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Characterization of Natural and Synthetic Waxes Using Combined Chromatographic Techniques

VINCENT ABATE, B.S., VIOLETTE BADOUX, B.S.,
S. Z. HICKS, B.S., and MONROE MESSINGER, B.S.*

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Synopsis—A detailed thin-layer, gas-liquid, and column CHROMATOGRAPHIC PROCEDURE for the evaluation of single WAXES and various mixtures normally found in cosmetic preparations is presented. The thin-layer chromatographic systems developed have been found to give the best possible class separations of the wax components, which has facilitated the analysis of mixtures. The gas-liquid chromatographic evaluation utilized short columns (16–18 in. long) on the hydrocarbon, alcohol, and acid components of the various waxes. Thus the alcohols with chain lengths to C_{32} were easily resolved without resorting to derivatives. This combination of the various techniques described resulted in the identification of individual waxes.

INTRODUCTION

The analysis and characterization of waxes have been receiving increased attention recently. Several workers (1–7) have attempted to characterize various waxes using the well-known chromatographic techniques. Recently, Holloway and Challen (8) have presented a systematic approach to the analysis of natural waxes using thin-layer chromatography (TLC) combined with various detection methods. However, a systematic approach to the characterization of natural and synthetic waxes, using combined chromatographic techniques, has not been reported. Plant and animal waxes are, generally speaking, compositions made up largely of nonglyceryl esters formed in nature by the union of higher alcohols with the higher fatty acids and with which are associated one or more of the following components: free fat and wax acids, free

* Chesebrough-Pond's Inc., Clinton, Conn.

monohydric alcohols, and sterols, hydrocarbons, lactones, and other condensation compounds. This paper describes a detailed systematic approach to wax characterization utilizing TLC, liquid chromatography, and gas-liquid chromatography (GLC).

The analysis proceeds in three steps:

1. The use of analytical or diagnostic TLC which separates the waxes into "characteristic" classes.

2. Column chromatography to separate quantitatively on a preparative scale the five main classes of constituents observed on the analytical TLC plate.

3. GLC to further characterize some of the fractions obtained *via* column chromatography.

In the TLC step, the waxes are looked at in their "natural" state, using three mobile phases, two developed by Carlier *et al.* (2) and one which was developed in this laboratory. The column chromatography, which was developed as a preparative step to reflect the class separation achieved *via* TLC, results in five main fractions: hydrocarbons, simple esters, a mixture of esters (containing possible diesters, triesters, acid esters, etc.), alcohols, and acids. GLC is used on some of the fractions obtained from the column to separate these complex mixtures into as many individual components as possible. Though the hydrocarbons can be easily fractionated *via* a GLC procedure, the less volatile and more polar compounds such as the alcohols and acids present greater difficulties. Downing *et al.* (9) circumvented these obstacles by converting oxygenated components of beeswax into hydrocarbons containing the same number of carbon atoms by a process of chemical reduction. In this laboratory, the GLC "short column"* (an 18-in. long $\times$ $^1/_4$-in. diameter copper tube filled with acid-washed Chromosorb G coated with 1.5% Apiezon grease) was developed. This simple column allows an efficient resolution of the hydrocarbons and alcohol fractions directly and the acid fractions as their methyl ester derivatives. An added advantage of the use of short columns is the decreased retention time. For example, the separation of the C_{24}–C_{32} alcohols requires less than 10 minutes.

The combined chromatographic techniques evolved have been applied to the study of many of the natural and synthetic waxes used by the cosmetic chemist, some of which will be presented here. Also, these

*Subsequent work has resulted in a more efficient column, a 24-in. long $\times$ $^1/_8$-in. diameter stainless steel tube filled with AW/DMCS Chromosorb W coated with 5% Silicone Gum Rubber SE30 (methyl).

techniques have been used to identify individual waxes in possible mixtures of waxes used in cosmetic products. In this report, beeswax will be used as a reference wax to illustrate the techniques evolved.

Experimental

Thin-Layer Chromatography

Glass plates (20 × 20 cm) were prepared by spreading a slurry of 30 grams of Silica Gel G in 60 ml of distilled water on five plates at a 0.25-mm thickness. The air-dried plates were further activated by heating at 105°C for 30 minutes. The prepared plates were stored in wooden cabinets over a drying agent and used within two days. Shortly before use, the prepared plates were predeveloped in a mixture of chloroform/methanol 80/20 v/v. The solvent mixture was run to the upper edge of the plate as a washing procedure. The predeveloped plates were then dried again for 10 minutes at 105°C, and cooled at room temperature before use.

