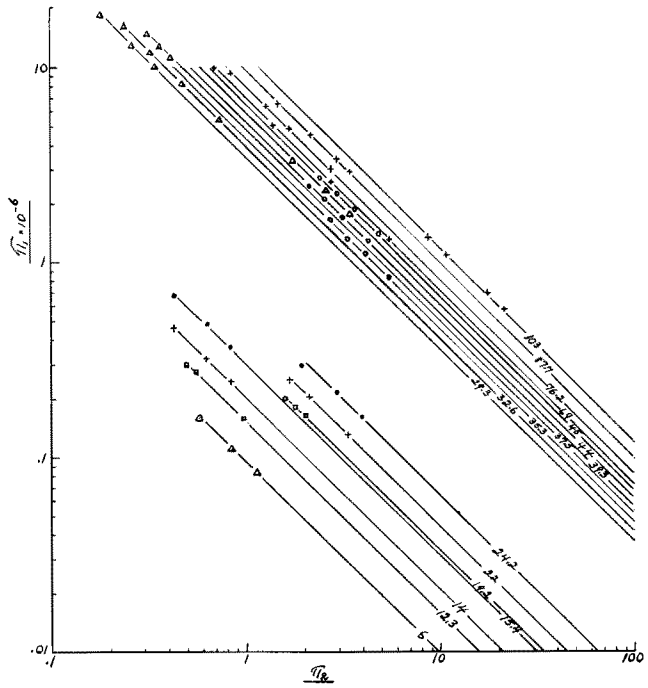

FIG. 2. Dimensional analysis No. 9-C

$$\pi_1 = \frac{D \cdot (\gamma_{CMC})^{4/5}}{DM^{8/5} \cdot C} \qquad \pi_2 = \frac{C}{CMC}$$

$$\pi_3 = \frac{V}{DM^{1/5} \cdot (\gamma_{CMC})^{2/5} \cdot CMC^{1/2}}$$

tional soil-surfactant systems using palmitic and linoleic acid soils.

It is now possible to form a still more useful set of dimensionless products from the set plotted in Figure 1″. A new π_1 can be obtained by dividing π_1 by π_2 of Dimensional Analysis 9–B of Figure 1″. When this is done the variable "C" replaces "CMC" in the denominator of π_1. Products π_2 and π_3 remain the same. Figure 2, a plot of these products, which we term the results of Dimensional Analysis 9–C, gives a much more convenient detergency diagram. The π_1–π_2 function on a log–log scale is a series of parallel lines with a negative slope of approximately 45°.

Further study will probably yield more suitable sets of dimensionless products of the detergency process. Improvement will result also from the use of more accurate constants (i.e., soil dipole moment and CMC). However, Figure 2 (Dimensional Analysis 9–C) now provides a valid, simple and practical detergency diagram that represents the mechanism by log–log curves of dimensionless products π_1 vs π_2 for fixed values of the parameter π_3. These products and the diagram are a vast improvement over the transcendental relationships established previously for selected groups of soil-surfactant systems (2). In fact it would seem that the eventual extension of the diagram's utility to prediction of detergency in various soil-surfactant applications is within reach.

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Studies on the Fatty Acid Composition of Crayfish Lipids[1]

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Abstract

The fatty acid composition of carcass and exoskeleton lipids was determined for the freshwater crayfish *Orconectes rusticus*. Lipid fractions were isolated by column and thin-layer chromatography. Fatty acid methyl esters and alcohol acetates were then prepared and analyzed by gas-liquid chromatography. Peak identities were established from retention time data for methyl esters, hydrogenated methyl esters, and saturated, monoene, diene, and polyene methyl esters separated as acetoxy-mercuri-methoxy derivatives. Minor component acids were estimated from their relative compositions in these fractions.

