Countercurrent Distribution of Sorghum Lipides in Leaf and Stem Extract

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Fractionation of the lipides of sorghum leaf and stem by countercurrent distribution yielded five distinct nonpigment components. Pigments in seven fractions accounted for 9% of total lipides. Phosphorus and nitrogen were found throughout the distribution. The range of phosphorus in various fractions was 0.04 to 1.2%; of nitrogen, 0.08 to 1.23%. The findings illustrate the complexity of the lipide extract and will serve as guides for further isolation of selected fractions for detailed study.

THE LIPIDES OF FEEDSTUFFS are important in animal nutrition, influencing not only the growth and well being of farm animals (*7*), but also the quality of meat produced (*15*). However, knowledge of the lipides of grasses is meager. The fatty acids of orchard grass (*Dactylis glomerata*) and rye grass (*Lolium perenne*) were examined by Smith and Chibnall in 1932 (*16*), who reported the percentage composition of each of five acids. Shorland, in 1944, fractionated the lipides of orchard grass into fatty acids, unsaponifiable matter, waxes, and phosphatides, and determined their percentage composition as well as that of the individual fatty acids (*14*). Hilditch and Jasperson, in 1945, analyzed mixed pasture grass for its fatty acids (*8*).

A thorough study on the waxes and fatty acids of sorghum grain oil has been carried out by Kummerow (*9*). He reported the oil to be similar to corn oil in composition. More recently, Cannon and Kummerow (*2*) have made comparative studies on the characteristics of the waxes from sorghum plant and grain.

The countercurrent distribution technique provides a new and efficient method for lipide and pigment separations (*6*). This technique has been used to fractionate lipides of the sorghum plant. As far as is known, this is the first time that countercurrent distribution has been applied to the fractionation of the lipide extract of plants.

Materials and Methods

Large Scale Preparation of Sorghum Extract. Forage sorghum (Atlas variety) at the early head stage was used for this study. After 20 pounds of heads were removed, the remaining 125 pounds of sorghum leaves and stems were cut to 1-inch lengths in a forage chopper. After 106 pounds were pulped in a double-disk refiner (Bauer mill), 84 pounds of this macerate—20.2 pounds of dry matter—were immediately placed in 134 pounds of anhydrous acetone. The sorghum tissue was steeped at room temperature for 3 hours, then the aqueous acetone extract (102 pounds) was drawn off. An additional 66 pounds of acetone were added in three portions and immediately drawn off. A total of 216 pounds of aqueous acetone extract was obtained. After the last draining of the acetone, 64 pounds of hexane were added to the residue; the mass was stirred and allowed to stand overnight. Subsequent to draining off 51 pounds of hexane extract, an additional 30 pounds of hexane were added in three portions and immediately drawn off. The hexane extract amounted to 104 pounds.

The dark green aqueous acetone extract was concentrated in vacuo to 156 pounds, united with the hexane extract (104 pounds), stirred for 15 minutes with an air stirrer, and allowed to stand for 1.5 hours. After this equilibration and settling, the two phases were separated—138 pounds of aqueous acetone layer and 123 pounds of hexane layer. Fourteen pounds of water were added to the hexane layer. This resulted in the extraction of acetone from the upper layer and the formation of 45 pounds of lower layer, which was withdrawn. The further addition of 11.5 pounds of water resulted in the removal of 24.5 pounds of lower phase. There then remained 77.9 pounds of hexane layer.

After overnight storage in contact with 5 pounds of anhydrous sodium sulfate, the washed hexane layer was decanted through glass wool. The sodium sulfate was washed with four portions of fresh hexane (5, 3.5, 2.5, and 3 pounds), and these washings were added to the decanted extract. The combined extract and washings were concentrated in vacuo in an all-glass circulating evaporator to 3155 ml., at a temperature not exceeding 40° C. This lipide concentrate contained 0.048 gram of solids per ml. and represented 152.4 grams of dry matter, or 1.66% of the original dry matter in the sorghum leaves and stems.

