THE CHROMATOGRAPHIC RESOLUTION OF BAYBERRY WAX

A Thesis

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Arthur Kraut

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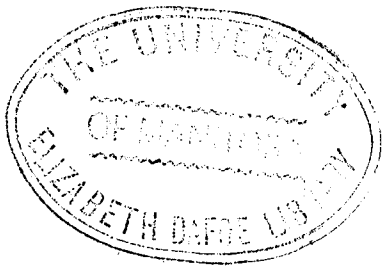

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ABSTRACT

Bayberry wax, a plant neutral lipid mixture, yielded eight fractions with thin-layer chromatography (TLC). Seven of these have been identified using the techniques of TLC, infrared spectroscopy, and column chromatography: hydrocarbons, two triglyceride fractions, free fatty acids, 1:3 and 1:2 diglycerides, and monoglycerides. The triglycerides were investigated by reversed-phase partition TLC. The fatty acids of Bayberry wax and of five fractions were analyzed by gas-liquid chromatography (GLC). Semi-quantitative results were obtained on five fractions by gravimetric analysis using TLC and column chromatography.

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INTRODUCTION

The term 'lipid' refers to esters of long-chain fatty acids and alcohols or closely related derivatives. Of importance in this study are the neutral lipids: hydrocarbons, cholesteryl esters, glycerides (fats), free fatty acids, cholesterol, and waxes. Though free fatty acids are acidic, they are classified as a neutral lipid here, for they are very weak acids and they are produced during the synthesis or degradation of glycerides.

Glycerides are esters of long-chain fatty acids and the trihydric alcohol, glycerol; waxes are esters of long-chain fatty acids and long-chain alcohols.

1	CH_2OCOR_1	CH_2OCOR_1	CH_2OCOR_1
2	$CHOCOR_2$	$CHOH$	$CHOH$
3	CH_2OCOR_3	CH_2OCOR_2	CH_2OH
	Triglyceride	Diglyceride	Monoglyceride

R: fatty acyl groups ranging from C_4 - C_{30} in chain length.

The chemical nature of the acyl group depends on the sources. The position of the acyl groups on the glyceryl residue depends to an extent on the nature of the fatty acid (39). If the acyl groups are classified as either saturated or unsaturated, there are then six triglyceride isomers. When the different kinds of saturated and unsaturated fatty acids are considered, the possible isomers greatly increase. The fractionation of these isomers is

difficult and a complete separation or detection is not yet possible. However, recent work has shown that chromatographic techniques, such as TLC and GLC, give a much better separation of isomers than other techiques in lipid analysis, such as countercurrent distribution and low temperature crystallization.

Bayberry wax contains at least 95% glycerides. Thus the name 'wax' is a misnomer. The fat is extracted from a shrub of the Myricaceae family of fruit-coat fats, i.e. fats which come from parts of the fruit other than the seed. The shrub is listed under several names: Candleberry, Bayberry, Candleberry myrtle, Tallow-tree, and Wax myrtle. The wax itself may also be called: Myrtle wax, Laurel wax, and Bayberry tallow. The shrubs are common to North America, South America, and South Africa. Myrica cerifera and Myrica carolinenis are the common species in North America. Unfortunately, the species from which our sample is extracted is unknown.

Commercial Bayberry wax is a green coloured, hard, solid, odoriferous fat, permeated with black or brown spots. On exposure to the air the outer layers are bleached to a whitish mass. The fat has little commercial use; it is used for making expensive candles, and it is a source of palmitic and myristic acids.

This work was introductory work for further chromatographic investigations in this laboratory of microgram quantities of plant lipids. Bayberry wax

was chosen because it has a simple lipid composition, it is easy to obtain in large quantities, and it has not been analyzed to this time, to our knowledge, by any chromatographic technique.

In the investigation of a natural lipid mixture a general procedure is followed:

1. The lipid is extracted from the source.
2. It is purified, i.e. removal of metal ions, surgars, amino acids.
3. It is fractionated into groups (i.e. neutral lipids, phospholipids) and classes (neutral lipids: hydrocarbons, glycerides, etc); the classes are identified.
4. Each class is analyzed, i.e. fatty acid analysis.
5. Each class is fractionated into its homologues and these are further analyzed.

The first step may be eliminated if a commercial sample is used (as with Bayberry wax). The fractionation and purification steps may be combined if column chromatography is used, at least in the case of the neutral lipids.

The ideal analysis should reveal the chemical nature and the amount of each constituent in the lipid mixture. This was accomplished in this laboratory in the study of Beeswax, in 1963 - the carbon numbers and the quantities of the homologues of the hydrocarbons, the esters, and the methyl esters of the fatty acids were found. With Bayberry wax only the chemical nature of the classes was studied. Quantitative work was not

attempted on homologues except for the fatty acid analysis of some of the classes.

Using TLC and column chromatography, Bayberry wax was fractionated into a number of classes. An attempt was made to identify each class using the same two techniques and also infrared spectroscopy. Semi-quantitative results were obtained on some of the classes using the above two chromatographic techniques. An attempt was made to fractionate the triglyceride class by reversed-phase partition TLC. Finally, using GLC, an attempt was made to analyse the fatty acids of some of the classes.

Recent reviews on the analysis of neutral lipids indicate that TLC, column chromatography, and GLC are among the best techniques for fractionation, isolation, and quantitative analysis.

Nevertheless, these methods should not be the only ones used in analysis of lipid mixtures. Other methods, such as infrared spectroscopy and chemical tests should be used to provide supporting evidence.

With chromatographic techniques certain lipid components may be chemically altered (i.e. 2-monoglycerides isomerize to 1-monoglycerides). Other methods such as low temperature crystallization, counter-current distribution, and fractional distillation reduce this possibility to a minimum. However, these methods either do not give as good separations

as the chromatographic techniques, or they are not as sensitive as chromatography to minor components or to small quantities of sample.

REVIEW OF LITERATURE

The *Myricaceae* family of fruit-coat fats consists of at least ten species. Hilditch (16) gives the most extensive summary, as given in Table I, of the results on the fatty acid composition of different species of this family.

TABLE I

		Fatty Acids				
		12	14	16	18	18:1
Myrica cerifera	U.S.A.		Ca.33	Ca.45	Ca.20	?
Myrica cordifolia	S. Africa	0.4	49.9	49.0	0.2	0.5
Myrica mexicana	Central Am.		61.1	37.5		1.4

Bayberry wax does not have the characteristic fatty acid composition of fruit-coat fats (16). The major component acids should be palmitic, oleic, and linoleic acids. The minor component acids should be myristic and stearic (linolenic) acids. Bayberry wax contains more myristic acid and less oleic acid than normally found in fruit-coat fats.

The investigation of Bayberry wax goes back to at least 1845, when Chevreul (32) reported the presence of stearic and myristic acids. Other workers (33) stated that lauric acid was also present. Benedict (34) reported

that the wax consists mainly of palmitin with some myristin and stearin. In addition to these, Lewkowitsch (35) said that a small amount of oleic acid is present. Allen (36) reported the presence of 0.12% free fatty acid (as palmitic acid). Myrica cerifera appears to be the species used in all these accounts, however this was not made clear.

In 1903, Smith and Wade (31) investigated Bayberry wax in an attempt to clarify the confusion concerning its composition. They concluded that the fat consists mainly of palmitin, some lower glyceride, and some free fatty acid. Stearic acid was concluded to be absent.

In 1932 Jamieson, McKinney, and Gertler (16) determined the fatty acid composition of Bayberry wax from Myrica mexicana by fractional distillation of the methyl esters. The results are given in Table I.

In 1948 McKay, using Bayberry wax from Myrica cerifera as a source of myristic and palmitic acids, found to his surprise Ca. 11% stearic acid (23). His results as given by Hilditch are shown in Table I (16).

Also in 1948 Schoeman and Hawke (12) analyzed the fruit-coat fat from Myrica cordifolia. Their results are given in Table I. They found in addition a hydroxyl value of 72, which indicates the presence of di- and monoglycerides. Two reports (21, 37) were found on a fat called Bayberry fat. At first the name was taken to

be another name for Bayberry wax. However, its fatty acid composition and Iodine Number are quite different from the values in the reports on Bayberry wax. This fat may be a member of the Lauraceae family of fruit-coat fats.

THIN-LAYER CHROMATOGRAPHY

Sensitivity, versatility, speed, and simplicity are the qualities that make TLC a useful qualitative and quantitative method of analysis. At present, it is one of the best techniques for the analysis of minute quantities of lipids and for the detection of minor constituents.

Many papers and several reviews have been published on TLC, even though it only came into extensive use after 1958. Mangold (19, 20) in two reviews discusses its theory and its application to the fractionation of lipid mixtures; Privett et al. (28) stress its quantitative aspects; Pelick et al. (26) emphasize its technical aspects. Books by Randerath (30) and Bobbitt (1) provide extensive information on the technique and application of TLC.

TLC is a technique of open column chromatography. To some degree, adsorption, partition, and ion-exchange chromatography influence the fractionation of the sample. However, one type can be made to predominate. Adsorption chromatography fractionates the sample into classes (i.e. a functional group separation); reversed-phase partition chromatography (i.e. the plates are impregnated with a non-polar phase) resolves a class into its constituents. Partition chromatography with silver nitrate impregnated plates resolves a class into fractions differing in degrees of unsaturation.

TLC can be divided into the following categories:

1. Diagnostic thin-layer adsorption chromatography

   The sample is fractionated into classes and an attempt is made to identify these classes.

2. Diagnostic thin-layer partition chromatography

   The classes are fractionated into their constituents and an attempt is made to identify these substances.

3. Preparative TLC

   The fractions on the plates are collected.

4. Quantitative TLC

   The present procedures can be divided into two groups:

   a) Analysis after the recovery of the fractions:

      1. Gravimetric analysis
      2. Spectroscopic analysis
      3. Radiometric analysis

   b) Analysis directly on the plates:

      1. Spot size method
      2. Photometric methods:

         photoreflectometry, spectrophotofluorometry, photodensitometry.

The following work was attempted using TLC:

1. Diagnostic adsorption chromatography

   To separate Bayberry wax into fractions and to identify these fractions.

2. Diagnostic partition chromatography.

   To fractionate the triglycerides of Bayberry wax using tetradecane and silver nitrate as impregnating agents.

3. Preparative TLC.

   To isolate the fractions appearing in diagnostic adsorption chromatography.

4. Quantitative TLC.

   To obtain semi-quantitative results using gravimetric analysis.

EQUIPMENT AND CHEMICALS

Adsorbents

Silica gel G(S.G.G.) - Kensington Scientific Corporation.

Camera

KOWA SE (F 1.9, 50 mm., single lens reflex) was used for the majority of the photographs; Royal Close-up lenses.

Detectors

a) 60% Sulfuric acid

b) Iodine chamber

c) 2'7' Dichlorofluorescein (0.1% solution in 95% ethyl alcohol).

Developing Chamber

a) Glass tanks - 12 in. (depth) x 10 in. (width), 10" x 6".

b) Glass cylinder - 8" x 2.5"

c) Coffee jars - 9" x 3".

Glass plates

Glass panes were obtained from a local hardware store and cut into various sizes in the laboratory: 5cm. x 20 cm., 10 x 20, 15 x 20, 20 x 20.

Samples

1) Bayberry wax - Fisher Scientific Company.

2) Laurin, myristin, palmitin, and stearin - Eastman Organic Chemicals; not chromatographically pure.

3) Laurin, myristin, and stearin - Hormel Institute; highly purified; used only in partition TLC.

4) Olein and linolein - Applied Science Laboratories; chromatographically pure.

5) Methyl palmitate - Hormel Institute; chromatographically pure.

All other chemicals used are not chromatographically pure.

Spot Applicator

Hamilton syringe - 10 microliters with a 2 in. fixed needle.

Thin-Layer Applicator

"Kensco Applicator" - Kensington Scientific Corporation.

Vacuum Aspirators

a) A glass tube (15 cm. long, i.d. 5 mm.) is drawn out to make a constriction at about 5 cm. from one end. A piece of glass wool is packed against the constriction on the short side of the tube. Both ends of the tube are then drawn out.

b) A larger aspirator than the above one (14).

Solvents

a) Adsorption TLC: petroleum ether (30-50°C), diethyl ether, acetic acid.

b) Partition TLC: chloroform, acetic acid, acetone, acetonitrile.

Except for acetonitrile none of the solvents are chromatographically pure.

EXPERIMENTAL PROCEDURES

Adsorption TLC

Preparation of the plate

All the plates are cleaned with Ajax cleanser, washed, and then dried in an oven before use. The applicator is always set at 0.33 mm. The adsorbent (13 gm. for ten 5 x 20 cm. plates) is applied in a slurry (two parts water to one part S.G.G.). After application, the plates are air-dried for one hour. Initially, plates were then activated for two hours at 100°C. However, this was later changed to one hour. If they are not used immediately, they are placed in a desiccator. If a plate is not used for a prolonged period of time, it is again activated by heating for twenty minutes.

Development of the plate

The sample (in solution) is generally applied two cm. from one end; the plate is developed by the ascending method.

Solvent - As previously discussed.

Developing Chamber - A glass container; a large piece of filter paper saturated with the required solvent surrounds its walls.

Solvent front - Ten or fifteen cm.

Development time - Thirty to sixty min.

Detector - One or more of the three kinds mentioned previously.

Preparative TLC

The procedure for the preparation of the development of a plate is the same as the above procedure except that a larger plate and a thicker layer of adsorbent (o.65 mm., 0.76 mm., 0.89) are used.

The other differences are:

Detector - Iodine chamber or 2',7' dichlorofluorescein.

Recovery of fractions - Vacuum aspirator technique; both types of aspirator are used.

Analysis - Gravimetric analysis.

Partition TLC

1. Silver Nitrate ($AgNO_3$) partition chromatography.

Preparation of the plate

A slurry of 12.5% silver nitrate in distilled water is used. The plate is not activated by heating. It must be used immediately for the best results, because the silver nitrate decomposes on standing.

Other differences:

Solvent - Chloroform: acetic acid (99.5/0/5 V/V).

Detector - 2',7' dichlorofluorescein.

2. Reversed-phase TLC

Preparation of the plate

The plate is prepared in the same manner as for adsorption chromatography. After the plate is air-dried

for two hours it is carefully dipped into a solution of tetradecane in petroleum ether (5 or 10% solution). The plate is air-dried for twenty min. before the application of the sample. Only about one-tenth the amount of sample can be applied before overloading, as compared with adsorption chromatography.

After development, the plate is heated at 200°C for one hour to evaporate the tetradecane.

Other differences are:

Solvent - Acetone: acetonitrile (8/2, 1/1, 10/1) saturated with tetradecane.

Development time - about sixty min.

Detector - 2',7' Dichlorofluorescein followed by charring with 60% sulfuric acid.

Some of the plates from adsorption and partition TLC were photographed for a permanent record.

In brief, the basic technique of TLC consists of the following steps:

1. A glass plate is coated with a finely divided powder. In partition chromatography the powder is impregnated with a substance which becomes the stationary phase.

2. A sample of the mixture, in solution, is applied near one end.

3. A moving phase, generally rising, passes over the plate by capillary action to a given distance.

4. The plate is air-dried and sprayed with a reagent to make the separated fractions visible.

Factors influencing the separation

The kind of solvent and adsorbent (or stationary phase) used are the prime factors influencing the separation. Small variations in the separation may be caused by the following factors:

1. Thickness of adsorbent
2. Method of activation, i.e. moisture content
3. Method of development
4. Fumes in the laboratory
5. Purity of adsorbent
6. Purity of solvent
7. Temperature

If the layer of adsorbent on a plate is not uniform, the migration rates of the classes in a sample may vary even on the one plate (Fig. 1). The amount of sample applied is also of importance. As seen in Fig. 2, there is a lower limit of concentration for the detection of certain classes of Bayberry wax, at least by the method of detection used.

