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Wax Esters of Mullet (*Mugil cephalus*) Roe Oil

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ABSTRACT: Lipids of mullet roe were separated and analyzed by liquid and gas chromatographic methods. Nearly 70% of the oil consists of wax esters in which saturated and monounsaturated fatty alcohols are bound to fatty acids with up to six double bonds. Alcohols and acids from wax esters were identified and quantified. Both contain appreciable amounts of odd-numbered straight-chain compounds. The structures of monoenoic alcohols are the same as those of monoenoic acids, *e.g.*, 9-heptadecenoic, 9- and 11-octadecenoic, and nonadecenoic. The total content of odd-numbered alcohols was between 10 and 25% while that of odd-numbered acids was consistently somewhat lower. Several polyunsaturated acids such as 4,7,10,13,16-heneicosapentaenoic or 4,7,10,13,16,19-docosahexaenoic and related acids were newly identified from mullet. They occur more in wax esters than in triglycerides of roe or of the body oil.

The fatty acids in wax esters of mullet are more typical for marine oils than those in any other wax esters of marine source so far reported. The extensive combination of polyunsaturated acids with saturated or monounsaturated alcohols emphasizes strongly the limitation of unsaturation in the latter. With acids being the most likely precursor for these alcohols, the biological reduction apparently cannot take place with polyenoic acids. Gas–liquid partition chromatography of hydrogenated wax esters showed that the alcohols and acids are combined to form C_{30} to C_{40} esters, with maximum amounts for C_{32} and C_{34}. The combination of chain lengths was random with a sample which contained about 10% each of C_{14} and C_{15} alcohol, but it was not random when each of these alcohols occurred at the level of 20%.

In a recent study of the fatty acids of mullet (*Mugil cephalus*) oil in this laboratory it was found that they contain 15–30% straight-chain odd-numbered components (Sen and Schlenk, 1964). The oil samples investigated had been obtained from the whole fish or from the fillets, and they consisted mainly of triglycerides. It appeared of interest to check the distribution of odd-numbered fatty acids in specific parts of the body and in lipid classes other than triglycerides. In the course of such investigation it was found that wax esters represent the major portion of the lipids in roe. The structures of fatty alcohol and acid components of the waxes are reported here and their possible biological correlation is discussed.

Procedures and Results

Mullet was caught on the coast of the Gulf of Mexico near Pascagoula, Miss., in the fall of 1964 and 1965. Fish containing roe were about 28–30 cm long, weighed 250–500 g, and had between 15 and 55 g of roe.

Lipids were extracted from the roe with $CHCl_3$–CH_3OH (2:1) in an Omni-Mixer. About 20% of the wet weight was obtained as a yellow oil which partly solidified in the refrigerator. Thin layer chromatography on silicic acid with hexane–diethyl ether–acetic acid (85:15:1) indicated the presence of wax esters, triglycerides, acids, alcohols, free cholesterol, and cholesterol esters, listed in order of decreasing amounts. A small

