Reversed-Phase Liquid Chromatographic Determination of Riboflavin in Feeds

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A method for determination of riboflavin in animal feeds using liquid chromatography (LC) was developed for feed samples fortified with riboflavin at 1 mg/lb or greater (up to 10 000 mg/lb). Feed samples were extracted in 0.1N HCl with heating on a steam bath for 30 min, followed immediately by mechanical shaking for 30 min. Sample extracts were diluted to target volume with 2% acetic acid and filtered; riboflavin was determined by LC on a reversed-phase C18 column with 2% acetic acid–acetonitrile (85 + 15) mobile phase for separation and fluorescence detection with excitation at 460 nm and emission at 530 nm. The extraction was compared with that of the AOAC Official Method for riboflavin in food and feed premixes. The 2 method extractions were not significantly different from each other at the 95% confidence level. The developed method also had good linearity over 4 orders of magnitude, recovery of 95–99% from spiked feed samples, a limit of detection of riboflavin at 0.00034 μg/mL in solution, a limit of quantitation of 0.023 mg/lb in feed, and good ruggedness.

Riboflavin (vitamin B2) is a water-soluble vitamin (Figure 1) that is needed in cellular metabolism for utilization of pyridoxine (vitamin B6) and folic acid and for transformation of tryptophan into niacin. Riboflavin is essential for proper animal nutrition and, therefore, its supplementation in animal feeds is usually required because cereals, used as a base for most feeds, are poor sources of riboflavin.

The current AOAC Official Method for determination of riboflavin in foods and vitamin preparations (1), including feed premixes (2), is a fluorometric method which uses pyridine in the extraction solution. Pyridine is flammable and dangerous to health, is irritating to the eyes, and has a noxious odor. The method is also susceptible to interference from other fluorescent compounds, such as nonenzymatic browning reaction compounds (3) and pigments (4), especially at lower concentrations (5). Many of these interfering compounds are removed by permanganate oxidation followed by treatment with hydrogen peroxide to remove excess permanganate. However, such treatment may destroy riboflavin (6). Additionally, the AOAC procedure reportedly overestimates total flavin content due to the presence of interfering artefacts (7, 8).

The use of liquid chromatography (LC) with fluorescence detection for determination of riboflavin has been reported in food (9, 10) and pet foods (11). LC separation of riboflavin provides greater selectivity and less susceptibility to interference than fluorimetry alone. However, the sample preparation for these methods is somewhat complex and time-consuming.

An LC method developed by J. Hillebrandt (personal communication, 1986) at the Agway Technical Center (now the Cornell University Diagnostic Laboratory, Ithaca, NY) used the basic extraction described here for riboflavin in feed samples at low levels (1–30 mg/lb) and the pyridine extraction solution of the AOAC Official Method (1) for high levels of riboflavin in feeds and premixes (1000–10 000 mg/lb). The goal of the Office of Indiana State Chemist (OISC) was to eliminate the use of pyridine in extraction by extending the range of extraction with 0.1N HCl to include all levels of riboflavin in feeds and premixes of interest. In addition, the performance of the developed method extraction should be comparable to that of the established AOAC extraction method for riboflavin.

METHOD

Apparatus

(a) Riffler (1-in.).—Carpeco Inc. (Jacksonville, FL; Model SS16-25X); used to subsample collected feed samples.

(b) Grinding mill.—Retsch GmbH & Co. (Haan, Germany; Model ZM100 or ZM 1000); used to grind subsamples.

(c) Liquid chromatograph.—Consisting of a quaternary gradient pump (Thermo-Finnigan, San Jose, CA; P4000), variable loop autosampler (Thermo-Finnigan; AS3000), and fluorescence detector (Shimadzu, Tokyo, Japan; RF-551); used for separation and detection of analytes.

(d) Glass volumetric flasks.—100, 200, 500, 1000, and 2000 mL, with polyethylene stoppers; used for sample extraction and standard preparation.

