Influence of Temperature on Changes of Some Constituents in Alfalfa Meal and Formation of Glycosamines, Glycoproteins, and Melanoidins

Ilija Delić,* Božidar Milić,[1] and Mirko Vlahović

The influence of different drying temperatures (600°, 700°, and 800° C) on changes in the concentrations of hemicellulose, cellulose, lignin, glycosamine, glycoproteins, and melanoidins in alfalfa herbage was studied. High drying temperatures caused a decrease of hemicellulose percentage, and an increase of cellulose and lignin percentage in alfalfa dry matter. The applied temperatures produced a considerable increase in the concentrations of glycosamine (about twice), glycoproteins (about 10 times), and melanoidins (about six times) in the artificially dried alfalfa.

Many workers have studied the influence of the drying temperature on the degradation and nutritive value of proteins and carbohydrates in plant material. Thompson and Wolfrom (1958), Kertesz (1951), Waite and Boyd (1953), and Reynolds *et al.* (1962) found that drying temperature influenced degradation of the matrix structure, causing a possible shift of the starch, cellulose, hemicellulose, and uronide linkages. By degradation of the more complex polymers, simple sugars and their derivatives were formed. Underwood and Deatherage (1952), Raguse and Smith (1965), and others found that simple sugars undergo degradation, decarboxylation, or dehydration, and may form various new forms by interaction with other constituents. Ekern *et al.* (1965), Bathler *et al.* (1960), Gordon (1967), Duckworth and Woodham (1961), Van Soest (1965), Wiering (1960), Richards (1956), Goering and Van Soest (1967), and others have shown in experiments with laboratory animals that drying temperatures affect considerably the digestibility and nutritive value of carbohydrates and proteins in the dried plant tissue.

Interaction of certain constituents, the browning process and the formation of glycoproteins, hexosamine, and melanoidins in plant material, under the influence of the temperatures and duration of drying are of special interest. Several methods have been developed for determination of hexosamine, glycoproteins, and melanoidins in various materials. The resistance against digestive enzymes, the mechanism of the formation, and the composition of these substances have remained unexplained.

Kass and Palmers (1940), Agrawal and Goldstein (1968), Koehler *et al.* (1969), Lea and Hannan (1950), Clegg (1964), Karel and Labuza (1968), Talley and Porter (1968), Wrench (1966), Clamp and Hough (1965), Liener (1958), Kawamura *et al.* (1968), Spark (1969), and others have shown that the brown substances resulting from the nonenzymatic browning process are formed by the primary interaction of amino groups (proteins and amino acids) and the carbonyl groups of pentoses, aldo-, and ketohexoses. According to Van Soest (1965, 1967), and Burton and McWeeny (1964), an amorphous substance from the nonenzymatic browning process is deposited in the lignin fraction of an acid-detergent fiber and renders it insoluble and indigestible. It is worth mentioning the view that hexosamines, glycoproteins, and melanoidins are formed in different ways, and that the interaction of nitrogen and carbohydrate components occurs in different ways by binding amino compounds with simple sugars, furfural, and lignin, as reported by Anett (1960, 1961, 1962), Burton *et al.* (1963), and McWeeny and Burton (1963). Karel and Labuza (1968) found that melanoidins could not be formed in the absence of organic acids which serve as the catalyst in the formation of hexosamines and melanoidins. Lea and Hannan (1950) showed that the relation of C-atom of the sugar to N-atom of the amino groups in substances of melanoidin type amounted to 6:1. However, Liener (1958) stated that this relationship was 1:1. Both workers have emphasized the low digestibility of these substances in the digestive tract of animals, which has been confirmed by the presence of a large amount of these constituents in the feces.

Because there were so few studies related to the browning process of feeds during heat drying, the current investigations were conducted to establish the concentrations of hexosamines, glycoproteins, and melanoidins in alfalfa meal resulting from different drying temperatures of fresh alfalfa herbage.

EXPERIMENTAL

Material and Methods. Alfalfa (*Medicago sativa*, cultivar Panonska) from the second cutting (June) at bud stage was used for these investigations. Two (200 g) samples of herbage were added to boiling 2-propanol until the final concentration of 2-propanol was about 70%, including the water from the herbage tissue. After boiling for 5 min, the tissue was macerated and extracted by the Soxhlet procedure for 5 hr.