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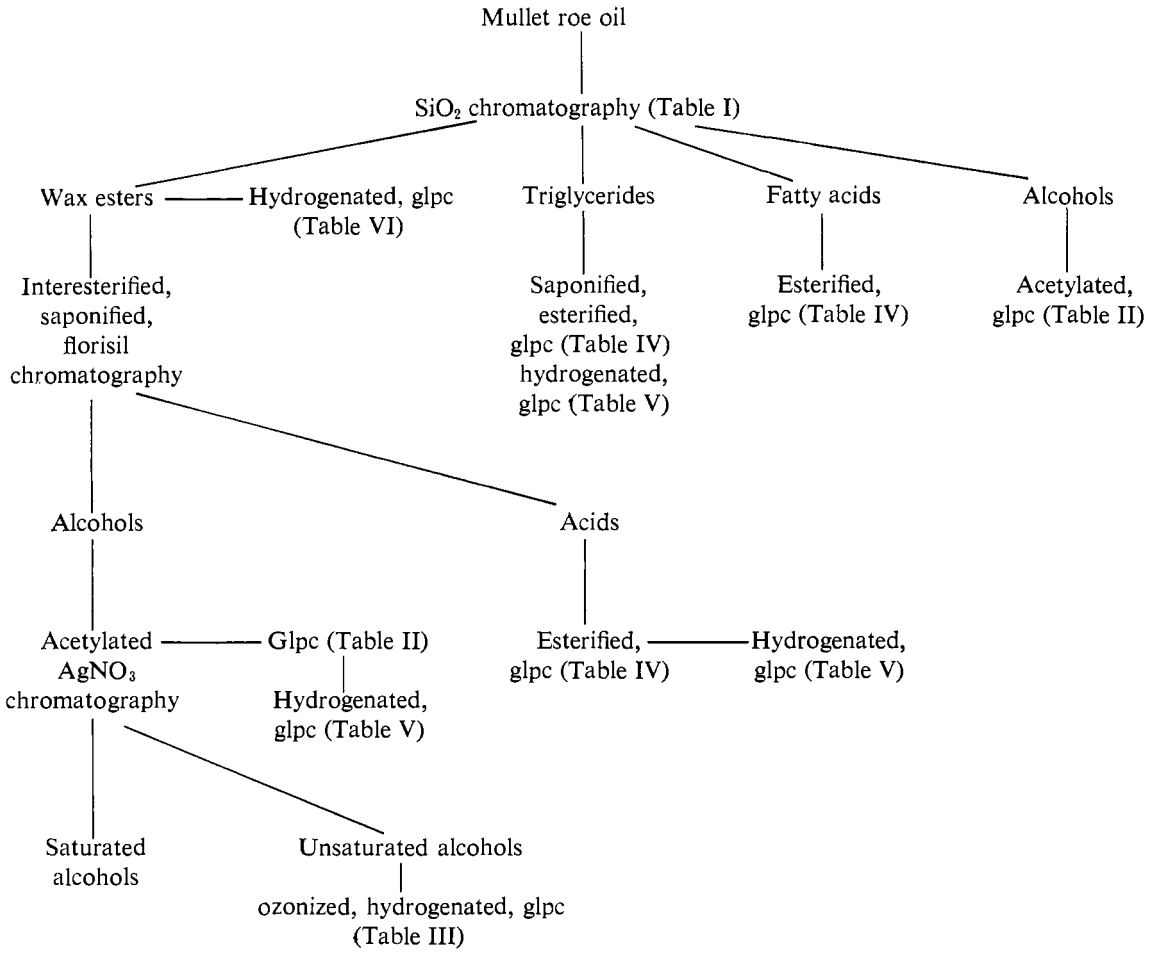

FIGURE 1: Summary of fractionation and identification procedures.

amount of more polar lipids remained at the starting point.

The scheme of fractionations and identifications is outlined in Figure 1 which also refers to the tabulation of results. Chemical and analytical procedures used have been reported adequately in the literature so that only a few details need to be described here.

A typical example for fractionation of roe lipids into classes is given in Table I. The separation of wax esters from cholesterol esters was not quite complete but the very minor amount of the latter did not interfere with the analyses of the wax esters. Values for cholesterol, its esters, and phospholipids were also obtained from the total oil by other common methods and they were in reasonable agreement with the chromatographic data. The polar lipids were not further investigated.

Analysis of Fatty Alcohols. Procedures used were essentially those described by Nevenzel *et al.* (1965). The wax esters were interesterified with CH_3OH by use of $NaOCH_3$, and the resulting methyl esters were saponified with KOH. Acids and alcohols were then separated from each other on a column of Florisil. An experiment using 190 mg of wax esters yielded 65 mg of alcohols and 90 mg of acids as pure fractions.

