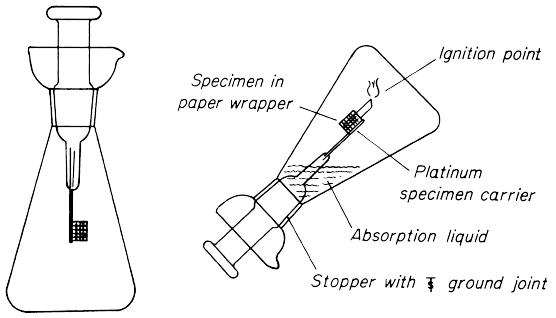
The caution statement given for *Procedure* covers minimum safety precautions only, and serves to emphasize the need for exceptional care throughout.

Apparatus—The apparatus[1] consists of a heavy-walled conical, deeply lipped or cupped 500-mL flask (unless a larger flask is specified), fitted with a ground-glass stopper to which is fused a test specimen carrier consisting of heavy-gauge platinum wire and a piece of welded platinum gauze measuring about 1.5 × 2 cm.



Apparatus for Oxygen Flask Combustion

Procedure—[*Caution—Wear safety glasses and use a suitable safety shield between yourself and the apparatus. Exercise care to ensure that the flask is scrupulously clean and free from even traces of organic solvents.*] Weigh the substance, if a solid, on a piece of halide-free filter paper measuring about 4 cm square, and fold the paper to enclose it. Liquid substances are weighed in tared capsules, polycarbonate capsules[1] being used for liquids in volumes not exceeding 200 µL, and gelatin capsules being satisfactory for use for larger volumes. [NOTE—Gelatin capsules may contain significant amounts of combined halide or sulfur. If such capsules are used, perform a blank determination, and make any necessary correction.] Place the specimen, together with a filter paper fuse-strip, in the platinum gauze specimen holder. Place the absorbing liquid specified in the individual monograph or general chapter in the flask, moisten the joint of the stopper with water, and flush the air from the flask with a stream of rapidly flowing oxygen, swirling the liquid to favor its taking up oxygen. [NOTE—Saturation of the liquid with oxygen is essential for the successful performance of the combustion procedure.] Ignite the fuse-strip by suitable means. If the strip is ignited outside the flask, immediately plunge the specimen holder into the flask, invert the flask so that the absorption solution makes a seal around the stopper, and hold the stopper firmly in place. If the ignition is carried out in a closed system, the inversion of the flask may be omitted. After combustion is complete, shake the flask vigorously, and allow to stand for not less than 10 minutes with intermittent shaking. Then proceed as directed in the individual monograph or general chapter.

[1] A suitable apparatus [Catalog Nos. 6513-C20 (500-ml capacity) and 6513-C30 (1000-ml capacity)] and suitable capsules [Catalog No. 6513-84 (1000 capsules)] are obtainable from Thomas Scientific, 99 High Hill Road, Swedesboro, NJ 08085.

⟨481⟩ RIBOFLAVIN ASSAY

The following procedure is suitable for preparations in which riboflavin is a constituent of a mixture of several ingredients. In employing it, keep the pH of solutions below 7.0, and protect the solutions from direct sunlight at all stages.

USP Reference Standards ⟨11⟩—*USP Riboflavin RS.*

Standard Riboflavin Stock Solution—To 50.0 mg of USP Riboflavin RS, previously dried and stored protected from light in a desiccator over phosphorus pentoxide, add about 300 mL of 0.02 N acetic acid, and heat the mixture on a steam bath, with frequent agitation, until the riboflavin has dissolved. Then cool, add 0.02 N acetic acid to make 500 mL, and mix. Store under toluene in a refrigerator.

Dilute an accurately measured portion of this solution, using 0.02 N acetic acid, to a concentration of 10.0 µg of the dried USP Riboflavin RS per mL, to obtain the *Standard Riboflavin Stock Solution.* Store under toluene in a refrigerator.

