The Fatty Acids of Degras[1]

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Abstract

Degras contains a complex mixture of lipids comprised of branched and normal chain fatty acids, hydroxy acids, sterols, sterol esters and long chain wax esters. There are no glycerides in degras. This paper is a report on the composition of the branched and normal chain fatty acids. Preparative techniques of thin-layer chromatography were used to isolate the fatty acids from the other lipid classes. Gas chromatography was used on three different stationary phase separations of the fatty acid methyl esters. Identifications of the composition were based on a combination of techniques and known standards.

Introduction

DEGRAS, A FATTY PRODUCT OF REFINED WOOL, contains no glycerol and is considered to be a wax rather than a fat. Chemically, it is chiefly a mixture of long chain esters of fatty acids esterified to alcohols and sterols (1). The fatty acids are distributed among four homologous series as Weitkamp first showed (2): a) normal chain; b) iso branched chain; c) anteiso branched chain; and d) α-hydroxy. Subsequent studies in wool wax have revealed additional fatty chain structures. Branched chain alcohols (3), diols (4,5), naturally occurring hydrocarbons, and ω-hydroxy fatty acids were also found to occur in wool wax (6–8).

In the present study the total fatty acids of degras have been determined semiquantitatively by a combination of modern chromatographic techniques: thin-layer chromatography (TLC) and gas-liquid chromatography (GLC). Infrared spectrophotometry and mass spectrometry analysis was used to determine the structure of various iso and anteiso fatty acid methyl esters.

The fatty acids of degras were isolated as a group and converted to their methyl esters. GLC of reference and unknown branched chain fatty acid compounds were determined under a variety of chromatographic conditions. These data permitted the identification of over 90 fatty acid compounds in degras, ranging in chain length 8 to 31 carbons and in unsaturation from 0 to 2 double bonds.

The amounts of branched chain fatty acids relative to other lipids and the types of fatty acids belonging to those lipids have not been fully established. TLC of intact wool lipid and GLC of fatty acid esters permit a more thorough and precise characterization of these lipid classes than is possible with traditional methods.

Materials and Methods

The specimen of degras used in this study was a sample purchased from R.I.T.A. Chemical Corporation, Chicago, Ill., under the brand name "Barre Common Degras." The exact source of the material is unknown. The method of preparation was by a chemical process requiring pH adjustments of the detergent before treating with sulfuric acid by which the wool grease and sludge are settled out and later separated by filtration. This wool grease is commonly known as "acid-cracked degras."

In this work, TLC is used for separating neutral lipid classes (9). The equipment used for making the plates was an adjustable plastic spreader, from Applied Science Laboratories, Inc., State College, Pa. The adsorbents used, Adsorbosil-1 and Adsorbosil-ADN-1, 25% silver nitrate-impregnated silica gel, were also products of Applied Science Laboratories.

The stationary phases used for GLC analysis are shown in Table I. Column temperatures were usually 185C; the temperature of the flash heater was about 300C and the detector about 240C. Helium or argon was used as carrier gas at flows of 75 ml/min, inlet pressures 30 psi. The individual peaks were calculated by triangulation. All quantitative work was performed by comparison of area responses to those of knowns and by equivalent carbon numbers (10) on polar and nonpolar stationary phases.

Preparative TLC was used to fractionate the methyl esters of the normal and branched chain fatty acids from other lipid classes of degras, mostly esters of hydroxy acids. Approximately 120 mg of methyl esters were isolated. The sample was subjected to three different GLC analyses using three stationary phases of different polarity for the purpose of determining the complexity of the sample being analyzed.

A 25% degras solution in benzene was analyzed by TLC using several solvent systems (9) that separate neutral lipids. The thin-layer chromatogram consisted of seven spots ranging from more polar material at the origin such as sterols to relatively nonpolar constituents nearer to the solvent front such as hydrocarbons and sterol esters. Standard lipid samples were used to establish the positions on TLC of the various classes that were reported to be present in degras (6), namely, hydrocarbons, a mixture of sterol esters and wax esters, free fatty acids, free sterols, and hydroxy fatty acids uncharacterized material.

