment authorized the Secretary to promulgate standards of quality, condition and fill of container for canned foods other than for meat products and canned milk. Senator Copeland introduced a bill in 1932 which was designed to provide a legal basis for a Food Standards

Committee and to give a legal status to food standards, but it was not enacted into law.

Most of the food standards which were recommended to the Secretary of Agriculture by the Joint Committee during its 25-year life from 1913 to 1938, and which were published by the Secretary in the Service and Regulatory Announcements, did not enjoy the force and effect of Federal law. However, we would be in serious error if we assumed that these so-called "advisory standards" were without value. They afforded reliable and practical means of measurement to both the regulatory official and the affected industry. The laws of many States adopted them as legal standards and almost all food regulatory officials had a high respect for them.

From 1933 to 1938 the Congress considered a succession of bills sponsored by Senator Copeland which culminated in the passage of the Federal Food, Drug, and Cosmetic Act of 1938. These bills were introduced in the following order and I find it convenient to refer to them by number: S. 1944 in 1933; S. 2000 in 1934; S. 2800 in 1934; S. 5 of the 74th Congress in 1935, S. 5 of the 75th Congress in 1937. The first bill, S. 1944, proposed to give legal status to food standards but did not provide for an official food standards committee. The next bill, S. 2000, continued the authorization for establishment of Food Standards and also carried a section which stated " . . . A Committee on Food is hereby provided which shall consist of five members. . . . " In S. 2800 the designation of the committee was changed to "Committee on Food Standards" and the membership was increased to seven. As introduced in the 74th Congress S. 5 authorized the promulgation of food standards and the appointment of a seven member Committee on Food Standards. The House Committee on Interstate and Foreign Commerce omitted the provision for the committee as unnecessary. The bill which became the present law was S.5 enacted by the 75th Congress. This bill provided a legal status for food standards but it carried no authorization for a Food Standards Committee.

After the new law was passed in 1938 Mr. Walter G. Campbell, who was then Chief of the Food and Drug Administration, recommended to the Secretary of Agriculture that he appoint a food standards committee to function within and for the Administration in the formulation of proposals for standards to be promulgated by the Secretary. This recommendation was adopted and a committee of six members, which we know as the Food Standards Committee, was appointed. The Chief of the Division of State Cooperation of the Food and Drug Administration was designated Chairman, and the Chief of the Food Division of the Administration was appointed as a member of the committee. The other four members were State regulatory officials and in a sense were also representatives of the A.O.A.C. and the Association of Food and Drug Officials of the

United States. In conformity with a resolution that had been adopted by the Association of Food and Drug Officials of the United States, the four members of the committee who were State regulatory officials recommended that a policy of rotating the State appointees be instituted. This recommendation was accepted and the matter was worked out so that each State official is appointed for a four-year term and one member changes each year. The Federal Food, Drug, and Cosmetic Act of 1938 established a detailed procedure for formal hearings in the promulgation of food standards. For that reason the Food Standards Committee now functions as a consulting body on policy making, doing its work in the formative and preliminary steps in the development of those food standards initiated by the government.

Let us in a sentence recapitulate the evolution of food standards by noting the publications used to announce them. To begin with, food standards were published in Association pamphlets, then in Office of the Secretary Circulars, thereafter in Service and Regulatory Announcements, and now they are published first in the Federal Register and then they are compiled in the official Code of Federal Regulations.

The standards for foods which have been promulgated to date under the 1938 Act are published in 187 numbered sections of the Code of Federal Regulations. It is not possible to state an exact figure for the number of these which reflect data accumulated by A.O.A.C. work, but a count shows that 117 sections explicitly prescribe that A.O.A.C. methods be followed to determine some one or more of the indices of compliance.

Food standards under the 1938 Act have stood the test of court review and interpretation,—witness the farina case, the catsup case, the canned oyster case. Court decisions in these first two cases unequivocally sustained the validity of that provision of the Act which stipulates that a food must comply with the requirements of the applicable established definition and standard of identity if it is represented as or purports to be a food so standardized.

I would not wish to permit this occasion to pass without suggesting that the present members of the A.O.A.C. have both an opportunity and an obligation to play a responsible role in the food standardization program. Food standards are required by the law to be based on facts. The data which A.O.A.C. investigators amass in working on food problems are facts which often have important applications to the development of good food standards. The methods which you develop and prove to be valid and workable by collaborative studies are very important for the establishment, maintenance, and enforcement of good food standards. You who contribute data and methods which enter into food standards are materially contributing to the development of the organic law which deals with the necessary regulation of the distribution of foods for consumption by the American public, a matter in which each of us as an

individual consumer has an important stake. For that reason the responsibility is great and the opportunity for service should be inviting.

I should like also to voice a note of caution. Sometimes there may be temptation to tinker with A.O.A.C. methods which have been incorporated into food standards. Let's think the problem through before we do this. A change just for the sake of change is unjustified. We must remember that the method which was adopted in the standard has contributed authentic data which enable the standard to carry its limiting specifications. For example, the moisture limits in the cheese standards reflect the data accumulated from the use of the A.O.A.C. method specified in the cheese standards. To change the method might well require the accumulation of a new body of authentic data with all that entails in the expenditure of time, and funds already too limited, not to mention the possibilities that may be raised for altering the effect of important court interpretations.

In opening I quoted a sentence from the Presidential address, which was given by Professor Myers in 1889, in which he stated the two aims of gaining court recognition for conclusions developed by A.O.A.C. work and of winning the respect of analytical chemists, both in this country and elsewhere, for A.O.A.C. methods. I have undertaken to develop the achievement of the former aim, but I would not have you believe that progress has not been made toward the achievement of the latter aim also. Two weeks ago today Mr. Charles A. Adams, Director, Food Standards Division of the Ministry of Food for the United Kingdom, spoke here in Washington to the Division of the American Bar Association specializing in food, drug, and cosmetic law. The position occupied by Mr. Adams in Great Britain is comparable to the position occupied by Dr. Paul B. Dunbar, the Commissioner of Food and Drugs here in the United States. In the course of his paper Mr. Adams spoke of the advisability of including analytical methods in food standards, and then he said,

> "In this connection one cannot fail to notice the wide acceptance given by chemists the world over to the methods of analysis published by the A.O.A.C. The obvious corollary seems to be that given adequate facilities for amendment, agreement to apply the same standard methods of analysis to the foods exchanged between our countries should be a reasonable target for our chemists to aim at. Were that goal attained, the inclusion of these methods in food standards legislation could quickly follow."

A similar tribute to the suitability of A.O.A.C. methods for incorporation in food standards was given in a paper delivered in Oslo University last June.

With this ever-broadening recognition of the worth of our endeavors and accomplishments, both at home and abroad, there rests upon us the inescapable obligation to maintain, and to improve as far as that may

be possible, the high quality of the work that has always characterized the efforts of our committees, referees, associate referees, and collaborative investigators, in the development of methods that will meet the essential requirements of accuracy and precision dictated by the critical demands of court action necessarily associated with the enforcement of the regulatory laws with which many of us are directly concerned. The success of our efforts in this direction must not be curtailed or circumscribed by inaction or indifference on the part of those of us who should be most concerned with the adequacy and validity of analytical methods, or those who are best able to contribute substantially to the achievement of this purpose. As Mr. Lepper, our esteemed Secretary, has pointed out, every official analyst, State or Federal, whether in a department of agriculture, experiment station, health department, or other designated agency, is by the terms of our constitution a member of the Association and privileged to participate in its important work. I should like to join with him and others, to whom the work has been a labor of love, richly compensated in realization of well rendered service, in soliciting your continued interest and earnest efforts in this most worthy undertaking.

ORDER OF PUBLICATION

The reports of the committees presented on the last day of the annual meeting are given at the beginning of the proceedings, not in their chronological order. This arrangement will assist the referees, associate referees, and collaborators in planning and developing their year's work. The remainder of the proceedings will follow in their usual order.

Third Day

WEDNESDAY—MORNING SESSION

REPORT OF THE EDITORIAL BOARD

HENRY A. LEPPER, *Chairman*

Since the last report of the editorial board, the 7th edition of *Methods of Analysis* has come into existence. It was the hope that copies, at least for demonstration purposes, might have been available at this meeting. It is realized now that in view of the magnitude of the work involved such expectation was too optimistic. However, the editorial work is completed and the book has been "on the press" since last week. It should be ready for public distribution this fall. The only regrettable feature concerning the book is that the unprecedented rise in the printing costs makes it necessary to price the book at $10.00 net (domestic postage prepaid, foreign postage 50 cents extra). The discussion of the details concerning the preparation, progress, and final format of the book will be presented by the Chairman of the Editorial Committee of Methods of Analysis, Dr. H. J. Fisher. The Association was fortunate indeed to have the services of Mrs. Marian Lapp Otis in the editorial preparation of the book who, together with Dr. Fisher, by diligent effort, have made possible its publication in a shorter time than any previous edition. The Association is grateful to them as well as to all the referees, associate referees, collaborators, committee workers and others without whose splendid cooperation the book could never have become a reality.

Dr. White, the Editor of our *Journal*, will give the report of the Editorial Committee of the *Journal*. The financial status of the *Journal* is being presented in the report of the Secretary-Treasurer.

Approved.

REPORT OF THE EDITORIAL COMMITTEE OF THE *JOURNAL*

W. B. WHITE, *Editor & Chairman*
(Food and Drug Administration, Federal Security Agency, Washington, 25, D.C.)

The increase in the subscription price of the *Journal* has had no appreciable effect upon the number of subscriptions; there are now 1857 compared with 1862 last year. This is of course encouraging, but we must expand our circulation considerably if the *Journal* is to become self-supporting. The Publicity Committee (W. A. Queen, H. A. Lepper, and W. B. White, all of the U. S. Food & Drug Administration) has as yet received no suggestions as to suitable advertising media or potential subscribers, but perhaps this is because we all recognize the superiority of personal salesmanship over the printed word. We would again urge every subscriber to point out the value of the *Journal* to every prospect on his list of acquaintances, and at every meeting where it seems appropriate. It seems to us that the May and August numbers of Volume 33 (357 and 434 pages, respectively) would win many subscribers on sight. The *Journal* will be glad to publish the names of new subscribers, giving credit to the persons who obtained them, in the hope that this will start a "chain reaction."

Volume 33 will run to nearly eleven hundred pages, which is an all-time record. The contributed papers will total 40 and there will be 4 notes and 14 book reviews.

The comprehensive paper by K. L. Harris in the August number deserves special mention. It deals with the recognition of insect fragments as they actually occur in contaminated foods and with the determination of the type of insects from which the fragments were derived. There are 36 pages, 25 figures, and 23 references in this pioneering monograph. Nearly all of the figures contain several individual drawings or photomicrographs. In anticipation of a heavy demand, arrangements have been made for a large supply of reprints, which we will sell at cost.

We again extend our thanks to our contributors and reviewers for their wholehearted co-operation in making the *Journal* indispensable to workers in the wide range of fields covered.

Adopted.

REPORT OF COMMITTEE ON REVISION OF *METHODS OF ANALYSIS*

Reading of proof of the seventh edition of *Methods of Analysis* has been completed except for the index, and it is hoped that the book will be printed and in your hands before 1951. The first portion of the page proof was received in July and the last portion in September, while for the sixth edition no page proof was received until November 1945 and the last proof did not arrive until January 29, 1946. Therefore, unless something unforseen happens to the printer's schedule the seventh edition should appear several months earlier than did the sixth.

The committee had estimated from the manuscript that the seventh edition would contain about 100 more pages than did the sixth edition, but actually the number of pages will be about the same in spite of the increase in the number of methods. This is partly due to the deletion of the three chapters on Naval Stores, Leathers, and Tanning Materials, but what is chiefly responsible is the new system of abbreviations. Chapters showing notable increases in content are those on Coloring Matters and Enzymes.

The dropping of three chapters made some renumbering of chapters inevitable, and since some renumbering was necessary the committee decided to do a real job and rearrange all the chapters according to a definite system. The result is that only one chapter, that on Fertilizers, bears the same number as it did in the sixth edition. The rearrangement made much more work for the committee because nearly every cross reference had to be changed, but we believe we have made the change successfully.

This is the fourth edition of *Methods of Analysis* in whose preparation the chairman of the committee has had some part, and the sixth that has had the invaluable services of Mrs. Otis. Neither Mrs. Otis nor the chairman will be available for editing the 1955 edition. Because the preparation of the eighth edition will be in entirely new hands the chairman of the present committee suggests that now is not too soon to begin to look for a person with part of the editorial abilities and experience that Mrs. Otis has, for without the assistance of such a person the chairman of the new committee will have a very hard time indeed.

The committee wishes to express its thanks to the referees for their able assistance in preparing the manuscript and reading the proof, and moves the adoption of the seventh edition.

HARRY J. FISHER, *Chairman*
E. F. GRIFFIN
MARIAN LAPP OTIS
W. F. REINDOLLAR
J. WALTER SALE
F. H. WILEY

Approved.

REPORT OF COMMITTEE TO CONFER WITH AMERICAN PUBLIC HEALTH ASSOCIATION ON STANDARD METHODS FOR THE EXAMINATION OF DAIRY PRODUCTS

Some progress has been made comparing the official Sanders-Sager method and the Cornell method for residual phosphatase in certain types of ripened cheeses of known composition. Because the work is incomplete, no report is available.

Practical specifications, with minor modifications of the tolerances authorized for bacteriological transfer pipettes, in Standard Methods for the Examination of Dairy Products, Ninth Edition (1948), by the APHA, have been agreed upon. The specifications will become effective on January 1, 1951. Both laboratory supply houses and glassware manufacturers have been informed of the change. A brief report on methods for determining conformance with specifications for Babcock and Gerber volumetric milk and cream testing glassware and of the APHA pipettes will be released within a few months. While basically such a record is not new, the release will describe certain worthy procedures.

Your Associate Referee on sediment in milk has made additional observations on the selection of ingredients for a standard sediment mixture which, when distributed on standard reference discs in appropriate amounts, more nearly resembles in appearance the sediment commonly found in retail milk supplies. A progress report of this activity will be made at the St. Louis meeting of the APHA next month.

The need to add one per cent of fresh or reconstituted skim milk to the official agar milk plating medium of the APHA has created problems of its (the skim milk) availability, its reconstitution, its solubility and subsequent partial precipitation in the sterilized medium and of its precipicated particles being mistaken for small bacterial colonies in the poured plates. Studies are now being made to assess the properties of certain candidate skim-milk-free substitute media. Studies are also in progress for the ultimate recognition of a synthetic reference plating medium, composed of various vitamins, amino acids, purine bases, and mineral salts, by which the growth promoting properties of all candidate media may be compared.

A. H. ROBERTSON, *Chairman*
GUY G. FRARY
J. O. CLARKE

Approved.

REPORT OF THE COMMITTEE ON RECOMMENDATIONS OF REFEREES

WM. F. REINDOLLAR, *Chairman*

Reports submitted by referees and associate referees are somewhat lower in number than those received in recent past conferences. The reason for this is probably twofold: (1) the early date of this year's meeting, resulting in decreased time in which to complete the work; (2) the fact that last year the groups were required to give extra time and service in order to make necessary changes in existing procedures for the new Book of Methods.

The seventh edition, soon to be available, classifies the methods contained therein, as follows: "Official," "First Action," and "Procedures." Inasmuch as it is highly desirable that all First Action methods be advanced to the status of Official as soon as conveniently possible, referees and associate referees should give early consideration to having collaborative work done on this group so that the task may be completed for the eighth edition in 1955.

Once again the chairman wishes to stress the valuable contributions made to this important work by the subcommittees, referees, associate referees, and collaborators. Theirs is the major role in the A.O.A.C.; it is through their efforts that our program advances.

Approved.

REPORT OF SUBCOMMITTEE A ON RECOMMENDATIONS OF REFEREES*

H. A. HALVORSON (Department of Agriculture, Dairy and Foods, St. Paul, Minnesota), *Chairman;* E. L. GRIFFIN; and J. B. SMITH

FEEDING STUFFS

It is recommended—

(1) That work on the short method for crude fibre be discontinued as recommended by the Associate Referee.

(2) That work on the following be continued:

(a) Calcium and iodine in mineral mixed feeds
(b) Lactose in mixed feeds
(c) Fat in fish meal
(d) Crude fat or ether extract
(e) Microscopic examination
(f) Fluorine

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* These recommendations submitted by Subcommittee A were approved by the Association. Unless otherwise given all references are to *Methods of Analysis*, A.O.A.C., 1950.

(g) Mineral constituents of mixed feeds
(h) Protein evaluation in fish and animal products
(i) Tankage (hide, hoof, horn, and hair content).

(3) That the method for sulfaguanidine as presented by the Associate Referee be adopted, first action.

(4) That studies be continued with the object of shortening the general method for sulfa drugs.

(5) That collaborative studies of methods for m-m-dinitrodiphenyldisulfide, nitrophenide® and 2-amino-5-nitrothiazole, enheptin® be undertaken.

(6) That studies on adulteration of condensed milk products be discontinued.

(7) That studies on sampling and analysis of condensed buttermilk be discontinued.

(8) That the methods on mineral constituents in mixed feeds, sections **22.49–22.51,** be made official.

FERTILIZERS

It is recommended—

(1) That the study of the Shuey method for the determination of nitrogen in high nitrate-chloride mixtures be continued.

(2) That alpha phosphate (defluorinated phosphate rock) and phosphate rock-magnesium silicate glass be evaluated by the neutral ammonium citrate method, preferably with continuous agitation during the citrate digestion, and made official.

(3) That work on methods for phosphoric acid in fertilizers be continued, with emphasis on:

(a) Aging of the molybdate solution to determine if a time limit should be put on its use or a preservative added.
(b) Preparation of solutions of calcium metaphosphate for determination of total P_2O_5.
(c) Use of perchloric acid in preparation of solutions for analysis.

(4) That the present official method, the modified Perrin method and the flame photometer methods for potash be compared with further collaborative samples containing magnesium, sodium, and other ions which might cause interference in analysis.

(5) That work on sampling and preparation of sample for analysis be continued.

(6) That the study of methods for sulfur in mixed fertilizers be continued, as recommended by the Associate Referee.

(7) That studies on free water be extended to other fertilizer materials, and studies on possibilities of other methods for determining water.

(8) That work on the following be continued:

(a) Acid-forming or nonacid-forming quality

(b) Boron
(c) Copper and zinc
(d) Inert materials
(e) Magnesium and manganese
(f) Determination of calcium metaphosphate by the neutral ammonium citrate method.

ECONOMIC POISONS

It is recommended—

(1) That an Associate Referee be appointed to study methods for pyrethrins with special attention to the mercury reduction method.

(2) That an Associate Referee be appointed to study methods for the determination of allethrin.

(3) That an Associate Referee be appointed to study methods for piperonyl butoxide.

(4) That collaborative study be made of the electrometric titration method and the adsorption indicator method (Fajan's method) for halides in quaternary ammonium salts.

(5) That the ferricyanide method be studied to determine its applicability to quaternary ammonium salts.

(6) That colorimetric methods be further studied with a view to their application to relatively dilute solutions of quaternary compounds in commercial preparations.

(7) That the Davidow and the Harris methods for determination of chlordane be further studied.

(8) That the polarographic and titration methods for parathion be subjected to collaborative study and that a more acceptable indicator for the titration method be sought.

(9) That study of methods for alpha naphthyl thiourea be discontinued, but that study of methods of analysis of other rodenticides be continued.

(10) That the Elmore method for the determination of organic thiocyanate nitrogen in livestock or fly sprays be revised as recommended by the Associate Referee and adopted, first action.

(11) That the methods for determination of 2,4-dichlorophenoxyacetic acid, previously adopted as first action, with added note, now be made official.

(12) That the study of methods for the determination of 2,4-dichlorophenoxyacetic acid be continued, paying special attention to products containing small amounts of it and to ester type compounds.

(13) That the method for determination of total chlorine in liquid herbicides containing 2,4-D, 2,4,5-T, or mixtures of both in presence of oils and emulsifiers, be subjected to collaborative study.

(14) That the method for determination of potassium cyanate in herbicides be subjected to collaborative study.

(15) That the method for determination of tetraethyl pyrophosphate, which was made first action last year, now be made official.

(16) That the modified partition chromatographic method for determining the gamma isomer in technical benzene hexachloride and in wettable powder and dust formulations, **5.149–5.153,** incl., be adopted as official.

(17) That the infrared spectrophotometric method for determining the gamma isomer in technical benzenehexachloride, **5.154–5.157,** incl., be adopted as official.

(18) That the work on phenolic disinfectants be continued.

DISINFECTANTS

It is recommended—

(1) That the study be continued of dilution methods for testing disinfectants with particular attention to evaluation of the quaternary ammonium compounds.

(2) That work on the standardization of culture media for disinfectant testing be continued.

(3) That the proposed changes in the method for fungicides be collaboratively tested during the coming year.

PLANTS

It is recommended—

(1) That the Associate Referees continue the study of their assignments.

(2) That collaborative study be made of the ortho-nitrocresol method for the determination of cobalt in plants as modified by Gregory, Morris, and Ellis.

(3) That the investigation of methods of clarification of plant materials preparatory to sugar analysis be continued.

(4) That further study be made of the method for starch as applied to plant materials containing less than 5% starch and that consideration be given to the establishment of appropriate factors for the method.

(5) That the study of the method for zinc be continued with particular regard to improvement of the ashing procedure and that the modified procedure be tested by collaborative studies.

SOILS AND LIMING MATERIALS

It is recommended—

(1) That studies on the "combination dithizone-spectrographic method" and on the polarographic procedure for the determination of zinc in soils be continued.

(2) That the study of the determination of copper in soils be continued.

(3) That the utilization of carmin as an indicator in the determination of boron content of soils be studied further and that p-nitro benzenazo-1, 8-dihydroxy naphthalene-3, 6-disulphonic acid, or "chromotrope-B," be studied as a suitable reagent for the determination of boron in soils.

(4) That studies on the *p*H in soils of arid and semi-arid regions, based upon soil systems of moisture content representative of aid-dried soil, be discontinued.

(5) That further studies on the *p*H of soils at a moisture content approximating the field capacity be carried out.

(6) That the double distillation procedure for fluorine, as set forth in the paper by MacIntire *et al.*, be made first action, and **3.31** and **3.32** deleted.

(7) That the Ca-acetate replacement method of exchangeable hydrogen determination in soil be studied collaboratively in comparison with the ammonium acetate method and with the paranitrophenol method on a number of soils to be selected as representative of the several types of soil colloids.

(8) That the exchangeable hydrogen indications by these methods be checked through the incorporation of finely divided calcite, and against determined *p*H values of the treated soils after allowing a sufficient period of contact for complete decomposition of the added calcite.

(9) That the survey and comparison of methods for the determination of phosphorus (a) that fraction in "available" state and (b) the proportion of organic-inorganic forms therein be continued (*This Journal,* **30,** 43, 1947).

(10) That the survey and comparisons of methods for the determination of exchangeable K in soils be continued (*This Journal,* **30,** 44, 1947).

(11) That the Associate Refereeship on Exchangeable Calcium and Magnesium be maintained.

STANDARD SOLUTIONS

It is recommended that work on this subject be continued.

VITAMINS

It is recommended—

(1) That the study of the method for Vitamin B_{12} content of foods and animal feeds be continued.

(2) That the method for Vitamin A in mixed feeds as described by the Associate Referee be adopted, first action, and subjected to further collaborative study.

(3) That the method for Vitamin D in poultry feed, previously adopted, first action, now be made official.

(4) That the microbiological method for folic acid, adopted first action last year, be made official.

(5) That the chemical method for the determination of nicotinic acid by the sulfanilic acid procedure, with phosphate buffer modification, be adopted, first action.

(6) That studies on carotene analysis be continued.

REPORT OF SUBCOMMITTEE B ON RECOMMENDATIONS OF REFEREES*

G. ROBERT CLARK (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Chairman;* F. O. WILEY; and H. J. FISHER

RADIOACTIVITY

The committee recommends the topic be continued.

SPECTROGRAPHIC METHODS

The committee recommends the topic be continued, as recommended by the Referee.

VEGETABLE DRUGS AND THEIR DERIVATIVES

It is recommended—

(1) *Theobromine and phenobarbital.*—That the proposed spectrophotometric methods be adopted, first action; that the proposed gravimetric method not be adopted; that the topic be closed.

(2) That the following topics be continued in accordance with the recommendations of the Referee:

Aminopyrine, ephedrine, and phenobarbital
Quinine and strychnine
Rutin in tablets.

SYNTHETIC DRUGS

It is recommended—

(1) *Carbromal.*—That the proposed methods for determination of carbromal and for carbromal and pentobarbital be adopted, first action; that the topic be closed.

(2) *Butacaine sulfate.*—That method **32.27** be revised to read "Ointments containing butacaine sulfate in petrolatum or other greasy base"; that the method so revised be adopted, first action; that the topic be closed.

(3) *Synthetic estrogens.*—That the proposed method for diethylstilbestrol in oil be adopted, first action.

(4) *Propadrine hydrochloride.*—That the topic be reassigned and continued.

(5) That the following topics be continued in accordance with the recommendations of the Referee:

Methylene blue
Sulfanilamide derivatives
Propylthiouracil
Diphenhydramine hydrochloride and tripelennamine hydrochloride
Synthetic estrogens
Spectrophotometric methods.

* These recommendations submitted by Subcommittee B were approved by the Association. Unless otherwise given, all references are to *Methods of Analysis*, A.O.A.C., 1950.

MISCELLANEOUS DRUGS

It is recommended—

(1) *Estrone and estradiol.*—That the proposed gravimetric method for the determination of total ketosteroids and the colorimetric methods for determination of estrone, equilin, equilenin, and alpha- and beta-estradiols be adopted, first action; that the topic be closed.

(2) That the following topics be continued in accordance with the recommendations of the Referee:

Microscopic tests for alkaloids and synthetics
Mercury compounds
Organic iodides and separation of halogens
Alkali metals
Glycols and related compounds
Preservatives and bacteriostatic agents in ampul solutions
Methyl alcohol.

COSMETICS

It is recommended—

(1) *Deodorants and anti-perspirants.*—That the proposed methods for chlorides, sulfates, and urea be adopted, first action.

(2) That the following topics be continued:

Deodorants and anti-perspirants
Cosmetic creams
Cosmetic skin lotions
Mascara, eyebrow pencils, and eye shadow
Sun tan preparations
Hair dyes and rinses.

(3) That the topic "Cosmetic Powders" be closed.

COAL-TAR COLORS

It is recommended—

(1) *Intermediates derived from phthalic acid.*—That the proposed methods for the determination of phthalic acid in FD&C Red No. 3, D&C Orange Nos. 5, 6, 7, 8, 9, 10, 11, 12, 13, and 17; D&C Red Nos. 21, 22, 23, 24, 25, and 26; D&C Yellow Nos. 7, 8, and 9; and Ext. D&C Orange No. 2; D&C Yellow No. 10; and D&C Yellow No. 11; and D&C Red No. 19, be adopted, first action.

(2) *Boiling range of amines derived from coal-tar colors.*—That the proposed method applicable to pseudocumidine in FD&C Red No. 1 be adopted, first action.

(3) *Unsulfonated phenolic intermediates in coal-tar colors.*—That the proposed method for β-naphthol be further tested collaboratively, using samples containing known amounts of added β-naphthol.

(4) That the following topics be closed:

Mixtures of colors for drug and cosmetic use
Hygroscopic properties of coal-tar colors.

(5) That the following topics be continued in accordance with the recommendations of the Referee:

Inorganic salts in coal-tar colors
Ether extracts in coal-tar colors
Halogens in halogenated fluoresceins
Identification of coal-tar colors
Volatile amine intermediates in coal-tar colors
Unsulfonated phenolic intermediates in coal-tar colors
Sulfonated amine intermediates in coal-tar colors
Intermediates in triphenylmethane colors
Non-volatile unsulfonated amine intermediates in coal-tar colors
Intermediates derived from phthalic acid
Lakes and pigments
Spectrophotometric testing of coal-tar colors
Subsidiary dyes in FD&C colors
Determination of arsenic and antimony in coal-tar colors
Boiling range of amines derived from coal-tar colors
Determination of heavy metals in coal-tar colors
Sulfonated phenolic intermediates in coal-tar colors
Subsidiary dyes in D&C colors
Paper chromatography of coal-tar colors.

(6) *Coloring matters in foods.*—It is recommended that the topic be closed as a subject of study by a Referee, and that the subject matter be referred to the Referee on coal-tar colors for recommendation of future action.

REPORT OF SUBCOMMITTEE C ON RECOMMENDATIONS OF REFEREES*

J. O. CLARKE (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Chairman;* PAUL A. CLIFFORD; and A. H. ROBERTSON

General Recommendation: The Committee recommends that each General Referee study all methods adopted as first action last year with a view to recommending appropriate action, with collaborative study if necessary, as soon as possible.

PROCESSED VEGETABLE PRODUCTS

It is recommended—

(1) That work on methods for determining quality factors in canned and frozen fruits and vegetables, determination of moisture in dried vegetables, and enzymatic action in frozen vegetables be continued.

(2) That the method for the determination of acetaldehyde as an index

* These recommendations submitted by Subcommittee C were approved by the Association. Unless otherwise given, all references are to *Methods of Analysis, A.O.A.C.*, 1950.

of quality deterioration in frozen vegetables be submitted to collaborative trial.

COFFEE AND TEA

It is recommended—

(1) That methods for chlorogenic acid in coffee be developed.

(2) That more modern methods for the determination of moisture in coffee and tea be developed.

DAIRY PRODUCTS

It is recommended—

(1) That study of the phosphatase test on all types of dairy products be continued.

(2) That the method for ash in milk be amended by changing the amount of sample to be taken for the determination from "about 10 gms." to "about 5 gms.", and that the method be made official.

(3) That methods for sampling of hard cheeses be further studied.

(4) That the method for the determination of acidity of milk be revised in accordance with the suggestions of the Associate Referee and made official.

(5) That the procedure for the preparation of butter samples be revised in accordance with the suggestions of the Associate Referee to allow the use of shaking machines. That at the discretion of the Referee and Associate Referee other methods of preparation, whether or not involving the use of mechanical mixers or use of different temperatures of softening, be studied collaboratively with a view towards a statistical appraisal of their effectiveness.

(6) That study of methods for the detection of reconstituted milk be continued.

(7) That the official acetic serum method and the copper serum method be modified by the deletion of references to specific limiting of refractometer readings and ash values. For interpretation of results the analyst is directed to compare readings with those obtained from local authentic samples, preferably from the same source.

(8) That consideration be given to the desirability of modifying interpretations in the cryoscopic method for the detection of added water in milk.

(9) That analysts take note of the necessity of the use of A. C. S. peroxide-free ether in the Roese-Gottlieb methods for dairy products as pointed out by the Referee. (See Definitions of Terms and Explanatory Notes in Methods of Analysis).

(10) That the description of the Babcock pipette be modified in accordance with the suggestions of the Referee.

(11) That study be undertaken of methods for the preparation of samples of soft cheeses.

(12) That studies be continued on methods of analysis for frozen desserts.

(13) That the topic "Lactic Acid" be transferred to Decomposition and Filth in Foods (Chemical Indices), and that work be continued.

EGGS AND EGG PRODUCTS

It is recommended—

(1) That further work be done on the quantitative determination of glycerol in mixtures of eggs and sugars.

(2) That work on the determination of ammonia nitrogen in eggs be continued along the lines indicated by the Associate Referee.

DECOMPOSITION AND FILTH IN FOODS (CHEMICAL INDICES)

It is recommended—

(1) That methods for the determination of water-insoluble acids and succinic acid in fish and other marine products be studied collaboratively.

(2) That methods for the detection of decomposition in fruits and vegetables be further studied.

(3) That work on the detection of animal and insect fecal matter be continued and subjected to collaborative study where feasible.

GELATIN DESSERT PREPARATIONS AND MIXES

(The work on this subject involves further study of first action recommendations made last year and is covered by the general recommendation of Committee C).

FISH AND OTHER MARINE PRODUCTS

It is recommended—

(1) That work be continued on methods for the determination of total solids in fish.

METALS, OTHER ELEMENTS, AND RESIDUES IN FOODS

It is recommended—

(1) That work on methods for copper and zinc be continued.

(2) That the two methods for mercury studied by the Associate Referee be evaluated by further collaborative study.

(3) That the effect of canning and other processes on the decomposition of the newer pesticides be further studied with respect to the nature of possible decomposition products and their effects on methods of analysis.

(4) That studies on the determination of parathion be continued.

(5) That the quantitative method for sodium fluoroacetate (1080) be further studied for the purpose of improving its accuracy and that further work be done on a sensitive qualitative test for 1080.

(6) That studies on the determination of methoxychlor and the differentiation between methoxychlor and DDT in plant and animal products be continued.

GUMS IN FOOD

It is recommended—

(1) That the method for the detection of gums in soft cheeses (except algin) as suggested by the Referee be adopted as official.

(2) That work be done on the detection and estimation of algin in cheese.

(3) That work be continued on methods for the detection of gums in cacao products, frozen desserts, starchy salad dressings, catsup and related products.

MEAT AND MEAT PRODUCTS

It is recommended—

(1) That collaborative studies be continued on the detection of horse meat. The Committee suggests that serological studies in addition to the methods already suggested be undertaken.

NUTS AND NUT PRODUCTS

It is recommended—

(1) That methods for moisture, crude fat, crude protein, crude fiber, ash, reducing sugar, and salt be further studied.

(2) That the changes suggested by the Referee for the preparation of sample be adopted.

(3) That sorting methods for moisture and fat be studied.

(4) That methods for added starch and other additives in peanut butter be studied.

(5) That methods for added glycerol and propylene glycol in shredded coconut be studied.

(6) That chemical methods for decomposition in nuts be studied.

MICROBIOLOGICAL METHODS

It is recommended—

(1) That work be continued on canned meats, canned acid foods, canned vegetables, eggs and egg products, nuts and nut products, frozen fruits and vegetables, sugar, and canned fishery products.

MICROCHEMICAL METHODS

It is recommended—

(1) That the methods proposed by the Referee for carbon and hydrogen be adopted, first action.

(2) That the Referee study methods for sulfur, the halogens, and phosphorus.

(3) That the method for nitrogen be further studied.

OILS, FATS AND WAXES

It is recommended that—

(1) That studies on quantitative methods for peanut oil be continued.

(2) That studies on spectroscopic methods for the analysis of oils be continued.

(3) That collaborative work on various methods for antioxidants in oils be conducted.

(4) That the present method for coal-tar colors in oils (**26.51, 26.52**) be deleted and that the method proposed by the Associate Referee be made first action; and that further collaborative work on the modified method for coal-tar colors be conducted.

SPICES AND OTHER CONDIMENTS

It is recommended—

(1) That studies of methods for the detection of caramel in vinegar be continued.

(2) That studies on the determination of tartrates in vinegar be continued.

(3) That the official method for "Permanganate Oxidation Number" be further studied.

(4) That methods for the determination of free mineral acids in vinegar be further studied.

(5) That methods for the determination of sorbitol be studied.

(6) That methods for the determination of ash, sugars, and ether extract in prepared mustard be continued.

(7) That studies of methods for the determination of pungent principles in prepared mustard and mustard flour be continued.

(8) That the official method for total nitrogen in mayonnaise and salad dressing be amended as suggested by the Associate Referee and further studied.

(9) That methods developed by the Associate Referee for the detection of seeds and stems in chili be further studied and submitted to collaborative trial.

(10) That methods of preparation of sample and sampling of french dressing be studied.

ENZYMES

It is recommended—

(1) That work on hydrocyanic glucosides be continued.

(2) That new or more rapid methods along the lines indicated be devised and studied.

(3) That methods for testing the activity of the various enzymes used in analytical procedures be studied for the purpose of inclusion in the chapter on enzymes.

(4) That methods for the preparation of enzymes, which may not be available commercially, be studied.

REPORT OF SUBCOMMITTEE D ON RECOMMENDATIONS OF REFEREES*

KENNETH L. MILSTEAD (Food and Drug Administration, Cincinnati, Ohio), *Chairman;* J. WALTER SALE; and C. S. FERGUSON

Malt Beverages, Brewing Materials, and Allied Products:

It is recommended—

(1) That the study of methods for the determination of essential oils and resins in hops be continued.

(2) That the methods described in the 1950 report of the Associate Referee on Color in Beer and Wort, for the spectrophotometric and photometric determination of color in beer, be adopted, first action.

(3) That study of beer turbidity methods be continued.

(4) That methods either of removing or compensating for turbidity in samples for color measurement and for color in samples for turbidity measurement be studied.

(5) That methods for the degassing of beer in such a manner as to result in no change in either color or turbidity be studied.

(6) That the Mathers test for caramel, **10.36,** be adopted as official for beer.

(7) That the methods for carbon dioxide in beer, **10.23–10.27,** be adopted as official.

(8) That work be continued on polarographic and spectrographic methods for tin in beer.

(9) That studies be continued on the determination of iron in beer, giving attention to both the wet-ashing orthophenanthroline procedure and the direct non-ashing procedure as suggested by the Associate Referee.

Wines:

It is recommended—

(1) That chromatographic studies of wine be continued.

Distilled Liquors:

It is recommended—

(1) That the study of colorimetric methods for fusel oil be continued.

(2) That the official method, **9.29,** for methanol by the immersion refractometer method, be studied in the light of the findings of Beyer and Reeves, *This Journal,* **28,** 800 (1945).

(3) That study be continued on the methods for methanol in distilled liquors and drugs with a view to their correlation.

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* These recommendations submitted by Subcommittee D were approved by the Association. Unless otherwise given, all references are to *Methods of Analysis, A.O.A.C.,* 1950.

(4) That the method for aldehydes be made official.

(5) That the Fulton test for caramel, **9.35, 9.36,** be made official for distilled liquors and for cordials and liqueurs.

Cordials and Liqueurs:

It is recommended—

(1) That the following methods be adopted as official:

Total solids by evaporation, 9.46(b).
Caramel (Mathers test), 9.54.

(2) That the method for citric acid described in the Associate Referee's report on Fruits and Fruit Products be adopted as first action for cordials and liqueurs to replace present first action method **9.58.**

CACAO PRODUCTS

It is recommended—

(1) That work on pectic acid be continued.

(2) That the modified acid hydrolysis method, **12.23,** be made official, and that the subject be closed.

(3) That the work on methods for determination of maltose in cacao products be continued.

(4) That work be continued on methods for the determination of lactose in cacao products containing dextrose, malt, or corn sirup solids.

(5) That work be continued on methods for the determination of cacao constituents, particularly theobromine, cacao red, and tannins.

(6) That the method for lecithin, *This Journal,* **32,** 168 (1949), be subjected to further collaborative study.

CEREAL FOODS

It is recommended—

(1) That the studies on the determination of starch in raw and cooked cereals be continued.

(2) That the study of the modification of method **13.30–13.32,** incl., and for the determination of reducing and non-reducing sugars in bakery products, as recommended in the Associate Referee's report for last year, be continued.

(3) That the method for benzoic acid in flour in the Associate Referee's report last year, be made official.

(4) That the methods for the determination of lactose in bread be studied further.

(5) That the work for the determination of proteolytic activity of flour be continued.

(6) That the study on methods for soybean flour, for moisture, ash, nitrogen, crude fiber, and oil or petroleum benzine extract, be continued.

(7) That the method referred to in *This Journal,* **25,** 83 (1942), for the

determination of unsaponifiable matter and sterols in noodles be studied to determine its applicability to bakery products containing eggs.

(8) That a study of methods for the determination of albumen in noodles and macaroni products be conducted.

(9) That the study on determination of moisture, fat, crude fiber, ash, and protein in bakery products be continued.

(10) That the procedure presented by the Associate Referee on the determination of moisture (loss of weight by drying) in flour products containing sodium bicarbonate as one of its constituents, be adopted, first action.

(11) That the study on the determination of bromates in flour be continued.

(12) That the procedure proposed by the Associate Referee for the determination of acetic and propionic acids in bread be adopted as first action, and the study continued.

(13) That Method I given in the Associate Referee's report on nitrites in flour, be adopted as first action, and that methods **13.43** and **13.44** be dropped.

(14) That the methods given in the Associate Referee's report on carotene in noodles be adopted as first action, and that methods **13.118,** and **13.119** be dropped.

(15) That the applicability to baked products of method for sugars in flour, **13.30–13.32,** be studied.

BAKING POWDERS AND BAKING CHEMICALS

It is recommended—

(1) That the methods for anhydrous monocalcium phosphate, monocalcium phosphate monohydrate, and sodium acid pyrophosphate, given in the Referee's report, be adopted as first action, and that methods **7.8** and **7.9** be dropped.

(2) That the qualitative test Method II, given in the Referee's report, be adopted as first action, and that methods **7.18** and **7.19** be dropped.

EXTRANEOUS MATERIALS IN FOODS AND DRUGS

It is recommended—

(1) That the method for mold in crushed pineapple, described in the report of the Associate Referee, be studied collaboratively.

(2) That the method for mold count in citrus juices, **35.5,** modified for use on pineapple juice, as described in the report of the Associate Referee on Extraneous Matter in Beverage Materials, be made first action for pineapple juice.

(3) That the modification described in the Associate Referee's report on Extraneous Materials in Drugs, Spices and Miscellaneous Products, for extraneous matter in onion powder, **35.85,** be studied collaboratively.

(4) That the method for staining with a combination Universal textile

stain of plant and dung fragments in dairy products, be studied further.

(5) That the method given by the Associate Referee for differentiation between plant and dung fragments in cheese be studied collaboratively.

FLAVORS AND NON-ALCOHOLIC BEVERAGES

It is recommended—

(1) That the photometric method for vanillin in imitation vanilla, as given in the report of the Associate Referee on Vanilla Extract and Imitations, be adopted as first action.

(2) That the photometric method for coumarin in imitation vanilla, as given in the report of the Associate Referee on Vanilla Extract and Imitations, be adopted as first action.

(3) That the study of the photometric methods for vanillin and coumarin be continued, particularly in regard to their application to true vanilla extracts.

(4) That the method for "Determination of Propylene Glycol in Vanilla Extracts," *This Journal*, **33**, 103 (1950), be studied collaboratively.

(5) That collaborative studies be continued on the following:

a. Reflux method for peel oil in citrus fruit juices.
b. Beta ionone where small amounts are present.
c. Method for isopropyl alcohol in the presence of acetone.
d. Method for oil in emulsion flavors.

FRUITS AND FRUIT PRODUCTS

It is recommended—

(1) That the method for citric acid described in the report of the Associate Referee be adopted as first action and that the present first action method, **20.37, 20.38,** for citric acid be dropped.

(2) That the work on methods for the separation and determination of fruit acids be continued.

(3) That procedure **20.2 (a),** Preparation of Sample, Juices, be changed to read:

"Mix thoroly by shaking to insure uniformity in sampling, and filter thru absorbent cotton or rapid filter paper. Prep. fresh juices by pressing well-pulped fruit and filtering. Express juice of citrus fruits by one of the common devices used for squeezing oranges or lemons, and filter."

(4) That work on methods for the examination of frozen fruits and fruit products be continued.

PRESERVATIVES AND ARTIFICIAL SWEETENERS

It is recommended—

(1) That the method for determination of monochloroacetic acid in beverage bases containing halogenated weighting oils, proposed by the Associate Referee, be studied collaboratively.

(2) That further work be done on the determination of monochloroacetic acid in fruit juices other than orange juice.

(3) That collaborative study be continued on the following quantitative methods for the determination of quaternary ammonium compounds.

(a) Method for Fruit Juices, *This Journal*, **29**, 318 (1946).
(b) Shorter Method for Fruit Juices, *This Journal*, **29**, 319 (1946).
(c) Method for Bottled Sodas, *This Journal*, **29**, 323, subject to increasing the volume of bromophenol blue reagent to 5–10 ml.
(d) Method for Milk, *This Journal*, **29**, 324, on samples containing preservative quantities of quaternary ammonium compounds.
(e) Method for Mayonnaise, Salad Dressings, and Sandwich Spreads, *This Journal*, **29**, 323 (1946).
(f) Method for Pickles and Relishes, *This Journal*, **29**, 326 (1946).

(4) That work be continued on the determination of quaternary ammonium compounds in shrimp.

(5) That work on formaldehyde be discontinued.

(6) That the subject of mold inhibitors, propionates, be transferred to Cereal Foods, with a cross reference under "Preservatives and Artificial Sweeteners."

(7) That work on formic acid as a preservative be discontinued and that the method be deleted from the chapter on preservatives.

(8) That studies on qualitative methods for fluorides be continued.

(9) That an Associate Referee be appointed to develop methods for the detection and determination of "cyclamate sodium" or "sucaryl."

(10) That studies be continued on the Denigès-Tourrou method and the modified LaParola-Mariani method for the detection of dulcin.

(11) That work be initiated on methods for the detection and estimation of propoxy, 2-amino, 4-nitrobenzene.

SUGARS AND SUGAR PRODUCTS

It is recommended—

(1) That the study of methods for the determination of moisture be continued.

(2) That the study be continued on tables of density of solutions of sugar at various temperatures.

(3) That the study of methods for the detection of adulteration of honey be continued.

(4) That methods for the determination of reducing sugars including those employing chromatographic separation be studied.

(5) That the Zerban and Martin values for refractive indices of dextrose and invert sugar solution, **41.10**, be made official.

(6) That the methods, **29.132–29.154**, incl., adopted as first action last year, be studied further.

(7) That the procedures for the measurement of transmittancy of solution of commercial sugar products be studied.

(8) That the first action, modified micro-method for dextrose, **29.61–29.63,** be adopted as official.

WATERS, MINERAL AND SALT

It is recommended—

(1) That the first action method for boron in water be studied collaboratively.

(2) That a colorimetric method for phosphates in water be studied.

(3) That further study on methods of fluorine in salt be dropped.

CHANGES IN OFFICIAL METHODS OF ANALYSIS MADE AT THE SIXTY-FOURTH ANNUAL MEETING, OCTOBER 2, 3, AND 4, 1950*

The changes recorded in the methods of the Association, as given below, become effective, as provided in Sec. 8 of the by-laws, on the thirtieth day from the date of publication of this Report, Feb. 15, 1951.

Chapter numbers and titles and references of sections are to those in the seventh edition of "Official Methods of Analysis, 1950," unless otherwise indicated.

1. AGRICULTURAL LIMING MATERIALS

No additions, deletions, or other changes.

2. FERTILIZERS

(1) The official citrate-insoluble phosphoric acid method, **2.16** and **2.17** (pp. 10, 11), using continuous agitation during the citrate digestion, was adopted as official for evaluating alpha phosphate (defluorinated phosphate rock) and phosphate rock-magnesium silicate glass.

3. SOILS

(1) The following method for fluorine was adopted, first action, and the first action method for fluorine, **3.31** and **3.32** (p. 40), was deleted.

REAGENTS

(a) *Buffer soln.*—Dissolve 9.448 g monochloracetic acid in 50 ml water and add 2 g NaOH dissolved in 50 ml water.

(b) *Sodium alizarin sulfonate indicator.*—0.05% aqueous soln.

(c) *Thorium nitrate soln.*—0.02 *N* aqueous soln; titrate against standard NaF soln and prepare standard curve. (Titration solns of 0.01 *N* and 0.05 *N* are desirable for low or high fluorine values, respectively.)

DETERMINATION

For soils of high F content, use 0.5-g charge; for those low in F, use 1 g. Transfer soil charge into 125-ml Claisen flask fitted with thermometer and steam inlet tube. Connect flask to condenser provided with delivery tube which dips below surface of a few ml of dilute NaOH in 600-ml beaker. Moisten charge with 5 ml water and add 50 ml 1+1 H_2SO_4. Close flask; apply heat and raise temperature to 165°C. Introduce steam current and collect 500 ml of distillate, which should be kept slighty alkaline to phenolphthalein by addition of dilute NaOH soln. Concentrate distillate to 10–15 ml and transfer to Claisen distillation flask. Add 25 ml 70% $HClO_4$ containing 0.2% Ag_2SO_4 and steam distill at 130°C.–135°C. Collect ca 200 ml of distillate in a wide-mouth Erlenmeyer flask and make to volume in volumetric flask.

Introduce 25-ml aliquot into 150-ml beaker; dilute to 100 ml with distilled H_2O. Add 2 ml of sodium alizarin sulfonate soln; neutralize with .05 *N* NaOH until pink color appears and then add 1 ml of the buffer soln. Titrate with .02 *N* $Th(NO_3)_4$ to

* Unless otherwise given all references in this Report are to *Methods of Analysis, A.O.A.C.*, 1950.

to faint salmon-pink color. Calculate the results by means of the curve and express as p.p.m. fluorine.

4. CAUSTIC POISONS

No additions, deletions, or other changes.

5. ECONOMIC POISONS

The following method for the determination of organic thiocyanate nitrogen in livestock or fly sprays was adopted, first action.

REAGENTS

(a) *Strong potassium polysulfide soln.*—Dissolve 180 g of KOH in 120 ml of water. Saturate 100 ml of this soln with H_2S (about 42 g) while cooling. Add the other 100 ml of KOH soln and 80 g of sulfur. Shake until dissolved.

(b) *Sodium sulfide.*—($Na_2S \cdot 9H_2O$).

(c) *Mixed sulfide soln.*—To 100 ml of (a) add 50 g of (b), 30 g of KOH, and 200 ml of water.

(d) *Sodium bisulfite.*—($Na_2S_2O_5$ or $NaHSO_3$).

(e) *Sulfur dioxide.*

(f) *Copper sulfate soln.* (20% $CuSO_4.5H_2O$).

(g) *Potassium hydroxide soln.*—(10%).

(h) *Sulfuric acid.*—(1+4).

(i) *Wash soln.*—To 300 ml of water add 1 ml of (h), 1 g of (d), 10 ml of (f), 12 g of Na_2SO_4, and pass SO_2 into the soln for 10 min.

DETERMINATION

Weigh an amount of sample preferably containing ca 0.03 g of thiocyanate nitrogen into a 250-ml glass-stoppered Erlenmeyer flask. (If the percentage is very low, the weighed amount should not be increased unduly without correspondingly increasing the quantity of mixed sulfide soln (c) used; 20–25 g of fly spray is usually sufficient.) Add 35 ml of the mixed sulfide soln (c). Shake vigorously at room temp. for 10 min., during which time reaction is nearly completed; next heat to 70°C. on a steam bath, carefully releasing the pressure resulting from heating; and shake at temp. of 70°C. for 15 min. more. Cool.

Removal of petroleum oil.—Dilute and transfer to a separatory funnel with about 200 ml of water. Add 50 ml of petroleum ether, shake, and draw off the aqueous layer into a 600-ml beaker. Wash the petroleum ether layer with two 10-ml portions of water, which add to the main soln. (If emulsions occur during the washing process, they may be broken by acidifying with H_2SO_4 (1+4). The aqueous layer may then be drawn off and the petroleum ether layer washed with water as directed.) Discard the petroleum ether layer.

Determination of thiocyanate nitrogen.—Dilute the water soln to about 300 ml and neutralize with H_2SO_4 (1+4), using a piece of litmus paper as outside indicator. Add 2 ml of H_2SO_4 (1+4). Bring the mixture to a boil quickly and boil for 8 min. to remove H_2S. Cool. If fatty acids or other oils are present at this stage, transfer to a separatory funnel, extract with petroleum ether, and return the aqueous phase to the original beaker. Filter thru a small Büchner funnel and transfer the filtrate to a beaker. Neutralize to litmus paper with KOH 10% and add 1 ml of H_2SO_4 (1+4). Add about 1 g of $Na_2S_2O_5$ and stir until dissolved. Add excess (about 15 ml) of 20% $CuSO_4.5H_2O$ soln and pass SO_2 into the soln for 10 min. Allow the precipitated cuprous thiocyanate to settle for 2 hours and filter with suction thru a 2-inch Büchner funnel. Coat the Büchner funnel with a layer of asbestos fibers,

upon which is placed a No. 42 Whatman filter paper, a second layer of asbestos fibers, a layer of diatomite, and finally a third layer of asbestos fibers. If the filtrate is not clear, centrifuge the sample at 2000 r.p.m. for 10–15 min. and pour thru the filter a second time. Wash the filter and precipitate once or twice with the wash soln, continue suction until filter pad is dry, and transfer to an 800-ml. Kjeldahl flask. (This may conveniently be done by folding it in a filter paper together with bits of moist filter paper used to wipe out the Buchner funnel. Then place the whole in the Kjeldahl flask.) Add a few glass beads, 35 ml of concentrated H_2SO_4, 10 g of K_2SO_4, and ca 0.7 g of HgO or 0.6 g of metallic Hg. Digest until white and for 15 min. thereafter. Determine the nitrogen as in method **2.22,** beginning with the words "After cooling, dilute. . . . " Run a blank analysis on the paper and filtering pad.

(2) The first action method for the determination of 2,4-dichlorophenoxyacetic acid, **5.129** (p. 77), was adopted as official with the addition of the following note:

"If the material is slightly acidic, transfer sample to a 250-ml beaker, add 50 ml of H_2O, neutralize with 10% NaOH and add 5 ml in excess. Proceed as directed in (b) beginning 'warm and stir 15 min'."

(3) The first action method for tetraethyl pyrophosphate, **5.158–5.160,** (p. 87), was adopted as official.

(4) The first action partition chromatographic method for hexachlorocyclohexane gamma isomer, in technical benzene hexachloride and wettable powder and dust formulations, **5.149–5.153** (p. 84), was adopted as official.

(5) The first action infrared spectrometric method for the gamma isomer in technical benzenehexachloride, **5.154–5.157,** incl. (p. 86), was adopted as official.

6. PLANTS

No additions, deletions, or other changes.

7. BAKING POWDERS AND BAKING CHEMICALS

(1) The first action methods for neutralizing values of monocalcium phosphate, **7.8** (p. 119), and of sodium acid pyrophosphate, **7.9** (p. 120), were deleted and the following adopted, first action.

Monocalcium Phosphate

Weigh 0.84 g of monocalcium acid phosphate into 375 ml casserole. Add 24 ml of cold H_2O, and after stirring for moment add exactly 90 ml of 0.1 *N* NaOH. Bring suspension to boil in exactly 2 min. and boil 1 min. While soln is still boiling hot add 1 drop of phenolphthalein indicator, **4.6(a),** and back titrate with 0.2 *N* HCl until all pink color has disappeared. Boil soln 1 min. and again add 0.2 *N* HCl until pink color has just disappeared. Multiply number of ml of 0.2 *N* HCl used by 2 and subtract from 90; difference is neutralizing value, parts of $NaHCO_3$ equivalent to 10C parts of the phosphate.

Anhydrous Monocalcium Pyrophosphate

Use 100 ml of 0.1 *N* NaOH and stir intermittently for 5 min. before bringing to boiling. Otherwise follow above method for monocalcium phosphate.

Sodium Acid Pyrophosphate

Weigh 0.84 g of sodium acid pyrophosphate and 20 g of pure NaCl into 375 ml casserole and add 25 ml of H_2O slowly while stirring. Stir and crush with flattened end of a stirring rod for 3–5 min. Add 90 ml of 0.1 *N* NaOH and 1 drop of phenolphthalein indicator, 4.6(a), and titrate with 0.2 *N* HCl to disappearance of pink color. If a "starch filled" or 50% neutralizing strength pyrophosphate is being titrated, use 70 ml of the NaOH. Multiply number of ml of 0.2 *N* HCl used by 2 and subtract from number of ml of 0.1 *N* NaOH used; difference is neutralizing value, parts of $NaHCO_3$, equivalent to 100 parts of sodium acid pyrophosphate.

(2) The following qualitative test for aluminum was adopted, first action, and first action methods for aluminum, **7.18,** and **7.19** (p. 121) were deleted.

Method II

Place 1 g baking powder in 250 ml beaker, add 5 ml HCl, 20 ml H_2O, heat until starch is hydrolyzed. Add 100 ml cold H_2O and 5 ml of 10% sodium ammonium phosphate. Add 3 drops 0.1% methyl orange. Add NH_4OH dropwise until ppt forms or until color changes and then add HCl dropwise until ppt dissolves or until color changes. Add 2 or 3 drops excess HCl. Add 5 ml 0.1% aurin tricarboxylic acid and let stand 1 min. Add 50% ammonium acetate dropwise until ppt forms or color changes and then add 1 ml excess. Let stand 5 min., stirring occasionally and filter a portion of soln. A bright red ppt on filter paper shows aluminum is present.

8. BEVERAGES: NON-ALCOHOLIC AND CONCENTRATES

No additions, deletions, or other changes.

9. BEVERAGES: DISTILLED LIQUORS

(1) The first action method for aldehydes, **9.18** (p. 130), was adopted as official.

(2) The first action method for citric acid in cordials and liqueurs, **9.58,** (p. 137), was replaced by the method adopted for citric acid in fruits and fruit products, first action (details given on p. 330).

(3) The first action Fulton test for caramel in distilled liquors, **9.35** and **9.36** (p. 134), and the Mather's test for cordials and liqueurs, **9.54** (p. 136), were made official.

(4) The first action method for total solids by evaporation in cordials and liqueurs, **9.46** (*b*) (p. 135), was made official.

10. BEVERAGES: MALT BEVERAGES, SIRUPS AND EXTRACTS, AND BREWING MATERIALS

(1) The following methods described in the 1950 report of the Associate Referee on color in beer and wort, for the spectrophotometric and photometric determination of color in beer, were adopted, first action.

I. SPECTROPHOTOMETRIC METHOD (S R C METHOD)

APPARATUS

A precision spectrophotometer having a band width of 1 mmμ or less at 430 mmμ and whose wave length and photometer scales have been checked and cor-

rected for inaccuracies in accordance with the instructions contained in the Bureau of Standards Letter Circular LC-929 of November 26, 1948.

PREPARATION OF SAMPLE

Partially degas the beer by opening the bottle at room temperature, pouring contents into a 1 liter Erlenmeyer flask and swirling gently. Avoid formation of turbidity and conduct the partial degassing and readings as rapidly as possible.

DETERMINATION

Place the degassed beer in a suitable cell and determine its optical density at 430 mmμ and at 700 mmμ.

