Improved Dye Method for Estimating Protein[1]

DOYLE C. UDY, Udy Analyzer Company, Boulder, Colorado 80302

ABSTRACT

Several improvements and extensions of the Udy dye method for estimating protein content in natural products are discussed. The many outstanding physical and chemical properties of the monosulfonic azo dye, acid orange 12, are presented. This dye is used in a stablizing pH 2 buffer system to react with the protein's basic groups that originate from the basic amino acids (BAA) histidine, arginine and lysine. A complete analysis requires less than 5 min. The ionic reaction rate is limited to the exposure rate of each binding site. Regression equations relating nitrogen content and bound dye are presented for several oilseeds, grains, legumes and animal products. Random mixtures of products, having wide differences in dye-binding capacity, are not amenable to this method of protein estimation. Because nonprotein nitrogen is not measured, and the critically essential amino acid, lysine, is measured only when it is nutritionally available, it is suggested that the amount of dye bound by the protein's BAA offers a better nutritional index than conventional nitrogen procedures.

INTRODUCTION

Subsequent to the orange G (OG) and amido black 10B (AB) dye-binding procedures, as first outlined by Udy (1,2) and Schober and Hetzel (3) in 1956, for estimating proteins, many investigators have successfully applied and favorably reported on these two azo dye systems.

Since OG has a ratio of two binding sites to one azo chromophore, its color sensitivity is essentially only half that of AB which has two binding sites and two color producing azo groups. Another azo dye, acid orange 12 (C.I. 15970), has some important advantages over either of the above dyes (4). Acid orange 12 (AO-12) is structurally the same as OG, as shown in Figure 1, with the exception that AO-12 has only one sulfonic acid group for binding dye. Consequently, the color change per protein binding site of AO-12 is roughly equivalent to AB and also about double that of OG. It has a broad absorption maximum at 482 mμ and bonds strongly with the proteins. Other important properties of AO-12 are its low moisture absorption tendency and the relative ease with which it can be completely purified. Thus, it serves exceedingly well as a primary standard and allows preparation of exact standard reagent dye solutions without recourse to new calibrations for each fresh batch of dye as is required by AB.

Rapid protein tests permit better quality control in the plant through faster acquisition of results and the use of more samples. The Udy dye method (UDM) offers a fast, inexpensive and accurate means of estimating the protein content of most commodities. Marketing of commodities with a high protein content is normally done on a guaranteed protein content, but the nutritional quality is often even more important than the quantity of protein. For sound, unaltered, high quality products, estimation of protein by the UDM correlates nearly perfectly with nitrogen content as measured by conventional methods. However, it is well known that when some materials are subjected to heat in processing (5), the labile, and often limiting amino acid, lysine, is chemically altered and becomes nutritionally unavailable. In these instances, the nitrogen content remains the same, but the amount of bound dye is reduced in proportion to the amount of lysine destroyed. Consequently, the correlation is correspondingly diminished. A primary purpose of having a guaranteed protein analysis on a given lot of any commodity is to provide the ultimate user with an index or guide indicating it's nutritional value. Dye-binding offers a means of determining both quantity of protein and nutritional value.

In 1963 this laboratory succeeded in developing a buffer system for the AO-12 dye solution that has responded well with most commodities investigated. Previous formulations were either unstable with some commodities or did not exhibit constant dye-binding characteristics. Along with AO-12, many other seemingly useful azo dyes were studied, but none of these had equal or better overall properties for this method of estimating protein.

Although the author has used AO-12 dye successfully on an extensive commercial basis since 1959, its use was first reported in a comparative study of the Udy and Kjeldahl methods applied to wheat in 1961 by Hart et al. (6) and 1962 by Olson and Heiges (7) on barley. Other workers have applied the UDM using the improved AO-12 dye-buffer system to wheat (8), barley (9), milk (10-12), rice (13), and soybeans (14). Mossberg (15) has utilized AO-12 dye to evaluate protein quality in cereals. Procedures for a host of other commodities have been developed by Udy Analyzer Company and utilized in various industrial capacities.

PROCEDURES AND EQUIPMENT

Principle

Experience has shown that the proteins of essentially all sound, unaltered, natural products, or uniform fractions thereof, bind dye in a regular but individually characteristic manner. Each protein has a definite dye-binding capacity (DBC). For the total protein complex of a given commodity, the DBC can be quantitatively related to total nitrogen content to enable an accurate estimate of the protein content. The UDM was adopted as an approved method in 1967 (16,17).

