TABLE III
GLC Analysis of Vernonia Oil Methyl Esters Employing Internal Standard

Ester	Total esters[a]		Composite[b]	
	Without factors	With factors	Without factors	With factors
	%	%	%	%
<14:0	0.29	0.29	0.23	0.23
14:0	0.19	0.19	0.03	0.03
14:1 } 15:0 }	0.05	0.05	0.14	0.13
16:0	1.99	1.87	2.24	1.91
16:1 } 17:0 }	0.09	0.09	0.20	0.18
18:0	1.00	1.03	1.22	1.14
18:1	1.80	1.87	2.06	1.95
18:2	6.78	7.42	8.07	8.01
20:0	0.21	0.23	0.07	0.07
18:3 } Conj. 18:2 *c,t* }	0.33	0.36	0.41	0.41
Conj. 18:2 *t,t*	0.08	0.09	0.07	0.07
18:1 Epoxy	50.5	71.0	57.5	73.3
Unknown	.....	.....	1.36	1.24
Unsap.	7.76	7.76	7.76	7.76
Total	71.07	92.25	81.36	96.43

[a] GLC directly on total esters of oil.
[b] GLC on fractions from crystallization summated to original total esters.

18:2 *t,t* was more readily detected in the F_1 fraction owing to concentration of these by crystallization. A combination of gas-liquid chromatography, ultraviolet and infrared spectrophotometry was employed to establish their identity.

Further study is being made of the alteration of epoxyoleate during CLC analysis with the objective of finding more satisfactory column and stationary phase for analysis of oils containing epoxyoleic acid and possibly other oxygenated fatty acids. It is expected that the results will be reported soon.

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[Received September 26, 1962—Accepted December 14, 1962]

Thin-Layer Chromatographic Separation of Some Bromo-and Hydroxyderivatives of Stearic Acid[1]

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Abstract

Addition of polar substituents to unsaturated fatty acids or their methyl esters enhanced the adsorption affinity contributed by each unsaturation site to such an extent that the resulting derivatives could be easily separated by differential adsorption on a silica gel coating. Saturated and unsaturated fatty acids could be separated by thin layer chromatography after either bromination of their methyl esters or oxidation of their mixture into unsaturated esters, monoenoates, dienoates, and trienoates. Furthermore, it was possible to separate the configurational isomers of the hydroxystearic acids derived from *cis* and *trans* hydroxylation of oleic and linoleic acids.

Introduction

MORETTI AND POLONOVSKI (1) have reported that it was possible to separate by column or paper chromatography the bromo derivatives of the higher fatty acids by utilization of their differences in solubilities in a set of solvent systems. Howton (2) was able to separate methyl stearate, methyl threo-9,10-dibromostearate and methyl threo, threo-9,10,12,13-tetrabromostearate by gradient elution on alumina columns. Ory et al. (3) separated the methyl polybromostearates on silicic acid impregnated glass paper with isooctane as the developing solvent.

Methods of significance from an analytical aspect have been proposed for the chromatographic separation of saturated and unsaturated fatty acids, after hydroxylation of the latter. Bergström and Pääbo (4) have hydroxylated the unsaturated fatty acids with performic acid and separated the esters on a silicic acid column. Kaufmann and Nitsch (5) employed paper partition chromatography for the separation of hydroxy and polybromo acids. Kaufmann and Khoe (6) have also reported the use of the bromination reaction on hydrophobic chromatoplates (gypsum plates impregnated with undecane) for the separation of saturated and unsaturated fatty acids. Bromine was dissolved in the mobile phase (acetic acid/acetonitril, 1/1) and the reaction was carried out directly on the chromatoplate.

In the present study the methyl polybromostearates corresponding to oleic, linoleic, and linolenic acids were separated from each other and from methyl stearate on glass strips coated with silica gel. The resolution of hydroxylated derivatives of mixtures of oleic and elaidic acids and of mixtures of diastereoisomers of the 9,10,12,13-tetrahydroxystearic acids has also been described.

Experimental

Methyl stearate, oleate, linoleate, and linolenate were used as received, purity checked by gas liquid chromatography (GLC).

Elaidic acid. Commercial oleic acid was treated with nitrous fumes (7). The product, in boiling alcohol, was neutralized with aqueous lithium hydroxide. The lithium salt, which separated on cooling, was removed by filtration and recrystallized

[1] Partial support of these studies by Grant No. H-1819 from National Institutes of Health.

from 60% aqueous alcohol, giving a solid, mp 213-218C (uncorrected sealed capillary). Regeneration of the acid and one crystallization from alcohol at 0C gave elaidic acid mp 42–43, n_D^{50} 1.4488.