Countercurrent Distribution of Sorghum Extract. Two hundred milliliters (9.6 grams of solids) of the lipide concentrate were subjected to a 395 transfer countercurrent distribution in a 200-tube automatic Craig-Post apparatus (*4*). The solvent system used was mutually saturated hexane and 90% ethyl alcohol (1 to 1). Alcohol, 40 ml., was placed in each of the 200 tubes, and 40 ml. of the lipide concentrate in hexane were placed in each of the first five tubes. The automatic operation of the instrument introduced 40 ml. of equilibrated hexane to tube 0 at each transfer stage. At the end of 195 transfers, collection of the hexane upper layers was begun. The distribution was continued until 200 collector tubes were filled with upper layer—a total of 395 transfers.

At this stage, there were six distinct color zones present in the Craig apparatus and in the collector tubes. The contents of the tubes believed to represent the middle of the zones were withdrawn. Spectral curves, in the region 3500 to 7500 A. obtained with a Cary spectrophotometer, were used to identify pigments and to indicate wave lengths for quantitative pigment analysis.

A weight distribution curve was obtained by evaporating to dryness aliquots from every fifth tube. The fractions from the instrument proper—fundamental series—were made to 100 ml. with absolute ethyl alcohol, which resulted in the formation of a single phase from the two phases withdrawn. A 25-ml. aliquot was taken from these solutions and the solvent was removed in vacuo in tared flasks. The contents of every fifth tube of the collector—withdrawal series—were made to 50 ml. with hexane. In this case, 10-ml. aliquots were taken for evaporation of solvent and weighing the residue. On the

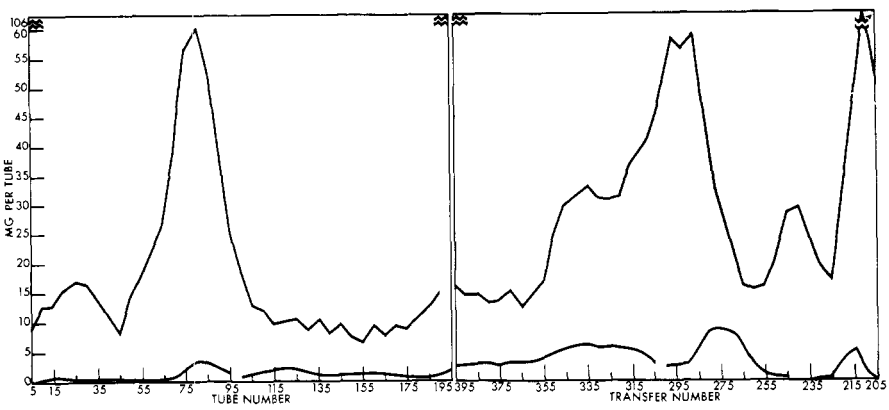

Figure 1. Countercurrent distribution of sorghum lipides

Upper curve, weight distribution. Lower curve, pigment distribution

basis of the weight curve data and pigment analyses (Figures 1 and 2), the contents of the adjacent tubes were combined into 13 major fractions. The solvents were evaporated in vacuo to yield concentrates containing approximately 10 mg. per ml. These concentrates of combined tubes served as the source of material for subsequent analyses.

Calculations of Pigment Concentration. Aliquots of solutions used for preparation of the weight distribution curve were also used in the estimation of pigment concentrations. The amounts of plant pigments in the various fractions were calculated from absorbancies at spectral peaks or critical wave lengths indicated below:

Pigment	Wave Lengths Used for Calculation, A.	Reference
Carotenoids, mg./100 ml.[a]	4450	(*18*)
Chlorophyll b, mg./liter	6475, 6650	(*10*)
Chlorophyll a, mg./liter (tubes 399–305)[a]	5350	(*12*)
Chlorophyll a, mg./liter (tubes 100–195)	6650, 6475	(*10*)
Pheophytin, mg./liter[a]	5350	(*12*)
Carotene, mg./liter[a]	4500	(*18*)

[a] Values approximate because solvents are not identical to those used by the authors referred to for calculations.

