Table V. Relative Magnitude of Variance Component

Tissue	Cattle	Swine	Chicken
	$(S_{DAR}^2/S_d^2)100$		
Fat	29.6	48.2	71.8
Lean	32.9	51.1	49.5
Liver	27.8	26.3	58.9
Kidney	46.4	57.0	44.0
Tripe	30.8	...	...
Av.	33.4	41.8	55.8
	$(S_{DR}^2/S_d^2)100$		
Fat	20.6	39.3	10.2
Lean	38.0	24.5	26.7
Liver	28.0	20.0	12.9
Kidney	30.0	25.0	31.6
Tripe	22.5	...	...
Av.	27.6	24.8	20.3
	$(S_{DA}^2/S_d^2)100$		
Fat	25.1	1.8	15.6
Lean	22.2	6.4	12.3
Liver	17.1	41.6	11.8
Kidney	14.0	14.3	12.9
Tripe	9.7	...	...
Av.	17.6	22.9	13.0
	$(S_A^2/S_d^2)100$		
Fat	13.2	10.7	9.8
Lean	4.0	3.2	8.8
Liver	17.1	10.1	7.3
Kidney	5.0	3.8	6.9
Tripe	29.0	...	...
Av.	13.7	7.4	6.2
	$(S_{AR}^2/S_d^2)100$		
Fat	11.3	0	1.5
Lean	3.6	14.9	2.7
Liver	10.0	3.7	9.0
Kidney	5.7	0	4.6
Tripe	8.1	...	...
Av.	7.7	3.2	4.6

apparent relationship between the variance components and the tissues studied on a within-animal basis.

Table VI. Results of 94 Independent Assays of Control Tissue plus Added Ingredient Indicated at Δ Level

Animal	No. of Independent Tests at Sensitivity Level	No. of Declared Residue	% Declared Residue
Cattle	33	26	78.7
Swine	4	3	75.0
Chicken	57	46	80.7
Total	94	75	79.8

The theoretical power of this procedure at the true Δ value is 50%. This means that there would be a β (beta) risk (failure to declare residue when truly there) of 50% at the Δ point if the sensitivity of the assay does not increase with increasing amounts of residue. It was of interest to check this empirically. As of this date, 94 independent studies were run at the parts per million estimated as the upper limit of the sensitivity ranges. These data are summarized in Table VI. These results indicate that the ability to detect residues at the Δ point is approximately 80%. This implies, therefore, that there is only a 20% β risk at the Δ criterion. It is realized that these data do not prove such power really exists for all tissues. However, studies will be continued and data will continue to be accumulated on the ability to detect residues at the approximate parts per million level indicated in the developmental studies.

A check on the reproducibility of the developmental system and method of analysis was attempted by replicating each of six 3 × 3 × 3 experimental systems twice. Table VII contains these results in terms of estimated parts per million of the test system. These data indicate that the approximate sensitivity of the test procedure is reproducible.

Table VII. Approximate Parts per Million Indicated by Replication of Development Assay Design

Test	Approximate P.P.M. Rep. 1	Rep. 2
1	0.03–0.05	0.05–0.08
2	0.20–0.40	0.30[a]
3	0.10–0.20	0.20[a]
4	0.05–0.10	0.05–0.10
5	0.20–0.30	0.10–0.30
6	0.03–0.05	0.01–0.03

[a] Dosage range failed to bracket Δ criterion.

Literature Cited

(1) Berkman, R. N., Richards, E. A., VanDuyn, R. L., Kline, R. M., *Antimicrobial Agents Ann.* **1960**, p. 595–604.
(2) Johnson, N. L., Welch, B. L., *Biometrika* **31**, 362–89 (1940).
(3) Kennedy, E. E., J. Agr. Food Chem. **11**, 393–5 (1963).
(4) Resnikoff, G. J., *Ann. Math. Stat.* **33**, 580–6 (1962).
(5) Roos, J. B., *Analyst* **87**, No. 1039, 832 (1962).
(6) Wilson, A. L., *Ibid.*, **86**, No. 1018, 72 (1961).

