A FLUOROMETRIC METHOD FOR DETERMINING THE RIBOFLAVIN CONTENT OF FOODSTUFFS

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A number of chemical methods have been described for the determination of riboflavin (1-13). These methods are in general limited in use because, when applied to certain products, either they are not specific for riboflavin or all the riboflavin present is not recovered (2). In most cases the authors of papers presenting riboflavin methods have indicated that the methods are of limited application either by the titles of the publications or in the descriptions of the methods. The various methods have proved of value both in the limited application for which they were intended and in forming the basis for the more general method presented in this paper.

Principle of Method—This method depends upon the following physical and chemical properties of riboflavin. (1) It fluoresces green when irradiated with blue light. (2) It is not destroyed by mild oxidation or by reduction. (3) It can be reduced to a non-fluorescing form with sodium hydrosulfite and reoxidized readily by shaking with air. (4) It is not reduced by stannous chloride. (5) The intensity of the fluorescence can be measured with a photoelectric cell.

This method for determining riboflavin in an unknown solution is an indirect one. This is necessary owing to the fact that in foodstuffs other pigments and fluorescing substances are usually present and accompany riboflavin on extraction. The pigments interfere with the accuracy of the determination by absorbing a part of both the activating and the fluorescent light and hence cause the direct determination of riboflavin by measurement of fluorescence to be too low. The fluorescent substances interfere with the accuracy of the determination by fluorescing in the
green portion of the spectrum and hence cause the direct determination of riboflavin to be too high. The interfering pigments can be gotten rid of to some extent and the interfering reducible fluorescent substances entirely by resorting to a preliminary reduction with sodium hydrosulfite and stannous chloride, since in the presence of the latter reagent none of the interfering substances encountered up to the present time is readily reoxidized to the fluorescent form, whereas riboflavin is.

The difficulty of getting rid of the stable interfering pigments is overcome by adding a known amount of a standard solution of riboflavin to the unknown solution after a preliminary measurement with the fluorometer and determining the extent by which these interfering pigments decrease the value of the added riboflavin. Then by applying a correction factor the quantity of riboflavin in the unknown solution is determined. Stable substances which fluoresce green are eliminated by reducing the riboflavin in the unknown solution with sodium hydrosulfite and determining the concentration of riboflavin by difference.

Reagents—

Sulfuric acid, approximately 0.25 N.

Trisodium phosphate solution; 65 gm. of Na₃PO₄·12H₂O dissolved in 1 liter of distilled water.

Acid-acetone solution; 3 volumes of commercial acetone plus 1 volume of N sulfuric acid.

Standard riboflavin solution; a weighed amount of riboflavin of the highest purity obtainable dissolved in distilled water and made to a known volume. A concentration of 50 micrograms per ml. makes a convenient solution. This solution should be preserved in a cool, dark place. The addition of a few drops of glacial acetic acid to the solution will insure an acid pH and help to stabilize it. Dilutions of the standard solution are used to standardize the instrument and in preparing a special standard solution for use in the determination of riboflavin in unknown solutions. The concentration of the special standard solution depends upon the sensitivity of the fluorometer.

Sodium hydrosulfite solution; 1 gm. of sodium hydrosulfite₁ and 1 gm. of sodium bicarbonate dissolved in 20 ml. of ice-cold

₁ Kahlbaum or Eastman.
distilled water and kept in an ice bath. The solution is stable for approximately 4 hours.

Stannous chloride. 10 gm. of stannous chloride are dissolved in 25 ml. of concentrated hydrochloric acid and stored in a brown, glass-stoppered bottle. For the determination dilute 1 ml. of the above stock solution to 200 ml. with water. A fresh preparation of the dilute solution should be made daily.