Standard Preparation—Dilute 10.0 mL of *Standard Riboflavin Stock Solution* with water in a 100-mL volumetric flask to volume, and mix. Each mL represents 1.0 µg of USP Riboflavin RS. Prepare fresh *Standard Preparation* for each assay.

Assay Preparation—Place an amount of the material to be assayed in a flask of suitable size, and add a volume of 0.1 N hydrochloric acid equal in mL to not less than 10 times the dry weight of the material in g, but the resulting solution shall contain not more than 100 µg of riboflavin per mL. If the material is not readily soluble, comminute it so that it may be evenly dispersed in the liquid. Then agitate vigorously, and wash down the sides of the flask with 0.1 N hydrochloric acid.

Heat the mixture in an autoclave at 121° to 123° for 30 minutes, and cool. If clumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture, with vigorous agitation, to a pH of 6.0 to 6.5 with sodium hydroxide solution,* then add hydrochloric acid solution* immediately until no further precipitation occurs (usually at a pH of approximately 4.5, the isoelectric point of many of the proteins present). Dilute the mixture with water to make a measured volume that contains about 0.11 µg of riboflavin in each mL, and filter through paper known not to adsorb riboflavin. To an aliquot of the filtrate add, with vigorous agitation, sodium hydroxide solution* to produce a pH of 6.6 to 6.8, dilute the solution with water to make a final measured volume that contains approximately 0.1 µg of riboflavin in each mL, and if cloudiness occurs, filter again.

Procedure—To each of four or more tubes (or reaction vessels) add 10.0 mL of the *Assay Preparation.* To each of two or more of these tubes add 1.0 mL of the *Standard Preparation,* and mix, and to each of two or more of the remaining tubes add 1.0 mL of water, and mix. To each tube add 1.0 mL of glacial acetic acid, mix, then add, with mixing, 0.50 mL of potassium permanganate solution (1 in 25), and allow to stand for 2 minutes. To each tube add, with mixing, 0.50 mL of hydrogen peroxide solution, whereupon the permanganate color is destroyed within 10 seconds. Shake the tubes vigorously until excess oxygen is expelled. Remove any gas bubbles remaining on the sides of

* The concentrations of the hydrochloric acid and sodium hydroxide solutions used are not stated in each instance because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

the tubes after foaming has ceased, by tipping the tubes so that the solution flows slowly from end to end.

In a suitable fluorophotometer, having an input filter of narrow transmittance range with a maximum at about 440 nm and an output filter of narrow transmittance range with a maximum at about 530 nm, measure the fluorescence of all tubes, designating the average reading from the tubes containing only the *Assay Preparation* as I_U and the average from the tubes containing both the *Assay Preparation* and the *Standard Preparation* as I_S. Then to each of one or more tubes of each kind add, with mixing, 20 mg of sodium hydrosulfite, and within 5 seconds again measure the fluorescence, designating the average reading as I_B.

Calculation—Calculate the quantity, in mg, of $C_{17}H_{20}N_4O_6$ in each mL of the *Assay Preparation* taken by the formula:

$$0.0001(I_U - I_B)/(I_S - I_U).$$

Calculate the quantity, in mg, of $C_{17}H_{20}N_4O_6$ in each capsule or tablet.

⟨501⟩ SALTS OF ORGANIC NITROGENOUS BASES

Standard Preparation—Unless otherwise directed, prepare a solution in dilute sulfuric acid (1 in 70) containing, in each mL, about 500 µg of the specified USP Reference Standard, calculated on the anhydrous basis, and accurately weighed.

