

## A Method for Determination of Nitrogen in Ruminant Feedstuffs and Products

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**ABSTRACT :** A method for the determination of nitrogen in ruminant feedstuffs, products and excreta (e.g. milk and urine) using a spectrophotometer is developed, where samples processed for P determination are also used to determine N. Samples are digested with sulphuric acid and subsequently with hydrogen peroxide in Kjeldahl tubes. Digested solutions along with phenol and buffered alkaline hypochlorite reagents are incubated in a water bath at 37°C for 30 min and presented in the spectrophotometer. The spectrophotometer set at 625 nm measures the concentration of N of each sample. Nitrogen in 261 of the samples was also determined by the classical Kjeldahl method in order to develop a relationship between N determined by the Kjeldahl method (Y) and the colorimetric method (X). The mean value of Y was as high as that of X (0.92 vs. 0.96;  $p > 0.05$ ). The colorimetric method predicted Kjeldahl N highly significantly ( $Y = 0.985X - 0.024$ ,  $R^2 = 0.993$ ,  $p < 0.001$ ; or more simply  $Y = 0.974X$ ,  $R^2 = 0.993$ ,  $p < 0.001$ ). An analysis of regression found no difference ( $p > 0.05$ ; both t-test and F-test) between colorimetric (0.96% N) and adjusted (0.96% N) N. In comparison with the Kjeldahl method, the analytical capacity of N by colorimetric method increases greatly, where 200-300 determinations of N are possible in a working day. In addition, the system provides an opportunity to use not only the same digested solution for both N and P determination of a particular sample, but also uses the same spectrophotometer to assay both N and P. Therefore, the system may be attractive in situations where both elements of a sample are to be determined. In conclusion, the speed of N determination, low cost, efficient use of labour, time and reagents, fewer items of equipment, and the reduction of environmental pollution by reducing effluent and toxic elements are the advantages of this method of N determination. (*Asian-Aust. J. Anim. Sci.* 2003. Vol 16, No. 10 : 1438-1442)

**Key Words :** Nitrogen, Phosphorus, Kjeldahl, Spectrophotometer, Feedstuffs, Phenol-hypochlorite Method

### INTRODUCTION

Nitrogen (N) is one of the key elements considered in formulating rations for ruminants and poultry. Due to the increasing environmental concern and possible public regulations on phosphorus (P) as well as N, attention has been paid to control their excretion by ruminants (Islam et al., 2002; Paik, 2003), which means that these two elements are likely to be assayed more than before in most animal nutrition laboratories. N and P are also key elements determined in plant nutrition laboratories. Two classical methods of N determination are used in most laboratories with some modifications (Revesz and Aker, 1977; Jones, 1991). One is the dry oxidation (combustion) method of Dumas (1831). The other one is the most widely used wet oxidation method of Kjeldahl (1883). Although the Kjeldahl method has had wider recognition than the Dumas method, the former is labour intensive, requires a large amount of potentially dangerous chemicals, and is less suitable for large-scale analysis such as the residues of *in situ* trials. Moreover, the Kjeldahl method generates a significant amount of effluent which poses a problem of disposal.

Several methods are also used to determine P. These include the method of Fiske and Subbarow (1925; Ammerman et al., 1982 and cited by Witt and Owen, 1983),

the molybdate-vanadate calorimetric method (Keonig and Johnson, 1942; cited by Martz et al., 1999) and the modified method of Cameron (1965). Emanuele and Staples (1989) used the acid-molybdate calorimetric method of Harris and Popat (1954). Khorasani et al. (1997) ashed samples (AOAC, 1984) and measured P by inductively coupled plasma emission spectroscopy. Although the procedures for the determination of N and P differed between laboratories, none of them determined N and P from a single digest. Had it been done, it would have been possible to reduce labour, time, and cost of reagents and apparatus. Moreover, during the process of determining N and P, both procedures generate effluent, which potentially pollute soil, air, surface and ground water. For example, the Kjeldahl procedure of N determination left about 100 ml of effluents, and the P determination, using the above methods, may produce about 25 to 100 ml of effluents per sample. If a single digestion method for both N and P could be adopted, these effluents per sample could be limited to 25 to 50 ml per sample instead of 125 to 200 ml per sample. Moreover, the Kjeldahl method uses toxic elements e.g. selenium which potentially pollute the environment. This study is therefore aimed at developing a method using a single digestion system to determine both N and P, with subsequent determination of N and P by a single spectrophotometer, which can reduce the risk of environmental pollution by reducing the amount of effluent and toxic elements (e.g. selenium).