Degras (100 g) dissolved in 100 ml of absolute ethanol was rapidly saponified with 25% aqueous sodium hydroxide solution (100 ml) mixed with n-heptane (150 ml). After 15 min, hot water (100 ml) was added. The n-heptane layer contained the bulk

TABLE I
Column Data Used for the GLC Analysis of Degras

Column dimensions	Per cent stationary phase on support	Polar characteristic
9 ft × 1/4 in. I.D., coiled stainless steel	17% Ethylene glycol succinate (EGS) on 80–100 Gas-Chrom P.	Polar
12 ft × 3/16 in. O.D., coiled copper	3% Ethylene glycol methyl silicone succinate (EGSS-X) on 100–120 Gas-Chrom Z.	Slightly nonpolar
6 ft × 1/4 in. I.D., coiled glass	1% Ethylene glycol phenyl silcone succinate (EGSS-X) on 80–100 Gas-Chrom P.	Intermediate polarity
6 ft × 1/4 in., glass U-tube	3% Silicone gum rubber (SE-30) on 80–100 Gas-Chrom P.	Nonpolar

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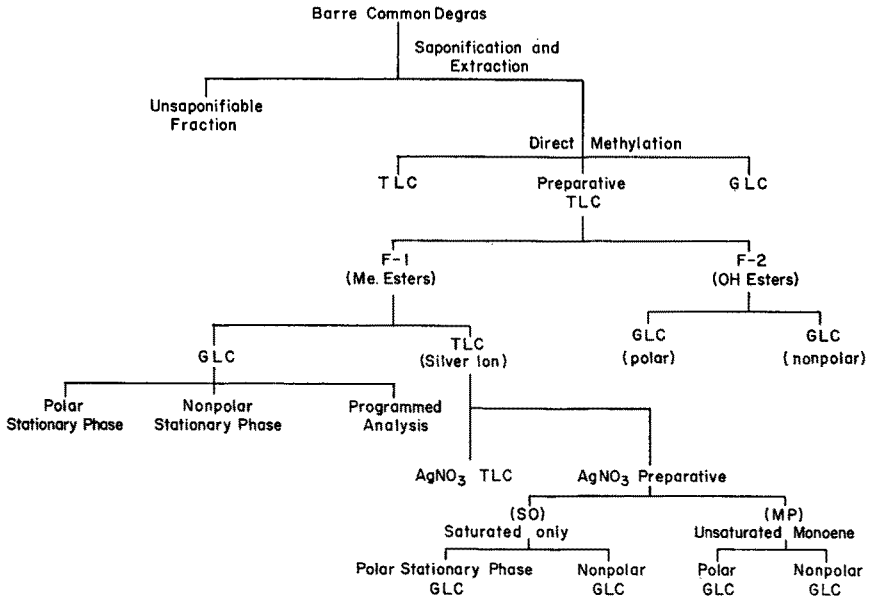

FIG. 1. Process for preparing methyl esters of degras, fractionation and characterization scheme.

of the unsaponifiable materials. This was removed and the aqueous layer extracted three more times with 50 ml portions of n-heptane. The n-heptane layers were combined and stored for future work. The aqueous layer was treated at 55C with an aqueous solution of $CaCl_2 \cdot 6H_2O$ (50 g). Heating the mixture on a steam bath for 4 hr according to the procedure of Downing (6) evaporated most of the ethanol and caused the calcium salts of the fatty acids to become granulated. The calcium salt granules were filtered on a Buchner funnel, dried, powdered and washed twice with 100 ml portions of water, and refiltered each time through a Buchner funnel. The dried powder weighed 42.5 g.

The methyl esters were prepared directly from the extracted calcium salts by refluxing them with methanol (100 ml), benzene (100 ml) and concentrated H_2SO_4 (5 ml) for 3 hr. The mixture was then filtered and the filtrate (consisting of the benzene solution of methyl esters) washed five times with a 25% aqueous ethanol solution (100 ml) until the filtrate was neutral to litmus. The remaining benzene solution was evaporated to give 42.0 g of methyl esters.