Apparatus—The fluorometer used most extensively in this work is a modification of the one designed by Hand (12) and is similar to the one described by Cohen (3). The following modifications were made in the Hand instrument. A Corning filter, H. R. lantern blue, No. 554, is used to give pure blue activating light. The light source is a General Electric mercury vapor lamp, type H3, 85 watt, stabilized with a Raytheon voltage regulator, type V4, to minimize the effect of line voltage variations. Because of the power factor of the special lamp transformer a General Radio variac transformer, type 200 CMH, connected for use on a 115 volt circuit, must be placed between the voltage regulator and the lamp transformer in order to step up the voltage. The variable transformer also permits control of the light intensity. The unknown solution is put in a 50 mm. cubical optical cell. The photocurrent is measured with a Lange multiflex galvanometer in order to obtain high sensitivity. It is essential that the fluorometer be highly sensitive, as the most effective means of determining riboflavin in the presence of large amounts of light absorbing impurities is to dilute the solution. It is sometimes necessary to use dilutions as great as 1000:1.

Standardization of Fluorometer—Before the instrument is standardized, it is necessary to regulate the intensity of the activating

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2 A fluorometer with suitable filters adaptable to the determination of riboflavin may be obtained also from Pfaltz and Bauer, Inc. This fluorometer has been used in obtaining part of the data presented in this report. When the line voltage is variable, it is necessary to use the voltage regulator and variable transformer in order to stabilize and control the lamp in the Pfaltz and Bauer instrument.

3 Corning Glass Works, Corning.

4 General Electric Vapor Lamp Company, Hoboken, New Jersey.

5 Raytheon Manufacturing Company, Waltham, Massachusetts.


7 Pfaltz and Bauer, Inc., New York.
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light so as to obtain a standard galvanometer response. This is accomplished by inserting a cube of canary glass which fluoresces in the green portion of the spectrum in the path of the activating light at such a position in front of the photocell that the galvanometer light beam comes to rest at an intermediate point on the scale. The position found for the glass cube must be easily reproduced; otherwise it will be difficult when the instrument is used in the future so to regulate the activating light as to obtain the standard galvanometer response. A solution of riboflavin or sodium fluorescein of appropriate concentration may also be used in obtaining the standard response in place of the canary glass cube.

After it has been adjusted to the standard response, the fluorometer is standardized by obtaining the galvanometer response to a series of riboflavin solutions of known concentration. These are prepared by diluting portions of the standard solution with distilled water. The solutions should vary in concentration from zero to a concentration which gives a full scale deflection of the galvanometer. Then the concentrations of riboflavin in micrograms per ml. are plotted against the galvanometer responses. The graph obtained is used to convert the galvanometer responses produced by unknown solutions into concentrations of riboflavin per ml.

If preferred, the concentration per unit of galvanometer deflection may be calculated and a factor obtained for converting the galvanometer deflection into the corresponding concentration of riboflavin in the unknown solution. This is possible because of the linear relationship between the concentration of riboflavin per ml. and the galvanometer response. That this relationship exists for low concentrations of riboflavin has been shown by Cohen (3). As an example of the linear relationship, a test on one fluorometer showed an average concentration of 0.00219 microgram of riboflavin per scale division with a maximum deviation of 2.3 per cent from an average of seven values spread over the entire galvanometer scale.

The fluorometer should be restandardized occasionally with a fresh solution of standard riboflavin. This is due to the fact that certain parts of the instrument change their characteristics slightly with use.
If one depends entirely upon the galvanometer response to a known amount of standard riboflavin solution added to the unknown solution after a preliminary reading is made, it is unnecessary to standardize the instrument in the manner just described. However, this is of value, as it permits of some check on the concentration of the standard riboflavin solution which is not entirely stable.