Adsorbent

Silica gel is the most widely used adsorbent for the chromatography of neutral and acidic lipids. It is a strong adsorbent, and it does not cause isomerization of glycerides (27). Though aluminum oxide is extensively

used, it cannot be used for glycerides for it causes their isomerization (22).

Silica gel G(S.G.G.) was used as an adsorbent for almost every plate. This material consists of fine grade (200 mesh) silica gel with about 13% calcium sulfate added as a binder.

Generally the thickness of the layer applied was 0.33 mm. The effect of the thickness of the layer on the resolution of Bayberry wax was not investigated. However, when the thickness was increased to 0.76 mm., the resolution was slightly better. (Compare Fig. 2 with Fig. 3).

Solvents

Adsorption TLC

For adsorption TLC of neutral and acidic lipids different ratios of the solvent system petroleum ether: diethyl ether:acetic acid are frequently used (19, 30). The migration pattern is essentially the same as the elution pattern with silicic acid in column chromatography (38): hydrocarbons, steryl esters, triglycerides, long-chain alcohols, fatty acids, sterols, diglycerides, and monoglycerides.

The above solvent system also gave a better fractionation of Bayberry wax than any other solvent system used: seven distinct fractions, one partially separated fraction, and a residue.

Other solvent systems used were:

Chloroform	4	fractions
Chloroform:ether (8/1)	4	"
Chloroform:ether (8/2)	5	"
Chloroform:acetic acid (30/.75)	6	"
Petroleum ether:benzene (100/6)	3	"
Petroleum ether:benzene:acetic acid (100/6/1.5)	3	"
Hexane:methyl acetate (50/25)	5	"
Hexane:ether (10/2)	2	"
Hexane:ether:acetic acid (10/2/1.5)	3	"
Ether:ethyl alcohol (40/1)	3	"
Carbon tetrachloride:benzene (5/2)	-	
Carbon disulfide:chloroform (30/1)	-	
Carbon disulfide:ether (30/1)	3	"
Carbon tetrachloride:acetone (30/2)	3	"
Benzene:chloroform (30/2)	2	"
Ether	3	"

The extent of migration of the different classes in the neutral lipid group can be regulated by varying the ratio of petroleum ether to ether (Fig. 4). The acetic acid prevents the smearing of the acid components as shown in Fig. 4. Also it appears to aid in the fractionation of the classes containing hydroxyl groups, i.e. diglycerides (Fig. 5). The results indicate that the fractionation of the neutral lipids is at a maximum,

only if certain ratios of the solvent system petroleum ether:ether:acetic acid are used, with the petroleum ether:ether ratio being the important factor. The petroleum ether:ether ratio was varied from 1:0.052 to 1:0.78. The proportion of acetic acid added was varied from 0.025 to 0.5. The best systems appear to be the ones with petroleum ether:ether between 1:0.44 and 1:0.56, and acetic acid varying from 0.025 to 0.042. Though Bayberry wax is fractionated into the same number of classes with the ratio 1/0.052/0.025, the separation between the classes is not as good as with the previous ratio. Fig. 6 shows typical separations using different ratios.

The ratios of the solvent systems termed the best ones produce an 'extra' spot in the fractionation of Bayberry wax. This development may be due to differences in the moisture content of the adsorbent or to other variations in the preparation of the chromatogram. However, many of the solvent systems shown were tried at various times and the only difference in the separations from one plate to another, using the same solvent system, was in the R_f values of the spots.

In summary, the most important factor in the petroleum ether:ether:acetic acid system appears to be the petroleum ether:ether ratio. High ratios produce a merging of spots; low ratios produce an incomplete separation.

Partition TLC

The solvent systems used in partition TLC are taken from Randerath (30).

a) Tetradecane impregnated plates

Acetone:acetonitrile (8/2,1/1, 10/1).

b) Silver nitrate impregnated plates

Chloroform:acetic acid (99.5/0.5).

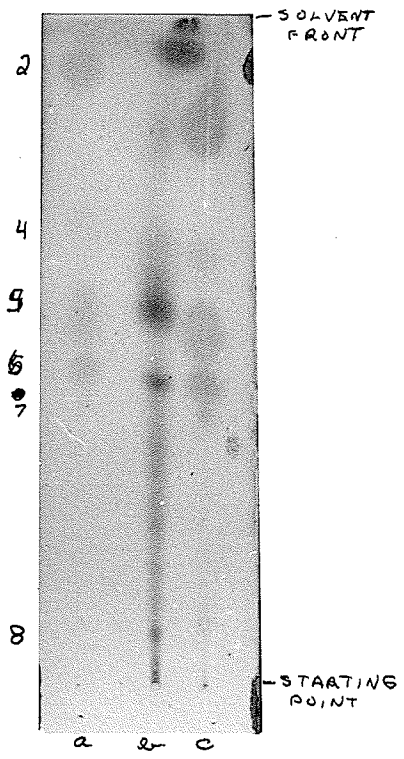


FIGURE 1.

TLC of Bayberry wax on an uneven layer of adsorbent (S.G.G.).

Solvent: Petroleum ether:diethyl ether:acetic acid (36/28/1.5, V/V/V).

Solvent front: 15 cm.

Development time: 58 min.

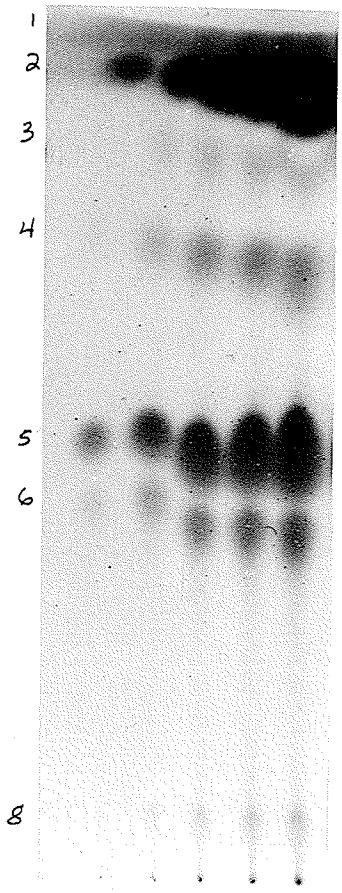
Detector: 60% aqueous solution of sulfuric acid.

Samples: a) Bayberry wax
b) Unsaponifiable fraction of Bayberry wax
c) Bayberry wax

Bayberry wax spots	R_f Value					
	2	4	5	6	7	8
	.85	.63	.51	.44	.40	.09

R_f value $\equiv$ $\frac{\text{Migration distance of spot}}{\text{Migration distance of solvent front}}$

Migration distance of spot $\equiv$ mean distance traveled.



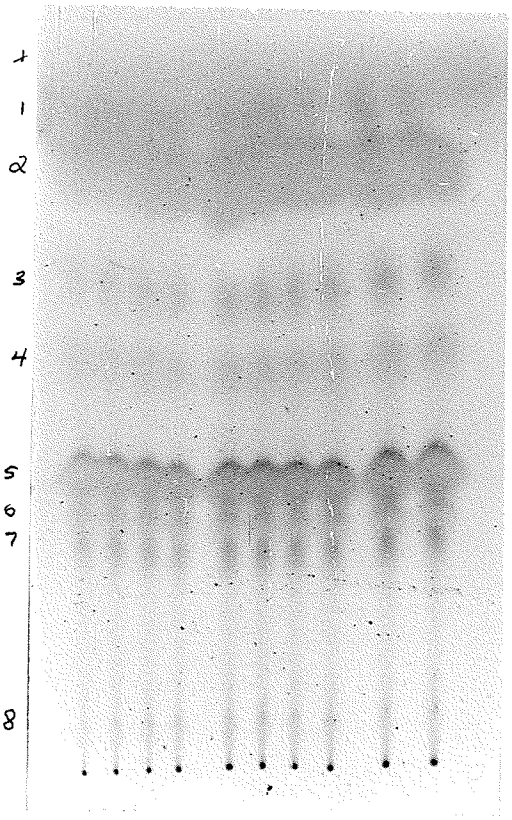


FIGURE 2.

TLC of different quantities of Bayberry wax on a 0.33 mm. layer.

Solvent: petroleum ether: ether:acetic acid (40/20/1.33).

Development time: 35 min.

Concentration of sample: 0.35 mg./ml.

Other conditions are the same as in Fig. 1.

FIGURE 3.

TLC of different quantities of Bayberry wax on a 0.76 mm. layer.

Solvent: petroleum ether:ether:acetic acid (40/20/1).

Same conditions as in Fig. 2.

	R_f Values							
Spots	1	2	3	4	5	6	7	8
Bayberry wax (Fig.2)		.84	.77	.66	.46	.37		.06
Bayberry wax (Fig.3)	.87	.78	.64	.52	.38	.33	.28	.06

FIGURE 4.

TLC of Bayberry wax using petroleum ether:ether mixtures.

Solvent: a) Petroleum ether: ether (30/70)
b) Petroleum ether: ether (50/50)

Concentration: 0.045 mg./micro-liter

Solvent front: 12 cm.

Development time: 25 min.

Detector: 2',7' Dichlorofluorescein (1% alcoholic solution)

FIGURE 5.

TLC of Bayberry wax using acetic acid.

Solvent: Petroleum ether:ether: acetic acid (6/4/0.5)

Same conditions as in Fig. 4.

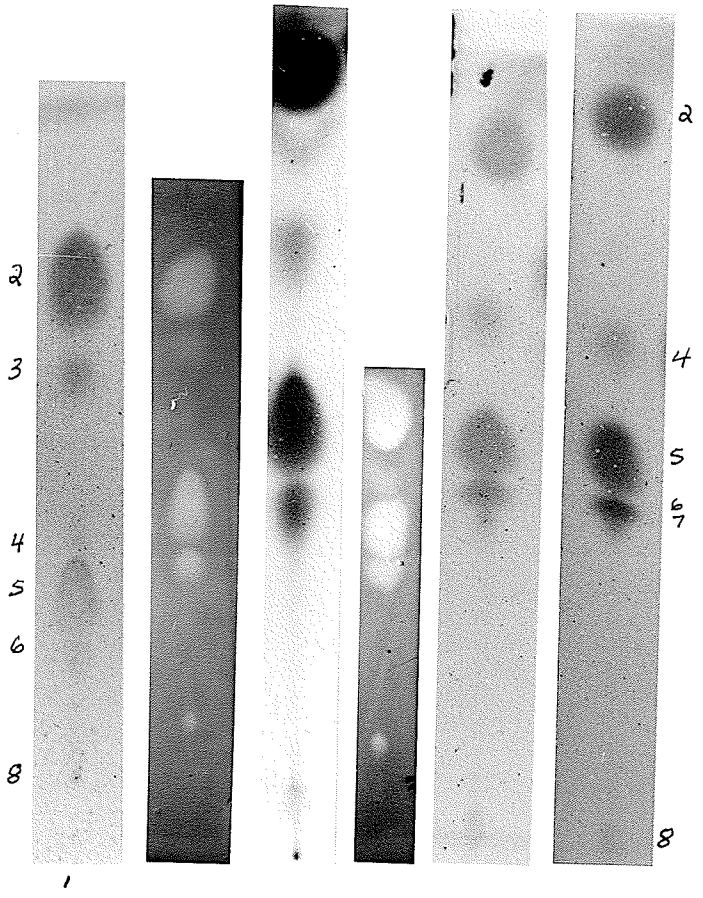


FIGURE 6.

TLC of Bayberry wax using different ratios of petroleum ether:ether:acetic acid.

Solvent:

1) Petroleum ether:ether (19/1/1.5) ; Solvent front: 11.5 cm., Time: 30 min.

2) (19/1/3) ; Solvent front: 14 cm., Time: 38 min.

3) (40/20/1.33) ; Solvent front: 12 cm., Time: 36 min.

4) (6/4/0.5) ; Solvent front: 12 cm., Time: 30 min.

5) (38/26/1.5) ; Solvent front: 15 cm., Time: 34 min.

6) (36/28/1.5) ; Solvent front: 15 cm., Time: 37 min.

Cont'd.....

	R_f Values						
Bayberry wax spots 1	2	3	4	5	6	7	8
Chromatogram 1)	.69	.55	.32	.24	.15		.10
" 2)	.82	.70	.51	.45	.35		.08
" 3)	.84	.77	.66	.46	.36		.06
" 4)	.79		.64	.61	.52		.15
" 5)	.82		.62	.49	.43	.40	.09
" 6)	.87		.61	.50	.44	.42	.10

THIN-LAYER ADSORPTION CHROMATOGRAPHY

Fractionation of Bayberry Wax and the Identification of the Fractions Without Prior Chemical Modifications

Introduction

Past analyses have shown that Bayberry wax consists mainly of triglycerides. Nevertheless the presence of diglycerides, monoglycerides, free fatty acids, and glycerol would not be surprising.

In its best separation, Bayberry wax is fractionated into seven completely separated spots, one partially separated spot, and a residue (Fig. 2). One spot and the residue were not identified. The spots are:

1. Hydrocarbons (H)
2. Triglycerides (T)
3. Triglycerides (T')
4. Free fatty acids (A)
5. 1:3 Diglycerides (D_A)
6. 1:2 Diglycerides (D_B)
7. Unknown (D_C)
8. Residue

As previously discussed, only certain ratios of the solvent system, petroleum ether:ether:acetic acid will fractionate Bayberry wax into the maximum number of spots. (Spot T' is the spot previously referred to as the 'extra' spot, and spot D_C is the spot appearing only in thick layers (Fig. 3) or in plates developed with a

solvent system where petroleum ether:ether is about 1:0.68 as shown by Fig. 6.) In most of the photographs shown these ratios were not used. Nevertheless, the numbers shown above will always be used for Bayberry wax whether or not all the spots are present.

For an initial identification of the fractions (or classes), representative substances from different classes are chromatographed along with Bayberry wax, and the migration distances (or R_f values) of the resulting spots are directly compared, i.e. using triglycerides, cholesteryl stearate, alcohols, acids and cholesterol. Since pure samples of di- and monoglycerides were not available, a comparison to these classes was made with impure triglycerides. These samples would contain, at the most, triglycerides, fatty acids, diglycerides, monoglycerides, and glycerol, depending on the completeness of esterification and purification procedures, and subsequent decomposition on standing.

Results and Discussion

The R_f values of Bayberry wax, lauryl alcohol, cetyl alcohol, palmitic and stearic acids (Fig. 7) suggest the following:

	Functional group
spot 4	- COOH
5	- OH
6	-OH

A chromatogram of laurin, myristin, palmitin, and stearin (Fig. 8) shows a number of spots other than triglyceride ones. They probably represent free fatty acids, and diglycerides. From Mangold (19) and from other chromatograms not shown here, the following sequence is the probable one for these impure triglycerides:

1. Triglycerides
2. Free fatty acids
3. 1:3 Diglycerides
4. 1:2 Diglycerides

A 1:3 diglyceride would be expected to migrate further than a 1:2 diglyceride, at least with only saturated acids present, because a secondary alcohol group would not be as strongly adsorbed on S.G.G. as a primary alcohol group (27). Monoglycerides are probably not present in these samples for no spot is in the proper range (R_f ca 0.06). However, one spot does appear with another triglyceride sample which corresponds to spot M. In all cases glycerol remains at the starting line.