The triglyceride, cholesteryl ester, and astaxanthin ester fractions exhibited a typical freshwater fatty acid composition. Carcass free fatty acids, present in unusually high amts, contained less 16:0 and 16:1, and more 10:0, 20:4, and 20:5 acids than the neutral lipids. The crayfish phospholipid fraction contained elevated amts of 20:4, 20:5, and 22:6 acids. A selective mobilization of triglyceride at lower temps may therefore contribute to the seasonal variation in relative C_{20} polyunsaturated fatty acid content reported for crustacea. Relative retention time data suggest that crayfish fatty acids belong to the 9,12-octadecadienoic acid and 9,12,15-octadecatrienoic acid structural types.

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Introduction

EARLY STUDIES BY LOVERN in 1935 (1) on crustacean fatty acid composition suggested that the previously observed differences between freshwater and marine fish oil composition could be traced directly to a diet consisting principally of planktonic crustacea. Marine copepod and freshwater branchipod crustacea exhibited the well-known marine and freshwater oil compositions, respectively. A typical marine oil composition was later found in a marine decapod, the rock lobster *Jasus lalandii,* which contained up to 50% C_{20} and C_{22} polyunsaturated acids (2). However, the marine branchiopod, *Artemia salina,* fed a pure diatom culture (*Chaetoceros simplex*) was similar to freshwater species in oil composition (3). Farkas and Herodek (4) stated in 1962 that the freshwater-marine relationship could not be applied to planktonic crustacea. They reported a seasonal variation in the fatty acids of the freshwater copepod *Cyclops vicinus.* The C_{20} and C_{22} polyunsaturated fatty acids rose to 51–59% of the total during the winter months with a corresponding decrease in the C_{16} and C_{18} components. This increment in C_{20} and C_{22} polyunsaturated acids was correlated with a lowered ambient temp (5). Furthermore, freshwater fish oil composition changed to a marine composition when the fish were fed winter-type freshwater planktonic crustacea.

In 1935, Karrer et al. (6) reported that the astaxanthin ester of lobster contained only palmitic acid. Several investigators have recently demonstrated that vitamin A esters in mammalian systems contain palmitic acid (7–10). The carotenol ester in dandelion flowers contained a large proportion of palmitic acid, and since the fraction studied was contaminated by triglycerides, Booth (11) concluded that the carotenol was present as the dipalmitate. These observations suggest a widespread fatty acid specificity in carotenol esterification. However, the vitamin A esters of various fish oils were found to exhibit compositions similar to the associated triglycerides (12). In this paper, we describe the fatty acid composition of several lipid fractions, including astaxanthin ester, from the freshwater crayfish *Orconectes rusticus.* A preliminary report of this work has appeared elsewhere (13).

Experimental

Crayfish were collected locally and sacrificed by freezing. The exoskeleton and carcass of the animal were separated and analyzed individually. The tissues were homogenized in a Waring blendor and extracted exhaustively with acetone. The extract was then taken up in hexane, dried over anhydrous sodium sulfate, and weighed. Total phospholipid was obtained by extracting tissues with chloroform-methanol (2:1, v/v). The extracts were analyzed for phosphorus (14), total cholesterol (15), and free fatty acid (16). Free cholesterol was separated as a digitonin precipitate or by column chromatography.

TABLE I

Lipid Content and Composition in *Orconectes rusticus*

Fraction	Whole animal[a]	Exoskeleton[b]	Carcass[b]	Carcass[b]
Collection date	6/25/64	7/1/63	7/1/63	9/18/63
Animals	325	100	100	161
Wet weight (g)	1,625	525	501	413
Total lipid (% wet weight)	3.2	0.4	2.1	2.1
Free cholesterol (%)[c]	2.8[d]	4.7[d]	3.7[e]	3.8[e]
Cholesteryl ester (%)[c]	0.6	0.5	0.3	0.4
FFA[f] (%)[c]	9.7	—	12.6	20.5
Phospholipid (%)[c]	13.3	7.7	10.5	3.7

[a] Chloroform-methanol extract.
[b] Acetone extract.
[c] Percent of total lipid.
[d] Digitonin precipitate.
[e] Column fraction.
[f] Calculated as oleic acid.