Threo 9,10-dibromooctadecanoic acid (I) was prepared from purified oleic acid according to the method of Nevenzel and Howton (8). The crude, viscous, brown oil (mp 20–24C, reported 28–29C for I) was esterified azeotropically within methanol-chloroform-sulfuric acid and the methyl ester was chromatographed on alumina with Skellysolve F as the eluting solvent. The pale yellow liquid was redistilled under vacuum three times (bp 194C at 0.2 mm) to constant refractive index $n_D^{20} = 1.4876$, M.W. 456 (theory 465.83), bromine content 34.62% (theory, 34.31%). Debromination with zinc and methanolic hydrochloric acid gave methyl oleate identified by comparison of its GLC and infrared (IR) spectra with those of an authentic sample of methyl oleate.

Erythro 9,10-dibromooctadecanoic acid (II) was obtained by reacting equivalent amounts of elaidic acid and bromine in carbon tetrachloride at 0C. The product crystallized at −15C and after two recrystallizations from Skellysolve F showed a mp of 29.5–30C. Its methyl ester was prepared with diazomethane. Debromination with zinc in methanolic hydrochloric acid gave methyl elaidate, identified by comparison of its GLC and IR spectra with those of an authentic sample.

Tetrabromostearic acid (III), mp 115.5C and hexabromostearic acid (IV) mp 185C was prepared from corn oil and linseed oil, respectively, according to well known procedures (9,10).

For the preparation of the erythro, erythro-9,10,12,13-tetrabromooctadecanoic acid (V), pure methyl linoleate (11), was elaidinized with nitrogen oxides (12) and the resulting linolelaidic acid (mp 28–28.5C) was brominated in petroleum ether at −5C (10). One crystallization from hexane produced crystals with a mp of 78C.

Acids II, III, IV, and V were esterified with diazomethane prior to chromatography. The conversion of acid to ester was verified in all esters by checking the carbonyl stretching frequencies in the I.R. region. Purities of acid were assessed by melting points, content of bromine, and thin layer chromatography (TLC). When only one spot was apparent in TLC a purity of 99% was assumed.

The strongly polar carboxyl group of the free fatty acids was esterified in order to make the contribution of the centers of the bromine substituents as dominant as possible. When the brominated free fatty acids were chromatographed by using Skellysolve B-ethyl ether-acetic acid 96:4:2 (v/v/v) as the developing solvent a similar separation was obtained but an overlapping of spots between dibromo and tetrabromo acid occurred when the substances were chromatographed in a mixture. The nonbrominated esters of stearic, oleic, linoleic, and linolenic acids did not separate when applied as a mixture.

Bromination was carried out by dissolving the mixture of methyl esters in sufficient dry diethyl ether to form a 10% solution. The mixture was kept at −10C and with constant shaking bromine was added very slowly from a finely drawn pipette until a persistent reddish-yellow color was obtained. The reaction mixture was kept at −10C for several hours for completion of the reaction and the excess of bromine then removed with sodium sulfite solution. The ether layer was washed repeatedly with distilled water, 2% sodium thiosulfate, water and dried over anhydrous magnesium sulfate.

9,10-Dihydroxystearic acid. The threo form mp 95C was prepared from technical oleic acid and peracetic acid (13). The erythro form mp 131C was prepared by oxidation of sodium oleate with alkaline permanganate (14).

Erythro, erythro, erythro-(mp 174C) and erythro threo, erythro-(mp 164C) 9,10,12,13-tetrahydroxystearic acids were prepared and separated as described by Riemenschneider et al. (15). Threo, erythro, threo-(mp 148C) and threo, threo, threo-(mp 126C) 9,10,12,13-tetrahydroxystearic acids were prepared as reported by McKay et al. (16). Purity of the acids was assessed by melting points, neutralization equivalents and thin layer chromatography (TLC). When only one spot was apparent in TLC a purity of 99% was assumed. Oxidation of mixtures of saturated and unsaturated fatty acids was carried out by permanganate oxidation (17).

Chromatography. Thin layers (250 to 275 μ) of silica gel on glass (20 × 20 cm) were prepared according to Stahl (18), using "Silica Gel G" a fine grade of silica gel containing 1% Plaster of Paris. A mixture of 96 vol of Skellysolve B (bp 67–68C) and 4 vol of anhydrous ether served as the developing system for the separation of the bromo derivatives. A more polar system of 90 vol chloroform, 10 vol methanol, and 2 vol acetic acid was used for the hydroxy derivatives. The acetic acid was added in order to prevent streaking of the fatty acids.