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## MATERIALS AND METHODS

### Digestion of samples

The digestion of samples used for N determination in this method is essentially the same as digestion of samples for P determination (Mizuno and Minami, 1980). Digestions are conducted in 300 ml of Kjeldahl digestion tubes of known weight ( $W_1$ , g). The size of sample depends on the substrates used. For solid samples, such as feed and faeces, about 0.5 g is taken while for liquid samples like urine or milk, 10 g or 3 g is taken and the weight of sample is recorded ( $W_2$ , g). Usually duplicate tubes per sample are used. In each tube, sulphuric acid (5 ml) is added and shaken gently to dissolve the sample in acid. Each tube is then covered with a glass funnel. The tubes (usually 20 tubes) are placed in a steel rack and set in the Kjeldahl digestion block (Tecator 2020 Digester, Perstorp Analytical Company) where the temperature is set at 300°C. The tubes are heated for 2 h after they have reached 300°C. Hydrogen peroxide (4 ml) is then added gently to each tube with a pipette, 2 ml at a time, until the solution is clear indicating completion of digestion. During the addition of hydrogen peroxide, the tubes should be shaken gently. Heavy protective gloves should be worn during shaking or addition of hydrogen peroxide to the tubes. Then the tubes are heated for another 2 h. Some more drops of hydrogen peroxide are added only to those tubes where the solution is not clear. After digestion, tubes are cooled to room temperature and distilled water (25 ml) is added to each tube and mixed by shaking. The tubes are left overnight at room temperature by covering them with the plastic film. However, it is not necessary to keep the solution overnight and it can be used as soon as the solution reaches at room temperature. The weight of the tube after the addition of water is recorded ( $W_3$ , g) to determine the weight of digested solution ( $W_3 - W_1 = DS$ , g). The supernatants are then transferred to tubes (30 ml) to analyse for P or N using this method. P is determined from this supernatant using a spectrophotometer set at 420 nm, the results of which are not included here.

### Nitrogen determination

In this laboratory, N was determined from the same digest using the same spectrophotometer (U-2001 Spectrophotometer, Hitachi, Japan) that was used to determine P. The spectrophotometer was connected to an auto-sampler (AS-1000, Hitachi, Japan) and both spectrophotometer and auto-sampler were connected to a printer. Four standard samples (0, 1.6, 3.2, 6.4 and 8.0 mg N l<sup>-1</sup>) were prepared. Stock N solution was prepared using ammonium sulphate (1.886 g in 1 litre of distilled water). Standard solutions were prepared using 1, 2, 4 and 5 ml of stock solution mixed with 4, 3, 1 and 0 ml of

distilled water, respectively. In addition, distilled water was used as a blank (0-mg N l<sup>-1</sup>). The concentration of N to be determined by the spectrophotometer should be within the range of the standard solutions. In cases where N content in digested samples is higher than in the standards, digests should be diluted with distilled water so that the N in digesta remains in the range of standards used (i.e. 0 to 8.0 mg N l<sup>-1</sup>). Two other solutions are needed to determine N by the spectrophotometer; phenol reagent and buffered alkaline hypochlorite solution. Phenol reagent is prepared by dissolving phenol (10 g) and sodium nitro-prusside (0.05 g) in distilled water to make 1 litre of solution. Buffered alkaline hypochlorite solution is prepared by dissolving sodium hydroxide (5.0 g), di-sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ) (53.6 g) and sodium hypochlorite (10 ml) in distilled water to make 1 litre. Now 0.2 ml of the standard or unknown solutions, all in duplicate was taken in spectrophotometer tubes (10 ml, Pyrex, Iwaki Glass, Japan) and phenol solution (2.5 ml) was added by autopipette (Shibata Nichiryo, Japan) and mixed (Touch Mixer, MT-31, Yamato, Japan). Then alkaline solution (2.5 ml) was added to the spectrophotometer tube and mixed as above. All tubes were then incubated in a water bath at 37°C for 30 min. Then the tubes were mounted on the auto-sampler of the spectrophotometer. Absorbance was measured at 625 nm for standards and unknown, and the calculated N concentration of each tube was printed automatically. The concentration of N (NC, mg N l<sup>-1</sup>) obtained against each absorbance by this method is used to calculate N (% DM) as follows:

$$N (\% \text{ DM}) = \frac{(\text{NC} \times \text{DS} \times \text{dilution rate})}{(\text{weight of sample, g} \times 1,000)} \quad (\text{equation 1})$$

Dry matter (DM) of all solid samples is determined in duplicate by drying at 60°C for 48 h.