TABLE II

Fatty Acid Composition of *Orconectes rusticus* Carcass Lipids[a]

Fatty acid	Total lipid	Triglyceride	FFA	Cholesteryl ester	Phospholipid[b]
10:0	2.7	–	6.4	–	–
12:0	0.6	–	–	–	–
14:0	2.1	3.5	1.0	5.9	0.9
15:0	–	–	–	0.7	0.4
16:0	19.7	29.2	11.9	25.4	18.7
16:1	15.1	16.3	11.4	18.4	6.0
(17?)[c]	6.1	7.9	3.4	–	2.0
18:0	3.1	2.9	5.0	5.5	7.3
18:1	21.5	21.5	25.9	25.7	20.5
18:2	7.4	7.5	7.6	9.6	6.3
(X)[d]	–	–	–	1.0	–
18:3	9.2	6.0	8.0	3.5	2.9
(Y)[d]	–	–	–	–	1.2
20:2	–	–	–	–	1.1
(Z)[d]	–	trace	trace	0.6	–
20:4	5.7	2.9	10.1	1.0	6.6
20:5	7.0	2.6	9.5	2.9	22.0
22:6	[e]	[e]	[e]	[e]	4.0

[a] Fatty acid composition reported in mole %.
[b] Phospholipid data from chloroform-methanol extract of whole animal included for comparison.
[c] Peak identity uncertain. The 17? component includes several unresolved peaks.
[d] Peak identity unknown. The Z component in the cholesteryl ester fraction may be a 20:3 acid.
[e] Not determined in these analyses.

Lipids were fractionated by column chromatography on activated silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.,). Cholesteryl esters, triglyceride, and free fatty acid (FFA) fractions were eluted, respectively, by 1%, 6%, and 10% ether in hexane. Unesterified cholesterol and astaxanthin were eluted together by 12–15% ether in hexane. Phospholipids from chloroform-methanol extracts were eluted from silicic acid columns with chloroform-methanol (1:1) and methanol following the elution of the neutral lipid with hexane-ether (1:1). Cholesteryl ester and triglyceride fractions were not separated completely on the silicic acid columns. Cholesteryl esters were purified further by thin-layer chromatography (TLC), being scraped from the solvent front after development with hexane-ether (9:1). FFA and triglycerides were not well separated on silicic acid columns, but were separated on alkaline silicic acid columns prepared by the method of McCarthy and Duthrie (17). Triglycerides eluted in this system with ether, while FFA remained at the top of the column. FFA were then eluted with 2% formic acid in ether. Alkaline silica gel G thin-layer plates were prepared to confirm the column separation. Coated plates were submerged in a solution of 5% potassium hydroxide in ether-isopropanol (1:1), rinsed by submerging briefly in ether, and dried at room temp. The plates were developed with hexane-ether (3:7). FFA remained at the origin while triglyceride migrated to the solvent front.

Astaxanthin ester from crayfish exoskeleton was prepared by chromatography on neutral alumina (M. Woelm, Eschwege, Germany) weakened by the addition of 5% water by weight and further purified by streaking the column fraction onto a thin-layer plate and developing with hexane-ether (3:2). The dense red band was scraped from the plate, eluted with ether, and rechromatographed on another plate. This astaxanthin ester fraction was free of triglyceride and FFA, as shown by a single spot on the plate. Free cholesterol still contaminated the fraction. All other lipid fractions were judged homogeneous by TLC.