Received for review July 1, 1964. Accepted February 18, 1965.

FORAGE ANALYSIS

Carbohydrate Content in Alfalfa Herbage as Influenced by Methods of Drying

C. A. RAGUSE[1] and DALE SMITH

Department of Agronomy, University of Wisconsin, Madison 6, Wis.

Qualitative and quantitative determinations of carbohydrates are performed in various studies of agronomic crop plants. Drying and storage of plant tissue are often necessary because of the numbers of samples to be processed and the length of time involved in many chemical analyses. For carbohydrate analyses to be of maximum value, changes in plant composition during preservation and storage must be minimized. Investigators have observed the effects of various preservation procedures on a variety of plant materials (*2, 3, 6, 7, 9, 11*). Changes in organic constituents have been attributed to respiratory losses, metabolic interconversions, and deleterious effects of high drying temperatures. Studies of changes occurring during wilting (*10, 15*) also are pertinent, because conditions within a mass of plant tissue during early stages of drying may be conducive to metabolic changes and accelerated respiratory losses similar to those occurring during wilting (*12*).

Changes that may occur in the content of some carbohydrate constituents during the drying and preservation of alfalfa herbage are considered here.

[1] Present address, Department of Agronomy, University of California, Davis, Calif.

Materials and Methods

Herbage from a stand derived from a single clone of Vernal alfalfa was harvested at or near bud stage on May 24, 1962, and again on October 11, 1962.

In a comparison of drying methods, fresh alfalfa herbage was dried at room temperature (ca. 27° C.), 60° C., 70° C., a combination of 100° followed by 70° C., or freeze-dried. Respectively, the drying treatments resulted in progressively higher percentages of total available carbohydrates and of carbohydrates nonextractable with 80% ethanol. Per cent of sucrose was highest under the 70° C. treatment. Percentages of glucose, fructose, and the total of the three sugars were less uniformly influenced.

Subsamples were subjected to one or another of the following five drying treatments: herbage allowed to dry in an open paper bag at room temperature (about 27° C.); herbage dried in paper bags in forced-draft ovens at 60° C., 70° C., or 100° C. for 90 minutes and then drying to completion in a similar oven at 70° C.; or herbage freeze-dried. Oven-dried samples were placed in the drying ovens within one-half hour after cutting. Freeze drying was accomplished by immediately pouring liquid nitrogen over freshly cut herbage and packing it in crushed dry ice. The dry ice–herbage mixture was coarsely ground through a thoroughly chilled meat grinder. The ground material was held in a freezer at −10° C. until most of the dry ice had evaporated. It was then freeze-dried in an apparatus similar in principle and design to that described by Davies, Evans, and Evans (*4*).

All dried samples were ground with a Wiley mill through a 40-mesh screen. The small amount of fibrous material remaining in the mill was combined with the ground portion of the sample. Air-dried and oven-dried samples were stored in tightly capped glass jars. Freeze-dried samples were stored in glass jars over P_2O_5 in a vacuum desiccator stored at −2° C.

Total available carbohydrates were extracted by the enzyme method of Weinmann (*14*), as modified by Lindahl, Davis, and Sheperd (*8*), or by the 2% H_2SO_4 method as described by Smith (*13*). Reducing power was determined and the results expressed as per cent total available carbohydrates.

Sugars were extracted with aqueous ethanol. Approximately 100 ml. per gram of sample of hot (80° C.) 80% ethanol was added to the sample in an Erlenmeyer flask. The flask was shaken vigorously on a Burrill shaker for 1 hour. The residue was allowed to settle by storing overnight in a refrigerator. The extract was decanted the following day, and the residue was re-extracted. The residue was then removed by filtration, dried, and stored. Ethanol was removed from the combined filtrates plus washings by evaporation.