Nitrogen and Phosphorus Determinations. Semimicro-Kjeldahl nitrogen analyses were carried out on countercurrent distribution fractions. Colorimetric phosphorus determinations were carried out according to the method of Truog and Meyer (*17*).

Hydrolysis of Countercurrent Distribution Fractions. A portion of the fraction estimated to contain 20 mg. of solids was placed in a 5-ml. test tube and the solvent was removed in vacuo. Each tube was treated with 1 ml. of 6*N* hydrochloric acid and then sealed. The tubes were placed in a 100° C. oven for 6 hours, removed, and cooled overnight in the refrigerator. The tubes were opened and the contents were filtered through sintered glass. The filtrates were taken to dryness in vacuo; the resulting residues were taken up in 0.1 ml. of water and subjected to paper chromatographic analysis.

Paper Chromatography of Acid Hydrolyzates. Two solvent systems were employed in these investigations with the ascending chromatographic technique. The solvent system butanol–methyl Cellosolve–water (2 to 1 to 1) was used for separation of components, which reacted with ammoniacal silver nitrate spray; the solvent system butanol–acetic acid–water (10 to 3 to water saturated) was used for separation of constituents which reacted with ninhydrin spray. Development was carried to a height of 11 cm. on sheets of Whatman No. 1 paper. The papers were air-dried and sprayed with the appropriate reagent.

Anthrone Tests. Two or three drops of hydrolyzate and 1 ml. of water were placed in a 5-ml. test tube. Two milliliters of anthrone reagent (*5*) were then added rapidly to the test tube, the resulting solution was stirred vigorously, and the formation of color observed. The intensity of the reaction was rated on the basis of green color developed.

Results and Discussion

Fractionation of sorghum lipides is shown in the countercurrent distribution curves of Figure 1. The uppermost line is the total weight of lipides in each tube plotted against the tube or transfer number. The weight of pigment determined spectrophotometrically in each tube is plotted similarly in the lower curve. Pigments identified on the basis of their characteristic absorption curves were carotenoids, chlorophyll b, chlorophyll a, pheophytin, and carotene. Pheophytin may be a destruction product of the chlorophyll and not a natural pigment. An unidentified green pigment was found in the tubes between pheophytin and carotene. Spectral absorption curves of representative tubes are indicated in Figure 2.

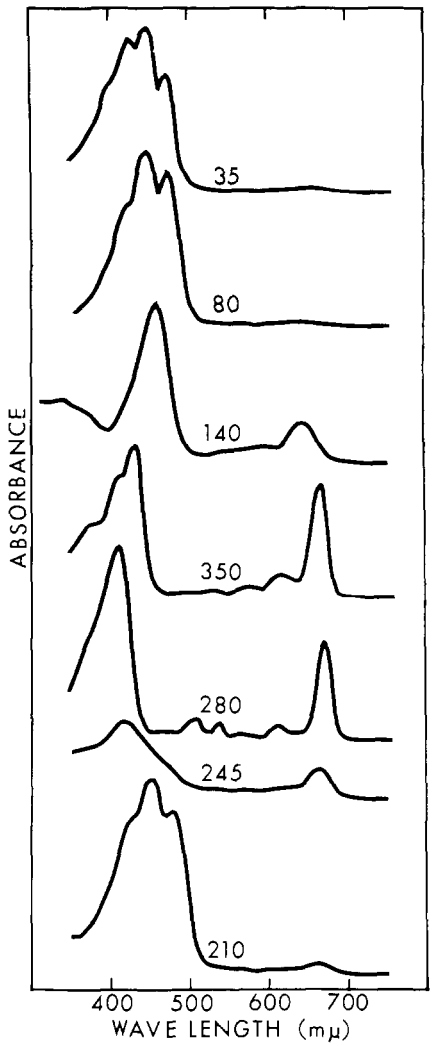

Figure 2. Spectral absorbance of pigmented fractions

Number of tube or transfer used for spectral analysis is indicated above each curve. The three upper curves were obtained on ethyl alcohol–hexane (60 to 40) solutions; the remaining curves on hexane solutions