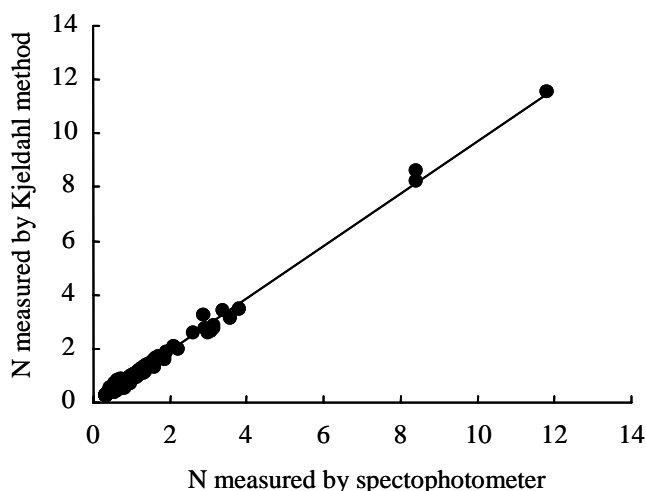
### Data analysis

A range of 261 samples was used to generate prediction equations. The samples include feed (forages and concentrates), faeces, urine, milk and *in situ* residues of silages incubated from 0 to 96 h in the rumen of Holstein steers. All 261 samples were assayed in duplicate for N using the Kjeldahl (AOAC, 1984) and colorimetric (spectrophotometer) methods. A simple linear relationship was developed to predict Kjeldahl N (Y) from the colorimetric N (X) i.e.,  $Y = a + bX$ . The  $R^2$  denotes the squared correlation co-efficient between the two methods of N determination. Since the relationship between X and Y was satisfactory and the intercept was low (-0.024), the intercept was set at 0 to develop the alternative equation  $Y = bX$  (Snedecor and Cochran, 1980). Moreover, the adjusted  $R^2$  ( $R^2_{\text{adj}}$ ) was estimated to correct for the effects of

**Table 1.** Values of N (% DM) used in developing relationship between colorimetric and Kjeldahl methods

Ingredients	n <sup>1</sup>	Spectrophotometer				Kjeldahl				Deviation, spectrophotometer minus Kjeldahl		
		Min.	Max.	Mean	S.D.	Min.	Max.	Mean	S.D.	Mean	S.D.	Significance
Feed, Orts, residue	237	0.30	11.78	0.89	1.08	0.27	11.58	0.87	1.08	0.02	0.07	ns
Feces	9	2.83	3.47	3.07	0.45	2.60	3.26	2.85	0.43	0.23	0.25	ns
Milk	8	0.49	0.64	0.56	0.03	0.40	0.46	0.44	0.00	0.12	0.03	ns
Urine	7	0.54	1.19	1.00	0.21	0.43	1.15	0.87	0.24	0.13	0.05	ns
Total	261	0.30	11.78	0.96	1.10	0.27	11.58	0.92	1.09	0.04	0.09	ns

<sup>1</sup> Each n is the mean of duplicate determinations in both methods.



**Figure 1.** Relationship between N (% DM) determined colorimetric and Kjeldahl method ( $Y=0.985X-0.024$ ;  $R^2=0.993$ ;  $p<0.001$ ). When intercept was set at zero then  $Y=0.974X$  ( $R^2=0.993$ ;  $p<0.001$ ).

reduction of degrees of freedom for error (SAS, 1988). Data on N content obtained by the two methods was analysed for variances using the paired t-test and F-test for two variances (SAS, 1988). The relationship between N of individual group of samples (e.g. feed, faeces, milk etc) obtained by the two methods was also examined. Analysis of regression between adjusted and regressed N obtained from both colorimetric and Kjeldahl N was tested for significance to investigate whether there was any difference between adjusted and regressed N as well as between colorimetric and adjusted N to determine the validity of the equation (Steel and Torrie, 1980).