Calculate the optical densities from the thickness at which read to $\frac{1}{2}$ inch. (O. D. $\frac{1}{2}$). Multiply the O. D. $\frac{1}{2}$ at 430 mmμ by 0.039. If the product exceeds the value for O. D. $\frac{1}{2}$ at 700 mmμ the beer is assumed "free of turbidity" and the color calculated as follows:

$$\text{Beer Color Intensity} = 10(\text{O. D. } \tfrac{1}{2} \text{ at 430 mm}\mu)$$

If the O. D. $\frac{1}{2}$ at 700 mmμ is more than 0.039 times the O. D. $\frac{1}{2}$ at 430 mmμ then use the following formula:

$$\text{Beer Color} = 10[(\text{O. D. } \tfrac{1}{2} \text{ at } 430 - \{(\text{O. D. } \tfrac{1}{2} \text{ at } 700) - 0.039\ (\text{O. D. } \tfrac{1}{2} \text{ at } 430)\}]$$

Report color values to the nearest 0.1 unit.

II. PHOTOMETRIC METHOD

APPARATUS

Use any commercially available filter photometer or abridged spectrophotometer utilizing a moderately broad spectral band and having adequate sensitivity. Use a light filter having peak transmission in the range 420–450 mmμ for maximum sensitivity and precision. The cell size should be of such size, if possible, as to give O. D. values between 0.187 and 0.699 (20–65% T) where maximum precision is achieved. Use the same size cell for both color measurement and calibration.

Calibration of Photometers

A. Beer calibration method

(1) For each color value of which measurements are to be made (Note 1) obtain four or more replicate bottles of beer which are low in air content (Note 2) and have been pasteurized.

(2) Determine the color value of the beer by averaging the readings obtained for at least two bottles by the Standard Reference Color method (Note 3).

(3) Determine the photometer reading of the beer by averaging the readings obtained for at least two bottles using the light filter and cell selected as above.

(4) Calculate a calibration factor in accordance with the photometer instructions. A calibration curve may be prepared by plotting the O. D. value or photometer scale reading versus the SRC for the beer, assuming that the curve passes thru the origin. This calibration will be accurate only for readings in the immediate vicinity of the calibration point (Note 1).

NOTE 1: If it is desired to measure accurately the color of more than one beer or colors over a range of values, it is necessary to calibrate the photometer for each of them or with beers having colors which cover the desired range. An average calibration factor can then be calculated or a calibration plot prepared.

NOTE 2: Air can be removed from the bottles before pasteurization by hand foaming and recapping three times. The bottles should be allowed to stand 15 to 30 minutes between each operation. An alternative method is to "jet" the bottles

with pure carbon dioxide so as to fill the headspace with fine bubbled foam with some overflow. Follow this by immediate capping before the foam has subsided.

NOTE 3: If it is necessary to obtain these values from another laboratory, the bottles should be shipped by the fastest method available and marked to avoid rough handling.

B. Potassium dichromate calibration method

(Restrict use to beers below 4 units of beer color intensity)

Prepare solutions of potassium dichromate in 0.01 *N* H_2SO_4 of the strength noted in the following tabulation:

Color Unit	*Grams per liter*
1	0.0451
2	0.0902
3	0.1352
4	0.1803
5	0.2254
6	0.2705

Read the various strength dichromate solutions in an abridged photometer equipped with a light filter of the proper characteristics.* Use the same size cell in the calibration as that used in the later color determinations. Calculate an average factor or plot a curve for converting instrument readings to color units by the method acceptable for the particular photometer.

PREPARATION OF SAMPLE

Prepare sample as described under I. Spectrophotometric Method.

DETERMINATION

Place sample in reading cell and determine the photometer reading. Calculate the color value using the calibration factor or calibration curve. Report color to the nearest 0.1 unit.

(2) The first action (Mather's test) for caramel in beer, **10.36** (p.147), was made official.

(3) The first action methods for carbon dioxide in beer, **10.23–10.27,** incl. (p. 142), were made official.

11. BEVERAGES: WINES

No additions, deletions, or other changes.

12. CACAO BEAN AND ITS PRODUCTS

(1) The first action method for fat, **12.23** (p. 184), was made official.

13. CEREAL FOODS

(1) The first action method for benzoyl peroxide bleach (benzoic acid), **13.45** (p. 205), was adopted as official.

(2) The official vacuum-oven, **13.3,** and air-oven, **13.4** (p. 192), methods for total solids were adopted as official for moisture (loss in weight by drying) in flour mixes containing sodium bicarbonate as an ingredient.

* A color filter having a peak transmission at 435 mμ ± 5 mμ and a band width at 50 per cent transmission of 35 mμ ± 15 mμ is required. The size of cell employed should be selected to give readings of acceptable precision.

(3) The following method for the determination of acetic and propionic acids in bread was adopted, first action.

I. PREPARATION OF SAMPLE

(a) *Air-dried bread.*—For analysis of the air-dried product prepare sample as directed in section **20.83**, 6th Ed. *Methods of Analysis.*

(b) *Fresh bread or cake.*—For analysis of the fresh product which may be difficult to air dry without spoilage or loss of volatile fatty acids, pass the sample thru a meat grinder equipped with a $\frac{1}{8}$" hole plate and reduce to a finely divided condition by rubbing thru an 8-mesh sieve. Proceed with the analysis promptly (24–48 hrs) or preserve with chloroform.

(c) *Fresh bread or cake, preserved with chloroform.*—To the bread prepared as in (b) in a Mason jar filled to $\frac{3}{4}$ capacity add washed chloroform adsorbed in ca 1 g of cotton (ca 5 ml $CHCl_3$ per pint of container size). Close the jar tightly (self-sealing lids are recommended) and mix the contents thoroly by rolling. Samples may be stored at temp. of ca 25°C. Refrigeration is recommended where higher temp. obtain.

II. REAGENTS

(1) *Silicic acid.*—Reagent grade.[1]

(2) *Indicators:*

(a) *Alphamine Red R.*—(also referred to as "RNH_4 indicator"); 0.2% in water.

(b) *Cresol Red.*—Dissolve 50 mg in 20 ml of ethyl alcohol, add 1.3 ml of 0.1 *N* NaOH and make to 50 ml with water.

(c) *Phenolphthalein.*—1% in 95% ethyl alcohol.

(3) *Solvents:*

(a) 1% *n-butanol-chloroform.*—Remove ethyl alcohol from U.S.P. chloroform by washing it three times with distilled water, using a quantity of water equal to about $\frac{1}{2}$ the volume of chloroform. To 1 liter of washed chloroform in a separatory funnel add 10 ml of *n*-butanol (acid free) and shake vigorously. Add 25 ml of water and shake again. Allow chloroform to stand until clear, draw off and discard water. (Referred to as "CB-1.")

(b) *10% n-butanol chloroform.*—Mix 900 ml U.S.P. chloroform, 100 ml *n*-butanol and 25 ml water in a separatory funnel. Shake vigorously and separate from water. (Do not wash chloroform to remove ethyl alcohol.) (Referred to as "CB-10.")

(4) *Alkali solutions:*

(a) *NaOH.*—ca 1 *N.*

(b) NH_4OH.—ca 1 *N.*

(c) $Ba(OH)_2$.—0.01 *N.* Store in a paraffin lined bottle protected from CO_2 by soda lime absorbent. Dispense from a 10 ml burette.

(5) *Acids:*

(a) H_2SO_4 (1+1) *or* H_3PO_4.—85%.

(b) *Formic, acetic, and propionic acids of reagent grade.*

(6) *Miscellaneous:*

(a) *Sodium sulphate.*—Anhydrous.

III. APPARATUS

(a) *Distillation app.* The Hillig volatile acid apparatus (6th Ed. **24.10**, Fig. 37) can be used to good advantage for the present purpose. Use a 150 ml distillation

[1] Mallincrodt's "Acid Silicic for Chromatography $SiO_2 \times H_2O$ Analytical Reagent Water-Max 20%" was found to be satisfactory. The Associate Referee used 1.5 ml H_2O per 5 g of the acid.

flask and so adjust the voltage (ca 80) that 200 ml is distilled in 35–40 min. An ordinary gas fired steam generator may also be used.

(b) *Chromatographic tube* (ca 15×250 mm) constricted at lower end to ca 4 mm inside diam.

(c) *Test tubes, glass stoppered*—(ca 16×150 mm.)

(d) *Eyedropper pipette* (sufficient length to transfer solvent from test tubes to top of silicic acid column.)

IV. DETERMINATION

Distillation:

Transfer 10 g of the air-dried bread or 15 g of the fresh bread to a 150 ml distilling flask. Add 50 ml of H_2O and 10 ml of ca 1 *N* H_2SO_4. Mix thoroly and add 10 ml of 20% phosphotungstic acid soln. Mix by swirling and add 40 g of $MgSO_4 7H_2O$. Swirl again to partially dissolve salt. The mixture should now be acid to Congo Red paper; if not, acidify with H_2SO_4 (1+1). Connect to condenser and steam generator, heat to boiling, and distill 200 ml in 35–30 min.[2] Maintain a volume of ca 60–80 ml in the distilling flask by means of a small burner. Transfer the distillate to a 400-600 ml beaker, add ca 10 ml of 0.01 *N* formic acid,[3] make alkaline to phenolphthalein with ca 1 *N* NaOH and evaporate to ca 5 ml. Transfer to a g.s. test tube of 25–30 ml capacity, rinsing beaker with three 5-ml portions of H_2O. If insoluble material adheres, add a few drops of ca 1 *N* H_2SO_4 with one rinse. Make alkaline to phenolphthalein and evaporate just to dryness by inserting the tube in a steam bath or in boiling water (air jet hastens evap.). Determine acetic and propionic acids by the following described chromatographic procedure.

Chromatographic Separation:

(a) *Preparation of partition column.*

To ca 5 g of silicic acid in a mortar add 1 ml of "RNH_4" indicator soln and sufficient ca 1 *N* NH_4OH to give the alkaline color of the indicator (1–2 drops are usually sufficient). Add the maximum quantity of H_2O that the silicic acid will hold without becoming sticky or agglomerating in the butanol-$CHCl_3$ soln. This quantity must be determined for each batch of silicic acid and usually varies from 50–75% of wt. of silicic acid. Mix thoroly with pestle until homogeneous. Add a few ml of the 1% butanol-$CHCl_3$; mix to form a paste, and then add sufficient solvent (ca 25 ml) to form a slurry that will pour readily. Pour this slurry into the chromatographic tube containing a small plug of cotton in the constricted end. To avoid air pockets, tilt the tube slightly while pouring. If air bubbles do form, eliminate by stirring suspension in tube with a long glass rod.

Clamp tube in ring stand in vertical position. In top insert 1-holed rubber stopper fitted with glass tube bent to 90° angle and held in place by Bunsen clamp. Connect glass tube to pressure source and adjust pressure to 5–10 lb/sq. in. so that excess solvent is forced thru column dropwise.

During removal of excess solvent the gel will pack down. After gel can no longer be poured, more excess solvent can be forced from the column. As column packs down particles of gel will adhere to walls of the tube, but eventually gel will leave walls of tube relatively clean. At this point optimum density for column has been reached, and column is ready for use. Do not allow column to dry below sur-

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[2] The steam source may be connected during the heating to boiling. This will prevent the steam tube from becoming clogged with bread solids.

[3] The small amount of formic acid normally present in bread is not sufficient to provide a distinct band in the chromatographic column that can be moved down to a definite threshold. The addition of ca 1 ml 0.1 *N* formic acid provides a band that can be moved to this point, thus insuring the complete elution of the preceding acetic acid.

face of the gel as such drying or "cracking" renders column useless. If column cracks before acids have been added, gel can be extruded from tube, re-slurried with solvent, and again poured into tube. If gel is not packed evenly or if air pockets are present, jagged fronts may occur where soln passes such points of separation.

(b-1) Test of silicic acid for suitability and standardization of column:

Prepare stock solns of formic, acetic, and propionic acids by diluting 5 ml to 250 ml and standardize the acetic and propionic acids as follows: Pipette 1 ml into a 125 ml Erlenmeyer flask; dilute to ca 15 ml with boiled water, and titrate with 0.01 *N* $Ba(OH)_2$ using cresol red indicator. End point is reached when soln assumes pink color that persists for ca 45 seconds. During course of the titration bubble stream of CO_2 free air or *N* thru soln.

Prepare the following dilutions from the stock solns with boiled water:

Formic acid—10 ml to 50 ml.
Acetic acid—20 ml to 50 ml.

Prepare the following mixtures of formic acid and known amounts of acetic and propionic acids:

A Formic acid—10 ml stock soln.
Acetic acid—10 ml stock soln.
Propionic acid—10 ml stock soln.
H_2O—20 ml.

Total of 50 ml of which take 1 ml for separation check.

B Formic acid—10 ml stock soln.
Acetic acid—10 ml stock soln.
Propionic acid—30 ml stock soln.

Total of 50 ml of which take 1 ml for separation check.

C Formic acid—1 ml diluted stock soln.
Acetic acid—1 ml diluted stock soln.
Propionic acid—1 ml stock soln.

Total of 3 ml for separation check.

The above mixtures are designed to cover the range of acetic and propionic acids usually present when 15 g of fresh bread, preserved with propionate, is used as the initial sample.

Pipette the above indicated aliquot of acids into the bottom of g.s. test tubes (ca 16×150 mm), neutralize with ca normal NaOH, using phenolphthalein indicator, and add 1 drop of NaOH soln in excess. Place test tube in steam bath or boiling H_2O bath and allow soln to evaporate to dryness. (Process can be speeded by carefully playing a stream of air on soln, or by carefully inserting vacuum line to a point a few cm above surface of soln; it is essential that none of the salts be lost during this process; spattering must be avoided.

(b-2) Separation technique:

When soln has evaporated to dryness add 2 ml of the 1% butanol in $CHCl_3$ to the test tube, and then add 2 drops of H_2SO_4 (1+1). Stopper tube immediately and shake gently until all Na salts are converted to free acids.[4] Take care to convert any of salts that may have been deposited on sides of tube without wetting stopper. (Too much H_2O will be indicated by several large drops of insoluble liquid in butanol-$CHCl_3$ mixture; this H_2O may be removed by the addition of a few mg of anhyd. Na_2SO_4; or if only a few small droplets of insoluble liquid are present, they

[4] The addition of several glass beads will aid in bringing the solids into contact with the sulphuric acid and solvent.

may be eliminated by rotating tilted test tube and shaking gently until insoluble liquid adheres to the glass walls.)

Place a 50-ml graduated cylinder under the prepared column to catch the forerun, and transfer the butanol-$CHCl_3$ extract of fatty acids to the top of the column with the eye dropper pipette. Exercise care to avoid disturbing the surface of the gel. If the surface of the gel is dented or splashed by careless addition of solvent, uneven band fronts will result which will make the accurate collection of each band difficult. Allow solution to sink into gel under pressure. Rinse test tube with three ml portions of the 1% butanol in $CHCl_3$, and transfer the washings to column allowing each to sink into gel before next is added. After last washing has sunk into gel, fill tube with the 1% butanol in $CHCl_3$ and renew pressure. Change receiver (50 ml graduated cylinder) each time the lower edge of a band reaches a point ca 2–5 mm above the cotton plug in the constricted end of the tube. Propionic acid will elute first, followed by acetic acid. At a definite total volume of 1% butanol-chloroform eluate and previous to the threshold of acetic acid,[5] release the pressure, pour off and discard any solvent above the column, and fill the tube with the 10% butanol-chloroform. Proceed with the elution of the acetic acid band to the threshold of formic acid.

Record the volumes of the forerun and the volumes in each eluate receiver and calculate threshold volumes. With respect to the trial mixtures, and in the usual bread analysis where propionate has been added, only three bands will appear on the column. In this event the forerun is the threshold volume for propionic acid and the forerun plus the volume required to elute the propionic acid is the threshold volume for acetic acid.

Transfer the forerun[6] and the eluates to 125 ml Erlenmeyer flasks, rinsing the graduated cylinder in each case with three 5 ml portions of boiled H_2O. Add 1 drop of the cresol red indicator and titrate with the 0.01 *N* alkali in CO_2 free atmosphere as described previously. However, as end point is approached, stopper flask and shake vigorously to extract acids completely from the solvent phase.

Correct titrations for a blank determined as follows: Collect 25 ml of the butanol-$CNCl_3$ mixture from the column before any acids are transferred, add 15 ml of boiled H_2O, and titrate as above with the 0.01 *N* alkali.

(c) Identification and Determination:

Proceed as described above under (*b-2*) on the basis of the evaporated distillates.

Identify the acids by comparing their threshold volumes with those for ca same quantities of known acids used in standardization procedure. However, if conditions are changed, such as by use of different batch of silicic acid or different quantity of same batch or different quantity of H_2O, the threshold volume of each acid must be redetermined.

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[5] Recent experiments have shown that the threshold volume—the amount of mobile solvent required to move each acid from the top of the column to the point of emergence—is a function of the concentration of the acid. In the amounts of formic and acetic acids in bread, the differences are not critical. However, the threshold volume of propionic may vary as much as 100%. Therefore, to use threshold volume as a means of identification it becomes necessary to determine what the threshold should be for the amount determined. The separation of the mixtures A, B, and C should supply sufficient data for the present purpose. It is suggested that threshold volumes be plotted against the titrations. Thresholds can then be predicted for intervening concentrations. After development of the bands with the "CB-1" solvent, the elution may be speeded by changing to the "CB-10" solvent. Due to the possibility of differences in the propionic threshold, this change should be made at a constant "CB-1" eluate volume rather than at the propionic threshold, preferably when the greater part of the propionic is eluted. By this means the acetic threshold will not be affected by changes in the propionic threshold. The Associate Referee used 40 ml of "CB-1" as the point of change to "CB-10."

[6] The titration of the forerun previous to the propionic threshold in the pure acid mixtures should be the equivalent of the "blank titration." However, in bread analyses, the forerun previous to propionic may titrate higher than the blank. This may be due to traces of higher acids.

The separated acids may be further identified by means of the formation of mercurous acetate and mercurous propionate crystals.

Calculate the results for acetic and propionic acids to mg/100 g. The following factors are based on 15 g of fresh bread and 10 g of air dried bread:

Fresh Bread	—Acetic	4.00 × ml 0.01 Normal
		Propionic 4.93 × ml 0.01 Normal
Air Dried Bread	—Acetic	6.00 × ml 0.01 Normal
	Propionic	7.40 × ml 0.01 Normal

(4) The first action method for nitrite nitrogen, **13.43, 13.44** (p. 204), was dropped and the following method adopted, first action.

Method I

Select a series of 100 ml volumetric flasks of uniform dimensions and color. (125 ml Erlenmeyer flask can be used.) Place 2 g of untreated (nitrite free) flour in each flask. Add to each flask, except one for blank, varying quantities of the standard $NaNO_2$ soln (b) (usually 0, 5, 10, 15, 20, 25, 30, 35 ml) cover probable nitrite content of unknown sample. Add sufficient H_2O to make total volume in each flask 80 ml. Shake while adding the standard soln and H_2O to get the flour moistened and well dispersed before too dilute. Add 2 g unknown flour to a similar flask, and add 80 ml H_2O. (Treat all flasks the same.) Digest all the flasks in water bath at 40°C. for at least 15 min., add 2 ml sulfanilic acid soln (dissolve 0.5 g sulfanilic acid in 150 ml of 20% acetic acid, warming slightly if necessary) from a Mohr pipette to each flask in succession, mix well, add 2 ml alpha naphthylamine hydrochloride soln (dissolve 0.2 g of the salt in 150 ml of 20% acetic acid, by heating, if necessary). Continue digestion at 40°C. for 20 min. from time of addition to the last flask. Shake the samples occasionally during the first part of the digestion. During the latter 10 min. the flour should be allowed to settle. Remove from bath without disturbing settled flour. Compare unknown with series of standards and estimate the closest match. Multiply number of ml of $NaNO_2$ soln (b) in flask by .05 to obtain p.p.m. of *N* (*i.e.*, unknown may be in between 30 and 35 ml, ca 32 ml, or $32 \times .05 = 1.6$ p.p.m. of *N*).

(5) The following method for carotene in noodles was adopted, first action, in place of **13.118** and **13.119** (p. 218), which was dropped.

Carotenoid and Carotene

Weigh 20 g of flour, semolina, or macaroni, or 10 g of egg noodles, or 2 g of egg yolk into 125 ml Erlenmeyer flask, add 50 ml of alcoholic KOH soln (dissolve 10 g of KOH/100 ml alcohol by warming on steam bath), and boil on steam bath for 30 min., with flask fitted to a reflux condenser. Rotate the flask occasionally during this time but be as careful as possible to keep the sample from collecting on the sides of the flask. Remove the flask and cool to room temp. Filter thru Büchner type medium fritted glass filter into a 250 ml suction flask using suctions, transferring most of the material with a few ml of alcohol from wash bottle. Turn off suction, rinse out flask with 25 ml of ether, pour rinsing on to glass filter, stir material with rod to allow ether to come in contact with all portions. Filter off and repeat this procedure twice more. Transfer filtrate to a 250 ml glass-stoppered separator, rinsing with ca 25 ml of ether, disregarding the soapy material in the flask. Add 175 ml of H_2O, carefully invert and rotate several times. When the aqueous-alcohol and ether layers have separated, remove lower aqueous-alcohol layer and extract

this layer again with 25 ml of ether. Discard lower layer. Add the ether to original ether soln. Wash the ether by pouring 50 ml of H_2O thru it. After separation of layers, withdraw H_2O layer and discard. Add to the ether soln 50 ml petroleum ether, and wash 5 times with 50 ml of H_2O carefully inverting and rotating the separator. Discard all H_2O layers (slight emulsions usually clear in a few min. but may be discarded especially if no significant yellow color). Transfer the ether petroleum ether mixture to a 250 ml distillation flask, place flask in beaker of H_2O at 45–50°C. Stopper flask, connect side arm with vacuum, and concentrate to ca 5 ml to remove the ether. Filter thru a Allihn type absorption tube with coarse fritted glass plate containing ca ⅛ in. layer of anhydrous powdered Na_2SO_4, or thru a 5.5–7.0 cm filter paper ½ filled with Na_2SO_4 (use a small long-stem funnel reaching thru neck of flask) into a 25 ml volumetric flask (100 ml for neutral wedge photometer). Make to volume with petroleum ether that has been passed portion-wise thru the filter and Na_2SO_4, mix by inverting a few times. Transfer to absorption cell of 1 cm thickness and read density at 436 mμ in spectrophotometer, making at least 3 readings. Compute from the av. reading the total carotenoid pigment in p.p.m. by multiplying the density by 13.05 for noodles, or by 6.52 for semolina and macaroni.

Separation of Carotene from Xanthophylls

Carotene by procedure A:

Transfer all soln from cell and volumetric flask quantitatively to 125 ml separator, rinse with petroleum ether and make to ca 100 ml volume. Add 15 ml of 92% CH_3OH (8 ml of H_2O and 92 ml of methyl alcohol), shake moderately ca 2 min. by hand, or on mechanical shaker 10 min., allow separator to stand in upright position a min. or so until alcohol and petroleum ether layers separate. Decant lower layer containing xanthophyll, repeat extractions 5 more times or until aqueous CH_3OH layer is nearly colorless for semolina. (Eight extractions is generally enough for noodles but higher than normal egg content may require 10 extractions.) Examine final CH_3OH layer recovered in a test tube over a white background to be sure of nearly colorless soln. Wash the petroleum ether with 25 ml H_2O, inverting the separator several times, discard H_2O layer and repeat twice more. Pour the petroleum ether thru the Allihn type absorption tube containing ¼ in. layer of anhyd. powdered Na_2SO_4, or thru a 9 cm filter paper ½ filled with Na_2SO_4 into the 250 ml distillation flask, washing the color from the filter with petroleum ether. Concentrate to 5 ml by vacuum as above and transfer to 10 ml volumetric flask using very small portions of petroleum ether, make to volume, mix by inverting, and read density in spectrophotometer as above. (For neutral wedge photometer make to 25–50 ml volume depending on concentration of carotene.) Compute carotene in p.p.m. by multiplying the density by 5.22 for noodles, or by 2.61 for semolina.

Carotene by procedure B:

Prepare column in absorption tube ca 18 mm O. D. × 240 mm with ca 5 cm tip for a rubber stopper. Loosely plug with small pad of cotton, place into 250 ml suction flask, turn on suction, add thru a funnel in small amounts from a spatula a (1+1) mixture of activated magnesia[1] and diatomaceous earth[2] to a height of ca 11 cm, pack the column by pressing down (*only once after all this mixture has been added*) with a cork stopper just fitting the tube on the end of a rod. Place on top 1–2 cm of anhydrous powdered Na_2SO_4. Transfer all soln from the cell and volumetric flask quantitatively to the 250 ml distillation flask, and concentrate as before to ca 5 ml. With the suction continuously applied to the flask 4 times, each time

[1] Micro Brand No. 2641, Westvaco Products Co., Newark, Calif.
[2] Hyflo Supercel, Johns-Manville, 22 W. 40th St., New York City.

with ca 5 ml petroleum ether to remove all the color. Finally rinse down the sides of the tube with a few ml of petroleum ether. After a few drops have come thru the absorption tube, change to another 250 ml suction flask. When nearly all petroleum ether is down to the Na_2SO_4 layer, add 50 ml of petroleum ether-acetone mixture (9+1) to wash thru the carotene. When all this solvent has passed thru the Na_2SO_4 turn off the suction. (Keep the top of the column covered sith solvent during the entire operation.) Transfer the carotene soln (which should be only a few ml) to a 10 ml volumetric flask, using very small portions of petroleum ether, make to volume with petroleum ether, mix by inverting and read as under procedure A and compute in p.p.m. likewise. (These solns should be read on same day of extraction.)

14. COFFEE AND TEA

No additions, deletions, or other changes.

15. DAIRY PRODUCTS

(1) The first action method for ash in milk, **15.16** (p. 231) was revised by substituting "ca 5 g" for "ca 10 g," in line 1, and adopted as official.

(2) The first action method for acidity of milk, **15.4** (p. 227), was revised as follows and made official:

Measure or weigh a suitable quantity (ca 20 ml or 20 g) of the sample into a suitable dish and dilute with twice its volume of CO_2-free water. Add 2 ml of 1% phenolphthalein soln, **39.31 (d)**, and titrate with 0.1 *N* NaOH to the first persistent pink shade. If a measured volume of sample was used determine its weight from the specific gravity of the sample. Report acidity as per cent of lactic acid by weight. If the Babcock milk pipette, **15.26 (b)**, be used, the number of ml of 0.1 *N* NaOH required ÷20 = per cent acid as lactic acid.

(3) The following revisions were made in the official acetic serum method, **15.28** (p. 234): Paragraph **(a)** delete "Reading below 39 indicates added H_2O; between 39 and 40, addition of H_2O is suspected. . . . When reading is 40 or below, det. ash in serum as directed under **(b)**"; and in paragraph **(b)** delete "Result below 0.715 g/100 ml indicates added H_2O."

(4) The following was deleted from the official copper serum method **15.29** (p. 235): "Reading below 36 indicates added H_2O. . . . When refractometer reading is 36 or below, det. acetic serum ash as directed under **15.28 (b)**."

(5) The lines "nozzle, straight" and "Delivery, 5–8 sec." were deleted from **15.26 (b)** (p. 234) in the official Babcock method for fat in milk, and the following substituted: "Nozzle parallel with axis of pipet, but slightly constricted so as to discharge in 5 to 8 seconds when filled with H_2O."

(6) A footnote was added referring to the ether used in the official Roese-Gottlieb method for fat, **15.25** (p. 233), as follows: "For the determination of fat in dairy products the ether should comply with requirements set forth under 'Definitions of Terms and Explanatory Notes,' p. XIV, paragraph (4) to assure freedom from peroxides."

(7) The first action method for gums in soft curd cheese, **15.139–15.142** (p. 266), was adopted as official and reworded as follows:

(Not applicable to the detection of alginates)

REAGENTS

(a) *Benedict's soln (qualitative).*—Dissolve 17.3 g of Na citrate and 10 g of anhydrous Na_2CO_3 in ca 80 ml of hot H_2O; dissolve 1.73 g of $CuSO_4.5H_2O$ in 10 ml of H_2O. Filter the alkaline citrate soln, add the $CuSO_4$ soln slowly, with constant stirring, and dilute with H_2O to 100 ml.

(b) *Sodium hydroxide soln.*—10%.

(c) *Trichloroacetic acid soln.*—50%. Use fresh soln made up just before use from non-hydrolyzed reagent.

(d) *Dilute trichloroacetic acid soln.*—10%. Note warning under (c).

(e) *Ammonium hydroxide.*—Reagent, 28–29% NH_3.

(f) *Acetic acid.*—Reagent, glacial, 99.5%.

(g) *Alcohol.*—95%.

(h) *Alcohol.*—70%.

(i) *Potassium aluminum sulfate soln.*—5%.

TREATMENT OF SAMPLE

Weigh into 250 ml centrifuge bottle 100 g of cheese. Add hot H_2O to make volume 170 ml. Heat in hot H_2O bath for 30 min. Cool to room temp., add 50 ml petr. ether, shake and centrifuge. Remove petr. ether layer by decantation or by use of a blow-off siphon. Repeat extraction with petr. ether at least twice. (Small amount of fat remaining will do no harm.) Warm bottle in hot H_2O bath to remove residual petr. ether from cheese. Centrifuge, if necessary, to break any foaming that may occur. Make volume to ca 190 ml with H_2O and add 3.5 ml NH_4OH a few drops at a time while stirring contents of bottle. Keep in hot H_2O bath and stir until all curd has dissolved. If curd fails to completely dissolve, add few more drops of NH_4OH, stir and macerate to secure soln. Add acetic acid, a few drops at a time, with shaking, until *p*H is ca 4.75 (nitrazine test paper or *p*H meter suitable). Use care in approaching *p*H point because isoelectric point for casein is ca *p*H 4.73. (If acid is added very slowly with constant shaking and the centrifuge bottle is kept hot, marked separation of casein and liquid will be noted at this point.) Stopper bottle, shake thoroly and allow to stand overnight in the hot H_2O bath as the H_2O cools. Check *p*H, and centrifuge at 1200 r.p.m. for 10 min. Decant supernatant liquid into 250 ml beaker with 40 ml graduation mark. Do not wash precipitate.

SEPARATION OF GUM

Evaporate decanted liquid on steam bath to 40 ml mark of beaker. Remove beaker from bath and cool to room temp. Disregard precipitate formed during concentration and add 10 ml of 50% trichloroacetic acid soln (note warning under Reagents). Replace on steam bath for at least 15 min. to coagulate protein. Remove beaker from steam bath, cool, transfer to 250 ml centrifuge bottle with 5 ml of the dilute trichloroacetic acid soln and centrifuge at 1200 r.p.m. for 10 min. Decant supernatant liquid into another 250 ml centrifuge bottle and add alcohol with stirring until bottle is full. Allow mixture to stand at least 1 hour to coagulate gums. Centrifuge at 1800 r.p.m., decant and discard liquid. (The volume before addition of alcohol should not exceed 50 ml and ca 4 volumes of alcohol should be added.)

Add to residue in bottle ca 50 ml of 70% alcohol, stopper and shake to thoroly break up material. Wash down stopper and sides of bottle with a little 70% alcohol centrifuge at 1800 r.p.m., decant and drain. Add 40 ml hot H_2O to bottle and

shake well to dissolve gum and disperse insoluble material. Add 10 ml of 50% trichloroacetic acid to bottle and heat on steam bath for 15 min. to coagulate any protein left after first treatment. Remove bottle, cool and centrifuge at 1200 r.p.m. for 10 min. Decant supernatant liuqid into another 250 ml pyrex centrifuge bottle. Fill bottle with 95% alcohol while stirring contents. Add 0.5 ml of 5% potassium aluminum sulfate soln (Reagent i). Shake and allow to stand at least 1 hour. Centrifuge at 1800 r.p.m. and decant. Add 50 ml of 70% alcohol, shake to disperse material and centrifuge at 1800 r.p.m. Decant off supernatant liquid and drain. Add 40 ml of hot H_2O and shake well to dissolve gum. Transfer to 50 ml capacity conical heavy duty centrifuge tube, keeping volume to 40 ml. Centrifuge at 1200 r.p.m. for 10 min. to remove any undissolved material and decant supernatant liquid back into 250 ml centrifuge bottle. Reprecipitate in bottle by filling with alcohol plus 1 drop of acetic acid. To insure precipitation of gum tragecanth and karaya, add 0.5 ml of potassium aluminum sulfate soln.

DETECTION OF GUM

Allow precipitate to coagulate as before. Centrifuge and decant off liquid. If the precipitate is small in amount and will not remain on bottom of 250 ml centrifuge bottle, centrifuge, a portion at a time, the alcohol and precipitated gum at 1500 r.p.m. for 15 min. in a 50 ml capacity conical heavy duty centrifuge tube, until all contents of 250 ml bottle have been transferred to 50 ml tube. After decanting supernatant liquid from last portion centrifuged, add 40 ml of 70% alcohol to tube (or bottle if tube is not used), shake until precipitate is dispersed, centrifuge, decant and drain.

Add to residue in tube or bottle 10 ml of hot H_2O, shake and transfer contents to 50 ml beaker. Rinse out tube or bottle with 10 ml of hot H_2O and add H_2O to beaker. Warm on electric hot plate to dissolve gum and evaporate to 10 ml. Add 2 ml conc HCl, cover beaker with watch glass and boil gently 5 min. Cool and transfer to 10 ml graduated cylinder. Adjust to 10 ml with H_2O and mix. Place 1 ml aliquot in 30 ml beaker and neutralize with NaOH soln using litmus paper as indicator. Remove litmus paper, add 5 ml of Benedict's soln and boil vigorously 2 min. Allow contents of beaker to cool spontaneously. Voluminous precipitate appearing on cooling, which may be yellow, orange or red, caused by reducing sugars formed by hydrolysis of the gums, indicates presence of gums.

16. EGGS AND EGG PRODUCTS

No additions, deletions, or other changes.

17. ENZYMES

No additions, deletions, or other changes.

18. FISH AND OTHER MARINE PRODUCTS

No additions, deletions, or other changes.

19. FLAVORING EXTRACTS

(1) The following photometric method for vanillin in imitation vanilla flavors was adopted, first action.

REAGENTS

(a) *Folin-Denis Reagent.*—To 100 g of pure Na tungstate and 20 g of phosphomolybdic acid (free from nitrates and NH_4 salts), add 100 g of sirupy H_3PO_4 (con-

taining 85% H_3PO_4) and 700 ml of H_2O. Boil over free flame 1½–2 hours, cool, filter, if necessary, and make up with H_2O to 1 liter. An equivalent amount of pure molybdic acid may be substituted for the phosphomolybdic acid.

(b) *Sodium carbonate soln.*—Dissolve 40 g of anhyd. Na_2CO_3 in 160 ml of water.

(c) *Standard vanillin soln.*—Weigh 0.1000 g of vanillin and transfer to a 100 ml volumetric flask. Dissolve with 3 ml ethyl alcohol and dilute to mark with water (1 ml = 1 mg).

(d) *Lead acetate soln.*—Dissolve 50 g each of neutral and basic lead acetate in hot water, dilute to 1 liter, cool, and filter.

PREPARATION OF GRAPH

From a 50 ml buret add 0.0, 2.5, 5.0, 7.5, 12.5, and 20.0 ml of standard vanillin soln to 250 ml volumetric flasks, and treat each as follows. Add water to make total volume 80 ml, and add from graduated pipet 2 ml of lead acetate soln. Dilute to mark with water and mix. Filter thru dry 18.5 cm folded filter paper, discarding first filtrate until clear, and pipet 10 ml of clear filtrate to a 100 ml volumetric flask. Add 5 ml of Folin-Denis reagent and let stand exactly 5 min. after mixing. Add 10 ml of carbonate soln, mix and let stand exactly 10 min. Dilute to mark with water, mix, and filter thru dry folded filter paper, discarding first filtrates until clear. Read clear filtrate at once using a 1-inch cell and a 61 or 65 filter in a neutral wedge spectrophotometer. Plot results on coordinate paper, and draw curve with a french curve or similar curved drawing aid. Curve does not obey Beer's Law.

DETERMINATION OF SAMPLE

Run sample concurrently with the standards for best results. Pipet 2 ml of sample into a 250 ml volumetric flask, and proceed as above for standards, beginning with "Add water to make total volume 80 ml, and add from graduated pipet 2 ml of lead acetate soln. Dilute to mark. . . . "

(2) The following photometric method for coumarin in imitation vanilla flavors was adopted, first action.

REAGENTS

(a) *Standard coumarin soln.*—Dissolve 0.2000 g of pure coumarin in 3 ml of ethyl alcohol and dilute to 100 ml with water (1 ml = 2 mg).

(b) *Lead acetate soln.*—Dissolve 50 g of neutral Pb acetate and 50 g of basic Pb acetate in hot water, dilute to 1 liter, cool, and filter.

(c) *Sodium oxalate.*—Anhyd.

(d) *Sodium carbonate soln.*—Dissolve 5 g of anhyd. Na_2CO_3 in water and dilute to 500 ml.

(e) *Diazonium soln:*

Soln A:—Dissolve 0.7 g of p-nitraniline in 9 ml of HCl and dilute to 100 ml. with water.

Soln B:—Dissolve 5 g $NaNO_2$ in water and dilute to 100 ml with water.

Chill a 100 ml flask and solns A and B to about 3°C. in a refrigerator or in chopped ice. Pipet 5 ml of each soln into the flask, mix, and let stand in refrigerator 5 min. Add 10 ml more of Soln B, return to refrigerator for 5 min., then fill the flask to the mark with ice-cold water. The diazonium soln is ready to use in 15 min. but must be discarded after 24 hours.

PREPARATION OF GRAPH

Place 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 ml portions of coumarin standard soln into 100 ml volumetric flasks, add water to bring volume to 80 ml, and treat each in the

following manner. Add 5 ml of lead acetate soln, fill to the mark with water, mix, and filter thru a folded filter paper, discarding the first filtrate until clear. To the clear filtrate add 0.2 g of anhyd. Na oxalate and dissolve in the filtrate by rotating the container. After dissolving completely, rotate again for a few seconds, let stand at least 5 min., and filter thru a 11 cm S & S 589 filter paper, discarding first filtrate until clear. Transfer 5 ml of filtrate to another 100-ml volumetric flask and add water to make volume to 20 ml. Add 10 ml of Na_2CO_3 soln and heat in a boiling water bath for 5 min. Allow soln to cool gradually; when room temp. is reached add 10 ml of diazonium soln, fill to the mark with water and mix. Let stand 1.5 hours, filter thru S & S 589 filter paper, and read in a neutral wedge spectrophotometer, using a No. 49 filter and a ½ inch cell. Plot results on coordinate paper.

DETERMINATION OF SAMPLE

Run samples for greatest accuracy along with the standards. Pipet 5 ml of imitation vanilla into a 100 ml volumetric flask, and proceed as above for standards, beginning with "add water to bring volume to 80 ml. . . . "

20. FRUITS AND FRUIT PRODUCTS

(1) The first action method for citric acid, **20.37** and **20.38** (p. 330), was deleted and the following method adopted, first action.

REMOVAL OF PECTIN

Measure or weigh accurately the desired quantity of prepared sample, **20.2**, into a 250-ml volumetric flask. Add water to make the total volume 70 ml. Add 2 ml HNO_3 to liberate acids and heat to 50°. Dilute nearly to neck of flask with alcohol and cool to room temp. Make to mark with alcohol, mix and filter on a funnel lined with cotton and collect at least 220 ml of filtrate. (Toward the end filtration is slow; by gathering the ends of the cotton and squeezing the incased residue the desired quantity of filtrate is secured.

ISOLATION OF POLYBASIC ACIDS

(The "Isolated Acid Solution")

Determine the titer (t) of 10 ml of the alcoholic filtrate in terms of ml 0.1 *N* NaOH, using phenolphthalein indicator.

Pipet 200 ml of the alcoholic soln into a 400 ml beaker, add "2t+2" ml normal NaOH, and place on the steam bath 30 min. Cool the mixture to room temp. add 5 ml of 1 *N* acetic acid, and rinse with alcohol into a 250 ml centrifuge bottle. Add ".6t" g of finely powdered lead acetate[1] and shake vigorously 5 min. Add 0.2 g of filter cel. fill the bottle with alcohol, and mix thoroly.

Centrifuge, decant, and discard the supernatant liquor. Completely disperse the lead salts by addition of portions of 50 ml of dilute alcohol (80 ml alcohol made to 100 ml with H_2O) and shaking.

Fill the bottle with 80% alcohol, mix thoroly, and centrifuge. Discard the liquor and repeat the washing with 80% alcohol. Disperse the lead salts in 50 ml of water, dilute to volume of 150 ml, and saturate with H_2S gas. Shake 1 min. and rinse into a 250 ml volumetric flask. Make to mark and filter thru a large fluted filter, *pouring back until bright.*

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[1] The quantity ".6t" is derived from .03t(200/10). It expresses the g of lead acetate required to form the lead salts of the acids contained in the 200 ml soln. The g of lead indicated is excessive by one-half and is generally sufficient. If an addition of lead to the supernatant liquor forms a further precipitate after 1 min., add more lead acetate and repeat centrifuging.

Dilute 20 ml of the isolated acid soln with 50 ml of water and boil a few min. to expel H_2S. Cool and titrate with 0.1 *N* NaOH using phenolphthalein indicator.

CITRIC ACID (NORMAL)

Evaporate 200 ml of the isolated acid soln to ca 20 ml, rinse into a 250–300 ml glass-stoppered Erlenmeyer flask, and adjust with water to a net weight of ca 40 g. Add 2 g of KBr and 5 ml of conc. H_2SO_4, and, if necessary, heat to about 50° and allow mixture to stand 5 min. Add 20 ml of 5% $KMnO_4$ soln from a pipet or burette slowly (1–2 ml at a time), swirling the flask a few seconds after each addition. Allow the mixture to stand undisturbed 5 min. and cool to 15°. Add a $FeSO_4$ soln (200 g of $FeSO_4.7H_2O$ to 500 ml+5 ml of conc. H_2SO_4) slowly with constant agitation until the mixture starts to clear. Shake 1 min., continue the addition of the $FeSO_4$ soln until the MnO_2 is dissolved, and add a few ml in excess. Add 20 g of anhyd. Na_2SO_4, with accompanying swirling to assure dissolution (if Na_2SO_4 remains substantially undissolved repeat the determination.) Cool to 15°, and shake vigorously 5 min. Immediately, while still cold, collect the pentabromacetone on asbestos in a gooch crucible and wash the residual precipitate from the flask with a portion of the filtrate. Finally wash the crucible with 50 ml of cold water and allow the crucible to remain under suction a few min. Dry the crucible overnight in a H_2SO_4 desiccator and weigh or place crucible in drying train (H_2SO_4 and soda lime) and aerate to constant weight. Designate the weight in g as "a." Remove the pentabromacetone from the crucible with alcohol followed by ether, filling the crucible 3 times with each solvent. Dry the crucible 10 min. in an oven at 100°, cool in a desiccator and weigh. Designate the weight in g as "b." Gm anhydrous citric acid (x) in the aliquot = 0.424 (a − b).[2] Gm anhydrous citric acid in the sample taken for analysis = x/64.

(2) The procedure **20.2 (a)** for preparation of sample, juices, was deleted and the following adopted as a procedure.

"Mix thoroly by shaking to insure uniformity in sampling, and filter thru absorbent cotton or rapid paper. Prepare fresh juices by pressing well-pulped fruit and filtering. Express juice of citrus fruits by one of the common devices used for squeezing oranges or lemons, and filter."

21. GELATINE, DESSERT PREPARATIONS, AND MIXES

No additions, deletions, or other changes.

22. GRAINS AND STOCK FEEDS

(1) The following method was adopted, first action, for sulfaguanidine.

REAGENTS

0.50 *N NaOH*
0.50 *N HCl*
1.00 per cent solution of $ZnSO \cdot 7H_2O$.
0.10 per cent solution of $NaNO_2$ (prepared fresh each day).
0.50 per cent solution of ammonium sulfamate.
0.10 per cent solution of N-(1-naphthyl)-ethylenediamine dihydrochloride. (Store in amber-colored bottle.)

[2] In the formula ".424(a −b)," 0.424 is the theoretical factor for converting pentabromacetone into anhydrous citric acid, and "a −b" is the weight of pentabromacetone in the aliquot.

DETERMINATION

Weigh 1 g of ground sample into 250 ml flask and add 100 ml of H_2O and 2.5 ml of 0.50 *N* NaOH. Heat in a water-bath 15 min. with occasional swirling, cool, make to volume, and mix well. Let material settle and pipet 25 ml into 100 ml flask, add 10 ml of the $ZnSO_4$ soln, dilute to mark, mix well, and let stand one min. before filtering thru 18.5 cm Whatman No. 2 paper. Discard first 10 ml of filtrate. (Filtrate *must* be free of turbidity.)

Pipet 2 ml of clear filtrate into 25 ml volumetric flask, add 2.5 ml of the 0.50 *N* HCl and 2 ml of the $NaNO_2$ soln. Let stand 3 min. Add 2 ml of the ammonium sulfamate soln and wait an additional 2 min. Finally add 2 ml of the coupling reagent and dilute to mark. Swirl contents of flask after each addition of reagent. Prepare blank using H_2O and same quantities of reagents made to volume of 25 ml. Shake vigorously.

Measure optical density of colored soln in spectrophotometer at wave length of 545 mμ against the reagent blank, and determine quantity of sulfaguanidine present by reference to standard curve.

PREPARATION OF STANDARD CURVE

Dissolve 0.010 g of pure sulfaguanidine in 2.5 ml of 0.50 *N* NaOH and 100 ml of H_2O in a 250 ml volumetric flask, by heating in boiling water-bath 15 min. Cool and make to volume with H_2O. Transfer 25 ml of this soln to 100 ml volumetric flask, add 10 ml of the $ZnSO_4$ soln, dilute to volume, and filter. Each ml of filtrate contains 10 mmg of sulfaguanidine. Dilute 1, 2, 3, and 4 ml portions of this filtrate, separately, (corresponding to 10, 20, 30, and 40 mmg of sulfaguanidine, respectively) to 10 ml with H_2O and treat each soln with 0.50 *N* HCl, 0.10 per cent $NaNO_2$ soln, etc., as directed under the method. Measure optical densities of final solns against reagent blank, and plot density readings against mmg of sulfaguanidine.

(2) The methods for calcium and phosphorus, **22.49–22.51,** incl. (p. 353) were adopted as official.

23. MEAT AND MEAT PRODUCTS

No additions, deletions, or other changes.

24. METAL, OTHER ELEMENTS, AND RESIDUES IN FOODS

No additions, deletions, or other changes.

25. NUTS AND NUT PRODUCTS

(1) The following sentence was added to the procedure on preparation of sample for nuts in shell, **25.2 (a)** (p. 425), at end of first sentence:

"The skin or spermoderm should be included with the meat in all nuts, including peanuts and coconuts unless specifically excluded by description."

26. OILS, FATS, AND WAXES

(1) The first action method for coal-tar colors, **26.51** and **26.52** (p. 447), was deleted and the following method substituted, first action.

REAGENTS

(a) *Acid Soln A.*—Mix 1 liter of acetic acid with 200 ml of HCl and 100 ml of H_2O.

(b) *Acid Soln B.*—Cautiously add 400 ml of sulfuric acid to 100 ml of H_2O. When cool, add 900 ml of acetic acid and mix.

(c) *Sodium hydroxide soln.*—ca 25%. Dissolve 250 g sodium hydroxide in H_2O and dilute to 1 liter.

SEPARATION AND IDENTIFICATION

Place 125 ml of oil and 250 ml of petroleum ether in each of 6 separatory funnels. Shake contents of the first funnel with 50 ml of soln A and, as soon as the layers have separated, transfer the lower layer to a flask containing 250 ml of H_2O. Mix and immediately extract this diluted acid soln by passing successively thru two 500 ml separatory funnels, each containing 75 ml of petroleum ether. After vigorous shaking, allow layers to separate and finally discard lower aqueous layer. Repeat this procedure with each of the other five funnels, using the same petroleum ether to re-extract the colors from the diluted acid solns. Combine the two petroleum ether extracts, wash with three 25-ml portions of water, and filter.

Extract the combined petroleum ether soln with two 25-ml portions of acid soln A. Treat each acid extract separately by mixing with 150 ml of water and re-extracting quickly by passing thru two 250-ml separatory funnels, each containing 50 ml of petroleum ether. Combine these petroleum ether solns, wash free from acid with 15 ml portions of water, and evaporate to dryness on the steam bath. Do not heat dish after removal of solvent. The residue may contain FD&C yellow No. 3 or No. 4 and possibly a trace of FD&C Orange No. 2, if this latter dye was originally present in large quantities. Dissolve residue in 10 ml of 70% alcohol, add 10 ml of water and dye color on small strip of silk previously washed with soap and rinsed thoroly with water. Dye in neutral soln by warming on steam bath until all alcohol is removed and color is transferred to silk. Rinse silk with water, dry in air and test by **21.11**, comparing reactions with authentic dyed silk of ca similar depth of color as unknown. Do not attempt to differentiate between FD&C Yellow No. 3 and No. 4, since reactions are almost identical.

Shake the contents of the first separatory funnel containing the diluted oil with 25 ml of acid soln B, allow to separate for 20 min. and then transfer the lower layer to a flask containing 200 ml of 25% NaOH. Mix and add 200 ml of water. Cool and remove the color from this alkaline soln by passing successive 100 ml portions thru two 250-ml separatory funnels, each containing 75 ml of petroleum ether. Discard the extracted alkaline soln. Continue this acid soln B treatment of the oil in the other 5 separatory funnels, and finally combine the two petroleum ether extracts. Wash with three 50 ml portions of water and extract with two 20-ml portions of acid soln B, allowing layers to separate for 5 min. Draw lower layers into a flask containing 300 ml of 25% NaOH, mix and add 300 ml of water. Cool and remove the color from this alkaline soln by passing successive 100 ml portions thru 2 separatory funnels, each containing 75 ml of petroleum ether. Combine the petroleum ether solns, wash free from alkali with water, transfer to an evaporating dish, and remove the solvent on the steam bath. The residue may contain FD&C Orange No. 2 and D&C Green No. 6. Remove the former color by dissolving in 3–5 ml portions of 60% alcohol and filtering each portion thru a small filter paper. Dye the color on silk as described above and identify by spot tests. Dissolve the residue on the filter paper in 2–5 ml portions of petroleum ether, collecting the filtrate in the original evaporating dish. Remove the solvent on the steam bath. A blue residue indicates D&C Green No. 6.

Dissolve the residue in 15 ml 95% alcohol and 10 ml H_2O, and add 0.5 ml acetic acid. Dye on washed silk strands (if evaporation occurs before strands well dyed add more H_2O and alcohol). Compare spot test reactions with authentic dyed silk (little or no change with HCl, NaOH, NH_4OH—olive green color with H_2SO_4).

(A pink color in the various acid extractions usually indicates coal-tar dye. However, corn oil sometimes produces a faint pink color in these extracts. This is readily differentiated from coal-tar colors by the absence of typical reactions in the dyeing tests. Chlorophyll may appear as a green scum at the interface in the acid extractions and as a green residue on the papers after filtration of the petroleum ether solns.)

27. PRESERVATIVES AND ARTIFICIAL SWEETENERS

(1) A method for acetic and propionic acids in bread was adopted, first action. See under Cereal Foods, p. 210.

(2) The official method for formic acid, **27.19–27.21** (p. 459), was dropped, first action.

28. SPICES AND OTHER CONDIMENTS

(1) In the official method for total nitrogen in mayonnaise and salad dressing, **28.44** (p. 485), the following, "50 ml. of H_2SO_4 (more if necessary)" was substituted for "35 ml of H_2SO_4," in line 5.

29. SUGARS AND SUGAR PRODUCTS

(1) The Zerban and Martin table for refractive indices of dextrose and invert sugar, **41.10** (p. 812) was adopted as official.

(2) The first action micro method for dextrose, **29.61–29.63** (p. 513), was adopted as official.

30. VEGETABLE PRODUCTS, PROCESSED

No additions, deletions, or other changes.

31. WATERS, MINERAL AND SALT

No additions, deletions, or other changes.

32. DRUGS

(1) The following method was adopted, first action, for carbromal.

Use portions for analysis weighing 0.25 g to 0.40 g. Proceed as directed in **34.90.**

1 ml 0.05 *N* $Na_2S_2O_3$ = 0.00593 g carbromal.

NOTE: Use 20 ml absorbing soln (15 ml of hydrazine sulfate and 5 ml 10% NaOH) if the apparatus has smaller absorption bulbs than those described in **34.90.**

(2) The following method was adopted, first action, for carbromal and pentobarbital in admixtures.

Transfer 0.5–0.7 g of sample to a separator, add 15 ml H_2O and 0.5 ml of *N* NaOH from pipet. Extract the carbromal with 25 ml portions of $CHCl_3$, washing each portion in a second separator containing 10 ml H_2O and 2 drops of NaOH. Filter the $CHCl_3$ thru cotton and transfer to a tared flask or beaker. Repeat the procedure until the carbromal is extracted. At least 4 shakeouts will be required. Evaporate the $CHCl_3$ soln nearly but not quite to dryness on the steam bath in a current of air. Remove the container and let stand in air to constant weight. Weigh the container and calculate per cent carbromal.

Combine the aqueous solns and proceed as directed in **32.116**, beginning "Acidify to litmus paper."

The weight of the barbiturate (pentobarbital) multiplied by 1.097, represents the weight of sodium pentobarbital in the portion taken for assay. Calculate the per cent of sodium pentobarbital in the mixture.

Determine the melting points of the dried extracts. Carbromal melts at 116°–119°C., and pentobarbital at 126°–130°C.

(3) The subtitle in first action method **32.27(a)** was changed by deletion of "ca 2%."

(4) The following method for diethylstilbestrol in oil was adopted, first action.

REAGENTS

Buffer soln.—Mix 90 ml of glacial acetic acid with 10 ml of 10% NaOH.

Alcoholic ammonium hydroxide soln.—Mix 450 ml of alcohol with 550 ml of ammonium hydroxide soln (1+1).

Sodium nitrite soln.—Aqueous saturated soln.

Skellysolve C or high-boiling petroleum ether.

Diethylstilbestrol standard soln.—0.1 mg/ml. Dissolve 50.0 mg of pure diethylstilbestrol in exactly 100 ml of alcohol, and dilute a 10 ml aliquot of this soln to exactly 50 ml with alcohol.

PREPARATION OF SAMPLES

Oil solns.—Place an accurately measured aliquot equivalent to 4–12 mg of diethylstilbestrol in a separator containing 30 ml of Skellysolve C and 20 ml of 10% NaOH. Shake vigorously, allow the layers to separate, and remove the sharply-defined alkaline phase to another separator. Repeat the extraction and partition process with two 10-ml portions of 10% NaOH. Reject the contents of the first separator. Acidify the combined alkaline extracts with H_2SO_4 (1+1), cool thoroly, and shake with 30 ml of chloroform. Transfer the chloroform layer to a separator containing 10 ml of wash water, shake, and filter the washed extract thru a pledget of cotton into a 100 ml volumetric flask. Repeat the extraction with three 20-ml portions of chloroform, dilute the combined extracts to exactly 100 ml with chloroform, and mix.

Tablets.—Place a representative portion of the powdered sample, equivalent to 4–6 mg of diethylstilbestrol, in a separator containing 40 ml. of chloroform. Add 20 ml of H_2SO. (1+9), and shake vigorously. Allow the layers to separate, and transfer the clearly defined chloroform extract to a second separator containing 20 ml of 10% NaOH. (Disregard a slight turbidity in the chloroform layer). Extract by shaking, remove the chloroform to a third separator containing 10 ml of 10% NaOH, and again shake. Discard the final chloroform layer. Repeat the double extraction process in the three separators using three 20 ml portions of chloroform. Discard the acidic mixture in the first separator, and add the alkaline mixture in the third separator to the contents of the second, rinsing with several portions of water. Proceed as directed under "Oil Solutions" beginning with the words " . . . Acidify the combined alkaline extracts."

DETERMINATION

Transfer duplicate aliquots of the sample soln, equivalent to 0.8–1.2 mg of diethylstilbestrol, to 50 ml beakers, and evaporate to dryness. Treat similarly duplicate 10 ml portions of the standard diethylstilbestrol soln, and a 20 ml portion

of chloroform as blank. Add to each of the residues 5.0 ml of the buffer soln, cover with a watch-glass, and dissolve by gentle warming on the steam bath. Cool to room temp., mix with 1.0 ml of H_2SO_4 (1+1), and add 2 drops of saturated sodium nitrite soln. Allow to stand for 45 min., with occasional mixing. Dilute to about 25 ml with the alcoholic ammonium hydroxide soln, wash quantitatively into a 50 ml volumetric flask with the alkaline reagent, and cool in an ice-water bath while swirling vigorously. Adjust to room temp., let stand one hour, and dilute to exactly 50 ml with the alcoholic ammonium hydroxide reagent. Mix, and filter thru dry filter papers, rejecting the first few ml of filtrate. Determine the absorbancies of the sample aliquots relative to the blank as reference at 440 m, and compare with the corresponding values for the standards. Compute the diethylstilbestrol content of the sample.

(5) The following spectrophotometric method was adopted, first action, for theobromine and phenobarbital.

APPARATUS

A *spectrophotometer* capable of indicating spectral bands of the order of 2 mμ or less in the region of 230–300 mμ.

Partition tube.—Fuse a 6 cm length of 5–6 mm tubing to a piece of 25 mm glass tubing about 200 mm in length. (A 25–200 mm test tube may be used.) Constrict the stem slightly about 2 cm below the joint.

Packing rod.—Flatten the end of a glass rod to a circular head with a clearance of about 1 mm in the partition tube.

REAGENTS

Dibasic potassium phosphate soln.—ca 2 *M*. Dissolve 35 g of the reagent grade anhyd. salt in water, cool to room temp., and dilute to 100 ml (K_2HPO_4).

