2. Cabanis, Y., L. Chabouis and F. Chabouis, Végétaux et Groupements Végétaux de Madagascar et des Mascareignes, BDPA, Tananarive, 1970, T2, pp. 699–701.
3. Watt, G., A Dictionary of the Economic Products of India, W.H. Allen and Co., London, 1893.
4. Badami, R.C., and C.D. Daultaladi, J. Karnatka Univ. 14:20 (1969); Chem. Abstr. 74:100826.
5. Pitke, P.M., P.P. Singh and H.C. Srivastava, JAOCS 54:592 (1977).
6. Artaud, J., M. Derbesy and J. Estienne, Ann. Falsif. Expert. Chim. 70:39 (1977).
7. Morad, M.M., S.B. El-Magoli and K.A. Sedky, Fette, Seifen, Anstrichm. 80:357 (1978).
8. Pitke, P.M., P.P. Singh and H.C. Srivastava, JAOCS 56:559 (1979).
9. Reddy, G.S., S.J. Rao, D.A. Ramayya, G. Azeemoddin and S.D.T. Rao, J. Oil Technol. Assoc. India 11:91 (1979).
10. Pitke, P.M., P.P. Singh and H.C. Srivastava, Proceedings of the Symposium on Production and Utilization of Forest Products, Regional Research Laboratory, Jammu and Tawi, Vol. III, 1979, p. 49. (taken in ref. 9).
11. Afnor, Recueil de Normes Françaises des Corps Gras, Graines Oléagineuses, Produits Dérivés, Afnor, Paris, 1981.
12. Folch, J., M. Lees and G.H. Sloane Stanley, J. Biol. Chem. 226:497 (1957).
13. Ramananarivo, R., J. Artaud, J. Estienne, G. Peiffer and E.M. Gaydou, JAOCS 58:1038 (1981).
14. Touche, J., Thèse d'Université, Aix-Marseille III, 1975.
15. Artaud, J., M.C. Iatrides, C. Tisse, J.P. Zahra and J. Estienne, Analusis 8:277 (1980).
16. Thompson, M.J., S.R. Dutky, G.W. Patterson and E.L. Gooden, Phytochemistry 11:1781 (1972).
17. Wright, J.L.C., A.G. McInnes, S. Shimizu, D.G. Smith, J.A. Walter, D. Idler and W. Khalil, Can. J. Chem. 56:1898 (1978).
18. Thompson, R.H., Jr., G. Patterson, M.J. Thompson and H.T. Slover, Lipids 16:694 (1981).

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Study of the Neutral Lipids of Sunflower Meal and Isolates

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ABSTRACT

Two types of sunflower protein isolates have been obtained from prepress and solvent extracted sunflower meal. The first was obtained by precipitation (at the isoelectric point) of the alkaline extract of the meal, and washing the curd with water. In the second, the alkaline extraction was carried out in the presence of sodium sulfite, and the curd was washed with water, ethanol and acetone. Both isolates were air-dried and then dried under vacuum at 50 C. From the total lipids, obtained with 86% ethanol, the neutral lipids were separated using a column of Florisil. The lipids studied were those of the two isolates mentioned above as well as those of the original meal. The following types of compounds were separated, identified and quantified: hydrocarbons, waxes, methyl esters, triglycerides, free fatty acids, diglycerides, free sterols, and hydroxy fatty acids.

INTRODUCTION

Due to the growth of world population, plant proteins will be more extensively used in the future. In developed countries, edible proteins are usually of animal origin, whereas in developing countries vegetable sources already account for more than 80% of protein supplies.

Traditionally, proteins from oilseeds have been byproducts of oil extraction, but now they are becoming increasingly important as foods. Oilseeds are one of the mainstays of the world food supply; sunflower being the second in importance, after soybean, as an oil source (1). Like other seeds, sunflower yields, after oil extraction, a meal which is a suitable source of proteins.

As far as we know, no previous studies have been reported on the lipid contaminants associated with sunflower proteins. Evidence for the presence of phosphatides in isolates from soybean meals was presented many years ago by Smiley and Smith (2). Eldridge et al. (3, 4) found phosphatidylcholine, phosphatidylethanolamine, saponines, sitosterol glycoside and genistein in soybean protein isolates. Neither free fatty acids nor hydroxy fatty acids were found in the lipids extracted with ethanol, which is surprising as they are rather polar compounds.