Subsequently, two methods were used to determine sugars. In the first, proteins were precipitated from the aqueous sugar solution (about 10 to 15 ml.) by adding 2 ml. of a 10% (w./v.) neutral lead acetate solution. The lead-protein precipitate was washed and removed by filtration, and the excess lead was precipitated with potassium oxalate. Lead oxalate crystals were washed and removed by filtration, and the filtrate was made to an appropriate volume. Reducing sugars were determined by testing an aliquot for reducing power. Total sugars were determined by testing the reducing power of an acid-hydrolyzed aliquot. Nonreducing sugars were estimated by subtraction. In the second method, proteins were precipitated (as above) with neutral lead acetate solution, and the lead-protein precipitate was removed by centrifugation. The aqueous sugar solution (about 50 ml.) was then mixed with about 10 meq. of a cation exchange resin [AG-50(H), obtained from Calbiochem] to remove excess lead. The resin was washed and removed by filtration. The sugar solution was adjusted with NaOH solution to approximately pH 6.8, evaporated to less than 3 ml. with a Rinco rotary evaporator, transferred quantitatively to a 10-ml. volumetric flask, and made to volume.

Aliquots consisting of 0.4 ml. of these concentrated solutions were applied as a streak to Whatman No. 1 chromatography paper and developed by descending chromatography with an ethyl acetate–acetic acid–water (3:2:1 v./v.) system. Side "marker" strips, obtained from spots of the sample solution applied at both ends of the sample streak, were sprayed with a *p*-anisidine phosphate solution and used to locate the separated bands of sucrose, glucose, and fructose. Bidimensional co-chromatography with known sugars had previously established that sucrose, glucose, and fructose were the three principal sugars of this alfalfa herbage. The bands were sectioned, placed in 10-cm. Petri dishes, and eluted by adding exactly 20 ml. of distilled water. About 30 minutes were allowed for complete equilibration, with occasional swirling. One-milliliter aliquots were used for anthrone determinations of the above three sugars.

Anthrone reagent was freshly prepared on the day of use by dissolving 100 mg. of anthrone crystals in 100 ml. of reagent grade concentrated sulfuric acid. The aqueous sugar solution was layered on 5 ml. of anthrone reagent in a 25×200 mm. test tube and the contents were mixed while in an ice water bath. After heating in a boiling water bath, 10 minutes for glucose and 8 minutes for sucrose and fructose, the test tubes were cooled rapidly to near room temperature by immersing them in an ice water bath. The cooled samples were transferred to 1-cm. cuvettes and absorptions were read at 580 mμ in a Beckman DU spectrophotometer. Duplicate or triplicate determinations were made on each duplicated sample. Duplicate blanks and sugar standards were included with each set of determinations.

The residue remaining after 80% ethanol extraction was further extracted with the enzyme procedure of Weinmann (*14*) as modified by Lindahl and coworkers (*8*). Aliquots were used to determine reducing power, and the results expressed as per cent of enzyme-extractable carbohydrates.

All determinations of reducing power were made with the Shaffer-Somogyi method as described by Heinze and Murneek (*5*). Glucose was used as the standard and results were calculated on a dry weight (70° C.) basis.

Results

Percentages of total available carbohydrates in the herbage, using the enzyme and 2% H_2SO_4 extraction methods, are shown in Figure 1. The lowest values obtained for both sets of samples and with both extraction methods were from the air-dried treatment. Progressively higher values were obtained in the following order: 60° C., 70° C., 100° + 70° C., and freeze-dried. Relative amounts of total available carbohydrates recovered in relation to drying treatment were similar for both sampling dates.

Percentages of sucrose, glucose, and fructose and the total percentages of these three sugars, based on anthrone determinations, are shown in Table I. Percentages of glucose, fructose, and the total percentages of the three sugars for

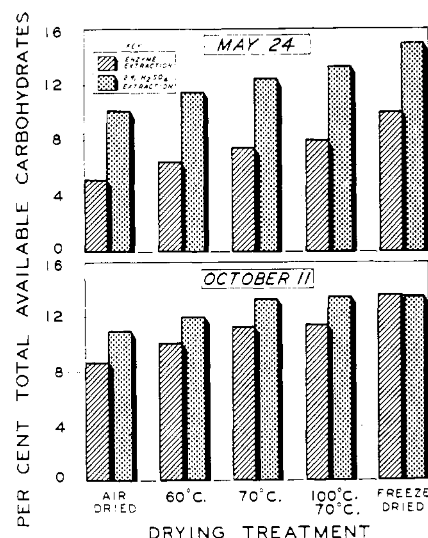

Figure 1. Influence of method of drying on total available carbohydrates in alfalfa herbage sampled twice at bud stage during 1962