The alcohols were converted into acetates and analyzed by gas–liquid partition chromatography (glpc). Ethylene glycol succinate (20%) stabilized with 2% phosphoric acid on Gas Chrom P (100–120 mesh) was the preferred phase in a column 6 ft × 1/8 in. using a F & M 810 instrument at 180° with flame ionization detector. Authentic saturated and mono- and diun-saturated fatty alcohol acetates were prepared for identification according to retention times. Table II lists the results for wax alcohol acetates from two roe samples and for the acetates of free alcohols contained in one of them. Infrared spectra did not indicate the presence of *trans* double bonds.

Saturated alcohol acetates were eliminated by column chromatography on $AgNO_3$–SiO_2–Celite, approximately 3:7:5 (De Vries, 1963; Subbaram and Youngs, 1964). After exploring proper conditions with model mixtures, 290 mg of alcohol acetates from roe wax esters were chromatographed on 40 g of adsorbent mixture with hexane (Skellysolve B) containing 15, 30, and 45% benzene. About 40 mg of monoenoic alcohol acetates was obtained from the chromatogram. Traces of saturated acetates had trailed into this fraction but they were irrelevant in the further treatment of the

TABLE I: Fractionation of Mullet Roe Oil into Lipid Classes.[a]

Fraction	Recov (% of total)
Wax esters	67
Cholesterol esters	0.3[b]
Triglycerides	8
Free acids	3
Free alcohols	2
Free cholesterol	2[b]
Polar lipids	10[b]
Not recovered	8

[a] Oil (700 mg) was chromatographed on a column of SiO_2, 1.8 × 40 cm, with hexane (Skellysolve B) containing 1%, then 4, 8, and 25% diethyl ether and, eventually, with 100% diethyl ether. [b] Values obtained from total oil by other common methods are cholesterol ester, 0.7%; free cholesterol, 1.8%; phospholipids, 7.6%.

monoenes. In agreement with the foregoing analyses by glpc, dienoic alcohol acetates were not detected when the $AgNO_3$ column was washed with 100% benzene and finally with diethyl ether.

The monoenoic acetates were separated according to chain length by glpc on a column, 8 ft × 3/8 in., packed with 20% β-cycloheptaamylose valerate (Schlenk *et al.*, 1962) on Chromosorb P (30–60 mesh). A Beckman GC-2 apparatus with thermoconductivity detector was used at 220° with 50-psi inlet pressure of He. The fractions were collected by gradient cooling (Schlenk and Sand, 1962) and subjected to ozonization–hydrogenation (Schlenk and Gellerman, 1965; Sand *et al.*, 1965). The resulting aldehyde–alcohol acetates and aldehydes were analyzed by glpc in reference to authentic compounds. The column of ethylene glycol succinate was used for identifying aldehyde–alcohol acetates under conditions as described above for long-chain alcohol acetates. Aldehydes were analyzed on a column, 10 ft × 0.25 in., of 20% Carbowax 4000 on Chromosorb W (60–100 mesh) at 160°.

The identifications and some retention values are listed in Table III. Proportions of isomers were estimated and are given in parentheses.

TABLE II: Composition of Alcohols.[a]

Alcohol[b]	From Wax Esters		Free
	Roe 1	Roe 2	Roe 2
14:0	21.7	9.9	10.4
15:0	18.6	6.2	7.7
16:0	42.0	54.3	61.6
16:1	6.5	14.4	7.0
17:0	1.6	1.7	2.2
17:1	2.8	1.5	1.3
18:0	2.8	6.4	5.2
18:1	3.3	5.3	3.8
19:0	0.2	0.2	0.3
19:1	0.5	0.2	0.6
$C_{15} + C_{17} + C_{19}$	24	10	12

[a] Weight per cent ≈ area per cent, using a flame ionization detector (Horning *et al.*, 1964). [b] Chain length:number of double bonds.