Celite No. 545.—Manufactured by Johns-Manville Corp.

Theobromine standard soln.—1.00 mg/100 ml. Dissolve 100 mg of pure theobromine in H_2SO_4 (1+4), and dilute to exactly 100 ml with the diluted acid. Transfer a 5.0 ml aliquot of this soln to a 500 ml volumetric flask, add 200 ml of 5% NaOH, and adjust to room temp. Dilute to the mark with water and mix thoroly.

Phenobarbital standard soln.—1.50 mg/100 ml. Dissolve 75.0 mg of pure phenobarbital in chloroform, and dilute to exactly 100 ml with the solvent. Dilute a 10 ml aliquot of this soln to exactly 50 ml. Finally, transfer a 10 ml aliquot of the latter soln to a 100 ml volumetric flask, dilute to the mark with chloroform, and mix.

SEPARATION OF THEOBROMINE AND PHENOBARBITAL

Transfer a representative portion of the well-mixed sample containing at least 15 mg of phenobarbital to a 125 ml separatory funnel, add 15 ml of 5% NaOH and extract with three 30 ml portions of chloroform. Wash the successive chloroform extracts with 10 ml of 5% NaOH in a second separator. Discard the chloroform layers.

Add 30 ml of H_2SO_4 (1+4) to the alkaline mixture in the first separator, cool thoroly, and shake with 50 ml of ether. Transfer the aqueous layer containing dissolved theobromine to the second separator, cool, and shake with 40 ml of ether. Remove the lower phase to a third separator, and wash with another 40 ml portion of ether. Repeat the extraction thru the three separators using two 40 ml portions of H_2SO_4 (1+4) and two 20 ml portions of water. Collect the aqueous extracts in a 250 ml volumetric flask, dilute to the mark with water, and mix carefully.

Filter the ethereal solns thru a pledget of cotton into a beaker, washing the

three separators and the filter successively with three 5 ml portions of ether. Evaporate carefully to dryness, and dissolve the residue in chloroform.

SPECTROPHOTOMETRIC DETERMINATION

Theobromine

Pipette an aliquot containing 4–8 mg theobromine into a 500-ml volumetric flask, add 200 ml of 5% NaOH and adjust to room temp. Dilute to the mark with water and mix. Determine the absorbancy of this soln at 274 mμ relative to a soln prepared by diluting 10 ml of the 5% NaOH soln to 25 ml. Compare this value with that obtained under identical conditions using the standard soln of theobromine. Compute the theobromine content of the sample.

Phenobarbital

Transfer the chloroform soln to a volumetric flask and dilute to yield a soln containing 20–40 mg phenobarbital per 100 ml. Place 5.0 ml of this soln in a 100 ml volumetric flask, dilute to the mark with chloroform, and mix. Transfer duplicate 20 ml aliquots of the latter soln to separatory funnels containing 25 ml of ammonium hydroxide soln (1+24). Treat similarly duplicate 20 ml aliquots of the standard phenobarbital soln, and a 20 ml sample of chloroform as blank. Shake vigorously at least one min., separate and discard the chloroform layers, and permit the aqueous extracts to stand 30 min. Determine the absorbancies of the clear aqueous solns at 241 mμ with respect to the blank using the same cell for standards and samples. Compute the phenobarbital content of the sample. In the presence of salicylates, proceed as directed below.

Phenobarbital in the presence of salicylates

Pack fine glass wool into the stem of a partition tube, and fasten a piece of rubber tubing with attached screw clamp to the outlet to limit the flow during packing. Cover 10 gm of Celite in a mortar with chloroform, and distribute uniformly over the mixture 10 ml of 2 *M* potassium phosphate soln. Mix carefully with a pestle until the Celite appears to be uniform in particle size and wetness. Transfer about a fifth of the Celite mixture to the tube, and form a flocculent suspension by working the packing rod up and down as a piston, adding chloroform if necessary. Gently compress the Celite with the packing rod to yield a uniform pack about 1.5 cm high. Transfer the remainder of the Celite mixture to tube in four portions, and repeat the packing process. The Celite must be covered with chloroform at all times. The flow rate of the mobile solvent is not critical, but it should be about 2.5–4 ml/min when the height of the chloroform is 2–5 cm above the top of the Celite.

Remove the rubber tubing from the stem of the partition tube, and wash off the outer surfaces with a stream of chloroform. When the chloroform just stops flowing from the tube, add by pipette 5 ml of the chloroform soln (equivalent to 1–2 mg of phenobarbital), and collect the eluent in a 100 ml volumetric flask. As the level of the chloroform soln reaches the top of the Celite column add about 5 ml of chloroform, and repeat with a second chloroform wash. Add sufficient chloroform to maintain a column of solvent 2–5 cm in height, and collect about 95 ml of eluent. Wash the outside surface of the tube stem with a stream of chloroform, and collect the washings in the volumetric flask. Dilute to 100 ml with chloroform and mix thoroly. Determine phenobarbital in the eluent soln, beginning with the words "Transfer duplicate 20 ml aliquots of the later solns. . . ."

(6) The following method for the determination of ketosteroids (gravimetric) was adopted, first action.

APPARATUS

Separatory funnels, 125 ml, with well-fitting stopcocks lubricated *only with water.*

REAGENTS

Skellysolve C or high boiling petroleum ether.

Sodium hydroxide soln.—ca. 2N.

Sodium carbonate soln.—10% W/V.

Sulfuric acid soln.—H_2SO_4 (1+1).

Ether.—U.S.P. grade freshly washed twice with an equal volume of distilled water.

Glacial acetic acid.—A. R. grade.

Chloroform.—U.S.P. grade.

Girard Reagent T (Trimethylacethydrazide Ammonium Chloride).—Recrystallize commercial samples twice from absolute alcohol and dry in a vacuum at room temperature. The recrystallized material should be white and practically odorless. Store in a tightly stoppered bottle in a desiccator.

PREPARATION OF SAMPLES

Tablets.—Weigh a counted number of tablets and reduce them to a fine powder without appreciable loss. Weigh accurately a portion of the powdered tablets containing (1) 5–10 mg ketosteroids, or (2) 0.2–0.5 mg estradiol, or (3) 50,000–100,000 I. U. of estrogens, and transfer to a 125-ml separatory funnel containing 25 ml of H_2O and 2 ml of H_2SO_4 soln. Extract with four 20-ml portions of $CHCl_3$. Evaporate the combined $CHCl_3$ extract to ca 5 ml, add 25 ml of Skellysolve C, and transfer to a 125-ml separatory funnel with the aid of several small portions of $CHCl_3$ and proceed as directed under "Oil Solutions," beginning . . . "add 10 ml of NaOH soln."

Aqueous Suspensions.—Measure a portion of the sample containing the quantity of estrogens directed under "Tablets" and transfer to a 125-ml separatory funnel. Extract with six 25-ml portions of ether (samples containing polyoxyalkylene of sorbitol monolaurate should be extracted with $CHCl_3$, as ether frequently forms emulsions difficult to break). Combine the ether extracts and evaporate to about 5 ml. Add 25 ml of Skellysolve C, transfer to a 125-ml separatory funnel with the aid of several small portions of $CHCl_3$, and proceed as directed under "Oil Solutions," beginning . . . "add 10 ml of NaOH soln."

Oil Solns.—Measure a portion of the sample containing the quantity of estrogens directed under "Tablets" and transfer to a 125-ml separatory funnel containing 25 ml of Skellysolve C. Add 10 ml of NaOH soln, shake vigorously for two min. and allow the two layers to separate completely. Transfer the aqueous layer to a second 125-ml separatory funnel. Repeat the extraction with two additional 10-ml portions of NaOH soln, adding each extract to the second funnel. (The NaOH extraction should be completed as quickly as possible. Long standing in strongly alkaline soln might cause some decomposition of the ketosteroids.) Discard the Skellysolve soln. Add H_2SO_4 soln to the combined alkaline solns until a permanent opalescence or precipitate forms (acid to litmus). Cool thoroly, add 25 ml of washed ether, shake carefully for one min. and allow the two layers to separate. Transfer the acid layer to a second 125-ml separatory funnel and repeat the extraction with another 25 ml of washed ether. Discard the acid layer. Extract the ether layers in succession with two 5-ml portions of Na_2CO_3 soln and two 5-ml portions of H_2O. Discard the aqueous layers. Transfer the ether solns to a small beaker and carefully evaporate to dryness on a steam bath in a current of air, adding a few ml of alcohol, if necessary to aid in removal of residual H_2O.

Dissolve the ether extract in a small amount of $CHCl_3$, warming if necessary, and transfer with the aid of a few ml of $CHCl_3$ to a 18×150 mm. test tube. Carefully evaporate the $CHCl_3$ on a steam bath in a current of air. Add 100 mg of Girard Reagent T and 0.5 ml of acetic acid to the test tube, stopper loosely with a foil-covered cork and heat in a boiling water bath for five min. At the end of the heating period cool the test tube in an ice bath. After cooling, dissolve the reaction mixture in a few ml of ice water and transfer to a 125 ml separatory funnel containing ca 25 ml of ice-water. Render the soln neutral to litmus paper by the addition of NaOH soln and extract *at once* with three successive 15-ml portions of $CHCl_3$. Drain each portion of $CHCl_3$ successively into a second separatory funnel containing 5 ml of H_2O, wash and filter thu a pledget of cotton wet with $CHCl_3$ into a 50-ml volumetric flask. Dilute to 50 ml with *alchohol* and retain for the estimation of alpha-estradiol. Add the 5 ml of wash water to the aqueous soln in the first funnel. Acidify the aqueous soln with 2 ml of H_2SO_4 soln and allow to remain at room temp. for one hour. Add 15 ml of $CHCl_3$, shake vigorously for one minute, and allow the layers to separate. Transfer the $CHCl_3$ layer to a second separatory funnel. Repeat the extraction with three additional 15-ml portions of $CHCl_3$. Wash the combined $CHCl_3$ extracts with 5 ml of H_2O, filter thru a pledget of cotton wet with $CHCl_3$ into a beaker, evaporate to a small volume and transfer with $CHCl_3$ to a tared 25-ml beaker previously dried in a vacuum desiccator to constant weight. Carefully evaporate to dryness on a steam bath in a current of air, adding a few ml of alcohol if necessary to aid in removal of residual H_2O. Dry in a vacuum desiccator to constant weight. Correct the weight of the ketosteroid residue by determining a reagent blank, following the above procedure.

NOTE: It is important that the gravimetric procedure be completed promptly once it has been started. However, it may be interrupted after obtaining the dry residue from the ether extract.

(7) The following method for the determination of ketosteroids (colorimetric) was adopted, first action.

APPARATUS

A spectrophotometer or photometer, capable of indicating spectral bands of the order of 2 mμ or less in the region of 400–700 mμ. (A Beckman Quartz Spectrophotometer fitted with matched 1 cm absorption cells is suitable.)

Glass-stoppered test tubes.—ca 16×150 mm.

REAGENTS

Benzenesulfonyl chloride.—reagent grade redistilled in vacuum in all-glass apparatus.

Sodium hydroxide soln.—ca 2N.

Pyridine.—redistilled over solid KOH in all-glass apparatus.

Buffer soln.—Dissolve 220 gm of crystalline sodium acetate, $CH_3COONa.3H_2O$ (or 133 gm of the anhydrous salt) in 600 ml of H_2O. Add 20.0 ml of glacial acetic acid, dilute to 1 liter and mix. The *p*H of the reagent is 5.2–5.4.

BQC reagent.—A 0.5% soln of Eastman reagent grade crystalline dibromoquinonechloroimide in ethyl alcohol, prepared just prior to use. The soln deteriorates progressively, yielding undesirable background colors. The solid reagent is sensitive to light and moisture and should be stored in a brown glass bottle in a desiccator.

Modified iron-Kober reagent.—Dilute 10 ml of stock iron-Kober reagent (Reagent A, Alpha-Estradiol Determination) to 100 ml with H_2SO_4 (2+1) just prior to use, shaking vigorously to homogeneity.

STANDARD SOLUTIONS

Estrone Standard.—50 mmg/ml. Dissolve 25 mg of pure estrone, accurately weighed, in alcohol, and dilute to exactly 500 ml with the solvent.

Equilin Standards.—50 mmg/ml. Dissolve 25 mg of pure equilin, accurately weighed, in alcohol, and dilute to exactly 500 ml with the solvent. Prepare the diluted standard (10 micrograms/ml.) required in the estrone determination from an aliquot of this solution.

Equilenin Standard.—50 mmg/ml. Dissolve 25 mg of pure equilenin, accurately weighed, in alcohol, and dilute to exactly 500 ml with the solvent.

Stored in the dark, in tightly stoppered containers, the standard solutions keep indefinitely.

SAMPLE SOLUTION

Prepare an alcoholic solution of the ketosteroids from the gravimetric determination to contain 90–120 micrograms of ketosteroids per ml.

DETERMINATION

Equilenin

Transfer 5 ml aliquots of the sample soln in duplicate to separatory funnels. Prepare a 5 ml alcohol blank and a set of standards—250 mmg of equilin, 100 mmg of equilenin, 200 mmg of equilenin—each made up to 5 ml. with alcohol in a separatory funnel. Add to each separator 5 ml of buffer soln and 1 ml of BQC reagent. Mix and let stand 2 hours. By means of a burette add 10 ml of $CHCl_3$ and mix by careful shaking. Add 20 ml of NaOH soln and shake vigorously at least 1 min. Remove the clear chloroformic extract and filter rapidly thru a dry folded paper. Determine the optical densities of the sample and standard solns at 535 mμ relative to the blank. Correct the sample reading for the equilin content as determined below and calculate the quantity of equilenin present.

NOTE: If the color obtained from 500 mmg of sample is less than that due to 25 mmg of equilenin, the ketonic residue may be presumed to consist entirely of estrone.

Equilin

Transfer 5 ml aliquots of the sample soln in duplicate to test tubes provided with glass stoppers. Add a chip of silicon carbide and evaporate *just* to dryness by immersing in a steam bath. Treat similarly a 5 ml alcohol blank and aliquots of the equilin standard containing, respectively, 100 mmg and 200 mmg of equilin. Cool in a vacuum desiccator for an hour. Dissolve the residue in 2 ml of dry pyridine, add 0.2 ml of benzenesulfonylchloride, stopper and let stand overnight. Mix with 10–15 ml of H_2O and wash into a separator. Rinse the tube with several 10-ml portions of H_2O, then with 15 ml of $CHCl_3$ and add each to the separator. Shake vigorously at least one min. and remove the $CHCl_3$ layer to a 50-ml glass-stoppered Erlenmeyer flask. Repeat the extraction with a 15 ml portion of $CHCl_3$, combine the extracts, and evaporate to dryness. Dissolve the residue in 2 ml of alcohol by gently warming in the stoppered flask, cool, and mix with 4 ml of buffer soln and 2 ml of BQC reagent. Let stand 4 hours (rapid development of a pink color indicates incomplete esterification of equilenin). Measure in 5 ml of $CHCl_3$ and mix carefully. Add 20 ml of NaOH soln and shake vigorously at least one minute. Separate the chloroformic extract and filter rapidly thru a dry folded paper. Determine the optical density of the sample and standard solns relative to the blank at 570 mμ, and compute the equilin content.

Estrone

Dilute 5 ml of the sample soln with 5 ml of alcohol. To duplicate 1 ml aliquots of this soln in test tubes provided with glass stoppers add 10 ml of modified iron-Kober reagent with thoro mixing. Stopper and mix vigorously. Treat similarly a 1 ml alcohol blank, duplicate 1 ml aliquots of the estrone standard soln and 1 ml each of the equilenin and the two equilin standard solns. Immerse the loosely-stoppered tubes in a hot water bath above the level of their contents, heat to 75–80°, and maintain at that temperature for 2 hours. Cool the tubes rapidly in cold water, mix by inverting and let stand at room temp 15 min. Determine the optical densities of the sample and standards relative to the blank at 510 mμ (maximum for estrone). Compute and apply the density corrections due to equilin and equilenin, using the 10 mmg/ml equilin standard for quantities up to 10 mmg of equilin in the aliquot and the 50 mmg/ml standard for greater quantities. Calculate the estrone content of the sample.

(8) The following method for the determination of alpha-estradiol was adopted, first action.

APPARATUS

A spectrophotometer or photometer capable of indicating spectral bands of the order of 2 mμ or less in the region of 400–700 mμ. (A Beckman Quartz Spectrophotometer fitted with matched 1 cm absorption cells is suitable.)

Separatory funnels, 125 ml, with well-fitting stopcocks lubricated only with water.

Silicon carbide.—20 mesh (obtainable from the Carborundum Company, Niagara Falls, New York).

Clean, dry, light rubber finger stalls, small or medium size (obtainable from drug supply houses or most drugstores).

Clean, dry, solid glass beads.—4 to 5 mm diameter.

Three burettes with stopcocks lubricated only with reagent. The orifice of one burette should be enlarged, if necessary, to deliver 1 ml of Reagent A in 30 seconds or less. Protect reagents in the burettes from absorption of moisture with suitable guard tubes.

Partition tube.—Select a 25×200 mm test tube of 3.85×200 mm test tube of 3.85–4.00 square centimeters cross-sectional area by measuring the height of a 50 ml column of H_2O in it. Fuse a 6 cm length of 5–6 mm tubing to the bottom of the tube and slightly constrict this stem about 2 cm below the tube.

Benzene reservoir.—A 500 ml separatory funnel with 3 mm or larger bore stopcock lubricated only with H_2O. The stem should be ca 10 cm long.

Packing rod.—Flatten the end of a glass rod to a circular head with a clearance of about 1 mm in the partition tube.

Leveling rod.—A sharp-edged rod about 1.5 cm in diameter.

REAGENTS

Reagent A—(Stock Iron-Kober reagent).—Dissolve 1.054 g of $FeSO_4.(NH_4)_2SO_4.6H_2O$ (Mohr's Salt) in ca 20 ml of H_2O, add 1 ml of H_2SO_4, and 1 ml of 30% H_2O_2. Mix, heat until effervescence ceases, and dilute to exactly 50 ml. To 3 volumes of the iron soln in a volumetric flask add H_2SO_4 (A.C.S. Reagent Grade) with cooling to make 100 volumes. Redistill U.S.P. phenol, discarding the first 10% and the last 5%. Collect the distillate with exclusion of moisture in a dry tared flask of about twice the volume of the phenol and equipped with a glass stopper. Place the stoppered flask in an ice bath to solidify the phenol, breaking the top crust with a glass

rod to be sure of complete crystallization. Dry and weigh the flask. Add to the phenol 1.13 times its weight of the iron-sulfuric acid soln, stopper the flask and allow to stand without cooling but with occasional mixing until the phenol is liquefied (ca 30 min. or less). Shake the mixture vigorously until homogeneous and allow to stand in the dark 16–24 hours. Add to the mixture 23.5% of its weight of H_2SO_4 (10+11). Shake the mixture vigorously to homogeneity. Transfer to dry glass-stoppered bottles. (Stored in the dark and protected from absorption of moisture this reagent is stable for months.)

Reagent B.—To a carefully measured volume of Reagent A in a glass-stoppered graduated cylinder add 0.45 volume of H_2O, mix, cool, and transfer to dry glass-stoppered bottles. Store in the dark and protect from absorption of moisture. Inspect before use and disperse any flocculent precipitate by vigorous swirling of the reagent. With this precaution the reagent may be used satisfactorily for weeks.

Reagent C.—To a carefully measured volume of Reagent A in a glass-stoppered graduated cylinder add 0.45 volume of 1 N hydrochloric acid, mix vigorously, and place at once in a water-bath at 25°–28°. Use the reagent preferably after 1 hour and not more than 3 hours from its preparation.

Sulfuric acid soln.—H_2SO_4 (7+13).

Packing material.—Celite No. 545 (Johns-Manville diatomaceous earth).

Sodium hydroxide.—0.400 N (carbonate-free).

Benzene.—A.C.S. Grade, redistilled in all-glass apparatus.

STANDARD SOLUTIONS

Alpha-Estradiol standard.—20 mmg/ml. Dissolve exactly 10 mg of pure alpha-estradiol in alcohol and dilute with alcohol to 100 ml in a volumetric flask. Pipette exactly 10 ml of this soln into a 50 ml volumetric flask and make to volume with alcohol.

Beta-Estradiol standard.—20 mmg/ml. Prepare in the same way from pure beta-estradiol.

Alpha-Dihydroequilin standard.—10 mmg/ml. Prepare in the same way from 5 mg of pure alpha-dihydroequilin.

Stored in the dark in tightly stoppered containers the standard solutions keep indefinitely.

PRELIMINARY DETERMINATION

Apply procedures (A) and (B) below to aliquots of the $CHCl_3$ soln of diols obtained from the Ketosteroid (Gravimetric) determination. (Very turbid solutions sometimes resulting from these aliquots should be filtered along with the blank and standard solutions through a pledget of fine glass wool packed tightly in the lower end of the stem of a Bunsen funnel.) For this purpose disregard the presence of alpha-dihydroequilin. If the weight of alpha-estradiol so determined is equal to one percent or less of the weight of ketosteroids in the same quantity of sample, report alpha-estradiol as "not over one percent of the ketosteroid content."

DETERMINATION

Preparation of partition column.—Pack fine glass wool into the constricted stem of the partition tube so that when the tube is filled with benzene the rate of flow is within 2.5–3.0 ml per minute. Before packing the column fasten a piece of rubber tubing with attached screw-clamp to the outlet to limit the flow during packing. To 1 g of Celite in a small beaker add sufficient benzene to cover the Celite. Pipette in 0.5 ml of H_2O and mix vigorously with a stirring rod until the Celite is uniformly wet. With the tube about one-quarter filled with benzene place a pad of glass wool (ca 1 cm high when gently compressed) at the bottom of the tube and then transfer

the Celite mixture to the tube. Form a flocculent suspension by slowly working the packing rod up and down as a piston thru the Celite mixture. Gently compress the Celite with the packing rod and finish off the edges with the leveling rod to form a level sharply defined surface on a uniform pack ca 1 cm high. Cover 8 g of Celite in a mortar with about 40 ml of benzene and distribute over the Celite from a pipette exactly 5 ml of 0.400 *N* NaOH. Mix carefully for several minutes with a pestle until the Celite appears uniformly wet. Open the screw-clamp enough to permit slow drainage during packing of the tube. Transfer the Celite to the tube with a spatula in about five portions, suspending each portion and gently packing as above. Finish off the top of the column, scraping down any Celite on the upper wall of the tube to form a sharply defined level surface on a column of ca 30 ml volume over the initial pack. (Packing too tightly may cause loss of estradiol in the fore-run, particularly at room temp much above 25°.) The Celite must be covered with benzene at all times. Nearly fill the separatory funnel with benzene and seal the stopper with a film of H_2O to prevent air leaks. Insert the stem of the funnel into the benzene over the Celite, fully open the stopcock and adjust the level of the benzene to produce a flow-rate of 2.0–2.5 ml per min. with the screw-clamp fully open. Mark the level of the benzene on the tube, close the stopcock and remove the benzene reservoir.

Partition.—Carefully evaporate to just short of dryness an aliquot of the $CHCl_3$ extract containing 100–250 mmg of total diols calculated as alpha-estradiol. If necessary, add a few ml of alcohol near the end of the evaporation to aid in removal of any residual H_2O. Remove the last portions of solvent in an efficient desiccator connected to the vacuum 1 hour or more. Dissolve the dry extract in 5 ml of benzene by warming gently, then cool the soln to room temperature or below. Remove the rubber tubing from the partition tube and when the benzene *just stops* dropping from the tube transfer the diol soln at once to the tube, allowing it to flow down the wall near the top of the Celite. (A 5 ml pipette is convenient for this purpose.) When the benzene just stops dropping from the tube, complete the transfer in like manner with three more 5 ml portions of benzene, discarding the effluent. Immediately place a 50-ml graduated cylinder under the tube, add benzene to the level marked on the tube and replace the benzene reservoir, supporting it at a height to maintain that level when the stopcock is fully opened. When exactly 30 ml of effluent collects in the cylinder, replace the cylinder as receiver with a dry 250-ml beaker previously marked at the 170 ml level. (Decrease the 30 ml of fore-run by 2 ml for each degree that room temp. exceeds 25°.) Collect 170 ml of effluent in the beaker, concentrate the soln to 30–40 ml, transfer to a 50 ml volumetric flask and make to volume.

Determine alpha-estradiol in the soln by Procedures (A), (B), and, if necessary, (C).

Procedure (A).—Transfer in duplicate to dry 18×150 mm test tubes (1) an aliquot of the sample soln containing 10–25 mmg of total estradiols (2) 1 ml of alpha-estradiol standard (3) 1 ml of beta-estradiol standard and, if necessary from Procedure (B) (4) 1 ml of alpha-dihydroequilin standard (see Note). Add several pieces of silicon carbide to each tube and rapidly evaporate the solvent in the steam bath (do not use a current of air) until ebullition from the boiling stones just stops. Instantly remove the tube, wipe dry quickly, and transfer to an efficient vacuum desiccator. Keep the tube in the desiccator connected to the vacuum at least 1 hour. To each tube and to a blank tube add a glass bead and measure into each tube from a burette 1 ml of Reagent A, quickly wiping the outside of the tip with a piece of absorbent paper before each addition. Immediately cap the tubes with rubber finger stalls and allow to stand for 30 min., shaking the tubes vigorously at 5-min. intervals. Place in a boiling water bath for 35 min., removing and shaking each tube for a few seconds after the first 5 min. Transfer to an ice bath for 2 min., then remove and add from a burette exactly 4 ml of the H_2SO_4 soln. Allow to stand 5 min., then mix by

shaking, first gently, then vigorously, to homogeneity. Measure the optical densities of the sample and standards relative to the blank at 525 mμ (midpoint between the maxima for alpha-estradiol and beta-estradiol) and at 420 mμ, making any necessary corrections due to cell variations.

Micrograms of total estradiols (calculated as alpha-estradiol) in aliquot = 20

$$\times \frac{E_{525m\mu} \text{ sample} - (E_{420m\mu} \text{ sample}/2)}{E_{525m\mu} \text{ alpha-estradiol standard} - (E_{420m\mu} \text{ alpha-estradiol standard}/2)} = T_a.$$

Micrograms of beta-estradiol (calculated as alpha-estradiol) in aliquot

= Micrograms beta-estradiol in aliquot (from Procedure B)

$$\times \frac{E_{525m\mu} \text{ beta-estradiol standard} - (E_{420m\mu} \text{ beta-estradiol standard}/2)}{E_{525m\mu} \text{ alpha-estradiol standard} - (E_{420m\mu} \text{ alpha-estradiol standard}/2)} = B_a.$$

Micrograms of alpha-dihydroequilin (DHQ) (calculated as alpha-estradiol) in aliquot

= Micrograms DHQ in aliquot (from Procedure C)

$$\times \frac{2E_{525m\mu} \text{ DHQ standard} - E_{420m\mu} \text{ DHQ standard}}{E_{525m\mu} \text{ alpha-estradiol standard} - (E_{420m\mu} \text{ alpha-estradiol standard}/2)} = D_a.$$

Micrograms of alpha-estradiol in aliquot = T_a minus B_a or D_a or both, depending on the composition of the aliquot.

NOTE: If the alpha-dihydroequilin in the aliquot exceeds 10 micrograms, a correspondingly higher standard should be used in determining the correction for alpha-dihydroequilin and the calculation corrected accordingly.

Procedure (B).—Transfer in duplicate to dry 18×150 mm test tubes (1) an aliquot of the sample soln containing 10–25 mmg of total estradiols (2), 1 ml of beta-estradiol standard, and (3) 1 ml of alpha-dihydroequilin standard (see Note). Add several pieces of silicon carbide to each tube, evaporate the solvent, and dry the residue exactly as described in Procedure (A). To each tube and to a blank tube add from a burette 1 ml of Reagent B, quickly wiping the outside of the tip with a piece of absorbent paper before each addition. Immediately cap the tubes with rubber finger stalls and place in a boiling water bath for exactly 2 min., shaking the tube after 30 sec for a few sec without removing from the bath. Transfer to an ice bath for 2 min., then remove and add from a burette exactly 4 ml of the H_2SO_4 soln. Mix by shaking vigorously to homogeneity. Promptly measure the optical densities of the sample and standard relative to the blank at 526 mμ (maximum for beta-estradiol), at 468 mμ (maximum for alpha-dihydroequilin), and at 420 mμ, making any necessary corrections due to cell variation.

If $E_{468m\mu}$ sample $- \left(E_{526m\mu} \text{ sample} \times \frac{E_{468m\mu} \text{ beta-estradiol standard}}{E_{526m\mu} \text{ beta-estradiol standard}}\right)$ does not exceed 20% of $E_{468m\mu}$ alpha-dihydroequilin standard, then disregarding the presence of alpha-dihydroequilin in a like aliquot will result in not more than 0.8 mmg of apparent alpha-estradiol in Procedure (A). This is normally a negligible quantity, in which case calculate beta-estradiol as follows and omit the dihydroequilin standard in Procedure (A):

Micrograms of beta-estradiol in aliquot

$$= 20 \times \frac{E_{526m\mu} \text{ sample} - (E_{420m\mu} \text{ sample}/2)}{E_{526m\mu} \text{ beta-estradiol standard} - (E_{420m\mu} \text{ beta-estradiol standard}/2)}.$$

Otherwise determine alpha-dihydroequilin by Procedure (C) and calculate beta-estradiol as follows, where DHQ = alpha-dihydroequilin:

$$E_{526m\mu} \text{ sample (corrected)} = E_{526m\mu} \text{ sample} - (E_{526m\mu} \frac{\text{DHQ Standard}}{\text{mmg DHQ in aliquot}/10})$$

$$E_{420m\mu} \text{ sample (corrected)} = E_{420m\mu} \text{ sample} - (E_{420m\mu} \frac{\text{DHQ Standard}}{\text{mmg DHQ in aliquot}/10})$$

Micrograms of beta-estradiol in aliquot

$$= 20 \times \frac{E_{526m\mu} \text{ sample (corrected)} - (E_{420m\mu} \text{ sample (corrected)}/2)}{E_{526m\mu} \text{ beta-estradiol standard} - (E_{420m\mu} \text{ beta-estradiol standard}/2)}.$$

NOTE: If the alpha-dihydroequilin in the aliquot exceeds 10 micrograms, a correspondingly higher standard should be used in determining the correction for alpha-dihydroequilin and the calculation corrected accordingly.

Procedure (C).—Transfer in duplicate to 18×150 mm test tubes (1) an aliquot of the sample soln containing up to 20 mmg of alpha-dihydroequilin and (2) 2 ml of alpha-dihydroequilin standard. Add several pieces of silicon carbide to each tube, evaporate the solvent and dry the residue exactly as described in Procedure (A). Place the tubes in a water-bath at 25°–28° and add rapidly near the bottom of each 5 ml of Reagent C. Using a long stirring rod, mix the residue vigorously with the reagent for at least one min. Leave the rod in the tube. Measure the optical densities of the sample and standard relative to the reagent (also held in the bath at 25°–28°) at 472 mμ just 30 min. after addition of the reagent and repeat the measurement at 5–10 min. intervals until the maximum optical density is reached (usually 35–55 min. after mixing).

$$\text{Micrograms of alpha-dihydroequilin in aliquot} = 20 \times \frac{E_{472m\mu} \text{ sample}}{E_{472m\mu} \text{ standard}}.$$

33. COSMETICS

(1) The following method for the determination of urea in deodorants was adopted, first action.

REAGENTS

Magnesium chloride hexahydrate, C.P.
Hydrochloric acid, concentrated.
Sodium hydroxide, 0.1 *N*.
Sulfuric acid, 0.1 *N*.
Methyl red indicator soln.

DETERMINATION

Pipet an aliquot containing 50–100 mg of urea into a 100-ml round-bottom flask having a ST 24/40 female joint. Acidify with concentrated HCl using 0.5 ml in excess; immerse flask in steam bath and evaporate to dryness. Add 10 g of crystalline $MgCl_2.6H_2O$, 1 ml of concentrated HCl, and connect the flask to a reflux condenser. Heat the mixture carefully with a small flame until the magnesium chloride dissolves in its water of crystallization, and reflux slowly for 2 hours so that the rate of return of liquid from the condenser is 9–14 drops per min. Allow the soln to cool, add water thru the top of the condenser, disconnect the flask, and if necessary heat to dissolve any solids. Transfer the soln to a 1-liter flat-bottom flask, dilute to ca 400 ml with water, make alkaline with 10% NaOH, and distill ca 275–300 ml into a suitable portion of 0.1 *N* H_2SO_4 containing several drops of methyl red. Titrate the excess acid with ca 0.1 *N* NaOH, using more indicator if necessary. Standardize the 0.1 *N* NaOH against the standard 0.1 *N* H_2SO_4, using methyl red as indicator.

Correct for a blank by refluxing 10 g of crystalline $MgCl_2.6H_2O$, 1 ml concentrated HCl, and proceeding as described above.

1 ml of 0.1 *N* H_2SO_4 = 3.003 mg of urea

(2) The following method for the determination of chlorides in deodorants was adopted, first action.

REAGENTS

Ammonium hydroxide, (1+1).
Nitric acid, (1+1).
Nitric acid, 0.01 *M*.
$AgNO_3$, 0.1 *M*.

Pipet an aliquot containing about 100 mg of chloride into a 250 ml beaker. Make volume 150 ml with water, neutralize to litmus with (1+1) NH_4OH, and acidify with 1 ml of (1+1) HNO_3. If any undissolved precipitate remains add additional (1+1) HNO_3 until a clear soln is obtained. Add dropwise and with constant stirring a slight excess of 0.1 *M* $AgNO_3$. (This excess should not exceed 5 ml.). The precipitation and succeeding operation must be carried out in subdued daylight. Heat the mixture to 90–95°C. and stir until the precipitate coagulates. Allow the precipitate to settle add 1–2 drops of 0.1 *M* $AgNO_3$ to the supernatant soln to make sure that an excess of precipitant is present. If there is no further precipitation, set the beaker aside in the dark, and allow the mixture to stand for 1–2 hours. Then decant the supernatant liquid thru a tared Gooch crucible, wash the precipitate 2–3 times with 0.01 *M* HNO_3 by decantation, and finally transfer the precipitate to the Gooch crucible using 0.01 *M* HNO_3. Continue washing the precipitate with 0.01 *M* HNO_3 until a washing gives a negative test for $AgNO_3$ when a drop of 0.1 *N* HCl is added. Complete the washing by removing most of the nitric acid with 1–2 portions of water. Dry the crucible in the oven at 120–130°C. for 2 hours. Repeat drying until successive weighings agree to 0.2 mg.

Weight of AgCl × 0.2474 = Weight of Chloride.

(3) The following method for the determination of sulfates in deodorants was adopted, first action.

REAGENTS

Ammonium hydroxide, (1+1).
Hydrochloric acid, concentrated.
$BaCl_2 \cdot 2H_2O$, 1% (w/v).

Pipet an aliquot containing about 100 mg of sulfate into a 600 ml beaker. Make volume 350 ml with water, neutralize to litmus using (1+1) NH_4OH, and acidify with 2 ml of concentrated HCl. If any undissolved precipitate remains add additional concentrated HCl until a clear soln is obtained. Heat 50 ml of a 1% soln of $BaCl_2.2H_2O$ almost to boiling and add rapidly with stirring to the sulfate soln which has also been heated near the boiling point. Allow the precipitate to settle, and test the supernatant liquid for complete precipitation by adding a little barium chloride soln. If there is no further precipitation set the beaker aside for 1–2 hours on the steam bath. After the digestion, decant the supernatant liquid thru a tared Gooch crucible, wash the precipitate 4–5 times with small portions of warm water by decantation, and finally transfer the precipitate to the Gooch crucible using warm water. Continue washing the precipitate with warm water until a washing gives a negative test for chlorides. Dry the crucible in the oven at 110–120°C. for 2 hours. Repeat drying until successive weighings agree to 0.2 mg.

Weight of $BaSO_4$ × 0.4115 = Weight of Sulfate.

34. COLORING MATTERS

(1) The following methods were adopted, first action, for intermediates derived from phthalic acid.

FD&C Red No. 3, D&C Orange Nos. 5, 6, 7, 8, 9, 10, 11, 12, 13, and 17; D&C Red Nos. 21, 22, 23, 24, 25, and 26; D&C Yellow Nos. 7, 8, and 9, and Ext. D&C Orange No. 2.

REAGENTS

Sodium hydroxide, 10% (*w/v*).—Dissolve 100 g NaOH in water and dilute to 1 liter.

Hydrochloric acid (1+9).—Dilute 100 ml of conc. HCl to 1 liter.

Ethyl acetate, absolute, C.P.

Standard phthalic acid soln.—Accurately weigh 0.130–0.135 gram of C.P. potassium acid phthalate, dissolve in water, and dilute to exactly 500 ml. Dilute 10 ml of this soln to 200 ml with ca 0.1 *N* HCl. Calculate the concentration of phthalic acid in this soln as follows:

$$\text{Conc. phthalic acid (mg/100 ml)} = \text{Mg of } KHC_8H_4O_4 \times 0.00813.$$

(*a*) *Water-soluble salts.*—Wash a 2-g sample (accurately weighed) of the dye into a 250-ml beaker with ca 100 ml of distilled water. Heat nearly to boiling and add slowly with stirring (1+9) HCl until precipitation appears complete. Add an additional 8.5 ml of (1+9) HCl, dilute to about 150–160 ml and digest on the steam bath 1–2 hours. Cool to room temp., wash into a 200-ml volumetric flask, and dilute to volume with distilled water. Filter thru a dry filter paper.

Pipette 50 ml of the filtrate into a 125-ml separatory funnel (use no grease on the stopcocks) and extract with 30 ml of ethyl acetate. Transfer the aqueous phase to another separatory funnel and extract with 25 ml of ethyl acetate. Again transfer the aqueous phase to a third separatory funnel and extract with 20 ml of solvent. Pass three successive 50 ml portions of distilled water thru the funnels in the same order that the extractions were made. Discard the ethyl acetate, combine the aqueous extracted soln, and evaporate to dryness. (It is convenient to reduce to a small volume on a hot plate with the aid of an air jet and then evaporate to dryness on a steam bath.) Dissolve the residue in distilled water and transfer to a 100-ml volumetric flask. Add 8.5 ml of (1+9) HCl and dilute to volume. Filter thru a dry filter paper and determine the extinction of the soln at 230, 262, and 276 mμ on a Beckman ultraviolet spectrophotometer against 0.1 *N* HCl as the blank. (If the soln is too concentrated for accurate readings, dilute an aliquot to a more suitable concentration with 0.1 *N* HCl and multiply the final result by the dilution factor.)

Measure the extinction of the standard phthalate soln at the same wave lengths.

(*b*) *Color acids.*—To 2 g of the color, add 6 ml 10% NaOH, a few ml of water, and mix until the color is dissolved. Dilute to ca 100 ml and proceed as directed above beginning with "Heat nearly to boiling . . . ."

CALCULATIONS

Calculate the quantity A for both sample and standard as follows:

$$A = [E_{230} - (E_{230} - E_{276}(0.7)] - E_{262}$$

then

$$\%\ \text{phthalic acid} = \left(\frac{\text{A sample}}{\text{A standard}}\right) \times \frac{\text{Conc. standard solution}}{\text{(in mg/100 ml)}} \times 0.2.$$

D&C YELLOW NO. 10

REAGENTS

Sodium hydroxide, 1% (w/v).—Dissolve 10 g of NaOH in water and dilute to one liter.

Hydrochloric acid (1+199).—Dilute 10 ml of concentrated HCl to one liter.

Diethyl ether, C.P.

Standard phthalic acid soln.—Accurately weigh 0.15 to 0.18 g of C.P. potassium acid phthalate, dissolve in water, and dilute to exactly 500 ml. Dilute 10 ml of this soln to 200 ml with 0.1 *N* HCl. Calculate the concentration of phthalic acid in this soln as follows:

$$\text{Conc. phthalic acid (mg./100 ml.)} = \text{mg. of } KHC_8H_4O_4 \times 0.00813.$$

DETERMINATION

Dissolve a 1-g sample of the color in water and wash into a continuous extractor. Add ca 1 ml of conc. HCl per 100 ml of soln and extract for 8 hours with ca 250 ml of diethyl ether. Transfer the ether to a separatory funnel. Rinse the extraction flask with two small portions of ether and add the washings to the main extract. Wash the ether extract with four 10-ml portions of (1+199) HCl. Combine the washings in a separatory funnel and extract with 50 ml of ether. Combine the ether fractions and extract four times with 10 ml of 1% NaOH soln. Collect the alkaline washes in a beaker and evaporate to dryness. Dissolve the residue in distilled water and transfer to a 200 ml volumetric flask. Add 2 ml of conc. Hcl and dilute to volume. Proceed as directed above under "(a) water-soluble salts" beginning "Filter thru a dry filter paper" in paragraph 2, line 10.

D&C YELLOW NO. 11 AND D&C RED NO. 19

REAGENTS

Sodium hydroxide, 1% (w/v).—Dissolve 10 g of NaOH in water and dilute to one liter.

Sodium hydroxide, 10% (w/v).—Dissolve 100 g of NaOH in water and dilute to one liter.

Hydrochloric acid (1+9).—Dilute 100 ml of conc. HCl to one liter.

Chloroform, U.S.P.

Standard phthalic acid soln.—Accurately weigh 0.15 to 0.18 g C.P. potassium acid phthalate, dissolve in water, and dilute to exactly 500 ml. Dilute 10 ml of this soln to 200 ml with ca 0.1 *N* HCl.

$$\text{Conc. phthalic acid (mg/100 ml.)} = \text{mg. of } KHC_8H_4O_4 \times 0.00813.$$

DETERMINATION

(*a*) *D&C Red No. 19.*—Weigh a 0.5-g sample into a beaker and dissolve in 20 ml of hot water. Cool to room temp. and transfer the soln to a 125 ml separatory funnel. Rinse the beaker with 5 ml of H_2O and add the wash water to the funnel. Add 80 ml of chloroform, 2 ml of 10% NaOH, and shake vigorously for one min. Draw off the chloroform layer and wash the aqueous phase twice with 30 ml portions of chloroform, discarding the chloroform. Add 7 ml of (1+9) HCl and wash with two 30-ml portions of chloroform, discarding the chloroform. Transfer the aqueous soln into a beaker, rinse the funnel with 10 ml of distilled water, and transfer to the same beaker. Evaporate to dryness on a steam bath with the aid of an air jet. Proceed as directed above under "(a) water-soluble salts" beginning with "Dissolve the residue in distilled water," in paragraph 2, line 9.

(*b*) *D&C Yellow No. 11.*—Wash a 0.5-g sample into a 125 ml separatory fun-

nel with 80 ml of chloroform. Add 20 ml of 1% NaOH. Proceed as directed above under "(a) D&C Red. No. 19" beginning with "Shake vigorously for one min.," line 5.

(2) The following method was adopted, first action, for pseudocumidine in FD&C Red No. 1.

APPARATUS

Steam distillation.—A three-liter, two-neck, round-bottom flask; one neck is fitted with a dropping funnel; the other with a steam distillation trap which has an inlet for live steam and which is connected to a water-cooled condenser.

Fractionating apparatus.—A 25 ml round-bottom flask fitted with an insulated distilling column, 10–12 inches long and ½ inch inside diameter, packed with 24 inches of ca 24-gauge nichrome wire coiled inside the column in the manner described by Podbielniak*. An insulated Vigreau column with a take off valve in the side-arm of ca the same dimensions is also suitable. Provide the column with a 200–260°C. Anschutz thermometer.

REAGENT

Sodium hydrosulfite soln.—A saturated soln of reagent grade $Na_2S_2O_4$ (ca 30%).

DETERMINATION

Dissolve ca 100 g of color in 2 liters of hot water contained in the round-bottom flask of the steam distillation apparatus. Add 10 g of sodium hydroxide and heat the soln to boiling, then while passing live steam thru the soln at a rate that will produce 5–10 ml of distillate per min., add the sodium hydrosulfite soln dropwise by means of the dropping funnel until the red color disappears (the soln will then be a light yellow-brown). Continue the steam distillation until no more oil distills. Extract the distillate with four 20 ml portions of ether and wash the combined extracts several times with 10 ml portions of water. Evaporate the major portion of the ether on a steam bath and dry the residue over sodium or potassium hydroxide pellets. Filter the residual soln into the 25 ml round-bottom flask of the fractionating apparatus. Heat cautiously with a heating mantle or a water bath until all the ether has been removed; then continue heating with a Wood's metal bath or an equivalent constant temp. bath. Reflux the material for 2 min. after the vapors reach the top of the column then distill at a rate of about one drop per second. The initial boiling point is the temp. at the end of the 2 min. reflux period. The final point is taken as the maximum temp. at which any material distills.

35. EXTRANEOUS MATERIALS: ISOLATION

(1) The first action method for mold count in citrus juices, **35.5** (p. 704), was adopted, first action, for pineapple juice, with the following revisions:

(a) Add "Read volume of sediment in centrifuge tube" after "tubes," line 10.

(b) Substitute "Add 0.5 ml HCl (to dissolve oxalate crystals) and add" in place of "Add," line 11.

(c) Add at end of method "In addition to checking microscopic fields, indicate those fields positive due to Oospora."

36. MICROBIOLOGICAL METHODS

No additions, deletions, or other changes.

* *Ind. Eng. Chem., Anal. Ed.*, 5, 119 (1933).

37. MICROCHEMICAL METHODS

(1) The following method for the determination of carbon and hydrogen was adopted, first action.

REAGENTS

Copper oxide.—Wire form, about 1 mm in diam. and 3–4 mm long; discard material finer than 20 mesh. Ignite at 800–900°C. for 1 hr before placing in combustion tube.

Platinum gauze, 52 mesh.—From three 3×5-cm sections, make 3 rolls, 30 mm long×7 mm O.D. Boil in 1-1 nitric acid for 15 min. and ignite in nonluminous Bunsen burner flame.

Asbestos.—Gooch crucible asbestos; ignite at 800–900°C. for 30 min. and store in wide mouth bottles.

Silver.—Fine wire or ribbon; if tarnished reduce in stream of hydrogen at 350–450°C.

Lead dioxide.—Pellets, 1–2 mm diam., special grade for micro analysis; or prepare by digesting commercial grade powder in concentrated nitric acid for 2 hrs, let stand for 1 hr, decant nitric acid, wash with distilled water until free of nitric acid, evaporate to dryness, and cut into 2 mm cubes. Roll cubes in jar to round corners and sieve out powder.

Glass wool.—Pyrex, pliable.

Dehydrite or anhydrone.—(Magnesium perchlorate, anhydrous). Break pieces to less than 3 mm long; discard portion passing 40-mesh sieve.

Ascarite.—(Sodium hydroxide on asbestos.) Use commercial preparation of 8–20 mesh.

APPARATUS (FIG. 1)

Oxygen.—Cylinder with pressure regulator adjustable from 0–10 lb. pressure on the low-pressure side and with needle-valve control.

Preheater.—As specified by American Chemical Society Committee on Standardization of Microchemical Apparatus[2] except with 12/2 ball joint.[3]

Bubble counter and U-Tube.—According to a A.C.S. specification except with ball joints.[3]

Combustion tube.—Fused quartz (or Vycor) glass,[4] dimensions as per A.C.S. specifications[5] but with 12/2 ball joint[3] on side arm and 5/12 or 7/15 inner joint[3] on exit end.

Absorption tubes.—Pregl type, as per A.C.S. specification, but with 5/12 joints[3] (alternative, Prater type, semimicro size with 7/15 joints[3]).

Bubble counter or flowmeter.—Any convenient arrangement which will measure 10–30 ml/min flow of gas from exit end of second absorption tube.

Preheater furnace.—Electric[6,7] 12–14 mm I.D. by 5 in. long; maintain at temp. of 600 ±25°C.

Burning furnace.—Electric[6,7] 13–14 mm I.D. by 4 in. long. Furnace should reach a temp. of 600–700°C. in 5 min, about 800°C. in 15 min, with max. of 850°C in 30 min.

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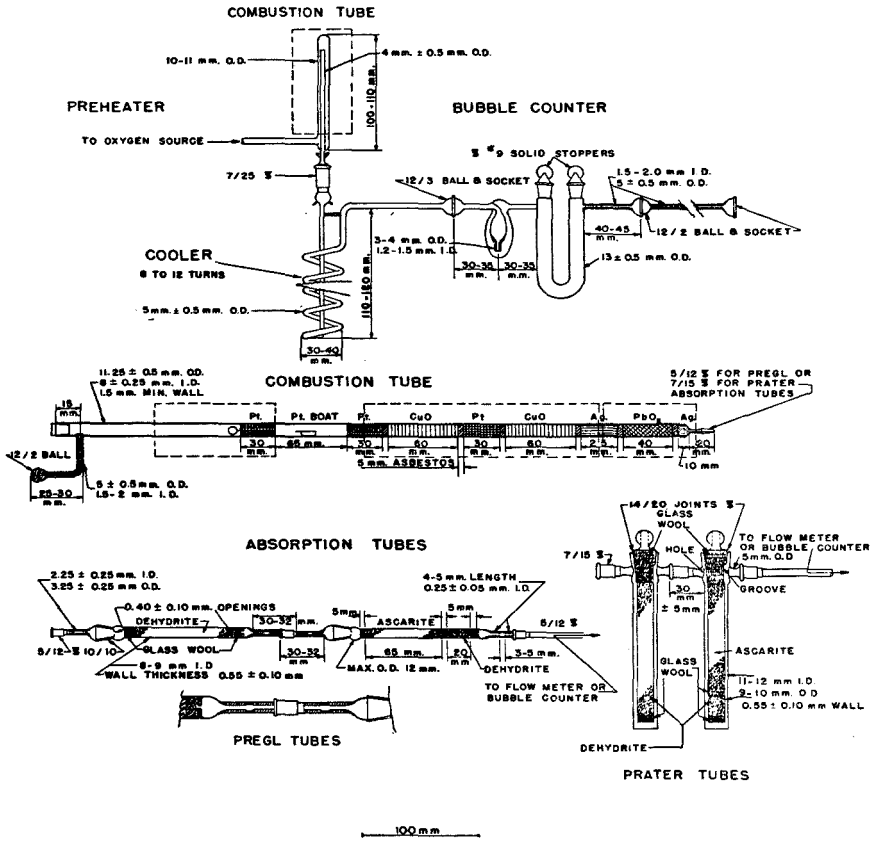
[2] *Analytical Chemistry*, 21, 1555 (1949).
[3] Rubber connectors may be used.
[4] If pyrex tubes are used, furnace temperatures should not exceed 725°C.
[5] 10 or even 12-mm I.D. tubes may be used, but oxygen flow rate must be increased proportionately.
[6] Gas heaters may be used but specified temperatures should be maintained.
[7] Temperature of furnaces measured at center of furnace inside empty combustion tube having one end stoppered.



CARBON & HYDROGEN APPARATUS

FIG. 1

Long furnace.—Electric[6,7] 13–14 mm I.D. by 8 in. long; maintained at a temp. of 775–800°C.

Constant temperature mortar.—Electric[6] 13–14 mm I.D. by 3 in. long, thermostatically controlled to maintain a temp. of 177 ±2°C.

Boat.—Platinum, micro.

Finger cots.—Chamois.

Tweezers.—Platinum tipped.

PREPARATION OF APPARATUS

Preheater.—Place copper oxide in preheater tube, connect spiral cooling coil, immerse coil in beaker of water, and support assembly by suitable clamps and stand. Place electric furnace over preheater tube and maintain at ca 600°C. Con-

nect side arm of combustion unit to needle valve of oxygen pressure regulator by suitable tubing, rubber or tygon.

Bubble counter-U-tube.—Fill bubble counter and U-tube by placing glass wool plug at bottom of U, fill side next to bubble counter with Dehydrite to within ½ in. of side arm and cap with another glass wool plug. Place Ascarite layer in other side to within 1½ in. of side arm, then insert a glass wool plug, ca 1 in. of Dehydrite and finally a second plug. Cement in stoppers with glass cement or paraffin, then with medicine dropper introduce concentrated sulfuric acid into bubbler until level is 3–4 mm above bubbler tip. Connect to preheater with pressure clamp.

Combustion tube.—Clean and dry combustion tube (Fig. 1). Place 10-mm roll of silver in exit end with one or two strands reaching to open end of ground joint. Insert a loose asbestos plug (not choking plug), 40 mm of lead dioxide, asbestos plug, and a second silver roll 25 mm long; which should extend into long furnace about ½ in. Introduce asbestos plug, 60 mm of copper oxide, asbestos plug, 30-mm platinum gauze roll, asbestos plug, 60-mm copper oxide, asbestos plug, and finally 30-mm platinum gauze, which should extend about 10 mm beyond end of long furnace. Place prepared tube in furnaces with exit end protruding beyond constant-temp. mortar sufficient to permit connecting absorption tubes. Connect side arm to bubble counter-U-tube.

Absorption tube.—Place glass wool plug in end of water absorption tube, fill tube to within ½ in. of other end with Dehydrite or Anhydrone and cap with second glass wool plug. If Pregl tubes are used, seal ground-glass joint with enough glass cement to give clear seal, and remove any excess on outer surface of tube with cotton dipped in benzene or other solvent. If Prater tubes are used, lubricate lower ⅔ of inner joint with minimum of light stopcock grease and insert in outer tube. Prepare carbon dioxide absorption tube by placing glass-wool plug in end and fill tube to about 1½ in. of other end with Ascarite. Insert ¼-in. glass wool plug, add ¾-in. layer of Dehydrite, and cap filling with another glass-wool plug. Complete assembly of absorption tube as directed for water absorption tube. Connect absorption tubes to combustion tube with ground joints (use no lubricant) or with special impregnated rubber tubing.

Attach bubble counter or flowmeter to exit end of carbon dioxide absorption tube. Counter or meter must be calibrated so that flow rate can be set at 15 to 20 ml per min.

DETERMINATION

Conditioning apparatus.—After various parts of apparatus have been prepared and assembled, condition combustion tube for 3–4 hours with long furnace at 775–800°C. and with oxygen flowing thru apparatus at rate of 15–20 ml per min.[8] At the same time, make two simulated sample burnings, without sample, with burning furnace at 825–850°C. (Temp. must be ca 100°C. lower if pyrex combustion tubes are used.)

Burn an unweighed 10–15-mg sample to condition combustion and absorption tubes. With absorption tubes connected, adjust needle valve on pressure regulator so that oxygen flow is 15–20 ml per min. and place burning furnace about 3 in. from long furnace. Place platinum boat containing sample in combustion tube about 2 in. from long furnace. Insert platinum flashback roll (Fig. 1) so that end of gauze is even with face of furnace next to sample, and stopper tube. Turn on burning furnace and allow it to reach temperature of ca 600°C. before starting sample combustion by moving furnace over sample at rate of 1 in. in 6–8 min. Move the burning furnace across sample only once, taking 18–24 min for full travel of furnace. Turn off burn-

[8] Use 3–4 lb. oxygen pressure head on low pressure side of pressure regulator.

ing furnace 5 min after it reaches long furnace but continue to sweep oxygen thru tube for an additional 15 min before disconnecting absorption tubes. Remove absorption tubes and place by balance to equilibrate. Handle tubes only with clean, chamois finger cots. If Prater tubes are used, turn joints ¼-turn to seal. If rubber connections are used, wipe only tips of tubes with moist, then dry, chamois before placing them by balance. Wait 10 min. if ground joints were used or 15 min. if rubber connections were made, then weigh crabon dioxide-absorption tube first and water-absorption tube second. A glass tare with a volume (surface) ca equal to that of absorption tubes should be used when weighing tubes. Record weights of tubes and then reconnect tubes to combustion tube for subsequent analysis.

Proving the apparatus.—Replace boat with one containing 10–15-mg sample of your own standard compound weighed to nearest 0.01 mg. Repeat combustion and weighing procedure described above. Calculate percentage of carbon and hydrogen in standard sample from increase in weight of carbon dioxide and water-absorption tubes. Repeat analysis until results from two consecutive runs are within 0.30 per cent of theoretical values and means of carbon and hydrogen results are within 0.20 per cent of theoretical value for the standard compound.

When apparatus has met this test, actual analyses of the collaborative sample should be made, using procedure described above. (Humidity conditions of room may make it necessary to correct apparent weight of water by subtracting a blank value.)

38. RADIOACTIVITY

No additions, deletions, or other changes.

39. STANDARD SOLUTIONS

No additions, deletions, or other changes.

40. VITAMINS

(1) The following method for vitamin A in mixed feeds was adopted, first action.

APPARATUS

(1) *Photoelectric Colorimeter.*—An Evelyn Colorimeter equipped with a 620 millimicron glass filter or a Coleman Spectrophotometer have been found to be desirable. An instrument with direct reading, deflecting type galvanometer is necessary.

(2) *Chromatographic tubes.*—Cylinder 23×200 mm, sealed to 5×80 mm tube. (Wilkens-Anderson Co., Chicago, Ill.)

(3) *Graduated cylinder.*—100 ml lipless or cut off top of a regular 100 ml graduated cylinder just below lip and fit with a suitable 2-hole rubber stopper (or use any other desirable receiver for eluate from adsorption column).

(4) *Fat extraction apparatus.*—Goldfisch, Bailey-Walker, or Soxhlet.

(5) *Automatic pipette.*—10 ml. This pipette should deliver rapidly thru an opening 3–4 mm in diam. (A suitable size graduated cylinder may also be used effectively for dispensing Carr-Price reagent.)

REAGENTS

(1) *Hexane.*—B. P. 60–71°C. (Skellysolve B).

(2) *Acetone.*—Reagent grade.

(3) *10% acetone in hexane.*—(Skellysolve B).

(4) *Adsorbent.*—Equal parts by weight of diatomaceous earth (Johns-Manville

Hyflo Super Cel) and Magnesia (Micron brand #2641—Westvaco Chlorine Products Corporation, Newark, Calif.).

(5) *Anhydrous sodium sulfate.*—Reagent grade.

(6) *Chloroform.*—Reagent grade (purify by distillation if necessary to prevent color interference).