Some of the herbage was cut into 5 cm segments and dried in an industrial dryer of Van der Broek type, with an inlet air temperature of 600°, 700°, or 800° C. The duration of drying of the given material was from 5 to 8 min, depending on the used temperatures. The dried alfalfa was ground to 60 mesh size. About 20 g of the fresh herbage and about 5 g of the meal in two replicates were used for the chemical analyses.

Analysis of Carbohydrates. After the sample had been extracted with 70% of 2-propanol, the preparation and treatment of the plant tissue and meal included extraction of the mono-, di-, and oligosaccharides, and the digestion of the starch with 1-amylase according the procedure described by Milić *et al.* (1971). The wet residue was used for hemicellulose and cellulose analysis.

Hemicellulose and Cellulose. The washed wet residue was hydrolyzed with 100 ml 0.7 *N* HCl under reflux for 5 hr in a boiling water bath. After hydrolysis, the mixture was filtered through a sintered glass filter, and packed with 2 cm layer of Celite 545 into a 500 ml volumetric flask. After fil-

Yugoslav Institute of Food Industry, Feed Technology Department, Novi Sad, Yugoslavia.
[1] Present address: Faculty of Technology, Novi Sad University, Novi Sad, Yugoslavia.

Table I. Influence of the Drying Temperatures on Changes in the Concentrations of Examined Constituents in Alfalfa Herbage (% d.w.)

Treatment	Moisture	Hemicellulose	Cellulose	Lignin	Hexosamines	Glycoproteins	Melanoidins
Fresh alfalfa	81.20	12.96	21.10	10.60	0.42	0.23	Traces
Heat-dried alfalfa							
600° C	7.48	11.80	22.37	12.50	0.68	1.73	0.42
700° C	7.09	11.62	23.40	12.16	0.77	2.22	0.54
800° C	7.88	10.27	25.09	12.93	0.93	2.12	0.63

tering, it was brought to volume with deionized water. A 25 ml aliquot was purified with anion and cation exchange resins (Lewatit MIH and Lewatit PN; Bayer AG, Leverkusen, Germany) and with an insoluble cross-linkage polyvinylpyrolidone adsorbent (Polyclar AT; General Aniline and Film Co., New York, N.Y.) with gentle agitation for 1 hr. The solution was filtered through dry filter paper and an aliquot tested for reducing sugars by the standard procedure. A portion of the purified filtrate was evaporated to a small volume and used for chromatographic evaluation. Amounts of individual sugars was determined from the chromatogram and were used to calculate the percentage of hemicellulose.

The residue on the glass filter, after hydrolysis with 0.7 *N* HCl, was dried at 45° C and transferred to a 500 ml round-bottomed flask, to which 25 ml 72% H_2SO_4 were added and thoroughly mixed with the glass rod. The reaction mixture was kept at room temperature for 5 hr with occasional stirring. The contents of the flask were transferred with 800 ml of deionized water, and the mixture was refluxed for 4 hr. The mixture was filtered through a sintered glass filter covered with asbestos into a 1000 ml volumetric flask. The pooled filtrate was neutralized with NaOH and brought to the volume with deionized water. A 25 ml aliquot was deionized and purified in the previously described manner. The percentage of reducing sugars was determined by standard procedure and the percentage of cellulose was calculated.

LIGNIN. About 10 g of the fresh herbage and about 2.5 g of the meal, in two replicates, were dried in vacuum, over P_2O_5 at 45° C, to the constant weights. After that, the percentage of lignin was determined, using the method of Thacker (1954).

MELANOIDINS. The amount of brown pigments was determined in separate fresh and meal tissue samples, using a model system as the standard substance. The model system was obtained by heating a mixture of 0.1 *M* glucose and 1.0 *M* solution of DL-aspartic acid at pH 2.3, at 93° C for 230 hr. The amorphous substance of brown color was purified in an ion exchange column of Permutit ES resin (2.5 × 35 cm), previously transformed into Cl′ form. The brown pigments were eluted with 5% NaCl solution until the color in the eluate had disappeared. The eluate was evaporated at 45° C in a rotary evaporator to small volume, dialyzed to liberate Cl′ ions, reevaporated to dryness under the same conditions, and dried over P_2O_5. The weighed substance was dissolved in 0.5 *N* NaOH. A series of graded concentrations was prepared, and a standard curve was plotted based on intensity of absorption at 420 mμ.