Each sample was applied in 5 to 10 γ quantities and the strips sprayed with a saturated solution of potassium dichromate in 50% sulfuric acid to detect the developing spots.

Results and Discussion

The results indicated that thin layer chromatography may be substituted for the conventional closed columns. It combined the reliability of glass paper chromatography with the advantage of speedier procedure and easier operation. The pattern of separation of a mixture was reproducible and reliable.

The Rf values obtained for the bromo derivatives (Fig. 1) are given in Table I. Since the Rf values obtained for the bromo derivatives fall very close to an extrapolated plot of molecular weight vs. log of the Rf values for saturated methyl esters, the bromine group contributed principally to the molecular weight and not to any basic change in chromatographs. Contribution of other factors, although of a lower order of magnitude than the weight factor, are responsible for the different behavior of configurational isomers. The methyl erythro-9,10-dibromostearate prepared from elaidic acid was eluted more rapidly than the methyl threo-9,10-dibromostearate prepared from oleic acid. Secondary effects also arose from diastereoisomerism when the methyl esters of threo, threo-, and erythro, erythro-9,10,12,13-tetrabromostearic acid were chromatographed on Silica Gel G plates with 4% ether in Skellysolve B as the resolving agent. Methyl threo, threo-9,10,12,13-tetrabromostearate was retained longer than the all erythro isomer.

It is not desirable with these limited data to discuss factors responsible for the variations in the adsorption behavior found among the diastereoisomers. No general conclusion may be drawn about the relative adsorbabilities of the two isomers which were used in each series of the bromo derivatives except

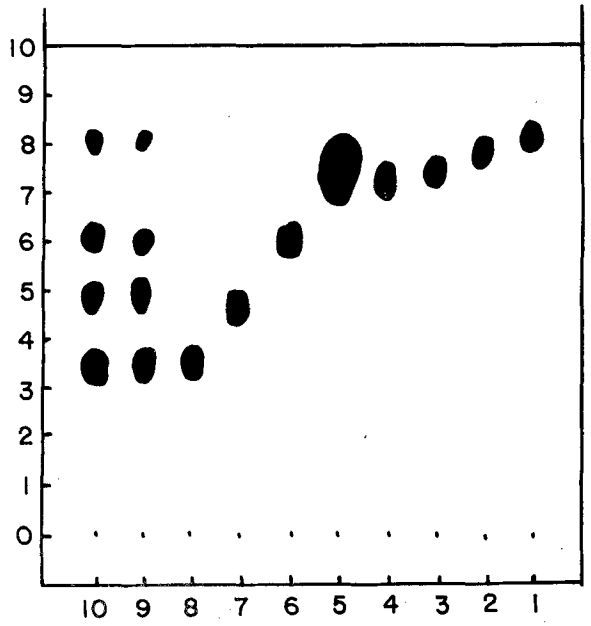

FIG. 1. Typical chromatogram showing separation of saturated and unsaturated fatty acids as brominated methyl esters. Solvent: Skellysolve B-diethyl ether, 96/4, v/v. 1. Methyl stearate. 2. Methyl oleate. 3. Methyl linoleate. 4. Methyl linolenate. 5. Mixture of 1, 2, 3, and 4. 6. Methyl dibromostearate.[a] 7. Methyl tetrabromostearate.[c] 8. Methyl henabromostearate. 9. Mixture of 1, 6, 7, and 8. 10. Mixture of 1, 2, 3, and 4 after bromination.

that the isomers with the higher dipole moments were adsorbed more strongly on the silica gel. The threo isomers being less symmetrical than the erythro, had a higher dipole moment. The increased dipole-dipole interaction between the vicinal pairs of bromine substituents and the hydrogen bond contributing type of adsorbent (silica gel) is of a higher order of magnitude in the threo isomers. This difference in migration of the two diastereoisomers is shown in Figure 2. As can be seen, mixtures of diastereoisomers in both series did not separate under the conditions used in the present studies. However, separation of pairs of diastereoisomers did occur when the hydroxy acids corresponding to oleic and linoleic were employed as the high polarity of the hydroxyl groups enhanced differences in the adsorption behavior of the diastereoisomers.

