The Lipids of Dehydrated Alfalfa Leaf Meal[1,2]

A. H. JACKSON[3] and F. A. KUMMEROW, Kansas Agricultural Experiment Station,[4] Manhattan, Kansas

STUDIES involving the lipid material of dehydrated alfalfa leaf meal have usually been carried out on the unsaponifiable rather than the saponifiable fraction. Petering and co-workers (1), for example, studied the carotene; Fernholz and Moore (2), the sterol; and Chibnall *et al.* (3), the wax fractions of the crude lipid material. This emphasis on the unsaponifiable fraction was probably due to the utilization of alfalfa leaf meal as a source of vitamin A in animal and poultry rations. However, the saponifiable fraction may also have nutritional significance. Various workers have shown that the major portion of the saponifiable fraction of lipids extracted from green plant materials was composed of highly unsaturated fatty acids. Hilditch and Jasperson (4) found a large amount of linolenic acid in the lipids of rye grass, and Shorland (5) found that the lipids of forage grasses and clover contained over 80% unsaturated $C_{16\text{-}18}$ fatty acids. Lovern (6) and Takahashi *et al.* (7) also found a large amount of unsaturated fatty acids in sea algae; Menke and Jacob (8) found that the lipids extracted from spinach had an iodine value of 198-210.

The presence of such large amounts of highly unsaturated fatty acids may affect the nutritional value of green plant stems and leaves in several ways. These plant materials could contribute significantly to the need for essential fatty acids. However, if they are fed to animals or poultry in excessive amounts, a decrease in the stability of the depot fat may result. Schreiber *et al.* (9) found that during cold storage the carcasses of birds which had been supplemented with alfalfa were less stable towards oxidative rancidity than the unsupplemented birds. Furthermore, various workers have shown that highly unsaturated fatty acids could be readily deposited in the depot fats of hogs (10), rats (11), and chickens (12), and that these depot fats contain arachidonic as well as linolenic acid (13).

The presence of highly unsaturated fatty acid could also enhance carotene destruction during storage of the meal and thus affect its nutritional value. Several workers (14, 15) have shown that the rate of carotene destruction was increased in the presence of rancid fats and that carotene actually functioned as an antioxidant in the presence of unsaturated fatty acids (16).

The importance of alfalfa as a livestock feed was shown by the fact that over 33 million tons of alfalfa hay were harvested in 1945 (17); of this amount approximately 1.3%, or 441,500 tons, were sold as dehydrated alfalfa leaf meal (18). As part of a study on the nutritional value of the dehydrated meal (19, 20) it seemed desirable to study the composition of the extracted lipids. The present study was primarily concerned with the characteristics of the saponifiable fraction.

[1] Financial support for this work was furnished by the Kansas Industrial Development Commission.

[2] A thesis presented as partial fulfillment of the requirement for the degree of Bachelor of Science in chemistry.

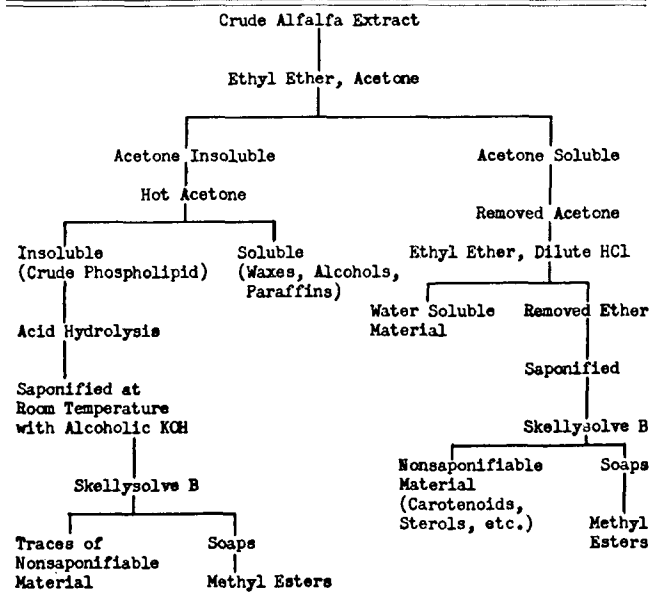
[3] Present address: Meharry Medical College, Nashville, Tennessee.

[4] Contribution No. 338 from the Department of Chemistry.

Experimental

The lipids were extracted from freshly dehydrated alfalfa leaf meal[5] with acetone, Skellysolve B, and ethyl alcohol at room temperature (Table I). Four

TABLE I



thousand grams of alfalfa meal were weighed into two 8-liter percolators and allowed to stand in contact with acetone a few hours. The acetone was then drained off at the bottom of the percolator and fresh acetone was poured over the alfalfa meal. This extraction procedure was repeated twice with Skellysolve B as a solvent and twice with ethyl alcohol. The extracts were filtered and most of the solvent removed at atmospheric pressure. The crude extracts were then combined and the remaining solvent removed under vacuum; the yield was 263.9 g. or 6.59% of the alfalfa meal extracted.