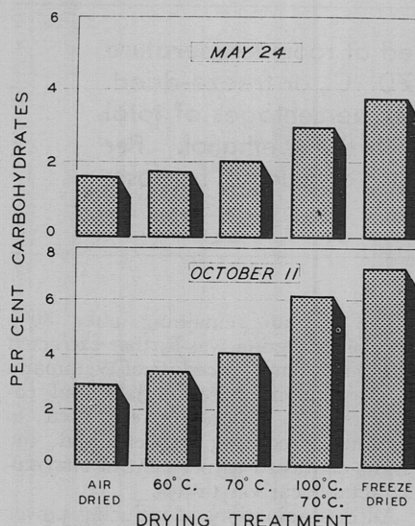

Figure 2. Influence of method of drying on enzyme-extractable carbohydrates in residues left after 80% ethanol extraction of alfalfa herbage sampled twice at bud stage during 1962

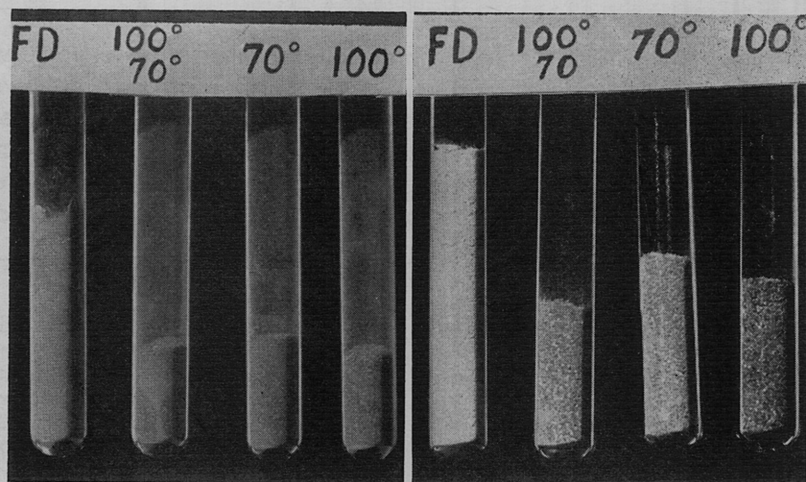

Figure 3. Effect of drying treatment on indentical sample weights of alfalfa herbage

Left. Whole samples
Right. Residues of samples following extraction with hot 80% ethanol

the May 24 sampling exhibited progressively higher values in the following order: air-dried, 60° C., 70° C., 100° + 70° C., and freeze-dried. This was not the case for the October 11 sampling, where all values for glucose and fructose were relatively low. The highest total percentage was found at the 70° C. treatment and appeared to be a reflection of the high value obtained for sucrose from that treatment. For both sampling dates, the highest value for sucrose was obtained with drying at 70° C. Values for reducing sugars, nonreducing sugars, and total sugars, based on Shaffer-Somogyi copper reduction determinations, presented essentially the same information as that given by the anthrone determinations and are not reported.

A possible artifact of the 70° and 60° C. treatments was noted during the chromatographic separation of sugars. The substance, presumably a carbohydrate, reacted with anthrone and with *p*-anisidine phosphate. Its position on the chromatogram in relation to sucrose and to the origin suggested a trisaccharide. Subsequent co-chromatography with raffinose, a commonly occurring trisaccharide, yielded only one spot on a single-dimension chromatogram. The substance was obtained in larger quantity, hydrolyzed with HCl, concentrated, and rechromatographed with known monosaccharides. The results suggested that at least glucose and fructose, and possibly a third monosaccharide, were present. The identity of the third monosaccharide was not established.

