The *component glycerides* of only about *100 seed fats and 20 animal fats* have so far been studied in any detail. The animal fats are largely those of ruminants and the most pressing need is for a survey of the glycerides in many fats of the non-ruminant mammalia and also of birds, reptiles and fish. To a somewhat less extent this lack of factual evidence also applies to the vegetable fats. No satisfactory picture of natural triglyceride constitution will be reached until the range of specimens examined at least approaches that for which the fatty acid compositions are already known. The reason for this paucity is evident: glyceride studies have so far been made mainly on fats of industrial or medicinal interest. Whilst understandable, this affords a very one-sided approach to knowledge of the wider range of natural fats. If, as has hitherto obtained, academic workers show little interest in natural triglycerides, it can only be hoped that some government or industrial institutions may be moved to see the need for fully comprehensive survey of the field irrespective of primarily utilitarian interests. After all, with the techniques presently available, a component glyceride study will occupy much less time than the older component acid analysis before GLC was at hand.

Besides the urgent need for studies over a much wider range of animal fats, it would be interesting to ascertain whether C_{18} polyethenoid acids such as elaeostearic, or even linolenic, cease to exhibit the same preferential attachment for the central glyceryl position as do oleic and linoleic in seed fats; whilst the general build of the 2-acyl groups in animal fats needs a good deal of further clarification and experimental study.

Above all, whilst the great assistance of selective enzyme hydrolysis, thin-layer chromatography and other recently devised procedures will no doubt be bettered by further advances in technique, the discovery of still more novel methods should be sought, especially to see if any new chemical method of attack still remains a possibility.

I wish, before concluding, to set on record my special thanks to two good friends: to Dr. Waldo C. Ault, who most kindly agreed to receive on my behalf your Award and to read this communication for me; and to Dr. F. D. Gunstone, for the great generosity with which he referred to me in the course of his prefatory remarks to the Symposium.

I would also like to repeat my deep appreciation of the manner in which my name has been linked with this Symposium, and to express the wish that it may fulfil all the hopes of its convenors and participants. May its deliberations be constructive, lively, provocative and a stimulant to all toilers in this sector of lipid chemistry.

The Triglyceride Composition of *Myrica carolinensis* Fruit Coat Fat (Bayberry Tallow)[1]

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Abstract

The triglycerides of *Myrica carolinensis* fruit coat fat (bayberry tallow) contain only three fatty acids: myristic (21.5 mole %), palmitic (77.5%), and stearic (1.0%). The component triglycerides of this simple fat were determined by gas-liquid chromatography and pancreatic lipase hydrolysis. Triglycerides with carbon numbers 42, 44, 46, 48, and 50 were found. Lipase hydrolysis showed a preferential but not exclusive esterification of myristic acid at the 2-position. The triglyceride composition calculated from the combined experimental results did not conform to a random or 1,3-random-2-random distribution pattern. Regional differences in fatty acid and triglyceride composition within the fruit coat were also observed.

Introduction

BAYBERRY TALLOW (also known as myrtle, candleberry, or capeberry wax) is a hard, solid fat with a gray-green color and a spicy fragrance. It has a small commercial importance as an ingredient of fancy candles. The fat comes from the fruit coat of several species of *Myrica,* a small tree which grows wild in America, Europe, and Africa. Youngken (1) has published a comprehensive monograph describing this genus.

The physical and chemical properties of bayberry tallow have been described by numerous workers (2-9). Several recent investigators have determined the fatty acid composition of a commercial product or of the fat from a specific *Myrica species* (3,4,8,10, 11). In all cases, myristic (33–61%) and palmitic (37–52%) acids composed 80–100% of the component fatty acids. Small amounts of lauric, stearic, and oleic acids have also been reported. The triglyceride composition of this fat has not been previously examined.

Since bayberry tallow has such a simple fatty acid composition, it must also have a relatively simple triglyceride composition which could be easily determined by modern analytical methods. This paper describes the application of pancreatic lipase hydrolysis and gas-liquid chromatography (GLC) techniques to the determination of the triglyceride composition of *Myrica carolinensis* fruit coat fat.

Procedures

Materials

Preliminary analyses of several commercial samples of bayberry tallow showed widely varying compositions, probably due to different sample origins and to fractional crystallization during processing. GLC results on one commercial product even indicated the presence of substantial amounts of petroleum wax. To insure the authenticity of the sample analyzed here, we obtained the intact *Myrica carolinensis* (also known as *M. pennsylvanica*) fruits from F. W. Schu-

[1] Presented at the AOCS meeting in Houston, Texas, 1965.