Other than triglycerides, spot T could contain steroid esters, for they have R_f values similar to triglycerides. However, Fig. 9 shows that Bayberry wax probably does not contain steroid esters or at the least not any cholesteryl esters.

Fig. 10 shows a chromatogram of Bayberry wax, laurin and myristin. The diglyceride spots of laurin

appear to correspond to spots D_B and D_C. Diglycerides containing myristic, palmitic or stearic acids would probably migrate further as shown by Fig. 8, for they would be more non-polar; therefore spot D_A could also be diglyceride. Since Bayberry wax consists largely of palmitin and myristin, two spots (D_A and D_B), at least, probably contain some diglyceride.

The above separations suggest:

Spot 2	Triglyceride (carbon number greater than 36)
4	Free fatty acid
5	1:3 Diglyceride or alcohol
6	1:2 Diglyceride or alcohol
7	Diglyceride
8	Monoglyceride

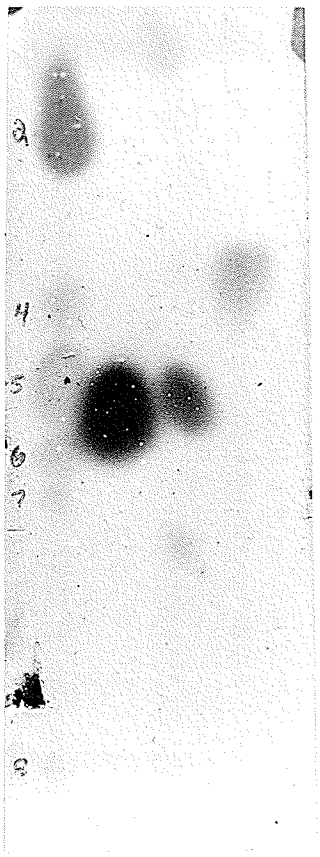


FIGURE 7.

A comparison of the R_f values of Bayberry wax with those of some alcohols and acids.

Solvent: Petroleum ether:ether: acetic acid (36/28/1.5)

Solvent front: 14 cm.

Development time: 34 min.

Samples:	R_f Values
a) Bayberry wax	
b) Lauryl alcohol	.48
c) Cetyl alcohol	.50
d) Palmitic and stearic acids	.64

	R_f Values					
Bayberry wax spots	2	4	5	6	7	8
	.83	.60	.49	.45	.40	.10

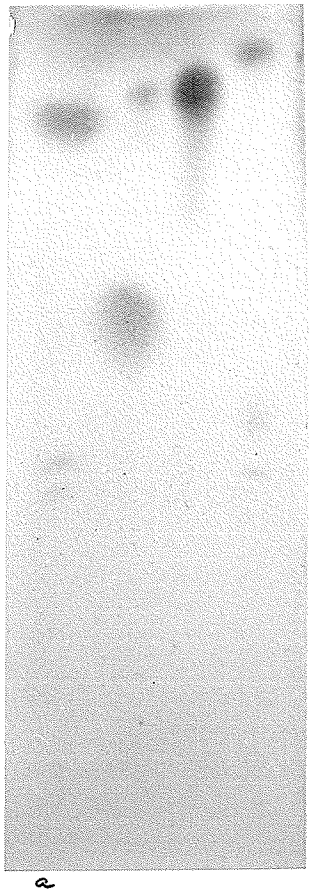


FIGURE 8.

TLC of impure triglycerides.

Solvent: Same as in Fig. 7

Solvent front: 15 cm.

Development time: 27 min.

Samples:	R_f Values
a) Laurin	.85
b) Myristin	.89
c) Palmitin	.89
d) Stearin	.94

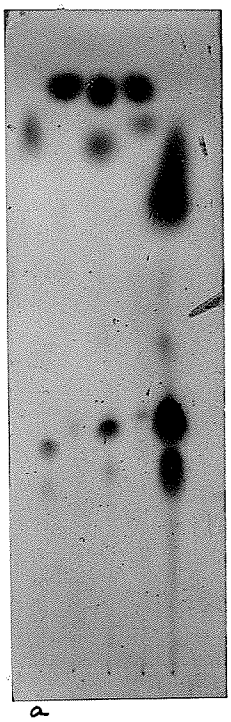


FIGURE 9.

TLC of Cholesteryl stearate with Bayberry wax .

Solvent: Petroleum ether:ether: acetic acid (36/16/1)

Solvent front: 15 cm.

Development time: 21 min.

Samples:
a) Bayberry wax
b) Cholesteryl stearate
c) Bayberry wax + b)
d) Stearin + b)
e) Bayberry wax oxidized by periodic acid

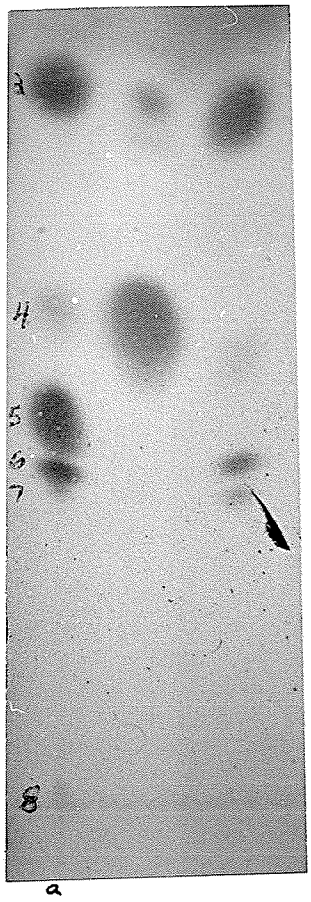


FIGURE 10.

TLC of Bayberry wax with laurin and myristin.

Solvent: Petroleum ether:ether: acetic acid (36/28/1.5)

Solvent front: 15 cm.,

Development time: 37 min.

Samples:
a) Bayberry wax
b) Laurin
c) Myristin

	R_f Values					
Spots	2	4	5	6	7	8
Bayberry wax	.87	.61	.50	.44	.42	.1
Myristin	.85	.59				
Laurin	.83	.57		.45	.42	

Chemical Modifications: Acetylation, Saponification, Methylation, and Periodic Acid Oxidation

Thin-layer chromatograms of Bayberry wax chemically modified by such procedures as acetylation, periodic acid oxidation, or saponification, provide further information on the nature of some of the classes fractionated by TLC.

Acetylation

Procedures

a) About 200 mg. of Bayberry wax and 2 ml. of acetyl chloride are refluxed together for 45 min.

b) About 600 mg. of Bayberry wax, 5 ml. of acetic anhydride and about 5 mg. of sodium acetate are refluxed together for 3 hrs. After the reaction mixture has cooled, the precipitate is filtered on a Büchner funnel and washed with water.

Results and Discussion

The fractionation of Bayberry wax acetylated by procedure (a) is shown in Figs. 11 and 12. The spots in Fig. 11 are more prominent than those in Fig. 12 because the applied sample was more concentrated.

A new spot, probably the acetylated lipids, appears below the triglyceride spot. Spot M completely disappears. However, the other spots in the diglyceride region are quite visible, especially D_C. These spots could represent substances other than glycerides, unacetylated D_A, D_B or D_C, or partial acetylation products

of spot M, i.e. monoglycerides.

The results obtained using procedure (b) indicate that acetylation by the previous procedure was probably not complete. All spots except for T, acetylated glyceride, and A are very faint. In a trial acetylation of lauryl alcohol a faint alcohol spot also remains.

The above observations allow for a more informative conclusion than that developed from class fractionations: all the constituents present in classes D_A, D_B, D_C, and M probably contain an hydroxyl group.

Saponification and Methylation of Bayberry Wax

Bayberry wax was saponified and was methylated a number of times using different quantities and different time limits. Samples of some of the classes of Bayberry wax as isolated by column chromatography were also saponified, methylated and chromatographed on thin layers.

Procedures

a) 5 gm. of Bayberry wax, 2 gm. of potassium hydroxide, and 25 ml. ethyl alcohol are refluxed together for 24 hrs. Before reaching the solidification point on cooling, the reaction mixture is extracted with diethyl ether. The aqueous layer is acidified with 60% sulfuric acid and then extracted with ether. To minimize the formation of an emulsion, the reaction mixture is divided into small portions and then extracted.

b) Between 9 and 450 mg. of sample, 0.6gm. of potassium hydroxide and 30 ml. of ethyl alcohol are refluxed together for two hrs. The reaction mixture is then acidified and extracted with petroleum ether. No attempt is made to separate the saponifiable from the unsaponifiable fractions.

Methylation

The extract from procedure (b) is reduced to a small volume and then methylated with BF_3 - methanol solution (2).

Results and Discussion

The acids produced by procedure (a) appear as one spot on a thin layer chromatogram. The unsaponifiable fraction, as shown by Figs. 1 and 13, contains at least five separate spots.

The results are the same for the saponification of Bayberry wax by procedure (b), except that all the spots, excluding the acid spot, are much fainter (Fig. 14). On methylation only the acid spot disappears (Fig. 15). In both figures a new spot, U, appears between classes D_C and M. From the above results two conclusions are possible: saponification is not complete and/or classes T, D_A, D_B, and D_C do not consist of glycerides alone.

Except for spots A and U, all the other spots present in Figs. 13-15 are concluded to be wholly unsaponified glycerides. The basis for this conclusion

is given in Fig. 16. Here different fractions separated by column chromatography have been saponified and methylated. The fractions contain the classes T, T + T' + A, D_A, $D_A + D_B$, $D_B + D_C$, and M. All fractions except for $D_B + D_C$, and T, show only acid spots after saponification and methyl ester spots after methylation.

Since class T' completely disappeared on saponification it probably consists only of glycerides (i.e. triglycerides). Since fraction $D_A + D_B$ only gives an acid spot, whereas fraction $D_B + D_C$ also gives an additional spot, U, i.e. representing unsaponifiable constituents, D_C is probably the only class of Bayberry wax that contains unsaponifiable constituents other than free fatty acids. Even though class D_C has not been further investigated, the evidence to this point suggests that:

1. It may contain diglycerides or other esters.
2. It probably contains an hydroxyl group.
3. The unsaponifiable constituents are not present in uncombined form, since its R_f value (.2) is lower than the R_f value of D_C (.32).

Possibly, certain components of D_C containing these constituents were saponified.

Spot T, as seen in Fig. 14, is quite dense, however, it is still concluded to represent only unsaponified triglycerides. If there were other classes present,

on the basis of R_f values and the colour and odor of the isolated spot, these would probably be the classes of hydrocarbons and waxes. With the solvent system used however, the hydrocarbons would migrate with the solvent front. As seen in Figs. 2 and 13, a small spot, presumably hydrocarbon, appears above the triglyceride spot. If waxes were present saponification would result in an acid and an alcohol spot. However only an acid spot is observed. Thus both possibilities are eliminated.

The ease of saponification of glycerides probably varies in the order: monoglycerides > diglycerides > triglycerides. With this in mind, the density of the charred spot T is not unexplainable, for in Fig. 14:

Spot T:	dense
Spot T':	absent
Spot A:	dense
Spots D_A, D_B, D_C:	faint
Spot M:	absent

In summary, saponification and methylation results suggest that:

Spot 1	Hydrocarbon
Spot 2	Triglyceride
Spot 3	Triglyceride
Spot 4	Free fatty acid

Spot 5 Diglyceride

Spot 6 Diglyceride

Spot 7 Diglyceride; other esters; unsaponifiable constituents

Spot 8 Monoglyceride

Periodic Acid Oxidation

By periodic acid oxidation 1-monoglycerides are changed to aldehydes while 2-monoglycerides remain intact. With oxidized Bayberry wax spot M became faint (Fig. 9). This shows that both isomers are present with 1-monoglycerides probably predominating. In terms of the stability of the isomers this qualitative result is in the correct range. At equilibrium, the ratio of 1-monoglycerides to 2-monoglycerides is 12:88 (22). This result would also occur if there were isomerization on S.G.G. However, Privett and Blank (27) did not find any such change on S.G.G., even though Borgström (27) found isomerization in silicic acid column chromatography.

FIGURE 11.

TLC of a concentrated solution of acetylated Bayberry wax.

Solvent: Petroleum ether:ether: acetic acid (38/26/1.5)

Solvent front: 15 cm.

Development time: 34 min.

Samples: a) Bayberry wax
b) Acetylated Bayberry wax
c) Acetylated Bayberry wax

	R_f Values					
Bayberry wax spots	2	4	5	6	7	8
	.82	.64	.49	.43	.40	.10

Acetylated Bayberry wax spots	2'	3'	4'	5'	6'	7'	8'
	.83	.74	.63	.55	.49	.45	.42

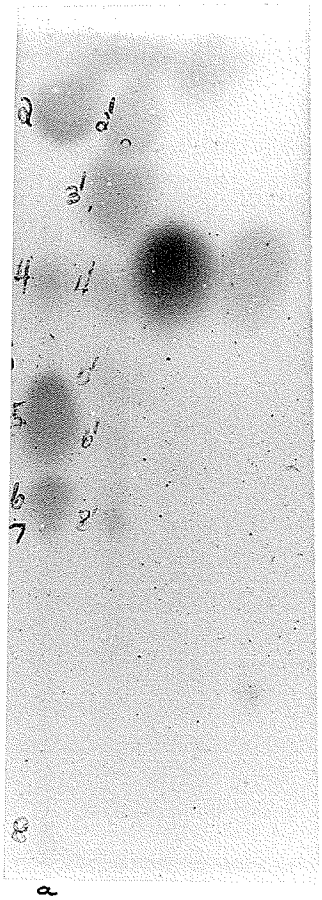


FIGURE 12.

TLC of a dilute solution of acetylated Bayberry wax.

Solvent: Same as above

Solvent front: 14 cm.

Development time: 40 min.

Samples: a) Bayberry wax
b) Acetylated Bayberry wax
c) Oleic acid
d) Palmitic and stearic acids

	R_f Values					
Bayberry wax spots	2	4	5	6	7	8
	.92	.71	.55	.46		.09
Oleic acid		.73				

Acetylated Bayberry wax spots	2'	3'	4'	5'	6'	7'	8'
	.88	.82	.71	.59	.51		.44

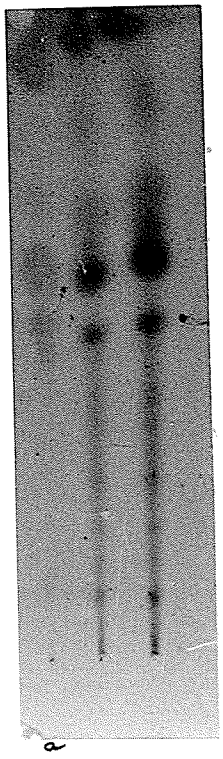


FIGURE 13.

TLC of the unsaponifiable fraction of Bayberry wax: Procedure A.

Solvent: Petroleum ether:ether:acetic acid (36/28/1.5)

Solvent front: 15 cm.

Development time: approx. 50 min.