(7) *Antimony trichloride reagent.*—(Carr-Price). Prepare by dissolving 20 g of antimony trichloride in sufficient chloroform to make 100 ml. Add 3 ml acetic anhydride. Filter if necessary.

PREPARATION OF ADSORPTION COLUMN

Pass the stem of the chromatographic tube thru a two-hole stopper of proper size to fit in the top of the lipless 100 ml graduated cylinder (eluate receiver). Thru the other hole in the stopper insert a bent glass tube and connect this to a source of vacuum. Ordinarily an efficient water pump will be sufficient. If another type of chromatographic assembly is employed an eluate receiver other than the lipless graduated cylinder may be used.

Place a *small* amount of cotton at the bottom of the chromatographic tube and pack tightly to a depth of 100 mm with a well-blended mixture of equal parts by weight of Hyflo Super Cel and magnesia. Inorder to assure a homogeneous blend of these two components, mix by rubbing out lumps by hand or run the mixture thru a kitchen flour sifter and roll on paper at least 50 times. Add the mixture in several portions, tamping well with a stopper or similar device. Keep suction on the column during packing. Add a 1 cm layer of anhydrous sodium sulfate to the top of the column. Do not wet column before passage of solution thru the column.

PROCEDURE

Extraction.—Weigh 10 g of feed directly into fat extraction flask. Weigh ca 1 g of dehydrated alfalfa meal into the same flask. (This serves as a source of carotene which facilitates locating by visual examination the carotene band in the chromatogram.) Add exactly 100 ml hexane and reflux on fat extractor for 30 min. Mark flask at 100 ml level with fine pointed china marking pencil. Remove flask from extractor, cover and cool for 10–15 min. Check for loss of solvent and add more if necessary to bring up to 100 ml level, mix, and allow to settle. Volume of soln may be checked also by weighing flask and contents before and after extraction.

Chromatography.—Draw a 50 ml aliquot of the supernatant liquid thru the adsorption column with suction. Elute with ca 35–40 ml of 10% acetone in hexane, using only enough of this reagent so that the first portion of the carotene band passes thru the column. A trace of carotene is eluted from the column at this point to insure complete elution of vitamin A. The eluate should, therefore, possess a distinct yellow color. Disconnect receiver from bottom of adsorption column, make up to a suitable volume, which is usually 50 ml, and mix.

Colorimetry.—Evaporate with mild heat and reduced pressure a suitable aliquot of this soln and dissolve the residue in sufficient chloroform so that 1 ml after the addition of the antimony trichloride reagent will give transmittance readings which are within the range of 30–65 per cent (absorbancies of ca 0.5–0.2). Set colorimeter at 100 per cent transmission, using a blank comprised of 1 ml of chloroform and 10 ml of Carr-Price reagent. Place the assay tube in the colorimeter and add rapidly (automatic pipette) 10 ml of Carr-Price reagent, using 620 mμ wave length light. Take the maximum colorimetric reading (color begins to fade within 3–5 seconds). Determine units of vitamin A in tube from standard curve and calculate units of A per g of feed.

(2) The first action method for vitamin D in poultry feed supplements, **40.58–40.60** (p. 792), was adopted as official.

(3) The first action method for folic acid, **40.40–40.43** (p. 784) was, adopted as official.

(4) The following chemical method for nicotinic acid was adopted, first action.

REAGENTS

Ammonium hydroxide.—5 ml conc. NH_4OH diluted to 250 ml.

Hydrochloric acid.—Dilute one part conc. HCl with 5 parts H_2O.

Cyanogen bromide.—10% aqueous soln. This should be prepared under a hood. Warm 370 ml H_2O in a large flask, add 40 g CN Br; warm and shake until soln is obtained. Cool and dilute to 400 ml. Do not allow CN Br to come into contact with the skin.

Sulfanilic acid.—10% soln. Place 20 g sulfanilic acid in 170 ml H_2O. Add conc. NH_4OH 1 ml at a time until soln is obtained. Adjust to *p*H 4.5 with 1:1 HCl, using bromocresol green as an outside indicator. Make up to 200 ml. If sulfanilic acid is pure, soln should be nearly colorless.

Sodium hydroxide.—10 *N*.

Nicotinic acid.—Stock soln (keep in refrigerator) 50 mg. U.S.P. Reference made up to 500 ml with 95% ethyl alcohol.

Nicotinic acid.—(Secondary standard) 2 ml stock soln diluted to 25 ml with H_2O. A small portion of the stock soln is removed each day and allowed to come to room temp. for preparation of the secondary standard.

Phosphate buffer.—17.6 g KH_2PO_4 + 10.24 g $Na_2HPO_4.12H_2O$. Dissolve in H_2O and dilute to 250 ml.

PROCEDURE FOR TABLETS OR CAPSULES

Preparation of sample.—Take for analysis at least 5 tablets or capsules. Dissolve by heating in a beaker with a little water. (Tablets must be ground then heated with water.) Transfer to a volumetric flask. Pipette 10 ml aliquot into a 250 ml Erlenmeyer flask. Add 10 ml conc. HCl. Evaporate on a hot plate to about 2 ml. Cool, add water, bring to *p*H 7–9 by addition of a few pellets of NaOH or KOH. Filter, if necessary, and make up to volume. (The final dilution should contain 5–8 mmg of nicotinic acid per ml. The first dilution usually contains 50–200 mmg per ml.)

Development of color.—Make up tubes as follows:

Blank	*Standard*	*Blank*	*Sample*
1 ml standard	1 ml standard	1 ml sample	1 ml sample
0.5 ml dil. NH_4OH	0.5 ml dil. NH_4OH	0.5 ml dil. NH_4OH	0.5 ml dil. NH_4OH
6.5 ml H_2O	1.5 ml H_2O	6.5 ml H_2O	1.5 ml H_2O
	5.0 ml CN Br		5.0 ml CN Br
2.0 ml sulfanilic acid	2.0 ml sulfanilic acid	2.0 ml sulfanilic acid	2.0 ml sulfanilic acid
1 drop conc. HCl or H Br			

A blank should be run for each sample.

Measure the sample soln, NH_4OH, and water into each tube from pipettes. Then add the sulfanilic acid to the blank tube and shake. Then add one drop of conc. HCl and again mix the contents. Place in photo electric colorimeter and adjust to 100% transmission at 450 mμ.

Add five ml cyanogen bromide soln from a pipette to the sample tube, while the tube is swirled vigorously. Exactly 30 sec. after completing the addition of the

cyanogen bromide add 2 ml sulfanilic acid from a pipette, while swirling the tube. Close tube with a rubber stopper. Place in colorimeter and read at 450 mμ. (The color reaches a maximum in about two min. and remains at the peak for about the same length of time. It then starts to fade slowly.)

(The addition of reagents should take place under a hood. The pipettes for addition of cyanogen bromide and sulfanilic acid should be mounted in a ringstand and should be filled by mechanical suction, since the cyanogen bromide is toxic.)

Method for enriched food products and feeds.—Weigh one ounce of material into a 1 liter Erlenmeyer flask. Add 200 ml 0.25 *N* H_2SO_4. Mix, heat in an autoclave at 15 lb pressure for 30 min. Cool, adjust to *p*H 4.5 with 10 *N* NaOH, using bromocresol green as an outside indicator, filter thru a rapid filter paper into a 250-ml volumetric flask, and mix. Wash residue with sufficient H_2O to fill flask to the mark. Weigh 17 g $(NH_4)_2SO_4$ into a 50 ml volumetric flask. Pipette 40 ml filtrate into the flask, shake vigorously, dilute to mark with water, mix. Filter and mix well. Take two ml for analysis.

Prepare the standard by diluting 2 ml of the stock soln to 50 ml with H_2O. Add 40 ml of this to 17 g of $(NH_4)_2SO_4$ in a 50 ml volumetric flask, and dilute to the mark with H_2O. (The standard now contains 3.2 mmg per ml and in the case of a feed with a niacin claim of 16 mg per lb., the final soln will also contain 3.2 mmg per ml. Prepare tubes as follows:

Blank	*Standard*	*Blank*	*Sample*
2.0 ml standard	2 ml standard	2.0 ml sample	2 ml sample
0.5 ml dil. NH_4OH	0.5 ml dil. NH_4OH	0.5 ml dil. NH_4OH	0.5 ml dil. NH_4OH
5.0 ml H_2O	5.0 ml CN Br	5.0 ml H_2O	5.0 ml CN Br
2.0 ml sulfanilic acid.	2.0 ml sulfanilic acid.	2.0 ml sulfanilic acid.	2.0 ml sulfanilic acid.
0.5 ml dil. HCl	0.5 ml dil. HCl	0.5 ml dil. HCl	0.5 ml dil. HCl

(The development of color is the same as in the case of tablets except that 0.5 ml of the HCl soln is added from a pipette immediately after the sulfanilic acid. The color, in this case, comes to a maximum at once and is less stable than in the previous case.)

(If the standard and sample are of ca same concentration, the nicotinic acid content is proportional to the photometric density.)

Modified Method of Color Development

Blank	*Standard*	*Blank*	*Sample*
2 ml standard	2 ml standard	2 ml sample	2 ml sample
2 ml phosphate buffer	2 ml phosphate buffer	2 ml phosphate buffer	2 ml phosphate buffer
5 ml H_2O	5 ml CN Br.	5 ml H_2O	5 ml CN Br.
2 ml sulfanilic acid.	2 ml sulfanilic acid.	2 ml sulfanilic acid.	2 ml sulfanilic acid.

(5) The word "international" was substituted for "A.O.A.C." in lines 1 and 3 of **40.60** (p. 793).

REPORT OF REPRESENTATIVE ON BOARD OF GOVERNORS OF THE CROP PROTECTION INSTITUTE

The function of the Board has continued to be advisory on the activities of the Institute acting as a liaison agency between the research workers at several Agricultural Experiment Stations and the producers of insecticides and fungicides that are subjected to screenings and experimental attack in the laboratory and trials in the field. Much of the exploratory work is conducted at the New Hampshire Agricultural Experiment Station, but the implementation of the exploratory findings and the outdoor trials are conducted at the several experiment stations through collaboration with the personnel of the stations. Two of the members of the Board are Directors of Experiment Stations, and they subscribe to the belief that the Institute has been, and is, an effective agency whereby the discoveries arrived at in industrial laboratories and organizations can be subjected to impartial and comprehensive tests. The Representative has found contact with the Institute to be informative and inspirational to him, and he has endeavored to prove helpful to the Institute.

Approved.

W. H. MacIntire

REPORT OF THE SECRETARY-TREASURER

Henry A. Lepper

The Executive Committee met in the Board Room of the Cosmos Club Sunday, October 1, 1950. All members were present. The audit of the accounts of the Association as reported by John Besselle and Company, Accountants, was presented and accepted.

For the second year the balance sheet shows a loss. As was the case last year the deficit in operating expenses is attributable to the cost of publishing our *Journal*. The increase in subscription price authorized at the last meeting and in effect during the past year has resulted in a lowering of the loss on the *Journal*. It has not, however, balanced the account, and it is not expected that the income from subscriptions this year will put the *Journal* on a self-supporting basis. None-the-less, the Executive Committee recommends that the Association continue to support the *Journal* from the surplus in its general funds and that no further increase in rates be made for the coming year. The only change in the price of the *Journal* authorized by the Executive Committee was a discontinuation of the 20% discount permitted to members.

Wm. F. Reindollar was reappointed chairman of the Committee on Recommendations of Referees. This year the terms of the chairman of Subcommittees A, B, C, and D expire. The Executive Committee recom-

mends the reappointment of G. R. Clark on Committee B, and K. L. Milstead on Committee D. In keeping with the wishes of H. A. Halvorson and J. O. Clarke the vacancies on Committees A and C, respectively, are to be filled by new appointments; and C. V. Marshall, Laboratory Plant Products, Canadian Department of Agriculture, Ottawa, Canada; and S. Alfend, Chief of the Kansas City District of the Food and Drug Administration, were appointed to Committees A and C, respectively, for a term of six years.

The Executive Committee passed the following resolution, which was presented to Dr. Fisher by W. B. White.

Whereas, Dr. Harry J. Fisher has twice served with marked distinction as Chairman of the Association's Committee on the Revision of Methods of Analysis; and

Whereas, this assignment is one of the most arduous among Association duties; and

Whereas, Dr. Fisher has brought to the task tireless energy, boundless enthusiasm, and excellent judgment, contributing in no small measure to the spectacular success of the Sixth Edition and to the early promise of an unprecedented demand for the forthcoming Seventh Edition, therefore be it

Resolved, that the Secretary-Treasurer be authorized to purchase and present to Dr. and Mrs. Fisher a silver service as an expression, however inadequate, of the gratitude of the Association for long, faithful, and able service at a heavy sacrifice of personal and domestic convenience.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS, INC.

BALANCE SHEET—SEPTEMBER 26, 1950

ASSETS

Current Assets:		
Cash, Lincoln National Bank		$11,561.82
Cash on hand		361.94
Office cash fund		67.56
Accounts receivable	$ 979.59	
Less reserve for doubtful accounts	51.30	928.29
Accrued interest receivable, Government bonds		250.00
Inventories		4,036.70
Total Current Assets		$17,206.31
Fixed Assets:		
Furniture and fixtures		964.47
Investments		51,292.00
Total Assets		$69,462.78

LIABILITIES		
Current Liabilities:		
Withholding tax collections		$ 25.90
Accrued salaries		229.72
Total Liabilities		$ 255.62
SURPLUS		
Balance, October 1, 1949	$71,438.00	
Add: Cancellation of outstanding checks applicable to prior years	20.50	
	$71,458.50	
Less: Net loss for the fiscal year ended September 26, 1950	*2,251.34*	
Balance, September 26, 1950		69,207.16
Total Liabilities and Surplus		$69,462.78

REPORT OF THE COMMITTEE ON NECROLOGY

Wm. C. Bainbridge

Mr. Bainbridge was a commercial chemist who took an active interest in the work of the A.O.A.C., serving for many years as Associate Referee on Coal-Tar Colors. He was a graduate of the Polytechnic Institute of Brooklyn, receiving his bachelor's degree in 1906. He was the author of many scientific publications and an instructor in chemical engineering at the Polytechnic Institute of Brooklyn.

Mr. Bainbridge participated in the preliminary work leading up to the certification of harmless coal-tar dyes under the Federal Food and Drugs Act of 1906, and during the years of his employment as a commercial chemist did outstanding work on color chemistry and research. He died July 6, 1950, in Brooklyn, New York, at the age of 67, after a long period of illness.

Royall O. E. Davis

Dr. Davis was born in 1880 at Newberry, South Carolina, and later moved to North Carolina, which he has since regarded as his home state. He attended the University of North Carolina, where he received the bachelor's degree in 1901 and a Doctorate in Chemistry in 1903. After further study at the University of Leipzig in Germany, he joined the staff of the Bureau of Soils of the U. S. Department of Agriculture in 1909, where he carried out research on fertilizers, paying especial attention to work on nitrogen. Dr. Davis directed construction of the first high pressure

pilot plant for the synthesis of ammonia in this country at the Arlington Farm Laboratories of the Department. He showed interest in our Association by collaboration on methods for nitrogen determination, by his contribution as joint author of a paper on the determination of Urea Nitrogen, and by his regular attendance at our annual meetings. He died October 30, 1949.

GEORGE W. HOOVER

Dr. Hoover died suddenly on July 26, 1950, aged 75. He joined the U. S. Bureau of Chemistry in 1904 soon after graduating from the Oklahoma Agricultural College. He served as analyst for a few years and subsequently became assistant chief of the Drug Division, chief of the Chicago Station, and eventually chief of the Office of Drug Control. He enjoyed the great privilege of working with Dr. Harvey W. Wiley and his famous "poison squad," and participated actively in the drafting of the Federal Food and Drugs Act of 1906. In the course of his career, he obtained the degree of M. D. from the Medical Department of George Washington University. He retired as chief of Drug Control in 1929 and became a successful private industry consultant. The many tributes to his work from his former associates in the Bureau of Chemistry, the Food and Drug Administration, and the drug industry attested to his outstanding service in the field of food and drug law enforcement. As a consultant to the drug industry, he consistently advocated adherence to both the letter and the spirit of the law.

BURTON J. HOWARD

Mr. Howard who was in charge of the Microanalytical Division of the Food and Drug Administration from 1928 to 1942, died at his home in Washington, D. C., on February 4, 1950, at the age of 77. At the time of his retirement in 1942 he had served 41 years with the Food and Drug Administration and its predecessor, the Bureau of Chemistry. He is probably best known as the originator of the "Howard mold count method" which is used throughout the food industry and by law enforcement officials.

Mr. Howard was a pioneer in educating food packers in the principles of sanitation in food factories. His work with the tomato industry in this country and in Italy was outstanding and the influence of his educational work spread to numerous other food industries. His enthusiasm was without limit and his tireless activity in canning factories in disregard of government work hours amazed many younger co-workers with whom he was associated in his field work. He was respected alike by food manufacturers and food officials and as a result of his activities, consumers received and are today receiving higher quality food.

Mr. Howard was born in 1872 in Ionia, Michigan. He received his Bachelor of Science degree from the University of Michigan in 1897 and did post-graduate work there in 1900 and 1901.

WILL J. MORGAN

Mr. Morgan, a chemist in the Insecticide Division, Livestock Branch, Production and Marketing Administration. U. S. Department of Agriculture, died March 12, 1949. He was born in Wales, May 28, 1880, and came to this country when a small boy. He was graduated from Morningside College and was appointed an assistant chemist in the Bureau of Chemistry in 1907. When the Insecticide Act of 1910 was passed, he joined the staff of chemists who enforced that law and was continuously employed in the analysis of insecticides and the investigation of methods of analysis of insecticides from that time until his death. He frequently acted as a collaborator in the study of methods of analysis, and was a regular attendant at the annual meetings of the Association.

CHARLES S. PURCELL

Mr. Purcell, a former chemist of the Boston District of the Food and Drug Administration, died January 18, 1950, while visiting at his daughter's home in New Jersey. A native of England, he came to Boston District in 1917 by transfer from the Bureau of Mines, Department of the Interior, and resigned from the Government service in 1921 to enter private employment. In 1943 he was appointed as a chemist and served again at Boston, until failing health caused him to retire in 1948. He participated actively in the work of the A.O.A.C. as a collaborating chemist on coal-tar dyes, etc. His pleasant manner, unfailing good humor, and gentle personality endeared him to his associates.

W. R. M. WHARTON

Mr. Wharton, retired chief of the Eastern District, Food and Drug Administration, died suddenly on September 8, 1949, after 41 years of Federal service. He was graduated from the University of Delaware and served 2 years as fellow and instructor in chemistry at the University of Maryland. He began his career in food and drug enforcement work in the Bureau of Chemistry, U. S. Department of Agriculture, in 1907, under Dr. Harvey W. Wiley. He was one of the original group of inspectors which pioneered in the field enforcement work of the Federal Food and Drugs Act of 1906.

During his career as a food and drug enforcement officer he worked successively in New Orleans., St. Louis, and New York City. He was chief of the Eastern District of the Food and Drug Administration for 28 years and during that period of his career delivered 52 addresses over the radio on the subject of "How to Read the Label." Over 30,000 letters

came in from listeners, and reprints of the talks ran into five editions. He retired from that position on April 30, 1948. He participated in many famous court cases and was outstanding in his ability to select and train personnel.

Dr. Dunbar and Mr. McKinnon, Jr., closed their article on "Bill" Wharton's career, published in the September, 1948, *Food, Drug and Cosmetic Law Quarterly*, with the following summary of his success: "The career of William Richardson Martin Wharton is proof positive that the work one loves is not work at all. He succeeded as a food and drug official because of this and, further, because he had a sincere, unwavering regard for the well-being of the consumer. The American public is fortunate to have had him in public service for so many years."

PHILIP ANSON WRIGHT

Mr. Wright died in Washington, D. C., September 13, 1950, at the age of 63 years. He was born in New Haven, Vermont, and was graduated from Middlebury College in the class of 1909. After post-graduate work at Yale University, he did research work at the University of Missouri, and came to Washington in 1913 ,where he resided until his death. He was employed in the laboratories of the Bureau of Dairy Industry, U. S. Department of Agriculture, and was interested in methods for the testing of milk and collaborated in the preparation of a revised manual for testing milk for that Bureau. He showed interest in the work of our Association by his frequent attendance at the annual meetings.

Approved.

J. W. SALE, *Chairman*
M. P. ETHERIDGE
J. J. T. GRAHAM

REPORT OF THE COMMITTEE ON NOMINATIONS

Your committee proposes the following nominees and moves their election to the respective offices, as designated:

President, H. A. Halvorson, Department of Agriculture, Dairy, and Food, St. Paul, Minn.

Vice-President, W. B. White, Food and Drug Administration, Washington, D. C.

Secretary-Treasurer, Henry A. Lepper, Food and Drug Administration, Washington, D. C.

As additional members of the Executive Committee: J. H. Fisher, New Haven, Connecticut; E. L. Griffin, U. S. Department of Agriculture, Washington, D. C.; Wm. F. Reindollar, Bureau of Chemistry, State

Department of Health, Baltimore, Md.; W. A. Queen, Past President, Food and Drug Administration, Washington, D. C.

Approved.

W. H. MACINTIRE, *Chairman*
GUY G. FRARY
J. O. CLARKE

REPORT OF THE COMMITTEE ON RESOLUTIONS

Whereas, the officers of our Association have capably and efficiently performed their duties during the past year and have carefully planned and successfully conducted this the 64th Annual Meeting at the close of the 66th year of this organization, therefore be it

Resolved, that we express our appreciation to President W. A. Queen, Vice-President H. A. Halvorson, and Secretary-Treasurer Henry A. Lepper, and to the members of the Executive Committee for their effective service.

Whereas, the accurate and prompt dissemination of new and improved methods is one of the principal services of this organization, therefore be it

Resolved, that we express our commendation of the excellent work done by Editor W. B. White and the Editorial Board of the Journal, and especially this year to H. J. Fisher and his co-workers for the vast amount of labor well performed in the preparation of the Seventh Edition of the *Book of Methods* of this Association.

Whereas, the primary purpose of this Association is adequate research in methodology which depends very largely upon the effective services of the members of the Committee on Recommendations of Referees, the members of Subcommittees A, B, C, and D, our Referees, Associate Referees, and collaborators, therefore be it

Resolved, that we express our appreciation to these workers who have given so generously of their time and thought to the successful conduct of this research.

Whereas, the successful conduct of our work and acceptance of the results obtained depends very largely upon the participation of personnel from many and widely varied State and Federal agencies and institutions in the United States and Canada, therefore be it

Resolved, that we hereby express our thanks to those agencies which have made possible the active participation of so many individuals in our research on methodology and conduct of this meeting.

Whereas, the success of any meeting of this type is in considerable degree dependent upon the availability of ample and satisfactory meeting places, therefore be it

Resolved, that this Association request its Secretary to express to the management of the Shoreham Hotel the thanks of our Association for making available rooms and other facilities for the succesful conduct of this annual meeting.

Approved.

J. F. FUDGE, *Chairman*
R. C. BERRY
V. E. MUNSEY

CORRECTIONS—NOVEMBER JOURNAL

In the paper on "The Co-determination of Indole and Skatole," by Charles S. Myers, published in the preceding number of *This Journal,* **33,** 971 (1950), the caption for Figure 1, p. 973, should read " . . . Reagent 1A," instead of "1B"; and the caption for Figure 2, p. 975, should read " . . . using 1B," instead of "1A."

In the paper on "Determination of Alcohol in Wines and Liqueurs," by A D. Etienne and G. F. Beyer, Table 1 (p. 1018), at the head of the second column of figures, "16%" should be "11%."

CONTRIBUTED PAPERS

A QUALITATIVE TEST FOR "TWEENS" IN COSMETICS*

By S. H. NEWBURGER (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Baltimore, Md.)

Tweens are water-soluble or water-dispersible fatty acid esters of anhydrosorbitols which have been solubilized by etherifying the free hydroxyl groups with ethylene oxide. Their increasing use as emulsifiers in all types of cosmetic formulations made it desirable to have analytical methods for their detection in cosmetics.

The most characteristic parts of the Tween molecule are the ethylene oxide chains. C. B. Shaffer and F. R. Critchfield (1) discovered that solid polyethylene glycols, which are ethylene oxide chain compounds, could be determined by the "formation in acid solution of highly insoluble complexes with the heteropoly acids, such as phosphomolybdic and silicotungstic, in the presence of a heavy metal cation such as barium."

The author observed that under the same conditions the precipitation also occurred with the Tweens. In the course of the investigation, it was learned that J. Oliver and C. Preston (2) recently developed a method, based on the work of Shaffer and Critchfield, for the determination of polyethylene glycol detergents. Subsequently, use was made of their work.

The procedures described by previous workers have been developed to provide for the quantitative determination of certain polyoxyethylene compounds. Quantitative analyses can, however, be obtained only when the identity of the compound is previously known. There is, at present, no way to differentiate among the various Tweens that may occur in cosmetics, and this investigation was therefore limited to the qualitative detection of Tweens in cosmetics.

The experimental work resolved into three phases: First, the qualitative tests were checked on all the Tweens. Secondly, the Tweens were isolated from the cosmetics in a detectable form. This was done by saponifying the cosmetic and extracting both the unsaponifiable matter and the fatty acids from an acid aqueous solution. Presumably, the Tweens were split into extractable fatty acids and polyoxyethylene anhydrosorbitol fragments which remained in the extracted aqueous solution. In general, these unextracted water-soluble fragments responded to the tests for Tweens. As the final step in the investigation, a number of commonly used cosmetic ingredients were tested to determine their possible interference with the test for Tweens.

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 2–4, 1950.

EXPERIMENTAL

The Tweens[1] used in the investigation were Tweens 20, 21, 40, 60, 61, 65, 80, 81, and 85. Tween 21, although not an emulsifying agent, was included for the purpose of completing the series.

REACTION OF TWEENS WITH SILICOTUNGSTIC ACID

REAGENT

Silicotungstic acid, 10% neutralized aqueous soln.—Dissolve 10 g of silicotungstic acid ($SiO_2.12WO_3.26H_2O$) in water and neutralize with 10% NaOH to a methyl red end point. Dilute to ca 100 ml.

PROCEDURE

Dissolve or disperse 20–30 mg of Tween, with heat if necessary, in 300 ml of water. To the soln at room temp., add 3 ml of HCl and 10 ml of 10% $BaCl_2$. If any precipitation occurs, filter and discard the precipitate. Add 10 ml of the silicotungstic acid reagent slowly with stirring. Heat to boiling to coagulate the precipitate and allow to stand overnight.

RESULTS AND COMMENTS

The aqueous solutions or dispersions of the Tweens were clear or slightly milky solutions which were little changed in appearance by the addition of the hydrochloric acid and barium chloride. As the silicotungstic acid was added the solutions became turbid, but with continued stirring flocculent precipitates separated. These coagulated on heating. The only exception was Tween 21. Its solution became very turbid on the addition of the silicotungstic acid but no precipitate coagulated on heating. However, on standing overnight a precipitate came down. With all the other Tweens the precipitates also settled out overnight leaving the supernatant liquid clear. The precipitates were usually coarsely granular. Because of adsorption of the methyl red used in the neutralization of the silicotungstic acid, the precipitates were colored pink.

REACTION OF SAPONIFIED TWEENS WITH SILICOTUNGSTIC ACID

PROCEDURE

Saponify about 50 mg of Tween for one hr. with 25 ml of alcohol and 1 g of KOH. Transfer the saponified soln to a separatory funnel with the aid of 50 ml of water, acidify with HCl, and extract with three 15-ml portions of benzene. Discard the benzene extracts, filter the extracted aqueous soln thru a cotton plug, wash cotton plug with 10 ml of water, and dilute the filtrate to 100 ml.

Dilute either a 50-ml aliquot or the entire soln to 300 ml with water and proceed as directed under "Reaction of Tweens with Silicotungstic Acid."

RESULTS AND COMMENTS

All the extracted aqueous solutions were clear and colorless. Using 50 ml. aliquots, Tweens 20, 40, 60, 65, 80, and 85 were unaltered by the hydrochloric acid and barium chloride, but with silicotungstic acid

[1] Descriptions of the chemical composition and general characteristics of the Tweens are to be found in the booklet, "Atlas Surface Active Agents," published by the Atlas Powder Company, Industrial Chemicals Department, Wilmington 99, Delaware.

flocculent precipitates formed which coagulated on heating. They settled out on standing overnight. Using the entire 100 ml. of solution, Tweens 61 and 81 were unchanged by the hydrochloric acid and barium chloride but the silicotungstic acid caused the solutions to become slightly milky. The solutions cleared on heating but on re-cooling became very turbid. Overnight small amounts of precipitate appeared. With the 100 ml. of the Tween 21 solution, neither hydrochloric acid, barium chloride, silicotungstic acid, heat, or standing overnight caused any precipitate or turbidity to appear.

On a relative basis Tweens 20, 40, 60, 65, 80, and 85 gave strongly positive tests, 61 and 81 weakly positive tests, and 21 was negative. The work of Shaffer and Critchfield suggests that the test is most positive for those compounds having the longest ethylene oxide chains.

SILICOTUNGSTIC ACID AS A REAGENT FOR THE DETECTION OF TWEENS IN THE PRESENCE OF OTHER COSMETIC INGREDIENTS

Each Tween was incorporated in a mixture of the following composition:

	per cent		*per cent*
Beeswax	8.0	Triethanolamine	6.3
Glycerylmonostearate	7.9	Sorbitol (Arlex)	6.5
Carnauba wax	7.9	Water	26.7
Paraffin	8.0	Boric acid	3.7
Ceresin	8.0	Tween	ca 1.0
Spermaceti	8.0		
Anhydrous lanolin	8.0	Total	100.0

The experiments were conducted with 5.4 gm. of this mixture.

PROCEDURE

Saponify the sample with 50 ml benzene, 25 ml alcohol, and 1 g of KOH for 2 hr. Transfer the saponified mixture to a separatory funnel with the aid of 50 ml hot water, acidify with HCl, shake well, and draw off the aqueous layer. Extract with 2 additional 25 ml portions of benzene and discard benzene extracts. Filter extracted aqueous soln thru a cotton plug, wash plug with 10 ml of water, and dilute filtrate to 100 ml.

Dilute either a 50-ml aliquot or the entire soln to 300 ml with water and proceed as directed under "Reaction of Tweens with Silicotungstic Acid."

RESULTS AND COMMENTS

The filtered solutions were clear and colorless. Essentially the same results were obtained with these solutions as those described under "Reaction of Saponified Tweens with Silicotungstic Acid." A blank run on the mixture, with the Tween omitted, gave a colorless solution unaffected by the addition of hydrochloric acid, barium chloride, silicotungstic acid, and heat. However, on standing overnight, the solution developed a slight turbidity but no precipitate could be observed. The cosmetic ingredients do not interfere with the detection of the Tweens.

REACTION OF TWEENS WITH PHOSPHOMOLYBDIC ACID

REAGENT

Phosphomolybdic acid, 5% aqueous soln.—Dissolve 5 g of phosphomolybdic acid (20 $MoO_3.2H_3PO_4.48H_2O$) in water, filter, and dilute to 100 ml.

PROCEDURE

Dissolve or disperse 20–25 mg of Tween, with heat if necessary, in 150 ml of water. With the soln at room temp., add 3 ml of (1+1) HCl and 5 ml of 10% $BaCl_2$. Filter to remove any precipitate that forms. Add 10 ml of 5% phosphomolybdic acid slowly with stirring. Heat to boiling to coagulate precipitate and allow to stand overnight.

RESULTS AND COMMENTS

The clear or slightly milky solutions were little changed by the hydrochloric acid and barium chloride, but the phosphomolybdic acid caused flocculent precipitates to form which coagulated on heating. With Tweens 20, 40, 60, 61, 65, 80, and 85 the coagulated precipitates were greenish-yellow; with Tweens 21 and 81 they were markedly greener. Overnight all precipitates settled, leaving clear supernatant liquids. Visually, the precipitates from Tweens 20, 40, 60, 65, 80, and 85 seemed to be much more abundant than those from Tweens 21, 61, and 81.

REACTION OF SAPONIFIED TWEENS WITH PHOSPHOMOLYBDIC ACID

PROCEDURE

Saponify and extract 50 mg samples as directed under "Reaction of Saponified Tweens with Silicotungstic Acid."

Treat 50 ml aliquots by the procedure described under "Reaction of Tweens with Phosphomolybdic Acid."

RESULTS AND COMMENTS

All solutions were clear and colorless. With the exception of Tween 21, the solutions, unaffected by the hydrochloric acid and barium chloride, reacted with phosphomolybdic acid to give flocculent precipitates. Heating caused coagulation; greenish-yellow precipitates settled out overnight. Visually, the precipitates from Tweens 20, 40, 60, 65, 80 and 85 seemed to be more abundant than those from Tweens 61 and 81. With phosphomolybdic acid the solution of Tween 21 became turbid and a very fine precipitate could be seen suspended in the liquid. Heating cleared the solution but the turbidity reappeared on cooling. Overnight a small amount of a greenish-yellow precipitate came down.

PHOSPHOMOLYBDIC ACID AS A REAGENT FOR THE DETECTION OF TWEENS IN THE PRESENCE OF OTHER COSMETIC INGREDIENTS

PROCEDURE

Mixtures identical with those described earlier in the paper were saponified and extracted in the usual manner. Fifty ml aliquots were tested for Tweens.

RESULTS AND COMMENTS

The observations were similar to those described under "Reaction of Saponified Tweens with Phosphomolybdic Acid." The clear solution of a blank, obtained from a mixture containing no Tween, did not become turbid or precipitate with the added reagents. However, on heating, the solution became slightly milky and overnight a very small amount of a fine precipitate came down. It could possibly be mistaken for Tween 21, but the manner in which the two precipitates formed was quite different.

CONCLUSIONS

In aqueous acid solution containing barium ions, the Tweens can be detected qualitatively by silicotungstic or phosphomolybdic acids.

In general, the fragments of saponified Tweens, which cannot be extracted from acid aqueous solution by benzene, will respond to the tests for the Tweens. However, the tests are not as positive as for unsaponified Tweens, and in the case of Tween 21, a negative test was obtained with silicotungstic acid.

Silicotungstic acid is less sensitive but more selective as a reagent than phosphomolybdic acid.

Under the specified conditions most cosmetic ingredients will not interfere with the test. However, false positive tests for Tweens will be given by the polyethylene glycols, other types of polyoxyethylene compounds, and some basic organic nitrogen compounds.

ACKNOWLEDGMENT

The author wishes to thank Dr. C. D. Pratt of the Atlas Powder Company, Wilmington, Delaware, for his generosity in supplying the samples of Tweens.

REFERENCES

(1) SHAFFER, C. B., and CRITCHFIELD, F. R., *Anal. Chem.*, **19**, 32 (1947).
(2) OLIVER, J., and PRESTON, C., *Nature*, **164**, 242 (1949).

STUDIES ON COAL-TAR COLORS, IX

D&C YELLOW NO. 7; D&C ORANGE NO. 5; D&C RED NO. 21; TETRACHLOROFLUORESCEIN; D&C RED NO. 27; AND FD&C RED NO. 3

By MEYER DOLINSKY and JOHN H. JONES (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D.C.)

This paper is one of a series* dealing with the preparation, purification and analysis of the certifiable colors. As in the previous studies, the spectrophotometric characteristics of solutions of these colors have been determined as an aid in the identification and analysis of colors submitted for certification. The following six colors, five of which are certifiable, are covered in this report.

Color	*Certifiable As* (1)
Fluorescein	D&C Yellow No. 7
Dibromofluorescein (4,5-Dibromo-3,6-fluorandiol)	D&C Orange No. 5
Tetrabromofluorescein (2,4,5,7-Tetrabromo-3,6-fluorandiol)	D&C Red No. 21
Tetrachlorofluorescein (12,13,14,15-Tetrachloro-3,6-fluorandiol)	(Not certifiable)
Tetrachlorotetrabromofluorescein (2,4,5,7-Tetrabromo-12,13,14,15-tetrachloro-3,6-fluorandiol)	D&C Red No. 27
Tetraiodofluorescein (2,4,5,7-Tetraiodo-3,6-fluorandiol)	FD&C Red No. 3 (Sodium Salt)

Methods for the preparation of all of these compounds have been described in the literature; in many cases suitable methods for purification are also described. The portion of this paper dealing with the preparation and purification of the compounds is, therefore, primarily a compilation of the methods that have been found most suitable in this laboratory.

PREPARATION OF PURIFIED COLORS

Intermediates

Phthalic anhydride (sublimed M.P. 130.5°C.)
Resorcinol (recrystallized from benzene) (M.P. 109°C.)
Tetrachlorophthalic anhydride (commercial grade).

The bromine used in the preparation of the brominated compounds was obtained as follows:

Commercial $KBrO_3$ was twice crystallized from H_2O. Part of the bromate was converted to KBr by cautiously heating in a platinum dish. A mixture of 33.0 grams of bromate plus 117.8 grams of the KBr was treated with 150 ml. of 85% H_3PO_4. The liberated bromine was distilled and collected in a flask cooled in an ice-bath. Yield, 91.1 grams.

* Previous papers have been published in *This Journal*, Vols. 27–33.

(*A*) *D&C Yellow No.* 7.—Phthalic anhydride (60 grams), and resorcinol (88 grams) were thoroughly mixed and heated at 210°C. until the melt solidified (approximately 1 hour). The reaction product was dissolved in alkali, filtered, precipitated with HCl, washed with H_2O and dried at 135°C. Yield, 115 grams.

The crude fluorescein was acetylated by refluxing 50 grams of the material with 250 ml. of acetic anhydride plus 25 grams of anhydrous sodium acetate for $2\frac{1}{2}$ hours. The reaction mixture was poured into water; the precipitate formed was separated by filtration, washed with alcohol, and dried at 135°C. Yield, 50 grams. The crude diacetate was recrystallized three times from alcohol to give a white crystalline product, M.P. 199.5°C. (literature 200°C.) (2). Further crystallization from alcohol or from benzene did not change either the melting point of the diacetate or the spectrophotometric characteristics of the dye obtained upon saponifying the diacetate.

The fluorescein diacetate (10 grams) was saponified by heating for 20 minutes on the steam bath with 200 ml of alcohol containing 4 grams of NaOH. The solution was filtered, diluted to approximately 1500 ml. with H_2O, and the D&C Yellow No. 7 precipitated by adding glacial acetic acid. The product was collected, washed with several portions of water, and dried at 135°C. Yield, 8.3 grams.

(*B*) *D&C Orange No.* 5.—Dibromofluorescein was prepared according to the method of Phillips (3), using 23.5 grams of fluorescein (purified; from diacetate), and 23 grams of bromine. The crude D&C Orange No. 5 was dissolved in dilute NaOH and reprecipitated by adding glacial acetic acid. The product was dried at 80°C. for 48 hours. Yield, 33.9 grams.

The dibromofluorescein thus obtained was acetylated using 160 ml. of acetic anhydride plus 16 grams of anhydrous sodium acetate. The mixture was refluxed for two hours, poured into water, and the solid reaction product collected and air-dried. Yield, 31.5 grams. The diacetate was crystallized first from a mixture of benzene and alcohol, and then twice more from alcohol. Yield, 13 grams, M.P. (capillary tube) 210°–211°C. (literature 211°C.).

The purified diacetate, 5.7 grams, was hydrolyzed with alcoholic NaOH (1.6 grams of NaOH in 200 ml. of alcohol) by heating on the steam bath for approximately 10 minutes. The solution was filtered, diluted to approximately 1500 ml with H_2O and the D&C Orange No. 5 precipitated with glacial acetic acid. The product was washed with water, dried over night at 80°C. and then for 3 hours at 135°C. Yield, 4.8 grams.

(*C*) *D&C Red No.* 21 (2).—Fluorescein, 20 grams (from diacetate), was heated with 19 ml. of bromine in 300 ml. of glacial acetic acid at 100°C. for 1 hour. The reaction mixture was poured into water, the solid product collected and washed with water. The crude D&C Red No. 21 was then dissolved in dilute NaOH, the solution filtered, and the color

precipitated with dilute H_2SO_4. The product was digested overnight, collected, washed with water, and dried at 135°C. Yield, 36 grams.

The tetrabromofluorescein thus prepared was acetylated by refluxing with 200 ml. of acetic anhydride plus 18 grams of anhydrous sodium acetate for 2½ hours. The reaction mixture was poured into water; the solid product collected, and washed with alcohol. Yield, 34 grams. When recrystallized twice from benzene, the product was white, M.P. 292°–295°C.

The purified diacetate, 6 grams, was saponified by heating for 10–15 minutes on the steam bath with 200 ml of alcohol containing 1.3 grams of NaOH. The solution was filtered, diluted to approximately 1500 ml. with H_2O, and the D&C Red No. 21 precipitated with glacial acetic acid. The product was collected, washed with H_2O, and dried at 80°C. for 48 hours, then at 135°C. for 2½ hours. Yield, 5 grams.

(*D*) *Tetrachlorofluorescein.*—Tetrachlorophthalic anhydride, 286 grams, plus resorcinol, 231 grams, were thoroughly mixed and heated in a stainless steel cup at 210°C. for two hours. The temperature was then raised to 230°C. and the heating continued for one hour at which time the reaction mixture had solidified. The crude product was dissolved in dilute NaOH, the solution filtered, and the dark red product precipitated with HCl. Yield, after washing with H_2O and drying at 135°C., 389 grams.

To 386 grams of tetrachlorofluorescein, 190 grams of anhydrous sodium acetate and 1600 ml of acetic anhydride were added and the mixture refluxed for 3 hours. The reaction mixture was then poured into H_2O, the solid product collected, washed with H_2O, and dried at 135°C. Yield, 380 grams. The color of the crude diacetate was very dark, but after three crystallizations from benzene, (using activated carbon) the product was colorless and melted sharply at 256°–257°C. (literature 256°C.) (5).

The purified tetrachlorofluorescein diacetate, 115 grams, was hydrolyzed with 250 ml of 10% alcoholic NaOH by heating on the steam bath for 15 minutes. The solution was then filtered, diluted with a large volume of H_2O and the tetrachlorofluorescein precipitated with glacial acetic acid. The precipitate was digested overnight, collected on a Büchner funnel, washed with H_2O, and dried at 135°C. Yield, 95 grams.

(*E*) *D&C Red No. 27.*—Tetrachlorofluorescein, 50 grams (from diacetate), was suspended in 190 ml. of acetic acid and a solution of 75 grams of bromine (from bromate) in 300 ml of acetic acid added. The mixture was heated on a steam bath for 2 hours and then poured into 500 ml of H_2O. The solid reaction product was collected on a Büchner funnel, washed with acetic acid and then with H_2O, and dried at 80°C. Yield, 88 grams of a light pink colored material having a faint odor of acetic acid.

Eighty-eight grams of the crude tetrachloroeosin plus 50 grams of anhydrous sodium acetate were refluxed with 500 ml of acetic anhydride

for 3 hours. The reaction mixture was poured into H_2O, the precipitated material collected on a Büchner funnel, and air-dried. Yield, 93 grams. The crude diacetate was crystallized twice from ethyl acetate and dried at 135°C. to give a white crystalline product, M.P. 308°C. (literature 298°–300°C.) (5). Purified D&C Red No. 27 diacetate, 10 grams, was hydrolyzed by heating for 15 minutes on a steam bath with 1.4 gram of NaOH in 150 ml of alcohol. The solution was filtered and the D&C Red No. 27 precipitated with acetic acid. The red product obtained was then dissolved in NH_4OH, precipitated with dilute H_2SO_4, collected, and washed with H_2O. The product was pink, but turned white after drying at 150°C.

(*F*) *FD&C Red No. 3.*—Commercial Erythrosine, 20 grams, was refluxed for $2\frac{1}{2}$ hours with 100 ml of acetic anhydride plus 10 grams of anhydrous sodium acetate. The reaction mixture was poured into H_2O, the solid product collected, and washed with alcohol. Yield, 20 grams. The product was recrystallized three times from bromobenzene and dried at 140°C; colorless crystals were obtained. M.P. 296°C. (literature 293°–294°C.) (4).

A mixture of 5 grams of purified diacetate, 300 ml of acetone, 22 ml of H_2O, and 45 ml of NH_4OH was stirred for 2 hours and then allowed to stand overnight. The solution was then heated to boiling for 5 minutes, cooled, filtered, and diluted with 100 ml of H_2O. The solution was acidified with acetic acid, the precipitate collected, washed with H_2O, and dried at 135°C. Yield, 4.2 grams of the color acid corresponding to FD&C Red No. 3. The color acid was used as the spectrophotometric standard as it is anhydrous and non-hygroscopic. (1 mg color acid = 1.074 mg FD&C Red No. 3.)

ANALYTICAL DATA

Analytical data for the purified colors are shown in Table 1.

The A.O.A.C. procedures for halogens in halogenated fluoresceins gave very satisfactory results for the iodine content of FD&C Red No. 3, the bromine content of D&C Orange No. 5, and the chlorine content of tetrachlorofluorescein. The results for bromine on D&C Red Nos. 21 and 27 by the A.O.A.C. procedure were consistently 1–1.5 per cent lower than the theoretical values. Considerable difficulty was experienced in obtaining consistently satisfactory fusion in the Parr bomb procedure, but when satisfactory fusions were obtained, the results appeared to be in close agreement with the theoretical values.

Titration of the purified fluorescein with titanium trichloride gave values which varied from 0.8 to 1.5 per cent higher than theoretical. The end point was somewhat difficult to determine; back titration with FD&C Green No. 2 indicator was necessary. The halogenated fluoresceins could not be titrated accurately with titanium trichloride.

TABLE 1.—*Analytical data*

MATERIAL	BROMINE		CHLORINE		CHLORINE+BROMINE (AS AgX)		IODINE		CARBON[c]		EQUIVALENT WEIGHT AS ACID	
	CALC.	FOUND	CALC.	FOUND	CALC.	FOUND	CALC.	FOUND	CALC.	FOUND	CALC.	FOUND
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*		
D&C Yellow No. 7											166	169 170 166
D&C Orange No. 5 Diacetate	27.84	27.64[a] 28.00[a]										
D&C Orange No. 5	32.61	32.21[a] 32.53[a]									245	241
D&C Red No. 21 Diacetate	43.68	42.32[a] 42.19[a] 43.61[b] 43.42[b] 43.43[b]										
D&C Red No. 21	49.34	49.03[b] 49.26[b]							37.07	37.18 36.93	324	322 323 323
Tetrachloro-fluorescein Diacetate			25.60	25.62[a] 25.72[a]								
Tetrachloro-fluorescein			30.17	30.09[a] 30.01[a]							235	233
D&C Red No. 27	40.7	40.2[a] 39.9[a]	18.1	17.5[a] 17.8[a]	168.46	165.77[a] 166.60[a] 169.12[b] 167.94[b] 168.14[b]			30.57	30.80 30.83	393	394
FD&C Red No. 3 Diacetate							55.20	55.84[a] 55.47[a]				
FD&C Red No. 3 (Color Acid)							60.66	60.59[a] 60.30[a]			418	414

[a] A.O.A.C. Procedure.
[b] Parr Bomb—Determinations by C. Graichen, Division of Cosmetics, Food and Drug Administration
[c] By Combustion.

Neutralization equivalents.—It was found that the fluorescein colors can be accurately titrated as dibasic acids in alcohol solution. An accurately weighed sample of the color (0.1 to 0.5 gram) is dissolved in 200–

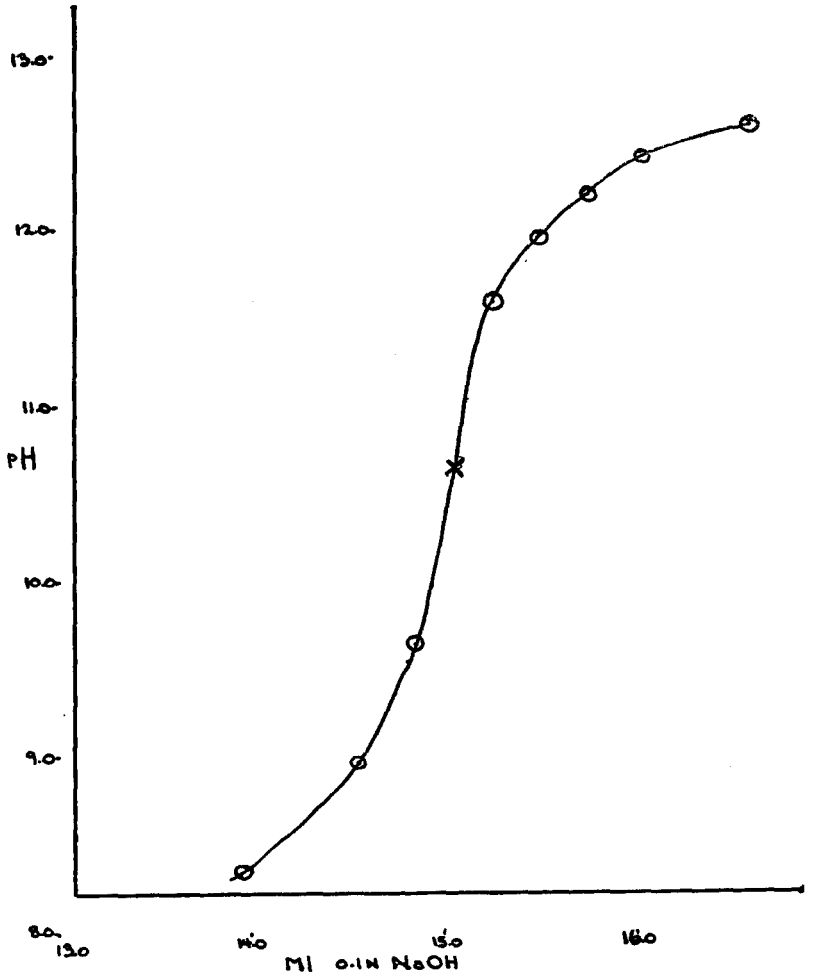


FIG. 1.—Potentiometric Titration Curve of D&C Red No. 21.

300 ml of alcohol and titrated potentiometrically with standard 0.1 *N* NaOH, using a Beckman *p*H meter equipped with a 290-E glass electrode and a sealed calomel electrode. (For very accurate results, it is necessary to determine the blank due to the alcohol.) The neutralization curves show a single sharp break at the point which corresponds to the addition

of two moles of NaOH per mole of color. Apparently the ionization constants of the phenolic and carboxy groups do not differ sufficiently to be distinguished by this titration.

A typical titration curve is shown in Figure 1. Typical results obtained by this method are included in Table 1.

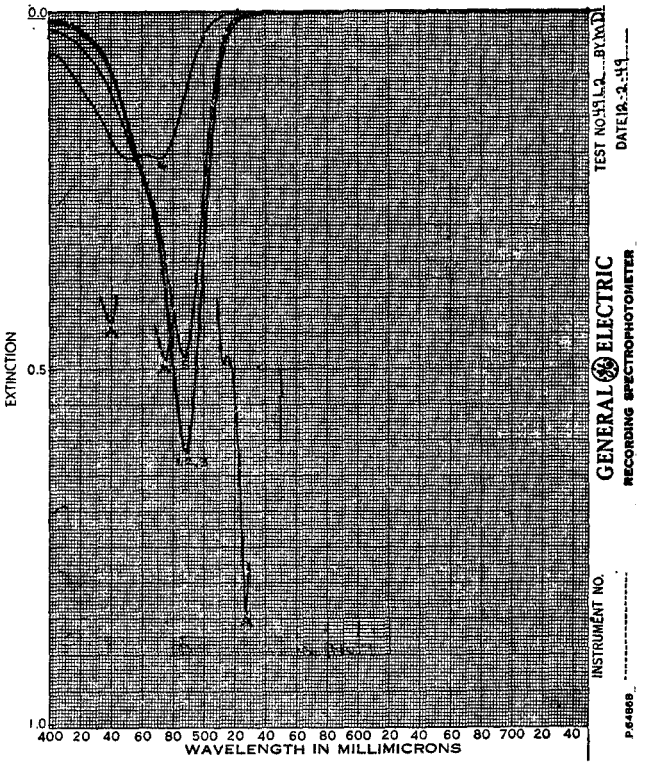


FIG. 2.—D&C Yellow No. 7 (Specially Purified Sample).
Conc.: 2.45 mg per liter
Solvent: Water
Curve 1—*p*H 11.6
Curve 2—*p*H 9.1
Curve 3—*p*H 13
Curve 4—*p*H 5.5
Curve 5—*p*H 7.3
Cells: 1 cm
A = Corning Didymium Glass 512, 6.0 mm (Absorption peaks at 400.4, 441.4, 477.1, 529.0 and 684.8 mμ).

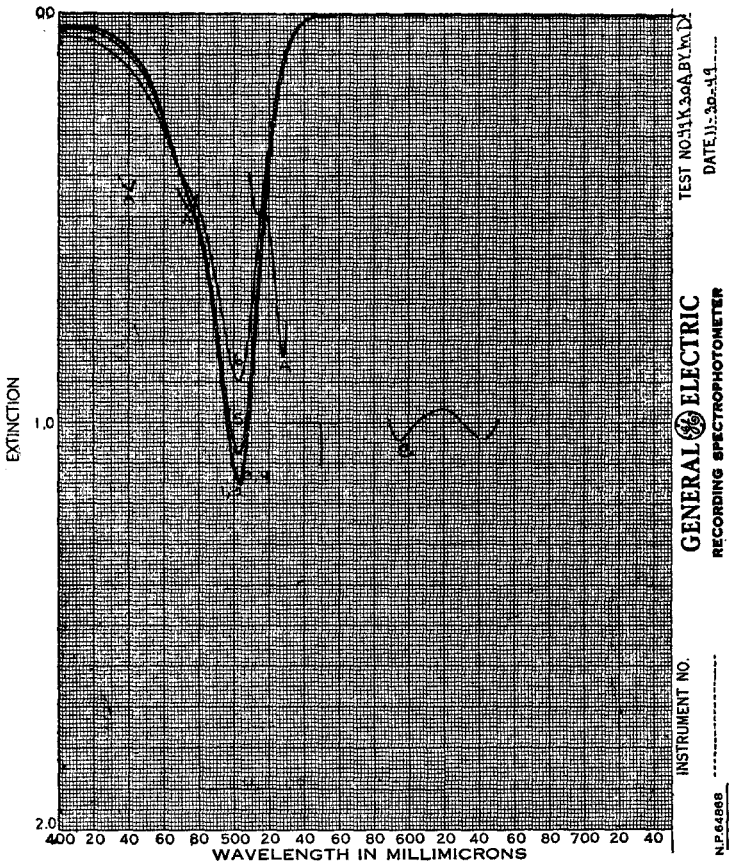


FIG. 3.—D&C Orange No. 5 (Specially Purified Sample).

Conc.: 7.46 mg per liter
Solvent: H_2O
Curve 1—*p*H 11.6
Curve 2—*p*H 13
Curve 3—*p*H 9.4
Curve 4—*p*H 7.0
Curve 5—*p*H 6.0
Curve 6—*p*H 5.2
Cells: 1 cm

A = Corning Didymium Glass 512, 6.0 mm (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ).
C = Signal Lunar White Glass-H-6946236.

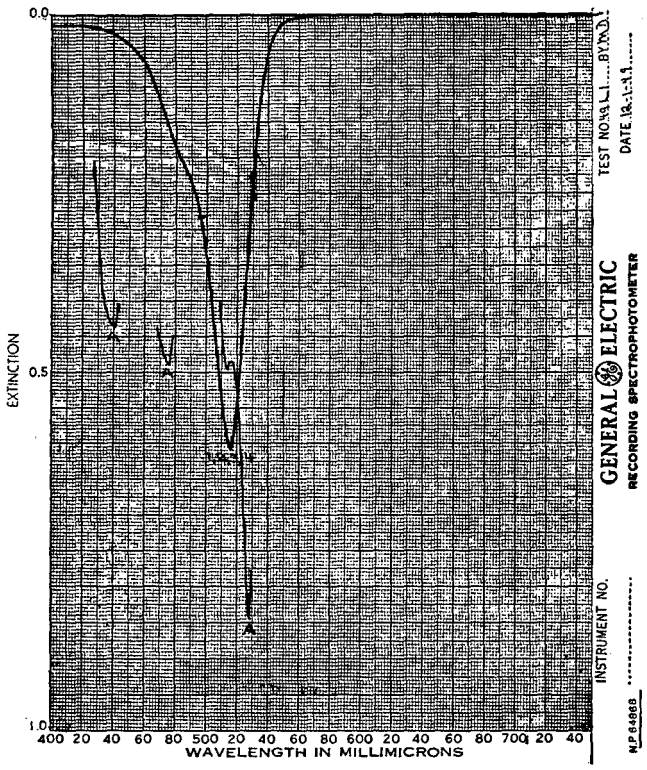


FIG. 4.—D&C Red No. 21 (Specially Purified Sample).
Conc.: 4.14 mg per liter
Solvent: Water (*p*H 5.3–13)
Cells: 1 cm
A = Corning Didymium Glass 512, 6.0 mm (Absorption peaks at 400.4, 441.4, 477.1 529.0, and 684.8 mμ).

SPECTROPHOTOMETRIC DATA

All spectrophotometric measurements were made with a General Electric recording spectrophotometer with slit adjustments for an 8 mμ wave length band. To minimize the effect of the fluorescence of the colors, the cells containing the solutions were placed at the forward end of the transmission compartment, approximately 5 inches from the integrating sphere. Under these conditions, less than 1 per cent of the fluorescent light emitted by the sample will enter the integrating sphere.

The solutions for spectrophotometric analysis were prepared as follows: An accurately weighed sample of the purified color* was dissolved in 1 per

* The purified diacetates may be used directly as the standards by saponifying an accurately weighed sample with a small volume of alcoholic NaOH and diluting to a suitable volume with H_2O.