Sample Preparation

Quantities of 0.5 g of waxes were dissolved and diluted to 10 ml with warm chloroform. The waxes which were slightly soluble in chloroform (e.g., carnauba wax) were maintained in solution in a lukewarm water bath until used. One or 2 μl of solution representing 50 and 100 μg of wax were applied at 2 cm above the bottom edge of the predeveloped plate, and after complete drying of the applied solutions, the plates were placed into the developing chambers.

Developing Chambers

The fully saturated atmosphere in the chambers was obtained by lining the walls with Whatman No. 1 filter paper. To obtain a fully equilibrated solvent system, the well-mixed mobile phases were transferred to the lined chambers at least one hour prior to development.

Mobile Phase (v/v)

The following mobile phases were prepared:

A. *n*-Heptane–ethyl ether anhydrous–acetic acid (90:10:2)
B. *n*-Heptane–ethyl ether anhydrous–acetic acid (95:5:2)
C. Chloroform–ethyl ether anhydrous–acetic acid (90:10:2)

The mobile phases A and B (2) were adopted to obtain the best resolution of the main classes of constituents. The fast moving spot adjacent

to the solvent front represents the hydrocarbon portion. The next spots, in descending order, are simple esters, mixture of esters, acids, and alcohols (Figs. 1 and 2). Mobile phase C was developed for the study of the polar portion of waxes: the alcohols, acids, etc., are moved to a higher R_f (Fig. 3). All plates were developed 12 cm above the origin. All solvents were analytical grade reagents used without further treatment.

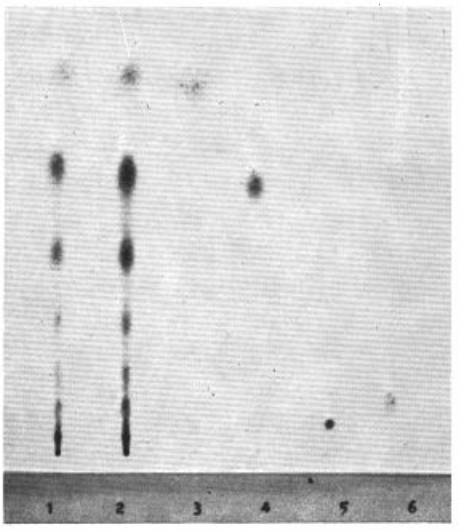

Figure 1. Thin-layer chromatographic separation of classes of constituents. Mobile phase: *n*-heptane, ethyl ether, acetic acid (90:10:2)

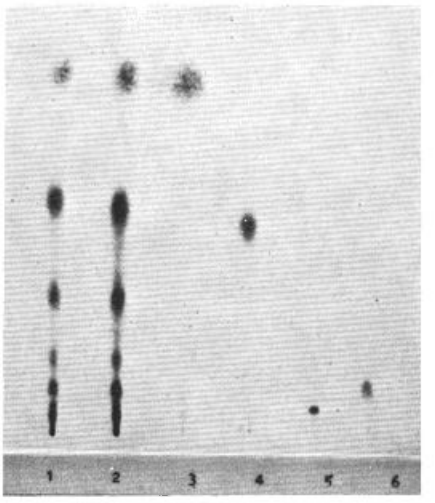

Figure 2. Thin-layer chromatographic separation of classes of constituents. Mobile phase: *n*-heptane, ethyl ether, acetic acid (95:5:2)

1. Beeswax, 50 μg
2. Beeswax, 100 μg
3. *n*-Docosane, 10 μg
4. Spermaceti esters, 10 μg
5. Docosanoyl alcohol, 10 μg
6. Docosanoic acid, 10 μg

Methods of Detection

Detection of constituents was accomplished by exposure to iodine vapors and by use of a sulfuric acid–potassium dichromate reagent. This reagent was prepared by slowly adding 180 ml of concentrated sulfuric acid, under cooling, to a solution of 2.25 g of potassium dichromate in 120 ml of distilled water.