## RESULTS

Table 1 shows the range, mean and SD of N of all samples determined by the Kjeldahl and colorimetric methods. Ranges, means and SD's of N determined by both methods are similar, although values of N obtained by colorimetric method were slightly higher than those obtained by the Kjeldahl method. However, both t-test and F-test found no significant difference of N obtained by the two methods (mean N (% DM): colorimetric, 0.96; Kjeldahl,

**Table 2.** Correlation ( $r$ ) and regression ( $R^2$  or  $R^2_{adj}$ ) for two equations used to predict N by Kjeldahl method (Y) from N determined by spectrophotometer (X)

Item	Equation 2 <sup>1</sup>	Equation 3 <sup>2</sup>
n	261	261
r	0.996	0.996
$R^2$	0.993	0.993
$R^2_{adj}$	0.993	0.990
Significance	***	***
RMS <sup>3</sup>	0.008	0.009

<sup>1</sup>  $Y=0.985$  (S.E. 0.005;  $p<0.001$ )  $X-0.002$  (S.E. 0.007;  $p<0.001$ );

<sup>2</sup>  $Y=0.974$  (S.E. 0.004;  $p<0.01$ )  $X$ ; <sup>3</sup> Residual mean square.

0.92;  $p>0.05$ ,  $n=261$ ). Despite a slight disparity in the mean value, the relationship between Kjeldahl N (Y) and colorimetric N (X) is highly significant ( $p<0.001$ ,  $Y=0.985X$  (S.E. 0.005)-0.024(S.E. 0.007)) (equation 2), where  $R^2$  and  $R^2_{adj}$  are 0.993 (RSD 0.008) and 0.993 respectively (Table 2). When the intercept is set at 0, the relationship between Kjeldahl and colorimetric N remains highly significant ( $p<0.001$ ,  $Y=0.974X$  (S.E. 0.004)) (equation 3; Figure 1) where  $R^2$  and  $R^2_{adj}$  are 0.993 (RSD 0.008) and 0.990 respectively (Table 2). Relationships within types of samples were also significant between Kjeldahl N and colorimetric N (Table 3). However, the relationship obtained with milk samples showed a poorer correlation between methods than with other types of samples (Table 3). Analysis of regression between regressed (0.96% N) and adjusted (0.96% N) values found no significant difference ( $p>0.05$ ; RSD 0.004) between them. The F-test and t-test between colorimetric and adjusted N obtained from the regressed value was insignificant ( $p>0.05$ ).

## DISCUSSION

The simple and highly significant linear relationship between N determined by Kjeldahl and colorimetric methods suggests that the N content of a wide range of feedstuffs, products and plant materials may be determined by colorimetric method with almost equal precision to that obtained by the Kjeldahl method. The similarity of N content as judged by the non-significant ( $p>0.05$ ) difference between the mean of N obtained by those two methods supports the validity of the relationship (0.96% N, colorimetric; 0.92% N, Kjeldahl). The principle of

**Table 3.** Equations to predict N by the Kjeldahl method (Y) from N determined by spectrophotometer (X), for four groups of samples

Item	n	R <sup>2</sup>	R <sup>2</sup> <sub>adj</sub>	Equation <sup>2</sup>	Equation <sup>3</sup>	RMS	Significance of regression slope
Feeds, orts and residues	237	0.996	0.996	0.997 (S.E. 0.004)X+0.023(S.E. 0.006)	0.987X	0.005	***
Feces	9	0.924	0.913	0.891 (S.E. 0.148)X-0.150 (S.E. 0.470)	0.922X	0.004	**
Milk	8	0.544	0.468	0.368 (S.E. 0.146)X-1.382 (S.E. 0.801)	0.775X	0.000	*
Urine	7	0.963	0.955	0.932 (S.E. 0.099)X-0.886 (S.E. 0.102)	0.869X	0.003	***

<sup>1</sup>Equation 3 (Y = bX) was used since all intercepts were significant (p < 0.05). RMS = Residual mean square.

determination in both methods is similar. Both methods use sulphuric acid to digest samples, but while the Kjeldahl method uses a catalyst reagent, the spectrophotometer uses an oxidising agent, hydrogen peroxide. The Kjeldahl method uses sodium hydroxide and boric acid to distil and then titrate with an acid to estimate N in the form of ammonia-N. The distillation and titration processes are, however, absent in the colorimetric method. Instead, the latter uses a phenol solution and subsequently an alkaline hypochlorite solution to generate colour with ammonia-N. Therefore, in both methods the released ammonia-N is used to determine N in a sample, and it is reasonable that the value of N obtained by either method would be similar.