Irregular protein systems, as present in a typical finished feed mix, also bind irregular amounts of dye. Consequently, a meaningful relationship between bound dye and nitrogen content is not possible for such mixtures. Commodities with nearly equal DBC could, however, be mixed. In this regard it is interesting to note in Table I that peanuts, sesameseed, soybeans and soybean cake show nearly identical DBC and could, therefore, be randomly mixed and still yield an accurate protein estimate by this method. Alfalfa,

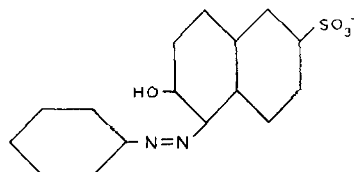

FIG. 1. Acid orange 12 dye (C.I. 15970) showing single anionic sulfonic acid binding site.

[1]One of four papers being published from the Symposium "Sampling and Process Control in the Oilseed Industry," presented at the AOCS Meeting, New Orleans, April 1970.

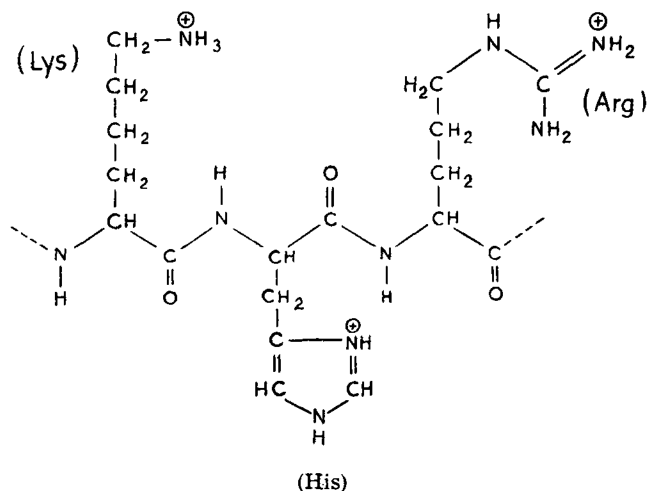

FIG. 2. Simulated protein chain showing cationic BAA binding sites at pH2.

coastal Bermuda grass and orchard grass constitute another example of commodities having essentially the same DBC.

In principle the buffered dye solution is mixed with the protein-containing sample: $Protein^{+} + (xs)\ dye^{-} \leftrightharpoons protein^{-}dye + dye^{-}$. The anionic, sulfonic acid dye bonds quantitatively with the cationic imidazol, guanidine and amino groups of the proteins (18). These groups, as depicted in Figure 2, originate from the basic amino acids (BAA) histidine, arginine and lysine, as well as from free amino end groups of the protein chains.

The resulting insoluble protein-dye complex is then readily filtered or centrifuged to allow colorimetric measurement of the unbound equilibrium dye concentration. By plotting this concentration against nitrogen content, a straight line regression is obtained.

Reagents

Reagent Dye Solution. Dissolve 1.300 g dry AO-12 (100% dye content) in about 100 ml warm 0.05 M phosphate buffer. Cool and dilute to 1 liter with buffer at 20 C.

Reference Dye Solution. Dissolve 0.600 g dry AO-12 (100% dye content) and 1 ml propionic acid in about 100 ml warm water. Cool and dilute to 1 liter with water at 20 C.

Phosphate Buffer (0.05 M). Dissolve 3.4 g KH_2PO_4 3.4 ml H_3PO_4 [1 (85%) + 1] v/v, 60 ml acetic acid, 1 ml propionic acid, and 2 g oxalic acid in about 800 ml water. Dissolve oxalic acid and KH_2PO_4 separately in hot water then combine with other components. Cool and dilute to 1 liter with water at 20 C. (Note: Volume of dilute H_3PO_4 in reference 16 is incorrect.)

Dye Purification

AO-12 dye has about 80% dye content as obtained from commercial suppliers (National Aniline Division, Allied Chemical Corp., Chicago). To purify, completely dissolve 100 g dye in 400 ml boiling distilled water. Immediately stir in 400 ml of boiling, denatured ethyl alcohol and allow to cool at 0 to 5 C for about 15 hr. Filter through a polypropylene or equivalent filter, fitted to a Buchner funnel and vacuum filter flask, with a water aspirator. Wash once with cold alcohol and continue vacuum filtration until all excess liquid is removed. Dry at 125 C. Yield is 75% to 80%, and purity is about 98%. Repeat recrystallization and dry in vacuum oven to obtain 100% purity. Regular oven drying reduces moisture to about 0.5%.