Methyl esters, prepared by saponification and ester-

TABLE III

Fatty Acid Composition of *Orconectes rusticus* Exoskeleton Lipids[a]

Fatty acid	Total lipid	Triglyceride	Astaxanthin ester
10:0	2.8	–	–
12:0	0.7	3.7	6.1
14:0	1.4	4.9	3.8
16:0	20.0	26.0	27.0
16:1	10.0	13.3	9.2
(17?)[b]	3.4	4.9	4.7
18:0	6.9	4.7	11.3
18:1	27.5	18.4	24.5
18:2	4.6	4.7	1.9
18:3	5.5	8.0	3.6
20:4	6.9	7.2	3.6
20:5	9.3	4.2	4.2

[a] Fatty acid composition reported in mole %.
[b] Peak identity uncertain. The 17? component includes several unresolved peaks.

ification, and alcohol acetates, prepared by hydrogenolysis and acetylation (18) were used for gas-liquid chromatography (GLC). Methyl esters from cholesteryl ester fractions were prepared by direct methanolysis with sodium methoxide (19). Unsaturated methyl esters were separated from saturated methyl esters by TLC of their acetoxy-mercuri-methoxy derivatives (20,21). Following reaction of the methyl esters with mercuric actate in methanol, the mercury adducts were streaked onto a silica gel G plate and eluted first with hexane-ether (4:1), then with n-propanol-acetic acid (100:1). Saturated, monoene, diene, and polyene fractions were then scraped from the plate and the double bonds regenerated by elution with methanol containing 5% (v/v) concd HCl. Methyl esters and alcohol acetates, about 100 mg lipid, were dissolved in 30 ml absolute methanol for hydrogenation. About 50 mg of Pd-charcoal catalyst was added, and the mixture was shaken for 6 hr under 35 psi hydrogen. The mixture was then filtered and the solvent removed.

An Aerograph A350-B chromatograph equipped with a Wheelco type A electronic integrator was used in these studies. Helium was the carrier gas. The columns were obtained from Applied Science Laboratories and were 10 ft stainless steel, 0.25 in. I.D., containing 10–13% ethylene glycol succinate polyester (EGS) on 60–80 mesh Gas Chrom P. Chart speed was 1 in./min.

Peaks were identified by correlating retention time data for standard mixtures (Applied Science Laboratories, State College, Pa.), total methyl esters, hydrogenated methyl esters, and the saturated and unsaturated ester fractions. Peak areas were corrected by the use of relative molar response factors (22). Peaks for which no factors have been reported were not corrected.

Results and Discussions

The compositions of lipid extracts from *Orconectes rusticus* are shown in Table I. Although phospholipid was present in the acetone extracts, analyses for phospholipid fatty acid composition were performed on chloroform-methanol extracts of the whole animal. The high FFA content, reported here as oleic acid, was noted even when the extraction was performed immediately after sacrifice of the animals, a procedure which eliminated the possibility of lipolysis during storage.

The fatty acid compositions of the various lipid fractions from the carcass are shown in Table II. These initial analyses revealed a typical freshwater composition for total and neutral lipid fractions with large amts of C_{16} and C_{18} acids. The relatively high 18:2 and 18:3 content is particularly significant. These acids, elevated in freshwater fish (23), are only

TABLE IV

Relative Retention Time for Methyl Esters of Standard Fatty Acids and Fatty Acids Isolated from Phospholipids

Fatty acid	rIS at 178C				
	Standard	Total	Mercury adducts Saturated	Monoene	Hydrogenated
12:0	0.216				
13:0	0.272				
14:0	0.342	0.338	0.339		0.348
14:1	0.408				
15:0	0.443	0.439	0.443		0.455
(16:0Br)[a]		0.497	0.489		0.495
16:0	0.568	0.568	0.568		0.568
(16:1Br)				0.571	
16:1	0.661	0.660		0.667	
17:0	0.750	0.780	0.791		0.769
(17:1)				0.886	
18:0Br)			0.881		0.900
18:0		1.000	0.995		1.000
(18:1Br)				1.01	

[a] No reference standard.

minor components in the marine branchiopod, *Artemia salina* (3). There are major differences between the fatty acid composition of crustacea reported by Farkas and Herodek (5) and the present study. For example, these investigators found only a small amt of the 18:1 acid, a major component of both *Orconectes rusticus* (Table II) and *Artemia salina* (3). Variations between species may explain the discrepancy; however, early data on the same species (1) do not correspond to the analyses obtained by Farkas and Herodek.