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Steam bath.—Atmospheric pressure, 100°C; used for heating sample extractions.

Horizontal reciprocating mechanical shaker.—Eberbach (Ann Arbor, MI; 6010) or equivalent; used for shaking during sample extraction.

Glass fiber prefilter pad.—13 mm (Gelman, Ann Arbor, MI; 66073) inserted into 0.5 in. id 5 mL syringe barrel [Becton Dickinson (B-D), Franklin Lakes, NJ; syringe, No. 5603], or a 25 mm 0.2 μm nylon syringe filter (Gelman 4436); used for sample extract clarification.

Reagents

Riboflavin analytical standards.—U.S. Pharmacopeia (Rockville, MD). Standard material was used as received.

Glacial acetic acid and concentrated HCl.—ACS reagent grade (Fisher Scientific, Pittsburgh, PA).

Acetonitrile.—LC grade (Fisher Scientific).

LC grade water.—From a Milli-Q water purifier (Millipore, Bedford, MA); used to prepare solutions.

Preparation

Class B volumetric glassware was used for reagent and sample preparation. Class A volumetric glassware was used for all standard preparations.

Extractant, 0.1N HCl.—Slowly pipet 17.1 mL concentrated HCl into 2000 mL volumetric flask containing ca 1 L water, and mix. Dilute to volume with water, and mix.

Diluent, 2% acetic acid (v/v).—Slowly pipet 40 mL glacial acetic acid into 2000 mL volumetric flask containing ca 1 L water, and mix. Dilute to volume with water, and mix.

Mobile phase, acetonitrile–2% acetic acid (15 + 85).—Instrument mix or premix 150 mL acetonitrile with 850 mL 2% acetic acid. Mobile phase proportions may need to be adjusted to resolve riboflavin peak.

Riboflavin standards.—Note: Protect all standard solutions from light (especially UV) to avoid degradation of riboflavin (12).

Stock standard.—Prepare stock solution (100 μg/mL) riboflavin by accurately weighing 50.00 ± 0.05 mg riboflavin analytical standard into 500 mL volumetric flask. Add 300–400 mL extractant (0.1N HCl) and heat on steam bath with occasional agitation until complete dissolution is obtained (30 min is usually sufficient). Cool to room temperature, dilute to volume with extractant, and mix. The stock solution is stable for ca 4 weeks if kept refrigerated and protected from light. Alternatively, the stock solution is stable indefinitely if refrigerated, protected from light, and stored under toluene.

First Intermediate Standard A.—Prepare First Intermediate Standard A solution (10 μg/mL) by pipetting 10.0 mL stock solution into 100 mL volumetric flask. Dilute to volume with diluent (2% acetic acid), and mix.

Second Intermediate Standard B.—Prepare Second Intermediate Standard B solution (1 μg/mL) by pipetting 10.0 mL First Intermediate Standard A solution into 100 mL volumetric flask. Dilute to volume with diluent, and mix.

High-level Working Standard I.—Prepare high-level Working Standard I (1 μg/mL) for sample guarantees >15 mg/lb by pipetting 10.0 mL First Intermediate Standard A into 100 mL volumetric flask. Add 20.0 mL acetonitrile, dilute to volume with diluent, and mix.

Low-level Working Standard II.—Prepare low-level Working Standard II (0.1 μg/mL) for sample guarantees from 1 to 15 mg/lb by pipetting 10.0 mL Second Intermediate Standard B into 100 mL volumetric flask. Add 20.0 mL acetonitrile, dilute to volume with diluent, and mix.

Sample Extraction

Split feed samples into subsamples by using a 1-in. riffler. Grind subsamples to pass through 0.75 mm mesh screen using grinding mill. Place ground samples in 4 oz glass jars and tumble to mix before weighing out sample portions. Note: Protect all sample extracts from light to avoid degradation of riboflavin.

