Cod Liver Oil Fatty Acids as Secondary Reference Standards in the GLC of Polyunsaturated Fatty Acids of Animal Origin: Analysis of a Dermal Oil of the Atlantic Leatherback Turtle

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Abstract

The use of the esters of whole cod liver oil fatty acids as secondary standards in the GLC identification of animal polyunsaturated fatty acids is feasible. This technique is exemplified by an analysis on several polyester substrates of the component fatty acids of a somewhat unusual marine-type oil from the Atlantic Leatherback turtle *Dermochelys coriacea coriacea* (*Linné*), with provisional identifications of minor components through the linear log plot and separation factor procedures.

Introduction

THE INCREASING USE of highly sensitive GLC detection systems means that routine analyses of lipid systems formerly regarded as simple may be complicated by small but significant amt of normal longer-chain polyunsaturated fatty acids. Thus in livestock or experimental animals fed fish meals as dietary supplements some of the characteristic fatty acids of marine lipids will be deposited in the depot fat. Particular lipids of microorganisms (1) and of commonly employed experimental animals may have complex fatty acid compositions as recently reported for mouse liver (2) or canine adrenals (3,4). Provisional identification of these materials, often present in such small proportions as to render isolation and degradation difficult, may be adequate for some experimental studies, and helpful as a guide in preliminary fractionation or in the application of further identification steps based on isolation and degradation.

Most common fatty acids from animal lipid systems and depot fats are remarkably homogeneous in that the double bonds are normally *cis*, follow a methylene-interrupted pattern in the polyunsaturated acids, and excepting certain C_{16} fatty acids in marine lipids, have the double bond farthest removed from the carboxyl group either three, six or nine carbon atoms removed from the terminal methyl group. This is only partly true, for example, in milk fats from ruminants (5), where *trans* double bonds and conjugated fatty acids occur, and animals fed structurally unusual fatty acids frequently deposit them unchanged in the depot fat. Certain animal lipid systems such as brain lipids are found to contain minor amt of numerous positionally unusual isomeric double bonds in addition to the characteristic hydroxy acids (6). If hydroxy or keto acids are present or suspected they may be provisionally identified in relation to the saturated acids by the equivalent chain length (ECL) system (7), but their removal by chromatographic procedures may then, in some instances, leave normal types of unsaturated fatty acids.

Subject to these limitations on the origin or nature of the sample the provisional identification of the normal polyunsaturated fatty acids is possible under certain conditions (cf. 8) on polyester columns by the ECL system. More generally the application of the linear log plot and separation factor procedures (8–12) offer a means of correlating normal polyunsaturated fatty acid structures on polyester columns independently of the type of polyester or operating conditions. The use of two polyesters of differing polarities eliminates most problems arising from chain length overlap or changes in the degree of chain length overlap associated with column ageing (13,14).

The ECL system, or the linear log plot and separation factor procedures as applied to the provisional identification of structurally normal polyunsaturated fatty acids in the GLC of samples of unknown composition, both require retention times for at least some of the longer-chain more highly unsaturated fatty acids in addition to readily available primary standards for commonly occurring fatty acid components such as 9-octadecenoic, 9,12-octadecadienoic, 9,12,15-octadecatrienoic and the normal saturated fatty acids. The longer-chain more highly unsaturated acids are not commonly available and their preparation may be difficult or prohibitively expensive (15–17) except as crude conc (11,18).

Naturally occurring fatty acid mixtures containing components which are well established as to identity and usually free from significant superimposed materials on GLC may be considered as secondary standards for comparison of relative retention times. As a secondary reference standard the use of whole marine oil methyl esters, especially of cod liver oil, offers several advantages. The starting material is inexpensive and readily available in a refined form substantially free of artifacts and oxidation products. Suitable esters may be prepared by transesterification since the chief non-saponifiable material, cholesterol, does not normally interfere with fatty acid ester GLC. The composition (19–22) of the major components (Table I), although subject to some seasonal variations, is sufficiently constant that the components in question may be recognized visually in most instances, simplifying preliminary identification. The presence of C_{16}, C_{18}, C_{20} and C_{22} monounsaturated fatty acids provides a good base line for the linear log plot, while the 5,8,11,14,17-eicosapentaenoic, 7,10,13,16,19-docosapentaenoic and 4,7,10,13,16,19-docosahexaenoic acids provide starting points for the application of separation factors in these series of acids.

