

# Colorimetric Determination of Urease Activity in Soybean Meals<sup>1</sup>

GUENTER SCHRAMM, The Procter and Gamble Company, Cincinnati, Ohio, and  
PHILIP D. AINES, The Buckeye Cellulose Corporation, Cincinnati, Ohio

IN THE PROCESSING of soybeans the meal is subjected to moist heat treatment after removal of the oil. During the application of heat and moisture most of the enzyme urease, present in soybean meal, is destroyed. Thus low levels of urease activity give an indication that the meal has been subjected to heat treatment.

Urea is frequently added to ruminant feed formulations containing soybean meal. The urease enzyme in soybean meal decomposes the urea in the feed. As a result the value of the feed is decreased. Consequently the use of soybean meal in ruminant feed rations containing urea requires that the meal be toasted sufficiently to inactivate urease.

Many methods for the determination of urease activity have been developed (1-5). The test most extensively used for detecting urease activity in soybean meals is the modified Caskey-Knapp method (1, 2). In this method the urease activity is measured by the pH change, resulting from incubation of soybean meal with urea in a buffered solution under specified conditions.

When the pH rise test is used to determine the compatibility of soybean meal and urea, a maximum pH increase of 0.1 unit is frequently specified by the trade. Such small differences in pH are measurable, but the experimental error is considerable. Since the pH change is measured in a buffered solution, the formation of a small amount of ammonia will produce only a very slight change in pH.

The present work was undertaken to develop a method which offers increased sensitivity and reproducibility, particularly in differentiating between the meals with low levels of urease activity. Since the urease method is to be used in mill laboratories, the procedure must be rapid and require a minimum in laboratory equipment. A colorimetric determination of the residual urea after incubation with soybean meal appeared to be the most satisfactory procedure. The method described in this paper is based on the incubation of soybean meal with urea in a buffered solution and colorimetric determination of the residual urea with p-dimethylaminobenzaldehyde (6, 7).

The incubation procedure is a modification of that used in the Caskey-Knapp test. To permit the urease in the soybean meal to decompose urea, the meal is incubated with urea solution for 30 min. The concentration of urea is 0.04%. For such urea concentrations, maximum urease activity is reported at pH 7.6-7.8 (8). Our experiments confirmed maximum urease activity at pH 7.7-7.8. Therefore in the proposed method this pH range is specified. Approximate adjustment of the pH within the pH 7.7-7.8 range is satisfactory. To maintain the pH in the desired

range a buffered solution containing 0.05 M sodium pyrophosphate, adjusted with hydrochloric acid to the desired pH, is used. This buffer was chosen because it has a high buffering capacity at the desired pH and does not inhibit the action of the urease enzyme. This solution is used for all determinations without further adjustment of the pH.

Urease activity determinations with the colorimetric method indicated that enzyme activity is markedly higher at 40°C. than at 30° but shows only a little additional increase at 50°. Therefore 40°C. was chosen as the preferred incubation temperature.

In the colorimetric determination of urea with p-dimethylaminobenzaldehyde, interfering materials are easily removed with potassium ferrocyanide, zinc acetate, and charcoal. This technique has been developed for the determination of urea added to mixed feeds (7). Potassium ferrocyanide and zinc acetate flocculate the suspended materials. Charcoal decolorizes the solution. The materials interfering with color development are removed completely. Filtration is rapid, and the filtrate is clear and colorless. Upon addition of the color reagent to the meal filtrate, which contains the residual urea, a yellow color with maximum absorption between 420 and 435 m $\mu$  develops immediately at room temperature. Maximum color is reached after 10 min. and is stable for several hours. The absorbance of the colored p-dimethylaminobenzylidene urea is sensitive to temperature differences, but it causes no problems since the calibration of the method can be made at the same temperature as the individual determinations.

The use of diacetylmonoxime (9, 10) or isonitrosopropiophenone (11) for the colorimetric determination of urea is reported in the literature. However materials in soybean meal other than urea interfere with these color reactions. Attempts to remove the interferences were not successful. In addition, the color was not stable. Therefore diacetylmonoxime and isonitrosopropiophenone are not suitable color reagents for the colorimetric determination of urea in the presence of soybean meal.

## Method

*Apparatus.* For the incubation of the sample a constant temperature bath is required, capable of maintaining a temperature of  $40 \pm 1^\circ\text{C}$ . A Coleman Junior spectrophotometer, model 6A, 6B, or equivalent, and cuvettes of 1-cm. diameter are used for the absorbance measurements. The use of a mechanical shaker is recommended but not required.