Methyl esters were found to be preferable to alcohol acetates in these studies, because triacetin interfered with the calculation of C_{16} and C_{17} components in the alcohol acetate chromatograms. Upon hydrogenation of the total extract, a C_{22} peak accounting for 1.9%of the fatty acids appeared. This minor component was not detected initially, and its concn in these neutral lipid fractions is not known.

FFA contained less C_{16} acids with corresponding increases in the C_{10}, 20:4 and 20:5 components. The FFA composition suggests that this fraction is not a lypolysis artifact. While the total extract contained several small unresolved components eluting between 16:1 and 18:0 acids, these acids were completely absent in the cholesteryl ester fraction. This fraction was, however, similar to the triglyceride fraction in fatty acid composition.

The fatty acid analyses of exoskeleton lipids are shown in Table III. The exoskeleton total lipid and triglyceride closely resemble the carcass total lipid and triglyceride, respectively. Alcohol acetates were used for the analysis of the astaxanthin ester fraction in order to demonstrate by the absence of triacetin that the fraction contained no triglyceride. The astaxanthin ester fraction was also similar to the total lipid, but contained a slightly higher proportion of 18:0 with a decrease in polyunsaturated acids. This finding is similar to that reported previously for the vitamin A ester composition of fish oils (12) and does not confirm the specificity for palmitate sug-

TABLE V

Relative Retention Time for Methyl Esters of Standard Fatty Acids and Fatty Acids Isolated from Phospholipids

Fatty acid	Standard	rIS at 192C		
		Mercury adducts		
		Monoene	Diene	Polyene
18:1	1.16	1.16		
18:2	1.43		1.42	
18:3	1.84			1.85
(19:1)[a]		1.46		
(20:1)		1.83		
(20:2)			2.36	
20:4	3.09			3.10
(20:5)				4.10
(22:6)				7.82

[a] No reference standard.

TABLE VI

Relative Retention Time (r_{18}) and Separation Factors (r) for Methyl Esters Isolated from Phospholipids

Fatty acid	r_{18} Total 192C	r_{18} Mercury adducts 192C	r_{18} Ackman (25) 197C
22:6 4,7,10,13,16,19-	7.93	7.82	7.75
20:5 5,8,11,14,17-	4.16	4.10	3.85
20:4 5,8,11,14-	3.19	3.10	3.04
20:2 8,11-	2.39	2.36	2.32
18:3 9,12,15-	1.93	1.84	1.72
18:2 9,12-	1.44	1.42	1.34
18:1 9-	1.15	1.16	1.12
Fatty acid ratio	r		
20:5/20:4	1.30	1.32	1.26
20:4/20:2	1.33	1.31	1.31
20:5/20:2	1.74	1.73	1.66
18:3/18.2	1.33	1.29	1.28
18:2/18:1	1.26	1.22	1.19
18:3/18:1	1.68	1.58	1.54

gested in the initial analysis of astaxanthin ester obtained by Karrer et al. (6).

The GLC analyses of total fatty acid mixtures were not adequate for the identification and estimation of minor components. Simplified methyl ester mixtures were therefore obtained by hydrogenation and the separation of acetoxy-mercuri-methoxy derivatives. Relative retention time data for typical mixtures (phospholipid fraction) and reference standards are summarized in Tables IV and V. When reference standards were not available, peaks were identified by their relative retention time and appearance in saturated, monoene, diene, or polyene fractions. For example, the 16:1Br component appeared in the monoene fraction and had a retention time between 16:0 and 16:1.

