Use of Near-Infrared Reflectance Spectroscopy and Dye-Binding Techniques for Estimating Protein in Oat Groats

R. BISTON and G. CLAMOT, Agricultural Research Center, Ministry of Agriculture, Gembloux, Belgium

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ABSTRACT

The techniques of near-infrared reflectance spectroscopy (NIRS) and dye-binding (DB) were applied for determination of protein in oat groats. Samples (584) ranging from 9.00 to 26.40% protein (N × 6.25) were subdivided into 37 standard samples to develop the calibration equations and 547 independent samples. Parameters a and b of the regression equations of NIRS and DB values on Kjeldahl values for the independent samples differed statistically from the same parameters of the theoretical calibration line (a = 0, b = 1). However, the observed deviations have no practical significance. Coefficients of determination of Kjeldahl and NIRS values were 0.992 and 0.943 for standard and independent samples, respectively, whereas for Kjeldahl and DB values, they were 0.994 and 0.977. Precision of analysis for the NIRS method, as measured by the residual standard deviation ($S_{y \cdot x}$) was 0.344 and 0.540% protein for standard and independent samples, respectively. Similarly, for DB method, the precision was 0.284 and 0.334% protein. Performances of the DB method indicate that the precision is less for high-protein samples than for low- or medium-protein samples. For the NIRS method, precision is not related to protein level. In conclusion, NIRS and DB are satisfactory techniques for estimating the protein content of oat groats in a breeding program.

Considerable interest has been shown in developing varieties of oats (*Avena sativa* L.) with higher protein levels. To accomplish this through varietal improvement programs, relatively quick, inexpensive, and accurate methods for determining protein are needed to facilitate screening of large numbers of oat lines and hybrids.

The usual Kjeldahl method of protein determination is expensive and time-consuming. Near-infrared reflectance spectroscopy (NIRS) and dye-binding (DB) methods offer simple, rapid, and inexpensive means for protein testing.

In 1971, NIRS was introduced in grain technology as a method of rapid analysis of oil, protein, and moisture content (Rosenthal 1971). NIRS instruments utilizing the original principle developed by K. F. Norris, U.S. Department of Agriculture, Beltsville, MD, were engineered by two U.S. companies. Both instruments claim to be capable of complete analysis of protein, oil, and moisture within a few seconds, with an accuracy equivalent to that obtained with standard laboratory procedures. The NIRS method has been extensively tested for measurement of oil and protein content of cereals, oilseeds, and grain legumes (Hunt et al 1977, Miller et al 1978, Pomeranz and Moore 1975, Hymowitz et al 1974, Watson et al 1976, Williams 1975). The fundamental importance of sample preparation for the successful use of NIRS techniques has been stressed by several workers (Graham 1975, Hymowitz et al 1974, Rinne et al 1975, Watson et al 1976).

In the DB method, an azosulphonic dye solution is mixed with a sample containing protein. The dye then bonds with the basic imidazol, guanidine, and amino groups of the protein. These groups may originate from the basic amino acids histidine, arginine, and lysine, and from free amino end groups of the protein chains. When the fraction of basic amino acids in the protein is reasonably constant, the dye-binding capacity can be quantitatively related to total nitrogen content, which gives an accurate estimate of the protein content. DB techniques have been tested for the estimation of protein content in wheat (Pomeranz and Moore 1975, Udy 1954, Udy 1956a, Wise et al 1965), barley (Olson and Heiges 1962, Pomeranz and Moore 1977), rice (Parial and Rooney 1970), soybeans (Hymowitz et al 1969, Moran et al 1963, Pomeranz 1965, Singh and Hymowitz 1971), herbage (Outen et al 1966), milk (Ashworth 1966, Ashworth and Chaudry 1962, Ashworth et al 1960, Udy 1956b), meat (Torten and Whitaker 1964), and common foods (Bunyan 1959). However, the usefulness of the DB method for determining protein in oats has not been fully explored.

This study considers the comparative efficiency of the NIRS and DB techniques on the basis of the values obtained through the Kjeldahl method for protein determinations.