This paper describes the isolation and characterization of neutral lipids associated with the protein isolates obtained by alkaline extraction and isoelectric precipitation from sunflower meals.

EXPERIMENTAL PROCEDURES

Materials

Meals from prepress hexane-extracted, partially dehulled sunflower seeds were supplied by the industry. Hydrocarbons and normal alcohols were obtained from olive oil unsaponifiable (5). Pure octadecyl octadecanoate was a gift of H.K. Mangold. Pure triglycerides were obtained from olive oil and sunflower by preparative thin layer chromatography (TLC) on Silica Gel G. 1,2- and 1,3-diglycerides and cholesterol were commercial samples. A qualitative mixture of methyl ester fatty acids with different degrees of unsaturation was prepared by transesterification of an equilibrated blend of coconut oil and linseed oil. 12-Hydroxyoctadecenoic acid (ricinoleic acid) was obtained from castor oil; hydrogenation of this compound gave the corresponding saturated acid. 9- and 13-hydroxyoctadecanoic acids were obtained from linoleic acid by oxidation with soybean lipoxygenase, followed by reduction ($NaBH_4$) and hydrogenation (6).

General Methods

Acid value and iodine value were determined with the micromethods of Gorbach (7). The colorimetric method of Vioque and Vioque (8) with N,N′-dimethyl-*p*-phenylendiamine was used for peroxide value determination. Standard methods for water, ash and fiber and the micromethod of Clark for nitrogen (9) were used. The total protein was calculated as total nitrogen × 6.25.

Free lipids refer to those extracted with hexane under continuous stirring for 6 h. Associated lipids were obtained, following the method of Nash et al. (10) by extraction with 86% ethanol at room temperature for 37 h and removing the nonlipid material according to Singh and Privett (11).

For the isoelectric point determination, 15 g of meal were extracted twice with 300 mL 0.2% NaOH solution. Forty-mL aliquots of the extract were titrated with 0.5 N HCl to various pH values ranging from 2.0 to 7.0. The curd formed was separated by centrifugation for 30 min at 4,000 rpm. The supernatant was decanted and its volume and nitrogen content were determined. The percentages of protein precipitated were plotted vs the different pH in order

to determine the isoelectric point.

The chlorogenic acid content was determined according to the method of Poments and Burns (12).

Usual methods were employed for hydrogenation, dehydration and acetylation.

Preparation of isolates. Isolate A: 1 kg of meal was extracted under stirring with 10 L of 0.2% NaOH solution for 1 hr. After centrifugation, two more extractions were carried out with half the volume of alkaline solution. The supernatants were pooled, the volume measured and analyzed for nitrogen. The final residue was air-dried, then oven-dried (50 C) in vacuum, weighed and analyzed for nitrogen. The total extract was taken to the isoelectric point (pH 4.3), and the curd formed was recovered by centrifugation. The curd was washed with distilled water at pH 4.3 using 15 L per batch. The isolate was first air-dried and then dried under vacuum at 50 C. The final color was dark brown.

Isolate B: 1 kg of meal was extracted as above but using for the extraction a 0.25% Na_2SO_3 solution at pH 11.0, to avoid darkening of the final product. The curd obtained after precipitation was successively washed with water at pH 4.3, ethanol and acetone, and dried as before. The isolate so obtained was almost white.

Fractionation of the lipids. A preliminary separation into types of compounds was achieved following the method of Singh and Privett (11), using a column (2 × 23 cm) of acid-washed Florisil (80–100 mesh). A chloroform solution of the lipids was poured into the column, and elution was effected with the following solvents to obtain the kind of compounds indicated: (a) chloroform for neutral lipids; (b) chloroform/acetone (1:1) for glycolipids, and (c) methanol for phospholipids. The separation of the different compounds was monitored by thin layer chromatography (TLC) on Silica Gel G. Hydrocarbons and waxes were separated with pentane as developing solvent. The other neutral lipids (i.e., methyl esters, triglycerides, free fatty acids, diglycerides, sterols and hydroxy fatty acids) were separated with hexane/diethyl ether/formic acid (70:30:1). The lipids were all visualized using either iodine vapors or by charring after spraying with 50% sulfuric acid. Glycolipids were separated with chloroform/methanol/7N ammonia (100:15:2) and detected with the abovementioned reagents. Phospholipids were developed with *n*-butanol/acetic acid/water (40:7:32), and detected with the reagent of Vaskovsky et al. (13).

