SEED LIPID CONSTITUENTS OF THREE SPECIES OF *PROBOSCIDEA*

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ABSTRACT.—The fatty acids, sterols, and tocopherols of the saponified seed lipids from three species of *Proboscidea* (Martyniaceae) have been determined. Linoleic acid was found to be the major fatty acid (ca 60%), along with oleic (ca 30%), palmitic (ca 6%) and stearic (ca 3%) acids as minor constituents. Traces of palmitoleic, linolenic, myristic, heptadecanoic, arachidic and behenic acids were detected and estimated. Beta sitosterol was the major sterol (ca 78%) along with five other common plant sterols. Among the tocopherols, γ-tocopherol was the major constituent (ca 60%), α- and δ-tocopherols being the other constituents. The fatty acid, sterol, and tocopherol compositions resemble closely that of soy bean oil.

Since the introduction of gas chromatography, much data on the fatty acid composition of various lipids has been published (1). During the past few years there has been much interest in the nature of the nonsaponifiable portions in these lipids with particular attention devoted to sterol and tocopherol composition (2–6). Since the discovery of vitamin E, most of literature has given data for total tocopherol content rather than for the individual forms. Only recently have the natural tocopherols been fully identified and methods developed for their determination (4–6).

The three species of *Proboscidea* (Martyniaceae) under investigation, *P. louisianica*, *P. althaefolia*, and *P. fragrans* are tropical plants commonly known as unicorn or devil's claw which grow wild in various parts of the United States (7). A preliminary study on the fatty acid composition of a seed sample of *P. louisianica* obtained from Kansas revealed the presence of linoleic acid in high proportions. The fatty acid profile was found to be similar to that of soy bean oil. These findings stimulated us to undertake the present investigation.

MATERIALS AND METHODS

SAMPLES.—The seeds of *Proboscidea fragrans* (Lindl.) Decne., were collected in 1976 at Alpine, Texas, and the seeds of *P. althaefolia* (Benth.) Decne. were collected at El Paso, Texas, in 1976. These seeds were collected by Dr. Barton H. Warnock, Chairman, Biology Department, Sue Ross State University, Alpine, Texas. Two seed samples of *P. louisianica* (Mill.) Thell. were collected by John Titus at Harper, Kansas, in 1975 and Anthony, Kansas, in 1976. A third seed sample of *P. louisianica* was collected by Steve Stephens at Stockton, Kansas in 1976. Botanical authentication of *P. louisianica* species were made by Dr. Ronald L. McGregor, Director, State Biological Survey of Kansas, The University of Kansas. Herbarium specimens of plants grown from the five seed samples are on file in the Herbarium of the Division of Pharmacognosy and Natural Products Chemistry, The Ohio State University.

EXTRACTION OF LIPIDS.—Seeds were ground and extracted successively in a Soxhlet with petroleum ether (30°–60°) and diethyl ether. The marc was then macerated at 40° for 30 minutes with constant shaking on a water bath with a mixture of chloroform and methanol (2:1), enough to cover the material, and then filtered. The three extracts were combined and solvents were removed in a rotary evaporator. The last trace of solvent was removed under a slow stream of nitrogen gas. The lipids were weighed and then stored in a freezer.

DETERMINATION OF IODINE VALUES.—The iodine values of the oil samples were determined by the Hanus method following the procedure of IUPAC (8).

SAPONIFICATION, EXTRACTION OF NONSAPONIFIABLES AND FATTY ACIDS.—The respective oils (10 parts by weight) were saponified (9) by refluxing with potassium hydroxide (3 parts by weight) in methanol (50 parts by volume), for three hours in an atmosphere of nitrogen gas. Following saponification, the methanol was removed by distillation, and the soaps were dissolved in water and extracted four times with diethyl ether. The combined ethereal extract

was washed four times with water and dried over anhydrous sodium sulfate. The ether was removed by evaporation and weighed to yield the nonsaponifiables. The aqueous layer left after the extraction of nonsaponifiables, was acidified with 4 N sulfuric acid in an atmosphere of carbon dioxide gas to prevent the oxidation of unsaturated fatty acids. The liberated fatty acids were extracted four times, using diethyl ether and petroleum ether (30°–60°), alternately. The combined extracts were dried over anhydrous sodium sulfate, and the solvent was then removed by evaporation.