The present demonstration of the use of cod liver oil esters as secondary reference standards for the provisional identification of fatty acids is applied to the analysis of a dermal oil of the Atlantic leatherback turtle *Dermochelys coriacea coriacea* (*Linné*). This is a low iodine value (I.V.) oil and found to be generally marine in character, but different from most marine lipid systems in having a high proportion of polyunsaturated fatty acids of the "linoleic" type. It is therefore more representative of animal fatty acid mixtures encountered in terrestial species.

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Experimental

Material. The turtle was taken at sea near Terence Bay, Halifax County, N.S., on September 11, 1961. The specimen was male, weighed 410 kg and measured 2.42 m overall. A sample of the dermal fat was taken from the mid-lateral region.

The fat sample was pale green in color and semi-solid at room temp. The material (8.8 g) was blended with light petroleum ether and anhydrous sodium sulphate in a Waring Blendor and the resulting suspension allowed to stand briefly before filtration and removal of the solvent.

The recovered oil (6.9 g) had the following properties: iodine value (Wijs) 96.7, n_D^{25} 1.46762, and non-saponifiables 1.5%. A sample of the oil was treated according to the Official and Tentative Method of the AOCS to determine the latter value. Part of the recovered fatty acids were converted to methyl esters with boron trifluoride-methanol reagent (23). The I.V. of the recovered esters as then employed in this study was 83.9. A subsequent further investigation of the handling procedures in this step (20) suggested that brief exposure to air had taken place with some oxidation of the sample. A further careful esterification of the remaining turtle oil fatty acids gave esters with an I.V. of 89.7 but comparison of GLC of the two samples indicated no substantial change in ester composition.

Gas-Liquid Chromatography. The basic apparatus used in the present study was a Barber-Colman Model 10. Columns were glass, 6 ft in length and 4 mm I.D., packed with either 20% diethylene glycol succinate (DEGS), type M 306, on 70–80 mesh Anakrom ABS, or 3% EGSS-X organosilicone polyester on 100–120 mesh Gas-Chrom P (polyester materials from Applied Science Laboratories, Inc., State College, Pa., USA). Operating conditions were: Column, 170C; injection port, 250C; detector cell, 230C; and argon pressure, 20 psig. The strontium 90 detector, type A-4147, was operated at full carrier gas throughput at 1,000 v and the electrometer at sensitivity 100 and appropriate attenuations. Another column employed in one experiment was packed with 3% SE-30 silicone polymer on 60–80 mesh Gas-Chrom Z. Other analyses were run in a Wilkens Aerograph using DEGS or neopentyl glycol succinate (NPGS) columns as described elsewhere (11).

Identification Procedure. The general procedure outlined permits the rapid setting up of relative retention time tables which include virtually all of the significant saturated, monounsaturated and polyunsaturated acids commonly found in animal lipids for any polyester column under any given operating conditions.

The methyl esters of the turtle fat were analysed on the DEGS column in the Barber-Colman apparatus giving a chromatogram essentially similar to that shown in Figure 1. Immediately following this analysis a small sample of linseed oil methyl esters, a sample of cod liver oil methyl esters and a sample of hydrogenated turtle oil methyl esters were run under the same conditions.

As a first step a list of adjusted retention times (i.e. measured from the air, solvent or first trace volatiles peak) relative to methyl octadecenoate (cf. Table II) was drawn up for all of the component peaks in the turtle oil analysis, for the C_{18} peaks in the linseed oil analysis, and for the major cod liver oil components as listed in Table I. The symbol *r* is used in this study to denote these adjusted relative retention times.

