THIN LAYER CHROMATOGRAPHY IN THE STUDY OF NATURAL WAXES AND THEIR CONSTITUENTS

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INTRODUCTION

Thin layer chromatography is already a well established technique in most fields of lipid analysis. However, one particular type of lipid, the waxes, has been studied in much less detail by this method. It has been established that waxes can be resolved by adsorption thin layer chromatography on layers of Kieselgel G[1–8] and Aluminium Oxide G[1] and that constituents can be separated into classes of compound but not into individual components. PURDY AND TRUTER[2] and TRUTER[3] chromatographed the surface waxes of many species of leaf and found that characteristic thin layer patterns were given by each species. In later publications[4a,b,c] the same workers performed a complete analysis of cabbage wax using the same techniques. This method was also utilised by KOLATTUKUDY[7] in tracer studies on cabbage wax biosynthesis. A detailed study of grape cuticle wax employing thin layer chromatography has been made by RADLER AND HORN[8]. German workers[5,6] have examined commercial waxes (beeswax, shellac, wool, Montan, and carnauba waxes) and some pure compounds which were examples of reported classes of wax constituents.

No thin layer investigation so far has been fully comprehensive in scope; published data are frequently obtained from one adsorbent with a single solvent system. R_F values for wax constituents have been quoted in one publication only[6]. Similarly, the examination of constituents which are more polar than fatty acids has only been reported once[8]. An assortment of detection methods have been employed.

This communication describes a detailed thin layer investigation of wax constituents with regard to the choice of adsorbent, solvent systems, and general and specific methods of detection. The techniques evolved were then applied to a study of natural waxes from various sources. The study also enabled the separation of mg quantities of wax classes from intact waxes by modification of the basic procedures to a preparative scale on thicker layers of adsorbent.

EXPERIMENTAL

Materials

Where possible at least two compounds of varying chain length were selected from the classes of compound reported to be present in waxes[9–11]. The various classes investigated are shown in Table I.

Esters were prepared from the fatty acid chlorides and the corresponding

TABLE I

CLASSES OF COMPOUND EXAMINED BY THIN LAYER CHROMATOGRAPHY

Class of compound	*Example*	*Supplier*
1. *n*-Alkanes	*n*-Hexatriacontane	Newton Maine Ltd.
	n-Eicosane	Koch-Light Labs. Ltd.
2. *n*-Alkenes	*n*-Nonadec-1-ene	Koch-Light Labs. Ltd.
	n-Eicos-1-ene	Koch-Light Labs. Ltd.
3. *n*-Alkyl esters	*n*-Octadecyl docosanoate	Newton Maine Ltd.
	n-Dodecyl docosanoate	
4. *n*-Alkyl ketones	*n*-18-Pentatriacontanone	Koch-Light Labs. Ltd.
	n-12-Tricosanone	Koch-Light Labs. Ltd.
5. *n*-Secondary alcohols	*n*-18-Pentatriacontanol	
	n-12-Tricosanol	
6. *n*-Primary alcohols	*n*-1-Docosanol	Newton Maine Ltd.
	n-1-Tetradecanol	Koch-Light Labs. Ltd.
7. *n*-Fatty acids	*n*-Docosanoic acid	Koch-Light Labs. Ltd.
	n-Octacosanoic acid	Newton Maine Ltd.
8. *n*-Monounsaturated fatty acids	*n*-Octadec-9-enoic acid	Koch-Light Labs. Ltd.
	n-Docosan-13-enoic acid	Koch-Light Labs. Ltd.
9. *n*-Hydroxy acids	12-Hydroxyoctadecanoic acid	Koch-Light Labs. Ltd.
10. *n*-ω-Hydroxy acids	22-Hydroxydocosanoic acid	
11. *n*-α,ω-Diols	*n*-Docosane-1,22-diol	
	n-Hexacosane-1,26-diol	
12. Sterols	β-Sitosterol	Aldrich Research Chemicals
	Cholesterol	Aldrich Research Chemicals
13. Sterol esters	Cholesteryl palmitate	Aldrich Research Chemicals
	Cholesteryl stearate	Aldrich Research Chemicals
14. Triterpenoids	Ursolic acid	
	Lupeol	
	β-Amyrin	
15. Triterpenoid esters	Lupeyl acetate	
	β-Amyrenyl acetate	