Percentages of carbohydrates obtained from enzyme extraction of the residue remaining after 80% ethanol extraction are shown in Figure 2. The pattern of response to drying treatments was the same as that discussed previously for total available carbohydrates.

Physical differences among the ground samples due to drying treatments were observed. The color, texture, and volume of the freeze-dried samples were markedly different from the oven-dried and air-dried samples, as shown in Figure 3. Freeze-dried material was greener in color, appeared to be more homogeneous and uniformly ground, and occupied a greater volume per unit weight. The air-dried and oven-dried samples, stored in glass jars in the laboratory, equilibrated with atmospheric moisture to the extent that when subsamples were later redried at 70° C. there was a weight loss of about 6%. The freeze-dried samples, stored in a desiccator over P_2O_5, suffered a weight loss of about 0.2%.

Table I. Per Cent of Sucrose, Glucose, and Fructose in Alfalfa Herbage Sampled Twice at Bud Stage during 1962 as Influenced by Methods of Drying

	Sampling Dates							
	May 24				October 11			
Treatment	Sucrose	Glucose	Fructose	Total	Sucrose	Glucose	Fructose	Total
Air-dried	2.2	0.7	0.8	3.7	2.5	0.8	0.7	4.0
60° C.	2.8	0.9	1.0	4.7	3.2	0.8	0.7	4.7
70° C.	3.2	1.0	1.1	5.3	3.9	0.7	0.7	5.3
100° + 70° C.	2.5	1.2	1.5	5.2	2.4	0.7	0.8	3.9
Freeze-dried	2.8	2.4	1.8	7.0	2.9	0.8	0.9	4.6

Discussion

Values for total available carbohydrates and for carbohydrates nonextractable with 80% ethanol were lowest from the air-dried treatment. Progressively higher values were obtained from the 60° C., 70° C., 100° +70° C., and freeze-dried treatments, respectively. Values for glucose and fructose and for the total of glucose, fructose, and sucrose did not follow a consistent pattern for the two sampling dates and probably depended in part on the relative proportions of mono- and disaccharides present at sampling. Evidence (*6, 7, 9, 10, 12, 15*) implicates respiratory losses, metabolic interconversions, and deleterious effects of heat as factors that may alter plant carbohydrate composition during conventional sampling and drying procedures.

The physical condition of the ground freeze-dried samples was different from that of the other drying treatments. Rapid freezing of the herbage probably resulted in freezing of the water in the plant as small crystals distributed uniformly throughout the tissue. Removal of this water by freeze drying left a more highly porous and less dense tissue than that resulting from conventional drying methods. This condition would seem to be conducive to more uniform grinding of the dry samples and more thorough penetration by extracting solutions. Both of these results occurred.

The freeze-dried material was assumed to be most nearly like fresh herbage in carbohydrate composition. This method appears to avoid some of the

disadvantages of oven drying (*7*). However, cost per sample is higher than when conventional drying methods are used, and conditions of sample storage are critical, since enzyme inactivation is minimal. Storage conditions must be carefully controlled to avoid chemical changes if freeze-dried samples are to be stored for more than short periods of time (*7, 11*).

The results of this study supported the conclusions of Link (*9*) that no universal method for drying plant tissue can be relied on for accurate results, because plant tissues vary so widely in chemical and physical nature and in enzyme content and that individual experimentation is required to determine an appropriate drying temperature. It seems clear, then, that an investigator must consider carefully sampling and drying procedures in relation to the objectives of a study, especially if small amounts of easily altered constituents are to be determined.