TABLE I

Major Components of Cod Liver Oil as Percentages of the Total

Fatty acid	Double bond positions	DeWitt (19) min	DeWitt (19) max	Ackman (20)	Kingsbury (21)	Klenk (22)
14:0	....	2.46	3.06	3.5	2.3	3
16:0	....	10.77	12.50	10.4	11.5	12
16:1	9[a]	6.56	10.31	12.2	7.8	5
18:1	9[a]	21.96	28.30	19.6	25.6	24
20:1	11[a]	9.69	17.01	14.6	11.7	9
20:5	5,8,11,14,17	7.19	12.03	5.0	8.2	8
22:1	11[a]	4.76	7.09	13.3	4.9	5
22:5	7,10,13,16,19	0.79	1.43	1.9	1.3	1
22:6	4,7,10,13,16,19	6.94	10.68	10.5	7.4	19

[a] Other isomers may be present.

Methyl octadecenoate is normally clearly distinguishable as a major peak in GLC of animal lipid fatty acid methyl esters. Visual comparison with the hydrogenated material analysis indicated which peaks in the cod liver oil esters were eicosenoate and docosenoate. Retention times were measured by the frontal tangent method (cf. 8) and in the case of overlapping peaks the leading edge of the second peak was determined by assumption of peak symmetry.

A linear log plot (8,11) was then drawn based on the established monoethylenic esters, the octadecadi- and octadecatrienoates, on the three polyunsaturated acid esters from the cod liver oil analysis and incorporating the C_{14}, C_{16} and C_{18} saturated ester *r* values. A number of *r* values from the turtle oil analysis could be obviously fitted into the log plots as peaks possibly corresponding to heptadecanoate, heptadecenoate, etc., while the *r* value of 2.37, taken as a C_{20} acid, was colinear with that for 9,12-octadecadienoate, immediately suggesting that the former peak was 11,14-eicosadienoate. Similar linear extrapolations of the log plot were made as further points could be plotted from the separation factor data (see below).

The *r* values for 9-octadecenoate, 9,12-octadecadienoate and 9,12,15-octadecatrienoate as determined from the linseed oil methyl esters provided accurate values for the 3/6, 6/9 and 3/9 type II separation factors. These systematic separation factors are in this instance (type II) defined by ratios of the number of carbon atoms between the center of the ultimate double bond and the terminal methyl group, inclusive, for acids of the same chain length where the first double bond is in a common position relative to the carboxyl group. The greater *r* value is divided by the lesser to obtain the numercial factors, in this case respectively 1.37, 1.27 and 1.73. Similarly the type I factors relate acids having ultimate double bonds in a common position relative to the terminal methyl group, while the type III factors relate acids with positionally isomeric groups of the same numbers of double bonds (9, 10–12).

Using these values a substantially complete table of *r* values was drawn up working initially from the established indentifications. For example, the known *r* of 5,8,11,14,17-eicosapentaenoate was 4.37. The 3/6 separation factor gave a calculated *r* of 3.19 for the biologically important 5,8,11,14-eicosatetraenoate and a large peak was found in this position (Fig. 1). Inspection of the linear log plot showed two points linearly related if taken as C_{18} and C_{20} acids (*r* 1.51 and r 2.80) for which the peaks occurred in positions suggesting that they were precursors (24) of 5,8,11,14-eicosatetraenoic acid, respectively 6,9,12-octadecatrienoate and 8,11,14-eicosatrienoate. On application of the type II separation factors confirmation for this suggestion was found in agreement for the peaks colinear on the log plot at *r* 2.06 and *r* 3.84, respectively, as 6,9,12,15-octadecatetraenoate and 8,11,14,17-eicosa-