METHYLATION OF FATTY ACIDS.—Aliquots of fatty acids (30 mg) were methylated using freshly prepared diazomethane (10). The fatty acid mixture was dissolved in diethyl ether-methanol (2 ml; 9:1) and treated with diazomethane, generated from N-methyl-N-nitroso-p-toluenesulfonamide, until a yellow color persisted. Excess of diazomethane was removed in a stream of nitrogen.

HYDROGENATION OF FATTY ACID METHYL ESTERS.—The fatty acid methyl esters (1–2 mg) from each of the samples were dissolved in methanol and hydrogenated in the presence of platinum oxide (1 mg) catalyst. Hydrogenation was carried out at room temperature and at atmospheric pressure for two hours, during which the mixture was agitated vigorously (11).

UV AND IR SPECTROPHOTOMETRY OF FATTY ACID METHYL ESTERS.[1]—The uv spectra of the fatty acid methyl esters were determined in ethanol to determine any conjugated unsaturation (12). The ir spectra of the methyl ester samples were determined in carbon tetrachloride solution to detect the presence of any unusual functional group or any trans- unsaturation (13).

SEPARATION OF STEROLS AND TOCOPHEROLS.—The sterols and tocopherols in the non-saponifiables were separated by preparative tlc (6). The plates (20 x 20 cm) were coated with 0.5 mm layers of silica gel G (E. Merck) containing 2'-7' dichlorofluorescein. The unsaponifiables were dissolved in chloroform and were applied (35 mg per plate) uniformly along a line 1.5 cm from one edge. A one inch panel along one side was spotted with a mixture of β-sitosterol and α- and δ- tocopherols. The α- and δ- tocopherols, respectively, define the maximum and minimum tocopherol mobility. Plates were developed by a solvent system consisting of petroleum ether (60°–80°)-ethyl ether-acetic acid (50:50:1). The solvent front was allowed to advance 17 cm before the plates were dried and viewed under uv light. Sterols appeared as a single white stripe (R_f=0.30–0.37), while tocopherols appeared as dark purple overlapping stripes (R_f=0.56–0.72). The sterol and tocopherol bands, defined by the reference spots, were scraped from the plate and transferred to 1.2 x 15 cm percolation tubes and eluted repeatedly with diethyl ether into glass stoppered Erlenmeyers. The solvents were removed under a stream of nitrogen on a water bath at 45°. After the sides of the flasks were rinsed with ether and the ether was evaporated, the flasks were dried to constant weight.

DERIVATIZATION OF STEROLS AND TOCOPHEROLS.—Trimethyl silyl (TMS) ether derivatives of the sterol and tocopherol fractions were prepared by adding 0.1 ml of a mixture of hexamethyldisilazane-trimethylchlorosilane-anhydrous pyridine (9:6:10) to each mg of sample in screw cap septum vials (14), shaking vigorously for 30 seconds, and allowing to stand for 30 minutes at room temperature. Authentic sterol and tocopherol samples were also silylated in the same way.

GAS CHROMATOGRAPHY OF FATTY ACID METHYL ESTERS.[2]—Glass U-tube columns (6 ft x 3 mm id) packed with 10% EGSS-X liquid phase coated on 80–100 mesh Gas Chrom-Q and 3% SE–30 liquid phase, coated on the same support, were used for gas chromatographic separation of the fatty acid methyl esters. The methyl ester samples and their respective hydrogenated products were determined under identical conditions of operation. Authentic methyl esters were also determined simultaneously. The SE–30 column was used only for analyzing the reduced fatty acid methyl esters along with appropriate authentic saturated fatty acid methyl esters. Identification was done by comparison of the retention times of the components with those of standards and by semilogarithmic plots of relative retention times (methyl oleate=1.00) against carbon chain length. The percent composition of the components was determined by the triangulation and peak area normalization technique.

GAS CHROMATOGRAPHY OF STEROL-TMS DERIVATIVES.—Gas chromatography of the TMS derivatives of sterols was performed with a 3% OV–17 column on 80–100 mesh Gas Chrom Q at 255°C, other conditions being the same as discussed previously. TMS derivatives of au-

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[1]The uv and ir spectra were recorded in Beckman UV5260 and Beckman IR4230 spectrophotometers, respectively.