Literature Cited

(1) Bath, I. H., *J. Sci. Food Agr.* **11**, 560 (1960).
(2) Bathurst, N. O., Allison, R. M., *New Zealand J. Sci. Tech.* **31(B)**, 1, (1949).
(3) Collins, F. D., Shorland, F. B., *Ibid.*, **26(A)**, 372 (1945).
(4) Davies, A. W., Evans, R. A., Evans, W. C., *J. Brit. Grassland Soc.* **3**, 153 (1948).
(5) Heinze, P. H., Murneek, A. E., Missouri Agr. Expt. Sta., Res. Bull. **314** (October 1940).
(6) Jones, D. I. H., *J. Sci. Food Agr.* **13**, 83 (1962).
(7) Laidlaw, R. A., Wylam, Clare B., *Ibid.*, **3**, 494 (1952).
(8) Lindahl, I., Davis, R. E., Sheperd, W. O., *Plant Physiol.* **24**, 285 (1949).
(9) Link, K. P., *J. Am. Chem. Soc.* **47**, 470 (1925).
(10) Melvin, J. F., Simpson, Beulah, *J. Sci. Food Agr.* **14**, 228 (1963).
(11) Perkins, H. J., *Can. J. Plant Sci.* **41**, 689 (1961).
(12) Pirie, N. W., in "Modern Methods of Plant Analysis," K. Paech, M. V. Tracey, eds., p. 50, Springer-Verlag, Berlin, 1956.
(13) Smith, Dale, *Crop Sci.* **2**, 75 (1962).
(14) Weinmann, H., *Plant Physiol.* **22**, 279 (1947).
(15) Wylam, C. B., *J. Sci. Food Agr.* **4**, 527 (1953).

Received for review May 11, 1964. Accepted February 10, 1965. Published with approval of the Director of the Wisconsin Agricultural Experiment Station. Part of a thesis submitted by the senior author in partial fulfillment of requirements for the Ph.D. degree.

PEANUT FLOUR CONSTITUENTS

Cyclic Imino Acid Derivative from Peanut Flour

LOUISE S. LEE, NELLE J. MORRIS, and VERNON L. FRAMPTON

Southern Regional Research Laboratory, New Orleans, La.

An imino acid derivative, melting at 236–8° C., which has not been reported previously as a constituent of an edible product was isolated from peanut flour (0.02% yield) in the course of a chromatographic investigation of the nonprotein fraction. Its identity as *N*-methylhydroxyproline was proved by elemental analysis, molecular weight determination, and comparison of the infrared spectrum of the compound with that of *N*-methylhydroxyproline synthesized in the laboratory. The structure of the synthetic product was confirmed by elemental and functional group analysis as well as by nuclear magnetic resonance.

The alcohol-soluble components of peanut flour are being investigated at this laboratory to assess their contribution not only to the biological value of peanut flour, but also to the formation of flavor and aroma in roasted peanut products. This paper describes the procedure used in the isolation of an imino acid derivative not previously reported as a constituent of an edible product and the methods used to establish its identity.

Experimental Procedure

Approximately 100 pounds of lightly roasted blanched peanuts served as the original material for this investigation. The fractionation procedure used to obtain the water-soluble fraction is identical to that used for the isolation of pinitol (*7*).

The column chromatographic separations were made using a 9-cm. i.d. × 40-cm. column, packed with Whatman cellulose powder. The powder was prewashed by extraction with methanol in a Soxhlet apparatus for 3 hours. It was applied to the column in a slurry and packed thoroughly by running the solvent, butanol–acetone–water (2:2:1), through the column. This solvent was used for all the column fractionations of material from peanuts. The synthetic product, *N*-methylhydroxypyroline, was prepared by the method of Leuchs and Felser (*8*) and separated from the reaction mixture by chromatography on the same column, using butanol–acetone–water (4:1:1) as the developing solvent. This fractionation was followed by paper chromatography on Whatman 3MM paper, using the same developing solvent. Only the fractions containing one component with an R_f identical to that of the natural crystals were combined.

Material containing the crystalline product from peanuts was obtained from a composite of 16 fractionations of the water-soluble fraction (*7*). This was divided into lots of about 4 grams each, which were rechromatographed separately using butanol–acetone–water (2:2:1) as the developing solvent. As the solvent evaporated, crystals formed in fractions which represented 0.02% of the peanut flour. The crystals were washed several times with methanol and the supernatant liquid was decanted.

When the melting behavior of this material was observed, it was noted that at 200–04° C. a sublimate separated as needle crystals, leaving plates in the residue. Consequently, the impure material was heated in a microsublimator to 180° C. under vacuum to separate this sublimate, which constituted ap-