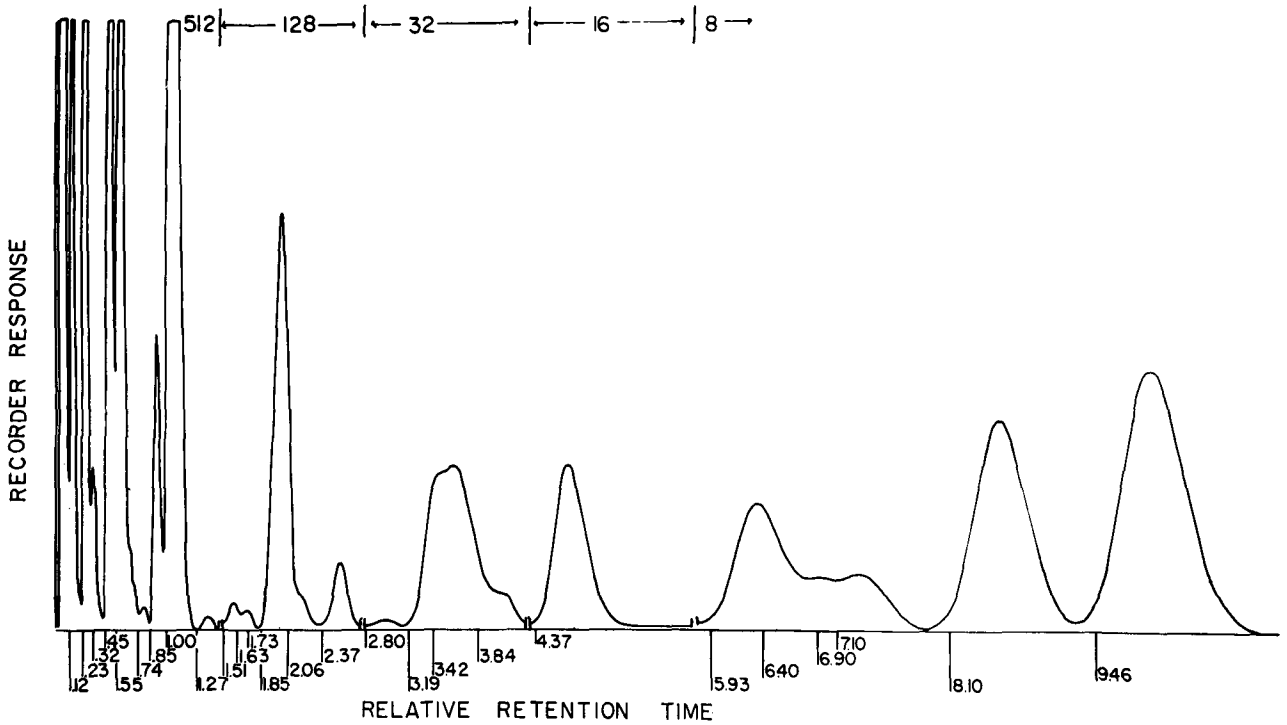

FIG. 1. Turtle oil methyl esters analysed on a DEGS column (20% on 70–80 mesh Anakrom ABS). Argon input 20 psig at 170C. Sample 0.005 ml of approx 1% solution in neohexane. Voltage 1,000 at electrometer setting S-100. Attenuations marked at top, retention times relative to methyl octadecenoate at bottom.

tetraenoate. Subsequently these four components were related to others through the types I and III separation factors.

It is thus possible to rapidly build up a comprehensive correlation of observed peaks and possible fatty acid structures using the linear log plot and the types I, II and III separation factors. Certain approximations may be made where necessary (10) but the three types of separation factors are mutually self-checking. In some cases retention times could be calculated for possible components and either the predicted peak position was obscured by another component, or no peak was found. In such instances the *r* values in Table II have been placed in parentheses.

Owing to the probability of chain length overlap and peak coincidence in complex lipid mixtures another polyester analysis is necessary in most cases. This can be the same polyester on a different support (12,25), but generally a completely different column is desirable to ensure a substantially different polarity. In the present study the same series of analyses were carried out on the EGSS-X column (Fig. 2) and the same approach followed with the linear log plot and separation factor calculations. A number of conflicting identifications provisionally assigned to peaks in the one polyester analysis could be resolved by reference to the other. For example, on the EGSS-X column the positions calculated for 5,8,11-eicosatrienoate and 11,14-eicosadienoate coincide, whereas in the DEGS analysis it is clear that no 5,8,11-eicosatrienoate is present. Similarly the presence of a tetracosenoate ester is confirmed, although on the EGSS-X column this peak could have been a possible 10,13,16,19-docosatetraenoate. The two polyester analyses also provide a clear means of distinguishing docosatetraenoate esters from 4,7,10,13,16-docosapentaenoate (26).