Lipide material remaining in the Craig apparatus and in the collector tray after the removal of every fifth tube as described was combined after considering both the weight and pigment curves in Figure 1. The pattern of combination, the amount of the lipide in the combined fractions, and its percentage of the total recovered weight are recorded in Table I.

Table I. Combination of Countercurrent Distribution Tubes

Fundamental Series	Weight, G.	% of Total[a]
0–17	0.20	2.09
18–27	0.18	1.21
28–45	0.23	2.33
46–65	0.37	3.87
66–100	1.46	15.10
101–140	0.44	4.55
141–185	0.40	4.09
186–200	0.24	2.51
Withdrawal Series		
399–306	2.12	21.90
305–291	0.87	8.97
290–251	1.25	12.87
250–231	0.51	5.32
230–200	1.48	15.30
Total	9.75	100.11

[a] Total amount of residue recovered by all tubes was obtained by adding weight curve values and multiplying by 5.

Table II. Pigments in Combined Fractions

Fraction[a]	Total Solids, G.	Pigment	Grams	Fraction, %	Total Lipide, % (9.6 G.)
0–95	2.36	Carotenoids	0.090	3.82	0.94
100–195	1.09	Chlorophyll b	0.103	9.48	1.07
100–195	1.09	Chlorophyll a	0.025	2.25	0.26
399–305	2.33	Chlorophyll a	0.353	15.13	3.68
300–260	1.74	Pheophytin	0.288	13.10	2.37
235–200	1.60	Carotene	0.056	3.50	0.58
	9.12		0.915		8.90

[a] Tubes 255 to 230 contained a pigment which could not be identified by its absorption spectrum. This portion is omitted from the calculations.

Table III. Pigment Content of Leaf and Stem

Fraction	Pigment	Fresh Basis, P.P.M.	Dry-Weight Basis, P.P.M.
0–95	Carotenoids	37.6	156.5
100–195	Chlorophyll b	127.5	1783
100–195 and 399–305	Chlorophyll a	1483	6173
300–266	Pheophytin	947.0	3950
235–200	Carotene	23.15 (10.25 mg./lb.)	96.6 (43.9 mg./lb.)

Table IV. Nitrogen and Phosphorus Contents of Selected Fractions

Fraction	Nitrogen[a], %	Phosphorus, %	Molar Ratio[a], N/P
Whole extract	0.74 (0.27)	0.27	6.3 (2.2)
0–45	0.46	0.67	1.5
46–65	0.65	0.20	7.2
66–100	0.24	0.05	10.6
399–305	1.00 (0.05)	0.04	46.0 (2.8)
305–291	0.35	0.07	11.1
290–251	1.23 (0.23)	0.09	31.0 (5.7)
250–231	0.08	0.09	2.0
230–200	0.18	1.20	0.33

[a] Figures in parentheses are corrected for pigment nitrogen in the fraction.

The total concentration of all the assayed pigments was approximately 9% of the total solids subjected to countercurrent distribution. Table II indicates the percentages of individual pigments in each fraction, calculated on the basis of their spectral characteristics. Percentages of pigments calculated on the basis of the original plant material are recorded in Table III.

The chlorophyll a to chlorophyll b ratio was calculated to be 3.45 to 1, which agrees with results obtained for other green plant material. According to Gortner and Gortner (*7*), the ratio of occurrence in higher plants is about three molecules of chlorophyll a to one molecule of chlorophyll b.

Table IV gives the results of nitrogen and phosphorus analyses and also the percentage nitrogen adjusted for chlorophyll and pheophytin content of various fractions. The high nitrogen content of fractions 399 to 306 and 290 to 251 is due to chlorophyll a and pheophytin, respectively.