TABLE III: Identification of Monoenoic Alcohol Acetates from Wax Esters.[a]

Fragments, C Atoms		
Aldehyde–Acetate[b]	Aldehyde	Original Compds[c]
9	7	9–16:1[d]
9 ≫ 11	8 ≫ 6	9–17:1[d] ≫ 11–17:1[e]
9, 11	9, 7	9–18:1,[d]11–18:1[d] (2:3)
9, 11	10, 8	9–19:1,[e]11–19:1[d] (1:7)
11, 13	9, 7	11–20:1,[d]13–20:1[d] (2:3)

[a] Roe sample 1 of Table II. [b] Equivalent chain length (Miwa *et al.*, 1960) for aldehyde–alcohol acetates are C_9, 9.05, C_{11}, 11.1, C_{13}, 13.2, on ethylene glycol succinate at 180°. [c] Position of double bond, counting from the functional group–chain length:number of double bonds. [d] Acids of these structures have been identified in earlier work (Sen and Schlenk, 1964). [e] Acids of these structures have not been identified in earlier work but their presence in minor amounts has not been ruled out.

Analysis of Fatty Acids. Fatty acids from wax esters, triglycerides, and the free acids of roe lipids were converted into methyl esters (Schlenk and Gellerman, 1960) and analyzed as such by a two-step glpc procedure. Between 10 and 50 mg of esters were separated first by chain length on β-cycloheptaamylose valerate and collected. The uniformity of chain length in these fractions was checked by hydrogenation of an aliquot and subsequent glpc. The fractions were then chromatographed on ethylene glycol succinate to determine their equivalent chain length (Miwa *et al.*, 1960). The actual chain length of the components was known from the first chromatogram so that identification by comparison with authentic samples (Sen and Schlenk, 1964; Hofstetter *et al.*, 1965) was possible for most of them. However, isolation of individual esters or of isomeric mixtures was necessary in some cases for subsequent identification by ozonization. They were isolated on larger scale by liquid–liquid chromatography

and subsequent preparative glpc (Sen and Schlenk, 1964).

Altogether more than 50 acids were identified from the roe lipids, but for brevity only part of them are listed in Table IV. The acids omitted are mostly minor components. They are insignificant for the later discussion and have been reported previously as components of mullet oil. However, several new acids have been identified which had not been reported from mullet, and they are incorporated into Table IV. After finding them in roe, they were also isolated after appropriate fractionation from body oil (Schlenk and Sand, 1966) and were identified by ozonization–hydrogenation.

TABLE IV: Fatty Acids in Mullet Roe Lipids.[a,b]

Acid[c]	Wax	Triglycerides	Free Fatty Acids
14:0	1.0	6.9	3.1
15:0	0.4	3.3	1.4
16:0	4.1	24.6	18.3
9–16:1	23.3	23.8	33.4
9,12–16:2	4.8	1.0	1.0
6,9,12–16:3	2.2	0.3	0.1
17:0	0.1	0.6	0.6
9–17:1	2.2	2.3	3.4
18:0	0.8	2.9	3.8
9–18:1	12.8	14.5	15.8
9,12–18:2	3.8	1.8	2.1
6,9,12–18:3	1.2	0.4	0.5
9,12,15–18:3	2.2	0.4	0.4
6,9,12,15–18:4	3.1	0.7	0.2
8,11,14–20:3[d]	0.4	0.2	0.1
5,8,11,14–20:4	1.3	0.6	1.7
8,11,14,17–20:4	2.4	0.7	0.5
5,8,11,14,17–20:5	8.8	2.2	2.8
7,10,13,16–21:4[d]	0.5	0.1	0.3
4,7,10,13,16–21:5[d]	3.4	0.2	0.3
7,10,13,16–22:4[d]	0.2	0.3	0.3
4,7,10,13,16–22:5[d]	0.4	0.3	0.3
7,10,13,16,19–22:5[d]	4.4	2.7	1.3
4,7,10,13,16,19–22:6[d]	5.9	3.2	1.9

[a] Incomplete listing. [b] Glpc area per cent of methyl esters using a flame ionization detector. [c] Position of double bond, counting from the functional group–chain length: number of double bonds. [d] Acid newly reported for mullet.