primary alcohols by the method of KAUFMANN AND POLLERBERG[12]. Secondary alcohols were prepared from the corresponding ketones by MEERWEIN–PONDORFF–VERLEY reduction in isopropanol solution[13]. Commercial waxes were supplied by Brohme and Schimmer Ltd. and A.F. Suter Ltd. Natural waxes were extracted from whole leaves by immersion in chloroform according to the method of MARTIN AND BATT[14].

Preparation of the plates

Aluminium Oxide G, Kieselgel G and Kieselguhr G (E. Merck AG) were employed as adsorbents. Adsorbent layers of 250 μ thickness were spread on 20 × 20 cm glass plates with the Desaga apparatus using 4 g of Kieselgel G and Kieselguhr G, and 6 g of Aluminium Oxide G, per plate. Kieselgel G and Kieselguhr G layers were activated at 105° for 60 min and Aluminium Oxide G layers at 110° for 30 min. The layers were then allowed to cool and the plates stored in a desiccator over self-indicating silica gel granules. Plates were always used within two days of activation, if not they were reactivated before use.

Preparative layers were prepared only from Kieselgel G and spread at a thickness of 1.5 mm using an adjustable spreader. 25 g of adsorbent was used for each 20 × 20 cm plate. After spreading, the layers were allowed to air dry for 3 h and then activated at 105° for 6 h.

Chromatographic procedure

Waxes and wax constituents were applied to the start lines as solutions in suitable solvents. Chloroform was found to be the most suitable solvent for general purposes. Hydrocarbons were best dissolved in petroleum spirit (60–80°) and polar constituents, *e.g.*, hydroxy acids and diols, in absolute ethanol. 1–2 % solutions were employed throughout and loads of 10–20 μg of wax constituents and 50–100 μg of waxes were applied to the layers using glass capillary pipettes. Loaded plates were developed at room temperature (20–25°) in glass tanks (22 × 6 × 22 cm) by the ascending solvent technique. Precautions were taken to ensure that the tank atmosphere was saturated with solvent vapour. Lids were greased and made airtight, and the walls of the tank completely lined with Whatman No. 1 chromatography paper. Solvents were placed in the tank at least 30 min before the development. The running time varied with the solvent and adsorbent, but a development of 12 cm was adopted. Solvent fronts were immediately marked on removal from the solvent and traces of solvent removed by a hot air drier. Chromogenic reagents were applied to the layers using an aerosol spray gun which gave a fine spray with the minimum disturbance of adsorbent.

For preparative work weighed wax samples (50–100 mg) were dissolved in warm chloroform and applied to the start lines as a band 18 cm long using a wide band pipette (Desaga). Narrow starting bands could be obtained if the plates were warmed immediately prior to loading. Development was carried out as previously, but a longer development of 18 cm was allowed.

Solvent systems

Carbon tetrachloride.

Methylene chloride.

Benzene–chloroform (7:3 v/v).

Chloroform–ethyl acetate (1:1 v/v).

All solvents employed were laboratory reagent grade and were dried over Molecular Sieve (Type 4A) for several days before use.

Methods of detection

(*A*) *General methods*

(*i*) *Charring with sulphuric acid*[4,7]. Developed plates were sprayed with conc. H_2SO_4 and heated at 120° until black spots appeared.

(*ii*) *Fluorescein*[4a,6], *2',7'-dichlorofluorescein*[1]. Two techniques were employed; firstly, spraying with an 0.1 % ethanolic solution of the reagent after development, and secondly, by incorporation into the layers by preparing the slurry with an 0.01 % aqueous solution of the reagent. After activation, loading and development were carried out as normal. Examination of plates was then carried out in long wave U.V. light (peak at 366 mμ) and in short wave U.V. light (peak at 254 mμ).