The crude extract was dissolved in 200 ml. ethyl ether and approximately 50-ml. portions were poured slowly into 250-ml. centrifuge tubes containing 200 ml. acetone which had been cooled to 10°C. The tubes were centrifuged and the acetone soluble fraction decanted. The acetone insoluble fractions were taken up in ethyl ether, transferred to a round-bottom flask, and freed from solvent under vacuum; yield, 55.0 g. or 21% of the crude extract. The acetone soluble fractions were combined and also freed from solvent under vacuum; yield, 208.9 g. or 79% of the crude extract.

The acetone insoluble fraction was subjected to further separation by repeated precipitation from hot acetone according to the procedure of Pollard, Chibnall, and Piper (21). The fraction, insoluble in hot acetone, was dried over P_2O_5 in a vacuum desic-

[5] First cutting at college farm and dehydrated in a pilot plant model through the courtesy of the Department of Chemical Engineering. This meal was identical to a high quality commercial dehydrated alfalfa leaf meal.

cator; yield, 9.7 g. A small amount of this residue was heated in the presence of dilute hydrochloric acid in order to hydrolyze any carbohydrates which may have been present. However, the aqueous phase gave a negative Molisch and Benedict test. The following characteristics of the fraction which had been insoluble in hot acetone were also determined. Total nitrogen according to the Kjeldahl method, calcium as the oxalate (22), choline as the reineckate (23), and total phosphorus according to the method of Gortner (24).

Five g. of the fraction which had been insoluble in hot acetone was dissolved in 50 ml. ethyl alcohol and refluxed with an equal volume of 1:1 hydrochloric acid for six hours. Hydrolysis of the phospholipids was completed by neutralizing with alcoholic potassium hydroxide and allowing the mixture to stand overnight in the presence of a slight excess of the alkali. Residual unsaponifiable material was removed by extraction with Skellysolve B. The soaps were then acidified with hydrochloric acid and the resulting fatty acids extracted and converted into methyl esters by refluxing in methyl alcohol with sulfuric acid as a catalyst. The methyl esters were pale yellow in color and had a saponification equivalent of 298.0 and an iodine value of 157.0; yield, 2.0 g. The percentage composition of the mixed methyl esters was determined by the spectrophotometric method of Brice *et al.* (25).

According to Pollard, Chibnall, and Piper (21) the fraction which was soluble in hot acetone contained wax, higher molecular weight alcohols, and paraffins. As Chibnall and co-workers (3) have already characterized the principal components of alfalfa wax, this fraction was not subjected to further characterization. It was, however, recrystallized from acetone and alcohol and found to melt between 68-70°C. Chibnall *et al.* found that the wax contained n-triacontanol melting point 86.3°C., and a paraffin fraction melting point 65.6°C.; the mixed fatty acids of the wax were not identified.

The 208.9 g. of crude extract which had been soluble in cold acetone contained chlorophyll and other water soluble material, triglycerides, and unsaponifiable material. Most of the chlorophyll and water soluble material was removed from this fraction by diluting it with one liter of ethyl ether and repeatedly shaking with dilute hydrochloric acid in a separatory funnel, according to the method of Willstatter and Fritzsche (26). The ether layer was then shaken free of acid with water, dried with anhydrous sodium sulfate, and freed from solvent. The residue was taken up in ethyl alcohol, subjected to mild saponification, and freed of unsaponifiable material with Skellysolve B. The soaps were then acidified, converted to methyl esters and characterized. The methyl esters had a saponification equivalent of 294.1 and an iodine value of 146.7; yield, 80.6 g.

Results

The crude alfalfa extract contained approximately five times more acetone soluble than acetone insoluble material. Of these fractions, the former contained 33.2% triglycerides and the latter 3.7% of phospholipid material respectively (Table II). The remainder was composed of unsaponifiable and water soluble materials. The phospholipid fraction was found to contain 0.61% nitrogen, 0.11% calcium, 0.98% choline, and 3.6% phosphorus. Assuming that all of the calcium had been present as calcium phosphotidate (mol. wt. 1366) and that all of the choline had been present as lecithin (mol. wt. 778) these percentages of calcium and choline represent 3.7% calcium phosphotidate and 7.0% lecithin respectively. The remainder of the fraction was probably composed of cephalin and phosphotidic acid.