[2]The instrument used was a dual column gas chromatograph by Hewlett and Packard, model 402, equipped with dual flame ionization detector. Carrier gas was helium with a flow rate of 45 ml per minute at 40 psig. Hydrogen and air flow rates were 25 ml and 150 ml per minute, respectively. Oven temperatures for EGSS-X and SE-30 columns were 170° and 200°C, respectively. The liquid phases, solid support, and authentic fatty acid methyl esters were obtained from Applied Science Laboratories, Inc., State College, PA, U.S.A.

thentic sterols[3] were also chromatographed under identical conditions. Brassicasterol and Δ^5-avenasterol were identified by determination of their retention indices as described by Knights (15), using authentic normal hydrocarbons. The retention indices thus determined were compared with published data (2), and the component sterols were identified and quantified, as described previously.

Gas chromatography of tocopherol-TMS derivatives.[4]—Tocopherol-TMS derivatives of the samples and standards were gas chromatographed on the 3% OV-17 column at 250°C. Other conditions were the same as stated earlier. Identification of peaks was made by comparison of the retention times of the component peaks from the samples with those of the standards, and quantitations were made by the triangulation technique.

RESULTS AND DISCUSSION

The oil content, iodine value, and nonsaponifiable content of the samples studied are presented in table 1. The oil of these three species are identical in respect to these values. The uv spectrum of the mixed methyl esters did not show any absorption for conjugated unsaturation. The ir spectrum of the samples did not show any absorption for unusual functional groups.

Table 1. *Oil content, iodine value and nonsaponifiable contents of* Proboscidea *seed oils.*

Species (Sample Source)	Year of collection	Oil content (% Seed)	Iodine value (Hanus)	Nonsaponifiable content (% Oil)
1. *P. louisianica*.......... (Harper, Kansas)	1975	40.4	119.0	2.2
2. *P. louisianica*.......... (Anthony, Kansas)	1976	42.3	122.0	1.9
3. *P. louisianica*.......... (Stockton, Kansas)	1976	43.5	121.5	2.0
4. *P. althaefolia*.......... (El Paso, Texas)	1976	39.8	123.5	1.8
5. *P. fragrans*............ (Alpine, Texas)	1976	39.0	117.0	2.4

The major product of hydrogenation was stearic acid (over 90%) along with palmitic acid (ca 6%) and traces of other saturated acids. These saturated acids had identical retention times with authentic acids, both on polar (EGSS–X) and non-polar (SE–30) liquid phases. The percentages of stearic acid in the hydrogenated samples were equal to the sum of the percentages of stearic, oleic, linoleic, and linolenic acids in the respective nonhydrogenated samples. The fatty acid compositions of the oils are presented in table 2. Linoleic acid (18:2) was found to be the major component in all of the samples and comprised about 60% of the total fatty acids. Among other constituent acids, oleic acid (18:1) constituted about 30% of the total fatty acids. Palmitic acid (16:0) was the major saturated acid, being about 5 to 6%. Traces of other constituent acids were also recorded, behenic acid (22:0) being the acid with the longest chain length. Palmitoleic acid (16:1) was the only unsaturated acid found with a chain length other than 18. Many seed oils are known to contain unsaturated monoenoic acids in traces, with a chain length of 14, 20 or 22, always with palmitoleic acid. Such acids could not be found in the present study. The only fatty acid with an odd number of carbon atoms recorded was heptadecanoic acid (17:0), although many seed oils

[3]The standard sterols were obtained from Applied Science Lab., Inc.

[4]The standard tocopherols were obtained from Eastman Kodak Company, Rochester, N.Y., U.S.A.

are known to contain acids with 13, 15 or 19 carbon chain lengths in trace amounts with the 17 carbon acid.

The last two columns of table 2 show the total saturated and unsaturated fatty acids. The total unsaturated fatty acid content is about 90% in all of the samples. There is one report (16) on the fatty acid composition of *P. althaefolia* which was determined by techniques other than gas chromatography. The values for oleic and linoleic acids reported in that study were 55% and 36%, respectively, which are almost the reverse of the values reported in the present study.