Apparatus

The apparatus used were: (a) Udy (Udy Analyzer Co., Boulder, Colo.) react-r-shaker provided with react-r-tube, or shaker assembly capable of shaking horizontally several plastic sample bottles, or both. (b) Automatic pipet calibrated to deliver 40 ml. (c) Syringe type pipet for measuring liquid samples. (d) Colorimeter (Udy Analyzer Co., Boulder, Colo.) or spectrophotomer equipped with a flow-through type short-light-path cuvet (19) and a 480 mμ color filter. (e) Cyclone type sample mill (Udy Analyzer Co., Boulder, Colo.) (20) having a perforated discharge screen with holes of 0.5 mm or less. Mill should be capable of reducing 30 g of sample in less than 1 min with a recovery of 99% or better and should not require clean out between samples. (f) Filtering equipment (Udy Analyzer Co., Boulder, Colo.) adapted for inert glass fiber filters or a suitable centrifuge capable of clarifying the protein-dye reaction mixture. If centrifuged, use a medicine dropper to transfer into cuvet. (g) Analytical balance with 1 mg sensitivity.

TABLE I

Regression Equations and Specific Procedures[a] for Several Commodities

Commodity	Sample weight, mg	Reaction time, min	Regression equation $P = (C_I - C)/k$
Alfalfa	500	3	P=(1.130-C)/.0310
Alfalfa haylage	500	3	P=(1.110-C)/.0247
Barley	800	3	P=(1.000-C)/.0333
Beans	320	3	P=(1.110-C)/.0200
Bermuda grass	500	3	P=(1.130-C)/.0310
Chickpeas	320	3	P=(1.210-C)/.0295
Corn	1000	3	P=(1.100-C)/.0545
Corn silage	800	3	P=(1.050-C)/.0380
Cottonseed	140	5	P=(1.300-C)/.0143
Cowpeas	320	3	P=(1.150-C)/.0240
Fishmeal	120	5	P=(1.300-C)/.0110
Gainesburger	320	3	P=(1.300-C)/.0286
Gram	320	3	P=(1.190-C)/.0275
Lentils	280	3	P=(1.150-C)/.0214
Linseed	280	3	P=(1.150-C)/.0200
Milk	2300	¼	P=(1.231-C)/.1700
Milk powder	240	2	P=(1.295-C)/.1865
Mungbeans	320	3	P=(1.200-C)/.0277
Mustardseed	200	3	P=(1.210-C)/.0192
Mustard meal	200	3	P=(1.300-C)/.0185
Oats	720	3	P=(1.150-C)/.0588
Oats groats	480	3	P=(1.230-C)/.0344
Peanuts	160	3	P=(1.300-C)/.0153
Peas	280	3	P=(1.260-C)/.0270
Pigeon peas	320	3	P=(1.300-C)/.0302
Rapeseed	340	3	P=(1.200-C)/.0260
Rice	800	3	P=(1.220-C)/.0655
Rye	720	3	P=(1.020-C)/.0300
Safflowerseed	240	3	P=(1.190-C)/.0185
Sesameseed	160	3	P=(1.300-C)/.0157
Sorghum	1000	5	P=(1.050-C)/.3750
Soybeans	160	3	P=(1.300-C)/.0151
Soybeans hulls	800	3	P=(1.300-C)/.0689
Soybean cake	160	10	P=(1.300-C)/.0147
Urd beans	320	3	P=(1.220-C)/.0280
Wheat	800	3	P=(1.000-C)/.0333
Wheat bran	500	3	P=(1.000-C)/.0281
Wheat flour	1000	3	P=(1.090-C)/.0408
Wheat gluten	200	3	P=(1.300-C)/.0108
Wheat midds	500	3	P=(1.300-C)/.0462
Yeast	200	3	P=(1.300-C)/.0149

[a]General procedure: weigh specified amount of finely ground and representative sample; dispense 40 ml Udy Reagent Dye solution into React-R-Tube; transfer weighed sample quantitatively into React-R-Tube; react in React-R-Mill for the indicated number of minutes; adjust temperature, set Color Analyzer reference position, filter and measure.

