(13) Dreze, A., *Bull. Soc. Chim. Biol.* **38,** 243 (1956).
(14) Duckworth, J., Hepburn, W. R., Woodham, A. A., *J. Sci. Food Agr.* **12,** 16 (1961).
(15) Duckworth, J., Woodham, A. A., *Ibid.*, **12,** 5 (1961).
(16) Dustin, J. P., Schram, E., Moore, S., Bigwood, E. J., *Bull. Soc. Chim. Biol.* **33,** 1137 (1953).
(17) Ellinger, G. M., *Proc. Tenth World's Poultry Congr. (Edinburgh)* **2,** 128 (1954).
(18) Leitch, I., Godden, W., *Imp. Bur. Animal Nutrition Tech. Comm.*, No. **14** (1941).
(19) McPherson, A. T., *Food Technol.* **16,** 34 (1962).
(20) Moore, S., Spackman, D. H., Stein, W. H., *Anal. Chem.* **30,** 1185 (1958).
(21) Moore, S., Spackman, D. H., Stein, W. H., *Federation Proc.* **17,** 1107 (1958).
(22) Morrison, J. E., Pirie, N. W., *J. Sci. Food Agr.* **12,** 1 (1961).
(23) Pirie, N. W., *Econ. Botany* **15,** 302 (1961).
(24) Pirie, N. W., *Eng.* **190,** 274 (1960).
(25) Pirie, N. W., *Food Manuf.* **32,** 416 (1957).
(26) Pirie, N. W., *J. Biochem. Microbiol. Technol. Eng.* **1,** 13 (1959).
(27) Pirie, N. W., *Lancet* **2,** 961 (1959).
(28) Pirie, N. W., *Rept. Rothamsted Expt. Sta.* **173** (1952).
(29) Pirie, N. W., *Research* **10,** 29 (1957).
(30) Rose, W. C., *Federation Proc.* **8,** 546 (1949).
(31) Rose, W. C., Wixom, R. L., Lockhart, H. B., Lambert, G. F., *J. Biol. Chem.* **217,** 987 (1955).
(32) Schram, E., Moore, S., Bigwood, E. J., *Biochem. J.* **57,** 33 (1954).
(33) Spackman, D. H., Stein, W. H., Moore, S., *Anal. Chem.* **30,** 1190 (1958).
(34) Waterlow, J. C., *Brit. J. Nutr.* **16,** 531 (1962).
(35) Wilson, R. F., *Expt. Progr. Grassl. Res. Inst. (Hurley)* **11,** 65 (1958).

Received for review June 6, 1964. Accepted October 19, 1964. Work supported in part by grants from the Herman Frasch Foundation. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

LEAF PROTEINS AS FOODSTUFFS

Fatty Acids in Some Leaf Protein Concentrates

IRACEMA H. LIMA, T. RICHARDSON, and M. A. STAHMANN

Departments of Biochemistry and Dairy and Food Industries, University of Wisconsin, Madison, Wis.

The total fatty acid composition of leaf protein concentrates from seven plant species was studied by gas-liquid chromatography of their methyl esters. In general, the concentrates contained from about 3 to 8% fatty acids, more than 75 to 80% of which were linolenic, palmitic, and linoleic acids. Lesser amounts of oleic, palmitoleic, and stearic acids contributed 12 to 17% of the total fatty acids. Small amounts of other saturated and unsaturated fatty acids were detected. There appeared to be no gross qualitative differences in the composition of fatty acids from the leaf protein concentrates from different species.

Leaf protein concentrates may furnish adequate amounts of many essential amino acids, although they are low in methionine and cystine (*6*). The same concentrates contain significant levels of lipid, which could make a substantial contribution to the nutritional quality of the concentrates.

Since the early work of Smith and Chibnall (*19, 20*) on the lipids of forage grasses, a number of papers have appeared on the fatty acids of leaf tissue. The advent of gas-liquid chromatography has greatly facilitated studies of this nature (*1–3, 5, 8, 17, 18, 22, 23*).

The present paper deals with the total fatty acid composition of leaf protein concentrates used in the previous amino acid studies primarily in terms of the polyunsaturated fatty acids (PUFA) which affect the nutritional and keeping qualities of the concentrates. To the authors' knowledge, no studies have been published on the fatty acids of these leaf protein concentrates.