<table>
<thead>
<tr>
<th>Guarantee, mg/lb</th>
<th>W₀, g</th>
<th>Extraction flask volume, mL</th>
<th>Further dilutions</th>
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Weigh 1–2 g sample into 100 or 200 mL volumetric flask (for guarantees of 1–2999 mg/lb; Table 1), or weigh 1 g sample into 1000 mL volumetric flask (for guarantees ≤3000 mg/lb). Add ca 50 mL extractant (for sample guarantees of 1–2999 mg/lb) or add ca 600 mL water and 8.0 mL concentrated HCl (for guarantees ≥3000 mg/lb). Mix and heat on preheated steam bath for 30 min. Immediately stopper the flask (use tape to hold stoppers on flask), and shake mechanically for 30 min. Dilute to volume or make any further dilutions with diluent according to Table 1, while ensuring that final sample dilution contains 20% acetonitrile. Target riboflavin concentration for diluted extract is 0.1 g/mL (for guarantees of 1–15 mg/lb) or 1.0 g/mL (for guarantees >15 mg/lb). Clarify diluted sample extract through glass fiber filter or nylon syringe filter.

Determination

(a) LC conditions.—Use any C_{18} analytical column with particle size ≤10 μm that will separate riboflavin from co-extracted materials, fitted with C_{18} guard cartridge, for LC analysis. A Versapack RP18, 10 μm, 4.1 × 250 mm (Part No. 28024; Alltech Associates, Inc., Deerfield, IL) and a Partisil ODS3, 5 μm, 4.6 × 250 mm (Part No. 8660; Alltech Associates) are suitable. Condition column by programming from storage solvent (100% acetonitrile) to mobile phase (acetonitrile–2% acetic acid, 15 + 85); then pump mobile phase through column at flow rate of 1.0 mL/min for 30 min to equilibrate. Use 20 μL injection volume and bracket every 2 sample injections with working standard injections. Use Working Standard I for samples with guarantees >15 mg/lb. Use Working Standard II for samples with guarantees of 1–15 mg/lb. Determine riboflavin peak areas by fluorescence detection with excitation at 460 nm and emission at 530 nm.

(b) Calculations.—Calculate riboflavin concentration (mg/lb) in sample by comparing peak areas of standards and samples using the following equation:

\[
\text{Concentration riboflavin, mg/lb} = \frac{R_u \times W_s \times D_u \times 453592}{R_s \times W_u \times D_s}
\]

where \(R_u\) is the average response (peak area) for the unknown feed sample, \(R_s\) is the average response (peak area) for 2 bracketing working standard injections, \(W_u\) is the weight (g) of unknown feed sample, \(W_s\) is the weight (g) of standard used to prepare the stock standard solution, \(D_u\) is the total dilution (mL) of the sample (Table 1), \(D_s\) is the total dilution (mL) of the original standard weighed to prepare the working (bracketing) standard, and 453 592 is the unit conversion factor from g/g to mg/lb.

(c) Dilution calculation.—The total dilution of standards (\(D_s\)) and samples (\(D_u\)) is calculated by multiplying the dilute-to volumes and dividing by the transferred volumes. For example, \(D_s\) for Working Standard I, where a weight of dry standard, \(W_s\), is diluted to 500 mL and then further diluted 10/100 and 10/100, is calculated as follows:

\[
D_s = \frac{500 \times 100 \times 100}{10 \times 10} = 50,000 \text{ mL}
\]

Similarly, \(D_s\) for Working Standard II is 500 000 mL.

Results and Discussion

Sample weights were reduced from the Hillebrandt method (personal communication, 1986) to minimize dilution volume errors, and the dilution solvent was changed to 2% acetic acid. The extraction conditions were optimized as indicated below, and the method range was extended from 1 to 30 mg/lb to cover the entire range (1 mg/lb –) of the 2 extraction methods used by Hillebrandt. Mobile phase acetic acid concentration was changed to 2%, and mobile phase proportions were adjusted to provide adequate resolution for the chosen LC column.

