Liberation and Determination of Riboflavin in Natural Feedstuffs

V. M. R. MURTHY, R. N. BURROUGHS, B. L. REID, and J. R. COUCH

Departments of Poultry Science and Biochemistry and Nutrition, Texas A&M College System, College Station, Tex.

Two simplified procedures are outlined for liberation and determination of riboflavin from natural feedstuffs: extraction of riboflavin by steaming with water and subsequent microbiological assay, and steaming with very dilute hydrochloric acid followed by fluorometric determination of the vitamin directly from this extract. Repeated pH adjustments, adsorption on Florisil, and treatment with potassium permanganate have been eliminated.

STANDARD RIBOFLAVIN METHODS involve Florisil adsorption subsequent to permanganate decolorization and are suitable for a wide variety of materials (*1–6, 8, 10, 11, 13*). Extraction and liberation methods applicable to various samples are varied and involved. Conner and Straub (*5*) and Hudson and Norris (*7*) have suggested mild acid as the extracting agent, and an acetone-acid mixture was suggested by Scott and associates (*13*). Autoclaving in decinormal acid is widely accepted (*9, 14, 15*), although digestive release with papain and takadiastase is often employed (*3, 12*).

The need for a simplified method for riboflavin extraction and estimation has been felt in many feed control laboratories for the routine determination in a large number of similar samples. The present work was directed toward the development of such a method.

Experimental Procedure

Material. Riboflavin was determined in feed samples containing soybean oil meal, cottonseed meal, alfalfa, fish meal, fish solubles, dried whey, milo, and corn. These form some of the main constituents of industrial mixed feeds and contain varied quantities of free and bound riboflavin. Such ingredients also offer a satisfactory test for the efficiency of extraction procedures.

The ingredients were assayed for riboflavin individually as well as in standard mixtures compounded in different proportions. Known quantities of either free riboflavin or a fermentation residue (rich in riboflavin) were added to ingredient mixtures and the analytical recovery of the added vitamin from such was determined. The samples were prepared by weighing the constituent materials, mixing thoroughly, and grinding the mixture to 40-mesh size.

Liberation and Extraction

The two procedures developed for the liberation and extraction of riboflavin by fluorometric and by microbiological determination, used AOAC methods for microbiological assay (*1*) and fluorometry (*2*) as standards to check the suitability of extraction procedures.

For fluorometric determination, samples of feed containing 30 to 40 γ of riboflavin (usually about 5 grams of the feed) were suspended in 300 ml. of 0.06% hydrochloric acid and, after mixing, were steamed in an autoclave for 15 minutes. Sudden cooling following steaming helped to reduce the turbidity, which otherwise produced a high blank value upon fluorometric measurement. The steamed mixture was then made up to 500 ml. with distilled water and centrifuged. The clear supernatant, with subsequent filtration if necessary, was read in the fluorometer.

The extracts for microbiological assay were prepared in the same way, except that distilled water, instead of 0.06% hydrochloric acid, was employed for extraction. The extracts were prepared fresh before assay and used without further treatment.

Determination

Fluorometric riboflavin was determined according to the AOAC method (*1*) using a Coleman Photofluorometer (Model 12) with PC_2 and B_2 filters. Microbiological assays were carried out with the modified Snell-Strong medium (*6*), using *Lactobacillus casei* ATCC 7469 and acid production at 72 hours as the criterion of growth.

Discussion

Riboflavin, added to a mixed feed in concentrations ranging from 1.5 to 12.0 mg. per pound, was recovered to the extent of 97 to 100%. The bound vitamin present in the feed ingredients was liberated by the steaming process. Hot water extraction was satisfactory for the microbiological assay of riboflavin, but it produced an excess turbidity which interfered with the fluorometric determination.

An acid concentration of 0.06% was adequate both for liberation and extraction of total riboflavin from feed mixtures and for fluorometric determination of the vitamin. Recoveries of 97.8% were obtained with this extraction procedure.

The standard fluorometric procedure yielded a value of 4.58 γ per gram of sample, compared with 4.55 γ by the proposed method. However, elimination of the Florisil and permanganate steps in the AOAC procedure resulted in a lower value 4.15 γ per gram. Thus, the use of 0.06% hydrochloric acid as the extracting solvent and steaming made it possible to eliminate these two steps in the standard procedures. Florisil and permanganate treatments of the 0.06% hydrochloric acid extracts failed to affect the riboflavin values obtained.