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MATERIALS AND METHODS

Plant Material

The oat collection of the Plant Breeding Station, Gembloux, Belgium, containing 584 accessions, was investigated. Samples of seed having moisture contents of 10–12% were dehulled in a Labor Ear Thresher, type LD 180 ST 4 (F. Walter and Wintersteiger, Ried, Austria).

Dehulled samples were ground to powder in a Cyclotec sample mill (Tecator Inc., Boulder, CO) having a perforated discharge screen with holes 1 mm or less in diameter.

Because the NIRS and DB methods do not directly measure the protein content, they must be calibrated against standards. To develop standard curves, 37 seed samples were selected that represented virtually the entire range of Kjeldahl protein estimations expected in the oat groats (Table I).

Methods of Analysis

Kjeldahl Method. The equipment designed for the study was the Digestion System DS 20 and Kjeltec Distilling Unit II (Tecator Inc., Boulder, CO). A 1-g sample was used. The digestion mixture consisted of 7 g of K_2SO_4 and 350 mg of HgO, 10 ml of acid (five parts of concentrated H_3PO_4 per 100 parts of concentrated H_2SO_4), and 5 ml of 35% H_2O_2. Digestion was performed for 30 min at 420° C. The resulting mixture was distilled with steam for 3 min after addition of 50 ml of 40% NaOH. Ammonia was collected in 25 ml of 4% boric acid and titrated with 0.2*N* H_2SO_4. Protein content was calculated by multiplying the nitrogen content by 6.25.

Near-Infrared Reflectance Spectroscopy Method. A Neotec Feed Quality Analyzer model 51 (FQA) was used for all NIRS analyses. The basic operating principles and procedures were described by Trevis (1974), and detailed instructions are provided in the operating manuals.

In the many cases in which only 1–2 g of oat groats was available, the FQA sample cup was modified by introducing a ceramic disk between the sample and the plastic back plate of the cell (Williams

TABLE I
Comparison of Means and Ranges of Protein Content of Standard and Independent Samples

Method	Standard (n = 37) Mean (%)	Standard (n = 37) Range (%)	Independent (n = 547) Mean (%)	Independent (n = 547) Range (%)
Near-infrared reflectance spectroscopy	15.42	9.58–23.22	13.52	8.35–26.33
Dye-binding	15.41	9.87–23.00	13.30	8.79–25.41
Kjeldahl	15.43	10.03–22.94	13.36	9.00–26.40

et al 1978). This prevented contact between the infrared incident light passing through the thin layer of meal and the plastic, which has absorbance bands in the area of the spectrum where protein measurements are made. In this manner, samples as small as 1 g were successfully analyzed. NIRS determinations were performed as single tests.

Dye-Binding Method. The DB technique was described in detail by Udy (1971). The equipment designed for this study was the Udytec Protein Analyzer, model M (Tecator Inc., Boulder, CO).

Ground oat groats (480 mg) was weighed and placed into 50-ml polyethylene bottles to which 40 ml of reagent dye solution containing 1.320 g per liter of acid orange 12 was added. Forty-four samples were shaken simultaneously in a mechanical shaker for 1 hr. After shaking, the reacted samples were filtered through glass fiber filters, and absorbance of the unbound dye was measured at 480 nm by a color analyzer equipped with a flow-through short-light-path cuvette. All determinations were duplicated, and the averages were used.