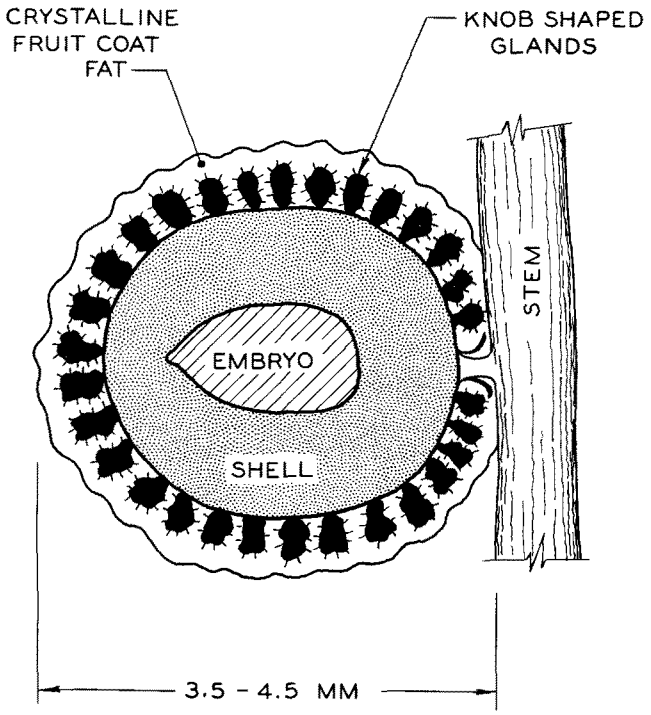

FIG. 1. Cross section of *Myrica carolinensis* fruit. See text for detailed description of morphology.

macher, Sandwich, Mass., and extracted the fruit coat fat in the laboratory.

Methods

Preparative thin-layer chromatography (TLC) on 0.25 mm thick Merck Silica Gel G plates was used to isolate the total triglycerides from crude bayberry tallow. Plates were developed in distilled diethyl ether prior to sample application to elute any possible lipid impurities to the top of the plate. After sample application, the plates were developed in an 80/20 (v/v) mixture of petroleum ether (bp 30–60C) and diethyl ether. After drying, the plates were sprayed with 2′, 7′-dichlorofluorescein solution to visualize the various lipid bands. The triglyceride band was scraped off and placed in a chromatography column containing 2–3 g of activated silicic acid. The triglyceride was then eluted from the column with distilled diethyl ether. The silicic acid at the bottom of the column prevented any 2′, 7′-dichlorofluorescein from eluting, but did not hold back the triglycerides.

Triglyceride samples were converted to methyl esters by H_2SO_4 catalyzed methanolysis (12).

Fatty acid compositions were determined by GLC of the methyl esters on a 6 ft by 0.125 in. column containing 20% (w/w) diethylene glycol succinate polyester coated on acid washed 60/80 mesh Chromosorb W as previously described (12). All fatty acid compositions are reported in mole percent to better show their relationship to triglyceride composition.

GLC of triglycerides was accomplished using the general procedure described by Kuksis and McCarthy (13). Analyses were run on an Aerograph Hy-Fi A-600-B gas chromatograph equipped with a flame detector and a 24 in. by 0.125 in. column packed with 2.7% (w/w) SE–30 silicone on acid washed 60/80 mesh Chromosorb W. The column was manually programmed from 170–300C at 1.3C/min with a nitrogen carrier gas flow of 150 ml/min. GLC on SE-30 separates triglycerides by mol wt only, and each peak is referred to by its carbon number (the number of carbon atoms in the fatty acid moiety of the triglyceride). Peaks were identified by comparison with the elution times and temperatures of known compounds. Quantitative response factors for the various triglycerides were determined using a known composition mixture made from 99+% pure trilaurin, trimyristin, tripalmitin, and tristearin (Applied Science Laboratories, State College, Pa.). All triglycerides of the same molecular weight were assumed to have the same quantitative response factor. Peaks were quantified by tracing them on vellum paper, cutting out the tracings, erasing the pencil marks, and weighing. Quantitative results are reported in mole percent values to better show the relationship between fatty acid and triglyceride composition.