Colorimeter Calibration

After the colorimeter has attained thermal equilibrium, adjust zero setting and fill the cuvet with reference dye solution. Set meter needle to the reference scale reading of 42.0. Determine relative scale readings of the following AO-12 dye concentrations: 0.350, 0.450, 0.750 and 0.850 g/liter. Using semi-log graph paper, plot log scale reading

TABLE II

Stabilizing Effect of Acetic Acid on DBC[a] of Milk

Phosphate buffer with 6% acetic acid		Phosphate buffer without acetic acid	
EDC[b]	DBC	EDC	DBC
0.446	3.08	0.642	3.54
0.547	3.12	0.692	3.64
0.955	3.15	0.749	3.70

[a]Dye-binding capacity as decigrams of dye bound per gram of milk protein (%N x 6.38).

[b]Equilibrium dye concentration in g/liter.

TABLE III

Effect of Unbound Dye Concentration on DBC of Milk

Grams sample	Dye conc.	DBC[a]	Per cent protein	Per cent protein error[b]
1.652	0.792	3.155	3.66	+ 0.04
2.022	0.685	3.138	3.66	+ 0.02
2.332	0.600	3.120	3.66	0
2.590	0.531	3.102	3.66	- 0.02
2.821	0.471	3.085	3.66	- 0.04
3.025	0.420	3.070	3.66	- 0.06
3.215	0.373	3.055	3.66	- 0.08

[a]Decigrams of dye bound per gram of milk protein (%N x 6.38).

[b]Error resulting from assumption of constant DBC of 3.120.

against dye concentration. This technique gives an expanded scale with much greater sensitivity than a standard per cent transmission procedure. Suitable colorimeters with narrow band color filters yield good straight line relationships.

Determination

For solid samples use A.A.C.C. Approved Method 46-14 (17) with modified sample weight, reaction time, regression equation and standard conversion table for the specific commodity being analyzed. These values are tabulated in Table I.

For liquid samples use UDM adopted by A.O.A.C. (16) with values tabulated in Table I and the standard conversion table for the specific commodity being tested.

Conversion Table Preparation

Choose 20 or more representative samples of a given commodity that cover as wide a protein range as normally encountered. Reduce a 30 g portion of each sample in the cyclone mill, and store the prepared samples in sealed containers. Select a sample size that will contain about 14 mg of nitrogen at the median protein range, and use the same sample weight for all samples. React each sample to equilibrium with 40 ml reagent dye solution. Determine the equilibrium dye concentration (EDC) of each sample dye mixture. Plot these EDC values against nitrogen or protein content for each sample (see equation 2 below). Using the method of least squares, calculate a regression equation that can be used for estimating the protein content in any unknown sample of this particular product. A useful conversion table relating scale reading to per cent protein can be readily prepared. A larger number of samples will, of course, give a more significant relationship. Typical plots are shown in Figure 3.

RESULTS AND DISCUSSIONS

Buffer System

The need for a better buffer system than that originally used with OG (21) became evident when AO-12 dye was employed to bind with the proteins of alfalfa, soybeans, cottonseed meal and milk. A steady fading or loss of dye continued after apparent equilibrium had been attained. Also, the change in DBC with EDC was too great. Table II shows the effect of 6% acetic acid on the DBC value as well as its stabilizing effect on DBC over a wide EDC range. For an equivalent change in EDC, the DBC changes about four times less in the presence of 6% acetic acid.

Different ions have pronounced effects on the DBC of proteins. Acetate ion causes a large decrease while phosphate and chloride ions increase the DBC. Consequently, DBC values are very dependent upon the buffer system used. Other unpublished observations in our laboratory confirmed an irreversible chelation of AO-12 dye with heavy metal ions. Thus, it is necessary to use distilled or demineralized water for dye solution preparations.

Addition of oxalic acid to the reagent dye solution helped to minimize or eliminate the gradual loss of color after the reaction. The effect of oxalic acid was especially dramatic with milk. Color loss was evident within 1 hr in the absence of oxalic acid; however, in its presence, the EDC remained unaltered for 10 months or longer. Propionic acid effectively inhibited mold growth over this period. Preferential bonding of the calcium and other heavy metal ions with the oxalate ion are probably responsible for this increased stability. This problem has not been completely resolved for all products. Sequestering agents, EDTA and DTPA have not been effective in this capacity.