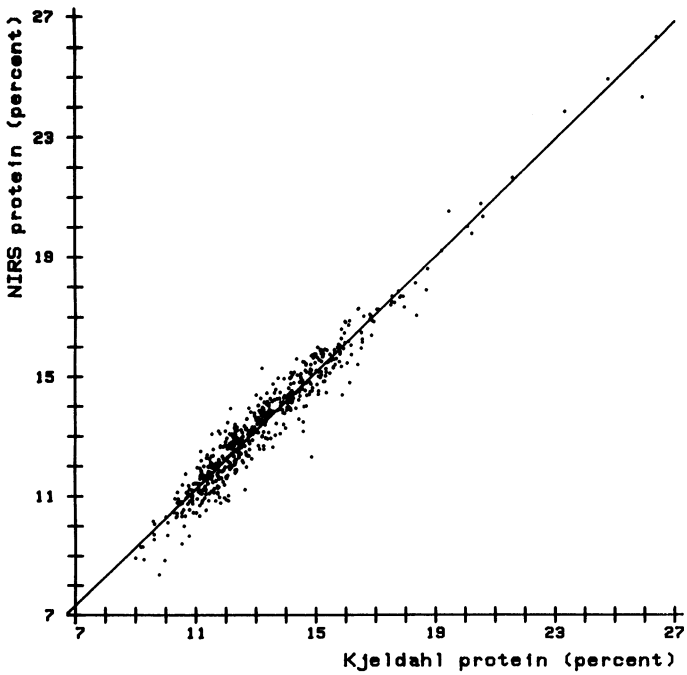

Fig. 1. Relation between near-infrared reflectance spectroscopy (NIRS) and Kjeldahl protein for 547 independent samples. y = 0.485 + 0.975 x.

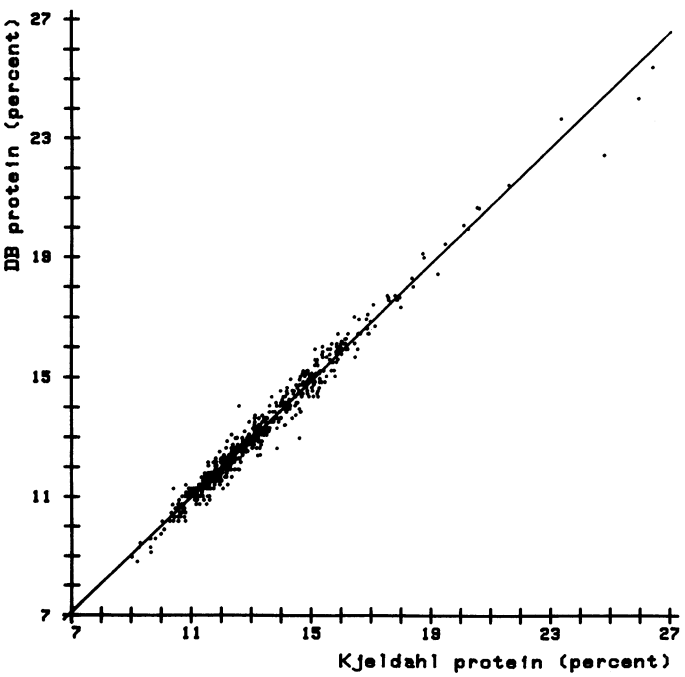

Fig. 2. Relation between dye-binding (DB) and Kjeldahl protein for 547 independent samples. y = 0.263 + 0.975 x.

RESULTS AND DISCUSSION

Calibration equations were developed for NIRS and DB methods using Kjeldahl values obtained from the 37 standard samples.

In the first method, the following equation was developed by multiple regression analysis:

$$\text{Protein percentage} = 19.8 - 697.6\,(\lambda_{2113} - \lambda_{2186}) - 448.4\,(\lambda_{2252} - \lambda_{2288}) + 940.6\,(\lambda_{2292} - \lambda_{2306}),$$

where λ_i (in nm) are the optical densities at the different wavelengths.

For the second method, the following regression equation was calculated using the method of least squares:

$$\text{Protein percentage} = (1.250 - C)/0.0346,$$

where C is the concentration of unbound dye at equilibrium. This equation is remarkably close to that of Udy (1971).

Table I shows the means and ranges of the protein content of the standard and independent samples obtained for the three methods. The standard samples were used to develop the calibration equations. The NIRS and DB values are very close to the Kjeldahl values for the standard samples. For the independent samples, the mean percent value for DB (13.30) is closer to the Kjeldahl value (13.36) than the NIRS value (13.52). The range values are very similar for the three methods.

Figures 1 and 2 present the relationship between the NIRS and DB values, respectively, and the Kjeldahl values for independent samples. A statistical evaluation of the performances of the first two methods for the standard and independent samples is shown in Table II.