The apparent absence of polyunsaturated C_{16} acids was somewhat surprising in a marine type depot fat, hence the turtle oil methyl esters were analysed on a NPGS column since even numbered chain lengths do not overlap on this polyester (11). No significant amounts of these acids were found. This column also gave somewhat better separation of branched chain fatty acids in the hydrogenated sample and small amt (0.1% or less), respectively, of C_{15}, C_{16}, C_{17} and C_{18} *iso*acids were tentatively identified by separations from the corresponding normal acid esters (11). Another peak may have been a multiple-branched C_{17} acid.

The C_{24} acids gave very flat peaks in the polyester analyses but the proposed identifications were satisfactorily corroborated by analysis on the SE-30 column, where the peak order is reversed in a fashion similar to Apiezon column separations.

Most of the provisional identifications assigned to observed components were regarded as satisfactory in agreeing with marine lipid fatty acid analyses and known fatty acid metabolic pathways (see below). However, 11,14,17-eicosatrienoate (1,27), a possible component, and 5,8,11,14-eicosatetraenoate normally nearly coincide in polyester analyses. Since this was a surprisingly large peak for a marine lipid analysis a large sample was injected into the Aerograph (DEGS column) and sufficient material collected to verify by alkali isomerization that only tetraene absorption existed for this peak. The 8,11,14,17-eicosatetraenoate peak was collected as a control.

Quantitative Analyses. Examination of standard mixtures (Courtesy of National Institutes of Health, Bethesda, Md., USA) of the C_{14} to C_{24} even chain acid esters suggested approximately mole per cent, and not wt percentage, response (cf. 28) at the sample loads (ca. 10^{-6} g/component) involved. The same detector in a different configuration subsequently again gave similar results for the saturated acid esters (29).

The turtle oil methyl esters were analysed at three different load levels to keep individual components at or below the desired level per component and the integrated areas were then proportionated through peaks of intermediate size. The wt percentages in Table II

TABLE II

Relation of Observed GLC Peaks to Fatty Acid Structures, Retention Times and Per Cent Composition

Fatty acid	Double bond positions	Relative retention time		Wt percentage		Remarks
		DEGS	EGSS-X	Mole % response	Factor response[b]	
12:0	....	0.12	0.10	10.2	10.2	
13:0	....	(0.17)	0.15	0.1	0.1	
14:0	....	0.23	0.21	18.7	14.2	
14:1	?	(0.29)	0.28	0.4	0.3	
15:0	....	0.32	0.30	0.3	0.2	
15:1	?	(0.40)	(0.38)			No significant amount present
16:0	....	0.45	0.43	15.7	11.8	Includes 0.1% branched chain C_{17} acid
16:1	9[a]	0.55	0.54	7.9	8.6	
16:2	6,9	(0.66)	0.62	....	....	Coincides with 17:0 on EGSS-X
16:2	9,12	(0.74)	0.71	0.2	0.3	
16:3	6,9,12	(0.88)	(0.81)	....	....	No evidence for this material
16:4	6,9,12,15	(1.19)	(1.03)	....		No evidence for this material
17:0	....	0.62	0.62	0.5	0.3	
17:1	?	0.74	0.75	0.2	0.2	
18:0	....	0.86	0.89	3.5	3.1	
18:1	9[a]	1.00	1.00	22.7	29.2	
18:2	6,9	(1.19)	(1.15)	....	....	No evidence for this material
18:2	9,12	1.27	1.27	0.6	0.9	
18:3	6,9,12	1.51	1.46	0.5	0.6	
18:3	9,12,15	1.73	1.68	....	....	No significant amount present
18:4	6,9,12,15	2.06	1.95	0.1	0.1	
19:0	....	(1.18)	1.27	....	....	No significant amount present
19:1	?	1.36	1.40	0.3	0.3	
20:0	....	1.63	1.81	0.2	0.2	
20:1	11[a]	1.85	1.95	3.6	4.2	
20:2	8,11	(2.22)	(2.24)	....	....	No evidence for this material
20:2	11,14	2.37	2.47	0.5	0.6	
20:3	5,8,11	(2.52)	(2.47)	....	....	No significant amount present
20:3	8,11,14	2.80	2.83	0.3	0.3	
20:3	11,14,17	(3.22)	(3.28)	....	....	No evidence for this material
20:4	5,8,11,14	3.19	3.16	1.7	1.6	
20:4	8,11,14,17	3.84	3.76	0.8	0.8	
20:5	5,8,11,14,17	4.37	4.20	2.8	3.4	
22:0	....	(3.10)	(3.65)	....	....	No significant amount present
22:1	13[a]	3.42	3.76	2.2	1.8	
22:4	7,10,13,16	5.93	6.20	0.6	0.7	
22:5	4,7,10,13,16	6.90	7.15	0.2	0.3	
22:5	7,10,13,16,19	8.10	8.26	1.4	1.6	
22:6	4,7,10,13,16,19	9.46	9.50	2.0	2.2	
24:1	15[a]	6.40	7.35	0.2	0.2	
24:6	6,9,12,15,18,21?	17.5	18.5	1.6	1.7	