**Extraction of Riboflavin**—A 5 gm. sample of the unknown is weighed into a 500 ml. Erlenmeyer flask and 50 ml. of 0.25 N sulfuric acid are added. The sample is thoroughly mixed with the solvent. Any lumps that form when the liquid is added are broken up and the mixture is gently refluxed under a condenser for 1 hour. Then the extract is allowed to come to room temperature, after which it is brought to pH 7.0 to 7.5 with a trisodium phosphate solution, with nitrazine paper* as an indicator. Sufficient water is then added to make the additions 50 ml. This makes the total volume of liquid added to the sample 100 ml., or a dilution of 1:20. After standing 30 minutes, the mixture is filtered through a fluted filter and an aliquot of the filtrate is pipetted into a 200 ml. volumetric flask and diluted to approximately 175 ml. with distilled water. Then 2 ml. of sodium hydrosulfite and 2 ml. of stannous chloride are added, and the solution mixed and diluted to the mark. This mixture is allowed to stand for 10 minutes and then is poured into a liter Erlenmeyer flask and shaken vigorously for 5 minutes with access to air. The extract may be shaken by hand or with a mechanical shaker. A definite volume is pipetted into an optical cell for the fluorometric determination. The solution should be filtered if a precipitate forms during the preliminary reduction and oxidation. A faint cloudiness does not seriously interfere with the fluorometric determination. In this work it is essential to avoid exposing the riboflavin extracts to light as much as possible.

The size of the sample and the amount of solvent may be varied, of course, but the amounts given above have been found convenient. The size of the aliquot taken for dilution depends on two factors, the concentration of riboflavin, and the amount of light-absorbing impurities. Sufficient riboflavin should be present

* E. R. Squibb and Sons, New York.
in the diluted solution to give a reasonable fluorometer response. On the other hand, it is preferable that the light-absorbing impurities be of such a concentration as not to decrease the intensity of the riboflavin fluorescence by more than one-half as determined by adding a known amount of riboflavin to the unknown solution. In some instances, however, satisfactory results have been obtained when the intensity of the riboflavin fluorescence was reduced to one-fifth.

In the extraction of bulky materials, such as alfalfa meals and other dried forages, it is desirable to double the quantity of 0.25 N sulfuric acid used to extract the dry sample. Samples of milk by-products containing casein should be extracted with the acid-acetone mixture rather than the 0.25 N sulfuric acid. When considerable casein is present, neutralized 0.25 N sulfuric acid extracts cannot be filtered. Experience has shown that with carefully prepared milk by-products the preliminary reduction with stannous chloride and sodium hydrosulfite may be omitted. With badly caramelized products the preliminary reduction must be included.

The method outlined above does not yield extracts in which the riboflavin can be satisfactorily determined if exceedingly large amounts of light-absorbing impurities are present, such as extracts of material containing a relatively large proportion of molasses. However, reasonably accurate determinations have been made on a few samples of riboflavin concentrates containing molasses by means of the following modification.

To 10 ml. of the neutralized extract are slowly added 90 ml. of methyl alcohol and the mixture is filtered with precautions to prevent evaporation. A suitable aliquot of the filtrate is transferred to a 200 ml. volumetric flask, diluted to 175 ml., and the usual procedure followed. Precipitation with methyl alcohol or other miscible solvents is not always advisable, as riboflavin is sometimes lost on the precipitate produced. If it is necessary to use this procedure, a check on the recovery of added riboflavin should be made. In the case of riboflavin concentrates containing molasses recoveries of riboflavin added to the extract before the methyl alcohol precipitation varied from 95 to 113 per cent with an average of 101 per cent on three different products.

**Determination of Riboflavin**—The intensity of the mercury vapor
light is adjusted by means of the variable transformer so that the canary glass cube gives the standard response. Then the optical cell containing the unknown solution is placed in the fluorometer and a reading (a) made. A known amount of the special standard riboflavin solution (not more than 2 per cent of the unknown volume) is added and a second reading (b) made. After this the riboflavin in the solution is reduced by adding sodium hydrosulfite solution (2 per cent of the volume of the unknown solution) and a third reading (c) made. The same amount of standard riboflavin solution is also added to a volume of water equal to that of the unknown solution and a fourth reading (d) made.