Samples: a) Bayberry wax
b) Unsaponifiable fraction
c) Unsaponifiable fraction

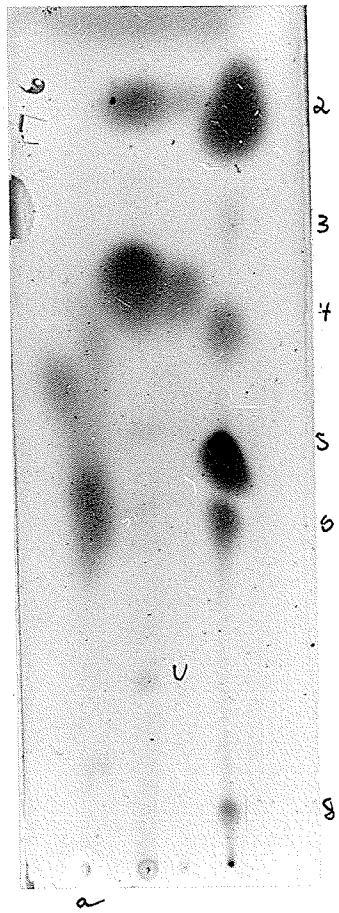


FIGURE 14.

TLC of the saponified mixture of Bayberry wax: Procedure B.

Solvent: Petroleum ether:ether: acetic acid (40/20/1.33)

Solvent front: 15 cm.

Development time: approx. 30 min.

Samples: a) Saponified mixture before acidification
b) Saponified mixture after acidification, extraction and concentration
c) Same as b)
d) Bayberry wax

	R_f Values					
Bayberry wax spots	2	3	4	5	6	8
	.81	.69	.58	.43	.36	.05

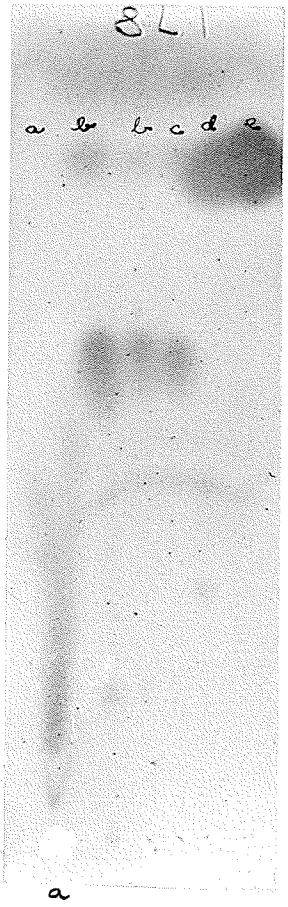


FIGURE 15.

TLC of saponified Bayberry wax after methylation: Procedure B.

Solvent: Petroleum ether:ether: acetic acid (40/30/1.33)

Solvent front: 15 cm.

Development time: 23 min.

Samples: a) Saponified reaction mixture before acidification
b) Saponified reaction mixture after acidification, extraction and concentration
c) Fatty acids of Bayberry wax separated from a portion of the reaction mixture
d) Methyl esters of Bayberry wax
e) Methyl palmitate

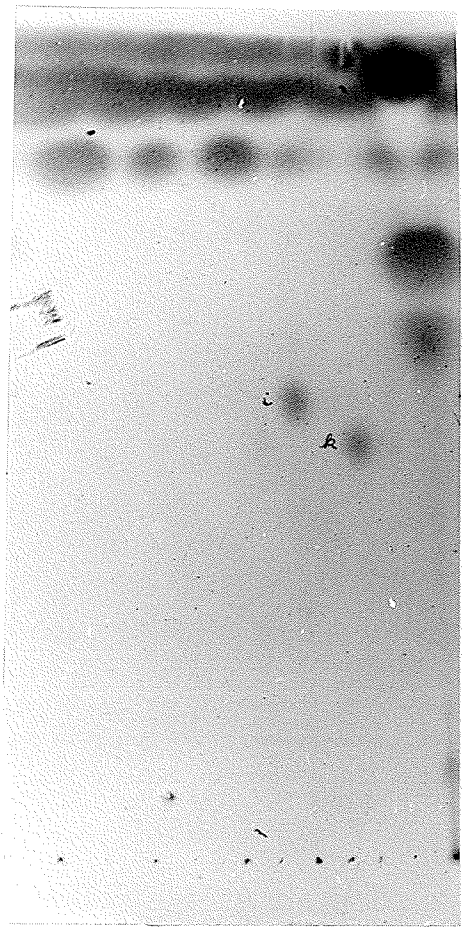


FIGURE 16.

Saponification and methylation of certain classes of Bayberry wax (Procedure B) as isolated by column chromatography.

Solvent: Petroleum ether:ether:acetic acid (40/20/1.33)

Solvent front: 15 cm.

Development time: 40 min.

Samples:
a) Fatty acids from Class T
b) Methyl esters from Class T
c) Fatty acids from Classes $D_A + D_B$
d) Methyl esters from Classes $D_A + D_B$
e) Fatty acids from Class D_A
f) Methyl esters from Class D_A
g) Fatty acids from Classes T + T' + A
h) Methyl esters from Classes T + T' + A
i) Fatty acids from Classes $D_B + D_C$
j) Fatty acids from Class M
k) Methyl esters from Classes $D_B + D_C$
l) Methyl esters from Class M
m) Bayberry wax

PREPARATIVE TLC - GRAVIMETRIC ANALYSIS

Gravimetric analysis (19) is a semi-quantitative method of recovery analysis, and it is primarily suited to the analysis of non-polar lipids that can be recovered rapidly using only small quantities of volatile solvent.

Spectroscopic and radiometric methods (19, 28) are other methods that depend on the recovery of the spots before analysis. Quantitative methods have also been developed for a direct spot analysis (19, 28), i.e. spot size method, photometric methods. The error for these procedures can range from 3-5%. Except for gravimetric analysis, these methods require special equipment.

Procedure

After the plate is developed, the spots are made visible by placing the plate in an Iodine chamber for a few minutes. The rows of spots are then marked with a glass rod, and the iodine is allowed to evaporate. The spots of each class are removed with a vacuum aspirator of type (a). To aid in the elution of the lipid, a water jacket, with warm water running through it, is attached to the glass tube. About 5 ml. of chloroform extract is collected from each batch of S.G.G. Air pressure is sometimes used to speed up the flow rate. The solvent is evaporated to a small volume and the test tube is then placed in a vacuum desiccator for a complete evaporation. Fig. 3 shows a typical chromatogram (it is

charred for illustrative purposes only). The size of the plate is 10 x 20 cm. The development is not as uniform on larger size plates.

The above methods of detection, recovery and elution were tedious, however they were still used for gravimetric analysis. When more material was again required, though, a larger aspirator was used (14), and 2',7' dichlorofluorescein was used as the detector (two lanes on each side of the plate were sprayed as guides). In this way one aspirator can collect the fractions of one class from at least ten plates (thickness of layer 0.76 mm.; size 10 x 20 cm., 15 x 20; ca. 7 gm. of S.G.G. per plate).

Results and Discussion

Class	% by weight
H	0.6
T	51.7
T' + A	<2.4
$D_A + D_B + D_C$	38.5
M	5.7
residue	0.6

Amount collected: 16.6 mg.

Theoretical yield: 14.5 mg.

A difference of 2.1 mg. exists between the actual and theoretical yields. There may be an error in the weighings, and in the quantitative application of the

spots. Also the difference may represent eluted impurities from S.G.G. Blank elutions of 10 gms. of S.G.G. yield between 0 and 0.4 mgs. of material. This amount itself then would not account for this difference.

The technique used for preparative TLC did not give a complete separation of the spots D_A, D_B, and D_C or A from T' (Fig. 17). Nevertheless, even though it was not demonstrated here, TLC is a good method for the isolation of classes, especially if the classes are close together. Its primary advantages over column chromatography are:

1. Speed
2. Sharp, easily detectable zones
3. Ease of isolation.

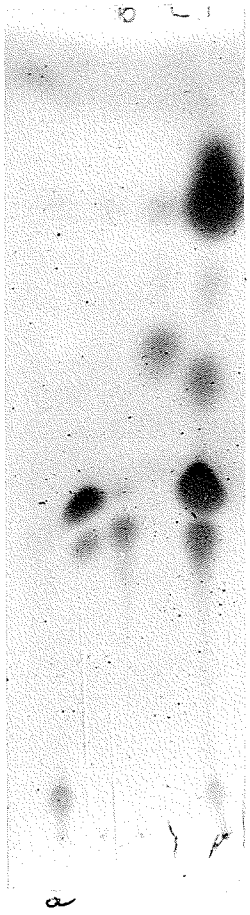


FIGURE 17.

TLC of fractions recovered by preparative TLC.

Solvent: Petroleum ether:ether:acetic acid (40/20/1.33).

Solvent front: 15 cm.

Development time: 16 min.

Samples:
a) attempted recovery of Class M
b) attempted recovery of Class D_A
c) attempted recovery of Classes D_B and D_C
d) attempted recovery of Class A;
e) Bayberry wax

THIN-LAYER PARTITION CHROMATOGRAPHY

Silver Nitrate Impregnated Plates

Silver nitrate fractionates esters according to their degree of unsaturation. On sample triglycerides, good separations occurred.

Triglyceride	R_f Value
Linolein	.12
Olein	.53
Palmitin and other saturated esters	.70
Spot T	.70

Only one spot with an R_f value of .7 appears with fraction T. Thus triunsaturated acids are not present. Mono- and diunsaturated triglycerides of oleic acid would probably migrate between R_f values .5 to .7. Since this is not observed, unsaturated acids themselves may be completely absent from Bayberry wax. Nevertheless the system used may not be sensitive to small quantities of these triglycerides. Bayberry wax methyl esters also gave just one spot.

Tetradecane Impregnated Plates

Tetradecane is used as a non-polar stationary phase to fractionate triglycerides according to chain length. Sample triglycerides gave good separations except for one case.

Triglyceride	R_f Value
Laurin (not the sample used in Fig. 8)	.33

Triglyceride	R_f Value
Myristin	.33
Palmitin	.23
Linolein	.69
Stearin	.00
Spot T	.26

Randerath (30) shows a chromatogram developed in a similar manner, and the saturated triglycerides were completely separated. This is not so in Fig. 18. The purity of the samples was checked by melting point determinations and the melting point of laurin did not correspond to the literature value. Thus this sample is not pure laurin. From its R_f value and M.P. it appears to be myristin.

M.P.	Lit. Value
Laurin 55-58°C	46.4°C
Myristin 57.5-60	56.5
Stearin 72.5-74	54.5, 70.8

The triglycerides of Bayberry wax give only one spot and appear to have a carbon number between palmitin and myristin. Thus two possible isomers are MMP and PMP (M: myristyl; P: palmityl).

In summary, by TLC of Bayberry wax:

		% by weight	
spot 1	Hydrocarbons	0.6	
2	Triglycerides	51.7,	possible types MMP and PMP
3	Triglycerides	<2.4	
4	Free fatty acids		
5	1:3 Diglycerides		
6	1:2 Diglycerides		
7	Esters plus unsaponifiable matter	38.5	
8	Monoglycerides	5.7	
9	Residue	0.6	

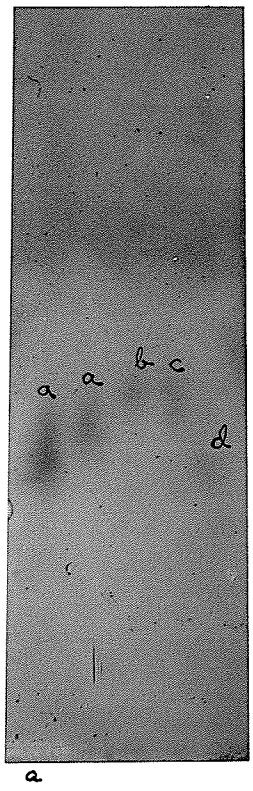


FIGURE 18.

TLC on a tetradecane impregnated plate.

Solvent: Acetone: acetonitrile (10/1) - saturated with tetradecane

Solvent front: 15 cm.

Development time: 65 min.

Samples:
a) Class T isolated by column chromatography
b) Laurin
c) Myristin
d) Palmitin

COLUMN CHROMATOGRAPHY

Introduction

Column chromatography is an effective method for separating mixtures of lipids into groups and classes. Three main adsorbents have been used for neutral lipid separations: alumina, silicic acid, and florosil.

Silicic acid is the most extensively used adsorbent. The work of Trappe (38), Borgström (38), Fillerup and Mead (15), and Lea, Rhodes and Stall (38), demonstrates the effectiveness of silicic acid column chromatography in the separation of neutral lipids from phospholipids and in the further fractionation of both of these into classes. The period between 1950-1959 covers the extensive work done with silicic acid. A review by Wren (38), articles by Creech (11), and a discussion by Hanahan (15), provide a good introduction to column chromatography.

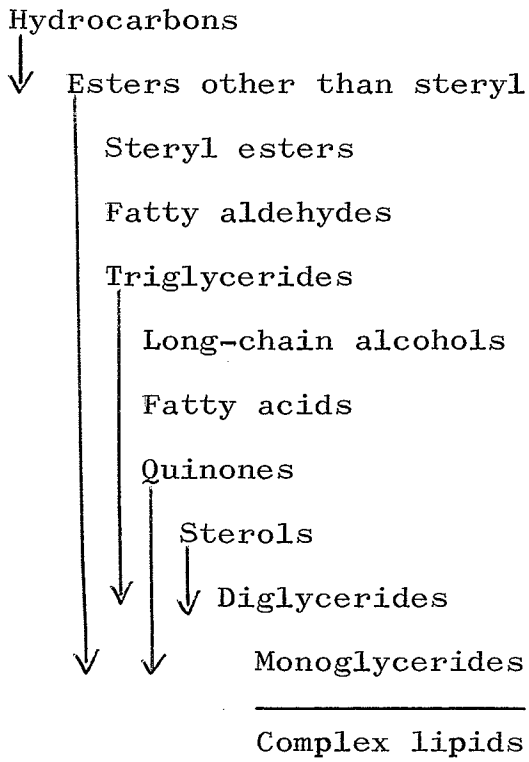
Adsorbent

Silica, silica gel and silicic acid are synonymous terms. The adsorbent consists of a powdered solid of the formula SiO_2xH_2O. This consists of porous three-dimensional siloxane (Si.O.Si) structures and surface silanol (SiOH) groups. Pure silicic acid has a neutral surface. Variations in the number of silanol groups, free water content, chemical impurities, topography, and particle size, affect the separations. Only in the case of polar lipids do the silicic acid and silica gel preparations show variations in the elution pattern. For the chromatography of

tissue lipids, several American preparations of silicic acid have proved superior to silica gel preparations (38).

Order of Elution

Wren (38) lists an order of elution for neutral and polar lipids on silicic acid. This should also be applicable to silica gel. The order is a general one and it is based on the polarity of the substance. The excerpt below shows the order of the most readily eluted member of each neutral lipid class. The arrows show the variation within each class.



Eluents

Petroleum ether:ether mixtures represent one of the better systems for the elution of neutral lipids. There

are two methods of elution:

1. Stepwise elution:

The column is eluted with pre-determined quantities of specific mixtures of the solvent pair.

2. Gradient elution:

The column is eluted by the addition of a continuously changing proportion of one solvent in the other member of the pair.

According to Rouser (29) gradient elution is useful for a rapid preliminary determination of the properties of a particular column packing, and for the rapid elution of substances of widely different polarities. Stepwise elution is best for a complete and quantitative separation, and this was the type used for the fractionation of Bayberry wax.