The developed plates were thoroughly dried to eliminate the acetic acid and cooled at room temperature before the exposure to iodine vapors. Any iodine reactive spots were rapidly recorded and the plates were heated again for a short period of time (10 min at 90–100°C).

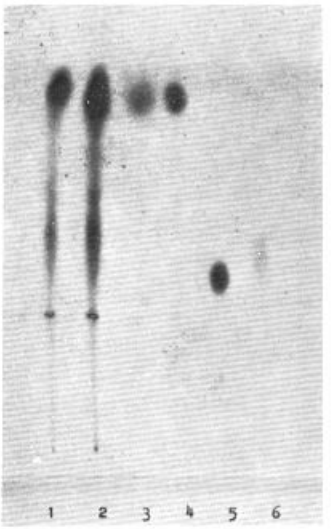

Figure 3. Thin-layer chromatographic separation of classes of constituents. Mobile phase: chloroform, ethyl ether, acetic acid (90:10:2). See Figs. 1 and 2 for code

The warm plates were evenly sprayed with the sulfuric acid–potassium dichromate reagent. At this point various constituents such as steroids and triterpenoids appeared as colored spots characteristic of lanolin and candelilla wax. They were recorded before the final charring of the spots was obtained by further heating (25 min at 120°C). Permanent records of each chromatograph were made by photography. The total time for the TLC and charring was 1.5 to 2 hours.

Column Chromatography

By monitoring the chromatographic column with TLC, a procedure was developed to reflect the separation of the five main classes of constituents observed on analytical TLC (mobile phase A). The use of column chromatography is superior to preparative TLC in collecting large amounts of the various fractions for further study and routine analysis.

Alumina* (225 g) was stirred for 10 minutes with 1 l. of 1% HCL aqueous solution. The mixture was allowed to settle and was decanted. The alumina was then dried at room temperature and activated at 110°C for 16 hours. The dry treated alumina (48 g) was poured into a 12-in. × 1-in. glass column (fitted with a stopcock) and rinsed with 100 ml of

* No. 9296, Matheson-Coleman, East Rutherford, N. J.

n-heptane. One gram of wax dissolved in a small volume of *n*-heptane was carefully introduced onto the top of the prepared column. The use of gradient elution resulted in five major wax fractions (Table I).

Table I
Wax Fractions Obtained using Gradient Elution Column Chromatography

Eluates	Fractions
A. 250 ml of *n*-heptane	Hydrocarbons
B. 400 ml of 4% ethyl ether in *n*-heptane	Esters I
C. 250 ml of 25% ethyl ether in *n*-heptane	Esters II
D. 250 ml of 3.5% *n*-propanol in *n*-heptane	Alcohols
E. 250 ml of 25% acetic acid in *n*-heptane	Acids

The eluates were taken to dryness without further treatment, except for eluate E, which was thoroughly washed with distilled water to eliminate the acetic acid prior to evaporation. The dried residues were weighed and then checked by TLC to control the efficiency of the separation and to make sure that no major hydrolysis had occurred. The residues were also checked by infrared spectrophotometry (IR) as a thin film between sodium chloride plates.

Gas-Liquid Chromatography

The separation of the class constituents into their individual components is best achieved *via* GLC. The objective was to analyze the fractions from the column without further chemical modification. This was partially realized by the development of a short column containing an inert support with a nonpolar coating. This one column effectively separates the "as is" hydrocarbon and alcohol fractions and the methyl ester derivatives of the acid fraction. Total time for all three separations is less than 45 minutes. Table II gives the instrument parameters and sample concentration used.

Results and Discussion

With TLC as a diagnostic tool, several natural waxes were studied as were synthetic waxes, wax substitutes, and mixtures of waxes. Most natural and synthetic waxes were separated by column chromatography (Table III). Depending upon the waxes analyzed, the total recovery of waxy material varied from 82–99%. Those waxes which showed low recoveries contained very polar substances that were retained on the column.