Assay Preparation—If the dosage form is a tablet, weigh and finely powder not less than 20 tablets, weigh accurately a portion of the powder, equivalent to about 25 mg of the active ingredient, and transfer to a 125-mL separator; or, if the dosage form is a liquid, transfer a volume of it, equivalent to about 25 mg of the active ingredient and accurately measured, to a 125-mL separator. Then to the separator add 20 mL of dilute sulfuric acid (1 in 350), and shake vigorously for 5 minutes. Add 20 mL of ether, shake carefully, and filter the acid phase into a second 125-mL separator. Shake the ether phase with two 10-mL portions of dilute sulfuric acid (1 in 350), filter each portion of acid into the second separator, and discard the ether. To the acid extract add 10 mL of sodium hydroxide TS and 50 mL of ether, shake carefully, and transfer the aqueous phase to a third 125-mL separator containing 50 mL of ether. Shake the third separator carefully, and discard the aqueous phase. Wash the two ether solutions, in succession, with a single 20-mL portion of water, and discard the water. Extract each of the two ether solutions with 20-, 20-, and 5-mL portions of dilute sulfuric acid (1 in 70), in the order listed, but each time extract first the ether solution in the third separator and then that in the second separator. Combine the acid extracts in a 50-mL volumetric flask, dilute with the acid to volume, and mix.

NOTE—Hexane or heptane may be substituted for ether if the distribution ratio of the nitrogenous base between water and hexane, or between water and heptane, favors complete extraction by the organic phase.

Procedure—Unless otherwise directed, dilute 5.0 mL each of the *Standard Preparation* and the *Assay Preparation* with dilute sulfuric acid (1 in 70) to 100.0 mL, and determine the absorbance of each solution at the specified wavelength, using dilute sulfuric acid (1 in 70) as the blank. Designate the absorbance of the solution from the *Standard Preparation* as A_S and that from the *Assay Preparation* as A_U, and calculate the result of the assay as directed in the individual monograph.

⟨503⟩ ACETIC ACID IN PEPTIDES

The following procedure is to be used to determine the amount of acetate or acetic acid in peptides. Acetate is a common counterion in many peptide preparations.

USP Reference Standards ⟨11⟩—*USP Glacial Acetic Acid RS.*

Strong Sodium Hydroxide Solution—Dissolve 42 g of sodium hydroxide in water, and dilute with water to 100 mL.

Solution A—Add 0.7 mL of phosphoric acid to 1000 mL of water, and adjust with *Strong Sodium Hydroxide Solution* to a pH of 3.0.

Solution B—Use methanol.

Diluent—Prepare a mixture of *Solution A* and *Solution B* (95:5).

Standard Solution—[NOTE—The concentration can be adjusted depending on the amount of acetate or acetic acid expected to be present in the test material.] Dissolve an accurately weighed quantity of USP Glacial Acetic Acid RS in *Diluent* to obtain a solution having a known concentration of about 0.1 mg per mL.

Test Solution—Prepare as directed in the individual monograph. The amount of material used can be adapted depending on the amount of acetic acid expected.

Chromatographic System (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 210-nm detector and a 4.6-mm × 25-cm column that contains not greater than 5-µm packing L1. The flow rate is about 1.2 mL per minute. The chromatograph is programmed as follows.

Time (minutes)	*Solution A* (%)	*Solution B* (%)	Elution
0	95	5	equilibration
0–5	95	5	isocratic
5–10	95→50	5→50	linear gradient
10–20	50	50	isocratic
20–22	50→95	50→5	linear gradient

Chromatograph the *Standard Solution,* and record the peak responses as directed for *Procedure:* the retention time of acetic acid is between 3 and 4 minutes; and the relative standard deviation for replicate injections is not more than 5%.

Procedure—Separately inject equal volumes (about 10 µL) of the *Standard Solution* and the *Test Solution* into the chromatograph, record the chromatograms, and measure the responses for the acetic acid peaks. Calculate the percentage of acetic acid in the portion of test material taken by the formula:

$$100(C_S/M)(r_U/r_S)$$

in which C_S is the concentration of acetic acid in the *Standard Solution*; M is the concentration, in mg per mL, of the *Test Solution,* based on the weight of test material taken and the extent of dilution; and r_U and r_S are the acetic acid peak