(*iii*) *Rhodamine B*[5]; *Rhodamine 6 G*[15]. Spray reagents consisting of 0.05 % solutions were employed. Rhodamine B was ethanolic and Rhodamine 6 G was aqueous. After spraying, plates were examined in U.V. light as for fluorescein detection.

(*iv*) *α-Cyclodextrin–iodine vapour*[16,5,7]. Plates were sprayed with a 1 % solution of α-cyclodextrin in 30 % ethanol and allowed to air dry. They were then treated with a 1 % solution of iodine in petroleum spirit (40–60°).

(*v*) *Antimony trichloride*[2]. A saturated solution of $SbCl_3$ was used in chloroform. After spraying, plates were heated at 120° until the coloured spots appeared.

(*B*) *Specific methods*

(*i*) *Detection of unsaturation.* Two methods were employed. A 1 % solution of iodine in petroleum spirit (40–60°)[1] and the sodium fluorescein–bromine test according to STAHL[17].

(*ii*) *Detection of carbonyl compounds*[4,5]. Two formulations of 2,4-dinitrophenylhydrazine were used. A saturated aqueous solution in 2 *N* HCl[18] and an 0.5 % ethanolic solution with the addition of 1 ml of 25 % HCl[18]. These were used as spray reagents and also allowed to react with samples on the start lines prior to development in the normal solvents.

(*iii*) *Detection of higher alcohols, ketones*[17]. A freshly prepared 3 % ethanolic solution of vanillin with 0.5 ml of conc. H_2SO_4 added, was used. After spraying, the plates were heated at 120° until the blue spots appeared.

(*iv*) *Detection of sterols*[17], *triterpenoids*[19]. Two reagents were used. The Liebermann-Burchard test was performed by spraying first with equal volumes of acetic anhydride and chloroform, and immediately spraying with conc. H_2SO_4. Plates were then heated at 120° until coloured spots appeared. A solution of chlorosulphonic acid (1 vol.) in glacial acetic acid (2 vols.) was similarly employed. After the development of colours with both reagents, the plates were examined in both short and long wave U.V. light.

(*v*) *Detection of acids.* A variety of indicators were employed for this purpose. Bromocresol green, Bromocresol blue[4], Bromothymol blue and Universal indicator (BDH Ltd.) were used. Ethanolic solutions (0.1 %) were sprayed except for Universal indicator which was used undiluted.

(*vi*) *Detection of –OH groups*[4a]. This test was found to be suitable for the identification of single compounds, synthetic compounds or fractions from preparative work. The formation of acetates with hydroxy compounds was carried out by reaction with samples on the start line with acetyl chloride. Acetyl chloride was applied by means of a capillary pipette and excess reagent then removed with a hot air drier. After reaction the plates were developed with suitable solvents and the acetates detected with a suitable reagent.

(*vii*) *Detection of –COOH groups.* This was similar to the above and was a modification of the method of METCALFE AND SCHMITZ[20] for the preparation of methyl esters. Samples were reacted on the start lines with boron trifluoride–methanol complex (BDH Ltd.) and heated by means of a hot air drier. When cool, the plates were developed in suitable solvents and the methyl esters detected with a suitable reagent.

RESULTS AND DISCUSSION

Choice of adsorbent

Layers of Kieselgel G and Aluminium Oxide G were both found to be suitable for the resolution of waxes and their constituents. Kieselguhr G alone was unsuitable; all constituents moving to the solvent front even in non-polar solvents. Kieselgel G gave the most consistent separation of all constituents and waxes. Aluminium Oxide

G was less adsorptive than Kieselgel G under the conditions employed and gave slightly better separations of non-polar constituents. Acidic constituents, however, were poorly resolved with Aluminium Oxide G, remaining on or streaking from, the start line even with polar solvent mixtures. The loading capacity of Aluminium Oxide G layers was not as good as Kieselgel G and streaking tended to occur with higher loads especially when waxes were chromatographed. A good loading capacity is a definite advantage in wax chromatography as this permits the examination of trace constituents in the thin layer pattern without masking by streaking.