Extraction Optimization

Optimal extraction of riboflavin occurred after 30 min of sample heating followed by 30 min of shaking. Longer heating and shaking times did not improve riboflavin recovery. For samples containing riboflavin at 3000 mg/lb or greater, increasing the extractant volume to 600 mL maximized the recovery of riboflavin from these higher-level samples. However, 50 mL extractant volumes were adequate for samples containing riboflavin <3000 mg/lb. These extraction conditions were selected for the present method.

Because riboflavin is almost exclusively added to animal feeds as HCl salt, enzyme digestion of the sample is usually not necessary. If riboflavin is added as one of the other flavins, such as flavin mononucleotide, then, after heating and shaking, the pH of the extracts should be adjusted and an enzyme digestion performed, as described elsewhere (10, 11). In over 20 years of riboflavin determinations at OISC, only one very unusual feed sample was encountered where riboflavin was added as the flavin mononucleotide.
Repeatability

The repeatability of the method was determined by 10 replicate injections of the 0.1 and 1.0 μg/mL riboflavin standards (Working Standards I and II). The coefficient of variation (CV) for the 0.1 μg/mL riboflavin standard was 1.98% (n = 10); the CV for the 1.0 μg/mL riboflavin standard was 1.45% (n = 10). The variation of the method for feed samples was estimated from determinations on a feed containing approximately 3000 mg/lb riboflavin. This sample was analyzed in duplicate on 18 days for 4 years. A CV of 3.03% was found. This variation includes all within-laboratory factors, including variation between days. The variation can be expected to be greater for feeds containing lower levels of riboflavin.

Linearity and Range

The linearity of the riboflavin standard response was established over the range of standard concentrations from 0.0005 to 5 μg/mL (4 orders of magnitude). Riboflavin standards were prepared at concentrations of 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 3, and 5 μg/mL. A riboflavin standard of 0.0001 μg/mL was also prepared, but was below the limit of detection (LOD).

A log–log plot of the riboflavin peak response (peak area) versus concentration (μg/mL) is shown in Figure 2. A linear least-squares regression analysis of the data reveals a correlation coefficient of 1.0000, which indicates good linearity of the data over the range examined. This is confirmed visually from the log–log plot.

A linear least-squares analysis of the peak response versus the concentration of riboflavin data reveals a slope of 1.126 × 10^{6} (95% confidence limits from 1.123 to 1.128 × 10^{6}) with a y-intercept of −2334 (95% confidence limits from −6070 to 1402, which includes 0).

Limits of Detection and Quantitation

Ten replicates of a solvent blank (20% acetonitrile in 2% acetic acid) were measured and a standard deviation (SD) of 115.2 was found for the peak areas. The LOD was calculated as (3.3 × σ / S) and the limit of quantitation (LOQ) was calculated as (10 × σ / S), where σ is the SD of the blank response and S is the slope of the calibration line. The LOD for riboflavin was 0.00034 μg/mL. The LOQ was 0.0010 μg/mL, which corresponds to a concentration of 0.023 mg/lb riboflavin in feed samples, well below the specified 1 mg/lb low end of the method range.

Recovery

Two feed samples were spiked at multiple levels with standard to determine riboflavin recovery. One feed, a mineral mix, was spiked at approximate concentrations of 20, 100, and 1000 mg/lb. The second feed, a cattle premix, was spiked at 1000, 3000, 5000, and 10 000 mg/lb. The average recovery of all levels from the first feed was 99% with a CV of 5.0%. The average recovery of all levels from the second feed was 95% with a CV of 1.8%. The recoveries were relatively consistent for the different feed matrixes and different levels. The observed CVs improved at higher concentrations, although this was not unexpected.