TLC of a 10 μl sample of a 10% solution of the methyl ester mixture showed that the saponification and methylation reactions were successful, leaving no unreacted fatty acids.

The methyl esters were analyzed as outlined in Fig. 1. Preparative TLC was employed to separate the esters of monohydroxy acids (Fraction F-2) from the normal and branched chain methyl esters (Fraction F-1). Each group was then subjected to TLC which helped to determine the extent of the fractionation. The spots representing the methyl esters and hydroxy methyl esters were scraped off the plate into a Soxhlet thimble and extracted with redistilled chloroform (11). Yields from F-1 and F-2 were 120 and 145 mg, respectively.

Gas-liquid chromatography of F-1 consisted of three separate analyses using three stationary phases of different polarity. The last analysis was a programmed study at temperatures between 120 and 220C with isothermal conditions prevailing after 220C (Fig. 2). Reference standards were run in all cases. Carbon numbers gave clues as to peak identity. Internal standards were also used in some analyses to improve the accuracy of the carbon number determinations. Comparative GLC data were the primary means used for identifying unknown fatty acids.

A 100 mg sample of F-1 was separated on four TLC plates into saturated, mono- and polyenoic acid methyl esters by the method of de Vries (12). Each plate had 500μ layers of silicic acid impregnated with 25% $AgNO_3$ (Adsorbosil ADN-1). The saturated methyl esters were collected as described above and labeled "saturated" (SO), while the entire small amounts of unsaturated methyl ester fractions were combined and labeled "monoene plus" (MP).

Results

Fifty-seven major components were noted when the fractionated methyl esters of degras lipid were gas-chromatographed at 185C. The identifications of these compounds by GLC proceeded in various steps, and the data obtained by one method were often confirmed by another.

Gas-liquid chromatographs of normal and branched chain fatty acids of degras were compared to known standards and from those prepared by synthesis. Comparison of polar and nonpolar columns at various temperatures permitted the tentative identification of the saturated normal and branch chain fatty acid esters. The final assignment was based on the accumulated evidence.

It can be seen by GLC that an iso branched chain ester is eluted before the corresponding straight chain ester in both polar and nonpolar phases. Anteiso esters could be shown to elute slightly later, i.e., nearer the straight chain ester of the same carbon content of the iso compounds. Unsaturated esters are eluted before the corresponding saturated esters on a nonpolar stationary phase, as is well known.

An interesting note in the gas chromatograms is the way the iso and anteiso acids appear in the sample. The iso compounds almost always show a smaller peak in company with the normal even carbon acids whereas the anteiso compounds show just the opposite in company with the odd carbon acids. From this pattern, one could almost predict the iso and anteiso compounds that appear in the sample. The signifi-

