Z. Ernährungswiss 15, 34–38 (1976)

John C. Weaver and Manfred Kroger, Division of Food Science and Industry The Pennsylvania State University, University Park, Pa. 16802 (U.S.A.)

Amido black dye binding of alfalfa and forage legume protein*)

M. Kroger

With 2 figures

(Received October 15, 1974)

An inexpensive, rapid method for the evaluation of proteins in forage crops would be desirable. Amido black dye binding, with equipment manufactured by *Foss* Electric Company, Hillerod, Denmark, is now routinely used for the quantification of milk protein and has been proposed for adoption by the Association of Official Analytical Chemists as an official method (15). Milk has a very accessible protein system that rapidly interacts with azo dyes. Milk is therefore most suitable in adapting it to automated protein determination by dye binding. Relatively inaccessible protein systems, as found in forage crops, would not exhibit such a complete and rapid reaction with the dye. Dye-protein interaction with such protein systems would be slower and subject to a number of conditions. The effects of several analytical parameters on the dye binding capacity of alfalfa protein remain to be investigated, in order to determine the optimum conditions and to propose a suitable method for accurate and precise results.

The chemistry of dye binding is not entirely understood. In working with dye binding it is necessary to control as many experimental variables as possible (14).

Reaction time with the dye, as well as particle size and uniformity, must be controlled to allow for complete dye binding in an equilibrium state (9). Reaction time depends on the solubility of the protein. Milk, containing dispersed and soluble protein systems, will attain equilibrium in seconds (3), while wool, with an insoluble protein, requires more than 24 hours (7). Equilibrium can be obtained faster by increasing the rate of agitation of the protein-dye system.

The temperature during dye binding is equally important and an optimum could be determined for each protein system. However, high heat treatment might cause a complexing between carbohydrates of the sample and the basic amino acids, thereby adversely affecting the dye-protein binding reaction (2, 13). Also, since the reaction is taking place at pH 2.2, heat denaturation would occur at a lower temperature than that at a higher pH (12).

MacKenzie and *Perrier* (10) used Orange G dye for the determination of protein in alfalfa and obtained a correlation of 0.88 with the *Kjeldahl*

*) Authorized for publication as Paper No. 4700 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Authorized 5/20/74.

method for crude protein. They concluded that dye binding was a suitable method for determining the protein in feed and forage crops. *Gengenbach* and *Miller* (8) also used Orange G dye in determining the protein content of alfalfa samples. Their results from the micro-*Kjeldahl* method of protein analysis and dye binding analysis showed a high correlation (0.96); the relationship was linear over the range of protein values expected of alfalfa herbage. A reaction time, with shaking, of at least 90 min. was required to obtain consistent results. *Outen* et al. (11) also compared forage dye binding with the *Kjeldahl* method and found significant differences between the results of the two methods and expressed doubt that dye binding was a satisfactory alternative to the *Kjeldahl* method.

In this paper, the use of amido black is demonstrated and those conditions are developed that are most suitable to suggest amido black dye binding as a simple, rapid screening test for protein content in alfalfa and other mixed legumes.

Materials and methods

Source of material. Forty-six samples of both alfalfa and other legumes from various areas were obtained from the Pennsylvania State University Soil and Forage Testing Laboratory. About 70 % of these samples were categorized "mostly mixed legumes".

Sample preparation. The samples were ground in a Wiley mill using a 2-mm screen and then reground in a small laboratory Wiley mill (40-mesh screen) (A. H. *Thomas* Co., Scientific Apparatus, Philadelphia, Pa.).

Dye binding analysis. The citrate-buffered amido black dye (pH 2.2) was the same as required in the dye binding method described for milk by the *Foss* Pro-Milk MKII apparatus (1). In general, the basic technique employed was as follows:

a) the sample, after grinding, was mixed with 20 ml of the dye in a Whirl-Pak plastic bag (NASCO, Inc., Fort Atkinson, Wisconsin)
b) several reaction periods and different reaction temperatures were selected to test for the optimum interaction conditions between the dye and protein of the sample
c) the supernatant was recovered by filtration
d) light transmittance through the supernatant was then determined.

The dye binding values recorded were obtained from the scale on the read-out unit of the Pro-Milk MKII. The lower scale indicates 0.0 for the unreacted dye and 100.0 for water.