Pancreatic lipase hydrolysis of triglycerides was carried out using the general technique of Mattson and Volpenhein (14). Since bayberry tallow triglycerides are solid at normal hydrolysis temperature (40C), they were mixed 1/4 (w/w) with pure triolein (Hormel Institute, Austin, Minnesota) to produce a liquid and emulsifiable fat. The addition of triolein did not interfere with subsequent GLC analysis of myristic and palmitic acids, but did prevent accurate determination of the stearic acid content. The diethyl ether extracted hydrolysis products were separated by TLC on 0.5 mm thick Merck Silica Gel G using a 66/33/1 (v/v/v) mixture of diethyl ether, petroleum ether, and acetic acid as the eluting solvent. The monoglyceride band was located by spraying with 2′, 7′-dichlorofluorescein solution and was scraped off the TLC plate. The monoglycerides were eluted with ether and converted to their corresponding methyl esters, which were analyzed by GLC as described above.

Results

Experimental

M. carolinensis fruits are drupelets about 3.5–4.5 mm in diameter and are covered with a crust of fat. A microscopic examination of the fruits revealed their unusual morphology shown in Fig. 1. The central dicotyledonous embryo is covered by a thin membrane (testa) which is surrounded by a very hard, thick shell (pericarp). The outer surface of the shell is covered with small, black, knob-shaped glands (trichomes) from which protrude short transparent hairs. A light gray, crystalline, fruit coat forms the outer surface of the fruit and fills the space between the knob-shaped glands. This fruit coat is completely soluble in benzene, indicating that it is composed entirely of lipids.

The usual method for extracting the fruit coat fat (bayberry tallow) is to boil the fruits in water (2). The melted fat rises to the top and solidifies upon cooling. Preliminary experiments showed that this technique left considerable residual fat between the glands on the outside of the shell. On the other hand, solvent extraction with benzene gave a very rapid and efficient extraction of the fruit coat fat. Microscopic examination of solvent extracted fruits showed no traces of bayberry tallow remaining, indicating that a representative fat sample had been obtained.

Two hundred *M. carolinensis* fruits having an intact fruit coat (i.e. no black glands showing) were individually selected. One hundred fruits weighing 4.093 g were placed in a 250 ml Erlenmeyer flask. Fifty milliliters of distilled benzene was added, and the flask was swirled continuously for 60 sec. The benzene solution of fat was immediately decanted off,

TABLE I

Fatty Acid Composition of *M. carolinensis* Fruit Coat Fat (Mole %)

Triglycerides	Total fat (100%)	Outer layer (20%)	Inner layer (80%)
14:0	21.5	11.9	23.4
16:0	77.5	87.2	75.4
18:0	1.0	0.9	1.2
Monoglycerides from lipase hydrolysis			
14:0	36.8	23.2	39.6
16:0	63.2	76.8	60.4

and the same benzene extraction procedure was repeated two more times. The three benzene extracts were combined, filtered, and the solvent evaporated on a rotary flash evaporator. The extraction yielded 1.187 g of fat, indicating that the original fruits contained 29.0% bayberry tallow.

The remaining 100 fruits were used to study the chemical homogeneity of the fruit coat fat. Each individual fruit was firmly held in a pair of tweezers and the outer layer of fruit coat fat was scraped off with a scalpel. Care was taken not to scrape so deeply that any of the black glands or the fat between them was removed. The recovered fat was taken up in benzene, filtered, and the solvent evaporated to obtain an "outer layer" sample of fruit coat fat. The 100 scraped seeds were then extracted three times with benzene as described above. The three benzene extracts were combined, filtered, and the solvent evaporated to obtain an "inner layer" sample of fruit coat fat.

Pure triglyceride fractions were prepared from the total fat, outer layer, and inner layer samples by preparative TLC. The fatty acid composition of each fraction was determined by GLC of the corresponding methyl esters. Results (Table I) showed only myristic, palmitic, and stearic acids were present in all three samples. No minor fatty acids constituting more than 0.1% of the total triglycerides were found. Myristic and palmitic acids constituted about 99% of the triglyceride fatty acids in each case. The outer layer triglycerides were 11.8% richer in 16:0 and 11.5% poorer in 14:0 than the inner layer triglycerides.

The carbon number distribution in each purified triglyceride fraction was determined by GLC on an SE–30 column. Quantitative results are presented in Table II along with a list of possible triglycerides which could be present in each peak. Triglycerides with carbon numbers 42, 44, 46, 48, and 50 were found in each sample. As expected, the major peak in each case occurred at carbon number 48 corresponding to tripalmitin. No carbon number 52 or 54 triglycerides were found, indicating that palmitodistearin and tristearin were not present.