Three hundred samples of commercial feed were obtained from feed manufacturers, in which both fermentation residues and the free riboflavin were used as additives. In all cases there was good agreement between the values obtained by 0.06% hydrochloric acid extraction and the standard AOAC method.

One feed manufacturer supplied daily samples of a standard boiler feed formulation, with estimated riboflavin content of 5.73 γ per gram. The average riboflavin content for the 30-day period was 6.38 γ per gram, obtained by the simplified fluorometric procedure. The daily determination varied from 4.82 to 8.40 γ per gram. This variation is

probably traceable to sampling procedures and not to errors within the method itself. The average for the 30-day period, 6.38 γ per gram, was in good agreement with the value claimed by the manufacturer, 5.73 γ per gram, when it is taken into account that 30 samples were taken, each from a separate day's run on a broiler feed formula in a commercial feed operation.

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FEED ADDITIVES ANALYSIS

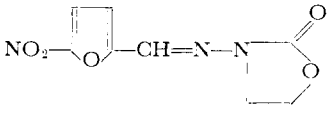
Microdetermination of the Medicaments Furazolidone and Nitrofurazone

HERMAN F. BECKMAN

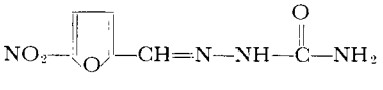
State Chemist Laboratory, Texas Agricultural Experiment Station, College Station, Tex.

Colorimetric procedures allow the assay of a feed containing a mixture of furazolidone and nitrofurazone. *N,N*-Dimethylformamide is used as the solvent for the compounds and as the medium in which the color of furazolidone is developed. The color is developed with nitrofurazone in mixture of alcohol and dimethylformamide. Furazolidone develops a blue and nitrofurazone a pink-orange color in the presence of alkali. A chromatographic separation of these feed medicaments is described.

FURAZOLIDONE, *N*-[(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone], is represented by the following formula:



Nitrofurazone, 5-nitro-2-furaldehyde semicarbazone, is represented by:



Several methods of determining furazolidone and nitrofurazone have been published (*1–3*). Some require reduction of the active materials for a blank reading in the absence of control samples. These procedures give variable results, partly inherent and partly due to the limited solubility of the compounds in the solvents used. Other methods give good results, but are somewhat more complicated and time-consuming than the procedure proposed here. No method for separating the components of a mixture of these medicaments has been published. Porter (*4*) used dimethylformamide as a solvent in a color test for mono- and dinitro compounds. He investigated aromatic compounds and utilized tetraethylammonium hydroxide, but did not include nitrofurans or other heterocyclics.

As these compounds are finding wide acceptance in the feed industry, a rapid procedure for routine laboratory analysis is of prime importance. This procedure requires but a few steps and reagents.

Materials and Equipment

Extraction tubes, 12 inches, made from 20-mm. glass tubing, drawn to a tip.

Chromatographic columns, a 50-ml. buret or one about 12 × 450 mm.

Spectrophotometer, suitable for reading at 490 and 600 mμ.

Skellysolve B is a satisfactory petroleum hydrocarbon solvent. *N,N*-Dimethylformamide, 95% ethyl alcohol, and potassium hydroxide (1*N*) in 50% alcohol with water.

Aluminum oxide, suitable for chromatographic adsorption. Each lot should be checked for recovery of the medicaments. An acid wash of the alumina generally corrects any difficulties.

Crystalline furazolidone and nitrofurazone, obtained from Eaton Laboratories, Division of Norwich Pharmacal Co., Norwich, N. Y., and Bifuran from Hess and Clark, Inc., Ashland, Ohio.

Procedures

Standards. To prepare stock solutions of each compound, weigh exactly 0.1000 gram and transfer to a 100-ml. volumetric flask, using dimethylformamide. One milliliter of this (1.0 mg.) is equivalent to the amount of medicament in 9.1 grams of feed at 0.011% concentration. Prepare other dilutions to correspond to various amounts of medicament in the feed or concentrate. Standards are more comparable to samples if the standard curves are made by using known amounts of medicament added to a blank feed.

Pre-extraction. All feeds should be mechanically ground to pass a 20-mesh sieve. As furazolidone is not very soluble in Skellysolve B, it is convenient to use it to pre-extract the feeds to remove interfering material. Add 9.1 grams of feed to an extraction tube containing, first, a glass wool plug, and then 1 cm. of Hyflo Super-Cel in the tip. Allow 50 ml. of Skellysolve to pass through the column. If necessary, ap-