Since reference standards for many of the C_{20} and C_{22} acids were not available, the relative retention time data reported by Ackman (24,25) were compared with values obtained in this study (Table VI). All values except the 20:5 peak were in reasonable agreement. This peak had a relative retention time of from 4.10 to 4.16 which proximated the 4.20 reported by Ackman for 22:2. The peak was identified as 20:5 since it appeared in a polyene fraction (Table V) and since the 20:0 and 22:0 components obtained after hydrogenation were in the same proportion as the C_{20} and C_{22} polyene acids tentatively identified in the original mixture.

Analyses, including minor component acids for total carcass lipid and phospholipid, are summarized in Table VII. The presence of branched chain C_{16} and C_{18} saturated and unsaturated acids was verified. These minor components were estimated after hydrogenation and reported as total C_{16} and C_{18} branched acids. The total C_{17} content was also estimated after hydrogenation. Carcass lipid contained small amts of 16:2, 16:3, 16:4 and 18:4 acids. These acids were not identified as components of the phospholipid fraction. It is interesting that an 18:4 acid was the principal C_{18} acid reported in the crustacea investigated by Farkas and Herodek (5). The phospholipids contained increased amts of the 20:5 and 22:6 acids. Other C_{22} acids which occur in freshwater fish (23) were not present in either phospholipid or carcass lipid fractions. Seasonal variations in fatty acid composition (4,5) may be explained in part by depletion of triglyceride reserves during the winter months. Total fatty acids would then resemble phospholipid fatty acids and show an enhanced C_{20} and C_{22} polyunsaturated acid content. This triglyceride depletion hypothesis is supported by seasonal variations in lipid content reported for marine zooplankton (26) and the enhanced mobilization of depot fat induced in animals such as hamsters by cold exposure (27). Environmental temps do not appear to have a direct effect on polyunsaturated fatty acid interconversions in teleost fish (28).

TABLE VII

Fatty Acid Composition of *Orconectes rusticus* Total Carcass Lipid and Phospholipid Fractions

Fatty acid	Total carcass lipid	Phospholipid
	Mole %	
10:0	2.3	–
12:0	Trace	–
14:0	2.1	0.5
15:0	1.4	0.1
16:0Br	0.4	1.1
16:1Br		
16:0	19.0	19.4
16:1	13.8	7.5
16:2	Trace	–
16:3	1.3	–
16:4	1.4	–
17:0	4.7	0.8
17:1		
18:0Br	–	1.2
18:1Br	–	
18:0	3.2	7.6
18:1	19.4	22.0
18:2	5.4	5.4
18:3	8.9	1.3
18:4	0.9	–
19:1	0.5	2.4
20:1	Trace	3.2
20:2	Trace	1.2
20:4	6.5	4.8
20:5	7.8	17.5
22:6	1.5	4.2

Relative retention time data are not sufficient to establish unequivocally the double bond structure of unsaturated fatty acids; however, tentative structures may be assigned with the aid of separation factors (24,25). Separation factors (Table VI) suggest that the 18:2 and 18:3 acids are 9-12-octadecadienoic (linoleic) and 9-12,15-octadecatrienoic (linolenic) acids, respectively. Linoleic acid is metabolized to 5,8,11,14-eicosatetraenoic (arachidonic) acid (29,30). This acid was tentatively identified from separation factor data (Table VI). Intermediates in the conversion process, 6,9,12-octadecatrienoic and 8,11,14-eicosatrienoic acids, were not identified, although one component in the cholesteryl ester fraction (Table II) showed the correct separation factor for the 20:3 intermediate. (The r values were 1.12 and 1.10 for 20:4/Z and 20:4/20:3, respectively).