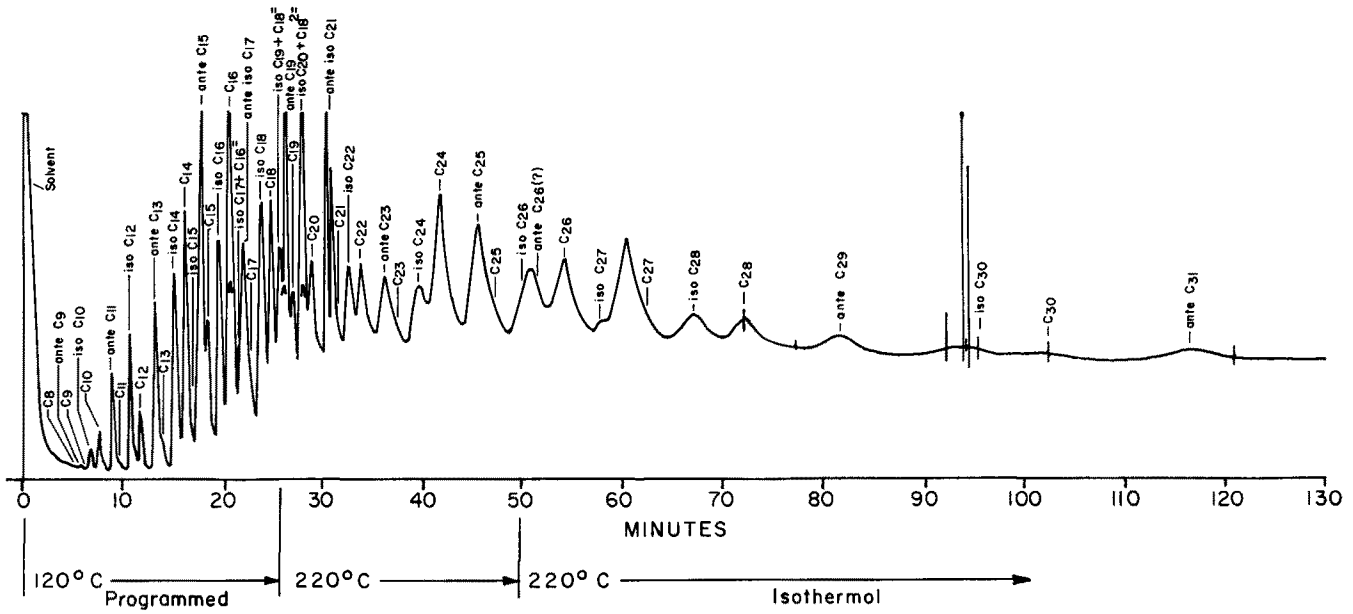

FIG. 2. GLC Analysis of F-1 methyl esters on 3% EGSS-X. Temperature programmed 4C/min between 120C to 220C and isothermal conditions after 220C. Hydrogen flame detector, 12-ft column.

cance of this is not fully understood. Since the iso acids appear to be associated only with the even carbon acids, the detection of odd carbon iso acids in this report can only be tentative until isolation and chemical analysis is possible. The presence of unsaturated C_{14}, C_{16}, and C_{18} monoenes were shown to be present during the fractional distillation and isolation of iso C_{14}, C_{16}, and C_{18}. The unsaturated component was easily removed by crystallization in acetone.

Table II shows the weight percent as determined by comparing the peak areas on chromatograms. The figures for the amounts of odd-numbered iso acids in the C_{15}, C_{17}, C_{19}, and C_{27} fatty acids are tentative because of the similarity in relative retention times to the even carbon unsaturated monoenes, and because of the lack of information concerning these compounds.

The detection of iso acids with an odd number of carbon atoms has been accomplished by GLC of Fraction F-1. Fatty acids of this type were also detected in the unsaturated fraction (MP) and in the hydroxy methyl ester Fraction F-2. The composite picture of the normal and branched chained fatty acids looks something like this: The normal chains are made up of acids ranging from C_8 through C_{31} with hexadecanoic acid as the largest single component (8.2%). The unsaturated fatty acids comprise only a small portion of the sample with octadecadienoic acid as the only component having the least percentage (0.5%) and the largest number of double bonds (two). The iso acids range from C_{10} through C_{30} with 14-methylpentadecanoic acid (iso C_{16}) as the largest component (3.9%) with the next highest being 18-methylnonadecanoic acid (iso C_{20}) and 24-methylhexacosanoic acid (iso C_{26}) each with (3.8%); the anteiso acids are all odd carbon and range from C_9 through C_{31} with 12-methyltetradecanoic acid (anteiso C_{15}), 22-methyltetracosanoic acid (anteiso C_{25}) and 24-methylhexacosanoic acid (anteiso C_{27}) having a percentage of 3.6%, 4.4%, and 4.6%, respectively.