Duplicates of each sample were prepared by weighing 0.300 g into the plastic bags, followed by the addition of 20 ml amido black dye solution. The bags were then sealed and hung on racks holding ten bags each. The loaded racks were placed in a *Dubnoff* metabolic shaking incubator (Precision Scientific Inc., Chicago, Illinois) covering a stroke of three inches and reciprocating at 44 strokes a minute. The study included reaction times of 8, 12 and 24 hours and temperatures of 50, 55, 60 and 65° C.

After the reaction the samples were poured into the mixing tube of the Pro-Milk MKII, passed with air pressure through its specific silicon-treated filter paper into the flow-through cuvette of the apparatus. Upon stabilization of the galvanometer needle, the dye binding value (DBV) was read.

All samples were also analysed for crude protein content by the macro-*Kjeldahl* method.

Results and discussion

A series of preliminary investigations was conducted to determine the parameters for the final evaluation of the 46 alfalfa samples. It was found that increasing reaction times (up to 24 hours) also increased the dye binding values obtained (Fig. 1). A time of 24 hours appeared to be the most suitable reaction time for a routine method, and 24 hours is believed to be the beginning of a plateau in Fig. 1. As the reaction temperature increased from 50 to 60° C, the dye binding values increased but at 65° C the dye binding values were lower again (Fig. 2).

The number of dye ions bound per molecule is always higher for denatured than native proteins (4, 5, 6). Therefore, it could be assumed that the temperature is changing the protein to a more denatured state as it increased from 50 to 60° C, since the dye binding values increased under these conditions. There must be an optimum to this relationship, since at 65° C a decrease in the dye binding values was observed. It is possible that heat denaturation or other phenomena occurred above 60° C causing conditions that lowered the protein's dye binding capacity.

Using 24 hours and 60° C the dye binding values were correlated to macro-*Kjeldahl* values. A correlation of 0.88 was obtained. The linear regression equation obtained was $K_m = 2.98 + 0.227$ (DBV). This equation would account for 78 % of the total variation in macro-*Kjeldahl* values. The standard error of estimate by the dye binding method was 1.67.

In summary, this study with amido black dye and the *Foss* Pro-Milk MKII apparatus was designed to determine the conditions necessary for maximum dye binding values with such forage crops as alfalfa and other legumes. These conditions are basic to a suitable method for the determination of protein in forage. Past work dealing with the measurement of alfalfa protein by dye binding shows investigations of temperature as a parameter that may influence dye binding values. From this study it can be concluded that:

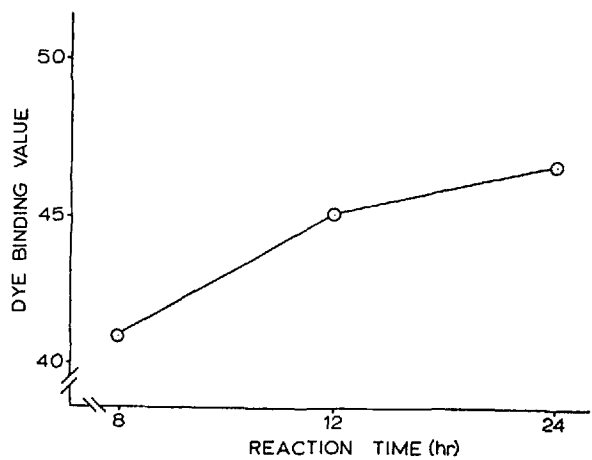

Fig. 1. (see text)

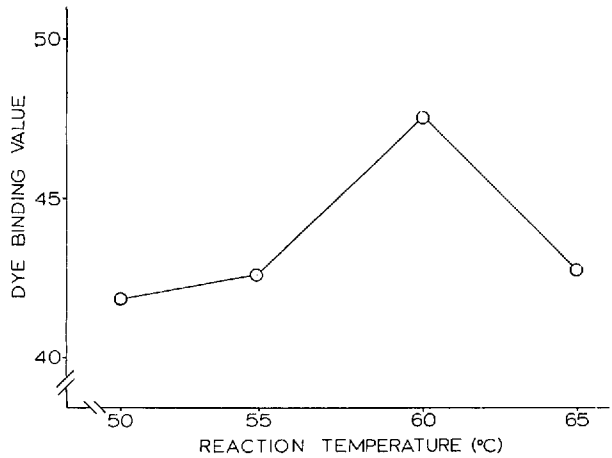

Fig. 2. (see text)

1. temperature plays an important role in obtaining dye binding values and must be strictly controlled; with 60° C yielding close to maximum values;
2. a reaction time of 24 hours yields close to maximum dye binding values;
3. amido black dye is suitable for measuring forage legume protein content;
4. the Pro-Milk MKII is adaptable to measuring alfalfa protein;
5. development of a standard method would be desirable.