Table II

Instrument Parameters and Sample Concentrations used for GLC Analysis

Instrument	Perkin-Elmer CG Model 800
Recorder	Leeds and Northrup (one mv)
Column	18 in. × ¼ in. O.D. copper, Apiezon L 1.5% on 60/80 mesh Chromosorb G—AW/DMCS
Carrier gas	Nitrogen at 105 ml/min
Air	50 psig
Hydrogen	26 psig
Detector temperature	220–320°C
Injector temperature	320°C
Sensitivity	100 ×
Temperature program	Hydrocarbons, 180°–290°C at 10°C/min Alcohols, 210°–290°C at 7.5°C/min Acid-derivatives, 160°–290°C at 10°C/min
Sample size	1.0 μl
Solution concentrations in chloroform	
Hydrocarbons	9.0 μg/μl
Alcohols	22.0 μg/μl
Methyl ester derivatives of the acids	15.0 μg/μl

Table III

Per Cent of Fractions A-E (Table I) Found using Column Chromatography

Waxes	A	B	C	D	E	Recovery, %
Beeswax, natural	14.5	25.4	15.5	22.0	13.4	90.8
Candelilla wax	42.7	6.1	5.3	22.4	16.5	93.0
Wool wax (lanolin)	1.0	26.3	12.7	44.9	14.4	99.3
Adulterated beeswax						
+20% Paraffin wax	30.8	20.7	11.0	20.3	10.4	93.2
+20% Carnauba wax	11.1	29.3	14.1	21.8	15.3	91.6
+20% Wool wax	13.1	26.4	18.1	24.2	11.6	93.4
Synthetic wax						
Sample #1[a]	21.5	...	...	13.2	47.6	82.3
Sample #2[a]	19.7	21.2	...	22.0	22.0	85.8

[a] Commercial samples of "unknown" composition.

Carnauba wax could not be eluted from the column at room temperature. It would require heat to keep it in solution throughout the procedure. However, it was found that carnauba wax when mixed with beeswax went through the procedure at room temperature without difficulty.

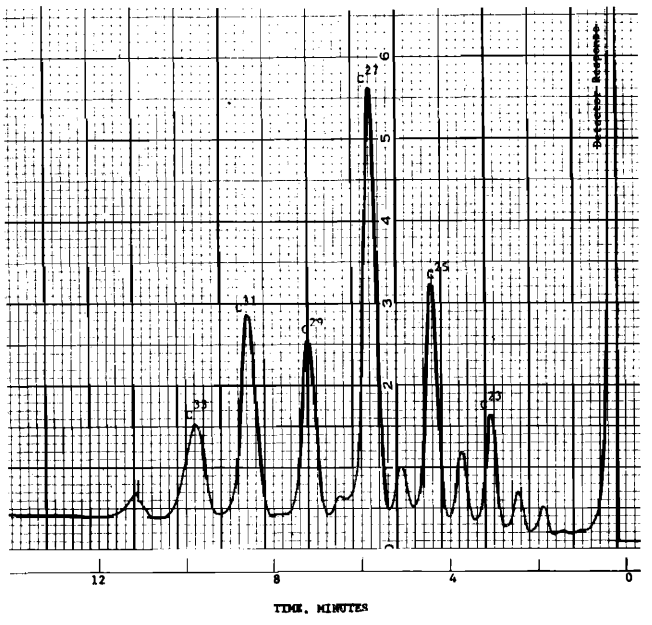

Figure 4. Gas-liquid chromatogram of natural hydrocarbons from beeswax. Temperature program: 180°−290°C at 10°C/min on 1.5% Apiezon L, 60/80 mesh Chromosorb G AW DMCS. Sample injection of 1.0 μl ≏ 9.0 μg

Though several waxes were examined *via* GLC, essentially the fractions of beeswax and candelilla wax will be discussed to demonstrate the effective separation of the classes of constituents. The identification of the separated constituents was made by known retention time of pure standards and by the "spiking" of the samples with pure standards. The hydrocarbons of beeswax (Fig. 4) consist mainly of the odd carbon-chain *n*-hydrocarbons C_{23-33}, with C_{27} being predominant. Those of candelilla wax consist mainly of *n*-hydrocarbons C_{29-33}, with C_{33} being predominant. The beeswax alcohols (Fig. 5) are mainly of the even carbon-chain *n*-alcohols C_{24-32} and the candelilla wax alcohols consist mainly of C_{30}. The beeswax *n*-acids (Fig. 6) are of even carbon-chain consisting of $C_{16\ 34}$, with C_{16} and C_{24} being predominant. The candelilla wax *n*-acids are also of even carbon chain, with C_{30} and C_{32} being predominant. The results of the above GLC analysis compare favorably with the work of other investigators (8).