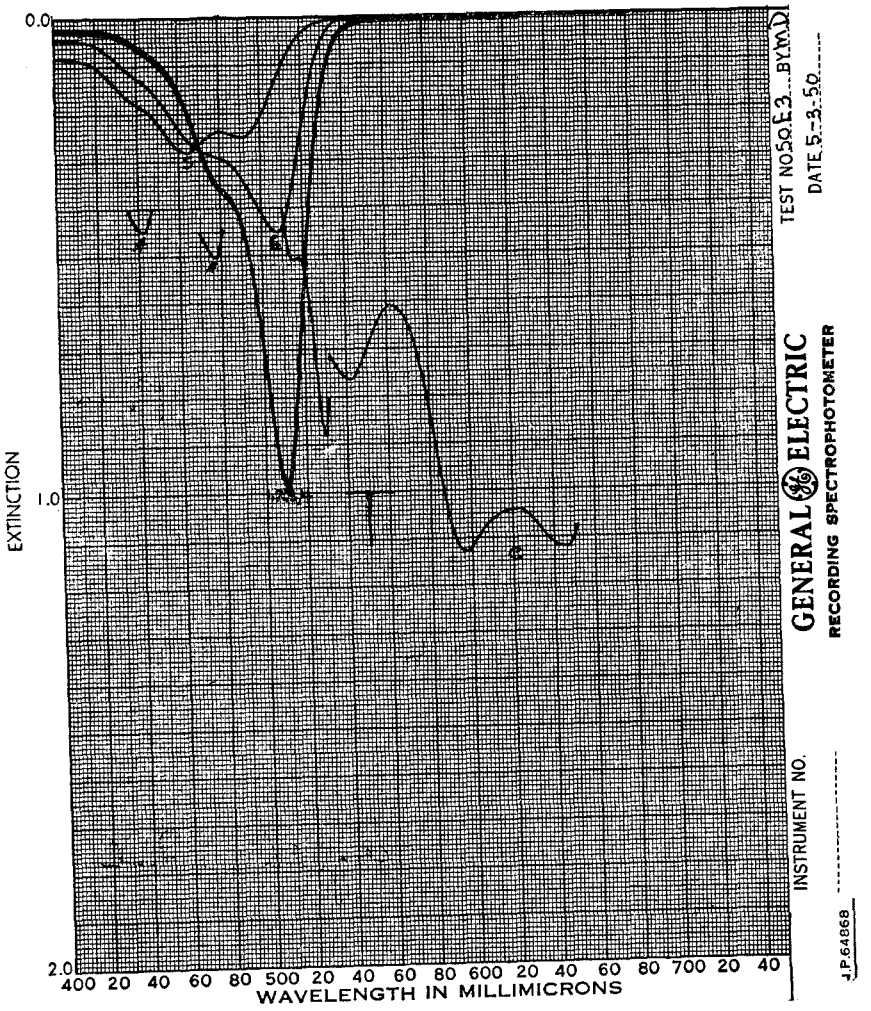


FIG. 5.—Tetrachlorofluorescein.

Conc.: 5.0 mg per liter
Curve 1—*p*H 8.1
Curve 2—*p*H 13.0
Curve 3—*p*H 10.0
Curve 4—*p*H 11.3
Curve 5—*p*H 4.3
Curve 6—*p*H 6.0

A = Corning Didymium Glass 512, 6.0 mm (Absorption peaks at 400.4, 441.4, 477.1, 529.0 and 684.8 mμ).
C = Signal Lunar White Glass-H-6946236.

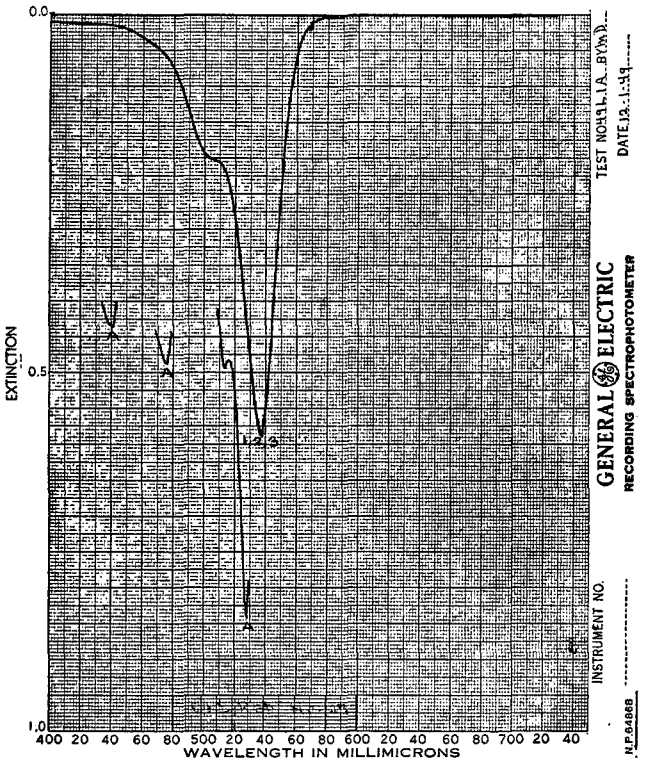


FIG. 6.—D&C Red No. 27 (Specially Purified Sample).
Conc.: 4.26 mg per liter
Solvent: Water (*p*H 5.1–13)
Cells: 1 cm
A = Corning Didymium Glass 512, 6.0 mm (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ.)

cent NH_4OH and the solution diluted to exactly 1000 ml with the same solvent. Suitable aliquots of this solution were then diluted to a definite volume with either 1 per cent NH_4OH or with buffer solution. All solutions were made to volume at the temperature of the room in which the optical measurements were made.

The effect of *p*H on the absorption spectra of these colors is shown in Figures 2–7. (The *p*H values of the solutions are those obtained with a glass electrode *p*H meter.) In general, as the *p*H of the solution is raised the extinction per mg. is increased. For each color, however, all solutions with a *p*H above a certain value gave essentially the same spectrum.

Solutions of each of these colors in dilute (1+99) NH_4OH follow Beer's

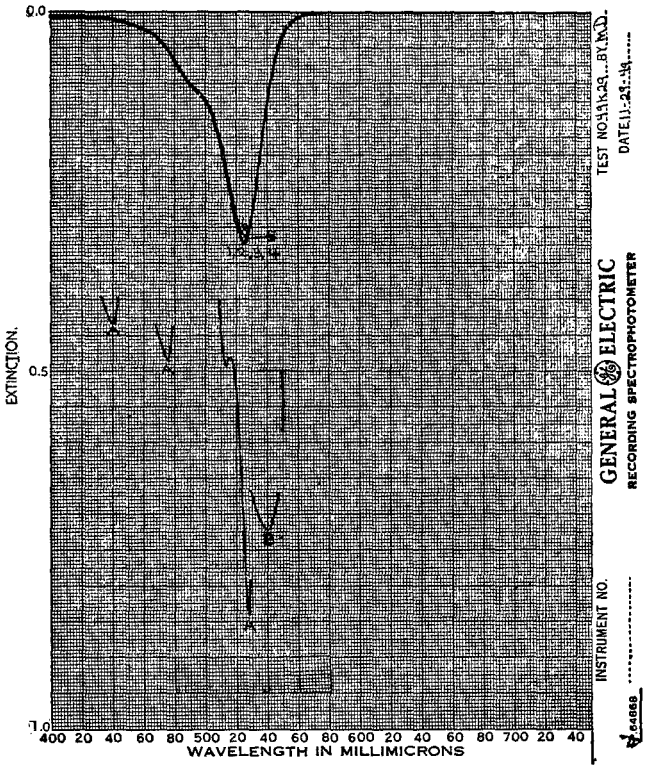


FIG. 7.—FD&C Red No. 3 (Color Acid) (Specially Purified Sample).
Conc.: 2.81 mg per liter
Solvent: Water
Curve 1—*p*H 8.5
Curve 2—*p*H 10.8
Curve 3—*p*H 13.0
Curve 4—*p*H 11.4
Curve 5—*p*H 6.9
Curve 6—*p*H 4.8
Cells: 1 cm
A = Corning Didymium Glass 512, 6.0 mm (Absorption peaks at 400.4, 441.4, 477.1, 529.0, and 684.8 mμ.)
C = Signal Lunar White Glass-H-6946236.

law to within ±0.3 per cent if the effect of the fluorescence of the solution is eliminated. The amount of concentrated NH_4OH in the solution for spectrophotometric analysis may be varied from 1 to 10 ml per 100 ml of solution without appreciably changing the spectrophotometric characteristics.

Ammoniacal solutions of the colors stored in the dark for 48 hours gave absorption curves identical with those of freshly prepared solutions. When exposed to light, however, the solutions faded appreciably in 24 hours.

The spectrophotometric data are summarized in Table 2.

TABLE 2.—*Spectrophotometric data*

COLOR	CONC. MG/LITER	AV. DEV. BEER'S LAW	ABSORPTION PEAK*	AVERAGE E/MG/L*	APPROX. pH RANGE OVER WHICH CURVE DOES NOT CHANGE
		Per cent	*Mμ*		
D&C Yellow No. 7	1.57 to 4.80	0.3	490 ± 2	0.252	9.1 to 13
D&C Orange No. 5	3.09 to 7.46	0.2	504 ± 2	0.156	7.0 to 13
D&C Red No. 21	3.56 to 7.12	0.2	517 ± 2	0.150	5.3 to 13
D&C Red No. 27	1.57 to 7.34	0.3	538 ± 2	0.131	5.1 to 13
Tetrachloro-fluorescein	2.25 to 4.75	0.2	510 ± 2	0.187	8.1 to 13
FD&C Red No. 3	3.71 to 9.28	0.2	526 ± 2	0.109	8.5 to 13

* In dilute ammoniacal solution.

SUMMARY

The preparation and purification of several fluorescein colors is described. Analytical data obtained on samples of the purified colors are given. Spectrophotometric data obtained on dilute aqueous solutions of the colors are presented as an aid in the identification and analysis of samples of these colors.

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DETERMINATION OF UNCOMBINED ALPHA- AND BETA-NAPHTHOL IN COAL-TAR COLORS*

By LEE S. HARROW (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D.C.)

Alpha- and beta-naphthol are intermediates used for the preparation of numerous certifiable coal-tar colors. The amount of uncombined alpha- or beta-naphthol permitted in batches of certified coal-tar colors are specified in the regulations governing their certification (1).

Numerous methods have been developed for the determination of these intermediates on both the micro- and semi-micro scale. Weiss (2) has proposed a method involving bromination with $KBrO_3$-KBr solution in the presence of KI and HCl with a final titration with $Na_2S_2O_3$. Procedures for the determination of these phenols by estimation of the compounds produced by coupling of the phenols with diazotized sulfanilic acid (5) or *p*-nitroaniline (4) (5) have been described.

Of all these methods the use of *p*-nitroaniline appears to give the most accurate results with the quantities of phenols dealt with in this work (4).

The proposed methods include procedures for extracting the intermediates from the colors, for coupling of the naphthol with *p*-nitrobenzene diazonium chloride, and for spectrophotometric determination of the color produced.

β-NAPHTHOL—METHOD

APPARATUS

A spectrophotometer suitable for use at 490 mμ.

REAGENTS

Standard 1-(4-nitrophenylazo)-2-hydroxy naphthalene soln. (2 mg/l).—Dissolve 10 mg of purified 1-(4-nitrophenylazo)-2-hydroxynaphthalene in 100 ml of warm acetone, cool, transfer to a 500-ml volumetric flask and dilute to volume with chloroform. Dilute a 10-ml aliquot of this soln to exactly 100 ml with chloroform.

p-Nitroaniline (M.P. 145–7°C.)—The commercial material can be purified by recrystallization from 70% alcohol.

PROCEDURE

A. Colors Soluble In Water

Dissolve 2 g of the color in 250 ml of water. Make the soln acid with 5 ml of 6 *N* HCl and extract with six 30-ml portions of isopropyl ether. Wash the combined ether extracts with 20-ml of 0.1 *N* HCl and extract with 30-ml portions of 0.1 *N* NaOH. Reserve the combined alkaline extracts for coupling with *p*-nitrobenzene diazonium chloride.

Dissolve 20 mg of *p*-nitroaniline in 2 ml of conc. HCl, dilute to 200 ml with water, add 100 g of crushed ice and stir until the temp. of the soln is 5–10°C. Add 2 ml of 10% $NaNO_2$ soln and stir for 10–15 min. At the end of this time add

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* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 2–4, 1950.

small portions of 10% sulfamic acid soln until the soln gives a negative test for nitrous acid with starch-iodide paper.

Cool the alkaline β-naphthol extract to 5–10°C. with crushed ice and add the *p*-nitrobenzene diazonium chloride soln slowly with constant stirring. Stir the reaction mixture for 15 min. and then heat to 90°C. on a steam bath. Remove from the steam bath, cool to room temp., and extract the soln with 20-ml portions of chloroform until all of the color is extracted. Wash the combined chloroform extracts with 30 ml of 0.1 *N* NaOH. Filter the chloroform soln thru a cotton pledget into a 500 ml volumetric flask and dilute to volume with chloroform. Determine the absorbency of the standard and unknown solns at 490 mμ.

$$\% \ \beta\text{-Naphthol} = \frac{A_{Unknown} \times C_s}{A_{Standard\ solution}} \times \frac{144}{293} \times \frac{1}{40}$$

where C_s = the concentration of the standard solution expressed in milligrams per liter.

B. Colors Soluble in Isopropyl Ether

Dissolve 2 g of the dye in 250 ml of isopropyl ether. (Warm on a steam bath to aid soln.) Extract the isopropyl ether soln with six 30-ml portions of 0.1 *N* NaOH. Wash the combined alkaline extracts with 30 ml of isopropyl ether and proceed as directed under (A) beginning with "Dissolve 20 mg of *p*-nitroaniline. . . . "

C. Colors Insoluble in Water or Isopropyl Ether

Extract a 10-g sample of dye with isopropyl ether for 8–10 hr. in a Soxhlet extraction apparatus. Transfer the ether extract to a 1-liter separatory funnel. Rinse the extraction flask with two 20-ml portions of isopropyl ether and add to the main extract. Extract the isopropyl ether with six 30-ml portions of 0.1 *N* NaOH. Combine the alkaline extracts and wash with 30 ml of isopropyl ether. Dilute the alkaline soln to exactly 500 ml with 0.1 *N* NaOH. Place a 100 ml aliquot of this soln in a beaker and proceed as directed under (A) beginning with "Dissolve 20 mg of *p*-nitroaniline. . . . "

UNCOMBINED α-NAPHTHOL IN FD&C ORANGE NO. 1—METHOD

APPARATUS

Soxhlet extraction apparatus.

A *spectrophotometer*, suitable for measurements at 464 mμ.

REAGENTS

Standard 4-(4-nitrophenylazo)-1-hydroxynaphthalene soln (2 mg per liter).—Dissolve 10 mg of 4-(4-nitrophenylazo)-1-hydroxynaphthalene in 100 ml of warm acetone, cool, transfer to a 500-ml volumetric flask and dilute to volume with chloroform. Dilute a 10-ml aliquot of this soln to exactly 100 ml with chloroform.

p-Nitroaniline.—M.P. 146–7° C.

DETERMINATION

Place 2 g FD&C Orange No. 1 in a cellulose extraction thimble and extract in a Soxhlet extractor with ethyl ether for at least 4 hr. Transfer the extract to a 500-ml separatory funnel, wash the extraction flask with two 10-ml portions of ethyl ether, and add these to the main extract. Wash the combined extracts once with 20 ml of water and extract with six 30-ml portions of 0.1 *N* NaOH. Couple a 100-ml aliquot of this soln with *p*-nitrobenzene diazonium chloride as directed under β-Naphthol. After the coupling mixture has been warmed on the steam bath, make the soln acid to litmus with 6 *N* HCl, cool, and extract with 20-ml portions of

chloroform until the chloroform layer is colorless. Wash the combined chloroform extracts with 20 ml of water, filter thru a cotton pledget into a 500-ml volumetric flask and dilute to volume with chloroform. Determine the absorbency of the standard and unknown solns at 464 mμ.

$$\% \ \alpha\text{-Naphthol} = \frac{A_{Unknown} \times C_s}{A_{Standard\ Solution}} \times \frac{144}{293} \times \frac{1}{20}$$

where C_s = the concentration of the standard soln, expressed in mg per liter.

EXPERIMENTAL

Preparation of purified 4-(4-nitrophenylazo)-1-hydroxynaphthalene.

Crude α-naphthol was dissolved in 0.1 *N* NaOH, filtered, and precipitated with 6 *N* HCl. The pink powder obtained was further purified by repeated recrystallization from water. The final product consisted of long white needles which melted at 93°–94°C. (Fisher block).

p-Nitroaniline (1 gm. 0.0072 moles), purified by two recrystallizations from 70% alcohol, was dissolved in 3 ml of conc. HCl (0.02 moles). To this solution was added 200 ml. of water and 80 gm of crushed ice. While stirring, 10% $NaNO_2$ solution was added dropwise until a positive test was obtained with starch-iodide test paper. The solution was stirred for ½ hour then the excess HNO_2 was destroyed with sulfamic acid. This solution was poured slowly into a beaker containing 0.72 gm (0.0050 moles) of the recrystallized α-naphthol, 200 ml of water, 100 gm of crushed ice and 15 ml. of 10% NaOH solution. The reaction mixture was stirred for ½ hour, warmed on a steam bath for 1 hour, cooled, and filtered. The filtrate was acidified with 6 *N* HCl and the precipitated dye filtered on a Büchner funnel, washed with water, and dried. This material, when prepared from purified intermediates, is sufficiently pure to be used as a standard.

Preparation of purified 1-(4-nitrophenylazo)-2-hydroxynaphthalene.

Crude β-naphthol was recrystallized 4 times from hot water. The product consisted of white leaflets which melted at 121.8–122.5°C.

The color is prepared in the same manner as 4-(4-nitrophenylazo)-1-hydroxynaphthalene except that it is filtered directly from the coupling mixture, washed and dried.

Known quantities of recrystallized alpha- or beta-naphthol were added to samples of four representative certifiable colors and the amount of alpha- or beta-naphthol determined by the methods given. The results are shown in the following tables.

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TABLE 1.—*β-Naphthol in D&C Orange No. 4 (color soluble in water)*

WT. COLOR	β-NAPHTHOL ADDED	β-NAPHTHOL FOUND (TOTAL)	ADDED β-NAPHTHOL RECOVERED	RECOVERY
Gm	*Mg*	*Mg*	*Mg*	*Per cent*
*2.0	0.00	1.80	—	—
2.0	5.20	6.86	5.06	97.3
2.0	6.10	7.52	5.72	94.1

TABLE 2.—*β-Naphthol in FD&C Orange No. 2 (color soluble in isopropyl ether)*

WT. COLOR	β-NAPHTHOL ADDED	β-NAPHTHOL FOUND (TOTAL)	ADDED β-NAPHTHOL RECOVERED	RECOVERY
Gm	*Mg*	*Mg*	*Mg*	*Per cent*
*2.0	0.00	0.73	—	—
2.0	2.26	2.73	2.00	88.5
2.0	2.26	2.77	2.04	90.4

TABLE 3.—*β-Naphthol in D&C Red No. 35 (color insoluble in water and isopropyl ether)*

WT. COLOR	β-NAPHTHOL ADDED	β-NAPHTHOL FOUND (TOTAL)	ADDED β-NAPHTHOL RECOVERED	RECOVERY
Gm	*Mg*	*Mg*	*Mg*	*Per Cent*
*10	0.00	3.31	—	—
10	19.20	21.70	18.39	95.8
10	28.61	31.69	28.38	99.2
10	41.35	39.97	36.66	93.5

* Blanks.

TABLE 4.—*α-Naphthol in FD&C Orange No. 1*

WT. COLOR	α-NAPHTHOL ADDED	α-NAPHTHOL FOUND (TOTAL)	ADDED α-NAPHTHOL RECOVERED	RECOVERY
Gm	*Mg*	*Mg*	*Mg*	*Per cent*
*2.0	0.00	0.34	—	—
2.0	1.025	1.24	0.90	88.2
2.0	2.05	2.22	1.88	91.6
2.0	3.86	4.23	3.89	100.8

* Blanks.

DETERMINATION OF β-NAPHTHYLAMINE IN FD&C YELLOW NOS. 3 AND 4*

By LEE S. HARROW (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

β-Naphthylamine is an intermediate used in the production of FD&C Yellow No. 3 and No. 4. The official A.O.A.C. method for the determination of aniline and *o*-toluidine in FD&C Yellow Nos. 3 and 4, respectively,[1] includes the steam distillation of the amine intermediates from alkaline aqueous suspensions of the dyes. Very little β-naphthylamine can be recovered by this procedure; β-naphthylamine can, however, be distilled from a saturated salt solution of the dye made slightly alkaline with sodium hydroxide. The intermediate in the distillate is determined spectrophotometrically.

METHOD

APPARATUS

A spectrophotometer, suitable for measurements at 305 mμ, 335 mμ, and 365 mμ.

REAGENTS

Standard β-naphthylamine soln.—Dissolve 25 mg of purified β-naphthylamine (M.P. 111–112°C.) in 20 ml of alcohol and dilute to 100 ml with H_2O. To a 10-ml aliquot add 25 ml of 0.4 *N* NaOH and dilute the soln to exactly 100 ml with water.

PROCEDURE

In a 500 ml round-bottom flask place the following in the order given: a few boiling chips, 80 g NaCl, 5 g FD&C Yellow No. 3 or 4, enough glass wool (presoaked in water)† to fill ca ¾ of the flask, 5 ml of 30% NaOH, and 100 ml of water. Fit the flask with a steam trap and condenser. Distill at a rate of about 2 drops per second into a flask containing 5 ml of (1+1) (v/v) HCl soln. After 125 ml has been distilled add 100 ml of water to the distillation flask and continue the distillation until a total volume of ca. 200–225 ml has been collected. Make the distillate alkaline to litmus with 30% NaOH and transfer to a 500-ml separatory funnel. Wash out the receiver with two 15-ml portions of H_2O and add these to the soln. Extract the soln with three 50 ml portions of ethyl ether, combine the ether solns and extract with four 10-ml portions of 0.2 *N* HCl. Combine the acid extracts and heat for 30 min. on a steam bath to remove the last traces of ether, then cool the soln to room temp. Add 45 ml of 0.4 NaOH, and dilute to exactly 100 ml with water. Determine the absorbency of the standard and unknown solns at 305, 335, and 354 mμ with a suitable spectrophotometer.

$$\% \ \beta\text{-Naphthylamine} = \frac{A_{\text{Unknown } 335m\mu} - \frac{A_{\text{U } 365m\mu} + A_{\text{U } 305m\mu}}{2}}{A_{\text{Standard } 335m\mu} - A_{\text{St } 365m\mu} + A_{\text{St } 305m\mu}} \times C_s \times \frac{1}{50}$$

where C_s = the concentration of the standard solution expressed in milligrams per 100 ml.

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 2–4, 1950.

† The glass wool will take up about 100 ml. of water.

[1] Caemmerer, Alice, *This Journal*, 31, 592 (1948).

EXPERIMENTAL

β-Naphthylamine.—A sample of pure β-naphthylamine was prepared by recrystallization of crude β-naphthylamine from hot water. The final product melted at 111–112°C. (lit. M.P. 111–112°C).

Recovery experiments.—Weighed samples of purified β-naphthylamine were added to 5-g. samples of FD&C Yellow Nos. 3 and 4 and analyses carried out following the proposed method. The results are given in Tables 1 and 2.

TABLE 1.—*Recovery of β-naphthylamine from FD&C Yellow No. 4*

β-NAPHTHYLAMINE ADDED	β-NAPHTHYLAMINE FOUND	β-NAPHTHYLAMINE FOUND NET	NET RECOVERY
Mg	*Mg*	*Mg*	*Per cent*
0.00*	0.12	—	—
2.85	2.63	2.51	88.1
2.85	2.64	2.52	88.4
2.85	2.62	2.50	87.5
5.70	5.30	5.18	91.0
5.70	5.37	5.25	92.1
8.55	8.14	8.02	93.8
1.42	1.33	1.21	85.1
		Av. Rec.	89.4

TABLE 2.—*Recovery of β-naphthylamine from FD&C Yellow No. 3*

β-NAPHTHYLAMINE ADDED	β-NAPHTHYLAMINE FOUND	β-NAPHTHYLAMINE RECOVERED NET	NET RECOVERY
Mg	*Mg*	*Mg*	*Per cent*
0.00*	0.11	—	—
0.99	0.94	0.93	84.3
2.85	2.69	2.58	90.6
2.28	2.15	2.04	89.5
4.56	4.27	4.16	91.2
		Av. Rec.	88.9

* Blanks.

DETERMINATION OF *m*-DIETHYLAMINOPHENOL IN D&C RED NO. 19 AND D&C RED NO. 37

By LEE S. HARROW (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

m-Diethylaminophenol is an intermediate in the production of the certifiable coal-tar colors, D&C Red No. 19 and D&C Red No. 37. Separation of the intermediate from the colors in which it appears is readily accomplished by steam distillation from saturated salt solution adjusted to a *p*H of 6.6 ± 0.2. In the case of D&C Red No. 37 it is necessary to make a second steam distillation to avoid interference due to the stearic acid present in the color. The intermediate, so separated, is identified and determined spectrophotometrically.

m-DIETHYLAMINOPHENOL IN D&C RED NO. 19—METHOD

APPARATUS

A spectrophotometer suitable for use at 295 mμ.

REAGENTS

Standard m-diethylaminophenol soln.—Dissolve 0.2 g of purified *m*-diethylaminophenol in 100 ml of 50% alcohol. Dilute this soln to 200 ml with water. Transfer a 20-ml aliquot to a 1-liter flask, add 100 ml of 1 *N* NaOH, and dilute to 1 liter with water.

PROCEDURE

In a 500-ml round-bottom flask place a 3-in. swab stick (boiling aid), 20 g of KH_2PO_3, and 35 g of NaCl. Weigh 2.5 g of D&C Red No. 19 into a 200-ml beaker and make a paste of the color with about 50 ml of H_2O. Add 50 ml of H_2O and wash this mixture into the 500-ml round-bottom flask with several 10-ml portions of H_2O. Make the volume to 250 ml and adjust the *p*H of the soln to 6.6 ± 0.2 with dilute NaOH. Fit the flask with a steam distillation trap and condenser and distill 175 ml. Dilute the distillate to 250 ml in a volumetric flask with 10 ml of 10% NaOH and enough water to make to the mark. Determine the absorbency of the known and unknown solns at 295 mμ.

$$\% \; m\text{-Diethylaminophenol} = \frac{A_{\text{Unknown } 295m\mu}}{A_{\text{Known } 295m\mu}} \times C_S \times \frac{1}{100}$$

where C_S = the concentration of the standard soln. expressed in mg. per liter.

EXPERIMENTAL

Weighed quantities of purified *m*-diethylaminophenol (recrystallized from (1+5) CCl_4-petroleum ether soln and then from 40% methanol-water soln) were added to samples of D&C Red No. 19 and the samples analyzed by the method. The results are shown in Table 1.

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* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 2–4, 1950.

TABLE 1.—*Results of analysis*

WT. D&C RED NO. 19	WT. *m*-DIETHYL-AMINOPHENOL ADDED	WT. *m*-DIETHYL-AMINOPHENOL FOUND	RECOVERY
Gm	*Mg*	*Mg*	*Per cent*
2.5	0.00	Trace (Undistinguishable from blank)	—
0.0	4.40	4.37	99.0
2.5	5.22	4.92	94.2
2.5	5.22	4.55	87.2
2.5	4.90	4.75	96.9
2.5	11.63	10.43	89.7
2.5	10.50	9.47	90.2
2.5	9.20	8.52	92.6
2.5	14.79	13.87	93.8
2.5	16.24	15.09	92.9
2.5	15.56	14.95	96.1
2.5	2.33	2.06	88.5
		Av. Rec.	92.2

m-DIETHYLAMINOPHENOL IN D&C RED NO. 37—METHOD

Proceed as directed for *m*-diethylaminophenol in D&C Red No. 19 up to and including " . . . and distill 175 ml." Transfer the distillate to a 500-ml round-bottom flask, rinse the receiver with several 10-ml portions of water, and add the washings to the flask. Add 30 g of NaCl, 15 g of KH_2PO_4, and adjust the *p*H to 6.5–6.7 with 30% NaOH soln. Distill ca 175 ml and filter the distillate thru a retentive filter paper into a 250-ml volumetric flask containing 10 ml of 10% NaOH. Wash the receiver with several 10-ml portions of water, filtering each washing thru the same paper. Dilute the filtrate to volume with water and proceed as directed for D&C Red No. 19 starting with "Determine the absorbency of the known and unknown solutions. . . . "

TABLE 2.—*Experimental results*

WT. D&C RED NO. 37	WT. *m*-DIETHYL-AMINOPHENOL ADDED	WT. *m*-DIETHYL-AMINOPHENOL FOUND	RECOVERY
Gm	*Mg*	*Mg*	*Per cent*
2.5	0.00	None	—
0.0	5.10	4.90	96.0
2.5	5.10	4.86	95.2
2.5	5.10	4.81	94.2
2.5	5.10	4.77	93.3
2.5	10.20	9.95	97.5
2.5	10.20	10.25	100.5
2.5	10.20	9.90	97.0
		Av. Rec.	96.2

EXPERIMENTAL

Recovery experiments.—Aliquots of a standard solution of purified *m*-diethylaminophenol were added to samples of D&C Red No. 37 and determined by the proposed method. The results are shown in Table 2.

ACKNOWLEDGMENT

The author wishes to express his appreciation to Meyer Dolinsky, of the U. S. Food and Drug Administration, for the preparation of the sample of purified D&C Red No. 19 used in this work.

A VARIABLE REFERENCE TECHNIQUE FOR ANALYSIS BY ABSORPTION SPECTROPHOTOMETRY

I. THEORY AND DISCUSSION

By J. H. JONES, G. R. CLARK, and L. S. HARROW (Division of Cosmetics, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

Probably the most common procedure in the spectrophotometric analysis of solutions is to compare the absorbancy of the unknown, at one or more wave lengths, with the absorbancies of standard solutions, in the same solvent, of the materials under examination (1). In effect, the known and unknown solutions are compared by comparing each to a third solution (*i.e.*, the "blank"), the optical properties of which are completely different from the optical properties of the standard and unknown.

It has been pointed out that greater precision can be obtained in certain cases by comparing the unknown solution to a reference solution containing the same material (2). In this case, the difference between the unknown and standard is compared to the "difference" between two standards. This procedure is not readily applicable to the analysis of multicomponent systems.

It is the purpose of this paper to point out the advantages of a technique in which the absorption of the unknown solution is compared to that of a known solution containing the same materials in the same concentration as in the unknown (or as nearly the same concentration as can be obtained with the available standards). Since this implies, and usually involves, changing the composition of the reference solution during the course of the measurement, the technique is called "Variable Reference Spectrophotometry."

Others have used reference solutions to "balance out" the absorption of one or more absorbing materials in a mixture, usually to emphasize the absorption of other constituents. So far as we are aware, however, the technique described in this report, that is, variation of the reference

solution as part of the determination, has not been described previously.

THEORY AND COMMENTS

The proposed technique is based on the following:

(1) If the spectrophotometric properties of two solutions, S_1 and S_2, are identical, the absorbance of S_1 measured against S_2, as the reference solution, will be zero *at all wave lengths.*

(2) If the spectrophotometric properties of S_1 and S_2 are not identical,

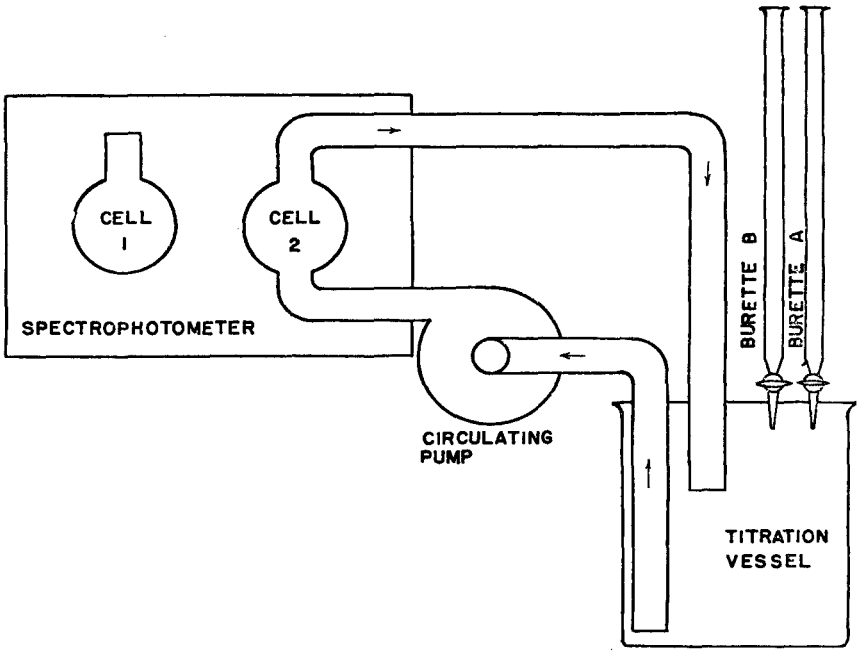

FIG. 1.—Schematic diagram of titration assembly.

the absorbance of S_1 measured against S_2, at any wave length, will be

$$D = As_1 - As_2.$$

where As_1 is the absorbancy of S_1 and As_2 is the absorbancy of S_2 at the same wave length.

(In this paper the term "absorbance" is used for the quantity, $\log_{10} T$, where T is the relative transmittance of the sample and standard, *i.e.*, T sample/T standard. The symbol D has been chosen to represent the difference in the absorbancies of two solutions and will be so used throughout this work.)

In the experimental set-up shown schematically in Figure 1 suppose

that cell C_1 is filled with a solution known to contain compounds 1, 2, 3—n all of which absorb light in the region covered by the spectrophotometer. If the titration vessel, V, contains the solvent used for the solution in C_1, it should be possible by the addition of compounds 1, 2, 3—n to V, to prepare a solution in V (and consequently in cell C_2) which will make the absorbancy of C_1 with respect to C_2 be zero at all wave lengths.[1] As will be shown, when this condition is fulfilled, the concentration of each of the compounds 1, 2, 3—n in cell C_2 must be the same as that in cell C_1, except in certain unusual cases. The concentration of each compound in C_1 can then be calculated from the amounts of compounds 1, 2, 3—n added to V and the total volume of the solution.

Case 1.—Suppose cell C_1 contains a solution whose concentration of compound X is x_1; if Beer's law is obeyed, the absorbency of the solution with respect to the solvent at any selected wave length will be:

$$As_1 = kx_1. \qquad \text{(Eq. 1a)}$$

If cell C_2 contains a solution of X whose concentration is x_2, the absorbancy with respect to the solvent at this wave length will be:

$$As_2 = kx_2. \qquad \text{(Eq. 1b)}$$

The absorbancy difference for the two solutions is, however,

$$D = As_1 - As_2 = kx_1 - kx_2 = k(x_1 - x_2) \qquad \text{(Eq. 2)}$$

and when the absorbancy difference, D, equals zero,

$$k(x_1 - x_2) = 0 \qquad \text{(Eq. 2a)}$$

which, if $k = 0$, will be true only when $x_1 = x_2$.

Case 2.—If cell C_1 contains a solution of two compounds, X and Y, and X and Y are added to the solution in cell C_2 then (if Beer's law holds and the compounds do not interact) the equation for D is, where x_1, x_2, y_1, and y_2 represent the concentration of X and Y in the respective solutions:

$$D = k_1(x_1 - x_2) + k_2(y_1 - y_2) \qquad \text{(Eq. 3)}$$

and when $D = 0$

$$k_1(x_1 - x_2) + k_2(y_1 - y_2) = 0. \qquad \text{(Eq. 3a)}$$

One solution of this equation is:

$$x_1 = x_2; \qquad y_1 = y_2.$$

Another possible solution is:

[1] It is assumed that the two cells are of equal length and are accurately matched at all wave lengths.

$$\frac{y_2 - y_1}{x_1 - x_2} = \frac{k_1}{k_2} \qquad \text{(Eq. 4)}$$

but if $D = 0$ at all wave lengths:

$$\frac{y_2 - y_1}{x_1 - x_2} = \frac{k_1}{k_2} = \frac{k_1'}{k_2'} = \frac{k_1''}{k_2''} = \cdots = \frac{k_1^r}{k_2^r} = C \qquad \text{(Eq. 4a)}$$

which can only be true if the absorbancy curve (*As vs.* λ) of X is the same or proportional to that of Y, and $C(x_1 - x_2) = y_2 - y_1$. Any such similarity would be apparent from the standard curves of the two components.

Case 3.—For a three component system, where Beer's law is obeyed and there is no interaction, the equation for D is:

$$D = k_1(x_1 - x_2) + k_2(y_1 - y_2) + k_3(z_1 - z_2) \qquad \text{(Eq. 5a)}$$

but if $D = 0$ at all wave lengths, then

$$k_1'(x_1 - x_2) + k_2'(y_1 - y_2) + k_3'(z_1 - z_2) = 0 \qquad \text{(Eq. 5b)}$$

$$k_1''(x_1 - x_2) + k_2''(y_1 - y_2) + k_3''(z_1 - z_2) = 0 \qquad \text{(Eq. 5c)}$$

. . . . . .

$$k_1^r(x_1 - x_2) + k_2^r(y_1 - y_2) + k_3^r(z_1 - z_2) = 0. \qquad \text{(Eq. 5d)}$$

Three possible solutions for these equations are:

(a) $x_1 - x_2$; $y_1 = y_2$; $z_1 = z_2$

(b) $z_1 = z_2$; $k_1 = Ck_2$; $C(x_1 - x_2) = y_1 - y_2$ (See Case 2—Eq. 4a)

(c) $k_1 = Fk_2 + Gk_3 = 0$; $y = Fx$; $z = Gx$

The third solution is possible if the absorbancy curve of one of the components can be obtained by a combination of the absorbancy curves of the other components.

Analogous equations may be set up for a system of n components.

Case 4.—So far, the discussion has been confined to systems which obey Beer's law. Suppose, however, we have two components which do not obey Beer's law. If the deviation from Beer's law is due solely to association, dissociation, etc., of the individual components, the absorbancy of the components may be represented by the equations:

$$As_x = k_1 f(x). \qquad \text{(Eq. 6a)}$$

$$As_y = k_2 f(y). \qquad \text{(Eq. 6b)}$$

For a two component system, if there is no interaction between the components, the equation for D is:

$$D = k_1[f(x_1) - f(x_2)] - k_2[f(y_1) - f(y_2)] \qquad \text{(Eq. 7)}$$

instead of that given as Eq. 3. An obvious solution of the equation when $D=0$ is: $x_1=x_2$; $y_1=y_2$ Another possible solution is:

$$\frac{f(y_2)-f(y_1)}{f(x_1)-f(x_2)}=\frac{k_1}{k_2}=\frac{k_1'}{k_2'}=\cdots\frac{k_1^r}{k_2^r}=C.$$

The possibility of obtaining such a solution to these equations is slight and can be predicted from the absorbancy curves of the individual components.

Obviously, the equations for mixtures of three or more components will be the same as for the simple case in which Beer's law is obeyed if one replaces x_1–x_2 with $f(x_1)$–$f(x_2)$; y_1–y_2 with $f(y_1)$–$f(y_2)$; etc.

Case 5.—In a two component system which deviates from Beer's law only because the components interact, *i.e.*, if $aX+bY\rightarrow Z$, the equation for the absorbancy where the concentrations are expressed in moles per liter is:

$$As = k_1(x_1-(1-b)z_1)+k_2(y_1-(1-a)Z)+k_3z_1 \quad \text{(Eq. 8a)}$$

or

$$As = k_1x_1+k_2y_2+[(k_3-k_1(1-b)-k_2(1-a)]z_1 \quad \text{(Eq. 8b)}$$

or

$$As = k_1x_1+k_2y_1+k_4z,$$

$$\text{where } k_4 = k_3-k_1(1-b)-k_2(1-a) \quad \text{(Eq. 8c)}$$

which is simply the expression for the absorbancy of a three component mixture such as discussed under Case 3. Therefore, if the reference solution contains the same concentrations of X, Y, and Z as the unknown, the absorbancy difference will be zero at all wave lengths.

Since Z is produced by a definite combination of X and Y, the concentrations of X, Y, and Z in the reference solution can be made equal to the concentrations of these materials in the unknown by adding X and Y, in proper proportions, to the solvent.

The usual purpose of a spectrophotometric analysis of a mixture is to determine the concentration of each component in the mixture. Any solution described above other than $(x_1-x_2)=0$, $(y_1-y_2)=0$, $\cdots$ and $(z_1-z_2)=0$, would therefore be of little value. Fortunately, the possibility of obtaining any other solution is slight and can usually be predicted from the curves of the standards.

Effect of Instrumental Errors

One of the causes of apparent deviation from Beer's law in certain spectrophotometers is that the detector (phototube) response is not linear. Since in the variable reference procedure both the sample and standard have the same absorption in the final determination, non-linearity of detector has no effect on the result.

Another instrumental cause of apparent deviation from Beer's law is

non-linearity of the measuring system. In the proposed procedure, however, the purpose is to bring about conditions such that the measuring system need only indicate balance of the absorptions due to sample and standard. In this case, linearity in the measuring system is not critical.

Stray light is probably the major cause of apparent deviation from Beer's law in most spectrophotometers. If the sample and standard each have the same absorbancy curve the stray light effects should cancel; the indicated balance point should be the same as if no stray light were present.

APPLICATIONS

Theoretically, when the variable reference technique is used, it is not necessary to measure the actual absorbancy of the unknown at any wave length; all that need be known is whether the absorbancy of the unknown with respect to the known is positive, zero or negative. In practical applications of the method, however, roughly quantitative absorbancy curves of standards and unknown are necessary. It is also desirable that the indicated value for the absorbancy differences be approximately equal to the actual difference in absorbancies. Since most of the spectrophotometers in use at the present time are capable of determining the absorbancy of a solution rather accurately, it is assumed in the subsequent discussion that this information can readily be obtained.

Analysis of Multicomponent Systems

Although variable reference spectrophotometry can be employed for the analysis of single component systems, the greatest advantage lies in the utility of the procedure for the analysis of multicomponent systems. (A discussion of the application of variable reference spectrophotometry to multicomponent systems in which the identity of only one component is known will be found in the section on analysis in presence of background.)

The most commonly used procedure for the spectrophotometric analysis of multicomponent systems is based upon a solution of simultaneous equations (1). In this procedure, one measures the absorbancy of the standards and unknowns at at least as many wave lengths as there are components in the solution and calculates the composition from the equations:

$$As\lambda_1 = a_1x + b_1y + \cdots + c_1z \qquad \text{(Eq. 9a)}$$

$$As\lambda_2 = a_2x + b_2y + \cdots + c_2z \qquad \text{(Eq. 9b)}$$

$$\cdots\cdots\cdots\cdots\cdots\cdots\cdots$$

$$As\lambda_r = a_rx + b_ry + \cdots + c_rz \qquad \text{(Eq. 9c)}$$

where $A_1, A_2, \cdots, A_r$ is the absorbancy of the unknown solution at $\lambda_1, \lambda_2, \cdots, \lambda_r$; $a_1, a_2, \cdots, a_r$; $b_1, b_2, \cdots b_r$; etc., are the unit absorbancies of the respective standards at $\lambda_1, \lambda_2, \cdots \lambda_r$, and $x, y \cdots, z$ are the concentrations of the materials in the unknown solution.

In the analysis of a two component mixture, for example, the absorbancies of both standards and the unknown are determined at two wave lengths and the values substituted in the above equation. This makes a total of six separate absorbancies that must be measured to solve the simultaneous equations. Since a small error in any of the absorbancies may cause a relatively large error in the calculated values for the components, the absorbancy determinations must be made precisely if the calculated values are to be reliable.

In the analysis of a two component system by the method of simultaneous equations, it can be shown that mathematically the best solution is obtained by making the absorbancy measurements at the two wave lengths where the difference between the absorbancy ratios of the two components,

$$\left(\frac{Asx_1}{Asy_1} - \frac{Asx_2}{Asy_2}\right)$$

is greatest (1). Similarly, it may be shown that the most accurate comparison of a standard and reference solution containing these two components can be made at the same two wave lengths.

When the method of simultaneous equations is used it is frequently not feasible to use the wave lengths most favorable from the standpoint of absorbancy ratios (1). In many cases, this would require that the absorbancies be determined at a wave length in a region where the change in absorbancy with wave length is very rapid, a condition which seriously decreases the accuracy with which the absorbancy can be determined. Consider, as an example, the curves shown in Figure 2 where Curves 1 and 2 are the absorbancy curves of two compounds, and Curve 3 is the absorbancy curve of the mixture of these two compounds. (These are the actual curves of two closely related compounds and a mixture of the two.) Mathematically, the most favorable wave lengths are approximately those indicated as λ_1 and λ_2; however, at these wave lengths the change of absorbancy with wave length is very great. If the wave lengths selected for the determination are those of the peaks of the two components, one of the absorbancies of each standard and both absorbancies of the mixture must be determined at a wave length where the absorbancy is changing rapidly with the wave length. Obviously, whatever wave lengths are chosen, an accurate determination of all six absorbancies is difficult, if not impossible.

Suppose, however, we place the solution whose absorbancy curve is given by Curve 3 in cell C_1 of Figure 1 and set the spectrophotometer at *approximately* the wave length indicated by λ_1. Any appreciable absorbancy in this region, relative to that at the peak, will be due principally to compound 2. Compound 2 may, therefore, be added to the reference solution until the absorbancy difference at λ_1 is considerably reduced.

Any absorbancy difference at λ_2 is now due primarily to compound 1 (most of the absorbancy due to compound 2 at this wave length has been "cancelled") and this difference can be reduced to a small value by the addition

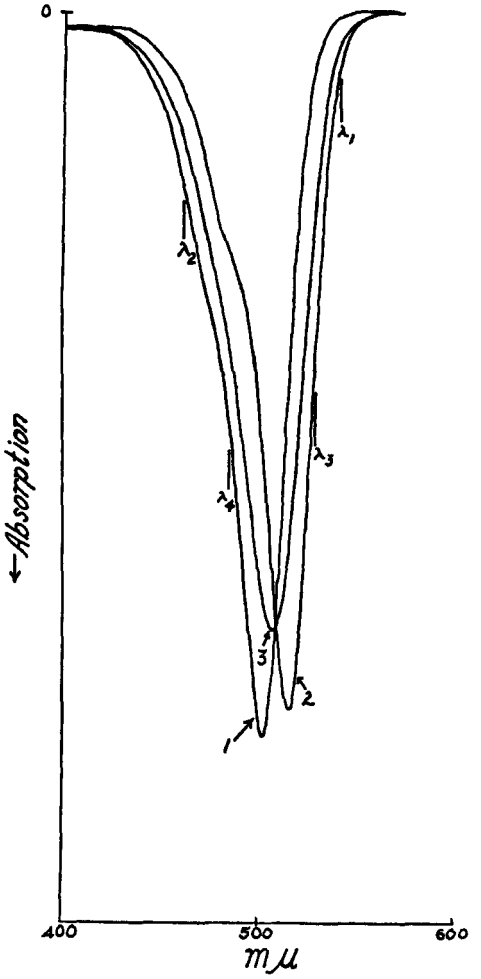

FIG. 2.—Absorption curves of two colors and a mixture of the two.

of compound 1 to the reference solution. Since the reference solution now contains approximately as much of compound 1 as does the unknown, the absorbancy difference at λ_1 will be due almost entirely to compound 2 and can be reduced to a very low value by the addition of compound 2 to the reference solution. Following this procedure, the absorbancy differ-

ence can be reduced to zero at both wave lengths. If the unknown contains only compounds 1 and 2, the absorbancy difference should then be zero at all wave lengths and the concentration of compounds 1 and 2 in the reference solution the same as the respective concentrations in the unknown solution.

In practice, it is preferable to make the second and subsequent additions of compounds 1 and 2 at approximately the wave lengths indicated by λ_3 and λ_4, where the actual absorbancies are higher even though the ratio of the absorbancies of the two is not as favorable as elsewhere.

It will be noted that in the above brief description of the variable reference procedure, it is stated that the instrument is set at *approximately* wave length λ_1. It is readily apparent that the actual setting is not critical. Although the absorbancy of the unknown is changing rapidly at this wave length, at any wave length in this region any appreciable absorbancy (relative to the absorbancy in the neighborhood of the peak) must be due to compound 2. The actual value of the absorbancy is not critical except at zero absorbancy. It is, therefore, logical to "cancel" most of this absorption by the addition of compound 2 to the reference solution. (The fraction of the absorption that may be "cancelled" without danger of adding too much of compound 2 depends upon the absorbancy ratio for the two components.) It is obvious that the same line of reasoning will apply to the addition of compound 1 at approximately wave length λ_2.

The two compounds whose curves are shown in Figure 1 are D&C Orange No. 5 (Dibromofluorescein) and D&C Red No. 21 (Tetrabromofluorescein). As is usually the case with closely related compounds, such as isomers or members of a homologous series, the curves of the two compounds are quite similar; the major difference in this case is a displacement of the wave length of maximum absorption. Ordinarily mixtures of isomeric or closely related compounds are difficult to separate by physical or chemical methods of separation; spectrophotometric methods of analysis of such mixtures are therefore quite useful. The variable reference procedure appears to be a very convenient technique for the analysis of such mixtures. Frequently, the use of this technique will permit the analysis to be made with considerably greater accuracy than can be obtained by the use of a method involving solution of simultaneous equations.

The procedure described above can be readily extended to the examination of mixtures of more than two components if:

(1) a wave length can be found at which the absorbancy of the mixture is due principally to the absorbancy of a particular component of the mixture, and

(2) after most of the absorbancy due to this particular component is cancelled, another wave length can be found at which the absorbancy difference is due primarily to only one of the remaining components.

Theoretically, the variable reference procedure can be used in any case where the adsorption ratios,

$$\frac{Asx}{Asy}, \quad \frac{Asx}{Asz}, \quad \frac{Asy}{Asz},$$

at a sufficient number of wave lengths, are favorable; however, the actual determination becomes largely a matter of trial and error unless the conditions stated in the previous paragraph are met.

Analysis in Presence of Background

So far the discussion has been confined to cases in which the identities of all the components of the mixture under investigation are known. It has been pointed out, however, that unless the unknown and final reference solution have the same composition it is improbable that the absorbancy difference can be reduced to zero at all wave lengths. If it is impossible to obtain zero absorbancy at all wave lengths by addition of the standards employed, it is obvious that the unknown must contain some material not present in the standards.

In spectrophotometric analysis, it is frequently desirable to estimate, as accurately as possible, the concentration of a certain component in the presence of unknown materials that also absorb in the same spectral region. Several procedures have been devised for the analysis of unknowns which have such "background" absorption in addition to the absorption of the desired components. Probably the most widely used procedure is to assume that the background is linear over a certain portion of the spectrum and to calculate the amount of the desired components from the deviation (of the actual absorbancy) from linearity measured in this portion of the spectrum (1). A more elaborate scheme is that of Tunnicliff *et. al.*, (3), in which it is assumed that the background can be defined by a certain function (usually a power series) of the wave length. Enough data is then obtained from the absorbancy curve of the unknown to calculate the constants of this equation and to correct accordingly the absorbancies used for calculation of the desired components.

If the unknown solution contains a known material X and an unknown material B the absorbancy curve, *i.e.*, As plotted against wave length for the unknown may be represented by the equation

$$As = x_1 f(\lambda) + b_1 f_2(\lambda).$$

When this solution is compared to a reference solution to which a concentration x_2 of X has been added, the absorbancy difference is, if compound X obeys Beer's law:

$$D = (x_1 - x_2) f(\lambda) + b_1 f_2(\lambda).$$

The quantity $(x_1 - x_2) f(\lambda)$ will be positive if $x_1 > x_2$; it will be negative if

$x_2 > x_1$. In other words as long as $x_1 > x_2$ the absorbancy difference will be the absorbancy due to the unknown material plus that due to the amount of X equal to $x_1 - x_2$. If, however, $x_2 > x_1$ the absorbancy difference will be the absorbancy of the unknown material minus that due to the amount of X equal to $x_2 - x_1$. When $x_1 = x_2$ the absorbancy difference will be $D = b_1 f_2(\lambda)$ or the absorbancy of the unknown material. It is obvious that the absorbancy difference curve will look the least like the curve of $Xf_1(\lambda)$, or its inverted curve, when $x_1 = x_2$.

Assume, for example, that the curve due to B is a straight line through the points λ_1, λ_2, λ_3, but the absorbancy curve of X has a peak in the vicinity of λ_2. Now, if we obtain the absorbancy difference curve for the unknown against various concentrations of X, as long as $x_1 > x_2$, the absorbancy difference at λ_2 will be positive with reference to the straight line through λ_1 and λ_3. When $x_2 > x_1$ the absorbancy curve will be negative with respect to the straight line through λ_1 and λ_2. But if $x_1 = x_2$ the absorbancy difference curve will be the straight line through λ_1, λ_2, λ_3.

Ordinarily, the "background" is not linear over any reasonable portion of the spectrum. In a great many cases, variable reference spectrophotometry can be used to obtain a fairly reliable estimate of the concentration of the desired constituent in the presence of a non-linear background. For example, suppose that the "background" gives a fairly smooth but not linear curve over the region λ_1, λ_2, λ_3, and that the absorbancy of the desired component is low at λ_1 and λ_3 and high at λ_2 (*i.e.*, the desired constituent, X has a peak at λ_2). Now if the unknown is compared to a reference solution containing approximately the same amount of X as the unknown, the absorbancy difference curve in the region λ_1 and λ_3 will be approximately that of the background alone and the background at λ_2 can be predicted fairly accurately. If the reference solution contains less of X than the unknown, the absorbancy difference in the region of λ_2 will be higher than expected from a smooth background curve through λ_1 and λ_3. If the reference solution contains an excess of the contituent X the absorbancy difference at λ_2 will be lower than expected.

When the variable reference procedure is employed, in the presence of a background, the amount of the desired constituent is actually determined as "not less than—and not more than—" the limits being determined by the amounts of the desired constituent in the reference solution which will produce definite positive and negative "distortions" of the background curve.

The amount of the desired constituent that will produce a definite "distortion" of the background curve will depend, of course, upon the degree of difference between the two curves and the unit absorption of the desired constituent. It is obvious that this method will fail, as will all others, if the background curve is quite "similar" to that of the desired constituent.

The chief advantage in the application of the variable reference technique to the determination of a single component in the presence of background absorption is that no assumption need be made as to the appearance of the background before starting the determination. In actual practice, the absorbancy difference curves are determined using the most likely range of concentrations of the desired constituent(s), and on the basis of these curves the most likely curve for the background is selected. The absorbancy values do not need to be accurate values; the determination is based on the differences between the various curves rather than the actual absorbancy values.

The existence of background presents a greater problem in the analysis of a multicomponent mixture than in those analyses in which a single component is to be determined. Frequently, the combined curve of the components has a relatively high absorbancy over a wide spectral range.[2] A careful study of the absorbancy data is required, therefore, even to establish the presence of the background; the application of a logical correction to the data is almost impossible.[3]

As will be shown in the experimental section, the presence of a moderate "background absorption" does not seriously decrease the accuracy obtainable by the variable reference technique in the analysis of many mixtures.

Precision and Accuracy

The precision and accuracy of a spectrophotometric determination depend so much on the particular system under investigation and the spectrophotometer used that a discussion of this topic, except for a particular case, is practically meaningless. In spectrophotometric determinations based on direct absorbancy measurements the precision depends upon the amount, Δx, of X required to produce a measurable change in the absorbancy of the solution. In the variable reference procedure, the precision of the determination depends essentially upon the amount, Δx, of the compound X which will produce a definite "distortion" of the zero absorbancy difference curve for the solution. These two quantities are not exactly the same; but for most solutions, the quantities are of very nearly the same magnitude. As explained previously, the variable reference procedure may eliminate, to a considerable degree, the effect of "mechanical" errors inherent in the spectrophotometer. It cannot, however, increase the accuracy with which a single measurement can be made by the spectrophotometer.

The inherent error in a spectrophotometric determination depends upon the value $\Delta x/x$. In general, the same techniques can be used in

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[2] In the visual and ultraviolet range, many mixtures have such spectra; in the infrared region, the peaks due even to closely related compounds are usually more easily resolved.

[3] The authors have not had occasion to test the method of Tunnicliff *et al.* (3); it is noted, however, that the mixtures they analyzed had very sharp "peaks" and "valleys" which would facilitate the calculation of the background curve.

variable reference spectrophotometry as are used in the more direct determination to reduce the quantity $\Delta x/x$ to a minimum value.

Qualitative Analysis

Variable reference spectrophotometry may be of considerable help in the qualitative analysis of solutions. For solutions which contain one known component, the procedure employed is the same as that used for the determination of a single component in the presence of a background; the absorbancy difference curves of the unknown *vs.* various concentrations of the known component are obtained. Of these curves, the one that least resembles the curve of the component in the reference cell (or its inverted curve) will be a fair approximation of the curve of the unknown material, or materials. Frequently, the difference curve so obtained can be used to identify the unknown component; this tentative identification can be confirmed or rejected by adding the compound selected to the reference solution in an attempt to obtain a zero difference curve.

ADVANTAGES AND DISADVANTAGES

The chief advantage of the variable reference technique is that the absorbancy difference values do not have to be determined accurately to insure the accuracy of the final result. It will be most useful, therefore, in those cases where for some reason, usually the slope of the absorbancy curve, it is difficult to obtain accurate (or more correctly, precise) absorbancy measurements at the most useful wave lengths.

The other major use for the technique is for the determination of known components in the presence of unknown materials. The advantage here is that the determination can be made without any prior assumption as to the nature of the absorption curve of the unknown materials.

It is obvious that the technique cannot yield any information which could not be obtained from perfectly reproducible absorption curves of the standards and unknowns. In many cases, however, it appears to be easier to obtain the information from absorbancy difference curves than from calculations based on even the most precise of direct measurements.