Calculation—The galvanometer readings are converted into micrograms of riboflavin per ml. by referring to the standardization graph. The converted reading $c_1$ multiplied by $y$ (the correction factor used to compensate for the dilution resulting from the addition of the standard riboflavin solution and the sodium hydrosulfite solution) is subtracted from the converted reading $a_1$. The result of $a_1 - c_1y$ gives the uncorrected micrograms of riboflavin per ml. of unknown solution. This result must be corrected for the absorbing effect of stable interfering pigments upon the activating and fluorescent light. It is accomplished by multiplying the result by $d_1/(b_1 - a_1)$, the converted reading $d_1$ divided by the converted reading $b_1$ minus the converted reading $a_1$. This calculation increases the result to the extent that the value of the standard riboflavin solution is decreased by the stable interfering pigments. The result of this calculation when it is multiplied by the proper dilution factor ($z$) gives the micrograms of riboflavin per gm. of sample. The complete formula is as follows:

\[
(a_1 - c_1y) \frac{d_1}{b_1 - a_1} z = \text{micrograms of riboflavin per gm.}
\]

It is, of course, not necessary that the calculation be made exactly as given, if the proper principles are followed and the proper corrections made.

**DISCUSSION**

The extraction methods presented in this report were chosen for efficiency of extraction, simplicity of operation, and the elimina-
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tion of interfering pigments and fluorescent substances. They are
the result of a somewhat detailed study of this problem. In this
study attempts were made to extract riboflavin from samples
of dried whey, dried skim milk, and dried buttermilk with 60
per cent methyl alcohol, 60 per cent ethyl alcohol, acetic acid
and trichloroacetic acid, 60 per cent methyl alcohol 0.25 N with
respect to HCl, 0.25 N sulfuric acid, 0.25 N acetic acid, and 0.25 N
hydrochloric acid. No solvent extracted more riboflavin than
the 0.25 N sulfuric acid or the acid-acetone mixture, and, when
applied to a variety of foodstuffs, the latter two proved to give
extracts that were better suited for the fluorometric determina-
tion. It has not been possible to make a comparison of all methods
of extraction on all types of foodstuffs because of the work in-
volved and because during extraction with solvents containing
ethyl alcohol, methyl alcohol, and acetone so much other pigment,
chlorophyll, carotenoids, etc., is often dissolved that a fluoro-
metric determination is impossible. The fact that the recovery
of riboflavin added to milk by-products varied from 95 to 102
per cent is additional, although not infallible, evidence of the
completeness of the extraction.

A comparison of results secured by the fluorometric method
with those secured by the microbiological method of Snell and
Strong (14) and the modified absorption method is presented
in Table I. Good agreement was obtained between the results
secured by the fluorometric method and the microbiological
method of Snell and Strong. These authors state that the ac-
curacy of their method is within ±10 per cent. Since the results
of the chemical methods occasionally vary by 10 per cent, it is
believed that the discrepancies can be largely explained on the
basis of the variability of the methods.

The fluorometric results are in agreement with results obtained
by modifications of the absorption photometric method of Sullivan
and Norris (13). The modifications in the Sullivan and Norris
absorption photometric method consist essentially of the use
of the improved extraction procedures and of stannous chloride
and sodium hydrosulfite to eliminate interfering light-absorbing
compounds as in the fluorometric method reported herein. The
modified absorption photometric method has not proved suitable
### Table I
Comparison of Results Secured by Fluorometric Method, Microbiological Method, and Absorption Photometric Method