It is expedient to refer to chain length rather than to individual compounds when comparing the amounts of odd- and even-numbered components. Such data were obtained by hydrogenation followed by glpc and are listed in Table V. Alcohols and acids of wax esters from three samples of roe were analyzed by chain length and, in addition, data are given for the acids from body oil of one of the fish which had yielded the roe. The body oil consists mainly of triglycerides so that these values can be taken as characteristic for them

Wax esters of two samples were hydrogenated and subjected to glpc in order to check for a specific pattern according to which odd and even chains may be combined. Glpc operating conditions were similar to those described for triglycerides by Litchfield *et al.* (1965). A glass column (6 ft × 1/8 in.), packed with 3.0% JXR on Gas Chrom Q (100–120 mesh), was programmed from 150 to 320° at 4°/min with a flow of 100 cc/min of He, using a flame ionization detector. Table VI shows data for hydrogenated wax esters by glpc and values calculated for these samples from Table VI assuming a statistical combination of all chain lengths.

Discussion

Oil from salted and dried mullet roe was investigated by Tsujimoto (1933) and a more detailed analysis of fresh mullet ovary oil was published by Kafuku and Hata (1934). Nearly one-half of the oil was unsaponifiable lipids from which mainly cetyl but also several other common fatty alcohols were identified. Besides saturated and monosaturated acids, the presence of polyenoic acids in the oil was indicated.

Although odd-numbered compounds were not identified at that time, the data are in sufficient accord with ours to conclude that the earlier authors had obtained from *Mugil japonicum* essentially the same material as we had from *Mugil cephalus*. The fatty alcohols which Kafuku and Hata had recognized came from wax ester which we know now represent 60–70% of lipids in the roe (Table I).

The wax esters are specifically located in the roe and do not occur elsewhere in the fish. Oil of total mullet, taken at different times of the year, showed that during the reproductive season a pronounced increase occurs of a thin layer chromatography (tlc) spot which corresponds to that of wax esters (Figure 2). Besides, such a spot was not encountered with body oil of the specimen from which the roe sample 3 had been taken.

The wax esters of mullet offer three novel features: (a) their occurrence is limited to the roe; (b) they have a higher content of polyunsaturated acids than ever reported for marine waxes, although they contain only saturated and monosaturated alcohols; (c) they have odd-numbered components at an unusually high percentage in alcohols as well as acids. These points will be discussed in sequence, followed by some considerations on the combination of alcohol and acid components.

(a) The classical source for marine waxes are lipids of the head cavity and of blubber of sperm whale. However, wax esters are found also in *Ruvettus pretiosus* (castor oil fish) (Nevenzel *et al.*, 1965), and *Latimeria chalumnae* (a coelacanth) (Nevenzel *et al.*, 1966), where they represent about 90% of lipids in total oil. The wax esters may be a phylogenetic vestige which is still prominent in *Ruvettus* and *Latimeria* as living fossils of early vertebrate development and which occurs in

TABLE V: Chain-Length Composition of Alcohols and Acids from Wax Esters and of Acids from Body Oil.

C Atoms	Wax Esters[a] Roe Sample 1 (mole %) Alcohol	Acid	Roe Sample 2 (mole %) Alcohol	Acid	Roe Sample 3 (mole %) Alcohol	Acid	Total Fish Oil, without Roe Sample 3 (mole %) Acid
14	23.4	3.0	11.0	1.7	8.0	1.1	5.2
15	19.1	2.4	6.5	1.0	16.8	2.0	15.6
16	47.3	34.8	68.6	40.7	57.6	36.8	41.2
17	4.1	14.0	3.1	4.4	6.5	17.5	10.6
18	5.4	24.7	10.6	25.6	7.8	18.2	13.3
19	0.6	2.3	0.3	2.0	2.1	3.7	2.3
20	+	9.2	+	12.1	1.2	12.4	6.3
21	+	1.5	+	3.4		1.5	1.1
22	+	8.2	+	9.1		6.8	4.4
$C_{15} + C_{17} + C_{19}$	23.8	18.7	9.9	7.4	25.4	23.2	28.5

[a] Mole per cent calculated from weight per cent ≈ area per cent, using a flame ionization detector (Horning *et al.*, 1964).

mullet only in early embryonic development.