Choice of solvent

The elutropic series of TRAPPE[21] was adopted as a general guide in the design of solvent systems; complicated multicomponent systems were avoided and only suitable single or two component solvent systems were selected. The solvent/adsorbent systems adopted are shown together with R_F values obtained for wax constituents in Table II. R_F values obtained were not consistent and values are therefore expressed as a range. These are based on observations made over a period of two and a half years. The constituents, however, always separated in a given system despite the fact that R_F value ranges overlapped in some cases.

TABLE II

R_F VALUES OBTAINED WITH THE VARIOUS CLASSES OF WAX CONSTITUENT

System I = Kieselgel G/carbon tetrachloride.
System II = Aluminium Oxide G/methylene chloride.
System III = Kieselgel G/benzene–chloroform (7:3 v/v).
System IV = Kieselgel G/chloroform–ethyl acetate (1:1 v/v).

Class	*I*	*II*	*III*	*IV*
n-Alkane	0.86–0.91	0.86–0.91	0.86–0.91	0.86–0.91
n-Alkene	0.86–0.91	0.86–0.91	0.86–0.91	0.86–0.91
n-Alkyl ester	0.36–0.42	0.86–0.91	0.82–0.86	0.86–0.91
n-Alkyl ketone	0.19–0.24	0.86–0.91	0.76–0.82	0.86–0.91
n-Secondary alcohol	0.02–0.08	0.65–0.70	0.49–0.53	0.82–0.85
n-Primary alcohol	0	0.35–0.39	0.17–0.23	0.50–0.55
n-Fatty acid	0	0	0.05–0.10	0.62–0.66
n-Monounsaturated fatty acid	0	0	0.05–0.10	0.62–0.66
n-Hydroxy acid	0	0	0 –0.02	0.37–0.41
n-ω-Hydroxy acid	0	0	0	0.25–0.30
n-α,ω-Diol	0	0	0	0.16–0.20
Sterols	0	0.19–0.21	0.12–0.16	0.56–0.60
Sterol esters	0.41–0.46	0.86–0.91	0.85–0.90	0.86–0.91
Lupeol, β-amyrin	0	0.39–0.43	0.21–0.25	0.61–0.65
Acetates of above	0.11–0.16	0.76–0.82	0.70–0.75	0.86–0.91
Ursolic acid	0	0	0	0.21–0.27

No single solvent system resolved all the fifteen classes of wax constituents studied in one development. A minimum of two solvent systems was found to be necessary for satisfactory resolution: System III for compounds of polarity between alkane and fatty acid, and System IV for compounds more polar than fatty acids. The order of R_F value was fairly easy to predict with aliphatic constituents, R_F value decreasing in a given system with increasing number and polarity of substituent

groups of the basic hydrocarbon skeleton. Fractionation was strictly by class, insignificant differences in R_F value were observed between homologues of a given class. Saturated and unsaturated homologues of a given class were not resolved. The R_F values of sterols and triterpenoids were less easy to predict. With triterpenoids there was a separation of "neutral" (*e.g.* lupeol, β-amyrin) from "acidic" (*e.g.* ursolic acid) triterpenoids.

From the examination of waxes a minimum of two systems must again be employed for a separation of all constituents. Separations obtained were found to depend on the proportions and numbers of constituents present in an individual wax. The overlap of certain groups of constituents, especially non-polar constituents, was frequently observed. The quality of resolution was improved partly by adjustments in loading and partly by using alternative solvent systems. Systems I and II were found useful in improving some separations of waxes. Separations achieved with some waxes are shown in Figs. 1 and 2.

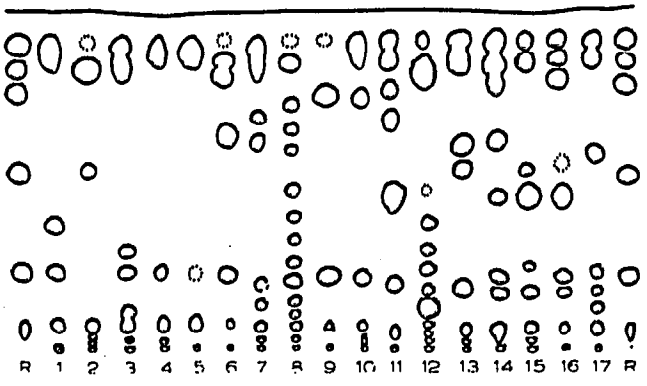

Fig. 1.