One of the procedures frequently used to increase the reliability of spectrophotometric determinations is to calculate each result from more than one set of independent data; the additional data is usually obtained by making measurements at more wave lengths than the minimum needed for the calculations. When the variable reference technique is used the result is calculated from measurements at all possible wave lengths; confidence in the reliability of the final result is thereby increased considerably.

Considerably more time is required for a determination by the variable reference procedure than for any reasonable number of direct absorbancy measurements. In many cases this time will be recovered in the calculation of the results. If results of the desired accuracy and reliability can

be obtained from a few direct absorbancy measurements, it would usually not be advantageous to use the variable reference procedure.

SUMMARY

If a solution is measured in a spectrophotometer against another solution as a reference, the instrument will indicate the absorbancy difference. If the composition of the two solutions is the same, the absorbancy difference will be zero at all wave lengths. If two solutions give zero absorbancy difference at all wave lengths under such conditions the composition of the two solutions must, therefore, be the same, except in certain unusual circumstances.

The procedure herein described consists of comparing an unknown solution with a reference solution, the composition of which is readily varied. Since the composition of the reference solution is always known, the composition of the sample solution is determined when the reference solution compensates for all of the absorption of the sample. In carrying out determinations by this technique, the spectrophotometer is used to indicate the balance of absorption and not to measure the actual absorption.

In certain applications of this technique, the absorbancy is not entirely compensated. These applications include systems showing background absorption and solutions containing one or more components not known to be present at the start of the determination. In the succeeding section of this study, application of the procedure to practical problems will be presented.

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A VARIABLE REFERENCE TECHNIQUE FOR ANALYSIS BY ABSORPTION SPECTROPHOTOMETRY:

II. PRACTICAL APPLICATIONS

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The theory of variable reference spectrophotometry has been discussed in Part I. The purpose of Part II is to point out practical examples of the use of the procedure. Most of the examples represent actual problems encountered in this laboratory, but it is felt that they illustrate the advantages and limitations of the technique.

A practical procedure for using the variable reference technique is shown schematically in Figure 1 (Part I). Concentrated solutions of the standard materials are added to a relatively large volume of the solvent which is being continuously mixed and circulated through the reference cell in the spectrophotometer. This might be considered as a "titration" of the unknown solution with the standard solutions using the spectrophotometer as an "indicator."

In such a titration when a zero absorbancy difference curve is obtained, the concentration of each component in the reference solution will be the same as in the unknown. In actual practice, the best zero difference curve will frequently lie between two of the absorbancy difference curves obtained in the course of the analysis. The concentration is actually determined, therefore, as "not less than—and not more than—." The results of analyses recorded in this paper are calculated on the basis of the concentration of the reference solution for the curve which is most nearly a zero curve or the average of the two curves which lie above and below the true zero curve.

APPARATUS

Circulating System

There is nothing novel about the circulation of a solution through the cell in the spectrophotometer. Such systems have been used for some time for continuous measurement of the changes in absorbancy occurring in a system. For the work reported here it was found most convenient to use a small centrifugal pump that could be immersed in the vessel, usually a large beaker, containing the reference solvent. Since the volume of solution in the cell and connecting tubing can be kept quite small (the actual volume in the two experimental assemblies used for most of this work was 10–25 ml), a flow rate of a few hundred ml per minute is adequate to give a rapid response to concentration changes in the reference solution. An auxiliary stirrer may be used if needed to obtain rapid mixing of the solution in the main container.

Two pumps that have been used successfully are a small brass pump, available commercially[1] and the all-glass pump shown in Figure 1a.[2] The latter can be constructed easily by any glass blower of moderate skill. This pump is not very efficient but is adequate for circulation of the reference solution through the reference cell. A well constructed pump of this size and design will operate against a head of approximately 12

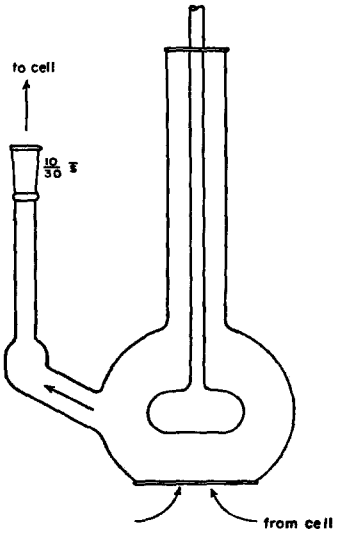

FIG. 1A.—All glass circulatory pump. (This unit is immersed in a large beaker containing the solvent.)

inches and will give a flow rate of several hundred ml. per minute at zero head. For work in the spectral range from 220–300 mμ it was necessary to use an all-glass pump and, as nearly as possible, all-glass connecting lines to avoid contamination of the reference solution.

The major mechanical difficulty encountered in the application of the procedure has been unsteadiness in the absorbancy reading while the pump is in operation. This unsteadiness is due almost entirely to air bubbles flowing through the cell. For this reason it was found advantageous to force the solution upward through the reference cell, and to turn off the pump to allow the air bubbles to escape from the cell before the difference curve is drawn.

The solvents used in the examples to be given were water and alcohol. Chloroform and hydrocarbon-type solvents have been used on occasion.

[1] Production Specialities, Inc., 296 Elm Street, New Haven, Connecticut.
[2] After the work given in this report was completed, the all-glass pump shown in Figure 1b was constructed. This pump has worked satisfactorily using 200–500 ml of solvent for the reference solution.

Cells

The most important factor insofar as the cells are concerned is that the cell length of the sample and reference cells be exactly the same. The easiest way to check this point is to run a "difference" curve with the same solution in both cells. For this test the absorbancy of the solution used should be as high as can be accurately measured in the spectrophotometer.

The cells used in this work were of the cylindrical type with the inlet

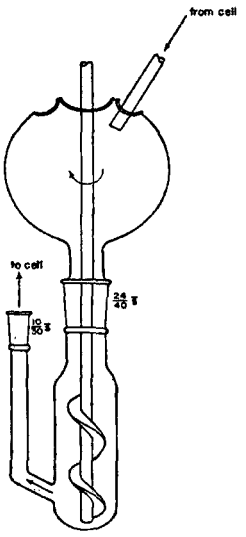

FIG. 1B.—All glass circulatory pump (for 200–500 ml of solvent).

and outlet tubes at 180° to each other. Quartz cells were used for the ultraviolet measurements and quartz or pyrex cells for visual measurements.

Spectrophotometers

Three spectrophotometers have been used in the experimental work reported here. The principles on which each of these spectrophotometers operate have been discussed elsewhere; however, the advantages and disadvantages of each for variable reference spectrophotometry will be discussed briefly.

The General Electric spectrophotometer has been used for a major portion of the experimental work in the 400–750 mμ range. This is a twin-beam, null indicating spectrophotometer and in principle is ideally suited for measuring the difference between two samples. Its major disadvantage is that it has a fixed slit width; therefore, the light passing through the

solution cannot readily be increased to obtain a measurable signal in the detector when solutions of high absorbance are to be compared. If solutions with absorbancies above 1 are placed in both the sample and reference beam the instrument is very sluggish, if not completely inoperative. The fixed slit is an advantage, however, in qualitative analysis since, when a fixed slit is used, the difference curve does not vary with the concentration of the reference solution.

Another minor disadvantage of the General Electric spectrophotometer is that the absorbancy scale does not go very far above zero; the instrument cannot draw an inverted curve. This can be partially overcome by "adjusting" the balance point to correspond to a high value on the absorbancy cam. The absorbancy values obtained when this is done are not accurate, but this is of no consequence when using the variable reference technique. When this instrument was used, the unknown solution was placed in the sample beam and the variable solution in the reference beam.

The Cary recording spectrophotometer has been used for most of the ultraviolet determinations made by the proposed technique. This is a twin-beam instrument which measures the ratio of the intensities of the light that reaches the sample and reference phototubes. In this instrument the slit width is automatically adjusted to give a uniform signal to the reference phototube. Since the slit can be opened up to offset the light absorbed by the solutions, solutions of relatively high concentration can be compared accurately on this instrument. When the absorbancy of the solution in the reference beam is changed the slit width will also change; the difference curve for two solutions may depend on concentration of the solutions as well as the difference in concentrations. This does not interfere in quantitative analysis by the variable reference technique although it may complicate the interpretation of the difference curves in qualitative analysis.

In practice the slit width, when using this instrument, will depend on the absorbancy of the solution in the reference beam. For this reason, it is preferable to place the unknown solution in the reference beam and the variable solution in the sample beam. When this is done interpretation of the difference curves is facilitated since the slit width at any wave length remains the same throughout the analysis. This instrument will draw an "inverted curve" up to an absorbancy of about 0.6.

A few determinations were carried out on the Beckman Model DU spectrophotometer. Since this is a single beam instrument provision must be made for placing first the unknown and then the reference solution in the light path. This requires a fairly flexible connection between the reference cell and the pump. Rubber tubing is satisfactory when only the spectral range from 300–750 mμ is of interest. No really satisfactory flexible connection was found that did not "bleed" materials which interfered in the 220–250 mμ region.

When the Beckman spectrophotometer is used it appears preferable to make the 100 per cent adjustments with the unknown solution in the beam in order to keep the same slit width and sensitivity settings at each wave length throughout the determination. Since the transmission scale on the instrument reads to only 110 per cent, the 100 per cent adjustment was made with the transmission set at 50 per cent and the switch at position 1; the transmission scale in thus compressed but "inverted" absorbancy differences can be measured. This adjustment also permits the comparison of solutions of greater concentration than can be compared when the 100 per cent adjustment is made in the usual manner.

The variable reference technique with the reference solution circulating through the cell has been used successfully on the Beckman in the visual range. In experiments using the ultraviolet range of this instrument a modified technique was employed in which the unknown and reference solutions were compared after each addition of standard to the reference solution. The regular cells for the instrument were used. Satisfactory results were obtained.

Although satisfactory results can be obtained in many instances, the variable reference technique is not readily adaptable to single beam or non-recording instruments. The time required to make determinations such as those described under "Determinations in Presence of 'Background' Absorption" would probably be prohibitive.

EXPERIMENTAL RESULTS

The examples given were analyzed as unknowns by one of the authors (L. S. Harrow). Most of the unknowns were prepared by dilution of the standard solutions used in the determination; thus, the error in the results is essentially that inherent in the procedure itself and does not include to any great extent the errors due to sampling and sample preparation. The curves shown are tracings of the curves as drawn in the course of the analysis by the recording instrument used. Usually the total volume of the reference solution was 800–1000 ml and the standard solution, which ordinarily contained 1–2 mg per ml of the standard, was added from an ordinary 10 ml burette.

Fluorescein Colors

The absorption curves of Fluorescein (D&C Yellow No. 7), Dibromofluorescein (D&C Orange No. 5), and Tetrabromofluorescein (D&C Red No. 21) are shown in Figure 2. These colors, and all of the other members of the group, give very similar curves which differ primarily in the location of the absorption peak. As noted in Part I of this report, at certain wave lengths the absorbancy ratios are mathematically favorable for resolution of mixtures of these colors. In the neighborhood of any wave lengths that could be used in an analysis of the mixtures by the method

employing simultaneous equations, the absorbancies are changing very rapidly. We have repeatedly tried to analyze mixtures of these colors by procedures involving calculation by simultaneous equations, using various techniques to increase the precision of the absorbancy measurements. The results so obtained have not been satisfactory. Examples of the results obtained by applying the variable reference technique to mixtures of fluorescein colors are shown in Figures 3–6.

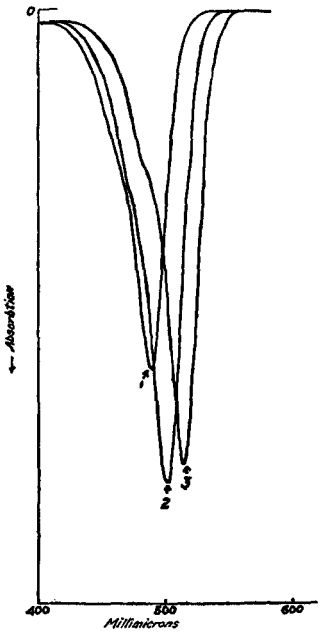

FIG. 2.—Absorption curves of fluorescein colors. Curve 1—Fluorescein, ca 2.5 mg/liter in dilute NH_4OH. Curve 2—Dibromofluorescein, ca 5 mg/liter in dilute NH_4OH. Curve 3—Tetrabromofluorescein, ca 5 mg/liter in dilute NH_4OH.

Curve 1 of Figure 3 shows the absorption curve of a mixture of fluorescein colors. Comparison of this curve with the curves shown in Figure 2 indicates that the mixture consists primarily of D&C Orange No. 5 and D&C Red No. 21 with a total concentration of about 5 mg per liter. At this concentration the absorbancy of D&C Orange No. 5 at 540 mμ is negligible. With the wave length dial set at about 540 mμ the standard D&C Red No. 21 solution was added to the reference solution until the absorbancy difference was quite low. The absorbancy difference curve was then that shown as Curve 2. From inspection of this curve, it can be concluded that the absorbancy difference must be due primarily to D&C Orange No. 5. The wave length dial was then set at about 440 mμ and

standard D&C Orange No. 5 solution added to the reference solution until the absorbancy difference at this wave length was quite low. The difference curve was now that shown as Curve 3. This curve indicates that the reference solution contains slightly less color than the unknown. (It can be inferred from Curve 3 that more D&C Red No. 21 than D&C Orange No. 5 will be required to give a zero difference curve, although this is

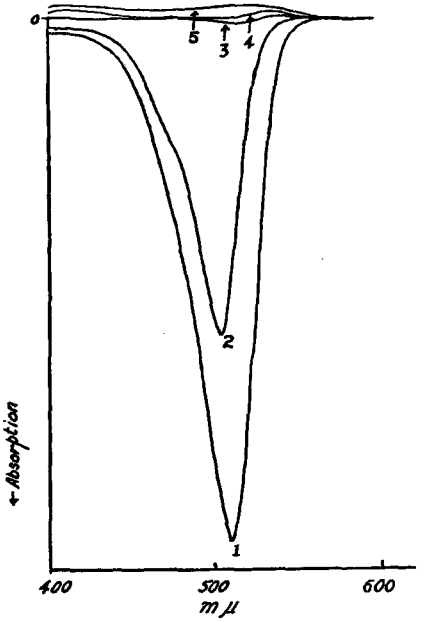

FIG. 3.—*Analysis of a mixture of fluorescein colors*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	D&C ORANGE NO. 5	D&C RED NO. 21	VOLUME
	mg	*mg*	*ml*
(1)	0.00	0.00	995
(2)	0.00	2.46	
(3)	2.49	2.46	
(4)	2.49	2.52	
(5)	2.52	2.52	1000
Calc. Comp. of Unknown	2.5 mg/l	2.5 mg/l	
Actual Comp. of Unknown	2.5 mg/l	2.5 mg/l	

admittedly somewhat of a guess.) Curves 4 and 5 were drawn after further addition of small amounts of D&C Red No. 21 and D&C Orange No. 5, respectively, to the reference solution. Curve 4 indicates that the reference contains more D&C Red No. 21 than the standard; Curve 5 shows an excess of D&C Orange No. 5 in addition to an excess of D&C Red No. 21. The true zero difference curve will lie somewhere between Curves 3 and 5. This titration indicates that the unknown contains 2.46–2.52 mg per liter of D&C Red No. 21 and 2.49–2.52 mg per liter of D&C Orange No. 5. The actual amounts present were 2.50 mg of each.

Curve 1 of Figure 4, obtained from another mixture of fluorescein

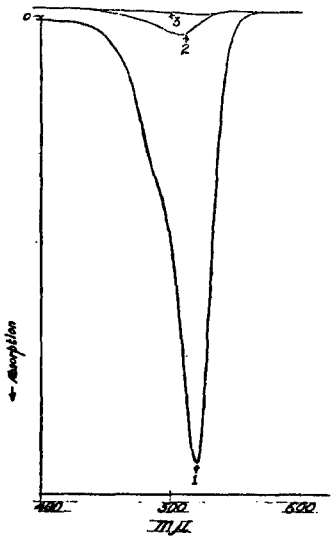

FIG. 4.—*Analysis of a mixture of fluorescein colors*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	D&C ORANGE NO. 5	D&C RED NO. 21	VOLUME
	mg	*mg*	*ml*
(1)	0.0	0.0	995
(2)	0.0	5.00	
(3)	0.26	5.00	1000
Calc. Comp. of Unknown	0.26 mg/l	5.0 mg/l	
Actual Comp. of Unknown	0.25 mg/l	5.0 mg/l	

colors, indicates that the unknown is chiefly, if not entirely, D&C Red No. 21. In the initial titration, therefore, practically all of the absorption at 540 mμ was cancelled by the addition of the standard solution of D&C Red No. 21. The absorbancy difference curve was then found to be Curve 2. This curve is not that of D&C Red No. 21 but appears to be that of D&C Orange No. 5. The absorption at 490 mμ was, therefore, cancelled by the addition of D&C Orange No. 5, to the reference solution. The absorbancy difference curve was then that shown by Curve 3. Curve 3 indicates that the unknown and reference have essentially the same composition. The reference solution for Curve 3 contained 5.00 mg per liter of D&C Red No. 21 and 0.26 mg per liter of D&C Orange No. 5. The actual composition of the unknown was 5.0 mg of D&C Red No. 21 and 0.25 mg of D&C Orange No. 5 per liter.

Curve 1 of Figure 5a indicates that the major component of the third unknown solution examined is D&C Orange No. 5. (Careful study of the curve will disclose the probable presence of D&C Yellow No. 7 and D&C Red No. 21.) After addition of increasing amounts of D&C Orange No. 5 to the reference solution, Curves 2, 3, and 4 were obtained. Curve 4 clearly indicates the presence of both D&C Yellow No. 7 and D&C Red No. 21. After addition of D&C Red No. 21 to the reference solution, Curve 5 was obtained. When sufficient D&C Red No. 21 was added to cancel the absorbancy difference at 540 mμ, Curve 6 was obtained. The reference solution for Curve 6 obviously contains more D&C Orange No. 5 than does the solution of the unknown. More solvent was, therefore, added to the reference solution and the new difference Curve 7 (Figure 5b) obtained. Curve 8 was obtained after a small additional amount of D&C Red No. 21 was added to the reference solution; Curve 8 appears to be essentially the curve of D&C Yellow No. 7. D&C Yellow No. 7 was then added to the reference solution. The final curve, Curve 10, corresponds to a concentration in the reference solution of 5 mg of D&C Orange No. 5, 1.0 mg of D&C Red No. 21, and 1.02 mg of D&C Yellow No. 7 per liter of solution; the concentrations in the unknown were 5.0, 1.0, and 1.0 mg per liter, respectively.

The third example illustrates two practical considerations that are frequently involved in analyses by variable reference spectrophotometry. It will be noted that in this case it was decided to add D&C Orange No. 5 to the reference solution before attempting to cancel out the D&C Red No. 21. This is usually desirable in case one component comprises the major portion of the mixture. It is easier to decide what components are present from the difference curve, after most of the absorbancy difference due to the major component is cancelled, than from the original curve. Also a more accurate appraisal of the amount of the minor components to be added to the reference can be made after cancelling most of the major component.

At one point in the above determination the concentration of D&C Orange No. 5 in the reference solution was higher than that in the un-

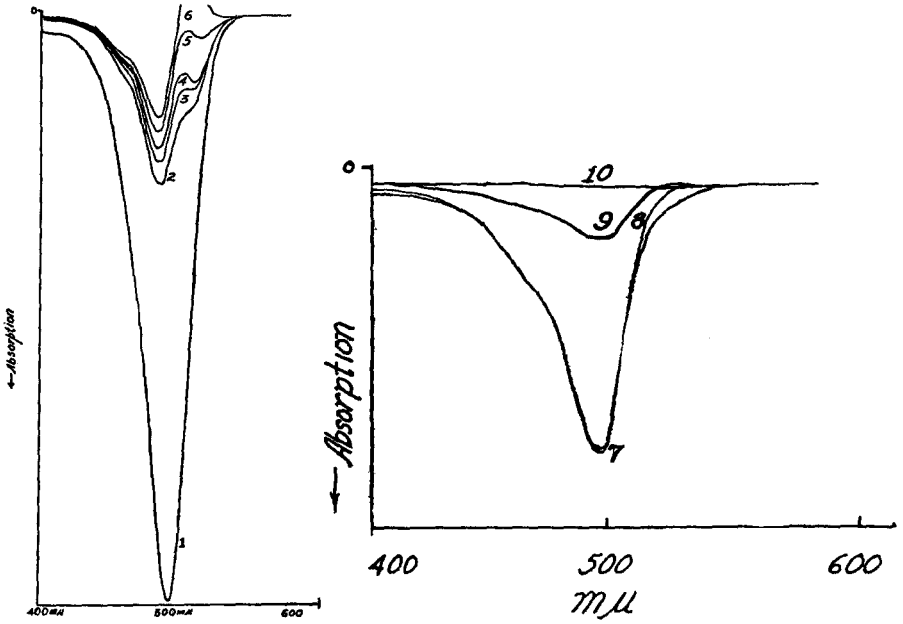

FIG. 5A and 5B.—*Analysis of a mixture of fluorescein colors*

CURVE	COMPOSITION OF REFERENCE SOLUTION			
	D&C ORANGE NO. 5	D&C YELLOW NO. 7	D&C RED NO. 21	VOLUME
	mg	*mg*	*mg*	*ml*
(1)	0.00	0.00	0.00	900
(2)	4.48	0.00	0.00	
(3)	4.80	0.00	0.00	
(4)	5.00	0.00	0.00	
(5)	5.00	0.00	0.50	
(6)	5.00	0.00	0.90	1000
(7)	5.00	0.00	0.90	
(8)	5.00	0.00	1.02	
(9)	5.00	0.76	1.02	
(10)	5.00	1.00	1.02	1000
Calc. Comp. of Unknown	5.00 mg/l	1.00 mg/l	1.02 mg/l	
Actual Comp. of Unknown	5.00 mg/l	1.00 mg/l	1.00 mg/l	

known. It is easy, of course, to reduce the total concentration by adding more solvent to the reference solution. Since the dilution required will depend on the relative excess of any component that has been over-titrated, less dilution will be required if the overage is in the major component only.

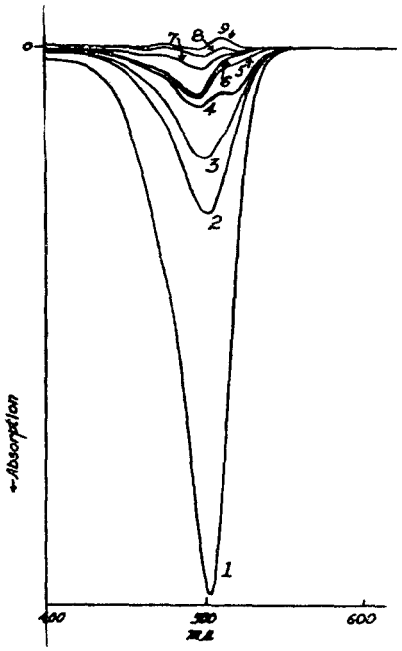

FIG. 6.—*Analysis of commercial sample of D&C Orange No. 5*

CURVE	COMPOSITION OF REFERENCE SOLUTION			VOLUME
	D&C ORANGE NO. 5	D&C RED NO. 21	D&C YELLOW NO. 7	
	mg	*mg*	*mg*	*ml*
(1)	0.00	0.00	0.00	1000
(2)	3.00	0.00	0.00	
(3)	3.50	0.00	0.00	
(4)	3.95	0.00	0.00	
(5)	3.95	0.29	0.00	
(6)	3.95	0.37	0.00	
(7)	3.95	0.37	0.14	
(8)	4.02	0.40	0.14	
(9)	4.08	0.40	0.16	1005
Calc. Comp. of Unknown	82%	8.0%	3.2%	

TABLE 1.—*Analysis of mixtures of fluorescein colors*

SAMPLE	D&C YELLOW NO. 7		D&C ORANGE NO. 5		D&C RED NO. 21		D&C RED NO. 27		FD&C RED NO. 3		D&C ORANGE NO. 10	
	FOUND	ADDED	FOUND	ADDED	FOUND	ADDED	FOUND	ADDED	FOUND	ADDED	FOUND	ADDED
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
(1)	1.00	1.00	5.00	5.00	1.02	1.00	—	—	—	—	—	—
(2)	—	—	0.26	0.25	5.00	5.00	—	—	—	—	—	—
(3)	—	—	2.5	2.5	2.5	2.5	—	—	—	—	—	—
(4)	—	—	—	—	—	—	—	—	5.0	5.0	0.23	0.25
(5)	—	—	2.5	2.5	2.4	2.5	—	—	—	—	—	—
(6)	—	—	0.5	0.5	6.0	6.0	—	—	—	—	—	—
(7)	2.5	2.5	2.5	2.5	—	—	—	—	—	—	—	—
(8)	0.25	0.25	5.0	5.0	—	—	—	—	—	—	—	—
(9)	0.17	0.15	—	—	—	—	—	—	7.7	7.6	0.72	0.72
(10)	0.49	0.50	—	—	—	—	—	—	4.0	4.0	0.51	0.50
(11)	0.25	0.25	0.5	0.5	0.9	1.0	4.9	5.0	—	—	—	—

Curve 1 of Figure 6 is that of a commercial sample of D&C Orange No. 5. The difference curves obtained when this sample was analyzed by the technique used for mixtures of D&C Orange No. 5 and D&C Red No. 21 and D&C Yellow No. 7 are shown as Curves 2–9. It is obvious from Curve 4 that the sample contains two other compounds in addition to D&C Orange No. 5. Curves 6, 7, 8, and 9 show that the absorbancy difference can be reduced to approximately zero at all wave lengths by the addition

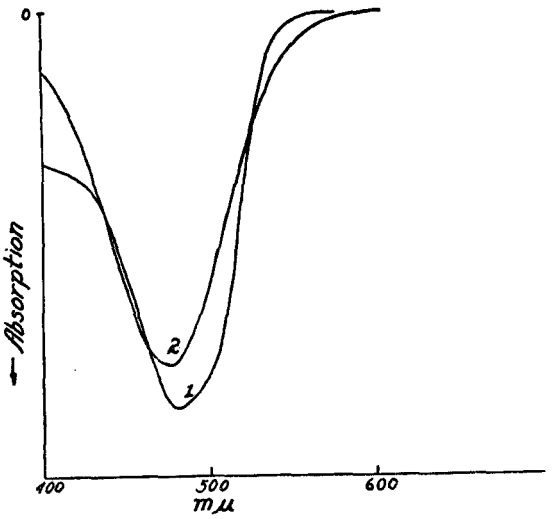

FIG. 7.—Absorbancy curves of FD&C Yellow No. 6 and FD&C Orange No. 1 in 0.02 *N* $NH_4C_2H_3O_2$ solution. Curve 1—FD&C Yellow No. 6 (18.5 mg/liter). Curve 2—FD&C Orange No. 1 (10.0 mg/liter).

of D&C Yellow No. 7 and D&C Red No. 21. It is highly probable, therefore, that these are the other two components present. (They are, of course the most likely impurities in commercial samples of this color.)

The results obtained in the analysis of a number of other mixtures of fluorescein colors are shown in Table 1.

Other Mixtures

Figures 7, 8, and 9 illustrate the application of the variable reference procedure to the determination of a two component mixture where the absorption peaks of the two compounds are at very nearly the same wave length but the shapes of the two curves are different. In each case, the FD&C Orange No. 1 was titrated first, using the absorbancy difference at 550 mμ as a guide, after which the FD&C Yellow No. 6 was titrated at 420 mμ. It is apparent that the curves marked 2 in both Figures

8 and 9 are essentially the curve of FD&C Yellow No. 6. Curve 3 of Figure 8 indicates that the reference solution is short in both components. Curve 4 of Figure 8 and Curve 3 of Figure 9 indicate that the reference is still short in one, or both, components but it is difficult to say which

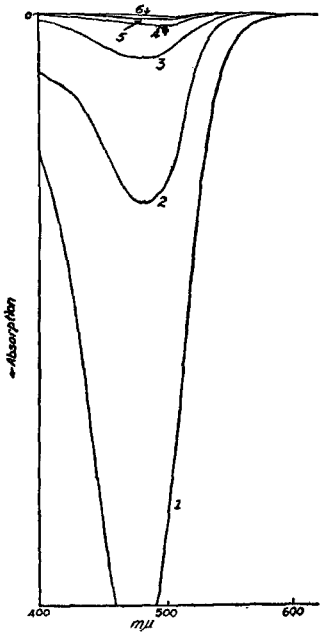

FIG. 8.—*Analysis of coal-tar color mixture*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	D&C ORANGE NO. 1	D&C YELLOW NO. 6	VOLUME
	mg	*mg*	*ml*
(1)	0.00	0.00	985
(2)	9.12	0.00	
(3)	9.12	4.92	
(4)	9.84	4.92	
(5)	9.94	4.92	
(6)	9.94	5.05	1000
Calc. Comp. of Unknown	9.94 mg/l	5.05 mg/l	
Actual Comp. of Unknown	10.00 mg/l	5.00 mg/l	

component should be added to the reference solution. This situation usually occurs when the two components have essentially the same absorption peak; in such cases, the curve due to a small amount of one component will not be distinguishable from the curve due to a small amount of the other. The extent of the uncertainty will depend, of course, on how similar the two curves are in shape.

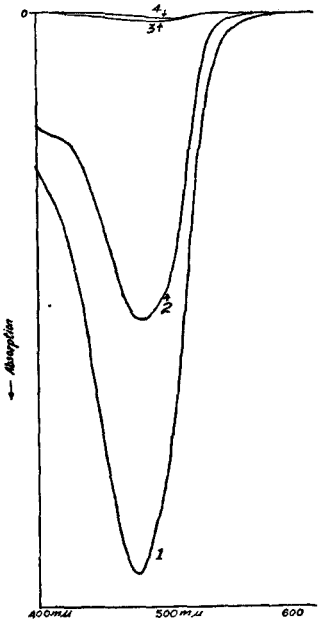

FIG. 9.—*Analysis of coal-tar color mixture*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	FD&C ORANGE NO. 1	FD&C YELLOW NO. 6	VOLUME
	mg	*mg*	*ml*
(1)	0.00	0.00	985
(2)	4.90	0.00	
(3)	4.90	9.98	
(4)	5.01	9.98	1000
Calc. Comp. of Unknown	5.01 mg/l	9.98 mg/l	
Actual Comp. of Unknown	5.00 mg/l	10.00 mg/l	

Figure 10 shows the curves of standard solutions of a yellow dye, C.I. No. 365, and a blue dye, C.I. No. 518. Curve 1, Figure 10, is the curve of a mixture of these two compounds. Since this curve obviously could not be the sum of the absorbancies of any combination of the two

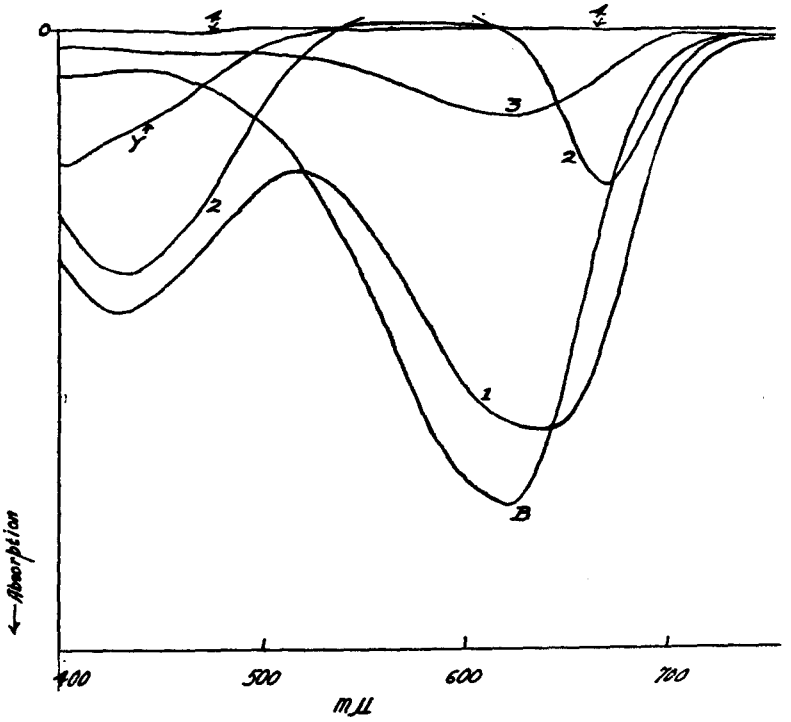

FIG. 10.—*Titration of two dyes which interact in solution*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	BLUE	YELLOW	VOLUME
	mg	*mg*	*ml*
(1)	0.00	0.00	840
(2)	6.80	0.00	
(3)	6.80	8.30	
(4)	8.40	8.30	
Calc. Comp. of Unknown	10.0	9.9	
Actual Comp. of Unknown	10.0	10.0	

components, the two components must interact in solution. When the absorbancy of the unknown at about 620 mμ was balanced by the addition of the blue dye to the reference solution, Curve 2 was obtained (the instrument used will not record the over portion of the curve). Next the absorbancy difference at about 430 mμ was reduced to a low value by the addition of the yellow dye to the solution; this gave Curve 3 for the absorbancy difference. It will be noted that this curve shows a substantial absorbancy difference at 620 mμ. Since there was no absorbancy difference at 620 mμ at the time Curve 2 was drawn, this indicates that part of the blue dye in the reference solution has reacted in some manner with the yellow dye. After the absorbancy difference now indicated at 620 mμ was reduced to zero by the addition of the blue dye, the absorbancy difference was as shown by Curve 4. At this point the concentration of yellow dye in the reference solution was 9.9 mg per liter and that of the blue dye 10.0 mg per liter. The actual composition of the unknown was 10 mg per liter of each dye. It was partly a matter of luck that the zero absorbancy difference curve was reached so quickly in this determination. In the analysis of another mixture of these two dyes, six difference curves were drawn before a good zero difference curve was obtained. The result of that determination indicated that the unknown contained 12.5 mg per liter of the blue dye and 17.3 mg per liter of the yellow dye. In this case, the actual amounts present were 12.5 and 17.0 mg per liter, respectively.

Analysis of Benzene-Toluene Mixtures

Figure 11 shows the absorption curves of solutions of benzene and toluene, in 95 per cent alcohol, as drawn by the Cary Recording spectrophotometer. Since the absorption of toluene at 268 mμ is very much greater than that of benzene, analysis of mixtures of these two compounds by the method of simultaneous equations should be quite accurate. The peaks for the two compounds are very sharp, however, so considerable care must be taken to have exactly the same wave length and slit width settings when determining the absorbancies. When the variable reference technique is used, on the other hand, the wave length and slit width setting is not critical. Any appreciable absorbancy difference in the region 265–270 mμ indicates that the reference solution contains less toluene than the unknown. If the absorbancy difference at about 254 mμ, compared to that at 268 mμ, is high the reference solution contains less benzene than the unknown.

Figure 12 shows the difference curves obtained in the analysis of a benzene-toluene mixture. At the time Curve 4 was drawn the reference obviously contained essentially the same amount of toluene as the unknown. When Curve 5 was drawn, the reference solution contained very slightly, but definitely, less benzene than the unknown. Curve 6 indicates a slight excess of toluene in the reference solution.

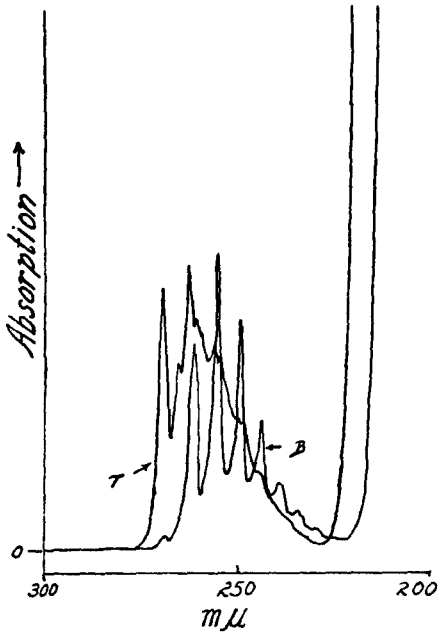

FIG. 11.—Standard curves for benzene and toluene—250 mg/liter in 95 per cent alcohol.

Table 2 gives the results of several analyses of benzene-toluene mixtures by the variable reference technique.

TABLE 2.—*Analyses of benzene-toluene mixtures*

SAMPLE	BENZENE		TOLUENE	
	FOUND	ADDED	FOUND	ADDED
	mg	*mg*	*mg*	*mg*
(1)	29.5	29.3	183.0	187.0
(2)	198.0	197.0	55.0	54.0
(3)	98.5	100.0	100.5	100.0
(4)	195.0	200.0	199.0	200.0

Analysis of a Mixture of Ortho- and Para-Aminoethylbenzene

Figure 13 shows the ultraviolet absorption curves of solutions of ortho-aminoethylbenzene and para-aminoethylbenzene in dilute aqueous alkali. Although the two curves are quite similar, the absorbancy ratios in the 240–250 mμ and 300–310 mμ regions are roughly 2/1 and 1/2, respectively. Figure 14, Curve 1, shows the absorption curve, at the same concentration of the material obtained by nitration of ethyl benzene followed by reduc-

tion of the nitration product. It appears to be, as would be expected, a mixture of the ortho- and para-amino compounds. When this mixture was analyzed by the variable reference procedure the series of curves

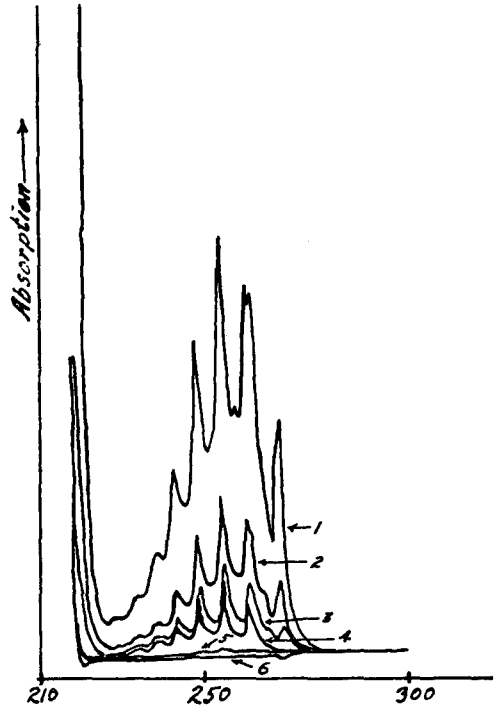

FIG. 12.—*Analysis of benzene and toluene mixture*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	BENZENE	TOLUENE	VOLUME
	mg	*mg*	*ml*
(1)	0.00	0.00	900
(2)	99.5	124.8	
(3)	125.0	161.8	
(4)	125.0	181.0	
(5)	174.8	181.0	
(6)	178.8	184.8	915
Calc. Comp. of Unknown	195.0 mg/l	199.0 mg/l	
Actual Comp. of Unknown	200.0 mg/l	200.0 mg/l	

shown in Figure 14 were obtained. The zero absorbancy curve would appear to lie between Curves 6 and 7. This corresponds to a mixture containing 49 per cent of the ortho compound and 51 per cent of the para compound. Curve 8 shows the curve obtained when the unknown was compared to a 45–55 ortho-para mixture (in the regular cells for this spectrophotometer); this curve does not appear to be as good a zero absorbancy curve as does the curve for the 49–51 ratio obtained in the

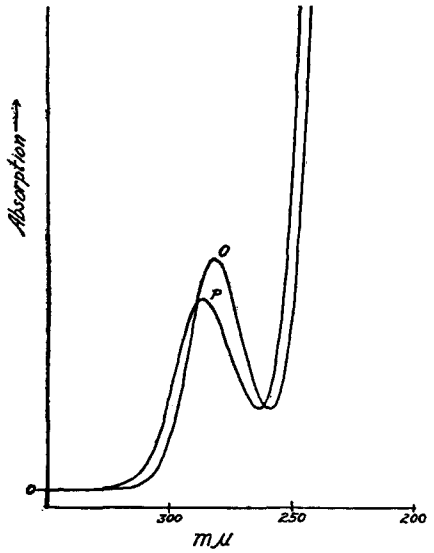

FIG. 13.—Standard curves for ortho- and para-aminoethyl benzene—50 mg/liter in 0.1 *N* NaOH.

titration. Analysis of the mixture by infrared spectrophotometry indicated that the unknown was a 48–52 ortho-para mixture.

DETERMINATIONS IN THE PRESENCE OF "BACKGROUND" ABSORPTION

Figure 15 shows the curves obtained in the determination of a coal-tar color (FD&C Yellow No. 6) in a mixture containing the color and caramel. If it is assumed that the background is a smooth curve, it is apparent that Curve 5 is the most likely curve for the background. This curve corresponds to a concentration of 8.9 mg per liter of dye in the reference solution; the actual concentration of the unknown was 9.0 mg per liter. The background absorption in this curve, at the wave length of maximum absorption due to the dye, amounts to about 15 per cent of the absorption due to the dye present. If the amount of dye present were calculated by

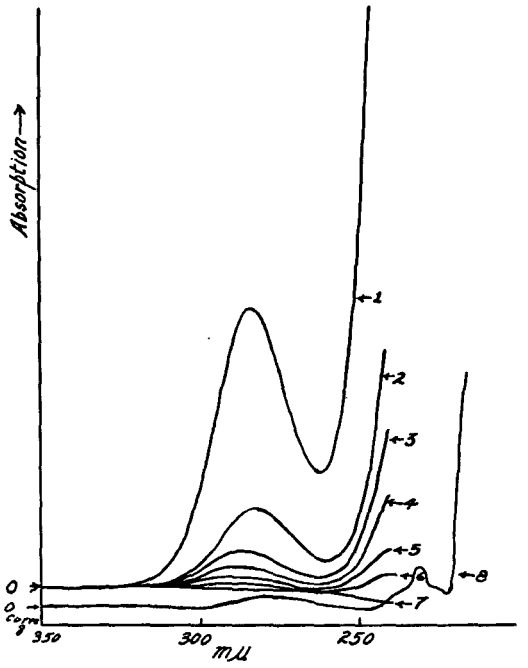

FIG. 14.—*Analysis of ortho-, para-aminoethylbenzene mixture*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	ORTHO	PARA	VOLUME
	mg	*mg*	*ml*
(1)	0.00	0.00	900
(2)	15.05	19.10	
(3)	22.00	19.10	
(4)	23.11	21.10	
(5)	23.11	23.12	
(6)	23.75	23.75	
(7)	23.75	25.00	948
Calc. Comp. of Unknown	25.00 mg/l	25.84 mg/l	
Calc. % Comp. of Unknown	49.20%	50.80%	

Curve 8 is the difference curve for the unknown solution vs. a solution containing 45 per cent o-ethylaniline and 55 per cent p-ethylaniline.

the usual method, assuming a linear background in the region, 420 mμ to 580 mμ, the result would be in error by about 5 per cent of the amount of dye present.

Figure 16 shows the curves obtained in the titration of an orange dye (FD&C Orange No. 1) in the presence of a background. Examination of

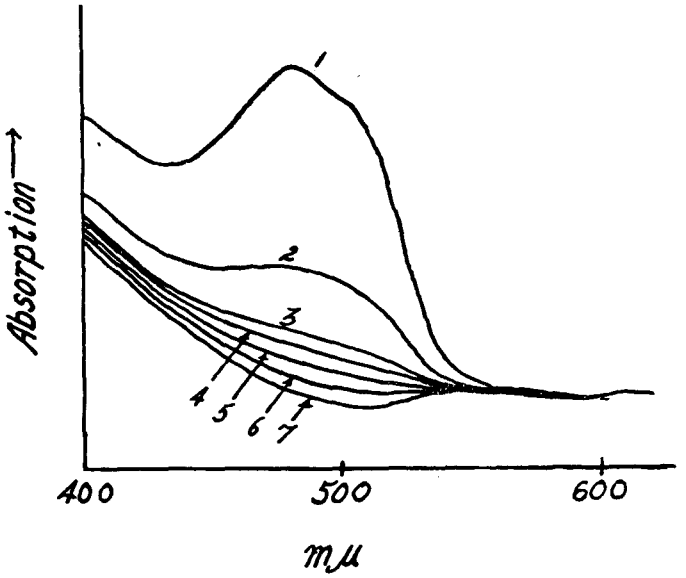

FIG. 15.—*Determination of a coal-tar color in the presence of "background"*

CURVE	COMPOSITION OF REFERENCE SOLUTION	
	FD&C YELLOW NO. 6	VOLUME
	mg	*ml*
(1)	0.0	1000
(2)	6.02	
(3)	8.00	
(4)	8.50	
(5)	9.00	
(6)	9.50	
(7)	10.00	1010
Calc. Comp. of Unknown	8.9 mg/l	
Actual Comp. of Unknown	9.0 mg/l	

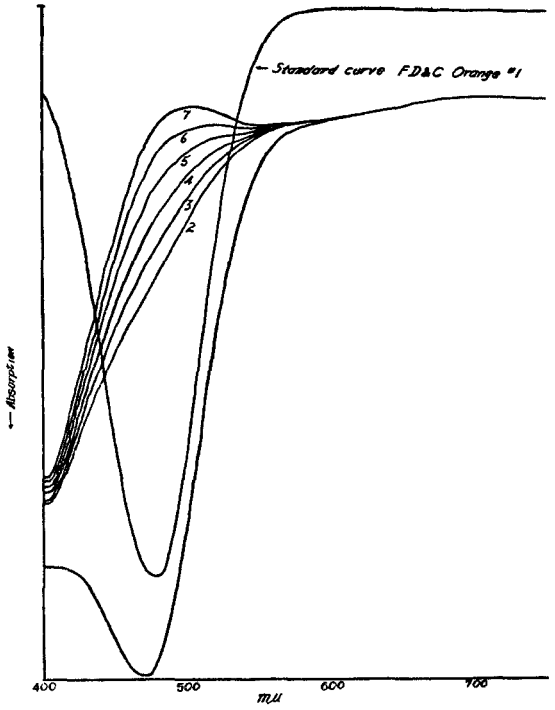

FIG. 16.—*Determination of coal-tar color in the presence of a "background"*

CURVE	COMPOSITION OF REFERENCE SOLUTION	
	FD&C ORANGE NO. 1	VOLUME
	mg	*ml*
(1)	0.00	900
(2)	6.00	
(3)	6.50	
(4)	7.00	
(5)	7.50	
(6)	8.00	
(7)	8.50	909
Calc. Comp. of Unknown	8.4 mg/l	
Actual Comp. of Unknown	8.8 mg/l	

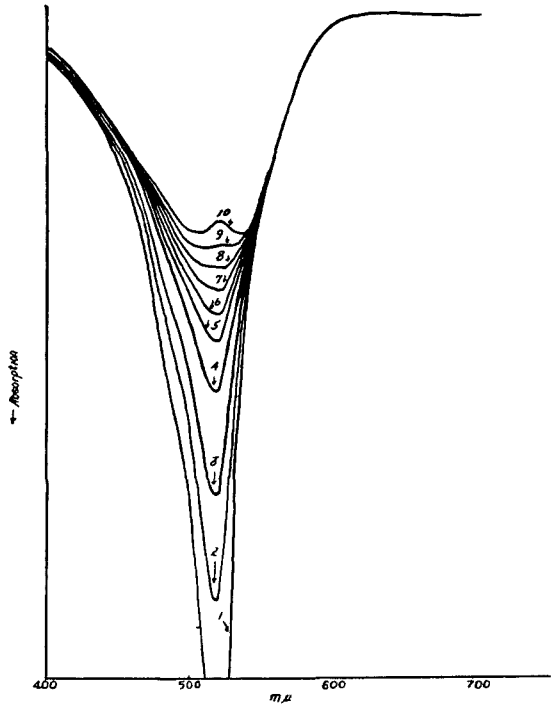

FIG. 17.—*Determination of a coal-tar color in the presence of a "background"*

CURVE	COMPOSITION OF REFERENCE SOLUTION	
	D&C RED NO. 21	VOLUME
	mg	*ml*
(1)	0.0	900
(2)	1.5	
(3)	2.5	
(4)	3.5	
(5)	4.0	
(6)	4.25	
(7)	4.50	
(8)	4.75	
(9)	5.00	
(10)	5.25	905
Calc. Comp. of Unknown	4.9 mg/l	
Actual Comp. of Unknown	5.0 mg/l	

the curve of the unknown (Curve 1) shows that it is highly unlikely that the background can be considered linear over any considerable portion of the spectrum. The difference Curves 2 through 7 were obtained after adding the indicated amounts of the color to the reference solution. If the background is assumed to give a smooth curve in the region of the peak absorption of the color, Curve 7 definitely indicates that the reference solutions contain more of the color than the unknown. The reference solution for Curves 2 and 3 apparently contained too little of the desired

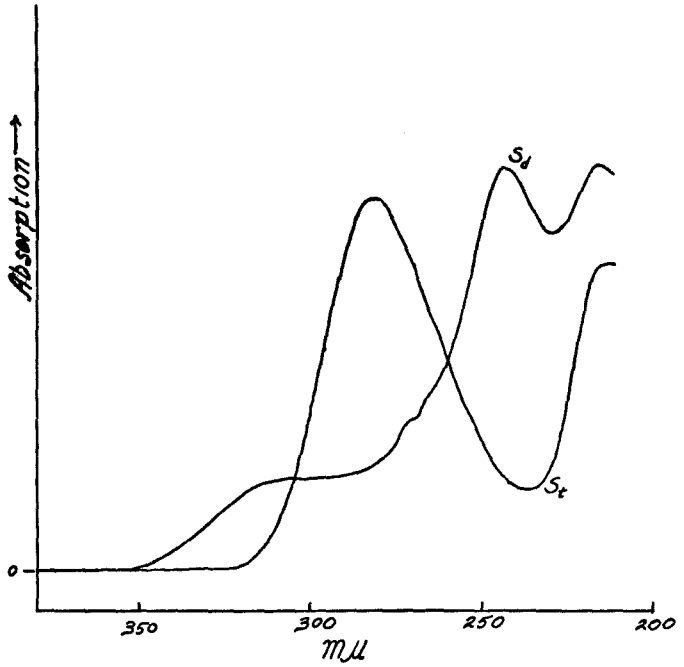

FIG. 18.—Standard curves for sulfadiazine and sulfathiazole—20 mg/liter in 0.05 *N* HCl.

component. The most likely "background" curve is, therefore, either Curve 4, 5, or 6. Close examination indicates that Curve 5 is probably the curve that least resembles the curve of the color or its inverted curve. This corresponds to a concentration of 8.3 mg per liter of the color in the unknown, actually the unknown contained 8.8 mg per liter. Curve 4 would correspond to 7.8 mg per liter and Curve 6 to 8.9 mg per liter of the desired constituent. The background absorption in this case is about 30 per cent of that due to the color alone.

Figure 17 shows the curves obtained in another determination in the

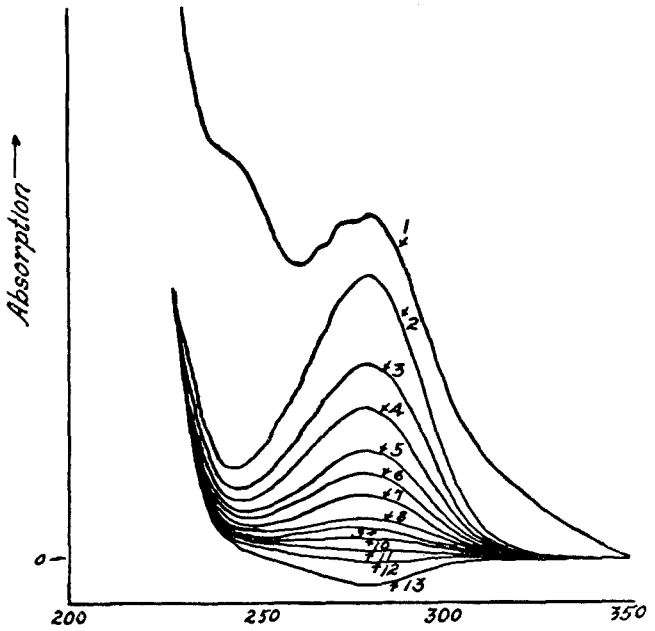

FIG. 19.—*Analysis of a sulfadiazine-sulfathiazol mixture in the presence of a "background"*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	SULFADIAZINE	SULFATHIAZOLE	VOLUME
	mg	*mg*	*ml*
(1)	0.00	0.00	900
(2)	6.30	0.00	
(3)	6.30	2.00	
(4)	6.30	3.00	
(5)	6.30	4.00	
(6)	6.30	4.50	
(7)	6.30	5.00	
(8)	6.30	5.50	
(9)	6.30	5.75	
(10)	6.30	6.00	
(11)	6.30	6.26	
(12)	6.30	6.50	
(13)	6.30	7.00	906
Calc. Comp. of Unknown	6.95 mg/l	6.86 mg/l	
Actual Comp. of Unknown	7.00 mg/l	7.00 mg/l	

presence of background. The red color to be determined has a sharp peak at 517 mμ with a slight shoulder at 480–490 mμ. Examination of the difference curves indicates that the most likely background curve is Curve 8. This corresponds to a concentration of 4.9 mg per liter; the actual concentration was 5.0 mg per liter. The only other curve that might be considered for the background is Curve 7, which would correspond to a concentration of 4.6 mg per liter. Curve 9, which would correspond to 5.2 mg per liter of the desired constituent, is definitely over titrated.

Figure 18 shows the absorbancy curves of solutions of sulfadiazine and sulfathiazole in 0.1 *N* HCl. Simple mixtures of these two drugs can be analyzed satisfactorily by the method of simultaneous equations from the absorbancies at 245 and 280 mμ. If, however, there is also background absorption present a logical correction for the background is difficult, if not impossible. Consider, for example, the curve of a mixture of these two drugs plus a background shown in Figure 19. It is obvious that there is considerable background in the 210–250 mμ range but just how much background is present at 245 mμ or 280 mμ is difficult to estimate. Since the background absorption at 350 mμ is obviously low, the absorbancy measurements might be made at 310 mμ and 280 mμ with greater confidence although the absorbancy ratios are not so favorable at these wave lengths.

This mixture was analyzed by the variable reference technique as follows: The absorbancy difference at 320 mμ was reduced to a low value by the addition of sulfadiazine to the reference solution. The residual absorbancy was then considered as that of a single component plus a background. The curves obtained in the course of the analysis are shown in Figure 19. The results indicate that the unknown solution contained 14.0 mg per liter of sulfadiazine and 13.8 mg per liter of sulfathiazole; the unknown actually contains 14 mg per liter of each component. The background absorption appears to be a typical end absorption curve (actually, the background was acetic acid).

In Figure 20 is shown the curve of another mixture of these two drugs. It is not possible to say with any certainty whether or not there is any background in this curve. The absorbancy difference curves, also shown in Figure 20, clearly show the presence of background absorption from 230–260 mμ. The absorbancy difference curves indicate that the most likely values for the desired components are 16.4 mg per liter of sulfadiazine and 15.5 mg per liter of sulfathiazole; the actual concentrations were 17.0 mg of sulfadiazine and 15 mg of sulfathiazole. If no background corrections were made the absorbancy values obtained at 245 and 280 mμ would be 5–10 per cent higher than the absorbancy due to the desired components only.