[a] May include other isomers.
[b] Correction fractors (22) applied.

are derived from a) assumption of mole percentage response for all components (30–32), and b) the use of literature specific correction factors (22) obtained on the same type of apparatus for these materials or homologues. The mole percentage calculation gives an I.V. of 79.1, and the specific factor responses an I.V. of 90.0 (vs. 83.9 found). Since the proportions of polyunsaturated longer-chain fatty acids are small (Table II) the errors in relative area measurement are probably as significant as those involved in the response corrections and the true proportions of fatty acids intermediate to those listed in Table II. For materials involving higher proportions of polyunsaturated acids an empirical correction system (29) was subsequently evolved for this detector.

Discussion

Cod Liver Oil. The biosynthetic pathways by which higher marine organisms convert 9,12-octadecadienoic and 9,12,15-octadecatrienoic acids into the more highly unsaturated longer-chain acids do not apparently differ from those found in mammals (24,33–37). The structural homogeneity of marine lipid fatty acids has recently been reviewed on this basis (38–40). The use of the major fatty acid components of marine oils as reference standards in most analyses of other animal lipids is therefore entirely feasible.

Although several analyses of cod liver oil have been published (19–22) there are some differences in the minor components indicated by various authors. It is thus better to examine the unknown sample for minor components by the linear log plot and separation factor techniques than to rely on comparisons with the corresponding peaks of components reported for the cod liver oil esters, since the observed peaks depend on polyester and support (12,40), age of column (13,14) and operating temp (41). Should, however, the sample being analysed not have a sufficient variety of components for the application of the log plot and separation factor procedures, then the retention times found or calculated from the cod liver oil analyses will normally be found sufficiently accurate. The use of two polyester columns of differing polarities, as shown in the case of the turtle oil, permits recognition of such important acids as 5,8,11,14- and 8,11,14,17-eicosatetraenoates, present in cod liver oil to the extent of ca. 1.0 and 0.5%, respectively, even in the presence of docosenoic acid (34,41). Octadecanoate peaks in marine lipid analyses are often distorted by superimposed C_{16} polyunsaturated ester peaks, and 9,12,15-octadecatrienoate is present only in trace amt, hence the relative retention times of these acids are best determined from the linseed ester analyses. The absolute retention times in the latter mixture may differ owing to the change in sample size, etc., but the retention times relative to octadecenoate are not altered.

Turtle Oil. The Atlantic leatherback turtle is regularly seen in Nova Scotia waters in summer but has no commercial value and little is known about it. The diet may consist of algae, jellyfish, crustacea, etc. (42). In analyses of lipids of amphibia, as has been pointed out (43), a distinction must be drawn between the fatty acid compositions determined on specimens kept for long periods in captivity on artifical diets, and those taken in the natural habitat.