Quantitative determination of the different compounds. For quantitative analyses, a known amount of lipid was applied to preparative TLC plates. After development with the proper solvent, the identified bands were scraped off the plate, extracted in a small column with chloroform, solvent evaporated and the components quantitatively determined. When the ester group was present in the compound to be quantified (waxes, methyl esters, tri- and diglycerides) the colorimetric method of Vioque and Holman (14), with a standard curve obtained with methyl myristate, was used. Free fatty acids were methylated with diazomethane (15) prior to their quantitative evaluation by the same method. Sterols and sterol esters were quantified by the colorimetric method of Huang et al. (16).

The relative proportion of the different fatty acids, hydrocarbons, fatty alcohols, and sterols was obtained by gas liquid chromatography (GLC), after hydrolysis of samples obtained from TLC plates as above. Waxes were hydrolyzed by boiling the sample for 5 min with a 0.2 N NaOMe solution; the methyl esters and free alcohols formed were separated by TLC with hexane/diethyl ether (8:2, v/v), scraped off the plate and analyzed by GLC. Other esters (tri- and diglycerides) were hydrolyzed in two steps: first the sample was boiled with a few mL of methanolic 0.2 N NaOMe, and then the resulting solution was neutralized (phenolphthalein) with a methanol/4% HCl mixture and boiled for 5 min. The methyl esters so obtained were analyzed by GLC.

As the hydroxamic method (14) for the ester group determination gives the number of moles in the sample, a mean molecular weight of each type of compound has to be estimated from the fatty acid composition obtained by GLC in order to calculate the weight percentages of the different compounds in the original sample.

Structural analysis of triglycerides. The structures of these compounds were established by the pancreatic lipase method (17). This requires the determination of the total fatty acid composition and the fatty acid distribution in the β-position of the glyceride. With these data, all the possible types of triglycerides were calculated by the Vander Wal formulae (18).

Gas chromatographic methods. Fatty acid methyl esters were prepared by the abovementioned method (15) and analyzed in a Perkin-Elmer F-17 apparatus with a column of BDS, 8% on Supercoport, 80–100 mesh, 2 m × 2.5 mm; oven temperature 180 C; detector and injector, 250 C. Hydrocarbons, alcohols and sterols, in a Carlo Erba Chromatograph, Model Fracto Vap GI, with a column of OV-17, 2.5% on Supercoport, 80–100 mesh, 2 m × 2.5 mm; oven temperature, 270 C; detector and injector, 300 C.

Ultraviolet, infrared and mass spectrometric methods. Some characteristics of the hydroxy fatty acids were studied with these physical methods. A spectrophotometer Beckman Model DK 2A was used for the ultraviolet studies. Infrared spectra were taken in carbon disulfide solution in an IR 20A Beckman apparatus and the mass spectra in a AEI Model MS 30 Spectrometer, EI fountain, introduction probe, source, 150 C, 70 eV.

RESULTS AND DISCUSSION

The recovery of nitrogen in the preparation of two sunflower protein isolates is shown in Table I. The total recovery of nitrogen reaches nearly 100% in both cases, about a fourth of the nitrogen remaining in the residue.

The chemical characteristics of the original meal and the protein isolates are shown in Table II. Most of the fiber and chlorogenic acid are eliminated during the isolation process, which yields products with almost 90% protein. The content of the free and associated lipids in the three samples is shown in Table III, together with some chemical characteristics. The percentages of free lipids are of the same order in the three samples, while all the other values are much lower in the isolate B when compared with those of isolate A. The peroxide values indicate decomposition of the peroxides present in the original meal during the different isolation steps.