Experimental

Samples. Leaf protein concentrates of *Brassica napus* (turnip), *Chenopodium amaranticolor*, *Curcubita ovifera* (marrow A), *Lolium perenne* (rye grass), *Trifolium pratense* (red clover), *Triticum vulgare* (wheat preparation 539), and *Zea mays* (corn preparations 577–d and 581–d) were obtained from N. W. Pirie, Rothamsted Experimental Station, Harpenden, Herts., England. Their preparation has been described (*12*).

Extraction of Lipids. One-gram portions of each sample, dried and finely powdered (60-mesh), were extracted with shaking at room temperature (23° C.) four times each for 2 hours with 20 ml. of chloroform-methanol (2 to 1, v./v.). After each extraction the samples were filtered through Whatman No. 1 paper on a Büchner funnel under suction. The final volumes of each extract were measured and 3-ml. aliquots were taken to determine total lipid extracted after complete removal of the solvent in vacuo. Four extractions removed all but negligible amounts of total lipid-solubles. The combined extracts were concentrated in vacuo at about 20 mm. of Hg and 30° to 40° C. The crude lipids were saponified by refluxing for 2 hours in 0.5*N* ethanolic potassium hydroxide (20 ml. per gram) as described by Weenink (*23*). After cooling, the unsaponifiable components were extracted from the soap solution by three extractions with 20 ml. of Skellysolve B. The remaining solution was acidified with hydrochloric acid and the total fatty acids were extracted with four 25-ml. portions of Skellysolve B. The solutions of fatty acids were transferred to tared tubes and yields were determined after complete removal of the solvent in a stream of dry nitrogen at 23° C.

Preparation of Methyl Esters. The fatty acids were converted to methyl esters by the procedure of Radin, Hajra, and Akahori (*14*) using 2,2-dimethoxypropane (DMP) as a water scavenger. To 30 mg. of fatty acid in a test tube, 2 ml. of DMP and 2 ml. of methanolic hydrochloric acid were added. The tube was stoppered and left for 1 hour at room temperature. The esters were isolated by addition of water and extraction with Skellysolve B. The extracts were washed with 0.2*N* sodium bicarbonate solution, the solvent was removed under nitrogen, and the methyl esters were examined by gas-liquid chromatography.

Gas-Liquid Chromatographic Analysis. Samples of methyl esters were chromatographed on an Aerograph Hi-Fy Model 600-B equipped with a hydrogen flame ionization detector. Stationary phases and solid supports were 15% diethylene glycol succinate (DEGS) on 60/80-mesh firebrick GC-22 and 15% Apiezon M on 60/80-mesh base-washed Chromosorb W. The columns were packed in a 5 foot × 1/8 inch stainless steel tube. The DEGS column was

Table I. Yields of Fatty Acids from Various Leaf Protein Concentrates

Samples	Age of Leaf, Weeks	Condition of Crop	Fatty Acid, % Dry Weight
Brassica napus Turnip	Unknown	Young, succulent	8.4
Chenopodium amaranticolor	Unknown	Dry tough	7.7
Curcubita ovifera Marrow A	12	Fresh	8.0
Lolium perenne Rye grass	2–3	Fresh lush	2.5
Trifolium pratense Red clover	Unknown	Wet plus elm leaves	3.7
Triticum vulgare Wheat 539	18	Fresh green	4.6
Zea mays			
Corn 577 d	14	Green stemmy	4.6
Corn 581 d	16	Fresh, more mature	3.6

Table II. Flame Ionization Detector Response Relating Area and Weight Per Cent (*11*)

Methyl Esters[a]	Wt. %	Av. Area, %[b]	S.D.[b]
10:0	15.4	15.0	(0.482)
12:0	14.4	14.4	(0.292)
14:0	13.5	13.7	(0.382)
16:0	14.9	14.8	(0.150)
18:0	14.2	14.4	(0.561)
18:1	14.0	14.3	(0.294)
18:3	13.6	13.3	(0.182)

[a] No. of carbon atoms: No. of double bonds.
[b] Standard deviation for six determinations.

operated at 190–192° C., the Apiezon M column at 200° C., and the injector block at 275° C.