Linolenic acid is metabolized to 5,8,11,14,17-eicosapentaenoic and 4,7,10,13,16,19-docosahexaenoic acids (29,30). These acids were tentatively identified from separation factor data (Table VI). The 18:4, 20:4, and 20:5 intermediates in this biosynthetic pathway were not observed. When triglyceride and phospholipid fractions are compared, a marked decrease in the relative 18:3 content is found to accompany a marked increase in 20:5 and 22:6 acids. A similar relationship was observed between 18:3 and 22:6 acids in the neutral and phospholipid fractions of tuna white muscle (31). This inverse relationship suggests that triglycerides resembles dietary lipid while phospholipids mirror the biosynthetic pathways operating in the animal.

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Methods for the Determination of Cyclopropenoid Fatty Acids. VI. A Direct Infrared Absorption Method

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Abstract

A simple rapid method for the estimation of the cyclopropenoid content of glycerides and methyl esters is described based upon the measurement of the characteristic infrared absorptivity of cyclopropenoids at 9.9 μ. Autoxidation products do not interfere, and the sample can be recovered. Equations are given for the calculation of the cyclopropenoid content of both glycerides and methyl esters.

Introduction

WHILE INVESTIGATING METHODS of isolation of cyclopropenoid methyl esters a need arose for a simple rapid method for estimating the cyclopropenoid content of concs. In general, available methods are either unsuitable or require some form of pretreatment of the sample. An infrared (IR) method based upon the characteristic absorptivity of cyclopropenoids at 9.9 μ was used by Varma et al. (1) in an attempt to measure the sterculic acid content of *Sterculia foetida* oil. However, the method involved converting the oil to the free acids and comparing their absorptivity in CS_2 solution with that of a standard. Their standard was a supposedly pure sample of sterculic acid. This procedure cannot be expected to give reliable results since pure sterculic acid is extremely unstable and polymerizes rapidly at room temp (2). Moreover, cyclopropenoid fatty acids are reported to react with carbon disulfide even at room temp (3). The present report deals with a simple rapid method of analysis for glycerides and methyl esters based upon the direct measurement of the 9.9 μ absorptivity and calibrated against a series of standards of accurately known cyclopropenoid concn.

Materials

Sterculia foetida oil, the fatty acids of which contain about 50% of sterculic acid and a small amt of malvalic acid, was used as a source of cyclopropenoid fatty acids for testing the applicability of the method. *Sterculia foetida* seed meats were extracted with several portions of petroleum ether (bp 30–60C) in an explosion-proof Waring Blendor at room temp. The extracts were combined, filtered, and freed from solvent on a rotary evaporator with a nitrogen leak at reduced pressure. Methyl esters were prepared from the oil by methanolysis catalyzed by sodium methoxide (5). The methyl sterculate concn of a portion of the *Sterculia foetida* methyl esters was increased by removal of the saturated esters by urea clathration. The oil, the methyl esters, and the methyl ester conc were passed in petroleum ether solution through an activated alumina column to remove interfering substances, dried over anhydrous sodium sulfate, stripped of solvent on a rotary evaporator at reduced pressure, and their cyclopropenoid acid content determined by HBr titration (4,5) just prior to their use in connection with the calibration measurements. The cyclopropenoid contents of the oil, the methyl esters, and the methyl ester conc so obtained were 52.05, 48.55, and 72.24%, respectively, calculated as sterculic acid.

Experimental and Results

Two series of methyl ester mixtures of accurately known cyclopropenoid content were prepared and used to establish a calibration curve. The first was prepared from the *Sterculia foetida* methyl esters, and the second from the methyl sterculate conc, by mixing with methyl oleate. The IR absorption curves of these mixtures from 9.0 to 10.7 μ were obtained on a Perkin-Elmer Model 21 IR spectrophotometer. The slits were fixed at 151 μ, and the instrument adjusted to have a slight drift upward. The scan was made at a speed of 1 μ per 3 min with a gain of 6, a response of 1, and a suppression of 0. The samples were analyzed in carbon tetrachloride using matched KBr cells of 0.5 mm length at a concn suf-

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