Though the combination of the three methods described above was used for a detailed study of waxes, it is preferable in certain instances to

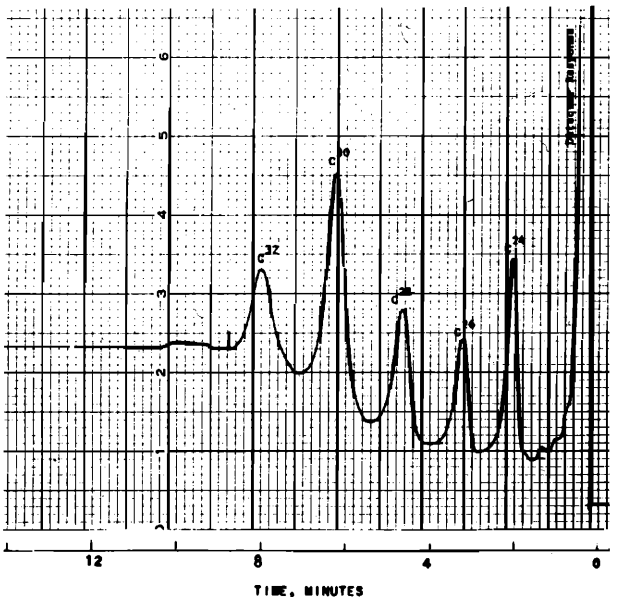

Figure 5. Gas-liquid chromatogram of monohydric alcohols from beeswax. Temperature program: 210°−290°C at 7.5°C/min on 1.5% Apiezon L, 60/80 mesh Chromosorb G AW DMCS. Sample injection of 1.0 μl $\simeq$ 22.0 μg

resort to only one method. For example, the GLC method can be used to detect the possible adulteration of a wax or TLC can be used for the investigation of mixtures of waxes in a cosmetic product.

Conclusion

A combined chromatographic technique for the characterization of natural and synthetic waxes has been developed. The described systematic approach can be used for the study of wax composition, for the control of raw materials, and for the investigation of finished products containing waxes singly or in mixtures.

Acknowledgment

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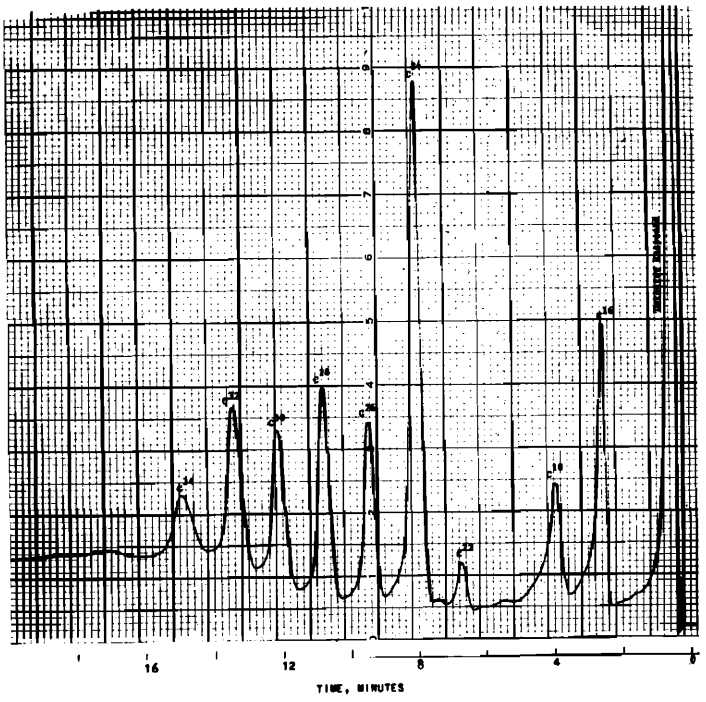

Figure 6. Gas-liquid chromatogram of methyl ester derivatives of straight-chain acids from beeswax. Temperature program: 160°−290°C at 10°C/min on 1.5% Apiezon L, 60/80 mesh Chromosorb G AW DMCS. Sample injection of 1.0 μl ≏ 15.0 μg

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