The ester sample from *Triticum vulgare* (wheat preparation 539) was also hydrogenated by the method of Farquhar *et al.* (*4*) and analyzed on the DEGS column.

Esters were identified by relative retention times compared with standard esters, by plots of log relative retention time against number of carbon atoms, by behavior of esters after hydrogenation, and by comparing the patterns with those in the literature run under similar conditions (*7, 10, 15, 21*).

The amount of each component was estimated from the area under its peak. The areas were calculated by counting squares and the esters were quantified by the area normalization method. Overlapping peaks were treated as described by Pecsok (*13*). Detector response was determined from a mixture of standard esters and area and weight per cent were correlated from these data.

Results and Discussion

Table I shows fatty acid yields ranging from 2.5 to 8.4% of dried concentrate and indicates a significant amount of fatty acids in the dried material. The relatively high levels of fatty acids in the concentrates would play a very important role in their nutritional properties. The fatty acid contents are in general agreement with data in literature on leaf tissues. Hawke (*8*), for example, reported approximately 3 to 6.5% fatty acids in dried rye grass tissue. Crombie (*2*) found 0.6 to 3.0% fatty acids in a variety of green plant tissues including *Zea mays*.

Table II illustrates the detector response relating area per cent to weight per cent. There is a close correlation for esters ranging from 10:0 through 18:3. As a result, no correction factors were applied to the area per cent data, and these data may be taken essentially as weight per cent methyl esters.

Figure 1 depicts a typical chromatogram of the plant methyl esters, which clearly shows 14 ester components. In all analyses, the number of total components varied from 12 to 15 with variations in the minor short-chain esters. The major fatty acids esters in leaf protein concentrates from the seven plant species studied were octadecatrienoate (linolenate), hexadecanoate (palmitate), and octadecadienoate (linoleate).

Table III shows that linolenate (21 to 54%) is the predominant unsaturated ester in all cases, corn having the lowest and wheat the highest percentage. Linoleate (3.8 to 25%) exhibits a very wide range of distribution with a low value for marrow of 3.8% but with most concentrations falling between 11.7 and 24.5%. Palmitate (15.4 to 40.3%) is the most abundantly distributed saturated component. Hexadecenoate (palmitoleate), octadecanoate (stearate), and octadecenoate (oleate) appear in smaller proportions, with *Chenopodium* containing considerably higher (10.6%) levels of oleate than the other concentrates. Negligible amounts of octanoate (caprylate), decanoate (caprate), dodecanoate (laurate), and tetradecanoate (myristate) were detected in addition to other minor unsaturated esters.

The distribution and relative concentrations of the major components are in general agreement with work in the literature on leaf tissue fatty acids. Hawke (*8*) found that the fatty acid content of rye grass depended upon the stage of plant maturity. The time of harvest affected the relative proportions of the palmitate (9.95 to 21.96 mole %), linoleate (6.8 to 13.6 mole %) and linolenate (58.6 to 79.1 mole %). Hawke concluded that the lipid content of new grass and yield of fatty acids in lipids were greater than in mature grass. Higher proportions of linolenic acid were present in new growth and, because of the lipid content at this stage, the total amount of linolenate was higher. The stages of maturity of plants used in the present study are uncertain; however, based on Hawke's work, changes in lipid patterns as a function of maturity are areas for future study. Crombie (*2*) also found palmitic (8 to 13.4%), oleic (6.9 to 10.1%), and linoleic (27.3 to 69.0%) as major acids in leaves of *Zea mays*. Jackson and Kummerow (*9*) in a spectrophotometric study of triglyceride and phospholipid fatty acids from dehydrated alfalfa leaf meal reported 34 and 36.8% oleic; 32.2 and 35.2% linolenic; and 16.9 and 14.7% linoleic acids, respectively. More recently, Debuch (*3*) established the presence of a *trans*-3-hexadecenoic acid in addition to the main components linolenic (56.2 to 57.9%),

Table III. Composition of Fatty Acid Esters from Leaf Protein Concentrates[a]