All samples (5 g of fresh tissue and 2 g of the meal) of alfalfa tissue were macerated, extracted with deionized water (100 ml), and left overnight to clarify. A 25 ml aliquot of the clear solution were purified through a Permutit ES resin column in the same manner as the model substance. The color intensity was read at 420 mμ and the percentage of brown pigments was calculated using the standard curve of the model substance. These tissue samples also were used for the determination of melanoidins by method of Choi *et al.* (1949).

A 2 g sample of meal (60 mesh size) or 5 g sample of macerated fresh tissue was extracted with 60 ml of deionized water to which 2.5 ml of 10% fresh trypsin suspension was added. The reaction mixture was incubated at 45° C for 1 hr. After incubation, 2 ml of 50% CCl_3COOH solution and 0.5 g Celite 545 were added. The solution was brought to 100 ml volume with deionized water. The mixture was shaken for 1 hr and left to clarify. Absorption of the centrifugally cleared solution was read at 420 mμ and the content of brown pigments was calculated from the standard curve of the model substance. Similar results were obtained from both fresh and dried tissues, using the above mentioned methods.

HEXOSAMINES AND GLYCOPROTEINS. Hexosamines were determined in 0.01 *M* sodium pyrophosphate extracts of both tissues with *p*-di-methylbenzaldehyde after the chromogen with acetylacetone had been formed in the basic medium, by the method of Rondle and Morgan (1955).

The procedure of Wrench (1966) was used to isolate and characterize the glycoproteins. Glycoproteins were extracted with 0.01 *M* sodium pyrophosphyte (100 ml) from all samples of herbage, at room temperature for 12 hr, and then they were precipitated from clear solutions by heating at 97° C. The obtained precipitates were separated by centrifugation at 10,000 r/min, and hydrolyzed in 2 *N* HCl (4 ml) at 100° C for 4 hr. The hydrolyzates were neutralized with 2 *N* NaOH, and the amount of hexosamines in glycoprotein fractions was determined according to the method of Rondle and Morgan (1955). The amount of glycoproteins was calculated on the basis of the obtained results of hexosamines.

RESULTS AND DISCUSSION

The percentage of hemicellulose decreased in alfalfa dry matter with increased drying temperatures (Table I). The concentration decreased from 12.96% (fresh alfalfa) to 10.27% (alfalfa dehydrated at 800° C), including a degradation of this polysaccharide in the drying process. In contrast, the percentage of cellulose was increased significantly in dry matter, with increased drying temperatures. The concentration of cellulose increased from 21.10% in fresh tissue to 25.09% in tissue dehydrated at 800° C. The marked increase in cellulose percentage probably was due to the relative changes and to the decrease of other carbohydrates and other compounds in alfalfa herbage during the temperature treatment. On the other hand, high drying temperatures, according to Van Soest (1965), can bring about the formation of heavy soluble polymers of cellulose and other constituents.

High drying temperatures also increased the percentage of lignin (Table I). The fresh alfalfa contained 10.60% of lignin, whereas the alfalfa dried at 800° C contained 12.93%. This increase probably was due to a change in lignin structure, possibly the binding of *N*-alkylamine on the phenylpropane radical. A higher amount of *N*-alkylamine in the dehydrated alfalfa was found in the isolated lignin fraction, as compared with the fresh tissue. Similar results have been

reported by Van Soest (1965). He found that high drying temperatures increased significantly the percentage of lignin and insoluble protein in dried herbage tissue.

High drying temperatures also affected the amount of brown melanoidin-type substances in dry matter of alfalfa. Melanoidins increased from trace amounts in the fresh tissue to 0.63% in that which was dehydrated at 800° C. Also, the content of hexosamines increased from 0.42% to 0.93%, about twice, and the glycoproteins increased from 0.23% to 2.12%, about 10 times. It was apparent that during the drying process of the plant tissue, complexes between carbohydrates and amino compounds or proteins occurred. Thus, considerable amounts of bound nitrogen remained biologically intact and inactive.