In Table II, measurement of the accuracy is obtained by comparing the a and b of the regression equations of NIRS or DB values on Kjeldahl values to the same parameters of the theoretical line of calibration (y = x, where a = 0 and b = 1). For the standard samples, using either method, a and b values are not statistically different from 0 and 1, respectively, at the 5% level of significance. By contrast, for the independent samples, a is statistically higher than 0, and b is statistically lower than 1 for both methods. The crossover point between the regression line and the theoretical line of calibration is 19.4 for the NIRS method and 10.5 for the DB method. This shows that the NIRS method overestimates protein values lower than 19.4% and underestimates the higher ones, whereas the DB method overestimates the protein values lower

TABLE II
Relationship Between Near-Infrared Reflectance Spectroscopy (NIRS) or Dye-Binding (DB) and Kjeldahl Determinations of Protein Percentage

Samples	n[a]	a	s_a	b	s_b	r^2	$s_{y\cdot x}$	$CV_{y\cdot x}$
NIRS method								
Standard	37	0.059	0.245	0.996	0.015	0.992	0.344	2.2
Independent	547	0.485	0.139	0.975	0.010	0.943	0.540	4.0
DB method								
Standard	37	0.140	0.202	0.990	0.013	0.994	0.284	1.8
Independent	547	0.263	0.086	0.975	0.006	0.977	0.334	2.5

[a] n = number of samples; a and b = intercept and coefficient of regression of NIRS values or DB values, respectively, on Kjeldahl values; s_a = standard error of a value; s_b = standard error of b value; r^2 = coefficient of determination; $s_{y\cdot x}$ = residual standard deviation; $CV_{y\cdot x}$ = residual coefficient of variation.

TABLE III
Performance of Near-Infrared Reflectance Spectroscopy (NIRS) and Dye-Binding (DB) Methods as Related to Protein Content Level

Kjeldahl Ranges (%)	n[a]	NIRS			DB		
		d	$s_{y\cdot x}$	$CV_{y\cdot x}$	d	$s_{y\cdot x}$	$CV_{y\cdot x}$
9.00–13.00	272	−0.034	0.535	4.0	−0.014	0.294	2.2
13.01–16.00	226	0.069	0.532	3.9	0.016	0.359	2.7
16.01–26.40	49	0.132	0.555	4.1	0.005	0.426	3.2
9.00–26.40	547	0.000	0.540	4.0	0.000	0.334	2.5

[a] n = number of samples; d and $s_{y\cdot x}$ = residual mean deviation and residual standard deviation, respectively, in relation to the calibration line established from all determinations; $CV_{y\cdot x}$ = residual coefficient of variation in relation to the general mean.

than 10.5% and underestimates the higher ones. The observed deviations, however, have no practical significance.

The coefficient of determination and the residual standard deviation, expressed either as absolute value ($S_{y\cdot x}$) or as the percent of mean ($CV_{y\cdot x}$), measure the precision of estimate of the NIRS and DB methods in relation to the reference Kjeldahl method. The observed values indicate that, for both methods, determinations made on the standard samples are more precise than ones made on the independent samples. They also show that the DB method is more precise than the NIRS method for both the standard and the independent samples.

The performances of NIRS and DB methods as related to protein level are presented in Table III.

Residual mean deviation takes into account the sign of the differences between NIRS or DB values and the corresponding values of the regression lines calculated from all observations. The fact that the residual mean deviations are negative for lower protein content and positive for higher protein content indicates the nonlinearity of the regressions. Conversely, comparison of values obtained for the residual standard deviation and the residual variation coefficient indicates that the precision of the determinations made by the DB method is less for high protein content than for low or medium protein content. By contrast, the precision of the NIRS method is not related to protein level.

Results of this study indicate that NIRS and DB are satisfactory techniques for estimating the protein content of oat groats in a breeding program. Although the NIRS method is less accurate than the DB method, it offers the distinct advantage of being simpler and quicker and of having fewer steps.

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