Weight distribution of neutral lipids isolated by TLC is shown in Table IV. The free fatty content in isolate B is lower than in isolate A but has a higher proportion of sterols. The other types of compounds are found in more or less the same proportions.

Figure 1 is a thin layer chromatogram of the total associated lipids of the samples studied together with some standards. With the solvent system used, the hydrocarbons and waxes ran with the solvent front and the glycolipids

TABLE I

Distribution of Nitrogen in the Preparation of Protein Isolates from Sunflower Meal in the Different Extraction Steps

	Recovered nitrogen (%)		Nitrogen in supernatant (%)[a]	
	Isolate		Isolate	
Extraction no.	A	B	A	B
1	45.9	45.0	7.1	8.7
2	15.7	19.9	2.9	2.2
3	9.5	5.4	1.5	0.8
Washing water	2.9	2.0		
Residue	25.3	23.5		

[a]Nitrogen in the liquor after isoelectric precipitation.

TABLE II

General Characteristics of Sunflower Meal and Protein Isolates A and B[a]

Sample	Moisture	Ash	Fiber	Lipids (ether)	Protein	Chlorogenic acid
Meal	8.6	6.1	14.4	0.3	36.7	1.9
Isolate A	6.8	0.9	1.3	1.4	87.1	0.3
Isolate B	4.6	0.8	1.2	0.3	89.0	0.1

[a]Percentage, moisture-free basis.

TABLE III

Content of Free and Associated Lipids and the Chemical Characteristics of the Associated Lipids of the Meal and Isolate Proteins

	Lipid[a]		Chemical characteristics of associated lipids		
Sample	Free	Associated	Acid value	Iodine value	Peroxide value[b]
Meal	0.04	1.04	13.5	79.0	30.1
Isolate A	0.04	0.36	57.1	97.1	8.5
Isolate B	0.02	0.04	10.5	57.6	4.8

[a]Percentage, moisture-free basis.
[b]meq/kg.

TABLE IV

Weight Distribution of Neutral Lipids of Meal and Protein Isolates (%)

Sample	Wax	Me esters	Triglycerides	FFA	Sterols	Di-glycerides 1,3-	Di-glycerides 1,2-	Hydroxy FA
Meal	1.1	1.6	1.1	19.6	2.7	2.7	3.2	0.7
Isolate A	3.5	2.1	6.4	42.2	1.7	5.3	5.7	1.8
Isolate B	3.8	3.6	8.8	15.2	3.8	5.0	4.6	1.0

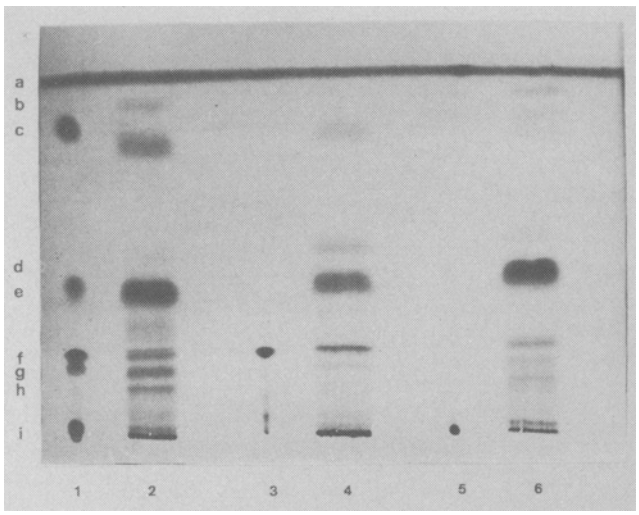

FIG. 1. Thin layer chromatographic separation of neutral lipids of meal and protein isolates on Silica Gel G. (1) Standard mixture, from top to bottom: triglyceride, free fatty acid, 1,3- and 1,2-diglycerides, monoglyceride; (2) associated lipids of isolate A; (3) cholesterol; (4) lipids of original meal; (5) esterified sterol glycoside; (6) associated lipids of isolate B. Letters on the figure refer to the identified compounds: (a) hydrocarbons and waxes; (b) methyl esters; (c) triglycerides; (d) unknown; (e) free fatty acids; (f) sterols and 1,3-diglycerides; (g) 1,2-diglycerides; (h) hydroxy fatty acids; (i) glycolipids and phospholipids. Eluent: hexane/diethyl ether/formic acid (70:30:1). Detection: charring after spraying with 50% sulfuric acid.

and phospholipids remained at the origin.