The presence of nitrogen and phosphorus in all of the countercurrent distribution fractions is an indication of the complexity of plant products that contain these two elements. Contaminating nitrogen and phosphorus compounds were found in all fractions in a distribution of unpurified flour lipides by Cookson and Coppock (*3*). These workers found molar nitrogen–phosphorus ratios greater than 2 to 1 in all fractions; it was sometimes as high as 6 to 1. An examination of the nitrogen to phosphorus ratio reported in this paper is in harmony with earlier findings, and even higher ratios were found. However, the absolute values for phosphorus and nonpigment nitrogen are so low in most fractions—for example, fractions 399 to 305 and 250 to 231—that little significance can be attached to the molar ratios. In the fraction 230 to 200, the high phosphorus content is noteworthy.

Table V. R_f Values for Spots Obtained by Paper Chromatographic Analysis of Hydrolyzates

Fraction	Ammoniacal Silver Nitrate	Ninhydrin
0–17	0.41; 0.63	0.24; 0.65
18–27	0.25; 0.40; 0.63	0.17; 0.61
28–45	0.24; 0.41; 0.62	0.23
46–65	0.42; 0.62	[a]
66–100	0.41; 0.62	0.38
101–140	0.61	[a]
141–185	[a]	[a]
186–200	[a]	[a]
399–306	0.40; 0.62	0.23; 0.43
305–291	[a]	0.23
290–251	0.47	0.23
250–231	0.47	0.23
230–200	0.47; 0.65	0.23; 0.45
Inositol	0.27	...
Galactose	0.39	...
Glucose	0.43	...
Arabinose	0.45	...
Glycerol	0.63	...
Serine	...	0.24
Ethanolamine	...	0.39

[a] No spots were obtained.

The results of the paper chromatographic analysis of the acid hydrolyzates are summarized in Table V. The probable presence of glycerol, monosaccharides, inositol, serine, and ethanolamine—all known constituents of phospholipides—is indicated.

Table VI. Anthrone Tests for Sugars

Fraction	Intensity of Color	Fraction	Intensity of Color
0–17	+++	399–306	++
18–27	+++	305–291	−
28–45	++++	290–251	++
46–65	++	250–231	+
66–100	++	230–200	−
101–140	−	...	...
141–185	+	...	...
186–200	+	...	...

Previous investigators found that both bound and free reducing sugars accompany phosphatides in a countercurrent distribution (*13*). The preliminary work reported in the present paper indicates that the highest concentration of reducing sugars is found in fraction 0 to 45 and confirms the findings of earlier investigators (*11*)—that the fractions from the more polar end of the distribution are high in reducing sugars.

The results of the anthrone tests for sugars given in Table VI show that this test was positive for all the fractions except 101 to 140, 305 to 291, and 230 to 200.