The division of *M. carolinensis* fruit coat fat triglycerides between the arbitrary inner and outer layers was calculated from the fatty acid and triglyceride compositions of the outer layer, inner layer, and total triglyceride fractions. Average results (Table I) indicated that the outer layer contained approximately 20 mole % and the inner layer approximately 80 mole % of the total triglyceride present.

Each purified triglyceride fraction was also hydrolyzed with pancreatic lipase, and the fatty acid composition of the resultant monoglycerides determined by GLC. Results (Table I) indicated a preferential though not exclusive esterification of myristic acid at the 2-position of the triglycerides.

M. carolinensis fruits contain two distinct fatty regions: the embryo and the fruit coat. Since the composition of the fruit coat triglycerides has proven so unusual, the fatty acid composition of the embryo fat was also studied. The knob-shaped glands were removed from a number of benzene extracted fruits by rolling against a hard surface. The bare-shelled seeds were then lightly crushed with an iron weight, and the exposed embryo and embryo pieces picked out with tweezers. The amount of 188.4 mg of embryo was ground in petroleum ether using a ground glass tissue grinder. The resulting slurry was quantitatively transferred to a Soxhlet extraction apparatus and extracted for 2 hr with petroleum ether. The evaporated extract yielded 80.5 mg of yellow liquid oil. The fatty acid composition of this fat was determined by GLC of its methyl esters: (mole %) 14:0; tr; 16:0, 6.7%; 16:1, 0.9%; 18:0, 0.8%; 18:1, 16.1%; 18:2, 75.5%; unknown, tr.

Calculations

The results from triglyceride GLC, fatty acid GLC, and lipase hydrolysis of the total triglycerides were combined into a series of simultaneous equations. With certain assumptions, these equations were solved to determine the component triglycerides of *M. carolinensis* fruit coat fat.

Since triglyceride GLC results had shown that no palmitodistearin (the most probable distearo-triglyceride) was present, it was reasonable to assume that myristodistearin (carbon number 50) was also absent. Table II lists the component triglycerides which might have been present in each GLC peak. From these results, five simultaneous equations were obtained:

$$MMM = 2.8$$
$$MPM + MMP = 12.3$$
$$PMP + MPP + MSM + MMS = 27.3$$
$$PPP + PMS + MPS + MSP = 56.1$$
$$PSP + PPS = 1.5$$

The triglyceride mixture contained 1.0% stearic acid only in the form of monostearo-triglycerides. Therefore, 3.0% of the total triglycerides contained stearic acid:

$$PSP + PPS + PMS + MPS + MSP + MMS + MSM = 3.0$$

Lipase hydrolysis results indicated what fatty acids were esterified at the 2-position of the remaining 97.0% nonstearo-triglycerides. Two more equations were derived from this information:

$$MMM + MMP + PMP = (0.970)(36.8)$$
$$PPP + MPM + MPP = (0.970)(63.2)$$

It was then assumed that stearodimyristin and myristostearopalmitin were formed in the same ratio that 14:0 and 16:0 were present in the total fat (i.e. that the diglyceride stearomyristin had a 21.5 to 77.5 chance of being esterified to either 14:0 or 16:0). This assumption was probably not entirely justified, but it did not introduce appreciable error into our calculations since stearic acid was such a minor component of the total triglycerides. The positional

TABLE II

Gas-Liquid Chromatography of *M. carolinensis* Fruit Coat Fat Triglycerides (Mole %)

Carbon number	Total fat	Outer layer	Inner layer	Possible triglycerides present
42	2.8	1.3	3.3	MMM
44	12.3	6.8	14.5	MPM, MMP
46	27.3	21.3	29.2	PMP, MPP, MSM, MMS
48	56.1	69.2	51.4	PPP, PMS, MPS, MSP
50	1.5	1.4	1.6	PSP, PPS, SMS, MSS

TABLE III

Triglyceride Composition of *M. carolinensis* Fruit Coat Fat Calculated from Experimental Results (Mole %)

Triglycerides[a]	Total fat	Outer layer	Inner layer
MMM	2.8	1.3	3.3
MPM	0–6.4	6.8	0–8.3
MMP	5.9–12.3		6.2–14.5
PMP	20.6–27.0	14.5–21.1	20.4–28.7
MPP	0–6.4	0–6.6	0–8.3
MSM + MMS	0.3	0.2	0.5
PMS + MPS + MSP	1.2	1.1	1.5
PPP	54.9	68.1	49.9
PSP + PPS	1.5	1.4	1.6