Extraction Method Comparison

The accuracy of the proposed method extraction was determined by comparison with that of the AOAC Official Method. The AOAC method of extraction was combined with LC determination to eliminate the possibility of interference (high bias) with straight fluorometric determination. A total of 21 different feed samples containing riboflavin at various levels were extracted by both the AOAC and the present OISC procedure and determined in duplicate by LC on separate days.

A 2-method comparison plot (Figure 3) can be used to graphically display the results of the paired sample study of the method extractions (13). Because the study encompassed a wide range of riboflavin sample guarantees, a log–log plot was used for the extraction method comparison. The log of the OISC result was used as the y value and that of the AOAC result as the x value for each sample represented as a single point. The solid line represents perfect correlation between the 2 method extractions (i.e., the same result is obtained by both method extractions, resulting in a regression line with slope equal to 1 and y-intercept equal to zero). From visual inspection of the plot, it appears that there is good correlation between the 2 methods. A least-squares linear regression fit of the data reveals a correlation coefficient of 0.9995 with a slope of 1.004 (95% confidence interval from 0.993 to 1.015) and a y-intercept of −0.0071 (95% confidence interval from −0.0339 to 0.0197), so that the 95% confidence limits for the slope include 1, and the 95% confidence limits for the y-intercept include zero. This is further indication that there is good agreement between the AOAC and OISC methods of extraction.

The results may also be compared as discussed by Wernimount (14). The difference between replicate determinations on each sample is calculated for each extraction procedure. The variances of the 2 procedures is compared by performing an F-test on the variances of the differences calculated above for each procedure. The calculated F statistic.
of 0.458 was less than the critical \( F \) statistic of 0.471 at the 95% confidence level. Therefore, we cannot say that the variances for the 2 extraction procedures are different at the 95% confidence level. Next, the differences between the mean results for the 2 extraction methods are calculated for each sample. The average of these differences is calculated along with a 95% confidence interval for the mean. The 95% confidence interval ranged from –78.35 to 11.54. Because zero is included in the confidence interval for the mean difference, the OISC and AOAC extraction procedures are not considered statistically different at the 95% confidence level.

### Method Ruggedness

A ruggedness study was performed on the proposed method to determine its sensitivity to minor changes in procedure. A 2\(^7\) partial factorial design was adopted, where combinations of 7 factors in the method were varied in a series of 8 experiments. The sample study design conforms to the formal ruggedness study design described by Youden and Steiner (15). Whereas Youden recommends variation from the norm in only one direction, this study incorporated both positive and negative variations from the normal method levels to examine the effects of changes in both directions.

The study examined the variability caused by changes in the amount of HCl used in the extraction (±12.5%), the amount of water used in the extraction (±8.3%), the extraction heating time (±16.7%), the use of low or high speed during extraction shaking, the extraction shaking time (±16.7%), the acetic acid concentration in the diluent (±10%), and the percentage of acetonitrile in the final dilution (±10%). The study used a feed sample guaranteed to contain riboflavin at 9900 mg/lb.

For each of the 7 factors, the difference between levels was calculated by subtracting the average of the 4 above-condition results from the average of the 4 below-condition results. The absolute values of all 7 differences were then ordered from largest to smallest. The results and the experimental conditions are summarized in Table 2. The overall CV of the results was 3.1%, which agrees well with the variability observed for a feed sample used by OISC for quality control. Therefore, the sample deviation, \( s \), was 310 mg/lb for the sample studied. When the absolute value of any factor difference exceeds \( \sqrt{2} \times s \) or 440 mg/lb, then the factor difference is said to be significant (13). Based on these conditions, none of the changes in the factors examined significantly affected the results. This is an indication that the method could be expected to transfer well to other laboratories.

### Conclusions

A method for the determination of riboflavin in animal feeds using reversed-phase LC was developed. The new extraction is simple, eliminates the use of pyridine, and performs well when compared with that of the AOAC Official Method. The method also has good linearity, recovery, and ruggedness.

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### References