TABLE II

Composition of Crude Alfalfa Extract

	Weight	Percentage on basis of:	
		Extract	Leaf meal
Insoluble in acetone:			
Crude phospholipid	9.7 g.	3.7%	0.24%
Wax, alcohols, paraffins	45.3	17.2	1.13
Soluble in acetone:			
Triglycerides	87.6	33.2	2.19
Unsaponifiable material	22.0	8.3	0.55
Water soluble material	99.3	37.6	2.48
Total	263.9 g.	100.0%	6.59%

The methyl esters of the mixed fatty acids isolated from either the triglyceride or phospholipid fractions were found to contain a large proportion of highly unsaturated fatty acids. The methyl esters obtained from the triglyceride fraction contained 80% and those from the phospholipid fraction almost 90% of unsaturated methyl esters respectively. This degree of unsaturation was similar to that noted for the lipids in rye grass (4), clover (5), and spinach leaves (8).

The mixed methyl esters of the phospholipid fraction contained approximately 2% less linoleic acid and 3% more linolenic acid than the triglyceride fraction. It also contained approximately 6% less saturated and 6% more oleic acid (Table III). These values were calculated from the maximum absorption at 2320, 2680, and 3160 Å according to the method of Brice *et al.* (25). The maximum absorption of the unisomerized methyl esters was much higher at 3160 Å than that of unisomerized corn, cottonseed, or soybean oil at this wave length. A 1-g. sample of the methyl esters was therefore subjected to molecular distillation in a semi-micro pot still. However, the amber colored distillate retained its high absorption at 3160 Å. One-half gram of this distillate was diluted with 100 ml. Skellysolve F and chromatographed on activated alumina.[6] A faint yellow band which formed at the top of the column was eluted with 100 ml. alcohol. This alcohol eluate had a much higher maximum absorption at 3160 Å than the original distillate or an absorption coefficient of 19.6 and 2.9 respectively. The Skellysolve F eluate on the other hand showed no detectable absorption at 3160 Å. Therefore the high absorption of the unisomerized esters must have been due to impurities and not arachidonic acid. Furthermore no methyl octobromoarachidate (28) could be isolated when 0.5 g. of the original methyl ester was brominated in ether at 0°C.

TABLE III

Comparison of Fatty Acids

Fatty acid	Phospholipid fraction	Triglyceride fraction
Linoleic	14.7%	16.9%
Linolenic	35.2	32.2
Oleic	36.8	31.0
Saturated	13.3	19.9

[6] Grade F-20, 80-200 mesh, Aluminum Ore Company, East St. Louis, Illinois.

The unisomerized methyl esters isolated from a sample of freshly cut alfalfa also showed a large amount of absorption at 3160 Å. The large absorption was therefore not due to the production of partially oxidized or polymerized linolenic acid during the process of dehydration.

Discussion

The contribution of alfalfa lipids to the total dietary fat intake is small as standard animal and poultry feeds usually do not contain more than 10% dehydrated alfalfa leaf meal. However, their contribution may become significant when animals and poultry are pastured on alfalfa, as is usually practiced with hogs and turkeys. Furthermore, Richardson and Abbott (27) found that when dairy cows were fed exclusively on alfalfa a crumbly butter resulted, which seemed similar to the results obtained when dairy cows were restricted to feeds containing too much oil meal.

A large amount of absorption of the unisomerized methyl esters seemed to be due to an unidentified faintly yellow compound. This was not extracted with the nonsaponifiable material. Furthermore it could not be removed from the methyl esters by high vacuum distillation. It is possible that this or a similar compound is partly responsible for the difficulties encountered in the spectrophotometric analysis of natural oils such as linseed oil.

Summary

Freshly dehydrated alfalfa leaf meal was repeatedly extracted with acetone, ethyl alcohol, and Skellysolve B in a percolator at room temperature; 6.59% of crude extract was obtained. This extract was composed of 3.7% phospholipids, 33.2% triglycerides, 17.2% crude wax, 8.3% unsaponifiable, and 37.6% water soluble material.

Spectrophotometric analysis of the mixed methyl esters from the triglyceride fraction indicated the presence of 32.2% linolenic, 16.9% linoleic, 31.0% oleic, and 19.9% saturated acids. The mixed methyl esters from the phospholipid fraction contained 35.2% linolenic, 14.7% linoleic, 36.8% oleic, and 13.3% saturated acids.