[a] PMP = 2-myristo-1,3-dipalmitin; PMS = 1-palmito-2-myristo-3-stearin; etc.

isomer terms of the monostearo-triglycerides were combined (i.e. MMS + MSM = one unknown), and the eight simultaneous equations were solved for MMM, MSM + MMS, PPP, PMS + MPS + MSP, and PSP + PPS. The remaining four unknowns were reduced to four equations having three degrees of freedom:

$$PMP + MPP = 27.0$$
$$MMP + MPM = 12.3$$
$$PMP + MMP = 32.9$$
$$MPP + MPM = 6.4$$

Since the minimum amount of any triglyceride was 0%, the range of values that PMP, MPP, MMP and MPM could have and still satisfy these equations was easily determined.

Similar calculations were carried out on the outer layer and inner layer triglyceride fractions. Combined results from all three samples are shown in Table III. As expected, all fractions showed different triglyceride compositions.

Numerous statistical methods have been proposed for calculating the triglyceride composition of a natural fat from its fatty acid composition. The random distribution (15) and 1,3-random-2-random distribution (16,16a) hypotheses were tested against the experimental data on *M. carolinensis* fruit coat fat triglycerides. Since regional differences in fatty acid and triglyceride composition existed, the integrated calculation method described by Litchfield and Reiser (17) was used to allow for this phenomenon. Experimental and calculated triglyceride compositions are shown in Table IV. Both distribution hypotheses showed substantial deviations from experimental results. When individual triglycerides were compared, too much MPP and too little MMM and PPP were predicted. When comparison was made on the basis of carbon number, too little carbon number 42 and 48 material and too much carbon number 46 material were predicted.

TABLE IV

Comparison of Experimental Results with Statistical Distribution Hypotheses (Mole %)

Individual triglycerides	Experimental results for total fruit coat fat	Integrated random distribution	Integrated 1,3-random-2-random distribution
MMM	2.8	1.3	0.9
MPM	0–5.2	3.7	1.2
MMP	7.1–12.3	7.4	9.0
PMP	20.6–27.0	12.3	25.4
MPP	0–5.2	24.5	13.7
MSM + MMS	0.3	0.2	0.1
PMS + MPS + MSP	1.2	1.2	1.0
PPP	54.9	47.9	46.6
PSP + PPS	1.5	1.8	1.8
SMS + MSS	....	....	tr
SPS + PSS	....	tr	tr
SSS	....	....	....
Carbon number			
42	2.8	1.3	0.9
44	12.3	11.1	10.2
46	27.3	37.0	39.2
48	56.1	49.1	47.6
50	1.5	1.8	1.8
52	....	tr	tr
54	....	....	....

Discussion

M. carolinensis fruit coat is unusually simple since it contains only three fatty acids, all of which are saturated. Moreover, 14:0 and 16:0 comprise 99% of the component fatty acids. As far as we know, only one other completely saturated natural fat, *Myristica canarica* seed fat, has been reported (18). The regional differences in fatty acid composition of the fruit coat are not particularly unusual, since this phenomenon has been encountered with many other fatty tissues (19–21). The embryo fat of *M. carolinensis* seeds has a fatty acid composition resembling safflower oil, and does not appear to have any exceptional properties.

Since *M. carolinensis* fruit coat triglycerides have such a simple fatty acid composition, the relatively simple component triglycerides can be determined in considerable detail using GLC and lipase hydrolysis techniques. The experimentally determined triglyceride composition does not conform to a random or 1,3-random-2-random distribution pattern. Unexpectedly, it is the simple triglycerides PPP and MMM which are present in greater than predicted amounts. A preferential but not exclusive esterification of 14:0 over 16:0 at the 2-position of the triglycerides is also observed.

The unusual composition of *M. carolinensis* fruit coat fat is undoubtedly related to the unusual botanical role which it plays. The fat appears in pure crystalline form rather than bound into cellular tissue. The morphology of the fruits suggests that the fat was excreted by the peculiar knob-shaped glands covering the outer surface of the shell. This outer crust of solid fat possibly serves as a protective coating for the seed. Certainly the fruit coat fat does not serve as a depot of reserve energy, since it has no vascular contact with the embryo in the mature seed. The enzyme system which can biosynthesize such unusual natural triglycerides certainly merits further study.

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