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Foodstuff</th>
<th>Fluorometric</th>
<th>Microbiological</th>
<th>Photometric</th>
</tr>
</thead>
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<tr>
<td>222</td>
<td>Dehydrated alfalfa meal</td>
<td>15.5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td>14.3</td>
<td>13</td>
<td></td>
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<tr>
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<td>&quot; &quot; leaf meal</td>
<td>17.6</td>
<td>17</td>
<td></td>
</tr>
<tr>
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<td>&quot; &quot;</td>
<td>13.1</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>171</td>
<td>&quot; &quot;</td>
<td>18.0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>233</td>
<td>Sun-cured &quot; &quot;</td>
<td>17.7</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>223</td>
<td>White fish-meal</td>
<td>10.6</td>
<td>11</td>
<td>9.7</td>
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<tr>
<td>229</td>
<td>&quot; &quot;</td>
<td>8.9</td>
<td>9</td>
<td>8.6</td>
</tr>
<tr>
<td>118</td>
<td>Meat scrap</td>
<td>8.4</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>&quot; &quot;</td>
<td>7.2</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>190</td>
<td>&quot; &quot; and liver meal</td>
<td>29.9</td>
<td>29.9</td>
<td>30.0</td>
</tr>
<tr>
<td>191</td>
<td>&quot; &quot;</td>
<td>28.9</td>
<td>34.3</td>
<td>28.2</td>
</tr>
<tr>
<td>193</td>
<td>&quot; &quot;</td>
<td>28.8</td>
<td>33.3</td>
<td>30.0</td>
</tr>
<tr>
<td>232</td>
<td>&quot; &quot;</td>
<td>29.1</td>
<td>25.0</td>
<td>28.9</td>
</tr>
<tr>
<td>235</td>
<td>&quot; &quot;</td>
<td>37.6</td>
<td>36</td>
<td>35.8</td>
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<td>Liver meal</td>
<td>78.8</td>
<td>72.0</td>
<td>74.5</td>
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<tr>
<td>225</td>
<td>Dried skim milk</td>
<td>20.0</td>
<td>17</td>
<td>21.4</td>
</tr>
<tr>
<td>236</td>
<td>&quot; &quot;</td>
<td>17.6</td>
<td>20</td>
<td>17.8</td>
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<tr>
<td>239</td>
<td>&quot; &quot;</td>
<td>19.2</td>
<td>19</td>
<td>19.6</td>
</tr>
<tr>
<td>5</td>
<td>&quot; &quot;</td>
<td>19.5</td>
<td>19.1</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>&quot; &quot;</td>
<td>22.2</td>
<td>21.7</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>&quot; whey</td>
<td>22.8</td>
<td>22.9</td>
<td></td>
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<tr>
<td>59</td>
<td>&quot; &quot;</td>
<td>25.7</td>
<td>26.6</td>
<td></td>
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<td>224</td>
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<td>231</td>
<td>&quot; &quot;</td>
<td>21.4</td>
<td>25</td>
<td>21.9</td>
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<tr>
<td>237</td>
<td>&quot; &quot;</td>
<td>20.7</td>
<td>21</td>
<td>20.8</td>
</tr>
<tr>
<td>226</td>
<td>&quot; yeast</td>
<td>32.4</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>238</td>
<td>&quot; &quot;</td>
<td>53.4</td>
<td>52</td>
<td>53.3</td>
</tr>
<tr>
<td>161</td>
<td>Ground soy beans</td>
<td>2.8</td>
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<td>234</td>
<td>Soy bean oil meal</td>
<td>4.6</td>
<td>4</td>
<td>4.7</td>
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<tr>
<td>182</td>
<td>Whole wheat</td>
<td>0.8</td>
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<tr>
<td>172</td>
<td>Wheat flour middlings</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>173</td>
<td>&quot; bran</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>193</td>
<td>&quot; flour</td>
<td>0.4</td>
<td></td>
<td></td>
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<tr>
<td>178</td>
<td>Yellow corn-meal</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>179</td>
<td>&quot; (granulated)</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>176</td>
<td>Oat groats</td>
<td>1.4</td>
<td></td>
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</tr>
<tr>
<td>184</td>
<td>Rolled oats</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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for determining riboflavin in alfalfa meal or in materials containing a relatively small amount of riboflavin.

SUMMARY

1. A fluorometric method for determining the riboflavin content of foodstuffs is presented.
2. Results secured by the method on a number of samples of foodstuffs are compared with results secured by the microbiological method of Snell and Strong and good agreement is shown.

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