Wax esters in fish roe may be a feature more common than presently known. The roe from five species has been investigated and, in one case, *Cynoscion nebulosus* (spotted weakfish), it was found that wax esters and triglycerides occur in about the same proportions. A further survey may contribute to the phylogenetic aspect.

TABLE VI: Chain-Length Composition of Wax Esters.

C Atoms	Sample 1, Mole % Ester[a] by Glpc	Calcd (%)[b,c]	Sample 2, Mole % Ester by Glpc	Calcd (%)[b]
30	5.0	10.0	5.5	5.6
31	6.2	11.1	4.0	3.9
32	23.0	24.2	30.4	31.2
33	10.2	13.3	7.0	6.3
34	17.9	16.7	21.6	23.5
35	5.4	5.0	3.0	3.9
36	12.4	8.1	13.1	12.3
37	4.4	2.9	2.5	3.6
38	11.7	4.5	10.1	7.6
39	1.1	0.5	0.8	0.7
40	1.9	0.5	2.0	1.0

[a] Hydrogenated esters were chromatographed (Litchfield *et al.*, 1965) using a flame ionization detector. Mole per cent were calculated from weight per cent ≈ area per cent (Horning *et al.*, 1964). [b] Data of Table V were used to calculate the chain-length composition according to statistical combination of alcohols and acids. [c] C_{28}, 0.7%; C_{29}, 1.2%.

(b) Unsaturation in marine wax esters was considered to be almost wholly confined to the monoethenoic state of alcohol and acid components (Hilditch and Williams, 1964). In consequence of improved analytical methods, this concept must be somewhat revised, in particular for the acids. For example, Mori and co-workers (1965a,b) reported about 8% polyenoic acids from wax esters of meat lipids of sperm whale. However, the triglycerides contained about 25% polyenoic acids and it appears that there is a pronounced distinction between acids of glycerides and wax esters from the same source (Hilditch and Williams, 1964; Tsujimoto and Kimura, 1928). In contrast to this, all polyunsaturated acids of mullet body oil are found also in the roe and their amounts exceed those encountered in triglycerides of the same sample of roe (Table IV). A similar trend becomes evident from Table V when keeping in mind that acids with chain lengths above C_{18} are predominantly polyunsaturated.

Unlike sperm whale, mullet seems to permit free exchange of fatty acids of wax esters with those of glycerides in roe or other parts of the body. Biological esterification of fatty alcohols with environmental fatty acids, among them linoleic, has been reported by Hansen and Mead (1965) from experiments with rats. Obviously, polyunsaturation of fatty acids does not prevent their incorporation into waxes.

(c) Odd chain acids are available in mullet at a relatively high level, and they are incorporated into wax esters like the even acids. However, odd chains are also prominent among the esterified alcohols. In the examples of Table V, the sum of odd-numbered alcohols is always slightly higher than that of odd-numbered acids. The differences would be even greater when

taking into account that there is probably no biosynthetic relation between saturated or monounsaturated alcohols and polyenoic acids. C_{14} and C_{15} acids contain very little of polyunsaturated components so that a more specific comparison of alcohols and acids is possible for these chain lengths (Table V). The values fluctuate greatly from sample to sample, but the ratio alcohol:acid of equal chain length is consistently about 8:1.

The structures of monoenoic alcohols (Table III) reflect those of monoenoic acids which have been found here and in previous analyses (Sen and Schlenk, 1964), except for 11-heptadecenol and 9-nonadecenol which are represented in small amounts. A specific search for these structures among acids has not been made and their occurrence as minor components is well possible. Regardless of odd or even chain, the principle of the double bond being in odd-numbered positions like Δ^9, Δ^{11}, or Δ^{13} is valid for alcohols as it is for the acids.