Fillerup and Mead (15) separated artificial mixtures, and plasma lipids using the following ratios:

1% Ether in petroleum ether	Steryl ester
3% Ether in petroleum ether	Triglyceride and free fatty acids
10% Ether in petroleum ether	Free sterol
25% Methanol in diethyl ether	Phospholipids

Hirsch and Ahrens (11) quantitatively separated synthetic mixtures of seven major classes of neutral lipids using the following ratios:

1% Ether in petroleum ether (60-70°C)	50 ml.	Paraffin
1% Ether in petroleum ether	75 ml.	Squalene
1% Ether in petroleum ether	250 ml.	Cholesteryl palmitate
4% Ether in petroleum ether	350 ml.	Stearin
8% Ether in petroleum ether	200 ml.	Caproin
8% Ether in petroleum ether	450 ml.	Cholesterol
25% Ether in petroleum ether	200 ml.	Dipalmitin
100% Ether	200 ml.	Monopalmitin

Barron and Hanahan (15) used hexane as the "basic" eluting solvent:

Hexane	Hydrocarbon
Hexane-benzene (6.5:1.5 V/V)	Steryl esters
Hexane-5% ether	Triglycerides plus free fatty acids
Hexane-15% ether	Sterol
Hexane-30% ether	Diglyceride
Hexane-ether (1:10 V/V)	Monoglyceride
80% Methanol in ether	Phospholipids

For Bayberry wax the following mixtures were used:

		Class expected
100% Petroleum ether(30-50°C)	100 ml.	Hydrocarbons
4% Ether in petroleum ether	300 ml.	Triglycerides
15% Ether in petroleum ether	100 ml.	Free fatty acids
20% Ether in petroleum ether	300 ml.	Diglycerides
30% Ether in petroleum ether	150 ml.	Diglycerides
50% Ether in petroleum ether	100 ml.	Diglycerides
100% Ether	100 ml.	Monoglycerides

Materials and Methods

Column

Pyrex glass column, 1.3 cm. (i.d.) x 37 cm.; 250 ml. bulb; sintered glass disk; ground glass stopcock.

Adsorbent

Silica gel: 100-200 mesh - Fisher Scientific Company.

Solvents

Petroleum ether - distilled between 30-50°C .

Ether - distilled.

Procedures

Packing of Column

About 25 gm. of silica gel is made into a slurry with petroleum ether. The column drains at 4 ml./min. as the slurry is added.

Preparation of adsorbent

Prior to making the slurry, the silica gel is washed with ether:acetone (1/1), withether, and finally with petroleum ether. Then the adsorbent is applied to the column to a height of 25 cm. Fifty ml. of ether:acetone (1/1) is run through the column, followed by 50 ml. ether, and then 50 ml. petroleum ether.

Application of sample

A weighed quantity of Bayberry wax (500-550 mg.) is applied in solid form to the top of the adsorbent. On top of this, an additional 1 cm. of adsorbent is applied.

Collection of eluent

Ten ml. quantities of solvent are collected in

weighed test tubes; the solvent is evaporated (40°C) to a small volume: then the test tube is placed in a vacuum desiccator. When dry, it is placed in a desiccator in order to reach room temperature, and then it is weighed.

Recombination technique

After weighing all the test tubes, a plot of weight vs. volume of effluent is made. Samples from the peaks and from the slopes of the peaks are chromatographed on thin layers. Based on these results, the samples are combined by transferring the material from the required test tubes to a main test tube, using small quantities of petroleum ether and chloroform.

Results and Discussion

Fig. 19 shows the variation in the weight of 10 ml. fractions with the volume of effluent from a 110 test tube system, in one attempted fractionation of Bayberry wax by a silica gel column. Six distinct peaks are visible. In addition, both the triglyceride and diglyceride peaks appear to be slightly split. Fig. 20 shows a thin layer chromatogram of samples from most of the peaks in Fig. 19.

The order of elution of the classes appears to correspond to the order in TLC: hydrocarbons, triglycerides, free fatty acids, 1:3 diglycerides, 1:2 diglycerides, and monoglycerides.

The cleft in the triglyceride and diglyceride peaks may indicate sub-fractionation of these classes. In the case of the diglycerides, the split comes where the 1:2 diglycerides are eluted from the column.

The weight percent of only certain classes can be calculated, for most of the classes were not cleanly separated. The results are shown in Table II. The triglycerides are eluted together with the hydrocarbons. Since class H is more completely separated by TLC, the higher percentage is probably more accurate. Also the free fatty acids are eluted together with the triglycerides. Though this is not obvious from Fig. 19, it is quite apparent from Fig. 20. Again TLC gives a better separation of the free fatty acids, thus the lower value is probably more

accurate.

Calculations of separate values for classes D_A, D_B, and D_C cannot be made. A sample from the apex of peak 5-6 shows that only class D_A is eluted to this point. However on the downward slope both D_A and D_B are eluted. About ten test tubes later D_C is eluted along with D_B. Here, for the first time, a fraction has both a colour and an odor. From the total weight of the sample in this peak, class D_C, probably represents less than 2% of Bayberry wax.

Perhaps modifications in the solvent system, and in the preparation of the column would give better separations. Lower petroleum ether:ether ratios, i.e. 7% and 10% were found to give a better separation of class A from classes T and T'. However traces of class D_A were present.

The merging of classes may indicate an overloaded column. For silicic acid, Wren (38) states that the weight of the sample should not exceed 50 mg./gm. of adsorbent. Hanahan (15) gives the load factor of 15-20mg./gm. of adsorbent, and the flow rate of solvent between 1.5-2ml./min. for the best resolution. The separation in Fig. 19 was obtained with a load factor of 22 mg./gm. of adsorbent and a flow rate between 1.3-2.7 ml./min. However, the column could also be overloaded if a constituent of a mixture were present in a large quantity.

In conclusion:

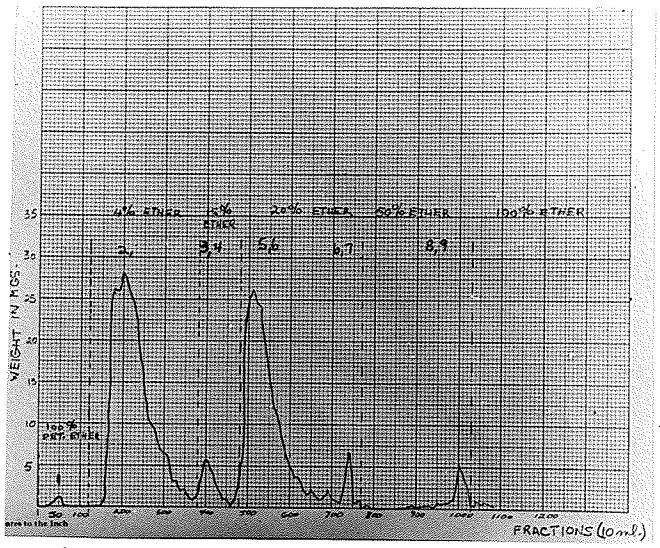
1. The fractionation of Bayberry wax on a silica gel column with petroleum ether:ether mixtures gave the same order of elution as TLC.

2. Chemical tests on some of the fractions confirmed the presence of 1-monoglycerides and the absence of cholesterol.

3. The colour and odor of Bayberry wax appeared to be concentrated in classes D_C and M, and in the residue.

4. Neither ether nor methanol completely removed the coloured material remaining at the top of the column.

5. The quantitative results were in fair agreement with the values from TLC.



Load factor: 22 mg./gm. of silica gel

Flow rate: 1.3-2.7 ml./min.

The numbers indicate the classes of Bayberry wax found in the above peaks by TLC.

FIGURE 19.

Column Chromatography of Bayberry wax - sample weight vs. volume of effluent.

Number	Class
1	Hydrocarbon
2	Triglyceride
3	Triglyceride
4	Acid
5	1:3 Diglyceride
6	1:2 Diglyceride
7	Unknown
8	Monoglyceride
9	Residue

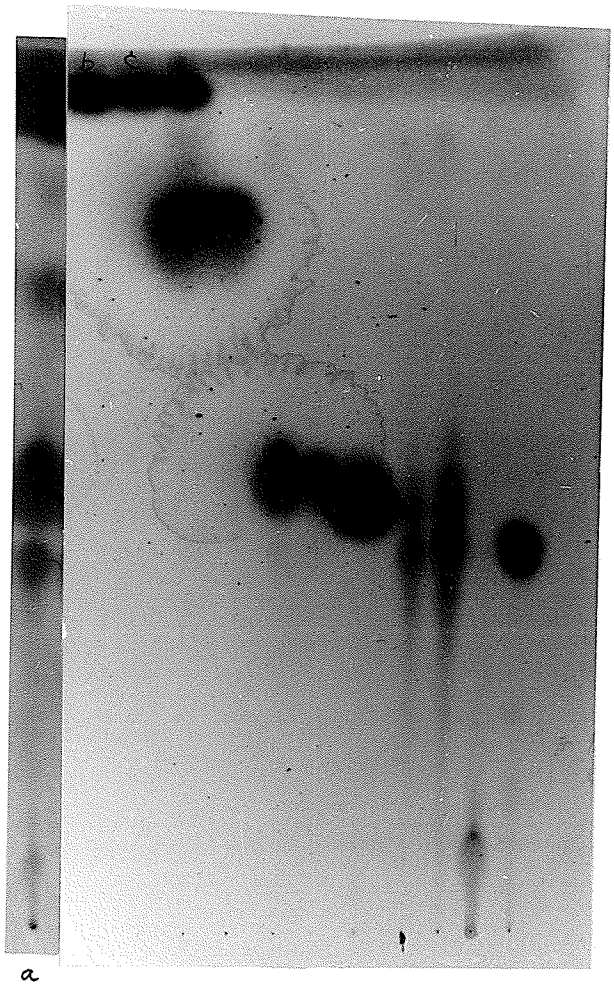


FIGURE 20.

TLC of some of the fractions isolated by column chromatography.

Solvent: Petroleum ether:ether: acetic acid (40/20/1.33)

Solvent front: 15 cm.

Development time: 36 min.

Samples:		Volume of effluent (as in Fig. 19)
a)	Bayberry wax	
b)	Class T	180 ml.
c)	Class T*	330
d)	Classes T, T' and A	400
e)	Palmitic acid	
f)	Class D_A	510
g)	Class D_A	510
h)	Lauryl alcohol	
i)	Classes D_B + D_C	740
j)	Classes D_B + D_C	740
k)	Class M	1000
l)	Cholesterol	

TABLE II

QUANTITATIVE RESULTS FROM TLC AND COLUMN CHROMATOGRAPHY

Class	Solvent	Volume of Solvent	% of the Total weight Recovered	% by TLC	Observations from the Classes Recovered
H	petroleum (pet.) ether	100 ml.	0.3	0.6	white colour; no odor
T + H	pet. ether:ether (4%)	300 ml.	48.6	51.7	white colour; no odor
T + T' + A	pet. ether:ether (15%)	100 ml.	4.2	2.4	white colour; no odor
$D_A + D_B$	pet. ether:ether (20%)	300 ml.	40.0	38.5	white colour; no odor negative Salkowski test
$D_B + D_C$	pet. ether:ether (20%)	300 ml.	2.3		green colour, and odor
M	ether	100 ml.	4.1	5.7	green colour and odor positive periodic acid oxidation test

The values are rounded off to the nearest tenth.

Weight of sample: 564 mg.
Recovered: 547 mg.
% Recovered: 97%

INFRARED SPECTROSCOPY

Introduction

Infrared light is light that has a wavelength range between 0.75 microns (μ) and 200 microns. However only the range between 2.5 μ and 16 μ is generally used, and this range is commonly called the infrared region. The shorter and longer wavelength regions are called the near and the far infrared regions.

The infrared region is used because different functional groups and different molecules absorb characteristic amounts of energy (over this region), and hence show a specific absorption pattern. The region above 8 μ is known as the "group frequency" region. Here absorption bands appear that are characteristic of certain functional groups. The region below 8 μ is known as the "finger print" region. Here the position of the bands are influenced by the environment of each group. Since small structural changes may affect the position of the bands, each substance has a unique pattern. The accurate correlation of the structure of an unknown substance with its absorption bands is difficult. However the identification of every band is not necessary to obtain sufficient information for identification.

Before 1955 infrared spectroscopy was not used extensively in the characterization of glycerides. In 1955 O'Connor *et al.* (25) took infrared solution spectra

of 21 pure glycerides between 2 and 12 μ. Starting in 1955 Chapman (3-8) began a series of papers discussing the polymorphic characterization of glycerides by infrared spectroscopy. He wrote reviews in 1960 (9) and 1965 (10). Though no book was found which offered extensive information on the infrared spectra of glycerides, a book by Nakanishi (24) provided a useful summary of absorption bands.

In the following presentation solution spectra of certain fractions of Bayberry wax as isolated by either column chromatography or TLC are shown, and the assignment of the major bands is discussed.

The spectra of the classes were interpreted on the basis of certain facts and possibilities derived from references and TLC (and column chromatography):

1. Bayberry wax supposedly consists largely of triglycerides .

2. Diglycerides and monoglycerides may be present .

3. Free fatty acids may be present .

4. Hydrocarbons may be present.

Equipment

Infrared Spectrophotometer

Model 21, Perkin-Elmer Corporation.

Solvent

Spectrograde carbon tetrachloride: Matheson, Coleman & Bell.

Cell

Sodium chloride (0.5 mm.).

Variables

Resolution: 927; response: 1; gain: 5.5; speed: 4; supression: 2.

Samples

1) Bayberry wax

2) Classes H, T, A, D_A, D_B + D_C, M, and R: from either TLC or column chromatography

3) Stearic acid N.F.: Fisher Scientific Company

4) Glycerol: found in the laboratory

5) Stearin: chromatographically pure; Hormel Institute, University of Minnesota.

Results and Discussion

O'Connor (25) noted a number of common bands in the spectra of tri-, di-, and monoglycerides. In addition to these, Chapman (4, 5, 8) noted that the bands in the region 730 cm^{-1} ($cm^{-1} = \frac{10,000}{\mu}$) and 1100 are also common to all glycerides. Table III gives the absorption bands of Bayberry wax, of most of its classes, and of some reference samples with the probable assignment of many of the bands.

Bayberry Wax

A comparison of the spectrum of Bayberry wax (Fig. 21) with spectra of impure triglycerides (Fig. 24) shows that there is good agreement for all of the major bands.

Bands indicating the presence of methylene ($-CH_2-$) or methyl ($-CH_3$) groups appear at 2930 cm^{-1}, 2870, 1465, 1378 and 710. O'Connor (25) and Chapman (6, 7) listed a band at 1261 - 1250, however no definite band is seen here. Instead, a gradual rising line appears with an inflection point at 1240.

A very strong band caused by the vibration of the carbonyl ($-C = O$) group in esters appears at 1750 cm^{-1}, with a small shoulder at 1710. The latter one may indicate free fatty acids. Very strong bands caused by ester group (C-O-) vibrations appear at 1164-1142 and 1110. A band of medium intensity appearing at 1419 may represent $-CH_2-CO$ groups.

Bands indicating unsaturation should appear at about 960 $cm.^{-1}$ and 1660. However, only very weak bands (if any at all) appear in these regions. This is expected for the Iodine number of our sample of Bayberry wax is between 0-1.

In a concentrated solution of Bayberry wax (Fig. 22) a medium band appears at 3500 $cm.^{-1}$. This probably indicates hydroxyl groups. A band at 4150 has not been identified. Both these bands almost disappear in a dilute solution.

Bands caused by vibrations of the hydroxyl (OH) groups of di- and monoglycerides are known to appear in the 1000 $cm.^{-1}$ region (4, 5, 8). Absorptions in this region are also expected for Bayberry wax since there has already been an indication of OH groups by the band at 3500. However only shoulders are produced: 1054, 1032, 952. Since the bands are known to be generally weak, the above result does not necessarily contradict the fact that di- and monoglycerides constitute about 40% of Bayberry wax.