The gas chromatographic analysis of the hydrocarbons of olive oil, used as standards, showed the presence of saturated hydrocarbons from C29 to C35, with C29, C31 and C34 as the main constituents, as reported by others (5). In the lipids of meal, isolate A and isolate B, saturated hydrocarbons from C25 to C40 were identified.

The fatty acid and alcohol components of the waxes are shown in Tables V and VI.

Methyl esters as such were present in all samples and their fatty acid composition is very similar to that of the free fatty acid (Table VI).

The main constituent of the neutral lipids is free fatty acids (Table IV). The free fatty acid composition of the original meal is quite different from those of isolates A and B (Table VI).

In the original sample, as well as in the isolate A, the main triglyceride types were monosaturated-diunsaturated (35.7% and 37.9%, respectively) and triunsaturated (56.7% and 52.7%, respectively), while in the case of isolate B, the trisaturated (27.2%), monounsaturated-disaturated (45.2%) and monosaturated-diunsaturated (23.6%) types of glycerides formed nearly the whole of these components, with only a small percentage (4%) of triunsaturated triglycerides.

The main component of the free sterols was β-sitosterol: 65.6%, 72.9% and 58.6%, in the original sample, isolate A and isolate B, respectively. Other sterols present were: campesterol, stigmasterol (the second main component), cholesterol and δ-7-stigmasterol.

TABLE V

Constituent Alcohols of Waxes from Lipids of Meal and Protein Isolates (%)

Sample	Chain length						
	14	16	18	20	22	24	26
Meal	10.4	5.6	9.4	28.2	19.9	21.5	4.9
Isolate A	–	5.2	12.3	20.9	56.1	1.5	4.1
Isolate B	1.1	4.3	7.4	16.5	45.9	24.7	–

TABLE VI

Constituent Fatty Acids of Waxes, Methyl Esters and Free Fatty Acids (%)

Sample	Chain length					
	14:0	16:0	16:1	18:0	18:1	18:2
			Waxes			
Meal	3.4	23.6	13.2	12.0	26.1	11.4
Isolate A	3.1	22.7	2.9	10.4	39.8	19.8
Isolate B	5.8	54.2	2.5	25.2	9.9	0.3
			Methyl esters			
Meal	4.2	16.7	–	4.8	15.9	58.4
Isolate A	0.2	14.2	–	3.8	12.7	65.1
Isolate B	1.4	37.8	–	4.1	16.7	39.9
			Free fatty acids[a]			
Meal	1.0	13.4	–	6.9	18.9	58.3
Isolate A	0.7	9.3	–	4.4	24.4	57.3
Isolate B	3.3	56.4	–	15.8	20.3	1.0

[a]The original meal and isolate A contain 1.4% and 1.6% of linolenic acid, respectively.

TABLE VIII

Constituent Fatty Acids of the 1,3- and 1,2-Diglycerides

Sample	Type	Chain length 14:0	16:0	18:0	18:1	18:2
Meal	1,3-	1.0	19.1	5.5	25.7	47.3
	1,2-	2.8	26.5	7.4	18.3	41.2
Isolate A	1,3-	13.1	11.2	6.2	17.1	51.4
	1,2-	4.1	42.6	15.9	12.9	8.8
Isolate B	1,3-	7.7	66.1	13.4	0.6	–
	1,2-	5.2	43.9	10.5	27.8	4.0

Both isomers, 1,3- and 1,2-diglycerides were present in about the same proportion, as could be anticipated as they probably result from the hydrolysis of the triglycerides. The relatively high proportion of free fatty acids (15–40%) is not in accordance with that of diglycerides (less than 6%), with no monoglyceride detected in any sample. The two isomers were easily separated on Silica Gel G plates, and after their transesterification were analyzed by GLC. Table VII gives the results obtained for the fatty acid composition of both types of diglycerides in the samples.