TABLE II
Composition of Normal, Iso- and Anteiso-branched Fatty Acids of Degras

Carbon No.	n-chain	Weight %	Iso-branched	Weight %	Anteiso-branched	Weight %
C8	n-octanoic acid	<0.1	.....	....	.....	....
C9	n-nonanoic acid	<0.1	.....	....	6-methyloctanoic acid	0.1
C10	n-decanoic acid	0.3	8-methylnonanoic acid	0.2	.....	....
C11	n-undecanoic acid	0.2	.....	....	8-methyldecanoic acid	0.7
C12	n-dodecanoic acid	0.6	10-methylundecanoic acid	1.2	.....	....
C13	n-tridecanoic acid	0.4	.....	....	10-methyldodecanoic acid	1.7
C14	n-tetradecanoic acid	2.7	12-methyltridecanoic acid	2.1	.....	....
C15	n-pentadecanoic acid	1.8	13-methyltetradecanoic acid	1.3?	12-methyltetradecanoic acid	3.6
C16	n-hexadecanoic acid	8.2	14-methylpentadecanoic acid	3.9	.....	....
C17	n-heptadecanoic acid	1.0	15-methylhexadecanoic acid	0.8?	14-methylhexadecanoic acid	2.5
C18	n-octadecanoic acid	2.5	16-methylheptadecanoic acid	2.7	.....	....
C19	n-nonadecanoic acid	0.7	17-methyloctadecanoic acid	1.0?	16-methyloctadecanoic acid	2.3
C20	n-eicosanoic acid	1.3	18-methylnonadecanoic acid	3.8	.....	....
C21	n-heneicosanoic acid	0.4	.....	....	18-methyleicosanoic acid	2.9
C22	n-docosanoic acid	1.5	20-methylheneicosanoic acid	1.4	.....	....
C23	n-tricosanoic acid	0.6	.....	....	20-methyldocosanoic acid	2.1
C24	n-tetracosanoic acid	4.4	22-methyltricosanoic acid	1.9	.....	....
C25	n-pentacosanoic acid	1.1	.....	....	22-methyltetracosanoic acid	4.4
C26	n-hexacosanoic acid	4.9	24-methylpentacosanoic acid	3.8	.....	....
C27	n-heptacosanoic acid	2.0	25-methylhexacosanoic acid	1.1?	24-methylhexacosanoic acid	4.6
C28	n-octacosanoic acid	2.5	26-methylheptacosanoic acid	3.1	.....	....
C29	n-nonacosanoic acid	T	.....	....	26-methyloctacosanoic acid	1.7
C30	n-tricontanoic acid	0.5	28-methylnonacosanoic acid	1.2	.....	....
C31	n-hentriacontanoic acid	0.5	.....	....	28-methyltriacontanoic acid	1.5
C_{14}-1	n-tetradecenoic acid	1.3				
C_{16}-1	n-hexadecenoic acid	1.0				
C_{18}-1	n-octadecenoic acid	2.0				
C_{18}-2	n-octadecadienoic acid	0.5				

TABLE III

Percentage Composition of Saturated and Unsaturated Fatty Acids of Fractions "SO" and "MP" by Comparative Gas-Liquid Chromatography