A variation of the technique used for one component in the presence

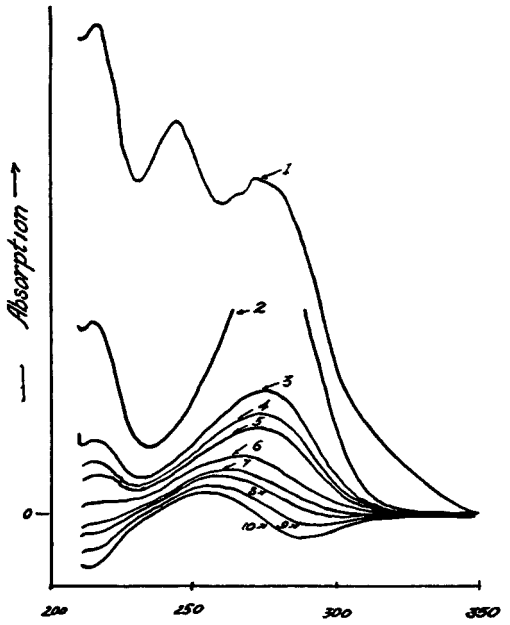

FIG. 20.—*Analysis of sulfadiazine-sulfadiazole mixture in the presence of a "background"*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	SULFADIAZINE	SULFATHIAZOLE	VOLUME
	mg	*mg*	*ml*
(1)	0.00	0.00	900
(2)	7.36	0.00	
(3)	7.36	4.30	
(4)	7.36	5.01	
(5)	7.36	5.50	
(6)	7.36	6.50	
(7)	7.50	7.00	
(8)	7.50	7.35	
(9)	7.50	7.85	
(10)	7.50	8.35	915
Calc. Comp. of Unknown	8.2 mg/l	7.7 mg/l	
Actual Comp. of Unknown	8.5 mg/l	7.5 mg/l	

of a background can frequently be employed in the analysis of mixtures where one component predominates. Suppose that we have two compounds whose absorption peaks are located in the same region of the

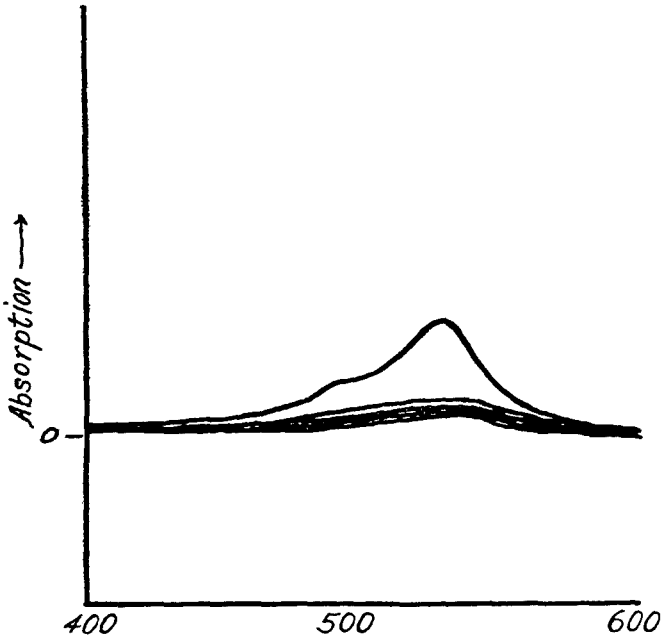

FIG. 21.—*Determination of a small amount of coal-tar color in the presence of a large amount of color having a similar absorption curve*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	FD&C RED NO. 2	FD&C RED NO. 3	VOLUME
	mg	*mg*	*ml*
(1)	81.0	0.0	900
(2)	81.0	0.72	
(3)	81.0	0.80	
(4)	81.0	0.90	
(5)	81.0	1.0	919
Calc. Comp. of Unknown	90.0 mg/l	0.88 mg/l	
Actual Comp. of Unknown	91.0 mg/l	0.85 mg/l	

spectrum and whose specific absorptivities are roughly the same. If one of the components is present in a low percentage, its contribution to the total absorption of the mixture will be small and the deviation of curve, when drawn in the usual manner, from that of the major component will be slight. If, however, we use a stronger solution of the unknown and approximately balance out the major component, the contribution of the minor component to the difference curve will be noticeable and the amount

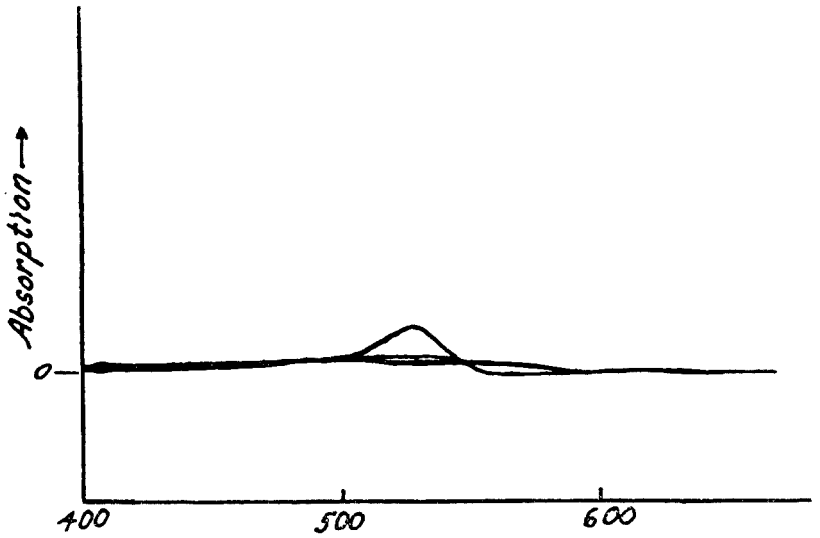

FIG. 22.—*Determination of a small amount of color in the presence of a large amount of color having a similar absorption curve*

CURVE	COMPOSITION OF REFERENCE SOLUTION		
	FD&C RED NO. 3	FD&C RED NO. 2	VOLUME
	mg	*mg*	*ml*
(1)	80.2	0.0	900
(2)	80.2	0.70	
(3)	80.2	0.80	
(4)	80.2	0.90	916
Calc. Comp. of Unknown	87.2 mg/l	0.9 mg/l	
Actual Comp. of Unknown	88.0 mg/l	0.88 mg/l	

of this component can be estimated by treating the residual curve as that of the minor component plus a background. Two examples of such determinations are shown in Figures 21 and 22. The apparent inconsistencies in the difference curves are caused by a reaction between the two colors which occurs at the concentrations used.

The lowest percentage of the minor component that this technique can determine is limited, of course, by the maximum concentration of the mixture which can be employed in the reference beam of the spectrophotometer.

SUMMARY

Practical methods of utilizing the variable reference technique in visual and ultraviolet spectrophotometry have been given. Several examples of the use of the procedure in the analysis of mixtures have been discussed.

REPORT ON VALUES OF THE CLERGET DIVISOR AND METHODS OF MULTIPLE POLARIZATION TO THE INTERNATIONAL COMMISSION FOR UNIFORM METHODS OF SUGAR ANALYSIS, TENTH SESSION, BRUSSELS, AUGUST 15–20, 1949 (Subject 14)

By F. W. ZERBAN (New York Sugar Trade Laboratory, New York, N.Y.)

The two previous reports by the referee and associate referees on the Values of the Clerget Divisor, presented at the Eighth and Ninth Sessions of the International Commission for Uniform Methods of Sugar Analysis, in Amsterdam, 1932, and in London, 1936, were combined and published as a supplement to Number 2 of Volume 2 of the *Archief voor de Suikerindustrie in Nederland en Nederlandsch-Indië* (1941). This journal ceased publication upon the invasion of Java by the Japanese. The present report prepared by the writer, as referee, in cooperation with the associate referees: J. Ancízar-Sordo, J. Dubourg, E. J. McDonald, W. Millar, V. Sázavský, W. M. Seaber, F. Tödt, and with S. J. Osborn of the United States National Committee, was rendered at the Tenth Session of the International Commission in Brussels, 1949, and has appeared only in abstract form in the official proceedings of this Session, published in the July 1950 number of the *International Sugar Journal.*

At the Ninth Session of the Commission this Referee repeated the recommendations made at the Eighth Session because little experimental work had been done in the interval between the two sessions. These recommendations were as follows:

(A) It is recommended that the principal inversion methods used in the various countries be studied and compared in each country, and that the conclusions arrived at in the referee's report be critically tested.

(B) It is recommended that the effect of ash on the Clerget Divisor be specially examined.

(C) It is further recommended that the temperature coefficients of the inversion constants be redetermined under various conditions, but that the polarization readings in sucrose determinations by inversion methods be made, as far as possible, at 20°C. in order to avoid temperature corrections altogether.

Since the last session of the Commission, World War II has interrupted not only international communications, but also nearly stopped non-military scientific investigations in most countries. As will be noted from the present report, only little work has been carried out on the subject of this report, and some of the publications which actually appeared have not been accessible to the referee. Fortunately, a few of the most

important of these have been made available in photostat form through the kind assistance of Dr. Sandera and Ing. Sazavsky, to whom the writer wishes to express his sincere thanks. If any publications having a bearing on the subject have been overlooked this is due entirely to the circumstances mentioned. Paar (1) has stated that the failure to make recommendations for changes in methods and constants at the last two sessions of the Commission has caused a widespread reluctance to take action even on those that are obviously obsolete. Thus, for instance, the Herzfeld value of the Clerget Divisor, 132.66, is still used in some places and reprinted in manuals of sugar analysis. In view of the criticism just mentioned, it appears advisable this time not to delay any longer, but to submit definite proposals for the adoption of certain methods and constants, even though they may require modification later on, as further experimental results become available. The referee therefore makes such recommendations at the end of the present report, for at least tentative adoption.

At the two previous sessions of the Commission, the subject of double polarization for the determination of sucrose, and that of the determination of sucrose and raffinose by multiple polarization, were treated separately. For the present session they have been combined into one, and both of them will be discussed in this report.

PART I

Determination of Sucrose by Double Polarization.

This part of the report is subdivided in the same manner as in the two previous reports by the writer (2).

Effect of the temperature of inversion.—Jackson and Gillis (3), in their classical investigation on the subject, had found that the temperature at which inversion with acid is carried out has no effect on the Clerget Divisor and that its value is always 133.25, as long as the temperature and time are chosen so that complete inversion is assured without destruction of invert sugar. The Schrefeld value 133.00 for inversion at 67–70° during 5 minutes was explained by destruction of invert sugar. But Spengler, Zablinsky, and Wolf (4) found the divisor 133.00–133.02 even when the solution was inverted at 60° for 5, 10, or 15 minutes, in which case, according to Jackson and Gillis, there is no destruction of invert sugar within 10 minutes. However, when the inversion was carried out at room temperature (22°) the divisor rose to 133.20, confirming the value of Schrefeld and of Zerban (133.18).

In a re-examination of the subject, Jackson and McDonald (5) observed that the divisor actually changes with the temperature of inversion, as shown in Table 1.

The lowering of the divisor with increase in temperature to 60° is according to these authors not due to gradual destruction of invert sugar,

TABLE 1.—*Variation in the negative constituent of the Clerget Divisor under different conditions of inversion*

TEMP. OF INVERSION	TIME OF INVERSION	ROTATION AT 20°C. ON NORMAL WEIGHT BASIS
°C.	*Minutes*	°S.
67–69	5.0	−33.00
67–69	2.0	−33.08
60	9.5	−33.18
49	38.0	−33.25
35	205.0	−33.25
25	1032.0	−33.29

but is explained by destruction of the fructofuranose formed initially upon the splitting of the sucrose molecule because at 60° the heating can be prolonged a full 3.5 minutes beyond the 9.5 minutes required for complete inversion, without effect on the divisor.

The basic divisors previously announced by Jackson and Gillis have accordingly been revised as follows:

	Inversion at 60°	*Inversion at Room Temperature*
Jackson and Gillis Method No. I	133.18	133.28
Jackson and Gillis Method No. II	133.27	133.37
Jackson and Gillis Method No. IV	132.56	132.66

Corrected divisors for Method III are not given, but they would analogously be 133.84 for inversion at 60°, and 133.94 for inversion at room temperature.

Effect of the temperature at which the saccharimetric readings are taken: Temperature coefficient for invert sugar.—This subject has also been studied by Jackson and McDonald (5). In preliminary experiments with a solution containing 28.656 g sucrose in 100 ml and inverted by the usual acid procedure at room temperature, they found temperature coefficients of 0.4960 and 0.4963, valid between 20 and 30°. Further experiments were to be made in both acid and neutral solution, but the results have not been published as yet. For the present the rounded off coefficient of 0.5 t may be retained for the invert sugar.

Effect of concentration: Concentration coefficient.—This phase of the subject has also been re-examined by Jackson and McDonald (5), with the result that the old Herzfeld factor 0.0676 for acid inversion was found to be appreciably too low. They observed an average factor of 0.0794, and for inversion with invertase the value 0.0824, also considerably higher than Paine and Balch's (6) factor 0.073. The rotation of the invert sugar in degrees S was found to increase approximately linearly with the concentration, between 5 and 26 g sucrose inverted with acid, and between 7 and 26 g sucrose inverted with invertase. Some experiments at con-

centrations below 7 g indicated that the concentration coefficient for acid inversion is somewhat higher than at greater sucrose concentration, namely 0.0834, but the difference was not considered large enough for practical purposes.

Previous experiments by Zerban (7), made originally for another purpose, gave for acid inversion an average coefficient of 0.0831 at concentrations between 6.5 and 13 g of sucrose in 100 ml, and of 0.0772 at concentrations between 13 and 26 g. These values agree fairly well with those of Jackson and McDonald, and also indicate that the concentration factor apparently increases with decreasing concentration. The average coefficient 0.0794 for acid inversion, reported by Jackson and McDonald, may be accepted tentatively.

TABLE 2.—*Concentration coefficient according to Jackson and McDonald (1942)*

WEIGHT OF INVERT SUGAR IN AIR	WEIGHT OF SUCROSE IN AIR	ROTATION	ROTATION ON SUCROSE NORMAL WEIGHT BASIS	INCREMENT FOR 1 g INVERT SUGAR
g/100 ml	*g/100 ml*	*°S*	*°S*	
8	7.60	− 9.240	−31.611	
9	8.55	−10.422	−31.692	0.081
10	9.50	−11.610	−31.774	0.082
11	10.45	−12.815	−31.884	0.110
12	11.40	−14.016	−31.966	0.082
13	12.35	−15.210	−32.021	0.055
14	13.30	−16.422	−32.102	0.081
15	14.25	−17.625	−32.158	0.056
16	15.20	−18.848	−32.239	0.081
17	16.15	−20.077	−32.322	0.083
18	17.10	−21.312	−32.403	0.081
19	18.05	−22.553	−32.486	0.083
20	19.00	−23.780	−32.541	0.055
21	19.95	−25.032	−32.623	0.082
22	20.90	−26.290	−32.705	0.082
23	21.85	−27.554	−32.787	0.082
24	22.80	−28.824	−32.869	0.082
25	23.75	−30.100	−32.952	0.083
26	24.70	−31.382	−33.033	0.081
27	25.65	−32.670	−33.115	0.082

In a later publication by Jackson and McDonald (8), the rotation of invert sugar in degrees S is given for concentrations from 8 to 27 g in 100 ml. From these values the negative component of the Clerget Divisor for invertase inversion has been computed by the writer, as shown in Table 2. It is found that the divisor increases about linearly with the concentration. The increments per gram sucrose average 0.08337 for the range from 7.60 to 17.10 g., 0.08327 for 17.10 to 25.65 g, and 0.08332

for the entire range. These figures check closely with those reported previously by Jackson and McDonald, and also show a slight drop in the higher range. The divisor for the inverted half normal of sucrose is 132.09, which is somewhat closer to the value 132.12 of Paine and Balch than the 132.065 previously given by Jackson and McDonald. In this later work, these authors also found that the Vosburgh rule does not hold exactly for the rotations of glucose and fructose in degrees S probably because of the effect of rotation dispersion when bichromate-filtered white light, instead of monochromatic light, is used.

The Clerget Divisor for inversion with invertase at various sucrose concentrations has also been determined directly by Salani, Kindt, and D'Orazi (9). They state that they have confirmed the concentration factor 0.073 of Paine and Balch. But their experimental values of the divisor check on the whole more closely with the figures of Jackson and McDonald (1942) than with that of Paine and Balch, except for the lowest concentration:

g. Sucrose	*S., K., and D'O.*	*J. and McD.*	*P. and B.*
2	131.36	131.17	131.32
4	131.34	131.34	131.46
6.5	131.54	131.55	131.64
13	132.10	132.09	132.12

Zablinsky and Wolf (10), on the other hand, found the divisor 131.40 for 3.25 g sucrose and 131.64 for 6.5 g sucrose, which values agree with those calculated from the formula of Paine and Balch, but for 13 g sucrose they found 132.06, which checks better with the figure of Jackson and McDonald (1942).

Taking all the available evidence into consideration, it appears that the concentration factor 0.0833, for inversion with invertase, established by Jackson and McDonald (1942), is essentially correct, and that it may be accepted as a tentative value, pending further investigation.

Data for evaluating the concentration coefficient for acid hydrolysis by independent methods, as has been done for invertase hydrolysis, are lacking. Until further evidence is obtained, it will be advisable tentatively to accept the value 0.0794, also given by Jackson and McDonald, and checking closely with the figures found by Zerban.

Clerget Divisors for invertase inversion, with the use of the polarimeter.— Since the advent of efficient sodium vapor lamps, the use of the polarimeter, instead of the quartz wedge saccharimeter, is expanding. It was therefore of interest to ascertain the value of the Clerget Divisor under this condition. It has been shown by Zerban (11) that under this condition Vosburgh's rule is valid for a concentration of 3 to 20 grams of invert sugar, and that the specific rotation is

$$[\alpha]_D^{20} = -(19.415 + 0.07065\,c - 0.00054\,c^2) \qquad (1)$$

The circular rotation of invert sugar has been calculated from this equation for increasing concentrations of invert sugar derived from 3 to 20 grams of sucrose weighed in air and dissolved to a volume of 100 ml. at 20°. The resulting values for the circular rotation have been converted into International Sugar Degrees by the factor 0.3462, because the sugar scale for the circular rotation polarimeter is based on this value. From these figures the following equation has been computed for the negative component of the Clerget Divisor:

$$^{\circ}S = -(30.712619 + 0.118455s - 0.000974542s^2), \qquad (2)$$

where s stands for grams sucrose weighed in air.

The values calculated from this equation are shown in column 4 of Table 3. The next column gives the increments per gram added sucrose. For comparison with the figures in column 4, the degrees S based on the equation of Jackson and McDonald (1939) are shown in column 6. It will be noted that up to a concentration of 11 g sucrose the values based on the circular rotation are lower than those based on saccharimeter values, but above that weight of sucrose they are higher except for 26 g sucrose. This discrepancy must be due to differences in rotation dispersion, as suggested by Jackson and McDonald, provided, however, that the formulas of Tollens for glucose, and of Vosburgh for fructose are correct. For a concentration of 13 g sucrose, the value −32.087 checks with that of Jackson and McDonald (1942), namely −32.09, but is slightly higher than the earlier figure of the same authors, −32.065.

The increments in column 5 indicate that the negative component of the Clerget Divisor for polarimeter readings does not increase linearly with the sucrose concentration, but that the increments become progressively smaller as the sucrose concentration increases.

Equation (1) for the specific rotation of invert sugar is strictly valid only for the range of 3 to 20 g in 100 ml. However, it is noted from column 5 in Table 3 that the total increment from 4 to 9 g sucrose, that from 9 g to 14 g, and also that from 14 to 19 g, all show the same decrease, 0.010. It therefore appears permissible to extrapolate the increments somewhat beyond 20 g of sucrose. In this manner the figures in Table 3 (a) were obtained.

It is evident that, when the readings are taken in a polarimeter, the concentration coefficient varies too much to permit the use of an average value per gram sucrose, and that in this case the Clerget Divisor should be found from the table.

The positive constituent of the Clerget Divisor.—This is generally taken to equal 100.00, but this assumption is not quite correct, since it is known that the specific rotation of sucrose does not increase linearly with the concentration. If it is desired to take this into consideration, the positive component of the divisor may be found from Table 4, based on the equa-

TABLE 3.—*Rotation in circular and sugar degrees for varying concentrations of invert sugar, and for readings with the polarimeter*

1 WEIGHT SUCROSE IN AIR	2 WEIGHT INVERT SUGAR IN VACUO	3 CIRC. DEGREES	4 SUGAR DEGREES NORMAL WEIGHT BASIS EQUATION 2	5 INCREMENT PER GRAM SUCROSE	6 SUGAR DEGREES NORMAL WEIGHT BASIS FOR SACCHARIMETER J. & McD. (1939)
g/100 ml	*g/100 ml*				
3.0000	3.1598	−1.241	−31.059		−31.241
4.0000	4.2131	−1.660	−31.171	0.112	−31.324
5.0000	5.2664	−2.083	−31.280	0.109	−31.406
6.0000	6.3197	−2.507	−31.388	0.108	−31.488
7.0000	7.3729	−2.935	−31.494	0.106	−31.571
8.0000	8.4262	−3.366	−31.597	0.103	−31.653
9.0000	9.4795	−3.799	−31.699	0.102	−31.736
10.0000	10.5328	−4.234	−31.799	0.100	−31.818
11.0000	11.5860	−4.672	−31.897	0.098	−31.901
12.0000	12.6393	−5.112	−31.993	0.096	−31.983
13.0000	13.6926	−5.554	−32.087	0.094	−32.065
14.0000	14.7459	−5.999	−32.179	0.092	−32.148
15.0000	15.7991	−6.445	−32.269	0.090	−32.230
16.0000	16.8524	−6.894	−32.358	0.089	−32.313
17.0000	17.9057	−7.344	−32.444	0.086	−32.395
18.0000	18.9590	−7.796	−32.529	0.085	−32.477
19.0000	20.0122	−8.250	−32.611	0.082	−32.560
20.0000	21.0655	−8.706	−32.692	0.081	−32.642

TABLE 3 (a).

1 WEIGHT SUCROSE IN AIR	2 WEIGHT INVERT SUGAR IN VACUO	3 CIRC. DEGREES	4 SUGAR DEGREES NORMAL WEIGHT BASIS EQUATION 2	5 INCREMENT PER GRAM SUCROSE	6 SUGAR DEGREES NORMAL WEIGHT BASIS FOR SACCHARIMETER J. & McD. (1939)
g/100 ml					
21.0000			−32.770	0.078	−32.725
22.0000			−32.846	0.076	−32.807
23.0000			−32.920	0.074	−32.889
24.0000			−32.992	0.072	−32.972
25.0000			−33.062	0.070	−33.054
26.0000			−33.130	0.068	−33.137

tion of Schönrock for the specific rotation of sucrose and the international conversion factor 0.34620. It should be noted that the results of Salani, Kindt, and D'Orazi (9) quoted above are based on the sum of the direct and invert polarizations *observed.*

TABLE 4.—*Rotation of sucrose in sugar degrees*

GRAMS SUCROSE (IN AIR) 100 ML.	°S	GRAMS SUCROSE (IN AIR) 100 ML.	°S
3	99.936	15	100.016
4	99.946	16	100.018
5	99.956	17	100.019
6	99.965	18	100.019
7	99.973	19	100.019
8	99.982	20	100.019
9	99.988	21	100.018
10	99.995	22	100.016
11	100.000	23	100.012
12	100.006	24	100.009
13	100.010	25	100.006
14	100.013	26	100.000

The positive component of the divisor differs from 100 by less than 0.02 for all concentrations above 8 grams in 100 ml. This difference is negligible. But at lower concentrations of sucrose the difference becomes greater, reaching 0.064 for 3 grams of sucrose and has a decided effect on the Clerget Divisor.

Effects of salts and other non-sugars on the Clerget Divisor.—The Referee (2) reported at the London Session of the Commission on this phase of the subject. The statement of Saillard that salts have an effect upon the Clerget Divisor was confirmed. It was shown that inorganic salts, such as sodium, calcium, and ammonium chloride, and potassium sulfate generally increase the value of the divisor, to a larger extent in invertase than in acid hydrolysis. The effect increases with the concentration of the salt. Potassium sulfate at low concentration actually depresses the divisor in acid inversion, but at higher concentrations it increases it. The sodium salt of aconitic acid, which is the principal organic acid in cane juice, has practically no effect on the divisor in invertase hydrolysis, but in acid hydrolysis it lowers the divisor appreciably. In other words, the effect is specific for each salt. The divisor may be higher or lower than for pure sucrose, depending on the nature of the salt, on its concentration, and on the method of inversion used. When known quantities of sucrose were added to distillery residue from cane molasses fermentation, or to cane molasses the sucrose in which had been completely inverted, the correct sucrose result was nevertheless obtained by the invertase method, provided that the divisor was based on the concentration of total solids, and not on the partial sucrose concentration. It appears that with actual cane molasses and with invertase hydrolysis the effects of the salts and other non-sugars are mutually compensating.

Researches on the influence of alkalis, salts, and organic solvents on the rotation of sucrose, glucose, and invert sugar have been published by Dubourg and Saunier (12), but the original publications have not become available to the writer.

The effect of the salts and other non-sugars on the rotation of invert sugar in the presence of inverting acid has been studied by Spengler, Zablinsky, and Wolf (4), but since this work was not extended to the effect of the same substances on the rotation of sucrose, the results need not be discussed here.

Experiments on the effect of various non-sugars on the Clerget Divisor in invertase inversion have been reported by Salani, Kindt, and D'Orazi (9), who used salts of inorganic and organic acids, glycine, betaine, asparagine, and various mixtures of these non-sugars. They confirmed the results of Zerban that these non-sugars generally increase the Clerget Divisor. They also found that the fermentation residue of beet juice, added to known amounts of sucrose, has very little effect on the Clerget Divisor. When the fermentation residue from various Italian beet molasses was added to pure sucrose to reconstitute beet molasses of 50 per cent sucrose content, the Clerget Divisor for the half normal weight of the artificial molasses was found to range from 131.95 to 132.13, and to average 132.00.

The subject was further studied by Zablinsky and Wolf (10). They found that caramel added to sucrose does not change the Clerget Divisor for invertase hydrolysis appreciably, but substantially lowers that for acid hydrolysis. Sodium glutamate, on the other hand, increased the value for invertase hydrolysis from 131.75, calculated on the basis of 8.5 g total solids, to 132.1 observed. The same authors repeated the experiments of Salani, Kindt, and D'Orazi, using the fermentation residue from various German molasses, and confirmed their results by finding Clerget Divisors of 131.90 to 132.10, and averaging 132.00. Since the half normal weight of beet molasses contains about 10.4 g of solids, the divisor, calculated with the concentration factor of Paine and Balch would be 131.93, and with that of Jackson and McDonald it would be 131.90. It appears, therefore, that there is a partial compensating effect of the non-sugars similar to that found for cane molasses.

PART II

Determination of Sucrose and Raffinose by Multiple Polarization

In the past the sucrose in beet products was generally calculated by the Clerget formula or the so-called "raffinose" formula. The latter formula, originally devised by Creydt, was later modified by Herzfeld, on the basis of the divisor 132.66 found by him, and with the factor 0.5124 for the ratio between the invert and direct polarization of raffinose. The formula has lately been recalculated by Paar (1) for the Schrefeld divisor

133.00, but without changing the raffinose factor 0.5124. This factor had previously been redetermined by Browne and Gamble (13) who found an average of 0.5142, which was rounded off to 0.514. The complete formulas, based on this factor and on the Schrefeld divisor, for the half normal weight and for 20°, are

$$\text{Sucrose } (S) = \frac{0.514\,P - I}{0.844}, \quad \text{and}$$

$$\text{Raffinose } (R) = \frac{P - S}{1.852}$$

For temperatures other than 20°, Browne and Gamble give the formulas:

$$S = \frac{P(0.478 + 0.0018t) - I(1.006 - 0.0003T)}{(0.908 - 0.0032t)(1.006 - 0.0003T)}, \quad \text{and}$$

$$R = \frac{P(0.43 - 0.005t) + I(1.006 - 0.0003T)}{(1.681 - 0.0059t)(1.006 - 0.0003T)}$$

where T is the temperature of the direct polarization and t that of the invert polarization.

Raffinose formulas have also been published by Jackson and Gillis (3) who used the Clerget Divisors found by them, 133.25 for Method No. I, and 133.91 for Method No. II, together with the old raffinose factor 0.5124, the Herzfeld concentration coefficient 0.0676, and temperature corrections based on values published by Herles long before the work of Browne and Gamble. Saillard (14) redetermined the rotation of raffinose after acid hydrolysis by his method of inversion, and found a raffinose factor of 0.5156, from which he calculated a new formula for calculating sucrose.

The raffinose formulas cited require further revision or amplification, either because they are valid for only one concentration and temperature, or use concentration coefficients and temperature coefficients which have since been superseded by later values, or require confirmation. These details are omitted here because it is now well known that neither the Clerget nor the raffinose formula give correct percentages of sucrose and raffinose in beet products. These, and especially the molasses, contain besides these two sugars still other optically active substances and it becomes necessary to add another equation, so as to solve for the three unknowns.

This problem has been attacked in two different ways. Paine and Balch (15) have developed a method in which the direct polarization is combined with the invert polarization after treatment with invertase from top yeast, and with invertase plus melibiase from bottom yeast, respectively. The method of Osborn and Zisch (16) is based on the observation

that, when the inversion is effected with acid, the nonsugars occurring in American beet molasses become optically inactive.

Vondrak (17) in his report on the determination of raffinose, rendered at the Ninth Session, states that "in the present state of our knowledge, the only methods that can be recommended on theoretical grounds, depend on the use of enzymes," and "the method of Osborn and Zisch must be judged by its capacity always to give results in close agreement with those obtained by the basic methods, working with enzymes." On the other hand, Saillard (18) in his report on the "Testing of Molasses" came to the conclusion that methods using enzymes can hardly be recommended for commercial purposes because of the time and care required, and because of the difficulty of securing suitable enzyme preparations. Regarding inversion with acid, he stated that "further experiments are needed to establish a formula for raffinose." For the sucrose determination he recommended his method of neutral double polarization.

Since the London Session the determination of sucrose and raffinose by one or both of the above methods has been studied in Italy and Germany.

Method of Paine and Balch.—If the direct polarization be designated by P, the polarization after treatment with top yeast extract by A, and that after treatment with bottom yeast extract by B, all at 20°, then the following equations of Paine and Balch serve to calculate both sucrose and raffinose.

$$\text{Raffinose} = 1.354(A - B), \quad \text{and}$$

$$\text{Sucrose} = \frac{100(P - 2.202A + 1.202B)}{132.12 + 0.073(m - 13)}$$

where m is grams solids in 100 ml. of solution. Thus, if the half normal weight of a beet molasses containing 80 per cent solids is used, the Clerget Divisor is 131.93. With the concentration coefficient of Jackson and McDonald (1942), it becomes 131.90. Temperature corrections for the above two formulas have not been determined, and it is necessary to operate at 20°.

Experimental work carried out in Italy by Salani and collaborators (19) has resulted in an average divisor of 132.00 for the half normal weight of beet molasses at 20°, as mentioned previously. This slight increase over the divisor 131.90 or 131.93, calculated on the basis of dry substance, is due to the effect of salts and other nonsugars. The value 132.00 has been confirmed in Germany by Zablinsky and Wolf (10). The same authors found that when the sum of sucrose and raffinose is determined by the copper reduction method according to Baumann (20) the results check closely with those obtained by the double enzyme method.

Method of Osborn and Zisch.—The formulas for calculating sucrose and raffinose by this method are:

$$S = \frac{0.514P' - I}{0.835 + 0.00009P}, \quad \text{and}$$

$$R = 0.54(P' - S),$$

where P is the direct polarization, and P' that corrected for the polarizing effect of the non-sugars by deducting N, which equals $I'-I-K$. I is the invert polarization in acid solution, I' that of the invert polarization after neutralizing with ammonia. K is a term which varies with P and with the amount of lead subacetate solution used for clarification, and which is found from a table. The factor 0.00009 represents the concentration correction. If the polarizations are made at temperatures other than 20°, the sucrose is calculated by the formula.

$$S = \frac{P'(0.478 + 0.0018t) - I(1.006 - 0.0003T)}{(0.899 + 0.00009P - 0.0032t)(1.006 - 0.0003T)},$$

where T is the temperature of the direct polarization, and t that of the invert polarization.

Finally, a correction for the volume of the lead precipitate is applied. In the case of molasses the result is multiplied by 0.99. The factor for raw massecuite is 0.993, that for white massecuite and saccharate sirup 0.998. The method is not applicable to products containing appreciable amounts of invert sugar.

This method was tested by Osborn and Zisch with a large number of American molasses from both Steffen and non-Steffen houses. It gave excellent checks with the method of Paine and Balch, for both sucrose and raffinose. But when Mastrogiacomo and Puliga (21) tried it on Italian beet molasses it gave in the majority of cases considerably higher sucrose results than the method of Paine and Balch. This work was extended to beet molasses from various other European countries. The sucrose results were in every case higher than those by the two-enzyme method. The differences averaged 0.71 per cent for German, 0.87 per cent for Hungarian, 0.32 per cent for English and Swedish, 0.64 per cent for French and Belgian, 0.74 per cent for Polish, and 0.31 per cent for Spanish molasses.

Mastrogiacomo and Puliga made an extensive study of the reasons for the discrepancies and were able to modify the method of Osborn and Zisch so as to give results in concordance with the method of Paine and Balch. They concluded from their experiments that the acid does not reduce the rotation of the optically active non-sugars to zero, but to about $-1.0°$. This value varies somewhat, but can be determined by fermenting the molasses and analyzing the unfermented residue. The experiments of these authors showed that the results check with those of the two-enzyme method if the method is modified as follows:

(1) The acid solution should not be neutralized until after standing for 3 hours at room temperature. To facilitate the reading of the rather

dark solution, a little sodium hydrosulfite is added one half hour before the polarization is made. The use of this decolorant had previously been suggested by Osborn and Zisch.

(2) The polarization of the inverted acid solution must be corrected for the residual rotation of the non-sugars. The correction may be found experimentally by fermenting the molasses produced at the beginning and the end of the campaign and analyzing the unfermented residue. As an alternative, the average figure of 1° may be deducted from the reading in acid solution.

(3) The reading of the neutralized invert solution must be corrected by deducting 0.2°, in addition to the correction prescribed in the method of Osborn and Zisch.

In comparative analyses of beet molasses the results of this modified method, but not those of the original method of Osborn and Zisch, checked satisfactorily with those of the method of Paine and Balch. The values in Table 5 are cited as an example:

TABLE 5.—*Comparative analyses*

	P. & B.	O. & Z. ORIGINAL	O. & Z. MODIFIED
Sucrose	52.58	53.32	52.61
Raffinose	0.62	—	0.53
Polarization of the non-sugars	− 3.50	− 2.41	− 3.21

RECOMMENDATIONS

On the basis of the reports presented at the Eighth, Ninth, and Tenth Sessions of the Commission the following recommendations are submitted for appropriate action:

A. General

(1) It is recommended that all polarizations be made at 20°C., or as close as possible thereto, and that the following temperature coefficients be used for correcting the Clerget Divisor: Invert sugar, −0.5; Sucrose, −0.03; Raffinose, −0.03.

(2) It is recommended that the concentration correction in the Clerget Divisor be based on the total solids concentration, not on the partial sucrose concentration.

B. Sucrose Determination in Sugar Cane Products

(3) It is recommended that in the determination of sucrose in cane products the solution for the invert polarization be of the same normality as that used for the direct polarization.

(4) It is recommended that the invertase method be adopted as the *standard* method for determining sucrose in sugar cane products, with the divisor $132.1-0.0833\ (13-g)-0.53\ (t-20)$, where g is grams solids from original sample in 100 ml. of the solution read in the saccharimeter, and t the temperature at which the readings are made.

(5) It is recommended that Jackson and Gillis method No. IV be adopted for the purposes of sugar factory control and trade analyses, as a temporary substitute for the standard method, because the results by this method check more closely with those by the invertase method than either the plain acid method or Jackson and Gillis method No. II. The revised Clerget Divisors for Jackson and Gillis method No. IV are, for inversion at 60°C., $132.56-0.0794\ (13-g)-0.53\ (t-20)$, and for inversion at room temperature $132.66-0.0794\ (13-g)-0.53\ (t-20)$.

(6) It is recommended that Horne's dry subacetate of lead or its equivalent be used for clarification of cane products. For deleading, dry ammonium dihydrogen phosphate is recommended for use with the invertase method, and anhydrous potassium oxalate with Jackson and Gillis method No. IV.

C. Determination of Sucrose and Raffinose in Sugar Beet Products

(7) It is recommended that the two-enzyme method of Paine and Balch be adopted as the *standard* method for determining sucrose and raffinose in beet products. The formulas of Paine and Balch, with the divisor 132.22, to correct for the effect of the non-sugars, and with the concentration factor 0.0833, are recommended. These give a divisor of 132.00, when the half-normal weight of beet molasses containing 80% solids and made to 100 ml, is used for the invert readings at 20°C.; the factor for A is changed to 2.201, and that for B to 1.201.

(8) It is recommended that, as a practical substitute for the two-enzyme method, the double acid method of Osborn and Zisch be accepted in principle but that the effect of the optically active non-sugars on the rotation in neutral and acid solution be further studied in different countries, in order to establish necessary corrections.

(9) It is recommended that for purposes of running factory control the method of Schrefeld be used, with the formulas of Browne and Gamble for calculating sucrose and raffinose.

(10) It is recommended that in the determination of sucrose and raffinose clarification be effected with lead acetate solution, and deleading with dry ammonium dihydrogen phosphate, as prescribed in the two-enzyme method of Paine and Balch, and in the double acid method of Osborn and Zisch.

(11) It is recommended that the concentration and temperature coefficients of the Clerget Divisor be studied upon actual cane and beet products.

(12) It is recommended that the specific rotation and saccharimetric polarization of raffinose be redetermined.

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REPORT ON REFRACTIVE INDICES OF SOLUTIONS OF SUCROSE, DEXTROSE, LEVULOSE, RAFFINOSE, INVERT SUGAR AND MIXTURES, TO THE INTERNATIONAL COMMISSION FOR UNIFORM METHODS OF SUGAR ANALYSIS, TENTH SESSION, BRUSSELS, AUGUST 15–20, 1949 (Subject 7.)

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This report prepared by the writer, as referee, in cooperation with the associate referees: H. C. S. deWhalley, E. Landt, J. R. Maybee, J. F. Snell, W. L. McCleery, P. J. H. Van Ginneken, O. G. deLima, J. Dubourg, E. J. Marais and J. Guild, was presented at the Tenth Session of the International Commission on Uniform Methods for Sugar Analysis in Brussels, 1949.

INTRODUCTION

A review of the accomplishments of the previous sessions of the International Commission with respect to this subject seems desirable due to the lapse of time since the last meeting. The subject of refractometric methods received little attention in the earlier sessions of the Commission. When the Commission was reconvened in Amsterdam in 1932, however, the subject was introduced into the agenda, under the refereeship of Mr. Hugh Main, of England.

EIGHTH SESSION, 1932

In Main's report, presented at this meeting, it was pointed out that with samples of high purity the determination of moisture (or solids) by (1) the usual methods of drying, (2) by specific gravity determinations, and (3) by refractometer, all give satisfactory and concordant results. The two latter methods are of course based on tables of specific gravity and refractive indices, respectively, of pure sucrose solutions. In regard to products of lower purity, attention was called to the effects caused by the presence of varying amounts and kinds of impurities which must be taken into account in the application of refractometric methods of sugar analysis.

In the consideration of the use of the refractometer in the estimation of moisture in raw sugars, mention was made of the method of Stanek of weighing 20 g of sample, dissolving in water, making up to 100 ml, and reading the solution on a dipping refractometer. The conversion to per cent water was made by means of a table accompanying the method.

Also discussed in this report was the method of I. Vondrak, who em-

ployed the normal solution (26 g. in 100 ml. of solution) and obtained the readings on the Zeiss dipping refractometer with interchangeable prisms, using the No. II prism. In the case of raw beet sugars, a correction was applied to compensate for the differences between the refractive indices of the non-sugars and of the sucrose.

The Main committee report called particular attention to the errors arising from the presence of invert sugar in such products as cane molasses when conversions are made with sucrose refractive index tables. On the basis of investigations of Main's co-workers and others, a correction of 0.02% for each 1% of invert sugar was suggested.

No recommendations were presented at the Eighth Session, the subject being referred to the Ninth Session.

NINTH SESSION, 1936

Previous to about 1936, the most widely used table of refractive indices of sucrose solutions at 20°C. was the Schönrock-Main Table. This table, giving the indices to four decimal places, consisted of the Schönrock (1) (1911) values from 100 to 34 per cent water and the Main (2) values from 34 to 15 per cent water. The table appeared in many of the sugar handbooks and in several editions of the *Methods of Analysis of the A.O.A.C.* The table for use with the tropical instrument at 28°C. was that of Prinsen Geerligs (3).

At the Ninth Session of the Commission, the Committee of Refractometry under the chairmanship of E. Landt of Germany presented two new tables of refractive indices of sucrose solutions, one at 20°C. and one at 28°C. The new tables were constructed on the best data available and the values were given to five decimal places for the range 0 to 24 per cent sucrose, the remainder given to four decimal places. The new 20°C. table consisted of the five place values of Schönrock and Landt (4) (1933) up to 24 per cent sucrose, the Schönrock (1911) values from 25 to 66 per cent, the extrapolated values from 66 to 70 per cent, and the Main values from 71 to 85 per cent. Using this table as a basis and the temperature corrections of Schönrock, the table of indices at 28°C. was computed.

Two temperature correction tables were in use at this time, the Stanek table (5) for instruments standard at 20°C., and the Prinsen Geerligs table (6) for the tropical model standard at 28°C. The Landt committee made a careful comparison of the values in these tables with the corresponding values calculated from the Schönrock temperature corrections and concluded that the latter values were to be preferred. Therefore, the two temperature correction tables based on the Schönrock values were constructed and presented before the Commission.

The Commission adopted the two refractive index tables for sucrose, namely, the International Scale (1936) of Refractive Indices of Sucrose Solutions at 20°C. and the corresponding one at 28°C. Also adopted were

the two temperature correction tables, the International Temperature Correction Table (1936) for the Normal Model of Refractometer, above and below 20°C., and the one for the Tropical Model, above and below 28°C.

In regard to the above tables, your present committee concurs fully with the action of the Ninth Session of the International Commission. It agrees with the opinions of many workers in the field, however, that it is essential that accurate values on indices of sucrose to five decimal places over the entire range be made available in order to fully utilize the precision of modern refractometers that are capable of readings accurate to a few units in the fifth decimal place. In an attempt to meet this need, the Bausch & Lomb Optical Company have issued a table for use with their precision instrument giving five place calculated values in the range 25 to 85 per cent sucrose. This was accomplished by methods of interpolation using the four place values on the International Scale (1936) for this range. In regard to these extended values, the present committee feels that further experimental data are required.

DEXTROSE, LEVULOSE AND INVERT SUGAR

The question of the use of sucrose tables of indices for products containing other sugars was mentioned in the Main report at the Eighth Session of the Commission. At the Ninth Session, H. C. S. de Whalley in his report on Subject 15 presented his results on the refractometric estimation of solids in sirups containing invert sugar in addition to sucrose (7). He proposed a correction factor of 0.021 for each per cent of invert sugar present in the solution. No formal action in regard to this factor was taken other than to suggest further study.

Zerban (8) carried out an investigation on this value for the correction factor and on the basis of de Whalley's data, calculated a table of refractive indices of invert sugar solutions. Later Zerban and Martin (9), using a Bausch and Lomb precision refractometer, determined the refractive indices of both dextrose and invert sugar solutions. The tables prepared by these authors were given to five decimal places with an accuracy of about three units in the last place. In the course of this investigation, use was made of the previously published values of Jackson and Mathews (10) of the indices of levulose solutions, who calculated a table from their data by means of three separate equations. Zerban and Martin, noting the trend of the residuals, suggested the use of a single equation instead of three. J. B. Saunders of the National Bureau of Standards devised such an equation, which fits the Jackson and Mathews data better than the values obtained by their three original equations.

The committee feels that the following tables of refractive indices are the results of careful and painstaking work and are the most accurate available for these materials:

1. Zerban and Martin table of refractive indices of Dextrose, 20°C.
2. Zerban and Martin table of refractive indices of Invert Sugar at 20°C.
3. Jackson and Mathews table of refractive indices of Levulose at 20°C. recalculated by the Saunders equation.

RAFFINOSE

Stolle (11) studied the refractive indices of raffinose solutions and prepared tables based on his data for the range 0 to 30 per cent for both raffinose hydrate and the anhydrous sugar at 17.5°C. No recommendation is made with respect to raffinose at this time.

MIXTURES

The present subcommittee feels that the refractometer is a valuable instrument for the approximate estimation of the per cent solids in various liquid sugar products containing several different sugars and related products but doubts the advisability of attempting to make any recommendations at the present time.

CONCLUSION

For detailed discussion of the various types of instruments and their applications to the analysis of sugars and sugar products, reference is made to the several sugar handbooks including Browne and Zerban, "Sugar Analysis," Spencer and Meade, "Cane Sugar Handbook" and N. B. S. Circular C440, "Polarimetry, Saccharimetry and the Sugars."

RECOMMENDATIONS

(1) It is recommended that in the determination of sucrose by the refractometer the tables adopted by the International Commission (1936) be employed.

(2) It is recommended that further study be made on the refractive indices of sucrose solutions in the range 25 to 85 per cent to obtain accurate values to the fifth decimal place.

(3) It is recommended that in the determination of dextrose by the refractometer the values of Zerban and Martin for dextrose be employed.

(4) It is recommended that in the determination of invert sugar by the refractometer the values of Zerban and Martin for invert sugar be employed.

(5) It is recommended that in the determination of levulose by the refractometer the values of Jackson and Mathews for levulose, recalculated by the Saunders equation, be employed.

(6) It is recommended that the refractive indices of raffinose be subjected to further study.

(7) It is recommended that the application of refractometric methods to the analysis of mixtures be further studied.

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REPORT ON DETERMINATION OF REDUCING SUGARS AND THE INFLUENCE OF OVERHEATING ON THE DETERMINATION OF INVERT SUGAR, TO THE INTERNATIONAL COMMISSION FOR UNIFORM METHODS OF SUGAR ANALYSIS, TENTH SESSION, BRUSSELS, AUGUST 15–20, 1949 (Subject 4.)

By EMMA J. McDONALD (National Bureau of Standards, Washington, D. C.)

This report prepared by the writer, as Referee, in cooperation with the associate referees: T. J. Mitchell, L. D. Hammond, F. Baerts, K. D. Dekker, J. Dabrowski, W. M. Seaber, A. H. Rheinlander, W. Buchanan, H. Hirschmuller, J. H. Lane, S. Ekelund, J. Ancizor-Sordo was rendered at the Tenth Session of the International Commission for Uniform Methods of Sugar Analysis in Brussels, 1949. Details of those methods appearing in *Methods of Analysis* are omitted, the references only being given. The report presented to the International Commission included the complete procedures.

The reducing sugar methods used throughout the sugar industry, according to our information, conform closely to the report presented at the Ninth Session of the Commission. Some work has been done on those methods which use copper reagents of low alkalinity. The Berlin Institute Method and Ofner's Method have been more generally adopted and that of Herzfeld for small amounts of invert sugar in sucrose has become obsolete. Detailed discussion of the individual methods is given later in the report. The Committee will appreciate any information concerning modifications of the methods that we have incorporated in this report. We also wish to have our attention called to oversights we may have made.

Determination of Invert Sugar in Refined Sugars, Factory White Sugars, Raw Beet Sugars, Beet Molasses, and Cane Sugars of Low Invert Sugar Content.—It has been recognized by many investigators that copper solutions of low alkalinity are more suitable for the determination of invert sugar in refined sugar because of their slight action on sucrose. Of such methods Ofner's is now official in Czechoslovakia, Luff-Schoorl's is official in Java, and the Berlin Institute Method has received considerable attention. At the Ninth Session of the International Commission it was recommended that a study be made of these methods and that the best features of the individual methods be embodied in a single method that would be acceptable as an International Standard Method for the determination of Small Quantities of Invert Sugar and Sugar Products. In accord with this recommendation and because of the need of replacing Herzfeld's Method,

which uses Fehling's Solution as a reagent, as an Official Method of the Association of Official Agricultural Chemists, Jackson and McDonald made an investigation of these methods and subsequently published their results. Preliminary experiments showed that Ofner's Methods and that of the Berlin Institute yielded results of about the same precision. Comparing the two methods, Ofner's Method is slightly more restricted in range, yet it is entirely adequate for the determination of high grade white sugars. This method is also slightly more convenient in that it specifies direct boiling instead of the use of a boiling water bath as prescribed by the German investigators. Jackson and McDonald found that the reproducability of the method was increased if the iodine was added after acidification with acetic acid but preceding the addition of hydrochloric acid. Ofner's Method, as modified by Jackson and McDonald, is now an Official Method of the A.O.A.C.

Defecation.—Defecation procedures form an important part of this section because of the effect they have on the reducing sugar analysis. The necessity of using neutral lead acetate when a lead acetate salt is used has long been recognized, but too frequently the exact amount of salt, whether it is to be added in the wet form or as a dry reagent, and proper detailed instructions for removal of the lead and other salts are completely lacking. There is a need for study in this field in order that detailed and specific procedures may be adopted as International Standards Methods for use when analyzing sugar products.

Determination of Invert Sugar in Raw Sugars.—The methods of Lane and Eynon, Luff-Schoorl, and Munson and Walker are used in general for the determination of invert sugar in raw cane sugar. Lane and Eynon's Method uses Fehling's Solution and therefore requires an appreciable correction for the sucrose present. It has found wide favor in industry because of its rapidity and simplicity of technique. Schoorl's Method employs a copper reagent of low alkalinity and, although it is more time consuming than that of Lane and Eynon, it is widely used. Munson and Walker's Method is most frequently used in laboratories where occasional analysis is required.

Determination of Invert Sugar in Cane Molasses.—At the ninth meeting of the International Commission it was recommended that comparative studies be made of Munson and Walker's; Lane and Eynon's; Schoorl's; and Brown, Morris, and Millar's Methods for the determination of invert sugar in cane molasses. Erb and Zerban (1) have shown that Lane and Eynon's Method and that of Munson and Walker gave results which agree for this analysis.

RECOMMENDATIONS

(1) That studies on sucrose products of low invert content be made comparing Ofner's Method as modified by Jackson and McDonald, and

Lane and Eynon's Method as modified for the determination of small amounts of invert sugar in the presence of sucrose.

(2) That studies such as those of Erb and Zerban on Lane and Eynon's and Munson and Walker's Methods on cane molasses be extended to include Schoorl's Method and that of Brown, Morris, and Millar.

(3) That defecation of various sugar products be studied with attention to (1) the necessity of defecating when analyzing a specific product, (2) the most suitable defecant, including a detailed study of the amount required and the form in which it shall be used, and (3) the exact procedure for removing the unused defecant.

DESCRIPTION OF METHODS

LANE AND EYNON METHODS (2)

See **29.32**, *Methods of Analysis, 7th ed.*

FORT MODIFICATION OF LANE AND EYNON

C. A. Fort has extended the scope of this method to include more dilute solutions than those used by the authors. His modification consists of a supplemental titration, by a standard invert sugar solution, of the residual strength remaining in a 10 ml portion of the copper reagent after it has reacted with a measured volume of the unknown.

CONTROL TITRATION

A standard invert sugar solution containing 2.1 mg per ml of solution is prepared. To a 10 ml portion of the Soxhlet soln add 25 ml of water if pure invert soln is to be analyzed or 25 ml of a sucrose soln having a concentration corresponding to the approximate average concentration of sucrose present in the product being analyzed, then add about 24 ml of standard invert and make a titration according to Lane and Eynon. This control titration need only be established once for a particular product, while the factor should be determined each day with pure invert sugar.

DETERMINATION

To 10 ml of Soxhlet soln add 25 ml of the sugar soln being analyzed, and make an addition of the standard invert soln from the burette. After the mixture has boiled 1½ min., the standard invert is added rapidly though dropwise to obtain a trial end point. Repeat the test making the preliminary addition of invert about 0.5 ml less than the trial titer, and complete the titration in the regular manner, adding the final amounts of invert very slowly. If the final titer obtained is more than 1 ml above the amount of the initial invert addition, the determination should be repeated.

CALCULATION

Subtract the titer of the unknown from the control titer. This difference represents the titer of the 25 ml of sample in terms of standard invert. This difference multiplied by .0021 represents the grams of invert sugar in the sample.

Tables may be found in the following:

1. *Methods of Analysis* of the Association of Official Agricultural Chemists.

2. Polarimetry, Saccharimetry, and the Sugars, Circular C440 of the National Bureau of Standards.
3. Sugar Analysis, by C. A. Browne and F. W. Zerban.
4. Cane Sugar Hand-Book, by G. L. Spencer and G. P. Meade.

MUNSON AND WALKER'S METHOD (3)

REAGENTS

See **29.32**, *Methods of Analysis, 7th Ed.*

DETERMINATION

See **29.35**, **29.36**, *Methods of Analysis, 7th Ed.*

DETERMINATION OF REDUCED COPPER

(1) *Reduction in methyl alcohol vapor.*—Reduction to copper in methyl alcohol vapor. Wash the cuprous oxide thoroly with water at a temp. of about 60°C., then with 10 ml of alcohol, and dry for 30 min. in a water oven at 100°C., and subsequently heat for 30 min. over a Bunsen burner. The precipitate is reduced to metallic copper in methyl alcohol vapor. This is done by placing about 25 ml of methyl alcohol in a 400 ml beaker containing a triangular support which will bring the crucible above the level of the alcohol. Heat the covered beaker on a hot plate to boiling, remove the cover, and place the hot Gooch crucible on the triangle. This ignites the alcohol vapor. Immediately cover the beaker with a watch glass and allow the crucible to remain for about 3 min., transfer to a 100°C. oven in order to remove any condensed vapor (ca 5 min. heating required), cool in a desiccator, and weigh. When using this procedure no precautions are made for removing occluded salts.

(2) Electrolytic deposition from nitric acid soln. *See* **29.42**, *Methods of Analysis, 7th Ed.*

(3) Copper by means of standard thiosulfate soln. *See* **29.38**, *Methods of Analysis, 7th Ed.*

(4) Cuprous oxide by direct weighing. *See* **29.37**, *Methods of Analysis, 7th Ed.*

(5) *Cuprous oxide weighed as cupric oxide.*—Wash the Cu_2O with hot water and heat a little above 100°C. to dry the precipitate. Place the crucible in an electric muffle furnace and heat at red heat for 15–30 min. to oxidize the cuprous to cupric oxide. Weigh the crucible as quickly as possible after removing it from the desiccator since cupric oxide is very hydroscopic. Multiply the weight of cupric oxide by 0.7989 to give the equivalent weight of copper.

Tables may be found in the following:

1. *Methods of Analysis* of the Association of Official Agricultural Chemists.
2. Polarimetry, Saccharimetry, and the Sugars, Circular C440 of the National Bureau of Standards.
3. Sugar Analysis, by C. A. Browne and F. W. Zerban.
4. Cane Sugar Hand-Book, by G. L. Spencer and G. P. Meade.

BERLIN INSTITUTE METHOD (4)

REAGENTS

Müller's Solution.—Dissolve 35 g of pure $CuSO_4 \cdot 5H_2O$ in about 400 ml of boiling water. Dissolve 173 g of Rochelle salt and 68 g of anhydrous Na_2CO_3 in about 500 ml of boiling water. After cooling, mix the two solns, make up to 1 liter and treat with active carbon. After several hours, filter thru a hardened filter paper. If any copper precipitate forms in the course of storage, the soln should be re-filtered.

DETERMINATION

Prepare a soln containing 10 g or less (so that the invert sugar present does not exceed 30 mg per 100 ml) of the sample in 100 ml. Pipette 100 ml of the sugar soln into a 300 ml Erlenmeyer flask and add 10 ml of the Müller's Solution. Heat for exactly 10 min. in a water bath boiling so vigorously that the immersion of the flask does not interrupt ebullition. The flask should be fixed in the bath so that the water level is at least 2 cm higher than the surface of the liquid in the flask. After exactly 10 min., the flask is removed from the bath and cooled rapidly without agitation by immersion in cold water. Add 5 ml of 5 *N* acetic or tartaric acid, and immediately after mixing add an excess of .0333 *N* iodine soln (*e.g.*, 20 or 40 ml) from a burette. After all of the copper precipitate has dissolved, titrate the excess iodine with .0333 *N* thiosulfate soln using starch indicator.

CALCULATION

Deduct the volume of excess iodine from the amount added. Three corrections must be applied to the volume of iodine soln consumed: (1) A small correction, determined occasionally, for the iodine consumption found by running a blank analysis on water instead of sugar soln. (2) A correction for the iodine consumption of the sugar sample under the conditions of the test but without heating. (3) A correction of 2 ml of .0333 *N* iodine soln for the reducing action of 10 g of sucrose, or proportionate corrections for smaller amounts of sucrose.

After these corrections have been made, each milliliter of .0333 *N* iodine solution consumed corresponds to 1 mg of invert sugar.

OFNER'S METHOD (JACKSON AND MCDONALD MODIFICATION) FOR THE DETERMINATION OF INVERT IN THE PRESENCE OF SUCROSE (5)

See 29.43, *Methods of Analysis, 7th Ed.*

LUFF-SCHOORL METHOD (6)

REAGENTS

Copper Solution.—Dissolve 17.3 g of $CuSO_4 \cdot 5H_2O$ and 115 g of citric acid crystals in about 200 ml of water by gentle heating. Add, while shaking, a soln of 185.3 g of anhydrous Na_2CO_3 dissolved in about 500 ml of water. Cool, make up to one liter, treat with kieselguhr, and filter under suction.

DETERMINATION

Dissolve 20 g of the sample in water and dilute to 100 ml. Transfer 25 ml of the soln containing not more than 45 mg of invert sugar to a 300 ml Erlenmeyer flask and add 25 ml of the copper reagent. Mix well, add 50–100 mg of pumice or talcum powder and fit the flask with a vertical condenser. Place on a wire gauze resting on an asbestos card having a central hole 6.5 cm in diameter. Bring to boiling in 3 min. and boil for exactly 5 min. Cool immediately without agitation by immersion in cold water. Add 15 ml of 20 per cent iodate-free KI soln, and then add 15 ml of 25 per cent H_2SO_4 slowly to avoid loss by effervescence. Titrate the free iodine with 0.1 *N* thiosulfate soln, using starch indicator. Run a blank determination using 25 ml of water instead of sugar soln.

CALCULATION

Find difference between the volumes of 0.1 *N* thiosulfate required by the blank, and the actual test. Convert milliliters of thiosulfate to milligrams of invert sugar by use of the following table.

Luff-Schoorl Method

0.1 *N* THIOSULFATE	NO SUCROSE	1.25 G SUCROSE	2.5 G SUCROSE	5.0 G SUCROSE
	INVERT SUGAR IN 25 ML SOLUTION			
ml	*mg*	*mg*	*mg*	*mg*
1	3.20	2.75	2.50	1.90
2	6.20	5.80	5.55	5.00
3	9.15	8.90	8.55	8.05
4	12.10	12.00	11.50	11.05
5	15.10	15.00	14.50	14.05
6	18.10	18.00	17.50	17.05
7	21.00	21.00	20.50	20.10
8	24.00	24.00	23.50	23.10
9	27.00	27.00	26.60	26.15
10	30.10	30.20	29.80	29.20
11	33.20	33.40	33.00	32.30
12	36.30	36.60	36.20	35.45
13	39.50	39.85	39.40	38.60
14	42.80	43.10	42.60	41.70
15	46.05	46.30	45.80	44.90
16	49.35	49.65	49.60	48.40

SCHOORL'S METHOD FOR THE DETERMINATION OF REDUCING SUGARS IN CANE MOLASSES (7)

REAGENTS

Soxhlet's modification of Fehling's soln:

A. Copper Sulfate Soln.—Dissolve 34.639 g of pure $CuSO_4 \cdot 5H_2O$ in water and dilute to 500 ml.

B. Alkaline Tartrate Soln.—Dissolve 173 g of Rochelle salt and 50 g of carbonate-free NaOH in water and dilute to 500 ml.