Summary

Although dye binding has been suggested for the determination of alfalfa protein content, a standard method has not been advanced. More data are necessary on the conditions required for the dye-protein reaction. Reaction times of 8, 12 and 24 hours and reaction temperatures of 50, 55, 60 and 65° C were selected to obtain dye binding values (DBV) with the *Foss* Pro-Milk apparatus. A correlation of 0.88 with macro-*Kjeldahl* values was obtained. 60° C and 24 hours reaction time can be considered conditions yielding maximum DBV. It is suggested to develop a standard method for the routine measurement of forage legume content.

Zusammenfassung

Obwohl Farbstoffbindung schon als Proteintest für Luzerne vorgeschlagen worden ist, ist ein Standardverfahren noch nicht entwickelt worden. Weitere Grundlagen über die Verhältnisse der Farbstoff-Protein-Reaktion sind erforderlich. Mit einem Foss-Pro-Milch-Mk-II-Gerät wurden Farb-Protein-Verbindungswerte ermittelt nach Reaktionszeiten von 8, 12 und 24 Stunden und mit Temperaturen von 50, 55, 60 und 65° C. Der Korrelationskoeffizient mit dem Kjeldahl-Standardverfahren war 0,88. Reaktionsbedingungen von 60° C und 24 Stunden brachten die höchsten Farb-Protein-Verbindungswerte. Es wird empfohlen, eine Standardmethode für die routinemäßige Erfassung des Proteingehaltes dieser Futtermittel auszuarbeiten.

References

1. A/S N. *Foss*, Electric, Instruction Manual, Pro-Milk MKII Milk Protein Analyzer (Hillerod, Denmark 1971). – 2. *Bjarnson, J., K. J. Carpenter*, Brit. J. Nutr. **24**, 313 (1970). – 3. *Dolby, R. M., J. Dairy*, Res. **28**, 43 (1961). – 4. *Fox, S. W.*, Science **132**, 200 (1960). – 5. *Fox, S. W., K. Harada*, J. Amer. Chem. Soc. **82**, 3745 (1965). – 6. *Fox, S. W., J. E. Johnson, M. Middlebrook*, J. Amer. Chem. Soc. **77**, 1048 (1955). – 7. *Fraenkel-Conrat, H., M. Cooper*, J. Biol. Chem. **154**, 239 (1944). – 8. *Gengenbach, B. G., D. A. Miller*, Crop Sci. **13**, 155 (1973). – 9. *Lakin, A. L.*, Evaluation of protein quality by dye-binding procedures. In: *J. W. G. Porter* and *B. A. Rolls* (eds.), Proteins in Human Nutrition (New York and London 1973). – 10. *MacKenzie, A. J., E. R. Perrier*, Agric. J. **61**, 332 (1969). – 11. *Outen, G. E., J. M. A. Tilley, R. F. Wilson*, J. Sci. Food Agric. **17**, 285 (1966). – 12. *Tanford, C.*, Protein denaturation. In: *C. B. Anfinsen* et al. (eds.), Advances in Protein Nutrition (New York and London 1968). – 13. *Udy, J. C.*, Cereal Chem. **31**, 389 (1954). – 14. *Weaver, J. C.*, Dye binding and its relationship to other methods of determining food protein quantity and quality (Master of Science Thesis, The Pennsylvania State University 1974). – 15. *Weik, R. W.*, J. Assoc. Off. Anal. Chemists **57**, 264 (1974).

Author's address:

Dr. *M. Kroger*, Div. of Food Science and Industry,
The Pennsylvania State University, 105 Borland Laboratory,
University Park, Pa. 16802 (USA)