Presently known biological syntheses of normal long-chain compounds lead to fatty acids, and it is suggestive to see in them the precursors of fatty alcohols (Chibnall and Piper, 1934; Deuel, 1957). The results from mullet wax esters support this: in both alcohols and acids, odd-chain components occur at a relatively high level; their amounts are interdependent, most obviously so with C_{14} and C_{15} compounds; their double bonds are in corresponding positions.

The data suggest more specific speculation on the biosynthesis of fatty alcohols. The combination of exclusively saturated and monounsaturated alcohols with a high percentage of polyunsaturated acids emphasizes that saturated and monounsaturated acids are preferentially amenable to biological reduction of their carboxyl group. One may speculate that an acid in activated form, as it occurs in *de novo* synthesis, or possibly in the elongation process, is the necessary starting point for reduction. The usual further conversions of acids, especially dehydrogenation, apparently do not bring about the appropriate activation to initiate the course of enzymatic reduction to an alcohol.

The combination of a great variety of acids with several alcohols poses a problem of distribution pattern similar to that of fatty acid combinations in glycerides. The wax esters of mullet were investigated by glpc of hydrogenated samples. Complete resolution of odd- and even-numbered esters was achieved, but isomers containing different alcohols and acids remained superimposed. Esters outside the range of C_{30}–C_{40} were less than 0.5% and are not considered in the data in Table VI.

Experimental and calculated values conform rather closely for sample 2. However, deviations exceed the experimental error for sample 1 where duplicate procedures and analyses of the original oil were carried out with very similar results. Statistical distribution of acids and alcohols in sample 1 would require more C_{30} and C_{31}, *i.e.*, more saturated wax esters than actually encountered. Correspondingly, other chain lengths are in discrepancy, in particular C_{36} and C_{38}, where the experimental values are higher than the calculated ones. It may be significant that in both samples the values found directly for C_{36} and C_{38} are between 10 and 13%. These chain lengths may have priority to reach this amount when statistical distribution does not provide for it.

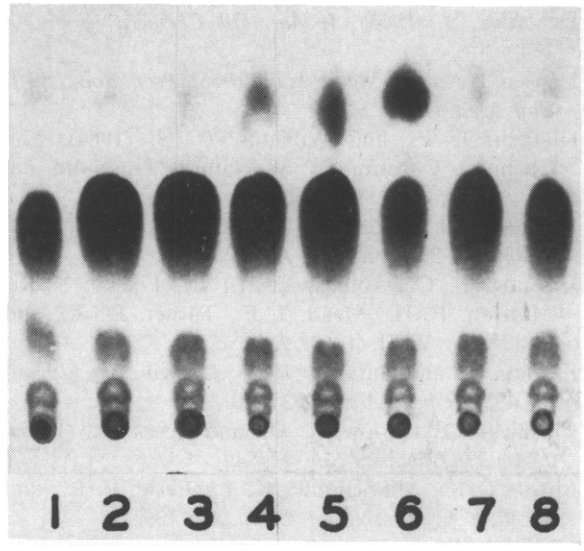

FIGURE 2: Thin layer chromatogram of total oil of mullet caught at different seasons: (1) May 5; (2) Aug 2; (3) Oct 7; (4) Oct 12; (5) Oct 14; (6) Nov 11; (7) Dec 21, 1965; and (8) Jan 27, 1966. Note the seasonal change of the highest spot which represents wax esters and/or cholesterol esters. The spots below are, in sequence, triglycerides, free fatty acids, cholesterol, and, at the starting point, polar lipids. Conditions: SiO_2 with hexane–diethyl ether–acetic acid (85:15:1).

Wax esters of *Ruvettus* have been analyzed in similar terms and the data conformed with a statistical combination of alcohols and acids (Nevenzel *et al.*, 1965). However, these waxes contain hardly any polyunsaturated acids. As in other lipids, selectivity of combinations may prevail with wax esters mainly for polyunsaturated *vs.* more saturated acids. A distinct grouping can then be detected only in mullet, but not in *Ruvettus*.

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