DETERMINATION

Treat a soln of 6 g of molasses in about 100 ml of water with 15 ml of 10 per cent neutral lead acetate soln. Make up to 250 ml and filter. Treat 50 ml of this filtrate with 5 ml of de-leading soln (7 g of $Na_2HPO_4 \cdot 12H_2O$ and 3 g of $K_2C_2O_4 \cdot H_2O$ per 100 ml), make up to 100 ml and filter. Pipette 50 ml of this filtrate, containing 0.6 g molasses into a 300 ml Erlenmeyer flask containing 50 ml of the copper reagent. Add one or two fragments of washed and ignited pumice. Heat the flask on a wire gauze resting on an asbestos card with a central hole, so that the liquid is brought to boiling in 4 min. Boil gently for exactly 2 min. Cool rapidly, without agitation, by immersion in a cold water bath. Add 25 ml of 20 per cent KI soln and 35 ml of H_2SO_4 (1 volume of conc. acid to 5 volumes of water). Titrate the free iodine with 0.1 *N* thiosulfate soln using starch indicator. Run a blank analysis using 50 ml of water instead of molasses soln.

CALCULATION

Subtract volume of 0.1 *N* thiosulfate required to titrate sample from that required by blank. Calculate this difference as mg of reduced copper (1 ml 0.1 *N* thiosulfate is equivalent to 6.357 mg of Cu) and refer to the table. The table enables

the percentage of reducing sugars, calculated as invert sugar, in the molasses to be ascertained from the mg of reduced copper and the polarization of the molasses.

Cu	REDUCING SUGARS				
	POL. 10°	POL. 20°	POL. 30°	POL. 40°	POL. 60°
mg	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
100	8.70	8.60	8.57	8.55	8.53
120	10.44	10.36	10.32	10.28	10.28
140	12.20	12.11	12.09	12.06	12.04
160	14.03	13.95	13.89	13.87	13.81
180	15.84	15.78	15.69	15.66	15.60
200	17.70	17.63	17.53	17.50	17.43
220	19.54	19.50	19.44	19.36	19.25
240	21.40	21.36	21.28	21.20	21.12
260	23.27	23.22	23.13	23.05	22.97
280	25.16	25.12	25.02	24.91	24.83
300	27.05	27.00	26.95	26.90	26.80
320	29.07	29.01	28.95	28.90	28.80
340	31.11	31.05	30.98	30.91	30.77
360	33.18	33.11	33.04	32.96	32.82
380	35.28	35.15	35.08	35.01	34.89
400	37.40	37.27	37.20	37.13	37.00

METHOD OF BROWN, MORRIS AND MILLAR (8)

REAGENTS

Fehling's Soln. Dissolve 34.6 g of $CuSO_4 \cdot 5H_2O$, 175 g Rochelle salts, and 65 g anhydrous NaOH in water in a 1 liter volumetric flask and make to volume.

DETERMINATION

Pipette 50 ml of the reagent into a 250 ml beaker and place in a gently boiling water bath for 6 min. At this time add 50 ml of 1 per cent molasses soln, cover the beaker with a watch glass and continue the heating exactly 12 min. Filter the Cu_2O and wash with 200 ml of boiling water. Convert the Cu_2O to CuO as described under paragraph 5 of Munson and Walker's Method.

CALCULATION

Correct the weight of CuO for the quantity obtained in a blank determination using water instead of molasses soln. Refer result to following table.

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CuO	INVERT SUGAR	CuO	INVERT SUGAR
gm	*per cent*	*gm*	*per cent*
0.1100	9.06	0.2900	24.02
.1200	9.84	.3000	24.92
.1300	10.62	.3100	25.82
.1400	11.40	.3200	26.74
.1500	12.20	.3300	27.64
.1600	13.02	.3400	28.56
.1700	13.84	.3500	29.54
.1800	14.68	.3600	30.54
.1900	15.50	.3700	31.50
.2000	16.30	.3800	32.38
.2100	17.14	.3900	33.30
.2200	18.00	.4000	34.28
.2300	18.88	.4100	35.24
.2400	19.74	.4200	36.24
.2500	20.58	.4300	37.24
.2600	21.42	.4400	38.24
.2700	22.28	.4500	39.26
.2800	23.14	.4600	40.32

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REFRACTIVE INDICES OF MALTOSE SOLUTIONS*

By F. W. ZERBAN and JAMES MARTIN
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Papers on the refractive indices of lactose hydrate solutions have recently been published by Emma J. McDonald (1), and by the writers (2). The latter authors had determined also the refractive indices of maltose hydrate solutions in the spring of 1946, but the results were withheld until Dr. McDonald could complete her investigations on the same subject so that the results obtained could be presented simultaneously.

The maltose used in this laboratory was originally prepared as a reference material for the purpose of studying methods for the analysis of mixtures of pure sugars. To insure the absence of higher saccharides, the maltose was made by a specialist in the preparation of organic compounds, by acetylation of Pfanstiehl c.p. maltose hydrate with sodium

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 2–4, 1950.

acetate and acetic hydride, followed by deacetylation with sodium methylate in anhydrous methanol according to the method of Zemplén (3). The maltose hydrate was recrystallized from strong ethyl alcohol, and dried in the air.

The greatest difficulty was experienced in precisely determining the residual moisture in the final product. Various methods of vacuum drying were tried (4), but it was found impossible to obtain strictly concordant results. Finally, R. F. Jackson, of the National Bureau of Standards, kindly agreed to determine the moisture in the product. He used a method which has not been published, and found 0.130 per cent moisture, which was accepted for correcting the weights of maltose hydrate taken for the experiments. The same Bausch and Lomb Precision Refractometer which had served for the determination of the refractive indices of solutions of dextrose (5), invert sugar (5), and lactose (2), was employed in this work, and the same procedure was followed as previously described. All solutions were allowed to stand for 24 hours to complete mutarotation. Attempts to prepare solutions containing 70, 75, and 80 per cent maltose hydrate were unsuccessful, the sugar crystallizing out in each case. The results of the measurements upon 16 solutions are given in the second column of Table 1.

TABLE 1.—*Refractive indices of maltose hydrate solutions; observed data and values calculated from Equation 1*

MALTOSE HYDRATE, PER CENT IN AIR	n_D^{20} OBSERVED	n_D^{20} CALCD. BY EQUATION 1	DEVIATION FROM FORMULA	SQUARE OF RESIDUALS $\times 10^{-10}$
2.029	1.33585	1.33583	+0.00002	4
4.995	1.34004	1.34006	−0.00002	4
8.027	1.34446	1.34447	−0.00001	1
10.021	1.34744	1.34742	+0.00002	4
13.087	1.35205	1.35205	0.00000	0
16.782	1.35778	1.35775	+0.00003	9
19.928	1.36266	1.36273	−0.00007	49
24.939	1.37094	1.37090	+0.00004	16
29.943	1.37938	1.37937	+0.00001	1
35.309	1.38883	1.38880	+0.00003	9
40.537	1.39834	1.39836	−0.00002	4
45.536	1.40743	1.40745	−0.00002	4
49.665	1.41598	1.41593	+0.00005	25
56.490	1.42989	1.42984	+0.00005	25
60.063	1.43729	1.43731	−0.00002	4
65.501	1.44917	1.44925	−0.00008	64
				223

The following equation was computed from the experimental data by the method of averages:

$$n_D^{20}, \text{ maltose hydrate} = 1.33299 + 0.0013920481p + 0.0000045908476p^2 + 0.000000023760493p^3 - 0.000000000070366691p^4. \quad (1)$$

The deviations of the experimental data from the formula are within 0.00003 in eleven of the sixteen experiments. The larger deviations are principally in the supersaturated range where there is always some danger of incipient destruction of maltose by the high temperature necessary for preparing such solutions.

The sum of the squares of the residuals for the 16 experiments equals 223×10^{-10}. The standard error is therefore 0.000039, slightly higher than that found for lactose hydrate, 0.000032, and for dextrose, 0.000029, but considerably lower than for levulose according to Saunders' equation based on Jackson's data, 0.000071. The varying sensitivity of the sugars towards heat is probably partly responsible for the differences in the standard errors found.

The refractive indices of maltose hydrate solutions, in steps of 1 per cent, calculated from Equation 1, are shown in Table 2.

TABLE 2.—*Refractive indices of maltose hydrate solutions at 20°C.*

MALTOSE HYDRATE, PER CENT IN AIR	n_D^{20}	MALTOSE HYDRATE, PER CENT IN AIR	n_D^{20}	MALTOSE HYDRATE, PER CENT IN AIR	n_D^{20}
1	1.33439	23	1.36771	45	1.40680
2	1.33579	24	1.36935	46	1.40874
3	1.33721	25	1.37100	47	1.41068
4	1.33863	26	1.37267	48	1.41264
5	1.34007	27	1.37435	49	1.41461
6	1.34151	28	1.37605	50	1.41660
7	1.34297	29	1.37775	51	1.41860
8	1.34443	30	1.37947	52	1.42062
9	1.34591	31	1.38120	53	1.42265
10	1.34739	32	1.38294	54	1.42469
11	1.34889	33	1.38470	55	1.42675
12	1.35040	34	1.38647	56	1.42882
13	1.35191	35	1.38825	57	1.43091
14	1.35344	36	1.39005	58	1.43301
15	1.35498	37	1.39185	59	1.43513
16	1.35653	38	1.39367	60	1.43726
17	1.35809	39	1.39551	61	1.43941
18	1.35967	40	1.39736	62	1.44157
19	1.36125	41	1.39922	63	1.44374
20	1.36285	42	1.40110	64	1.44593
21	1.36445	43	1.40298	65	1.44811
22	1.36607	44	1.40489		

Refractive indices of maltose hydrate solutions at 20°C. have previously been reported by Tolman and Smith (6) and by Körner, Reischel, and

Höppner (7); and at 25°C. by Pulvermacher (8). The values given by Tolman and Smith, to the fourth decimal place, check fairly well with those of the writers up to 10 per cent concentration, but above that point become increasingly higher, as may be noted from Table 3.

TABLE 3.—*Comparison of the refractive indices of Tolman and Smith with those calculated from Equation 1*

MALTOSE HYDRATE, PER CENT IN AIR	n_D^{20} TOLMAN AND SMITH	n_D^{20} EQUATION 1
1.00	1.3343	1.3344
2.07	1.3357	1.3359
5.07	1.3402	1.3402
10.07	1.3477	1.3475
15.12	1.3555	1.3552
20.17	1.3637	1.3631

The results of Körner, Reischel and Höppner are available to the writers only through the abstract in *Chemical Abstracts*. This gives the value 1.3910 for the lowest percentage of maltose used, 33.60; and 1.5000 for the highest percentage, 82.37. The equation for the n_D^{20} of maltose between these limits is given as follows:

$$p = -18277.67 + 36082.638n - 23893.57n^2 + 5332.331n^3. \quad (2)$$

This equation has been converted by the writers into the usual form by the method of averages:

$$n_D^{20} = 1.35328 + 0.0004715140p + 0.00002208982p^2 - 0.00000007492341p^3. \quad (3)$$

This formula gives $n = 1.39122$ for 33.60 per cent maltose, and 1.50012 for 82.37 per cent, in fair agreement with the figures reported by Körner, Reischel and Höppner.

If the value 1.3910 for 33.60 per cent maltose refers to maltose hydrate, the corresponding figure from Equation 1 is 1.3858, very much lower. If it refers to anhydrous maltose the corresponding percentage of hydrate would be 35.57, for which Equation 1 gives an n of 1.3889.

In either case the n of Körner, Reischel and Höppner is higher, but the difference is only 0.0021 under the assumption that their values refer to anhydrous maltose, whereas it amounts to 0.0052 under the assumption that they are for maltose hydrate. Taking the first assumption as correct, the comparison between the values of Körner, Reischel and Höppner and those calculated from Equation 1 is as shown in Table 4.

While the refractive indices of Körner, Reischel and Höppner for dextrose were found to agree with those of the writers within 0.0003, the dis-

TABLE 4.—*Comparison of the refractive indices of Körner, Reischel and Höppner with those calculated from Equation 1*

ANHYDROUS MALTOSE, PER CENT IN AIR	MALTOSE HYDRATE PER CENT IN AIR	n_D^{20} K., R., AND H.	n_D^{20} EQUATION 1
35.00	36.84	1.3936	1.3916
40.00	42.10	1.4027	1.4013
45.00	47.37	1.4124	1.4114
50.00	52.63	1.4227	1.4219
55.00	57.89	1.4336	1.4328
60.00	63.16	1.4449	1.4441

crepancies are much greater for maltose, owing probably to contamination of the maltose of Körner, Reischel and Höppner with dextrin, which is known to have higher refractive indices than maltose at equal concentrations.

Pulvermacher's values of refractive indices of maltose were determined at 25°C., and no direct comparison with the writers' values is possible. But since Pulvermacher's values for n_D^{25} of sucrose and lactose are considerably higher than those found by other investigators (2) it may be expected that his values for maltose are also in error.

SUMMARY

The refractive indices of maltose hydrate solutions containing up to 65 per cent of this sugar have been determined at 20°C. to the fifth decimal place with a Bausch and Lomb Precision Refractometer. The results obtained by previous authors are compared with those of the writers and discussed.

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(1) *This Journal*, **31**, 687 (1948); *J. Research Natl. Bur. Standards*, **41**, 63 (1948).
(2) *Ibid.*, **32**, 709 (1949).
(3) *Ber.*, **69B**, 1827 (1936).
(4) CLELAND and FETZER, *Ind. Eng. Chem., Anal. Ed.*, **14**, 27 (1942).
(5) *This Journal*, **27**, 295 (1944).
(6) *J. Am. Chem. Soc.*, **28**, 1476 (1906).
(7) *Z. anal. Chem.*, **122**, 321 (1941); through *C.A.*, **36**, 5049 (1942).
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REFRACTIVE INDICES OF MALTOSE SOLUTIONS*

By EMMA J. McDONALD (National Bureau of Standards)

INTRODUCTION

The disaccharide, maltose, has found extensive use in the food industry. Starch is converted into "soluble starch" by mild acid treatment and this in turn is hydrolyzed to a mixture of maltose and dextrins by the enzymes of barley flour. Much of the maltose is not further purified but is used as a constituent of this mixture. Upon hydrolysis maltose yields two glucose units which are joined in the maltose molecule to form a 4-D-Glucose D-glucoside. In the present investigation refractive index measurements have been made of aqueous maltose solutions at 20°C. and at 25°C. It is believed that these values will be of use in estimating the quantity of maltose present in solutions of the pure sugar as well as in those containing maltose along with other sugars.

PURIFICATION OF MALTOSE HYDRATE

Maltose crystallizes from aqueous solution and from aqueous alcohol solution as maltose $\cdot H_2O$. Considerable care is required to remove the last traces of dextrins. The preparation of the pure sugar by crystallization from alcohol, from acetic acid[1] and by deacetylation of the acetate[2] have been discussed in the literature.

The sugar used in this investigation was prepared by three recrystallizations of maltose from aqueous solution at approximately 5°C. The starting product was the C. P. maltose of Pfanstiehl and of Eastman Kodak Company. The sugar was dissolved in water and the solution filtered through carbon, evaporated under reduced pressure, and crystallized in an ice bath with constant stirring. After the first crystallizations the carbon was omitted. The final crystals were dried at room temperature, and, finally, at 50°C. They gave a very clear solution, were ash free, and had a specific rotation of +130.5 (water, C=4.73). This crystallization from water solution gives lower yields than when alcohol is used but has the advantage that small amounts of dextrose or dextrins remain in solution.

The maltose used for the refractive index determinations was found to contain 0.14 per cent moisture in excess of the theoretical 5 per cent for maltose hydrate. This moisture content was determined by a procedure used previously in this laboratory by R. F. Jackson, who found that carefully prepared maltose hydrate dried at room temperature, and finally at 50°C. contained 5.13 per cent of moisture rather than the theoretical 5.00 per cent. He dried maltose to constant weight in a vacuum over

* This paper appears also in the March 1951 issue of the Journal of Research of the National Bureau of Standards.

[1] T. S. Harding, *Sugar*, 25, 350 (1923).

[2] G. Zemplén, *Ber.*, 59, 1258 (1926); *Ber*, 60, 2, 1555 (1927).
G. Zemplén, and E. Pacsu, *Ber*, 62, 1613 (1929).
W. A. Mitchell, *J. Am. Chem. Soc.*, 63, 3534 (1941).

P_2O_5. The initial drying temperature of 64°C. was slowly increased to 105°C. In order to be assured that the loss of weight, in excess of 5.0 per cent, was not due to decomposition, a sample of the sugar hydrate was allowed to come to equilibrium in an atmosphere whose vapor pressure was that of pure maltose hydrate. This vapor pressure was main-

TABLE 1.—*Drying of maltose hydrate in vacuum over P_2O_5*

SAMPLE 1, 1.16355 g				SAMPLE 2, 1.1093 g			
TEMPERATURE	TIME	LOSS	TOTAL LOSS	TEMPERATURE	TIME	LOSS	TOTAL LOSS
°C.	*hours*	*mg*	*mg*	°C.	*hours*	*mg*	*mg*
76	26	42.1	42.1	76	26	44.1	44.1
76	19	2.3	44.4	76	19	2.1	46.1
100	3	2.0	46.4	100	3	1.6	47.8
100	7	1.8	48.2	100	7	1.4	49.2
100	3	0.3	48.5	100	3	0.5	49.7
100	3.5	.6	49.1	100	3.5	.6	50.3
105	4	2.2	51.1	110	3	3.8	54.1
105	8	2.5	53.6	110	2.2	1.9	56.0
105	8.2	1.7	55.3	110	2	0.4	56.4
105	6.4	1.3	56.6	110	2	.3	56.7
105	10.3	1.8	58.4	110	4	0	56.8
105	6.8	0.4	58.8				
105	7.2	.5	59.3	Total	74.7		
105	6	.25	59.5				
105	4	.15	59.7				
105	4.5	.1	59.8				
105	4	0	59.8				
Total	130.9						

Moisture 5.14 per cent

Moisture 5.12 per cent

TABLE 2.—*Maltose hydrate sample in atmosphere having vapor pressure as maltose H_2O*

TEMPERATURE	TIME	LOSS	TOTAL LOSS
°C.	*hours*	*mg*	*mg*
36	40	0.65	0.65
56	40	.3	.95
56	40	.1	1.05
56	45	.2	1.25
54	45	−.1	1.15
55	45	0	1.15
Total Time	255		

Loss 0.13 per cent

tained by means of a mixture containing equal amounts of maltose hydrate and partially dehydrated maltose hydrate. A relatively small sample of the sugar hydrate after standing 255 hours at 56°C. in this environment reached a constant weight and had lost 0.13 per cent of its weight.

Tables 1 and 2 record data obtained by Dr. Jackson.

MEASUREMENTS AND DISCUSSION OF RESULTS

The refractive index measurements were made on solutions whose concentrations varied from approximately one to sixty-five per cent of maltose hydrate. All measurements were made under carefully controlled temperature conditions. Observations over the entire range of concentration were made on a Bausch and Lomb Precision Refractometer, and at the lower concentrations a Zeiss Dipping Refractometer was also used. These instruments were calibrated at this Bureau. A measurement of the refractive index of water[3] was made before and after that of a maltose solution and proper corrections were applied. The used value was the average of four or five readings on a given solution. Each solution stood at least four hours to allow the maltose to reach equilibrium.[4]

TABLE 3.—*Refractive indices of maltose solutions at 20°C.*

PERCENTAGE OF MALTOSE HYDRATE	n_D^{20} OBSERVED	n_D^{20} CALCULATED[1]	OBS.-CALC.
1.563	1.33518	1.33517	+0.00001
5.015	1.34008	1.34008	.00000
7.003	1.34294	1.34296	− .00002
12.929	1.35180	1.35179	+ .00001
14.410	1.35404	1.35406	− .00002
20.512	1.36362	1.36366	− .00004
25.586	1.37200	1.37197	+ .00003
25.197	1.37135	1.37132	+ .00003
31.034	1.38126	1.38125	+ .00001
36.666	1.39128	1.39124	+ .00004
40.995	1.39918	1.39921	− .00003
44.801	1.40640	1.40641	− .00001
50.713	1.41807	1.41800	+ .00007
55.537	1.42781	1.42782	− .00001
59.798	1.43674	1.43677	− .00003
65.529	1.44920	1.44922	− .00002
	Average deviation ±0.000024		

[1] Equation (1) used in obtaining calculated values.

[3] L. W. Tilton and J. K. Taylor, *NBS J. Research*, **20**, 419 (1938).
[4] H. S. Isbell and W. W. Pigman, *NBS J. Research*, **18**, 183 (1937).

Sixteen observations were made at 20°C. and eight observations at 25°C. Equations relating the refractive index to the per cent concentration of sugar hydrate (p) were calculated by the method of averages from the observed data. Equations (1) and (2) express this relationship.

$$n_D^{20} = 1.33299 + 1.38914 \times 10^{-3}p + 4.76016 \times 10^{-6}p^2 + 2.0933 \times 10^{-8}p^3 - 6.124 \times 10^{-11}p^4 \quad (1)$$

$$n_D^{25} = 1.33250 + 1.38275 \times 10^{-3}p + 3.93026 \times 10^{-6}p^2 + 5.2527 \times 10^{-8}p^3 - 3.499 \times 10^{-10}p^4. \quad (2)$$

TABLE 4.—*Refractive indices of maltose solutions at 25°C.*

PERCENTAGE OF MALTOSE HYDRATE	n_D^{25} OBSERVED	n_D^{25} CALCULATED[2]	OBS.-CALC.
3.312	1.33709	1.33712	−0.00003
10.180	1.34707	1.34703	+ .00004
12.530	1.35051	1.35054	− .00003
25.530	1.37026	1.37023	+ .00003
39.837	1.39633	1.39626	+ .00007
40.405	1.39725	1.39732	− .00007
50.992	1.41777	1.41783	+ .00006
65.551	1.44842	1.44836	− .00006
	Average deviation ±0.000049		

[2] Equation (2) used in obtaining calculated values.

Tables 3 and 4 contain the observed data along with the calculated values. At 20°C. the average deviation of the calculated values from those observed amounts to ±0.000024 while at 25°C. the corresponding average deviation is ±0.000049. At both temperatures the deviations at the higher concentrations are greater than in the lower range. This may be attributed to the increased difficulty in the preparation and the handling of the solutions of higher concentration. The refractive indices presented in Table 5 were calculated by means of equations (1) and (2). The temperature coefficient of maltose solutions appears to be very similar to that of sucrose solutions in this temperature range. The apparent decrease in temperature effect above 44 per cent concentration is small and may be a result of experimental error. It is of the same order of magnitude as the deviation of the observed values from the calculated values. At 21°C. a saturated maltose solution contains 46.4 per cent of sugar; thus the solutions with concentration above 46.4 per cent are supersaturated and are more subject to error due to shifting of the equilibrium products. It appears that a temperature coefficient of 0.00016 would be within the

TABLE 5.—*Refractive indices of maltose hydrate solutions at 20°C. and 25°C.*

PER CENT	n_D^{20}	n_D^{25}	Δn	$\Delta n/\Delta t$
1	1.33438	1.33389	0.00049	0.00010
2	1.33579	1.33528	.00051	.00010
3	1.33720	1.33668	.00052	.00010
4	1.33862	1.33810	.00052	.00010
5	1.34006	1.33952	.00054	.00011
6	1.34150	1.34095	.00055	.00011
7	1.34295	1.34239	.00056	.00011
8	1.34442	1.34384	.00058	.00012
9	1.34589	1.34530	.00059	.00012
10	1.34738	1.34677	.00061	.00012
11	1.34887	1.34825	.00062	.00012
12	1.35039	1.34974	.00065	.00013
13	1.35190	1.35124	.00066	.00013
14	1.35343	1.35276	.00067	.00013
15	1.35497	1.35428	.00069	.00014
16	1.35652	1.35582	.00070	.00014
17	1.35808	1.35737	.00071	.00014
18	1.35965	1.35893	.00072	.00014
19	1.36124	1.36051	.00073	.00015
20	1.36283	1.36209	.00074	.00015
21	1.36444	1.36369	.00075	.00015
22	1.36606	1.36530	.00076	.00015
23	1.36770	1.36692	.00078	.00016
24	1.36934	1.36856	.00078	.00016
25	1.37100	1.37021	.00079	.00016
26	1.37267	1.37187	.00080	.00016
27	1.37435	1.37355	.00080	.00016
28	1.37604	1.37524	.00080	.00016
29	1.37775	1.37694	.00081	.00016
30	1.37946	1.37865	.00081	.00016
31	1.38120	1.38038	.00082	.00016
32	1.38294	1.38213	.00081	.00016
33	1.38470	1.38388	.00082	.00016
34	1.38647	1.38565	.00082	.00016
35	1.38825	1.38744	.00081	.00016
36	1.39004	1.38924	.00080	.00016
37	1.39185	1.39105	.00080	.00016
38	1.39367	1.39287	.00080	.00016
39	1.39551	1.39471	.00080	.00016
40	1.39735	1.39656	.00079	.00016
41	1.39922	1.39843	.00079	.00016
42	1.40109	1.40031	.00078	.00016
43	1.40298	1.40221	.00077	.00016
44	1.40488	1.40411	.00077	.00015
45	1.40680	1.40603	.00077	.00015
46	1.40873	1.40797	.00076	.00015
47	1.41067	1.40992	.00075	.00015
48	1.41263	1.41188	.00075	.00015
49	1.41460	1.41385	.00075	.00015

TABLE 5—(*continued*)

PER CENT	n_D^{20}	n_D^{25}	Δn	$\Delta n/\Delta t$
50	1.41658	1.41584	.00074	.00015
51	1.41858	1.41784	.00074	.00015
52	1.42059	1.41986	.00073	.00015
53	1.42262	1.42189	.00073	.00015
54	1.42466	1.42392	.00074	.00015
55	1.42672	1.42598	.00074	.00015
56	1.42878	1.42804	.00074	.00015
57	1.43087	1.43012	.00075	.00015
58	1.43296	1.43221	.00075	.00015
59	1.43508	1.43431	.00077	.00015
60	1.43720	1.43643	.00077	.00015
61	1.43934	1.43855	.00079	.00016
62	1.44150	1.44069	.00081	.00016
63	1.44367	1.44283	.00084	.00017
64	1.44585	1.44499	.00086	.00017
65	1.44805	1.44716	.00089	.00018

experimental error of the data for the concentration range above 23 per cent.

Work by F. W. Zerban and J. Martin* on the refractive indices of maltose solutions at 20°C. is in good agreement with the results obtained in this investigation. Previous work may be summarized as follows. Tolman and Smith[5] reported the refractive indices of maltose solutions of relatively low concentrations. In their discussion they state that the sugars were dried at 70°C. under a diminished pressure of about 27 inches; no other comment is made as to the preparation of the sugar. The following values are reported by these authors and a comparison is made to the values obtained in this investigation.

Percentage of maltose	n_D^{20} T & S	n_D^{20} McD
1.00	1.3343	1.3344
2.07	1.3357	1.3359
5.07	1.3402	1.3402
10.07	1.3477	1.3475
15.12	1.3555	1.3552
20.17	1.3637	1.3631

Pulvermacher[6] determined the refractive indices of maltose solutions whose concentrations varied from 1.16 to 19.40 per cent of anhydrous sugar at 25°C. His results are reported to the fourth place and the values are all higher by one in the third place than the results here presented.

* See paper immediately preceding this one.
[5] L. M. Tolman and W. B. Smith, *J. Am. Chem. Soc.*, **28**, 1476 (1906).
[6] G. Pulvermacher, *Z. anorg. Chem.*, **113**, 141 (1920).

Refractive indices of maltose hydrate solutions at 20°C—comparatice figures

PER CENT MALTOSE HYDRATE	n_D^{20} ZERBAN	n_D^{20} MCDONALD	DIFFERENCE	PER CENT MALTOSE HYDRATE	n_D^{20} ZERBAN	n_D^{20} MCDONALD	DIFFERENCE	PER CENT MALTOSE HYDRATE	n_D^{20} ZERBAN	n_D^{20} MCDONALD	DIFFERENCE
1	1.33439	1.33438	0.00001	23	1.36771	1.36770	0.00001	45	1.40680	1.40680	0
2	1.33579	1.33579	.00000	24	1.36935	1.36934	.00001	46	1.40874	1.40873	0.00001
3	1.33721	1.33720	.00001	25	1.37100	1.37100	.00000	47	1.41068	1.41067	.00001
4	1.33863	1.33862	.00001	26	1.37267	1.37267	.00000	48	1.41264	1.41263	.00001
5	1.34007	1.34006	.00001	27	1.37435	1.37435	.00000	49	1.41461	1.41460	.00001
6	1.34151	1.34150	.00001	28	1.37605	1.37604	.00001	50	1.41660	1.41658	.00002
7	1.34297	1.34295	.00002	29	1.37775	1.37775	.00000	51	1.41860	1.41858	.00002
8	1.34443	1.34442	.00001	30	1.37947	1.37946	.00001	52	1.42062	1.42059	.00003
9	1.34591	1.34589	.00002	31	1.38120	1.38120	.00000	53	1.42265	1.42262	.00003
10	1.34739	1.34738	.00001	32	1.38294	1.38294	.00000	54	1.42469	1.42466	.00003
11	1.34889	1.34887	.00002	33	1.38470	1.38470	.00000	55	1.42675	1.42672	.00003
12	1.35040	1.35039	.00001	34	1.38647	1.38647	.00000	56	1.42882	1.42878	.00004
13	1.35191	1.35190	.00001	35	1.38825	1.38825	.00000	57	1.43091	1.43087	.00004
14	1.35344	1.35343	.00001	36	1.39005	1.39004	.00001	58	1.43301	1.43296	.00005
15	1.35498	1.35497	.00001	37	1.39185	1.39185	.00000	59	1.43513	1.43508	.00005
16	1.35653	1.35652	.00001	38	1.39367	1.39367	.00000	60	1.43726	1.43720	.00006
17	1.36809	1.35808	.00001	39	1.39551	1.39551	.00000	61	1.43941	1.43934	.00007
18	1.35967	1.35965	.00002	40	1.39736	1.39735	.00001	62	1.44157	1.44150	.00007
19	1.36125	1.36124	.00001	41	1.39922	1.39922	.00000	63	1.44374	1.44367	.00007
20	1.36285	1.36283	.00002	42	1.40110	1.40109	.00001	64	1.44593	1.44585	.00008
21	1.36445	1.36444	.00001	43	1.40298	1.40298	.00000	65	1.44811	1.44805	.00006
22	1.36607	1.36606	.00001	44	1.40489	1.40488	.00001				

Since results reported in the same article by Pulvermacher are higher for glucose than the accepted values it appears that perhaps some discrepancy persisted throughout his measurements.

SUMMARY

The refractive indices of maltose hydrate solutions have been determined at 20°C. and at 25°C. Equations relating the reference index to the per cent concentration of sugar have been prepared from the observed data by the method of averages. A table, giving the refractive indices of maltose solutions at one per cent intervals over a range of 1 to 65 per cent of maltose hydrate at both 20° and 25°C., is included.

The table on the preceding page gives values for the refractive indices of maltose solutions which were obtained independently by F. W. Zerban of the New York Sugar Trade Laboratory, and by Emma J. McDonald of the National Bureau of Standards, which are given in the two papers which immediately precede.

A PROPOSED MODIFICATION OF THE A.O.A.C. METHOD FOR CAROTENE IN ALFALFA

By C. R. THOMPSON and E. M. BICKOFF

(Western Regional Research Laboratory,[1] Albany, Calif.)

INTRODUCTION

A collaborative study of the A.O.A.C. method for carotene in alfalfa meal by about 70 laboratories has been recently (1) completed. Results of the assays reported by the various laboratories differed considerably. In general, the average values obtained by the A.O.A.C. method were lower than by other methods studied. Studies in this Laboratory have indicated that the method as now outlined may result in incomplete recovery of carotene under certain conditions. A method is proposed which eliminates these sources of error and yields reproducible results.

METHODS

The method approved as official by the A.O.A.C. for carotene determination in dried plant materials is essentially as follows: A 1–2 gm sample is refluxed for one hour with 30 ml of a mixture of 3 parts acetone and 7 parts Skellysolve B, is transferred quantitatively to a 100-ml volumetric flask, and the entire sample is chromatographed on a 10-cm. column of magnesia No. 2642.[2] The carotene is eluted with 50 ml of a mixture of 1

[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.
[2] Westvaco Chlorine Products Company.

part acetone in 9 parts Skellysolve B, collected in a 100-ml flask, and the density determined at 440 mμ. Recently the A.O.A.C. has adopted overnight extraction as an alternative to the one-hour refluxing as a means of extraction (2).

The proposed method uses the latter means of extraction and is as follows: A 1–2 gm sample is extracted overnight in the dark in a 100-ml volumetric flask with 30 ml of a mixture of 3 parts acetone and 7 parts Skellysolve B at room temperature. After standing, the extract is diluted to 100 ml with Skellysolve B, and a 5-ml aliquot is chromatographed on a column 12 mm in diameter packed to 40 mm in height with an equal parts mixture by weight of magnesium oxide No. 2642 and filter aid. The carotene is eluted with a mixture of 1 part acetone and 9 parts Skellysolve B until 25 ml is collected in a volumetric flask. The density of the solution is read at 440 mμ in a colorimeter or a spectrophotometer. When a series of 10 replicate samples was run by this method the coefficient of variation was 1.4 per cent.

In this method quantitative transfers are eliminated, a smaller column is used, and a larger ratio of eluant to sample put on the column, is employed.

RESULTS

Preliminary studies with the A.O.A.C. method on a given sample showed that when 50 ml of 10 per cent acetone in petroleum ether was used as eluant (as recommended) the total carotene content of the sample was 287 μg/gm. Since carotenoid, presumably carotene, was still coming through the column when the eluant was exhausted, another 25 ml of eluent was added. This increased the total carotene to 293 μg/gm. An additional 25 ml gave values of 299 μg/gm. These studies suggested that all of the carotene had not been removed by the prescribed volume of eluant. In continued work, a series of samples of three lots of alfalfa meal were extracted by the A.O.A.C. and the proposed procedure. Different means of chromatographic separation were tried (Table 1). When the two procedures were compared, the A.O.A.C. method gave results which varied from 3 to 16 per cent lower than those obtained with the proposed procedure.

When the pigment on the column in the A.O.A.C. method was eluted with an additional 50 ml of 10 per cent acetone, more carotene was eluted (Table 1). The similarity of the carotenes in the first and second eluates was shown by a determination of their absorption with a recording spectrophotometer (Fig. 1). The shift in maxima shown by the carotene in the second eluate (A.O.A.C. method) was caused by a higher percentage of neo-beta-carotene U, which is more tightly adsorbed than the other stereoisomers. This was demonstrated by a determination of the amount of each of the three principal stereoisomers in the second and the combined

eluates. The method used has been described previously (4). The combined eluate had 40 per cent all-trans, 43 per cent neo-beta-carotene B, and 17 per cent neo-beta-carotene U. The second eluate had 3 per cent neo-beta-carotene B, 33 per cent all-trans, and 64 per cent neo-beta-carotene U. It should be noted that the concentration of the carotene in the solutions used was adjusted to give comparable densities and does not represent amounts proportional to that obtained in given eluates.

The use of the hot extraction according to the A.O.A.C. procedure followed with chromatography by the proposed procedure (Table 1, No. 3)

TABLE 1.—*Comparison of A.O.A.C. method for carotene analysis with proposed procedure*

A.O.A.C. PROCEDURE	CAROTENE IN MICROGRAMS PER GRAM			AVERAGE PER CENT OF HIGHEST VALUE
	SAMPLE A	SAMPLE B	SAMPLE C	
1) No change	188	106	162	84
2) Develop with additional 50 ml solvent	221	117	173	93
3) Simplified chromatography of AOAC Extract	235	122	180	98
Proposed Procedure				
4) No change	246	123	181	(100)
5) Extend column from 4 to 7 cm ht.	244	121	178	98
6) Use of AOAC column (10 cm)—develop with 50 ml	232	106	165	91
7) Use of AOAC column (10 cm)—develop with 100 ml	246	114	170	95
8) Use of short AOAC column (5 cm.)—develop with 50 ml	246	112	177	96

gave an average recovery of 98 per cent. To determine whether or not an increase in column length in the proposed procedure would decrease carotene recovery, the column was extended to 7 cm. A 98 per cent recovery was obtained. The meal remaining after chromatography by the A.O.A.C. method was re-extracted overnight in the dark and chromatographed by the proposed procedure. Two per cent more carotene was obtained.

With overnight extraction on another sample, followed by use of the large A.O.A.C. column, a low value was obtained (91 per cent), but development with an additional 50 ml of eluant increased the recovery to 95 per cent. A reduction in the length of the A.O.A.C. column to 5.0 cm gave essentially the same recovery as increasing the amount of eluant (Table 1).

Since the exposure of alfalfa extracts to light and heat have been shown (3) to produce rapid changes in the ratio of stereoisomers of beta-carotene, the isomer content of an extract prepared by the A.O.A.C. method was

compared with one from the proposed procedure. Both were compared with the original extract, which was prepared by adding 25 ml of Skellysolve B to 10 gm of meal in the dark and soaking for 30 min. (4).

The results showed that beta-carotene in the original extract from this sample was 53 per cent all-trans, 33 per cent neo-beta-B, and 13 per cent neo-beta-U. After overnight extraction by the proposed method the solu-

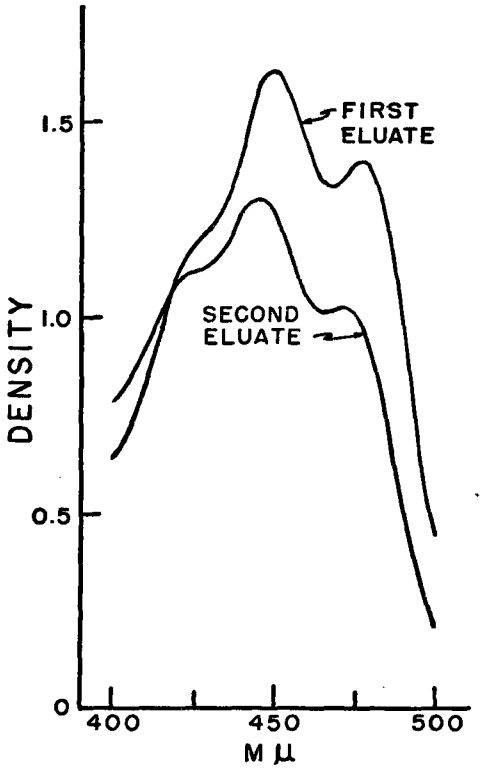

FIG. 1.—Comparison of absorption spectrum of fraction collected in A.O.A.C. procedure (first eluate) with that collected by passing an additional 50 ml of eluant through the column (second eluate).

tion contained 52 per cent all-trans, 30 per cent neo-B, and 17 per cent neo-U. With the A.O.A.C. extraction, because of the exposure to light on the steam bath (3), the neo-B was 5 per cent, while the all-trans and neo-U were 74 and 21 per cent, respectively. Since neo-B and neo-U have 78 and 95 per cent, respectively, of the absorptivity of all-trans (5), the total absorption for this sample would be increased about 6 per cent when determined by a filter-type instrument at 440 mμ. With a spectrophotometer

at 436 mμ, the specific absorption coefficients for all-trans, neo-B, and neo-U are 196, 176, and 191, respectively (6). The change in the isomer content would give about 2.5 per cent increased absorption.

DISCUSSION

These results show that recovery of carotene from the chromatographic column by the A.O.A.C. method may not be complete. This may be caused by two factors. The chromatographic step in the A.O.A.C. method does not provide enough eluant to give a margin of safety which will assure complete removal of carotene from the column. A change in the adsorbent activity of the magnesia from batch to batch, or because of lowered humidity, carbon dioxide content, etc., may cause appreciable retention of carotene on the column. Further, unless an efficient condenser is used in the procedure, solvent may be lost during reflux and the resulting solution when diluted will have too little acetone. This also will prevent complete removal of carotene from the column if the specified volume of eluant is used.

The hot extraction of pigments which is performed in laboratory light may cause enough isomerization of the neo-B to all-trans to increase the total absorption sufficiently to compensate, partially, for the low recovery of pigment from the column. The small amount of carotene (2 per cent) remaining in the meal after hot extraction accounted partially for the low results by the A.O.A.C. method. Chromatography of the hot extract by the proposed procedure gave 98 per cent recovery. Actually about 106 per cent should have been obtained because of the increased content of all-trans carotene in the extract. Since this higher value was not obtained, some destruction of carotene must have occurred during the hot extraction.

The specification of a 1–1 mixture of adsorbent ingredients by weight in the proposed procedure gives a standard mixture which speeds chromatography and avoids changes in the ratio of components caused by the variable density of the filter aid. The use of an aliquot simplifies manipulations because the need for a quantitative transfer of the entire extract to the column is eliminated.

SUMMARY

The present A.O.A.C. method for carotene in plant materials contains several inherent sources of error. These include the possibility of re-isomerization, partial destruction, and incomplete removal of the carotene during the hot extraction of the meal.

The specification of an insufficient amount of eluant coupled with the possibility of variation in the degree of adsorptive power of the magnesia used in the chromatographic adsorption contributes to incomplete elution of the carotene and consequent low carotene assays. If loss of acetone

occurs during reflux, increased retention of carotene during chromatographic adsorption will result, with consequent low carotene assays.

A procedure is proposed which eliminates these sources of error. It eliminates the need for quantitative transfer of extract, employs smaller columns and specifies sufficient eluant to remove carotene completely from the column. The chromatographic step is more rapid and requires less solvent. Because of increased simplicity, it lends itself better to the routine determination of large numbers of samples.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of S. Friedlander for spectrophotometric determinations.

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A NOTE ON THE MICROBIOLOGICAL ASSAY FOR VITAMIN B_{12}*

By H. W. Loy, Jr., and O. L. Kline (Division of Nutrition, Food & Drug Administration, Federal Security Agency, Washington 25, D. C.)

In the microbiological assay of products for their vitamin B_{12} potency, the standard of reference used is a solution of crystalline vitamin B_{12}. Liver extract preparations, and vitamin B_{12} concentrates prepared from fermentation sources, are said to contain primarily vitamin B_{12b}. The stability of vitamin B_{12b} in the microbiological procedure has been questioned.

Of the various modifications proposed for the microbiological estimation of vitamin B_{12} potency, the one recently subjected to extensive collaborative study under the auspices of the Anti-Pernicious Anemia Preparations Board and the Vitamin Advisory Board of the U. S. Pharmacopeia, has been found most successful in this laboratory. This method employs the microorganism *L. leichmannii* A.T.C.C. No. 7830, with titration of acidity formed during a 72 hour incubation, in a basal medium containing 20 mg. ascorbic acid per assay tube. In view of the known instability of vitamin B_{12b} in the presence of ascorbic acid, it was important to determine whether or not this form of the vitamin is accurately measured by this assay method.

Vitamin B_{12b} is readily converted to the more stable cyano form in the presence of excess cyanide.[1] The vitamin B_{12} potencies of the following solutions were determined: (a) a solution of B_{12b};[2] (b) an aliquot of this solution converted with cyanide; (c) a second aliquot similarly converted, then aerated to remove excess cyanide.

A solution of Vitamin B_{12}[2] was treated similarly, assays were conducted with sterilization times of 2 minutes and 20 minutes, and results in micrograms per ml. are given in Table 1.

TABLE 1.—*Vitamin B_{12} potency*

TREATMENT	SOLUTION B_{12}		SOLUTION B_{12b}	
	2 min.	*20 min.*	*2 min.*	*20 min.*
None	20.0	20.0	19.9	20.1
CN conversion	19.9	19.9	19.9	20.1
CN conversion with aeration	20.0	19.9	19.9	20.3

Assuming complete conversion of the B_{12b} (hydroxo-) to the B_{12} (cyano-) form in the B_{12b} solutions, it is demonstrated here that under conditions of this assay B_{12} and B_{12b} are equally stable and promote equal microbiological response.

To further determine the stability of vitamin B_{12b} to heat during the sterilization process, we made aseptic additions of solutions of B_{12} and B_{12b} to the assay tubes and compared the microbiological response with that of the same solutions added before sterilization. The comparisons are given in Table 2.

It is clear that both forms of the vitamin, after being heated in the basal medium, have an enhanced microbiological value. Other trials have indicated that B_{12} added aseptically has 50 to 65%, and B_{12b}, 80 to 90%, the potency of the heated forms.

The lowered potency of the aseptically added solutions does not result from agi-

* Approved for publication, February 9, 1951.

[1] The procedure for cyanide conversion was that obtained from Dr. W. E. Gaunt, E. R. Squibb and Sons (personal communication).

[2] We are grateful to Dr. D. V. Frost, Abbott Laboratories, for a generous supply of vitamin B_{12b} solution, and to Dr. A. B. Scott, Merck and Co., for crystalline vitamin B_{12} used in these studies.

TABLE 2.—*Vitamin B_{12} potency*
(micrograms per ml.)

	STERILIZED IN MEDIUM	ADDED ASEPTICALLY
Solution B_{12}	20.0	13.1
Solution B_{12b}	19.9	17.7

tation of the medium by the mechanical addition of the solutions. This was demonstrated by substituting water for the vitamin solutions. When the B_{12} was sterilized in the medium at 10 times the concentration used in the assay tubes, then diluted with sterilized basal medium, aseptic additions gave the enhanced potency value. However, when such solution was diluted with sterile water rather than with sterile medium, aseptic addition resulted in the lowered potency.

TABLE 3.—*Vitamin B_{12} potency*
(micrograms per ml.)

B_{12} + medium + water, sterilized	20.0
B_{12} + medium, sterilized then H_2O added aseptically	20.2
Medium sterilized, then B_{12} added aseptically	12.0
B_{12} + medium, sterilized, diluted 1:10 in sterile medium, then added aseptically	20.1
B_{12} + medium, sterilized, diluted 1:10 in sterile water, then added aseptically	12.0

We may conclude from these microbiological observations that vitamin B_{12b} is fully as stable under the conditions of the assay procedure as vitamin B_{12} itself. From the evidence that both forms of the vitamin have an increased microbiological potency after heating in the basal medium, we may postulate the conversion of both under these conditions to a form that is more readily utilized by the microorganism.

Spectrophotometric studies of these solutions support such a conclusion. We are indebted to John H. Jones and Lee S. Harrow of the Division of Cosmetics of this Administration for making spectrophotometric measurements of the two forms of the vitamin, heated and unheated, in the basal medium. It was observed that the characteristic absorption peak of B_{12} at about 360 mμ is not changed when B_{12} is added aseptically to the sterilized medium; but when B_{12} is sterilized with the basal medium the characteristic absorption peak disappears and a broad band with a maximum between 340 and 350 mμ is observed. With B_{12b}, whether added aseptically or before sterilization of the medium, the characteristic absorption peak at about 351 mμ does not appear. However, the density of the absorption for the sterilized mixture, between 350 and 370 mμ is considerably greater.

A more detailed report of these studies will be described later.

BOOK REVIEWS

Pocket Encyclopedia of Atomic Energy. By Frank Gaynor. Philosophical Library, New York, N. Y., publishers. 204 pages, 34 charts and tables. Price $7.50.

As the author so aptly states in his preface, this is not a textbook, but a sort of tourist's guide to the new and strange realm of the atom. The definitions and explanations are concise and sufficiently simple so that they can be of help to those who are not very conversant with this new field of physics, but who wish to acquire a "speaking" acquaintance without the extensive labor of recourse to original papers and texts. By the same token, however, this book will probably not appeal to the specialist in the atomic energy field.

The author is to be congratulated on his attempt to make this encyclopedia as up to date as possible. Probably, the chief criticism will be that certain parts of the book will be out of date within less than a year, and it is hoped that this inherent difficulty can be forestalled by frequent editions containing the necessary revisions.

Three interesting and useful features of this book are (1) listings of the German equivalents to many of the topics, (2) short biographical sketches of the outstanding authorities in the field and (3) listing of the radioactive as well as the stable isotopes of each element with percentage frequency of occurrence of the latter.

Perhaps it became necessary in order to keep this volume within "pocket" bounds to limit bibliographic references to charts and figures. In the opinion of the reviewer, the value of this encyclopedia would be considerably enhanced if some bibliographic references could also be appended to the textual contents.

E. P. LAUG

The Technology and Chemistry of Alkaloids. By Frank E. Hamerslag, Consulting Chemist, D. Van Nostrand Company, Inc., 1950, viii+319 pp. Price $6.50.

A single volume covering the many aspects of alkaloids, including the chemistry, natural sources, manufacturing methods, pharmacological properties, therapeutic uses, and analytical methods, cannot be comprehensive for any individual topic. To be of value, however, it must present a general picture of all the topics involved, with special emphasis directed in accord with the author's concept of the interest of those who will read the book.

"This book is primarily designed," the author states in the preface, "to help those engaged in or planning the manufacture of alkaloids." Accordingly, principal emphasis is given to manufacturing procedures, from the initial treatment of the crude drug to purification of the final product. The principles involved in the various steps are well covered, and alternate processes are described. Frequently, however, the descriptions are over-detailed; some sections of a single process are presented much like experiments in a chemistry laboratory manual, while other sections of the same process include only a general survey of the problem.

The presentation of the chemistry of the alkaloids is essentially a development of the salient reactions leading to the determination of structure. All reactions are well documented with references to original source. The value of this presentation is impaired by the faulty use of structural formulas. It is frequently difficult to identify the formulas with their subject matter in the text; simply numbering the formulas would add greatly to the clarity of this portion of the book. More seriously, indiscriminate use is made of symbols; e.g., the hexagon at times represents the aromatic ring, and at other times the saturated ring. As an example, in the formula of a diphenyl ether derivative depicted on page 85, one of the two benzene rings is symbolized by the conventional Kekule ring with three double bonds, while the other is a simple hexagon. In two other formulas on the same page, the benzene ring has only two double bonds.

The description of the botanical sources of the alkaloids is concise, and generally includes the descriptions given in such works as the National Formulary, the Dispensatory, and the Pharmacopeias. A shorter presentation of the pharmacological and therapeutic action of the alkaloids is given, while the description of analytical methods is quite sketchy.

Other than those noted above, errors are few. Inversion of one of the two asymetric carbon atoms of d-pseudoephedrine is incorrectly termed racemization (page 109); on page 159, the term "iodic acid" is used for hydriodic acid.

Conspicuous by their absence are the opium, the cinchona and the purine alkaloids. Despite the statement in the preface that "since this book deals mainly with the commercially practical alkaloids, . . . the purpose of the book would not be materially affected if the morphine and cinchona alkaloids were omitted," the reviewer feels that the inclusion of alkaloids of such wide commercial importance would be of value. Exclusion of the opium alkaloids might be explained on the basis of their illicit application; but the equally proscribed cocoa alkaloids are included.

This book will be of interest to a wider circle than those for whom it was primarily designed (as quoted above). Those engaged in all phases of pharmaceutical chemistry will find the over-all coverage to provide a good background for this important family of natural products.

JOSEPH LEVINE

Principles of Ionic Organic Reactions. By Elliot R. Alexander. vii+318 pages. 1950. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, N. Y. $5.50.

In recent years the tendency to apply a more theoretical approach to explain the mechanism of organic reactions has become more and more popular. So far this trend has not progressed far beyond an explanation of observed experimental facts; *i.e.*, to predict with any reasonable accuracy how an untried reaction will proceed may be possible in a few cases, but, in general, the true facts can be determined only by the well established but time-consuming experimental approach. However, the application of this relatively new theorecital knowledge can suggest in many cases what reaction products one should look for in a hitherto untried reaction.

For most of those who studied organic chemistry before the advent of some of these new approaches, ionic organic reactions were practically limited to the reaction of organic acids and bases. Today a great variety of organic reactions are recognized to be ionic in nature. Whereas earlier books on the subject tended toward physical and mathematical treatment which was not too easily digested by the ordinary organic chemist, this book avoids these hindrances and with careful reading can be of considerable help to the understanding of the "why" of organic reactions.

The book is designed as a text "for advanced undergraduates and first-year graduate students. First an attempt has been made to present from the point of view of an organic chemist the mechanisms which seem to be most reasonable for a number of organic reactions together with the pertinent data which support them." The author has "attempted to present the material in a sequence that will stress the *similarities* rather than the differences between seemingly diverse organic reactions."

The language and tools used to discuss the principles of reactions may not be too familiar to older chemists. Some of the terms used are "polarization," "hyperconjugation" and especially "resonance." The data from which reaction mechanisms are deduced may be obtained from reaction rates, from the extent of racemization, in a given reaction, or particularly from the use of isotopes.

As the title of the book specifies, only organic reactions which are most likely ionic in nature are discussed. Examples are the Friedel-Crafts reaction, the alkylation of acetoacetic ester, aromatic substitutions, dehydrations, the aldol or Claisen condensations, and esterification, including the reverse reaction—hydrolysis of esters. Esterification is ordinarily written as $RCOOH+R'OH=RCOOR'+H_2O$. Such a picture tells nothing about the mechanism of the reaction; *i.e.*, about the intermediate products which may have a transitory existence in the total process. In the earlier days of organic chemistry a much discussed question was: where does the water formed in esterification originate? That is, does the hydroxyl (OH) come from the acid (RCO/OH) or from the alcohol (R'/OH). Even though the answer was reasonably certain before the advent of isotopes, the use of methanol containing isotopic oxygen (O^{18}) proved that the hydroxyl comes from the acid, since the water formed contained no oxygen of atomic weight 18.

The scope of this book is defined by its chapter headings: Factors Influencing Distribution and Mobility of Electrons; Carbonium Ions; Displacement Reactions; Elimination Reactions; Carbanions; Addition Reactions of Carbon-Carbon Double Bonds; Noncarbanion Additions to Carbon-Oxygen and Carbon-Nitrogen Multiple Bonds; Carbanion Addition to Carbonyl Groups; Carbanion Displacement Reactions; Formation and Cleavage of Ethers; Esterification and Hydrolysis; Substitution on the Benzene Nucleus; Diazonium Reactions and the Wolff-Kishner Reduction; Miscellaneous Reactions Involving the Migration of a Double Bond.

For those chemists who have an interest in a more understandable approach to the principles of ionic organic reactions without the complications of mathematics, this volume is to be recommended.

W. I. PATTERSON

Melting and Solidification of Fats. By Alton E. Bailey. Interscience Publishers, Inc., New York, N. Y., 1950. xiv+357 pages. $7.00.

Melting and solidification phenomena are extremely important in the oil and fat industry, particularly in these days of "tailor-made" fats. Although considerable information on these physical properties of fatty substances has appeared in some recent texts, this new monograph offers the most comprehensive collection of data on melting and solidifying behavior. Interrelationships between the different phenomena are stressed by correlating and interpreting the large amount of experimental data. Information on the solubility of fatty substances in organic solvents is included in this volume, thereby enhancing its usefulness greatly. The author considers the distinction between melting and solution as illogical and arbitrary, since both are processes of dissolution governed by the same principles.

The monograph opens with an elementary review of the basic physical chemical principles concerned with crystal structure, melting and solidifying phenomena and solubility. This is followed by a brief chapter on the methods used in this field. These procedures are discussed fully and critically analyzed, but no details of the techniques are given. However, adequate references to source material are provided. Chapters III and IV are concerned with the melting and solidifying of pure compounds and of mixtures, respectively. These include x-ray diffraction data since melting data are related to the polymorphism exhibited by fats. Chapter V is devoted to solutions of fatty material in non-fatty organic solvents. The final chapter discusses the influence of melting and solidification phenomena on the consistency of commercial plastic fat products.

This monograph is clearly printed on good paper and is relatively free from typographical errors. Adequate indexes are provided and numerous illustrations, graphs and tables increase the value of the recorded data. As the author states in

the preface, the text has been written primarily for the practicing oil and fat chemist or technologist. However, all chemists interested in the physical chemistry of fatty products will find this book a valuable addition to their reference libraries.

J. FITELSON

Advances in Agronomy, Vol. II. Edited by A. G. Norman, prepared under the auspices of the American Society of Agronomy, Academic Press, Inc., New York, 1950. xi+407 pp. Price $7.50.

The subject matter of Volume II covers an even wider range of topics than did Volume I. Titles of chapters are as follows: Cotton, Soil Nitrogen, Vegetable Production, Prairie Soils of the Upper Mississippi Valley, Ladino Clover, The Control of Soil Water, Preservation and Storage of Forage Crops, The Reclamation of Coal Mine Spoils, and Irrigated Pastures.

This is a splendid book. However, the reviewer thinks that more copies of this volume, as well as of Volume I, might be sold if the subject matter of each were more closely related. Books devoted to such important topics as advances in crop production, crop processing, and soil science might well be redone at least once in a decade.

In this latest volume the chapter on Cotton has a section on ginning practices reminding the reader of the fact that the processing of a crop is a matter of interest to the agronomist. The chapter on Forage Crops points out the relative importance of constituents of quality which must be examined in determining the magnitude of losses incurred during preservation and storage. The old practice of making silage is examined, as well as are modern methods for crushing green hay at time of cutting in the field and artificial drying in barns.

The apparently unlimited potentialities in the use of heterosis in breeding vegetable crops is emphasized. The future is bright for development of new vegetable varieties of high quality. Many of these will have improved resistance to some of the diseases difficult to control otherwise. A breeding program is also desirable to develop greater uniformity of certain plants for mechanical harvesting.

Soils topics are well represented. The Control of Soil Water is written by English authors who take a world-wide view of the subject. All forms of soil water control rely upon control of water movement in the soil. Literature covering the vexing question of a definite wilting point is reviewed.

The Reclamation of Coal Mine Spoils reviews much of what the general citizenry as well as the agronomist, should know. The total area stripped for coal is only about a quarter million acres but at least an equal area of neighboring land is affected. The majority of graded spoils can be used for pasture and some of them, after a period of improvement, may properly be used for crops in rotation.

In the chapter on Irrigated Pastures the author sets forth the western situation from the standpoint of soils, their management, crops and the economic factors involved. Probably eastern readers would have appreciated a brief comment by the author on methods used for the limited supplemental irrigation of pastures now practiced in humid areas.

The discussion of Prairie Soils includes their chemical and physical composition, classification, and crop-producing powers. The name "Brunigra" is suggested by the authors as a substitute for the term "Prairie Soils" as a genetic group.

Agronomists of wide interests will appreciate this book.

M. S. ANDERSON