REFERENCES

1. Petering, H. G., Morgal, P. W., and Miller, E. J., Ind. Eng. Chem., *33*, 1298 (1941).
2. Fernholz, E., and Moore, M. L., J. Am. Chem. Soc., *61*, 2467 (1939).
3. Chibnall, A. C., Williams, E. F., Latner, A. L., and Piper, S. H., Biochem, J., *27*, 1885 (1933).
4. Hilditch, T. P., and Jasperson, H., J. Soc. Chem. Ind., *64*, 109 (1945).
5. Shorland, F. B., Nature, *153*, 168 (1944).
6. Lovern, J. A., Biochem. J., *30*, 387 (1936).
7. Takahashi, E., Shirahama, K., and Tase, S., J. Chem. Soc., Japan, *54*, 619 (1933).
8. Menke, W., and Jacob, E., Zeit. Physiol. Chem., *272*, 227 (1942).
9. Schrieber, M. L., Vail, G. E., Conrad, R. M., and Payne, L. F., Poultry Sci., *26*, 14 (1947).
10. Banks, A., and Hilditch, T. P., Biochem. J., *26*, 298 (1932).
11. Spadola, J. M., and Ellis, N. R., J. Biol. Chem., *113*, 205 (1936).
12. Cruickshank, E. M., Biochem. J., *28*, 965 (1934).
13. Brown, J. B., and Sheldon, C. C., J. Am. Chem. Soc., *56*, 2149 (1934).
14. Monaghan, B. R., and Schmitt, P. O., J. Biol. Chem., *96*, 387 (1932).
15. Lease, E. J., Lease, J. G., Weber, J., Steenbock, H., J. Nutrition, *16*, 571 (1938).
16. Newton, R. C., Oil and Soap, *9*, 247 (1932).
17. Crops and Markets, U. S. Dept. of Agr. Ec., *23*, 117 (1946).
18. Lantz, R. Z., American Dehydration Assoc., Chicago 4, Ill., personal communication.
19. Silker, R. E., Schrenk, W. G., and King, H. H., Ind. Eng. Chem., *36*, 831 (1944).
20. Kummerow, F. A., Avery, T. B., Vail, G. E., and Conrad, R. M., Poultry Sci., in press.
21. Pollard, A., Chibnall, A. C., and Piper, S. H., Biochem, J., *25*, 2110 (1931).
22. Official and Tentative Methods of Analysis, A.O.A.C., 5th Ed., p. 240 (1940).
23. Jacobi, H. P., Baumann, C. A., and Meek, W. J., Jour. Biol. Chem., *138*, 571 (1941).
24. Gortner, W. A., Jour. Biol. Chem., *159*, 97 (1945).
25. Brice, B. A., Swain, M. L., Schauffer, B. B., and Ault, W. C. Oil and Soap *22*, 219 (1945).
26. Willstatter, R., and Fritzsche, H., Ann., *371*, 33 (1910).
27. Richardson, G. A., and Abbott, F. H., Proc. 21st Ann. Meeting Western Div. Am. Dairy Sci. Assoc., p. 63 (1935).
28. Ault, W. C., and Brown, J. B., J. Biol. Chem., *107*, 607 (1934).

Processing of Cottonseed. IV. Effect of Preparation and Cooking of Meats on the Bleach Color and Storage Properties of Screw-Pressed Oils[1]

P. A. WILLIAMS and R. P. HADDEN, South Texas Cotton Oil Company, and C. M. HALL, L. E. CASTILLON, W. A. GUICE, R. T. O'CONNOR, and C. H. BOATNER, Southern Regional Research Laboratory,[2] New Orleans 19, Louisiana

Introduction

AS THE result of a series of mill-scale tests previously reported (1, 2), it was found that hydraulic-pressed oils had lower initial bleach colors,[3] which increased less rapidly during storage than did those of screw-pressed oils produced from the same seed. The lower bleach color of the hydraulic-pressed oils was attributed to the presence of water added to the meats during cooking prior to pressing. It was postulated that the presence of moisture during cooking of the meats caused deep-seated changes in the pigments contained in the water-sensitive pigment gland walls. It was therefore predicted that non-reverting[4] screw-pressed oils of low bleach color could be produced by wet-cooking of the meats prior to expression of the oil.

Conditions during preparation and cooking of the meats prior to expression of oil which might affect the bleach color of the oils are as follows:

1. Particle size of meats: a) whole, b) ground, c) rolled.
2. Moisture in the meats: a) originally, b) added before and during cooking.
3. Cooking conditions: a) temperature, b) duration of cooking, c) extent of agitation during cooking, d) venting during cooking.

[1] Presented before the 54th Annual Meeting of the National Oil Mill Superintendents' Association, Dallas, Texas, June 9-11, 1948.

[2] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[3] The term "bleach color" is used to designate the residual color, in terms of Lovibond red and yellow units of an oil which has been alkali-refined and bleached by American Oil Chemists' Society official methods.

[4] "Non-reverting oils" are oils which do not develop high bleach color during storage at moderate temperatures.