TABLE 2. *Fatty acid composition of* Proboscidea *seed oils.*

	Component fatty acids[a] (% w/w)										Total saturates (%)	Total unsaturates (%)
	14:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:0	22:0		
1. *P. louisianica*......	Tr[b]	6.6	0.1	Tr	2.3	30.7	59.0	0.3	0.5	0.5	9.9	90.1
2. *P. louisianica*......	0.1	5.0	0.1	0.1	2.2	30.5	60.6	0.5	0.2	0.7	8.3	91.7
3. *P. louisianica*......	Tr	5.6	Tr	Tr	2.2	30.7	60.5	0.2	0.2	0.6	8.6	91.4
4. *P. althaefolia*......	0.1	4.5	Tr	0.1	2.8	27.9	63.7	0.4	0.2	0.3	8.0	92.0
5. *P. fragrans*........	Tr	6.7	Tr	Tr	3.0	32.5	56.8	0.5	0.1	0.4	10.2	89.8

[a]The figures represent, carbon chain length: number of double bonds.
[b]Trace amounts, components below 0.05%.

The sterol composition of the oils is given in table 3. Beta-sitosterol was found to be the major component in all of the samples. Campesterol comprised 12 to 18%, other sterols being present in minor quantities. It is known that cholesterol occurs in sterol fractions of many vegetable oils, generally in extremely minor proportions; such was also observed in the present study.

TABLE 3. *Sterol composition of* Proboscidea *seed oils (% w/w).*

	Proboscidea seed oil samples[a]				
	1	2	3	4	5
Cholesterol...............	0.7	0.4	0.6	0.5	0.8
Brassicasterol............	3.0	1.4	2.4	1.8	2.5
Campesterol..............	12.5	14.4	18.1	16.7	17.8
Stigmasterol..............	0.2	0.2	0.1	0.1	0.1
Sitosterol................	81.1	82.1	76.8	79.0	76.6
Δ^5-Avenasterol............	2.5	1.5	2.0	1.9	2.2
Total (mg/g oil)..........	2.52	2.61	2.65	2.85	2.70

[a]See Table 1 for sample identifications.

The results for the tocopherol content and composition of the oils are summarized in table 4. In all the samples γ-tocopherol was the major component and ranged between 50 and 65%. Other tocopherols found α- and δ-tocopherols, being about 15% and 30%, respectively. The eight naturally occurring forms of tocopherols are: alpha-, beta-, gamma- and delta-tocopherols and alpha-, beta-, gamma- and delta-tocotrienols. All natural tocopherols are derived from plant sources and are concentrated in the seeds. None of the tocotrienols were detected

in the present study, and β-tocopherol is absent in the samples studied. Usually vegetable oils have simpler tocopherol patterns and contain principally the saturated forms, viz., alpha-, gamma- and delta-tocopherols (4).

Although the analytical procedures used in the present study did not distinguish the free and esterified forms, it is commonly assumed that tocopherols occur mainly as the free alcohols. Chow *et al.* (17) have determined both free and esterified tocopherols in a number of food oils and found only the free alcohols in oils from barley, corn, oats, soybean and wheat bran, indicating the absence of any significant amount of tocopherol esters.

TABLE 4. *Tocopherol composition of* Proboscidea *seed oils.*

Species	Total tocopherol (mg/g oil)	Component tocopherol (% w/w)		
		α-tocopherol	γ-tocopherol	δ-tocopherol
1. *P. louisianica*	1.02	15.2	50.4	34.4
2. *P. louisianica*	1.15	16.4	57.1	26.5
3. *P. louisianica*	1.21	14.5	61.2	24.3
4. *P. althaefolia*	1.13	15.0	59.5	25.5
5. *P. fragrans*	1.34	17.1	64.7	18.2

The oil content of the seeds studied totals about 40%, which is a significant amount. The fatty acid make up is similar to that of soy bean oil (6, 18) except for a few differences. For example, the linolenic acid content of soy bean oil is about 6 to 8%, whereas the *Proboscidea* seed oils contain this acid in traces (0.2–0.5%). The major component acids, viz., linoleic and oleic acids, are almost the same as in soy bean oil. The contents of unsaponifiables in the samples studied ranged between 1.8 and 2.4%, which is very close to that of soy bean oil (6). The total sterol content found was about 2.65 mg/g of oil; this is less than that in soy bean oil, which contains about 3.65 mg/g of oil. The total tocopherol contents are very close to that in soy bean oil. Regarding the sterol composition, soy bean oil contains about 55% of β-sitosterol, whereas that found in the present study is about 80%; this is a significant difference. Moreover, the stigmasterol content in the present study is between 0.1 and 0.2%, whereas that in soy bean oil is about 24%; this is also a notable difference. The tocopherol compositions of *Proboscidea* seed oils are very similar to that of soy bean oil (6).

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