However, the slight disparity between the mean values of N obtained by two methods is likely to be related to the liquid samples, particularly milk, where N obtained by the colorimetric method gave a greater estimate than the Kjeldahl method. This disparity was reflected in the relationship developed for milk N, determined by those two methods, where R<sup>2</sup> was about half of that with other types of samples (Table 3). This overestimation has been reflected in the mean and range used to develop the relationship for milk, although in the case of other types of samples mean, range and s.d. are in closer agreement with each other (Table 1). The reason why milk samples behave like that is not clear, but it should be mentioned that milk is foamed and burnt during digestion may be the reason for lower N, which needs further study.

The simplicity of the relationship between N by Kjeldahl and colorimetric methods lies in the fact that when the intercept is forced to 0, the relationship obtained for equation 3 (R<sup>2</sup><sub>adj</sub>=0.993) remains the same as that of equation 2 (R<sup>2</sup><sub>adj</sub>=0.993). Therefore, the applicability of equation 3 is expected to be broader than that of equation 2. Even when data with high N values are excluded, the relationship between Kjeldahl N and colorimetric N remains similar (R<sup>2</sup>=0.977, p<0.001, n=258; for both equation 2, Y=0.952X+0.003; and equation 3, Y=0.954X). However, high values are included in the equation to show that they remain on the same straight-line relationship as low values. The same regressed and adjusted N (0.96% N; RSD 0.004) obtained from analysis of regression and the insignificant difference (p>0.05) between colorimetric and adjusted N suggest that the result is likely to be acceptable.

Dilution of digested samples with distilled water may be needed when determining N by the colorimetric process. Since the N content in the digested solution should be no greater than the standard solution (i.e. N≤8 mg N l<sup>-1</sup>), the N content of the digested solution should be roughly determined or guessed and then diluted by distilled water (i.e. N free water) so that the digested solution is reduced to the range of 0 to 8 mg N l<sup>-1</sup> before being placed in the spectrophotometer. Alternatively, an entire set of samples may be diluted to the same extent before being placed in the spectrophotometer when high concentrations of N are encountered. For example, in a test run if the highest concentration of N of digested solution in the spectrophotometer appears as approximately 32 mg N l<sup>-1</sup>, the digested solution would be diluted 41 times with distilled water (e.g. 0.1 ml of digested solution diluted with 4 ml of distilled water). This dilution should be entered into equation 1.

The colorimetric method of N determination offers an opportunity for P to be determined from the same digest as N. Therefore, digestion cost, time, labour, reagents used and effluent produced may be minimised. Digestion of samples requires 4 to 5 h using the colorimetric method, which is similar to the time taken to digest the sample in the Kjeldahl method. However, it seems possible to speed up digestion in the new method if the digestion is conducted at 400-420°C instead of 300°C. This, however, needs further study. Moreover, the colorimetric method excludes distillation and titration and hence reduces the cost of apparatus, time and the possibility of error. The spectrophotometer needed to determine N is a cost for the alternative method, but most laboratories that determine P possess a spectrophotometer. A total of 100 samples can be run in a batch to determine N by spectrophotometer and a total of two to three batches (200-300 samples) can be run in a working day. These, and reductions in the quantity of effluents, are of the method for N determination used in the present study.

## CONCLUSIONS

The determination of N used in the present study is likely to be a step forward because there is no need to digest a sample separately for N and for P. Moreover, distillation and titration procedures are eliminated, and a total of 300

determinations of N are possible in a day using the method described in this paper. The new method is suitable for laboratories without funds to purchase modern distillation and titration apparatus, but that have a spectrophotometer. In situations where N and P need to be analyzed in the same sample, the method described here takes almost half the time of N determination by Kjeldahl and P determination by any of the methods described in the literature. Even if only N is determined following the method described here, total labour time is less and, reagents required and effluent produced are lower compared to the Kjeldahl method. Determinations can be made with almost equal accuracy to the Kjeldahl method.

The method has advantages over other methods in reducing cost, labour, time and reagents. Above all, it can determine two components, N and P, at the same time, which is always needed in animal and plant nutrition laboratories. Furthermore, the new method avoids distillation and titration steps saving apparatus and possibly errors. Also the new method does not need to use toxic metals e.g. selenium used by the Kjeldahl method.

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