For the resolution of these more polar fatty acids, a polar solvent system was employed. As expected, the more hydroxyl groups a molecule contained, the more strongly it was retarded on the adsorptive material of the plate. Erythro isomers which had hydroxyl groups in positions where hydrogen bonding between the adsorbent and hydroxy groups occurred more easily were retarded longest. Erythro-, and threo-9,10-dihydroxystearic acids could be separated by thin layer chromatography (Fig. 3), their Rf values are given in Table II. Under conditions of

TABLE I

Thin-Layer Chromatography of Brominated Methyl Esters of Fatty Acids

Substances	Rf values
1. Methyl stearate	0.82
2. Methyl oleate	0.78
3. Methyl linoleate	0.75
4. Methyl linolenate	0.73
5. Methyl dibromostearate[a]	0.61
6. Methyl dibromostearate[b]	0.63
7. Methyl tetrabromostearate[c]	0.47
8. Methyl tetrabromostearate[d]	0.49
9. Methyl hexabromostearate	0.35

[a] Derived from oleic acid. [c] Derived from linoleic acid.
[b] Derived from elaidic acid. [d] Derived from linolelaidic.

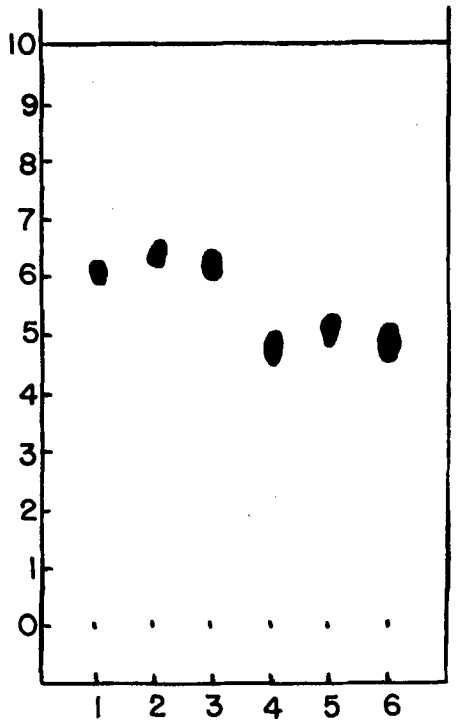

FIG. 2. Thin layer chromatography of methyl tetrabromostearates. Solvent: Skellysolve B-diethyl ether, 96/4, v/v. 1. Methyl threo-9,10-dibromostearate. 2. Methyl erythro 9,10-dibromostearate. 3. Mixture of 1 and 2. 4. Methyl threo-9,10,12,13-tetrabromostearate. 5. Methyl erythro-9,10,12,13-tetrabromostearate. 6. Mixture of 4 and 5.

the oxidation set forth by Robinson and Robinson (19) oleic acid produced the high melting (131C) whereas peracid oxidation yielded only the low melting (95C) 9,10-dihydroxystearic acid. On the other

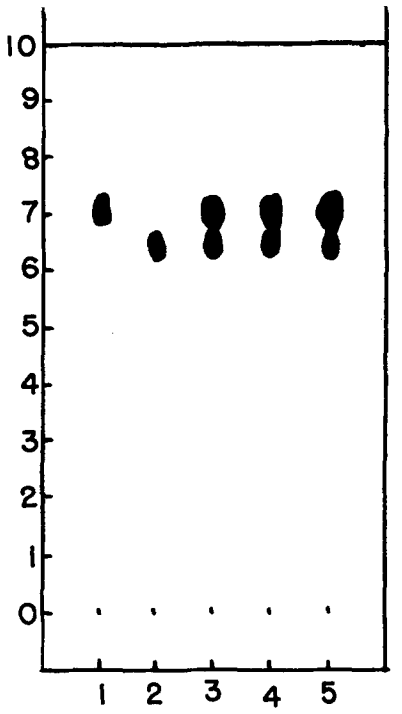

FIG. 3. Thin layer chromatographic separation of 9,10-dihydroxystearic acids. Solvent: Chloroform-methanol-acetic acid, 90/10/2, v/v/v. 1. Threo-9,10-dihydroxystearic acid. 2. Erythro-9,10-dihydroxystearic acid. 3. Mixture of 1 and 2. 4. Mixture of elaidic in oleic acid (10%) after alkaline permanganate oxidation. 5. Eutectic mixture of erythro in threo 9,10-dihydroxystearic (25%).

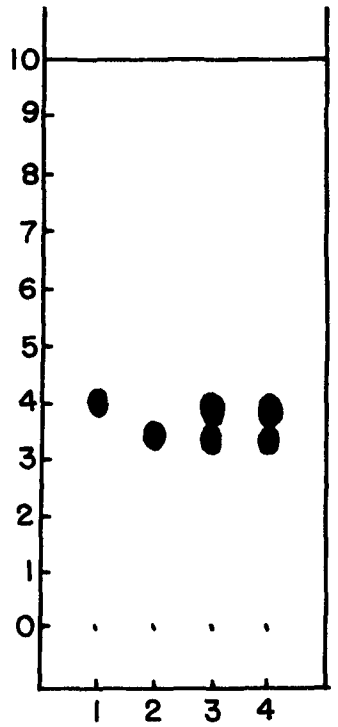

FIG. 4. Thin layer chromatographic separation of 9,10,12,13-tetrahydroxystearic acids. Solvent: Chloroform-methanol-acetic acid, 90/10/2, v/v/v. 1. Erythro, threo, erythro-9,10,12,13-tetrahydroxystearic acid. 2. Erythro, erythro, erythro-9,10,12,13-tetrahydroxystearic acid. 3. Eutectic mixture of 1 and 2. 4. Artificial mixture of 1 and 2.