Hydrocarbons (H)

The hydrocarbon class was isolated by TLC. However not enough was collected for an accurate analysis (in solution) by infrared spectroscopy. Nevertheless bands at 2930 $cm.^{-1}$, 2850, 1750, 1460, and 720 are visible. The 1750 band is the weakest one. If this sample consisted of just triglyceride, the 1750 band would have been at least as strong as the hydrocarbon bands.

Triglycerides (T)

Class T from both TLC (Fig. 23) and column chromatography give the same major bands. The $-CH_2-$ and $-CH_3$ groups account for bands at 2930 $cm.^{-1}$, 2850, 1465, 1378, and about 700. The ester bands appear at 1155 and 1110. The -C = O stretching vibration band appears at 1750. Several weak bands are also present but these have not been used to characterize triglycerides.

Acids (A)

The acid spectrum contains bands characteristic of both acids and esters (Fig. 27). This result is probably due solely to the impurity of the isolated sample (Figs. 17 and 20). Nevertheless, characteristic acid bands appear at 1712 $cm.^{-1}$, 1281, and 935. Several other prominent bands have not been identified.

Diglycerides A (D_A)

The infrared spectrum shows that this class consists at least partially of 1:3 diglycerides (Fig. 25).

The spectrum of a concentrated solution of D_A shows sharp bands at 4105 $cm.^{-1}$ and 3500. Only the peak at 3500, though much smaller, is still visible in the spectrum of a dilute solution. The band at 4105 is left unidentified. The band at 3500 is probably caused by an -OH stretching vibration. In the concentrated solution, bands at 2410, 2330, 2040 are also visible but they disappear completely in a dilute solution. They also remain unidentified.

O'Connor stated that a band at 1042 $cm.^{-1}$ is caused by the -OH vibration of a 1:3 diglyceride. Chapman (5) did not list this band. Instead the band at 940 $cm.^{-1}$ (in the case of 1:3 dilaurin) is considered as the unique band. In the spectrum of D_A, bands are present at 1035, and 934. These bands are not present in the spectra of classes T, D_B, or M. However, a band at 935 $cm.^{-1}$ does occur in the acid spectrum, but since there is no peak at 1710 in the spectrum of D_A the possibility of acid contamination is ruled out. Also TLC of D_A after isolation shows only a very faint acid streak. Thus the bands at 934 and 1035 are taken to represent the secondary hydroxyl group of 1:3 diglycerides.

Diglycerides B (D_B)

The infrared spectrum shows that at least part of class D_B as isolated by both TLC and column chromatography is a 1:2 diglyceride (Figs. 25 and 26).

A weak band at 3450 $cm.^{-1}$ is taken to indicate the presence of the OH group. This band may also be the first overtone of -C = O vibration. However, TLC indicates the presence of the OH group, and the band increases in intensity with increased concentration of sample.

O'Connor did not present any spectra on 1:2 diglycerides. Perhaps a characteristic band for the primary OH groups would appear at about the same position as the band for the primary hydroxyl group of 1-monoglycerides, i.e. 980 $cm.^{-1}$.

Chapman (8) reported the presence of two bands: 1097 and 1054. The spectra of D_B from samples isolated by column chromatography and TLC show a medium band at 1048 cm.$^{-1}$ and weak bands at 1082 and 952. Since these bands are in a different position from the ones in class D_A, and since diglycerides have been shown to be present by TLC, these bands are concluded to represent 1:2 diglycerides, even though the position of the bands are not in good agreement with the reference values.

Class D_C

No identification was made.

Monoglycerides (M)

Infrared spectroscopy again in this case provides further evidence to show that fraction M contains both 1- and 2-monoglycerides (Fig. 27).

O'Connor did not show any spectra of 2-monoglycerides. However, in the case of 1-monoglycerides, unique bands appeared at 1053 cm.$^{-1}$ (with an inflection at 1064 in some cases) and at 980. The first band is caused by a secondary alcohol vibration. The band at 980 is caused by a primary alcohol vibration. In the spectra of 2-monoglycerides, Chapman (4) found bands at 1048 and 975. In the spectra of 1-monoglycerides bands appeared at 1048, 975 and about 935. The spectrum of class M shows a band of medium intensity at 3450 cm.$^{-1}$. Again, this indicates the presence of OH

groups. Ester bands appear at 1165 and 1112. The spectrum of fraction M from column chromatography shows a strong band at 1050 and a weak band at 925. The spectrum of class M from TLC shows a band only at 1050. Though a band appears at 935 it is probably due to acid impurities, for a band also appears at 1710. Thus the monoglyceride band is probably hidden.

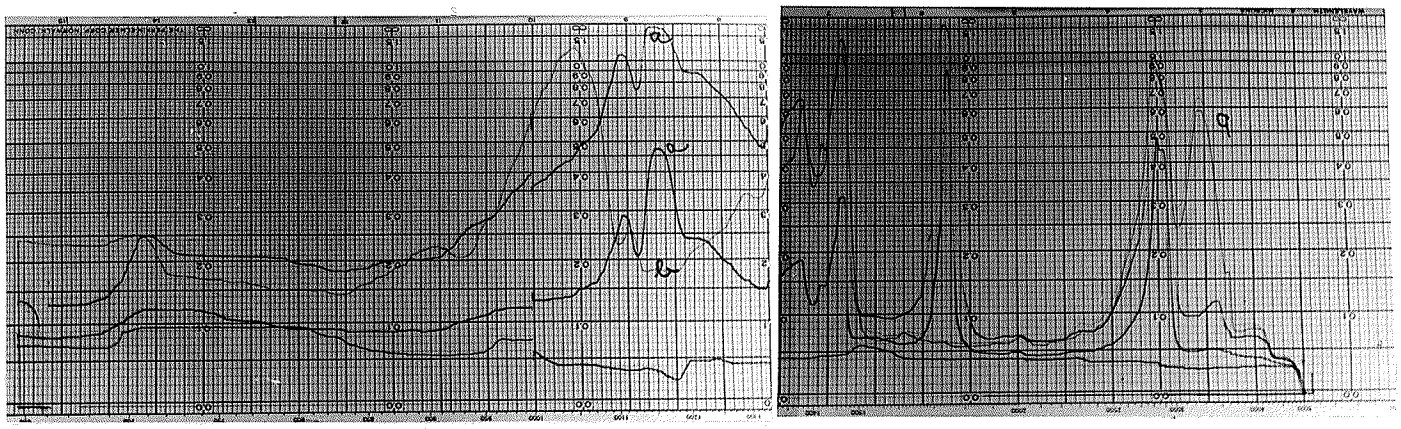
The reports by O'Connor and Chapman do not necessarily conflict, for O'Connor did not analyse 2-monoglycerides. Thus his assignment may be wrong. Since Chapman analyzed both isomers, his results are probably more conclusive.

Residue

Since only 1 mg. of the residue was collected, the amount was not enough to provide a good spectrum. Nevertheless noticable bands appear at 3400, 2900, 2850, 1740, 1460 and 770-720 $cm.^{-1}$. These indicate the presence of a methylene chain, and perhaps hydroxyl and ester groups.

Class T'

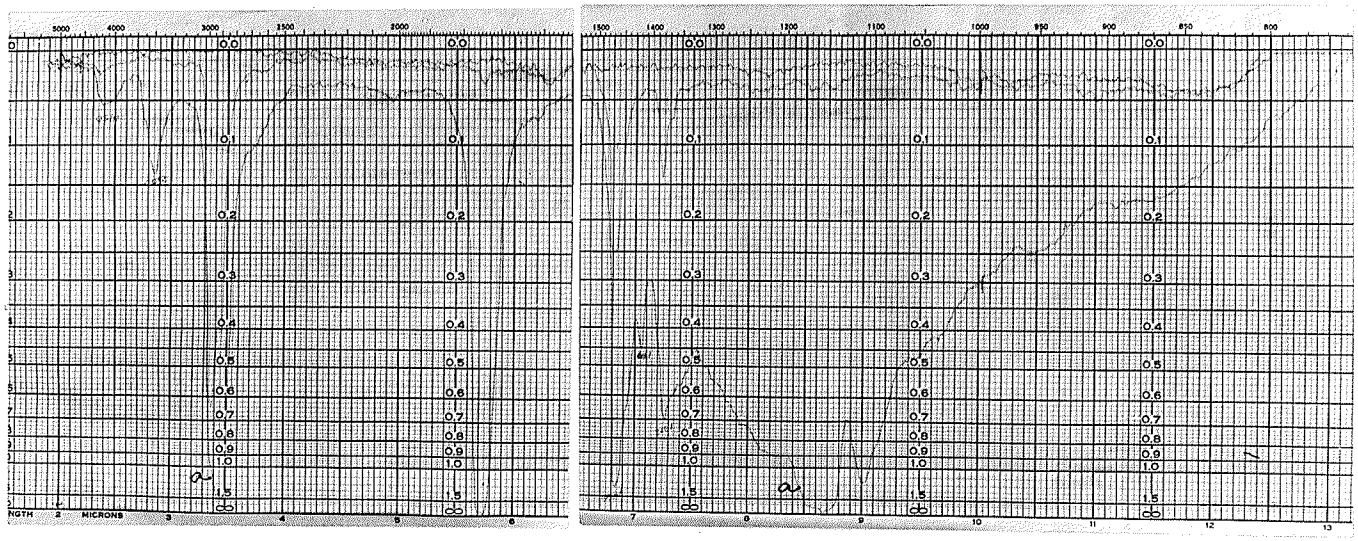
No spectrum was taken of this fraction.



a) Bayberry wax b) Lauryl alcohol

FIGURE 21.

Infrared spectrum of a dilute solution of Bayberry wax.



a) Bayberry wax

FIGURE 22.

Infrared spectrum of a concentrated solution of Bayberry wax.

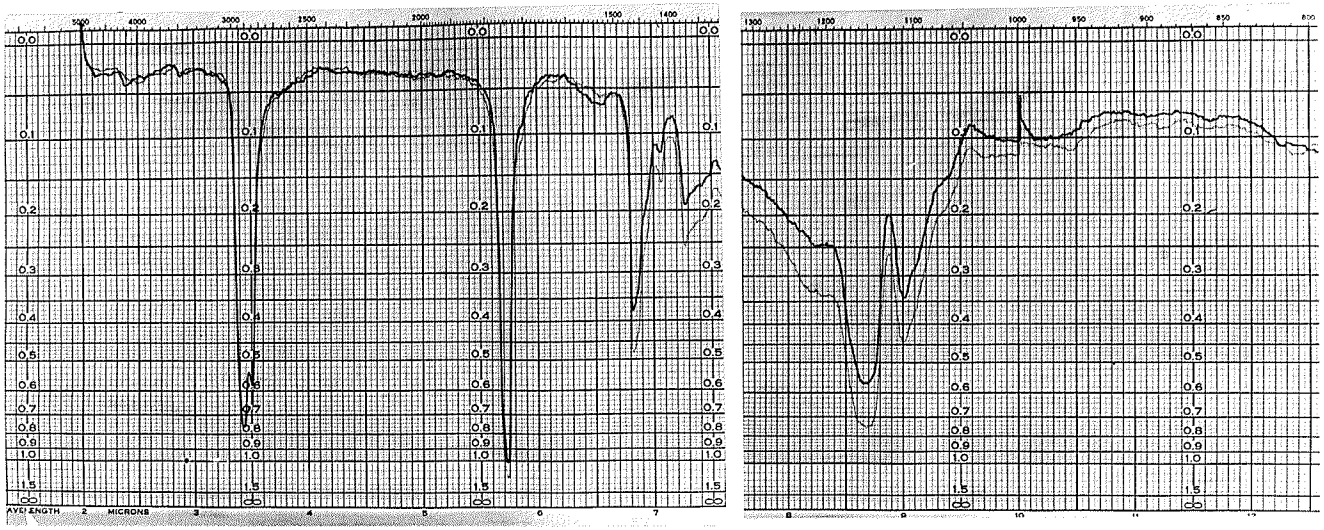
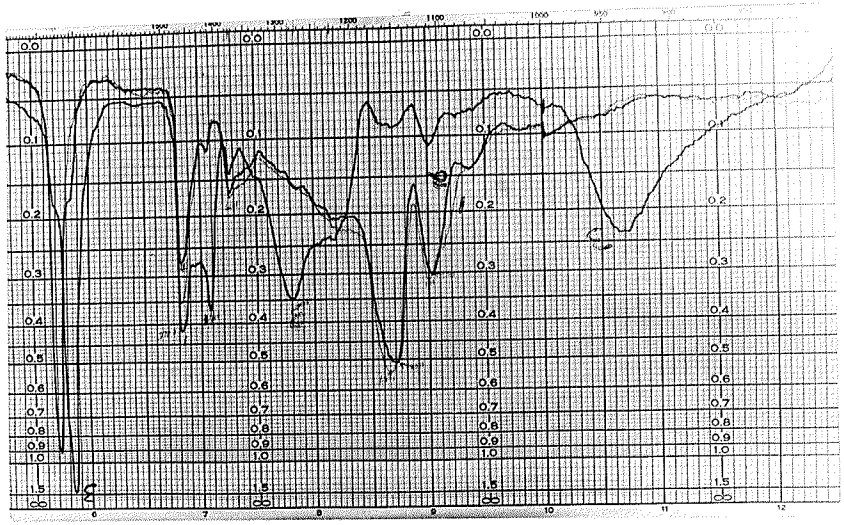


FIGURE 23.

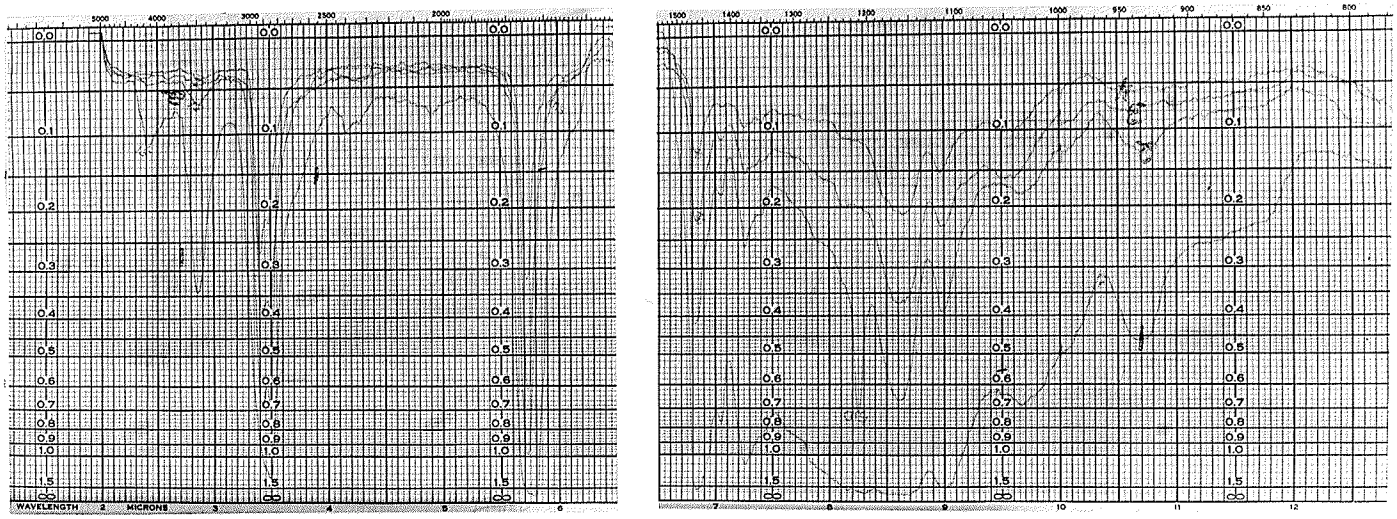
Infrared spectrum of class T isolated by TLC.