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Literature Cited

(1) Bourne, G. H., Kidder, G. W., "Biochemistry and Physiology of Nutrition," Vol. I, pp. 257–64, Academic Press, New York, 1953.
(2) Cannon, C., Kummerow, F. A., *J. Am. Oil Chemists' Soc.* **34,** 519–20 (1957).
(3) Cookson, M. A., Coppock, J. B. M., *J. Sci. Food Agr.* **7,** 72–87 (1956).
(4) Craig, L. C., *Anal. Chem.* **22,** 1346–52 (1950).
(5) Dimler, R. J., Schaefer, W. C., Wise, C. S., Rist, C. E., *Ibid.*, **24,** 1411–14 (1952).
(6) Dutton, H. J., in "Progress in the Chemistry of Fats and Other Lipides," (Holman, Lundberg, and Malkin, eds.), Vol. II, pp. 292–325, Pergamon Press, Ltd., London, 1954.
(7) Gortner, R. A., Jr., Gortner, W. A., "Outlines of Biochemistry," 3rd ed., p. 849, Wiley, New York, 1953.
(8) Hilditch, T. P., Jasperson, H., *J. Soc. Chem. Ind.* **64,** 109–11 (1945).
(9) Kummerow, F. A., *Oil and Soap* **23,** 167–70 (1946).
(10) Lancaster, C. R., Lancaster, E. B., Dutton, H. J., *J. Am. Oil Chemists' Soc.* **27,** 386–90 (1950).
(11) McGuire, T. A., Earle, F. R., *J. Am. Oil Chemists' Soc.* **28,** 328–31 (1951).
(12) Mackinney, G., Weast, C. A., *Ind. Eng. Chem.* **32,** 392–5 (1940).
(13) Olley, J., Lovern, J. A., *Biochem. J.* **57,** 610–19 (1954).
(14) Shorland, F. B., *Nature* **153,** 168 (1944).
(15) *Ibid.*, **165,** 766 (1950).
(16) Smith, J. A. B., Chibnall, A. C., *Biochem. J.* **26,** 218–34 (1932).
(17) Truog, E., Meyer, A. H., *Ind. Eng. Chem., Anal. Ed.* **1,** 136–9 (1929).
(18) Zscheile, F. P., *et al.*, *Plant Physiol.* **17,** 331–46 (1942).

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NUTRIENTS IN NUTS

The Nutritive Value of Fresh and Roasted, California-Grown Nonpareil Almonds

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The effect of blanching and of dry-oven and oil roasting on the proximate composition, protein quality, calcium, phosphorus, iron, thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, and biotin in California almonds of the Nonpareil variety was determined. The removal of the skins by the blanching process did not produce significant change in any of the nutrients measured, except for calcium and iron, which appear in greater amounts in the skin. Dry-oven roasting resulted in 69% loss of thiamine and 23% loss of pantothenic acid. There was additional loss of 15% of thiamine and 19% of pantothenic acid as a result of oil roasting. There was insignificant change in the other vitamins as a result of either type of roasting.

SEVERAL STUDIES of the B vitamins in California - grown products have been completed in this laboratory. A recent study included three varieties of walnuts (*9*). California also produces 80 to 100% of the almonds (*Prunus Amygdalus* Batsch) grown in the United States, and the Nonpareil (soft shell) variety represents a major part of this crop.

Few reports are available on the composition of known varieties of almonds. Early studies by Hart (*10*) and Pitman (*24*) reported the proximate composition of European and California almonds, but very little has been reported for the newer B vitamins—pantothenic acid, folic acid, vitamin B_6 (pyridoxine), and biotin. The values recorded for almonds in current food composition tables represent a compilation of the results of determinations of only a few nutrients, made in the various laboratories, and do not represent a complete analysis of any one sample of nuts. None of the tables contains values for almonds in the forms in which they are most frequently consumed—i.e., blanched, dry-oven, and oil-roasted.

The quality of almond protein has been studied by Morgan, Newbecker, and Bridge (*22*). Using mice as the experimental animals, these workers obtained a protein efficiency—grams gain per gram of protein eaten—of 0.63 on a diet containing 17.2% protein supplied by ether-extracted almond meal. Three rats fed almond residue proteins—fat-free almond meal from which the globulin protein had been extracted—at the 4.5% level, gained 3.8 grams per gram of protein eaten. The wide range between these values, the high level of protein in the diet fed the mice, and the small number of rats used in the experiment with almond residue proteins, leave doubts as to the significance of these results. Mitchell and Beadles (*21*) measured the biological value and digestibility of beef round and of five varieties of nuts, including almonds and English walnuts. Neither study measured the effect of roasting on the quality of protein in almonds. As heat is known to affect the nutritive value of protein, it seemed important to determine the effect of dry- and oil-roasting on the protein quality as well as on retention of the vitamins.

This study reports the proximate composition, including moisture, fat, and protein; protein efficiency as compared with that of English walnuts and beef;