*Reagents.* All reagents either are reagent grade or meet A.C.S. specifications. p-Dimethylaminobenzaldehyde (DMAB) solution is prepared by dissolving 16.00 g. DMAB (Eastman 95) in 1,000 ml. of 95%

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ethyl alcohol and adding 100 ml. of concentrated HCl. The solution is stable for one month.

Pyrophosphate buffer is prepared by dissolving 22.3 g.  $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$  in about 980 ml. of distilled water and adding 3 ml. of concentrated HCl. To obtain the desired pH more concentrated HCl is added dropwise until the pH is adjusted to 7.7-7.8, as measured on a pH meter. The solution is then diluted to 1,000 ml. The buffer may be used as long as it remains free of biological growth.

Buffered urea solution is prepared by dissolving  $0.400 \pm 0.0005$  g. of urea in exactly 1,000 ml. of pyrophosphate buffer. The solution is stable for one week. Zinc acetate solution is prepared by dissolving 22.0 g. of  $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$  in distilled water, adding 3 ml. of glacial acetic acid, and diluting to 100 ml. Ferrocyanide solution is prepared by dissolving 10.6 g. of  $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$  in distilled water and diluting to 100 ml. Vegetable charcoal Darco G-60, is used for decolorization.

**Preparation of Calibration Curve.** The calibration graph is prepared once and then used for all determinations. Aliquots of 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 ml. of buffered urea solution are pipetted into 25-ml. volumetric flasks. These aliquots correspond to 32, 48, 64, 80, 96, 112, 128, 144, 160, and 192 mg./liter urea. Exactly 10 ml. of DMAB solution are added to each aliquot and diluted to 25 ml. with distilled water. A reagent blank is prepared by diluting 10 ml. of DMAB solution with distilled water to 25 ml. The solutions are mixed and allowed to stand for at least 10 min. in tap water at 25°C. A different temperature may be used, provided the same temperature is maintained for both the calibration and routine determinations. Aliquots of the diluted urea solutions then are transferred into cuvettes. The absorbance (optical density) is measured at 430  $m\mu$  wavelength, using the reagent blank to set the spectrophotometer to zero absorbance.

To obtain the calibration curve the absorbance of each standard urea aliquot is plotted against the concentration of urea in mg./liter on regular coordinate paper. The calibration graph is slightly curved.

**Preparation of Sample.** The meal sample is ground without raising the temperature appreciably. It is preferable that at least 60% of the sample pass a 40-mesh screen.

**Procedure.** 1.000 g. of soybean meal is weighed into a 125-ml. Erlenmeyer flask. (For meals with known high urease activity a smaller sample is used). Exactly 50 ml. of standard urea solution are added and mixed. The sample is then placed in a constant temperature bath for 30 min. at 40°C. and shaken at 5-min. intervals. After  $30 \pm 1$  min. the sample is removed, and 0.5 ml. each of concentrated HCl, ferrocyanide solution, and zinc acetate solution, and about 0.1 g. of charcoal are added immediately. Medical droppers, marked at 0.5-ml. capacity, may be used to measure the 0.5-ml. volumes. The mixture is shaken for 15 min. mechanically or by hand, then filtered. If the filtrate is colored, the analysis is repeated, using more charcoal for decolorization. Exactly 10-ml. aliquots each of filtrate and DMAB solution are transferred to a 25-ml. volumetric flask and diluted with distilled water to volume. To prepare the reagent blank, 10 ml. of DMAB solution are diluted with distilled water to 25 ml. in a volumetric flask. To prepare the urea standard, exactly 10 ml. each of

buffered urea solution and of DMAB solution are pipetted into a 25-ml. volumetric flask and diluted to volume with distilled water. The solutions are allowed to stand for at least 10 min. in a container of tap water of the same temperature used for the preparation of the calibration curve. Aliquots of the reagent blank, standard, and samples are transferred into cuvettes. The absorbance is measured at 430  $m\mu$ , using the reagent blank to set the spectrophotometer to zero absorbance. The concentration of urea is determined on the calibration curve. The analyst can analyze six or more samples at one time.

**Calculation.** The urease activity of the soybean meal sample is: (mg./liter urea in standard solution) ÷ (mg./liter urea in sample). The urease activity is reported as mg./liter urea decomposed. If more than 130 mg./liter urea are decomposed, the analysis is repeated with a smaller sample and the calculation is made correspondingly.

**Urease Activity Unit.** The unit of urease activity is defined by the authors as one milligram per liter of urea decomposed by urease under the conditions of this colorimetric method.