1. Palmitin
2. Laurin
3. Myristin

FIGURE 24.

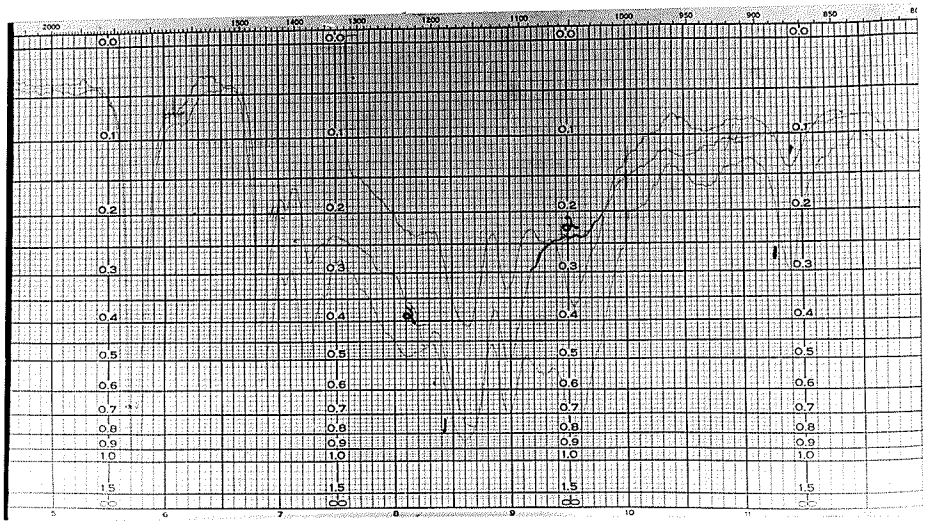
Infrared spectra of impure triglycerides.



1. A concentrated solution of class D_A isolated by column chromatography.
2. A dilute solution of class D_A.
3. A concentrated solution of classes D_B and D_C isolated by TLC.
4. A dilute solution of classes D_B and D_C.

FIGURE 25.

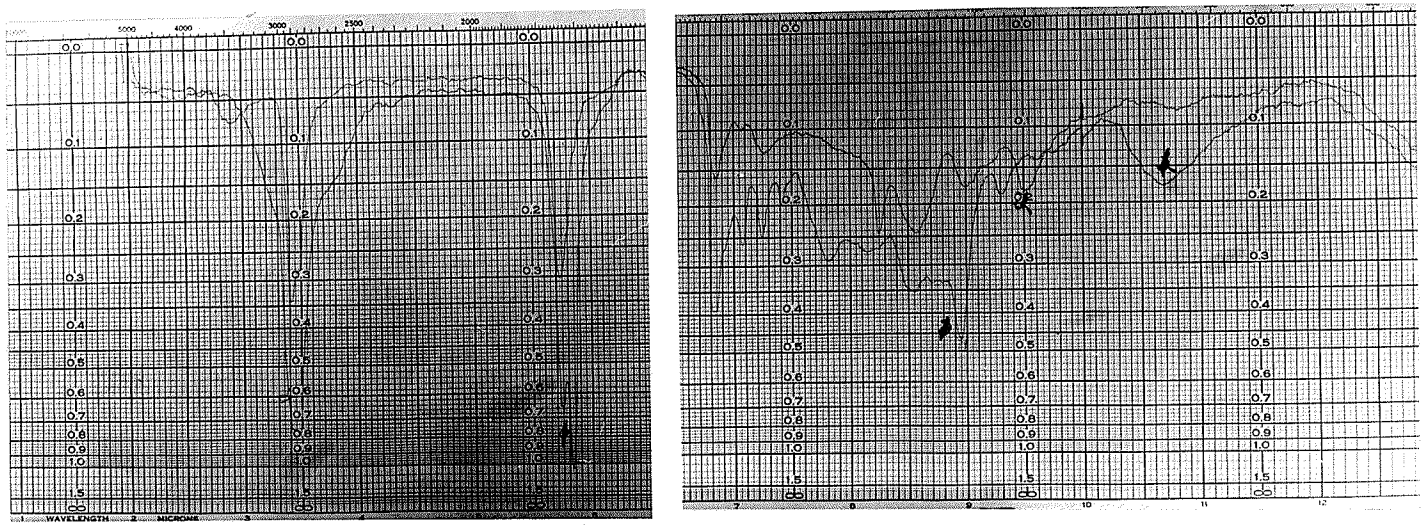
Infrared spectra of classes D_A, and D_B plus D_C.



1. A concentrated solution of classes D_A and D_B isolated by TLC.
2. Classes D_B and D_C isolated by column chromatography.

FIGURE 26.

Infrared spectra of classes D_A plus D_B and D_B plus D_C.



1. Class A isolated by TLC.
2. Class M isolated by column chromatography.

FIGURE 27.

Infrared spectra of classes A and M.

TABLE III

INFRARED ABSORPTION BANDS

l	Ba	H	T	A	D_A	D_B+D_C	M	R	T_S	S_A	G	Assignment
	4150 W.				4105 W.		4105 W.					
	3500 W.				3500 M.	3450 W.	3450 M.	3400 (?)				-OH
3030-2967V.S.	2930 V.S.	2930 S.	2930 V.S.	2930 V.S.	2940 V.S.	2810 V.S.	2940 S.	2900 V.S.	2940 V.S.	2930 V.S.	2950 M.	-CH_3
	2870 V.S.	2850 S.	2850 S.	2860 V.S.	2870 V.S.	2860 V.S.	2860 S.	2850 S.	2870 V.S.	2860 V.S.	2850 M.	-CH_3
1751-1733V.S.	1750 V.S.	1750 W.	1750 S.	1741 S.	1745 V.S.	1740 V.S.	1745 S.	1740 W.	1752 V.S.			-C = O in esters
	1710 Sh.			1712 V.S.		1709 Sh.				1710 V.S.		-C = O in acids
	1640 W.				1640 Sh.	1639 W.			1640 W.			-C=C-
1464-1453S.	1465 S.	1460 M.	1465 S.	1465 S.	1465 S.	1465 S.	1465 M.	1460 W.	1468 V.S.	1462 V.S.	1445 M.	-CH_3 and -CH_2-
	1419 M.		1419 M.	1415 M.	1419 M.	1419 M.	1415 W.		1419 M.	1410 S.		-CH_2-CO-
1383-1361S.	1378-1352S.		1378 S.	1381 M.	1378 M.	1378 S.	1375 M.		1381 S.	1380 M.	1380 M.	-CH_3

TABLE III (Continued)

INFRARED ABSORPTION BANDS

1	Ba	H	T	A	D_A	D_B+D_C	M	R	T_S	S_A	G	Assignment
				1349 W.								
											1345 M.	
				1281 M.						1285 S.		-OH (?)
1261-1250S.					1265 Sh.	1265 Sh.						$-CH_2-$
	1228 B.;Sh.			1227 M.					1225 M.			
	1210 B.		1210 M.		1213 S.	1212 M.	1212 S.					-OH (?)
1179-1166V.S.	1164-1144V.S.		1155 V.S.	1171 M.	1163 S.	1156 M.	1165 S.		1162 V.S.			-C-O-C of ester
											1115 V.S.	
	1110 S.		1110 S.	1118 S.	1113 M.	1115 V.S.	1112 M.		1115 S.	1114 M.		-C-O-C of ester
				1075 M.		1082 W.				1090 Sh.	1074 M.	-OH
	1054 ?					1048 M.	1050 W.					-OH

TABLE III (Continued)

INFRARED ABSORPTION BANDS

1	Ba	H	T	A	D_A	D_B+D_C	M	R	T_S	S_A	G	Assignment
	1032 ?				1035 M.				1030 W.	1032 W.		-OH
	952 ?		960 M.			952 W.			960 M.			-OH
				935 B.	934 M.		925 W.			935 M.		-OH
	710 W.	700 W.	700 W.	725 B.	720 W.	719 B.	725 M.	770-720?			725 W.	$-CH_2-$
					668 S.	668 M.	668 M.					-OH (?)

Abbreviations:

1: As given in reference 20
Ba: Bayberry wax
H: Hydrocarbon of Ba from TLC
T: Triglycerides of Ba from column chromatography (C.C.)
A: Acids of Ba from TLC
D_A: Diglycerides of Ba from C.C.
D_B: Diglycerides of Ba from C.C.
M: Monoglycerides of Ba from C.C.
T_S: Pure tristearin
S_A: Stearic acid
G: Glycerol
V.S: Very strong
S.: Strong
M.: Medium
W.: Weak
B.: Broad
Sh.: Shoulder

GAS-LIQUID CHROMATOGRAPHY

Introduction

Gas-liquid chromatography (GLC) is used to separate a class into its components, however even under optimum conditions it does not give a complete resolution. Nevertheless it is a rapid and a sensitive method, (quantities necessary range from 1 to 500 mg. depending on the detector), and it gives both qualitative and quantitative results.

Since its first use in 1952, GLC has been widely used for the analysis of long-chain fatty acids in the form of their methyl esters. In addition, acetylated mono- and diglycerides up to a molecular weight of 625 can be chromatographed at 300°C (2). Triglycerides up to a molecular weight of 800 can be chromatographed with temperature programming between 200-300°C (18). In this study, only the fatty acids of Bayberry wax were analyzed.

Though GLC gives the carbon number of the fatty acids, the corresponding structures can only be tentatively assigned (17). The separations are made quantitative by measuring the areas under the peaks with a planimeter.

To attain the best separation of a mixture of fatty acids, more than one stationary phase should be used. Non-polar stationary phases such as Apiezon L and Methyl-substituted Silcone gum (SE-30) give good separations of the saturated fatty acids (2). The saturated fatty acids are eluted according to increasing carbon number. The unsaturated fatty acids

are eluted before the saturated analogues. The retention volumes (the quantity of gas necessary to elute each substance) for a mixture of fatty acids are in the order: saturated > monenes > dienes = trienes. Also the separation of monenes from dienes is only partial even under optimum conditions. Apeizon L can withstand temperatures up to 300°C without extensive cracking or loss. Using this liquid, a good separation of fatty acids between C_{10}-C_{30} is achieved between temperatures 197°-286°C. SE-30 appears to give the same type of separation, however higher temperatures can be used since it is stable up to 400°C.

Polar liquids such as polyesters are better stationary phases for the resolution of unsaturated fatty acids. These liquids also separate members of a homologous series by boiling point, but they are not as efficient as nonpolar liquids. The retention volumes of a mixture of fatty acids are in the order: trienes > dienes > monenes > saturated. Peaks arising from polyenes may overlap with saturated ester peaks having the next highest carbon numbers.

Equipment and Chemicals

Carrier Gas

Helium

Column

Copper columns $\frac{3}{16}$ in. o.d. and $\frac{1}{8}$ in. i.d.; length varied from 56 cm. to 98 cm.

Connections

Swagelok fittings.

Detector

Thermal conductivity cell.

Flowmeter

Twin rotometers, 6" scale, with needle value calibration 0 - 100 cc/min.

Gas Chromatography

Dynatronic Chromalyzer-100.

Methyl ester standards

Heart Institute, Bethesda, Maryland.

Planimeter

Compensating polar planimeter - 620000, Keuffel and Esser Co.

Power Supply

"12 volts, D.C., solid state, 0.02% regulation, 400 milliamps".

Recorder

1 to 50 mv., and 1 to 10 volts, Zener diode reference system; speeds 1"/minute, 16"/hour.

Soap bubble flowmeter

Measures exit flow rates at atmospheric termperature and pressure.

Solid Support

Firebrick, not acid washed, $\frac{30}{60}$ mesh; washed in lab. with conc. HCl.

Solvents

Distilled:petroleum ether (30-50°C), chloroform, and ether.

Stationary phase

a) Apiezon L grease (15:85).

b) Silicone gum rubber (8:92).

Experimental Procedures

Coating of stationary phase

The stationary phase is dissolved in ether. Then the solid support is added to the flask and the ether is evaporated in a rotary evaporator. To remove the ether completely, the flask is heated in an oven at 70°C.

Packing the column

The bottom of the column is stoppered with a small piece of glass wool. Then the column is supported in a vertical position and a small funnel is attached to the top with a short length of plastic tubing. As the packing is added, the column is sharply tapped with a file. The column is filled to within 1 cm. of the top and sealed with a plug of glass wool. After bending it to the required shape it is connected to the chromatograph with Swagelok fittings.

Conditioning the Column

All columns are heated for at least 12 hours at a temperature 20-50° above the working temperature. In this way, any volatile substances are driven off.

Injection of Sample

All samples are injected as petroleum ether, ether or chloroform solutions. A 10 microliter syringe with a 2 in. fixed needle is used.

Reading of retention times

The times are taken from the elution of the solvent to the maximum height of the peak.

Saponification

Procedure C: as described on page 34.

Methylation

As described on page 35 .

Results and Discussion

Neither the Apiezon L nor the SE-30 column gives a separation of the saturated from the unsaturated fatty acids. The failure to resolve these types in the fatty acid analysis of most plant fats would very seriously limit the value of the results. With Bayberry wax the error is small. Different species of Bayberry wax (16) contain at the most only oleic acid, and in quantities from 0-1.4%. The Iodine Number is generally between 0.6-3.9 (12). Our sample of Bayberry wax has an Iodine Number of less than one (Hanus method). Also unsaturated constituents are not detected by either TLC or infrared spectroscopy. Even if there were enough unsaturated 18 carbon fatty acids to be detected by GLC, they probably would be eluted only together with stearic acid since the separation is by carbon number.

Chromatography of Fatty Acid Methyl Ester Standards

In an article on the quantitative analysis of fatty acids by GLC (17), the authors discussed instrument design, operating conditions, and the use of fatty acid methyl ester standards. In this study, standard mixtures E and F are used to determine the degree of resolution of the saturated fatty acids, to correlate peak area to percent composition, and to obtain retention times for a series of saturated fatty acids from C_8 to C_{24}.

Both the Apiezon L column and the SE-30 column give a complete resolution of the saturated fatty acids (Figs. 28 and 29). However the quantitative analysis shows only

fair agreement with the actual values. Table IV shows two experimental determinations for each column. In a report on fatty acid analysis by GLC (17) the authors stated that the "Composition values from 1 to 5% should have a maximum relative error of about 10%, and composition values above 5% a maximum relative error of about 5%. This level of accuracy is not attained in many laboratories".

TABLE IV

QUANTITATIVE RESULTS OF STANDARD METHYL ESTER MIXTURES

Mixture E

Methyl Esters	True wt. %	Experimental wt. % Apiezon L	
8:0	6.28	5.77	4.96
10:0	9.26	13.45	8.19
12:0	12.08	14.41	12.41
14:0	23.29	23.09	26.05
16:0	49.09	43.29	48.41

Mixture F

Methyl Esters	True wt. %	Experimental wt. % SE-30	
14:0	2.52	2.87	2.16
16:0	4.18	5.74	4.32
18:0	7.31	8.64	8.64
20:0	13.64	16.50	15.84
22:0	25.35	25.31	25.90
24:0	47.00	41.10	43.20

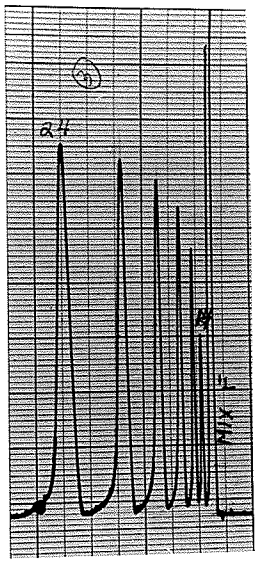


FIGURE 28

GLC of Mixture F on SE-30.