Methyl Ester[b]	Chenopodium	Clover	Corn 577	Corn 581	Marrow	Rye Grass	Turnip	Wheat
8:0	0.8	0.6	1.0	1.5	0.8	1.3	0.5	0.4
10:0	0.6	0.2	0.4	0.8	0.3	0.9	0.7	0.2
11:0 (10:1)	0.6	0.4	0.4	0.9	0.7	0.9	0.0	0.3
12:0	0.6	0.5	0.6	1.9	1.0	0.8	0.8	0.2
12:1	0.6	0.4	0.0	0.0	0.0	0.7	0.0	0.2
13:0	0.6	0.4	0.3	0.6	0.2	0.7	0.0	0.0
14:0	1.0	0.9	0.7	1.3	2.4	0.9	1.2	0.5
14:1	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15:0	1.3	1.0	0.4	0.9	1.1	0.7	1.6	0.5
16:0	22.8	15.4	40.3	34.4	29.9	18.2	24.8	15.9
16:1	5.2	5.2	4.3	5.1	8.0	6.6	6.7	4.9
17:0	0.0	0.8	0.5	0.7	0.0	0.0	1.1	0.0
18:0	1.2	2.5	2.6	2.2	2.9	1.5	4.5	2.5
18:1	10.6	4.5	4.9	4.5	2.6	4.3	2.4	5.9
18:2	18.4	18.9	22.0	24.5	3.8	11.7	16.8	13.8
18:3	33.5	48.4	21.8	20.7	46.3	50.9	38.9	53.7

[a] Per cent by weight of each fatty acid ester calculated from area of each peak.
[b] No. of carbons: No. of double bonds.

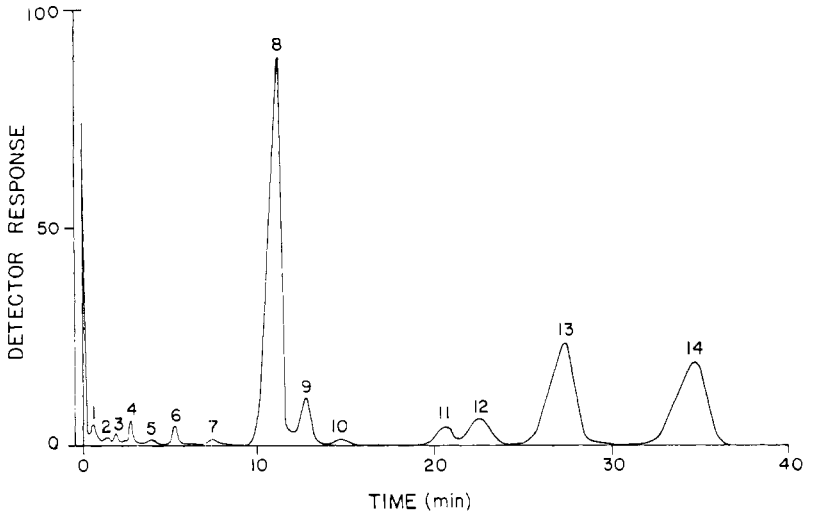

Figure 1. Gas chromatogram of fatty acid methyl esters (DEGS)

1. 8:0	6. 14:0	11. 18:0
2. 10:0	7. 15:0	12. 18:1
3. 11:0 (10:1)	8. 16:0	13. 18:2
4. 12:0	9. 16:1	14. 18:3
5. 13:0	10. 17:0	

linoleic (16.3 to 17.7%), and palmitic (12.9 to 13.4%) acids in green leaves of *Spinacia oleracea* and *Antirrhinum majus*. Burnet and Lohmar (*1*) reported high concentrations of oleic (21.0%), linoleic (12.9%), and linolenic (39.0%) acids in sorghum tissues. Garton (*5*) analyzed a mixture of forage plants and found high levels of unsaturated fatty acids. Sakai (*16*) did not use gas-liquid chromatography but reported that oleic and linoleic acids contributed 87% to the total fatty acids of *Chenopodium album*. Shorland, Weenink, and Johns (*18*) examined the fatty acids of clover-rich pasture and reported values of octadecatrienoic acid as high as 58.9% of total fatty acids.

The high levels of essential linoleic acid and other fatty acids in leaf tissues in general and the leaf protein concentrates in particular indicate that the leaf lipids are valuable human nutrients in terms of both energy contribution and essential nutrients. Attempts should be made to include these nutrients in leaf protein concentrates used for animal and human nutrition and still eliminate undesirable flavors and color. Studies on the peroxidation of these highly unsaturated fatty acids and the effect on protein quality would also be desirable.