Although hydroxy fatty acids represent a small proportion in all samples, their presence is quite interesting as they may affect the chemical properties of the proteins. Probably they originate from either epoxy compounds or hydroperoxides formed primarily in the autoxidation of the lipids.

The presence of hydroxy fatty acids was confirmed by comparison of the acetylated samples with standards on TLC, and by infrared, ultraviolet and mass spectrometry.

The infrared spectra of the hydroxy fatty acids from the meal and isolate B showed, in addition to a peak at 2.76 μ (nonassociated hydroxyl group) a broad band at ca. 2.9 μ due to associated or hydrogen-bonded hydroxyl group. The latter absorption suggests that the hydroxyl group might be adjacent to double bonds: $-CH(OH)-CH=CH-$. The ultraviolet spectra of the hydroxy fatty acids showed absorption of conjugated diene at 234 nm and this absorption disappeared after dehydration of the acids by boiling with acetic acid. At the same time, a triene absorption (three peaks about 270 nm) appeared thus confirming the presence of a hydroxyl group adjacent to a conjugated double bond: $-CH(OH)-CH=CH-CH=CH-$.

In order to locate the position of the hydroxyl group in the chain, a portion of sample was hydrogenated. The mass spectra of the hydrogenated methyl esters of the hydroxy fatty acids from the original meal and isolate B were similar to the spectra of a mixture of 9- and 13-hydroxyoctadecanoates, with fragments: m/e, 55, 74, 87, 155, 158, 187, 211, 214 and 243.

It is surprising that the hydroxy acid from isolate A is quite different from those of original meal and isolate B. On TLC, the R_f of the hydroxy acid showed a value between those of 9- and 13-hydroxyoctadecanoates. Its ultraviolet spectrum showed no maximum of absorption and the R_f on TLC-$AgNO_3$ plates of the original and the hydrogenated sample were the same. It seems then to be a saturated hydroxy fatty acid. The mass spectrum with fragments at m/e 55, 74, 87, 169, 172 and 201, showed that the hydroxyl group is on carbon atom number ten of the chain.

The chain length of the hydroxy acids from the original meal and isolate B was confirmed by hydrogenation of the dehydrated samples. They gave methyl octadecanoates when analyzed by GLC.

The hydroxy acids of the protein lipids may have originated from hydroperoxides formed by autoxidation of linolenic acid present in all the associated lipids of the proteins.

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REFERENCES

1. Baudet, J., and J. Mosse, JAOCS 54:82A–86A (1977).
2. Smiley, W.G., and A.K. Smith, Cereal Chem. 23:288 (1946).
3. Eldridge, A.C., P.K. Hall and W.J. Wolf, Food Technol. 17: 120 (1963).
4. Eldridge, A.C., W.J. Wolf, A.M. Nash and A.K. Smith, J. Agric. Food Chem. 11:323 (1963).
5. Vazquez, A., Grasas Aceites 6:262 (1963).
6. Vioque, E., and M.P. Maza, Ibid. 22:22 (1971).
7. Gorbach, G., Fette Seifen 47:499 (1940).
8. Vioque, E., and A. Vioque, Grasas Aceites 13:203 (1962).
9. Clark, E.P., in Semimicro Quantitative Organic Analysis, Academic Press, New York, 1943, p. 37.
10. Nash, A.M., A.C. Eldridge and W.J. Wolf, J. Agric. Food Chem. 15:102 (1967).
11. Singh, H., and O.S. Privett, Lipids 5:692 (1970).
12. Pomenta, J.V., and E.E. Burns, J. Food Sci. 36:490 (1971).
13. Vaskovsky, V.E., E.Y. Kostetsky and I.M. Vasendin, J. Chromatogr. 114:129 (1975).
14. Vioque, E., and R.T. Holman, JAOCS 39:63 (1962).
15. Schlenk, H.K., and J.L. Gellerman, Anal. Chem. 32:1412 (1960).
16. Huang, T.C., C.P. Chan, W. Mefler and A. Raftery, Ibid. 33: 1405 (1961).
17. Savary, P., and P. Desnuelle, Biochim. Biophys. Acta 21:349 (1956).
18. Vander Wal, R.Y., JAOCS 37:18 (1960).

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