Column length: 84 cm.

Stationary phase: 8% SE-30

Column temperature: 271°C

Exit flow rate: 15 ml./min.

Inlet Pressure: 15 lb./sq. in.

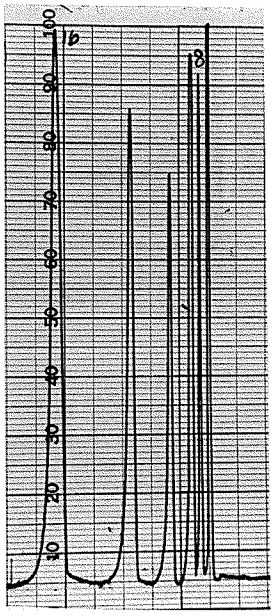


FIGURE 29

GLC of Mixture E on Apiezon L.

Column length: 98 cm.

Stationary phase: 15% Apiezon L

Column Temperature: 265°C

Exit flow rate: 20 ml./min.

Inlet Pressure: 15 lb./sq. in.

The Fatty Acids of Bayberry Wax and its Classes

The fatty acids of Bayberry wax itself, and the fatty acids of classes T, T + T' + A, D_A, $D_B + D_C$, and M as isolated by column chromatography, were analyzed on SE-30 and Apiezon L columns.

The areas of the major peaks can only be measured for dilute solutions, but then the areas of the minor peaks are found to be too small to give reproducible values with the planimeter. Fig. 30 shows the resolution of the fatty acids of Bayberry wax on an Apiezon L column. Fig. 31 shows a similar resolution on a SE-30 column.

The minor peaks may be produced by the decomposition of the fatty acids and by impurities. However Bayberry wax itself does not give any peak under the same conditions. Also these peaks are only found in certain classes of Bayberry wax. This then appears to indicate that the minor peaks are probably due to the presence of fatty acids themselves.

Tables V and VI show the retention times of the fatty acid esters of reference mixtures, of Bayberry wax, and of some of its classes, separated by Apiezon L and SE-30 columns. Graphs of the log (retention time) versus carbon number, drawn from the reference values, are used to determine the carbon numbers of the fatty acids of Bayberry wax (Figs. 32 and 33). The results show that the two major fatty acids are probably myristic and palmitic acids. This is expected for a species of the Myricaceae family. Also lauric and stearic acids appear to be present. In addition, peaks appear with

the retention times of odd carbon number fatty acids, i.e. 15 to 17. These peaks can also be caused by branched chain isomers or other isomers of even carbon number fatty acids. Though the possibility of an 18 carbon number unsaturated fatty acid has been previously eliminated, lower carbon number unsaturated acids may be present and can also produce these peaks. There is at least one more possible cause; these fatty acids are only found in fraction D_B+D_C and this is also the fraction in which the only detectable unsaponifiable material appears. Perhaps the peaks are due to that material. Thus the two odd carbon number peaks cannot be definitely taken to represent 15 and 17 chain fatty acids or perhaps even fatty acids. Only the results from fraction D_B+D_C varies widely on the two columns. However, since the concentration of the sample applied on the SE-30 column was much less than that on the Apiezon L column most of the smaller peaks would not be expected to appear. Also low concentrations of stearic acid were difficult to detect on the Apiezon L column.

In any case, the four minor components probably represent less than 1% of the fatty acids of Bayberry wax. The quantitative results from GLC are shown in Table VII. The values for the 1:3 diglycerides, free fatty acids, and the 1:2 diglycerides are quite different from the fatty acid distribution in Bayberry wax itself. In the case of the diglycerides, this suggests preferential bonding of the fatty acids on the glyceryl residue, i.e. myristic acid in the 2-position. This may also be accounted for by partial decomposition of

the methyl esters on injection into the column. However the same method of injection was used for each sample, and the results could be duplicated for each peak.

Since the species of Bayberry wax is unknown, no definite comparison can be made with another species. However the results on the fatty acid composition do correspond closely with the results for Myrica cordifolia as given in Table I.

TABLE VII

QUANTITATIVE RESULTS FROM GLC

Column	Carbon Number	Bayberry Wax	T	A	D_A	D_B+D_C	M
Apiezon L	14	53	48	33	40	66	52
T:265°C	16	47	52	67	60	34	48
SE-30	14	52	45	23	40	56	46
T:268°C	16	48	55	77	60	44	55

The values are rounded off to the nearest whole number.

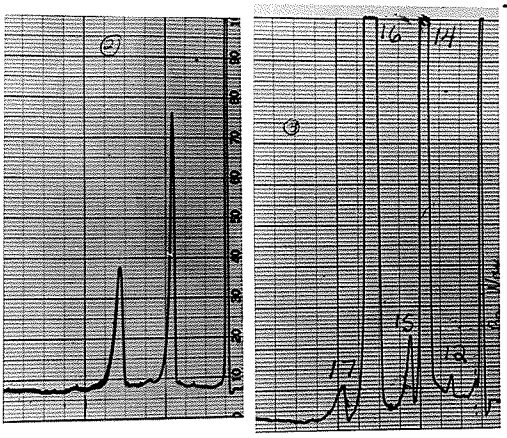


FIGURE 30

GLC of concentrated and dilute solutions of the methyl esters of Bayberry wax on an Apiezon L column.

Stationary phase: 15% Apiezon L

Column length: 98 cm.

Column temperature: 265°C

Exit flow rate: 20 ml./minute

Inlet pressure: 15 lb./sq. in.

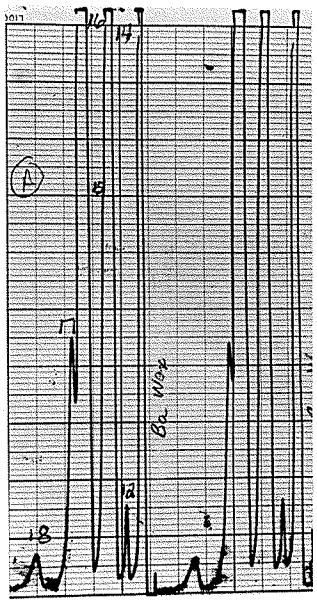


FIGURE 31

GLC of a concentrated solution of the methyl esters of Bayberry wax on a SE-30 column.

Stationary phase: 8% SE-30

Column length: 84 cm.

Column temperature: 243°C

Exit flow rate: 16 ml./min.

Inlet pressure: 15 lb./sq. in.

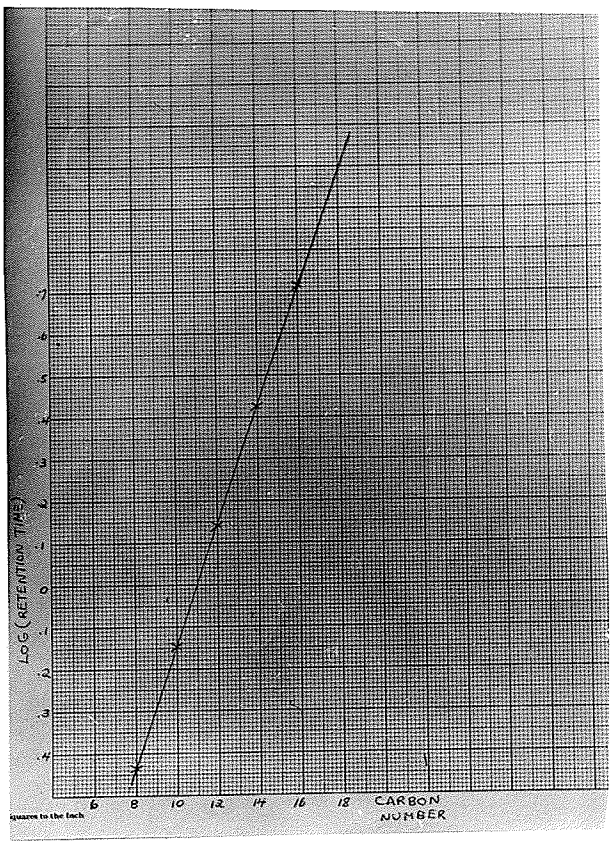


Figure 32.

Log (retention time) vs. carbon number for mixture E: Apiezon L column.

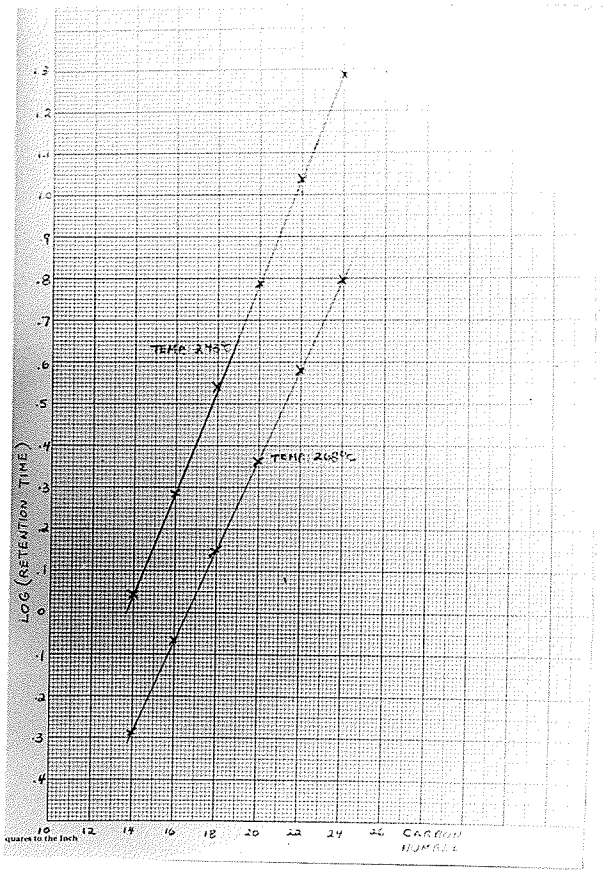


Figure 33.

Log (retention time) vs. carbon number for mixture F: SE-30 column.

TABLE V

LOG (RETENTION TIMES) OF THE FATTY ACID ESTERS ON APIEZON L

Carbon Number	Mixture E	Ba	T	A	D_A	D_B+D_C	M
8:0	-0.44						
10:0	- .15						
12:0	+ .14	0.20	0.19			0.19	
13:0	(.29)						
14:0	.43	.44	.43	.44	.40	.44	.43
15:0	(.57)	.54				.57	
16:0	.71	.73	.71	.70	.69	.72	.71
17:0	(.85)	.83				.84	
18:0	.99						

Ba: Bayberry wax
Stationary phase: 15% Apiezon L
Temperature: 265°C
Exit flow rate: 20 ml./min.

TABLE VI

LOG (RETENTION TIMES) OF THE FATTY ACID ESTERS ON SE-30

Carbon Number	Mixture F 243°C*	268°C	Ba	T	A	D_A	D_B+D_C	M
12:0	(-0.22)	(-0.51)	-0.18*	-0.18*				
13:0	(-0.12)	(-0.39)						
14:0	+ .04	- .29	-0.28	-0.29	-0.26	-0.30	-0.29	-0.30
15:0	(.16)	- .18	0.12*					
16:0	.28	- .08	-0.07	-0.09	-0.06	-0.07	-0.05	-0.05
17:0	(.40)	(+ .04)	0.39*					
18:0	.54	.15	0.56*	0.57*		0.58*	0.19	
20:0	.78	.36						
22:0	1.04	.58						
24:0	1.29	.80						

Stationary phase: 8% SE-30
Temperature: 243°C*
268°C
Exit flow rate: 16 ml./min.*
30 ml./min.

The brackets indicate the values of the standard mixtures extrapolated from Figs. 32 and 33.

CONCLUSION

A chromatographic approach to the study of the composition of Bayberry wax has been found to give extensive information with a minimum of equipment and of tedious work. However a complete analysis of Bayberry wax, i.e. the identification of homologues was not attempted. Thin-layer chromatography, column chromatography, and GLC were the chromatographic techniques used. Infrared spectroscopy, the only other major technique, was used to confirm the identification from chromatography of the constituents of Bayberry wax.

Qualitative Results

Thin-layer Chromatography

A functional group fractionation of Bayberry wax yielded eight classes and a residue. Class 7 was left unidentified.

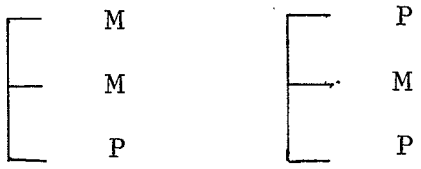
1. Hydrocarbons
2. Triglycerides
3. Triglycerides
4. Free Fatty acids
5. 1:3 Diglycerides
6. 1:2 Diglycerides
7. Esters and unsaponifiable constituents
8. 1- and 2-Monoglycerides
9. Residue

The unsaponifiable matter and the residue appear to

contain the constituents that give Bayberry wax its colour and odor. This was also found by column chromatography.

Reversed-phase partition chromatography and silver nitrate partition chromatography did not show any unsaturated constituents.

Also reversed-phase partition TLC indicated two major triglyceride isomers:



M: myristyl

P: palmityl

Infrared Spectroscopy

Infrared Spectroscopy confirmed the suggested identity of the classes of substances separated from Bayberry wax by TLC. The bands produced by glycerides, acids, alcohols, and hydrocarbons were easy to identify. However, the di- and monoglyceride bands were less well defined and more difficult to identify.

Gas-Liquid Chromatography

As expected GLC showed that myristic and palmitic acids are the only major component acids of Bayberry wax. Lauric, stearic and two possible odd chain fatty acids were also found. The distribution of the fatty acids in the different classes suggests selective bonding of myristic acid in the 2-position of the glycerol molecule. The other

anomaly found was palmitic acid greatly predominating in the free fatty acid fraction.

Quantitative Results

Thin-Layer Chromatography

By gravimetric analysis, Bayberry wax was found to contain 97% glycerides. Only 52% were triglycerides. The unsaponifiable matter probably represented less than 2% of Bayberry wax.

Column Chromatography

Column chromatography was used as a preparative and a quantitative technique. It would probably have been a tedious method if TLC were not used for determining the class of lipid eluted from the column at any stage of the procedure. The separations of neutral lipid mixtures are generally not as good as with preparative TLC. However in this case the separations were better. The quantitative results obtained by gravimetric analysis are in fair agreement with those from TLC. The results are shown in the following table.

Class	% by TLC	% by Column Chromatography
Hydrocarbon	0.6	0.3
Triglyceride	51.7	48.6
Triglyceride and free fatty acid	2.4	4.2
Diglycerides	38.5	40.0
1:2 Diglycerides		2.3
Monoglycerides + residue	5.7	4.1

Gas-Liquid Chromatography

Myristic and Palmitic acids represent about 98% of the fatty acids of Bayberry wax. The four minor component acids detected were only visible in a concentrated solution, in which case the major peaks went off scale.

The results obtained in this study cannot be compared to any other work, not only because no other extensive study has been completed on Bayberry wax, but also because the species of our sample is not known. Still the qualitative results were expected since triglycerides were known to be present; and the quantitative results from the fatty acid analysis were in the correct range for a species in the Myricaceae family of fruit-coat fats.

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