General Equation

Some commodities have shown essentially complete absence of interfering dye binding from nonprotein constituents, notably milk and soybeans. For these substances we can write the following general equation:

$$P = [VC_I - (V+v)C]/KW \qquad [1]$$

where P is the percentage protein, V is the milliliters of stock dye solution added, v is the milliliters of sample added, C_I is the concentration of stock dye in grams per liter, C is the concentration of unbound dye at equilibrium, K is the decigrams of dye bound per gram of protein, and W is the grams of sample. This equation is very useful for calculating the protein content when it becomes advantageous to vary the sample weight. Use of the UDM in accounting of milk products by federal regulatory laboratories (12) is an important example of this technique. As previously shown in Table II, the DBC can vary greatly with some buffer systems. However, with the buffer system above designated, the DBC remains sufficiently constant to allow the use of this equation over a wide practical range of EDC. Tables III and IV illustrate the modest effect of EDC on the DBC of milk and soybeans respectively. These results, along with those of Table V, confirm the premise

TABLE IV

Effect of Unbound Dye Concentration on DBC of Soybeans

Grams sample	Dye conc.	DBC[a]	Per cent protein	Per cent protein error[b]
0.120	0.860	3.83	38.6	+ 0.90
0.140	0.784	3.81	38.6	+ 0.65
0.160	0.712	3.78	38.6	+ 0.40
0.190	0.610	3.75	38.6	0
0.220	0.509	3.72	38.6	- 0.40
0.240	0.441	3.70	38.6	- 0.65
0.260	0.378	3.67	38.6	- 0.90

[a]Decigrams of dye bound per gram of soybean protein (%N x 6.25).

[b]Error resulting from assumption of constant DBC of 3.75.

TABLE V

Changes in Equilibrium Dye Concentration With Temperature

Temperature (F)	ΔT	Median dye concentration, mg/liter: 750 ΔC	750 ΔP	600 ΔC	600 ΔP	450 ΔC	450 ΔP
55	22	33	+0.20	34	+0.20	35	+0.22
65	12	17	+0.11	17	+0.12	18	+0.11
77[a]	0	0	0	0	0	0	0
85	8	12	- 0.09	14	- 0.08	12	- 0.07
95	18	29	- 0.18	28	- 0.17	29	- 0.18

[a]Reference temperature.

TABLE VI

Effect of Heating on Dye-Binding-Capacity

Hours heated[a]	Commodity: Whole soybeans	Extracted soybeans	Wheat flour	R. Meal bread
0	3.78	3.78	2.23	2.54
1	3.78	3.67	2.23	2.39
5	3.59	3.47	2.09	2.25
10	3.40	3.39	2.07	2.19
20	2.77	3.09	2.01	2.17
30	2.65	2.96	1.97	2.17
45	2.31	2.57	1.93	2.17
75	2.18	2.42	1.93	2.17

[a]Finely ground sample was oven-heated in an open vessel at 130 C.

that proteins from different commodities behave in an essentially identical manner, as one would expect, relative to complexing with dye. Because of this uniform behavior, a small EDC correction sufficient to yield an apparently constant DBC can be conveniently applied to cover all commodities. Even though the absolute DBC values may differ widely among commodities, the relative EDC corrections remain essentially equal because the different DBC values are reflected in each specific regression equation.

The shift of equilibrium DBC with temperature is quite pronounced and cannot be ignored in measurements of EDC. For example, the experimental error is approximately one fourth scale division or about 0.02% protein in milk. Also, the variation of EDC with temperature is equivalent to about 0.02% protein in milk per degree centigrade or 0.01% protein per degree farenheit as shown in Table V.

Nonprotein Binding

From Table I it is evident that many commodities do have constituents other than proteins that bind small