Although the possible variations in percentage composition of this oil in terms of particular fatty acids or chain lengths as given are unfortunate, the results in this paper compare generally with previous observations (44) on the fat of the green turtle *Chelone mydas mydas* (*Linné*). The latter, however, is not truely pelagic and is primarily herbivorous. The amt of dodecanoic acid in the fat of the green turtle

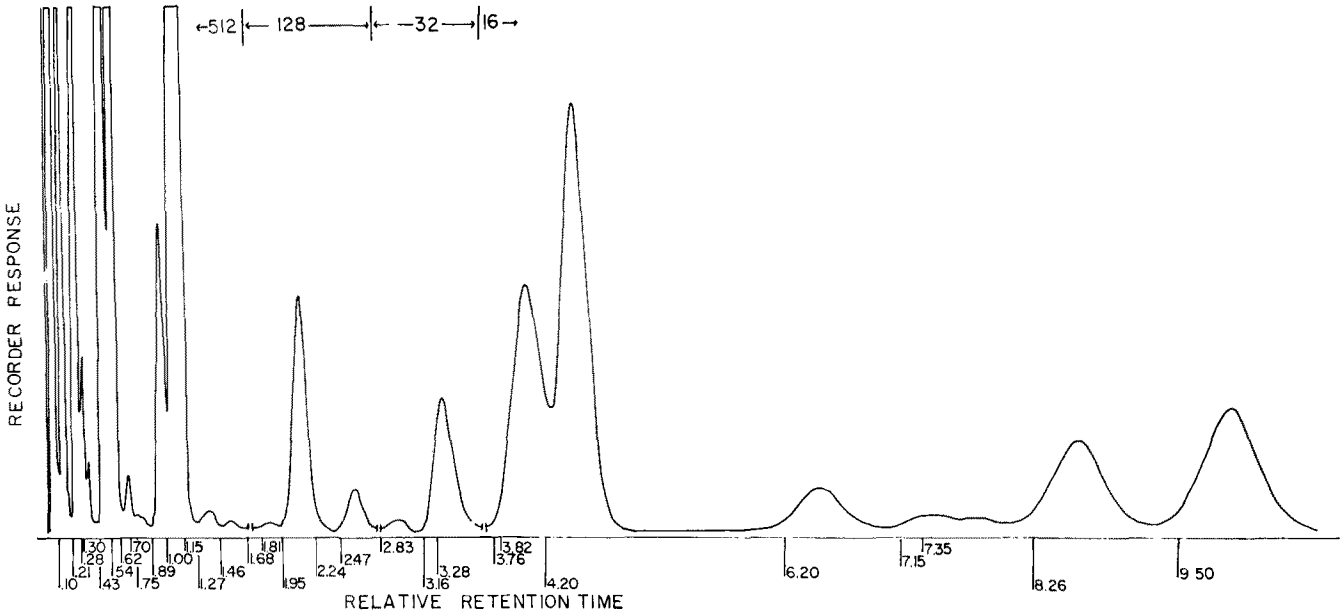

FIG. 2. Turtle oil methyl esters analysed on EGSS-X organosilicone polyester column (3% on 100–120 mesh Gas-Chrom P). Argon input 20 psig at 170C. Sample size 0.005 ml of approx 1% solution in neohexane. Voltage 1,000 at electrometer setting S-100. Attenuations marked at top, retention times relative to methyl octadecenoate at bottom.

is also usually stated to exceed the amt of tetradecanoic acid, differing from the present observations. The C_{20} acids and C_{22} acids reported (43) for an Indian Ocean turtle *Eretmochelys imbricata sqaumata* (*Agassiz*) are also comparable, although this omnivorous type contains only traces of dodecanoic acid in the fat.

It must be pointed out that the dermal fat of this species may be primarily intended for buoyancy, and hence be different in composition from the more metabolically active subcutaneous fats. When compared to marine oils in general, the presence of dodecanoic acid and of a high percentage of tetradecanoic acid are noteworthy. The comparatively high proportion of C_{20} and C_{22} tetraene acids in proportion to other acids of these chain lengths is also unusual, although the ratio of 5,8,11,14-eicosatetraenoic acid to the 8,11,14,17 isomer is not exceptional.

Summary

The present study has presented in some detail the use of different polyesters and of systematic approach to provisional peak identification in the GLC of normal fatty acid esters of complex animal lipid systems. It is suggested that marine oils such as cod liver oil could be employed as secondary reference standards in the GLC of polyunsaturated fatty acid mixtures of animal origin. In the present case, the use of auxiliary techniques has been limited, but the employment of prior fractionation techniques to eliminate chain length overlap, or of isolation and detailed examination of each component may equally well be benefitted by consideration of the probable identifications obtained by these procedures.

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