## Results and Discussion

To evaluate the recovery of urea, six different soybean meals were analyzed by the outlined procedure. To eliminate decomposition of the added urea by urease, the enzyme in the samples was inactivated with HCl prior to incubation. Data on the recovery of urea are shown in Table I. The average recovery was 99.8%. The results demonstrate that the recovery of urea is quantitative; the presence of soybean meal and

TABLE I  
Recovery of Urea

Soybean meal, inactivated	Urea (mg./liter)		Recovery %
	Added	Found	
A.....	179	180	100.5
B.....	179	179	100.0
C.....	179	182	101.5
D.....	179	179	100.0
E.....	163	161	99.0
F.....	163	160	98.0

the addition of potassium ferrocyanide, zinc acetate, and charcoal have no effect on the recovery of urea. Therefore the decrease of urea results from the action of urease only.

To check the specificity of the proposed method, a composite sample of soybean meal and urease enzyme (Matheson, Coleman, and Bell, L817), both with known urease activity, was analyzed by the outlined procedure. The results are shown below:

Sample	Urease activity units (mg./liter urea decomposed)
Soybean meal.....	23
Urease enzyme.....	20
Meal and enzyme.....	43

The urease activity of the composite sample was identical with the sum of the urease activities of the components. These data confirm that the colorimetric method is quantitative and specific for urease.

The reproducibility of the method was studied on seven different soybean meals. Six determinations were completed on each of the seven meals. The de-

TABLE II  
Reproducibility of the Method

Sample	Urease activity unit (mg./liter urea decomposed)							Standard deviation (mg./liter urea decomposed)
	Analyst 1			Analyst 2			Mean	
A.....	59	59	65	61	62	65	62	3.0
B.....	35	35	39	32	37	37	36	2.4
C.....	22	27	27	21	24	30	25	3.5
D.....	14	18	23	11	17	18	17	4.0
E.....	6	10	11	6	10	11	9	2.4
F.....	0	3	3	0	1	2	2	1.4
Untoasted meal.....	1090	1100	1180	1120	.....	.....	1120	40.0

TABLE III  
Comparison of Urease Activities

Sample	Colorimetric method			Caskey-Knapp method		
	Mean mg./liter urea decomposed	Standard deviation mg./liter	Coefficient of variation % rel.	Mean pH units	Standard deviation pH units	Coefficient of variation % rel.
A.....	62	3.0	5	0.10	0.01	10
B.....	36	2.4	7	0.07	0.01	14
C.....	25	3.5	14	0.04	0.01	25
D.....	17	4.0	24	0.05	0.0	0
E.....	9	2.4	26	0.03	0.02	67
F.....	2	1.4	70	0.01	0.01	100
Untoasted meal.....	1120	40.0	4	1.65 <sup>a</sup>	.....	.....

<sup>a</sup> One determination.

terminations were run on different days and with different working solutions. To obtain representative data the urease activities were determined independently by two analysts. The data are summarized in Table II. The over-all standard deviation from the mean was  $\pm 3$  mg./liter. These data demonstrate that the method is very reproducible.

To compare the proposed method with the modified Caskey-Knapp method, pH increases were determined on the same soybean meals used for the reproducibility tests with the colorimetric method. The reproducibility was evaluated on four determinations of each sample. In Table III the results of the urease activities on the seven meal samples are summarized. Data on the coefficients of variation are included.

Since the reproducibility of the colorimetric method was determined by two analysts, the data are representative for typical routine analyses. The reproducibility of the pH rise test on the same meals was determined by one experienced analyst only. The data on the Caskey-Knapp method show a better reproducibility than is generally experienced.

Under the conditions of this comparison the coefficient of variation, expressed in per cent relative units, in the colorimetric method is about half of the coefficient of variation in the Caskey-Knapp method. Consequently the proposed method is at least twice as precise as the currently used Caskey-Knapp method.

Table III shows that urease activities for meals with low enzyme activity range from 2 to 62 mg./liter urea decomposed in the colorimetric method and from 0 to 0.10 pH units in the Caskey-Knapp method. It is evident that the range of urease activity values is much larger with the colorimetric method than with the Caskey-Knapp method. Therefore the proposed method is more sensitive.

The results demonstrate that the colorimetric method offers higher reproducibility and sensitivity than the modified Caskey-Knapp method. Because of the high sensitivity the method can distinguish between well processed and over-heated meals and differentiate between meals of low urease activity. The method is simple and adaptable to multiple determinations. No unusual equipment is required. The method is intended for, but not limited to, meals with low urease activities.

### Summary

A quantitative method for the determination of urease activity in soybean meals is described. The procedure is based on the incubation of the meal sample with a known amount of urea, followed by colorimetric determination of the urea with p-dimethylaminobenzaldehyde after incubation. Urease activity is expressed as the amount of urea decomposed under the specified conditions. The test is both accurate and specific for urease. The method is very reproducible and sensitive, especially at low urease activities.

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