Melanoidins are formed as the final products of the interaction between carbonyl compounds and amino groups of the protein or amino acid, in addition to many other intermediates. The mechanism of their reaction is not completely understood, and it is a subject of interest to many workers. Formation of melanoidins in natural products under different conditions will be the subject of further investigations in our laboratory.

LITERATURE CITED

Agrawal, L. B. B., Goldstein, J. I., *Can. J. Biochem.* **46,** 1147 (1968).
Anett, E. F. L. J., *Aust. J. Chem.* **13,** 396 (1960).
Anett, E. F. L. J., *Aust. J. Chem.* **14,** 295 (1961).
Anett, E. F. L. J., *Aust. J. Chem.* **15,** 503 (1962).
Bathler, J. W., Keck, E., Yoerger, R. R., *J. Animal Sci.* **19,** 1186 (1960).
Burton, H. S., McWeeny, D. J., Biltchiffe, D. O., *J. Sci. Food Agr.* **14,** 911 (1963).
Burton, H. S., McWeeny, D. J., *Chem. Ind.* **11,** 462 (1964).
Clamp, J. R., Hough, L., *Biochem. J.* **94,** 145 (1965).
Clegg, M. K., *J. Sci. Food Agr.* **15,** 878 (1964).
Choi, R. P., Koncus, A. F., O'Malley, C. M., Fairbanks, B. W., *J. Dairy Sci.* **32,** 580 (1949).
Duckworth, J., Woodham, A. A., *J. Sci. Food Agr.* **1,** 25 (1961).
Ekern, A., Blaxter, K. L., Sawyers, D., *Brit. J. Nutr.* **19,** 417 (1965).
Goering, H. K., Van Soest, P. J., Ann. Meeting Amer. Dairy Sci. Ass., Ithaca, p. 103 (1967).
Gordon, C. H., Ann. Meeting Amer. Dairy Sci. Ass., Ithaca, p. 79 (1967).
Karel, M., Labuza, P. T., J. AGR. FOOD CHEM. **16,** 717 (1968).
Kass, J. P., Palmers, L. S., *Ind. Eng. Chem.* **32,** 1360 (1940).
Kawamura, S., Kasai, T., Honda, A., *J. Jap. Soc. Food Nutr.* **20,** 478 (1968).
Kertesz, Z. I., "The Pectic Substances," Interscience, New York, p. 387 (1951).
Koehler, P. E., Mason, M. E., Newel, J. A., J. AGR. FOOD CHEM. **17,** 393 (1969).
Lea, H. C., Hannan, S. R., *Nature* (London) **165,** 438 (1950).
Liener, E. J., "Effect of heat on plant proteins," Hannon M. Altschull, Ed., p. 172 (1958).
McWeeny, D. J., Burton, H. S., *J. Sci. Food Agr.* **14,** 291 (1963).
Milić, B., Vlahović, M., *J. Food Sci.*, in press, (1971).
Raguse, C. A., Smith, Dale, J. AGR. FOOD CHEM. **13,** 306 (1965).
Reynolds, T. M., Anett, E. F., Ingles, D. L., Comm. 1st Inter. Congress of Food Sci. and Technology, p. 196, London (1962).
Richards, E. L., *Biochem. J.* **64,** 639 (1956).
Rondle, C. M. J., Morgan, W. T. J., *Biochem. J.* **61,** 586 (1955).
Spark, A. A., *J. Sci. Food Agr.* **20,** 308 (1969).
Talley, E. A., Porter, W. L., J. AGR. FOOD CHEM. **16,** 264 (1968).
Thacker, E. J., *J. Animal Sci.* **13,** 501 (1954).
Thompson, A., Wolfrom, M. L., *J. Amer. Chem. Soc.* **80,** 6618 (1958).
Underwood, C. E., Deatherage, F. E., *Food Res.* **17,** 419 (1952).
Van Soest, P. J., *J. A.O.A.C.* **48,** 785 (1965).
Van Soest, P. J., Ann. Meeting Amer. Dairy Sci. Soc., College Park, Maryland, p. 276 (1967).
Waite, R., Boyd, J., *J. Sci. Food Agr.* **4,** 197 (1953).
Wiering, C. W., Proc. 8th Inter. Grassland Congress, London, p. 497 (1960).
Wrench, P. M., *J. Sci. Food Agr.* **17,** 403 (1966).

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