Acknowledgment

The authors thank N. W. Pirie and Marjorie Byers, Rothamsted Experiment Station, Harpenden, England, for samples of leaf protein concentrates used in this study.

Literature Cited

(1) Burnet, M. C., Lohmar, R. L., J. Agr. Food Chem. **7,** 436 (1959).
(2) Crombie, W. M., *J. Exptl. Botany* **9,** 254 (1958).
(3) Debuch, H., *Z. Naturforsch.* **9,** 561 (1961).
(4) Farquhar, J. W., Insull, W., Jr., Rosen, P., Stoffel, W., Ahrens, E. H., Jr., *Nutr. Rev. (Suppl.)* **17,** 29 (1959).
(5) Garton, G. A., *Nature (London)* **187** 511 (1960).
(6) Gerloff, E. D., Lima, I. H., Stahmann, M. A., J. Agr. Food Chem. **13,** 139 (1965).
(7) Hawke, J. C., *J. Dairy Res.* **24,** 366 (1957).
(8) *Ibid.*, **30,** 67 (1963).
(9) Jackson, A. H., Kummerow, F. A., *J. Am. Oil Chemists' Soc.* **26,** 26 (1949).
(10) Magidman, P., Herb, S. F., Barford, R. A., Reimenschneider, R. W., *Ibid.*, **39,** 137 (1962).
(11) Moore, J. L., Richardson, T., Amundson, C. H., *J. Gas Chromatog.* **2,** 318 (1964).
(12) Morrison, J. E., Pirie, N. W., *J. Sci. Food Agr.* **12,** 1 (1961).
(13) Pecsok, R. L., "Principles and Practice of Gas Chromatography," p. 135, Wiley, New York, 1959.
(14) Radin, N. S., Hajra, A. K., Akahori, Y., *J. Lipid Res.* **1,** 250 (1960).
(15) Richardson, T., Tappel, A. L., Smith, L., Houle, C. R., *Ibid.*, **3,** 344 (1962).
(16) Sakai, R., *Kumamoto Pharm. Bull.* **4,** 77 (1954); *C. A.* **50,** 501 (1956).
(17) Shorland, F. B., *J. Sci. Food Agr.* **12,** 39 (1961).
(18) Shorland, F. B., Weenink, R. O., Johns, A. T., *Nature (London)* **175,** 1129 (1955).
(19) Smith, J. A. B., Chibnall, A. C., *Biochem. J.* **26,** 218 (1932).
(20) *Ibid.*, p. 1345.
(21) Smith, L. M., *J. Dairy Sci.* **44,** 607 (1961).
(22) Weenink, R. O., *Biochem. J.* **82,** 523 (1962).
(23) Weenink, R. O., *J. Sci. Food Agr.* **12,** 34 (1961).

Received for review June 4, 1964. Accepted October 19, 1964. Work supported in part by grants from the Herman Frasch Foundation and the Rockefeller Foundation. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

LEAF PROTEINS AS FOODSTUFFS

Nutritive Value of Leaf Protein Concentrate, an In Vitro Digestion Study

WALTER R. AKESON and MARK A. STAHMANN

Department of Biochemistry, University of Wisconsin, Madison, Wis.

The amino acid composition of leaf protein concentrate (LPC) was reported to be as good as or better than that of many common foodstuffs (*16*). With the exception of methionine, which was low, all the essential amino acids were present in sufficient quantities to constitute a protein of good nutritive value in LPC. This indicated that plant leaves may be an important source of food for human consumption.

Feeding experiments have been conducted. Many early studies suggested that the nutritive value of LPC was low (*5, 7, 8, 10–12, 15*). The poor quality of the LPC used in early studies may have resulted from improper processing. Recent results by Duckworth *et al.* (*13, 14*), Barber (*2*), and Waterlow (*28*) were more encouraging, as they reported that LPC prepared under different conditions may be equal to or better in nutritive value than proteins such as soybean meal, white fish meal, and milk.

On the basis of amino acid composition, LPC from a wide variety of plant species may have high nutritive value (*16*). However, amino acid composition alone may not give a true picture, since processing may have a deleterious effect on the nutritive value of some proteins (*6, 17*). Carpenter (*6*) showed