Methyl esters	Saturated						Monounsaturated					
	Normal chain		Iso-branched		Anteiso-branched		Normal chain		Iso-branched		Anteiso-branched	
	EGS	SE-30	EGS	SE-30	EGS	SE-30	EGS	SE-30	EGS	SE-30	EGS	SE-30
C12	0.3	0.5	0.2	0.5	0.4	0.7	1.3	1.0	....	....	....	....
C12	0.8	0.6	0.5	0.8	....	....	2.5	1.7	....	....	....	....
C13	0.4	0.4	....	....	1.1	1.7	trace	0.6	....	....	....	....
C14	2.6	2.7	2.1	1.9	....	....	2.8	1.9	6.1	3.0	....	....
C15	1.2	1.8	....	....	3.5	3.6	trace	1.8	....	....	3.4	2.4
C16	6.8	5.7	2.2	2.7	....	....	11.6	10.1	5.9	3.9	....	....
C17	0.6	1.0	....	....	2.9	2.5	2.0	1.4	2.3?	0.6?	1.3	1.0
C18	2.7	2.5	3.2	3.7	....	....	34.6	28.8	3.6	1.5	....	....
C18-2a	....	....	....	....	....	....	3.9	....	....	....	....	....
C19	0.8	0.7	....	....	5.3	4.7	trace	0.9	2.3?	1.2?	4.0	3.4
C20	2.6	2.3	4.0	4.9	....	....	3.3	2.3	3.8	3.0	....	....
C21	1.1	0.9	....	....	4.7	2.9	1.8	0.5	....	....	....	2.8
C22	3.5	2.8	3.4	2.4	....	....	....	trace	....	2.5	....	....
C23	0.6	0.6	....	....	2.1	2.1	....	1.3	....	....	....	2.8
C24	6.7	5.4	4.7	3.9	....	....	....	0.3	....	2.1	....	....
C25	1.8	1.1	....	....	6.0	5.4	....	trace	....	....	....	3.0
C26	5.9	4.9	7.5	5.0	....	....	....	1.2	....	2.5	....	....
C27	2.1	1.0	....	....	3.9	3.1	....	....	....	....	....	2.1
C28	....	2.5	....	3.1	....	....	....	....	....	1.3	....	....
C29	....	trace	....	....	....	2.7	....	....	....	....	....	2.2
C30	....	0.5	....	1.2	....	....	....	....	....	....	....	....
C31	....	0.5	....	....	....	1.5	....	....	....	....	....	....

[a] Octadecadienoic acid.

The saturated (SO) and unsaturated (MP) samples were analyzed by GLC on EGS and SE-30 stationary phases at 185C and 220C and the results compared in Table III. The SE-30 columns in both cases gave better resolution of the components because of thermal stability of the silicone gum product and the use of a higher temperature in the analysis.

The unsaturated (MP) sample showed the normal chain dominating the chromatograms with some branching. The major constituents were hexadecenoic acid (11.6%) and octadecenoic acid (34.6%). What we suspected to be odd carbon iso branched fatty acids also show up on both polar and nonpolar stationary phases. An equivalent carbon number chart for EGS and SE-30, constructed just for this analysis, was instrumental in determining the branched acids. Carbon number values of unsaturated iso an anteiso fatty acids having the same number of carbon atoms (C_{17} shown in Table IV) are 16.40 and 16.55, respectively, when using EGS at 185C. SE-30 at the same temperature gave values of 16.20 and 16.30, respectively. This was done by taking into account the separation factors of the saturated normal and branched compounds that were run with authentic standards on EGS and the peak reversals that occur with unsaturated components when using SE-30. Isolation and ozonization are needed to elucidate these findings, but we suspect that these rare compounds are present in this sample of degras. Nicolaides et al. (13) isolated unsaturated fatty acids from human hair fat. They identified iso, anteiso, and normal unsaturated fatty acids and showed the position of the double bond to be Δ^6.

The hydroxy esters were analyzed on EGSP-Z and SE-30 stationary phases. The presence of α-hydroxytetradecanoic (4.7%) and a large amount of hydroxyhexadecanoic acids (50.3%) was observed and confirms the work of Weitkamp (2). In addition it establishes the presence of odd carbon number members, unsaturated α-hydroxyoctadecanoic acid and 14 components ranging in chain length of C_{14} to C_{23}. The identifications of the hydroxy ester have also been determined by equivalent carbon numbers.

The structures of five branched fatty acid esters (iso C_{14}, C_{16}, C_{18}, and anteiso C_{15}, C_{17}) were further substantiated by isolation and by GLC, and chemical property studies.

When these samples were examined by infrared spectrophotometry (Perkin-Elmer, model 337) the normal, iso, and anteiso acid methyl esters exhibited identical spectra. Absorbance at 1170 cm^{-1} with a shoulder at 1136 CM^{-1} and a doublet at 1368 and 1382 CM^{-1} were attributed to an isopropyl terminal grouping (14) for iso branched fatty acids. These bands were found in each spectrum. It is likely that normal and branched chain iso and anteiso fatty acids with just a lone methyl branch could be differentiated by infrared analysis.