hand, alkaline permanganate oxidation of elaidic acid produced exclusively the low melting 9,10-dihydroxystearic acid. With 9,10,12,13-tetrahydroxystearic acids, any procedure for their preparations which is based upon linoleic acid and which doesn't provide

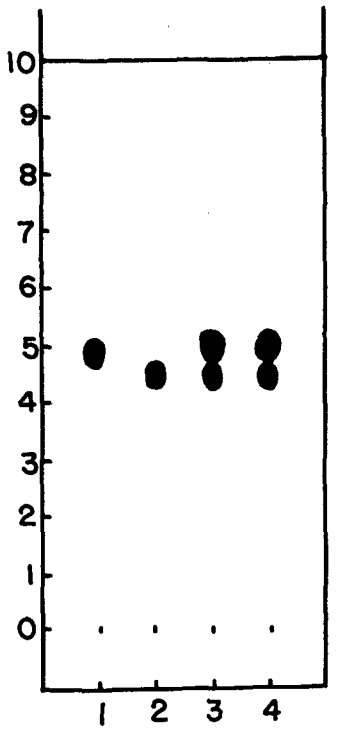

FIG. 5. Thin layer chromatographic separation of 9,10,12,13-tetrahydroxystearic acids. Solvent: Chloroform-methanol-acetic acid, 90/10/2, v/v/v. 1. Threo, threo, threo-9,10,12,13-tetrahydroxystearic acid. 2. Threo, erythro, threo-9,10,12,13-tetrahydroxystearic acid. 3. Eutectic mixture of 1 and 2. 4. Artificial mixture of 1 and 2.

TABLE II
Thin-Layer Chromatography of Di- and Tetrahydroxystearic Acids

Substances	Rf values
1. Threo-9,10-dihydroxystearic acid	0.70
2. Erythro-9,10-dihydroxystearic acid	0.65
3. Threo, threo, threo, threo-9,10,12,13-tetrahydroxystearic acid	0.48
4. Threo, erythro, threo-9,10,12,13-tetrahydroxystearic acid	0.44
5. Erythro, threo, erythro-9,10,12,13-tetrahydroxystearic acid	0.40
6. Erythro, erythro, erythro-9,10,12,13-tetrahydroxystearic acid	0.35

for maintaining a fixed stereochemical relationship between the 10 and 12 carbon atoms will result in a mixture of two diastereoisomers. A mixture of erythro, threo, erythro-9,10,12,13-tetrahydroxystearic acid (mp 174C) and erythro, threo, erythro-9,10,12,13-tetrahydroxystearic acid (mp 164C) were produced by *cis* addition and a mixture of thero, erythro, threo-(mp 148C) and threo, threo, threo-9,10,12,13-tetrahydroxystearic acids (mp 126C) by *trans* addition.

The Rf values for the separation of the pairs of tetrahydroxystearic acids which resulted from *cis* hydroxylation (dilute alkaline permanganate) of linoleic acids (Fig. 4) and the pair of acids resulting from *trans* hydroxylation (peracid oxidation) (Fig. 5) are given in Table II. Resolution of these pairs of hydroxy acids by thin layer chromatography provided a good criterion of the purity of these substances and a means of detecting eutectic mixtures. Witnauer et al. (20) in a study of the binary system of erythro- and threo-9,10-dihydroxystearic acids have shown eutectic formation at about 25% of the erythro form. The melting point of the eutectic mixture of 93C was only about 2C below that of the pure threo form. Obviously, the melting point was not a good criterion for the purity of this substance. However, a mixture of the above composition gave two spots on thin layer chromatography which corresponded to its components. The same was true for the eutectic mixture (mp 156C) of the two tetrahydroxy stearic acids of mp 174C and mp 164C and for the eutectic mixture (mp 122C) of the two acids mp 148C and 126C. Furthermore, the described method might be helpful in investigating the oily by-products resulting from the partial oxidation of linoleic acid by both permanganate and peracid routes.

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[Received June 15, 1962—Accepted November 26, 1962]