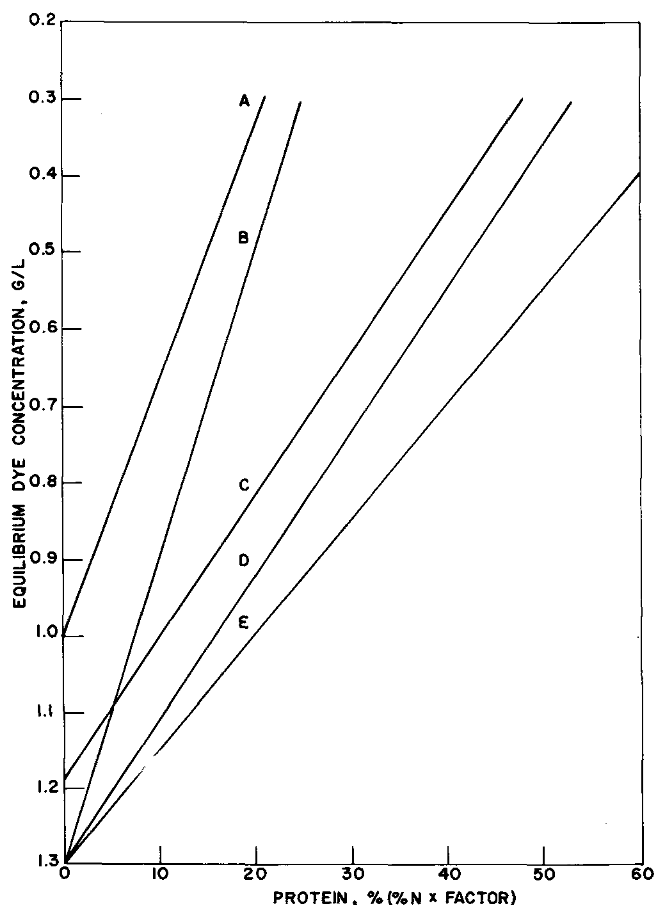

FIG. 3. Regression curves per equation 2: A, wheat; B, alfalfa; C, safflower seed; D, skim milk powder; E, soybeans.

amounts of dye. This is indicated by an intercept at a point other than the origin. Small grains are in this category. The principal nonprotein component binding dye is starch. Purified starch has a residual amount of nitrogen, and this is generally assumed to be protein. However, the amount of dye bound per unit of nitrogen would suggest the presence of a BAA group. On the other hand, the starch may simply physically adsorb a certain amount of dye. Rearrangement of equation 1 above gives:

$$C = VC_I/(V+v) - [KW/(V+v)]P \qquad [2]$$

This is the equation of a straight line, and as plotted in Figure 3, C is the ordinate, P the abscissa, $VC_I/(V+v)$ the intercept, and the coefficient of P is the slope.

For those commodities whose intercept, C_I, is not at the origin (1.300 g/liter, 0% protein), assuming a negligible sample volume, a regular variation of DBC is indicated. This is mostly caused by a change in the per cent of starch as the per cent of protein varies. The DBC of the proteins per se must remain essentially constant over the whole protein range, otherwise, the principle of the method is violated. Hageberg and Karlsson (22) have implied a regular variation of DBC with protein content in barley. Dye bound by nonprotein components must be taken into consideration. Also, the effect of EDC on DBC can be quite large for unstabilized buffer systems and could contribute significantly to an apparent but unreal change in the DBC of proteins at a given EDC and temperature.

Heating Effect

A substantial loss in DBC was observed when samples of soybeans and wheat products were heated in an oven at 130 C. Table VI shows a more rapid loss for the wheat products. This can most probably be attributed to a greater number of starch aldehyde groups available to react with the epsilon amino groups of lysine. A limiting DBC value was also attained with each material heated. When lysine was blocked (unpublished procedure for lysine screening developed by Udy Analyzer Co.) (23), the resulting DBC was essentially the same as the limiting value obtained by heating in each instance. Thus, heating can destroy all available lysine.

Earlier observations (21) indicated that the water soluble protein complex of wheat-flour had nearly double the DBC of the remaining acid dispersable protein complex. Since lysine is one of the BAA binding dyes, high DBC could be of significant nutritional value. Evidence for high correlations between BAA and DBC as well as between feeding value and DBC of heat damaged barley has been presented by Mossberg (15). Loss of available lysine in cottonseed meal is quite dependent upon the various processing methods used. These losses are associated with the amount of heating involved (5). Available lysine in cottonseed meal has been shown to be highly correlated with feeding value (24).

Nutritional Index

Soybeans and fishmeal have shown (25) good correlation between DBC, available lysine and nutritional value. High lysine corn has also been shown (26) to be comparable to soybeans in value in feeding experiments. Thus it would appear that DBC as a nutritional index could offer a substantial improvement over the present system of total nitrogen content, which does not reflect quality losses resulting from drum drying and other process heating procedures. In addition to this, the nutritional quality associated with BAA, such as found in high lysine corn, is not indicated by nitrogen measurements.

Milligrams of dye bound by proteins per gram of sample should be a useful nutritional index (U). This would represent the total milligrams of dye bound less the milligrams of dye bound by nonproteins per gram of sample. In terms of the symbols used in equation 1, U could be expressed as follows:

$$U = (V+v)\,(C_I - C)/W = KP \qquad [3]$$

Since both $(V+v)/W$ and C_I are constants for any given commodity and procedure, U would be a simple function of EDC.

For mixtures, the actual value of $(V+v)/W$ would be used, and C_I could be set at a fixed, agreed upon figure, e.g., 1.150 g/liter so as to yield uniform U values in commercial trading. Since sample weight does influence the C_I value because of nonprotein binding, this could be controlled by setting appropriate EDC limits, e.g., 0.4 to 0.8 g/liter.

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