Mass spectrometry (Nuclide model 12-90-G1.1) was used to examine iso C_{16} and anteiso C_{15}. Methyl esters of iso acids give mass spectra that are also very similar to those given by normal chain esters (15). The mass spectrum of iso C_{16} methyl ester, M = 270 shows a peak at m/e = M-43 characteristic for the isopropyl terminal group of the molecule as well as the strong peak at m/e 226–227. The anteiso C_{15} methyl ester, M = 256 shows a peak at m/e = M-57 indicating an isobutyl terminal group and a peak at m/e − 198–199. The molecular ion was also the base peak in both analysis. The occurrence of peaks at m/e = M-43 and m-57 in the high mass range is consistent with the presence of methyl side chains at positions 2 and 3, the ions being formed through the loss of the molecule-ion.

TABLE IV

Equivalent Carbon Number of Unsaturated Iso-, and Anteiso-Methyl Esters on Polar and Nonpolar Stationary Phases

Methyl ester	Equivalent carbon number					
	EGS (185C)		SE-30 (185C)		SE-30 (220C)	
	Iso-	Anteiso	Iso-	Anteiso	Iso-	Anteiso
C12	11.40					
C13		12.50				
C14	12.40		13.20			
C15		14.55		14.30		
C16	15.40		15.20			
C17	16.40	16.55	16.20	16.30	16.14	16.22
C18	17.40		17.20		17.15	
C19	18.40	18.55	18.20	18.30	18.15	18.25
C20	19.40		19.20		19.18	
C21				20.30		20.27
C22			21.20		21.25	
C23				22.30		22.29
C24			23.20		23.30	
C25				24.30		24.30
C26					25.32	
C27						26.32
C28					27.45	
C29						28.35

Discussion

This study establishes an updated quantitative fatty acid composition of degras. In agreement with reports of earlier investigators (2,16) degras fatty acids were found to contain normal and branched chain fatty acids. The pattern was one of odd- and even-numbered acids, both saturated and monounsaturated, ranging from 8 to 31 carbon atoms. This general type of fatty acid composition is similar to that reported by Weitkamp.

Recently, Nicolaides and Ray (17) have reported that human skin possesses a fatty acid composition of similar type. These unusual fatty acids were found to be products of the skin itself rather than products of external bacterial contamination. These same types of fatty acids were reportedly found in vernix caseosa (the lipoidal material covering the fetus) (17). This information was used by Nicolaides to differentiate between endogenous and exogenous components in human skin surface lipids. Degras lipid is also found in the sebaceous glands of sheep (1). This lipid shows no visible triglycerides when subjected to TLC analysis whereas human lipid shows some triglyceride to be present. This indicates a greater enzymatic lipase activity in the sebaceous glands of sheep than in human beings. A waxy substance, i.e., a long chain wax ester, would probably protect and be more suitable for sheep than the oily substance found excreted on human skin. Downing (18) compared the branched chain fatty acid content of wool lipid and depot fat of newly born lambs to those found in adult sheep. He also noted the relatively high content of branched fatty acids in vernix caseosa.

It must be realized that this report could differ from other investigations since the exact nature of the wool fat is unknown, and the refining process could cause some alteration to the composition.

ACKNOWLEDGMENTS

The fatty acid standards and stationary phases for the GLC used in this investigation were either donated by or purchased from Applied Science Laboratories, Inc. The samples of 14-methylpentadecanoic (iso C_{16}), 16-methylheptadecanoic (iso C_{18}), and 18-methylnonadecanoic (iso C_{20}) fatty acids were gifts from Nicholas Nicolaides, University of Southern California Medical School; methyl esters of 15-methylhexadecanoic (iso C_{17}) and 14-methylhexadecanoic (anteiso C_{17}) were synthetic products provided by Hermann Schlenk, The Hormel Institute, University of Minnesota. Mass spectrometry data were interpreted by M. Shamma of The Pennsylvania State University.

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