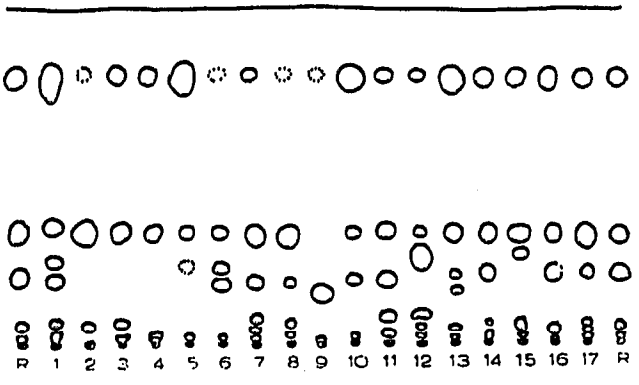

Fig. 2.

Fig. 1. Chromatography of natural and commercial waxes using System III. R (reference mixture), 10 μg each of *n*-alkane, *n*-alkyl ester, *n*-alkyl ketone, *n*-secondary alcohol, *n*-primary alcohol, *n*-fatty acid. 1–17, waxes approx. 50 μg of each. 1 = Candelilla; 2 = Spermaceti; 3 = Beeswax; 4 = Montan; 5 = Fibre; 6 = Shellac; 7 = Ouricury; 8 = Carnauba; 9 = Sugar cane; 10 = Esparto; 11 = *Papaver somniferum*; 12 = *Eucalyptus globulus*; 13 = *Pisum sativum*; 14 = *Allium porrum*; 15 = *Aquilegia vulgaris*; 16 = *Clarkia elegans*; 17 = *Acer pseudoplatanus*. Detection: Rhodamine 6 G 0.05 % aqueous.

Fig. 2. Chromatography of natural and commercial waxes using System I. Samples and detection as Fig. 1.

Methods of detection

Published general methods of detection were in general found to be successful but responses to the antimony trichloride reagent were very variable. An 0.05 % aqueous spray of Rhodamine 6 G was found to be most suibable for routine analysis. The reagent was easy to prepare, involved no heating stages and was sensitive to less than 1 μg of most wax constituents in daylight. Spots appeared bright red against a pink background which slowly faded. Under long wave U.V. light spots appeared orange-brown against a yellow background. Fluorescein was a useful alternative to Rhodamine 6 G and the best response was obtained with the reagent incorporated in the adsorbent layers. Visibility of the spots was improved by lightly spraying the plates with water[22]. Spots appeared pink against a yellow background in daylight and yellow-green fluorescent under long wave U.V. light.

The design of specific tests proved a difficult task as most wax constituents are long chain aliphatic compounds and therefore generally unreactive. The detection of unsaturation in wax constituents was successfully accomplished with the fluorescein–bromine test. Alkenes, monounsaturated fatty acids, sterols and triterpenoids all gave yellow spots against a pink eosin background after bromination. Iodine vapour was found to give a non-specific response. Unsaturated compounds were detected in two Eucalyptus waxes, apple wax and woolwax. The detection of carbonyl compounds was accomplished by reaction with 2,4-dinitrophenylhydrazine. The alcoholic formulation of this reagent was the most satisfactory, but was found to be non-specific (many compounds giving a yellow colour) unless allowed to react with compounds on the start line. Heating was necessary for the reaction to proceed and the 2,4-dinitrophenylhydrazones formed in the reaction were clearly visible as bright yellow spots on development. On spraying with Rhodamine 6 G it was seen that the derivatives moved in conjunction with the unreacted carbonyl compound. The results of such a test are shown in Fig. 3. Carbonyl compounds were detected in cabbage, clarkia, sugar cane and shellac waxes.

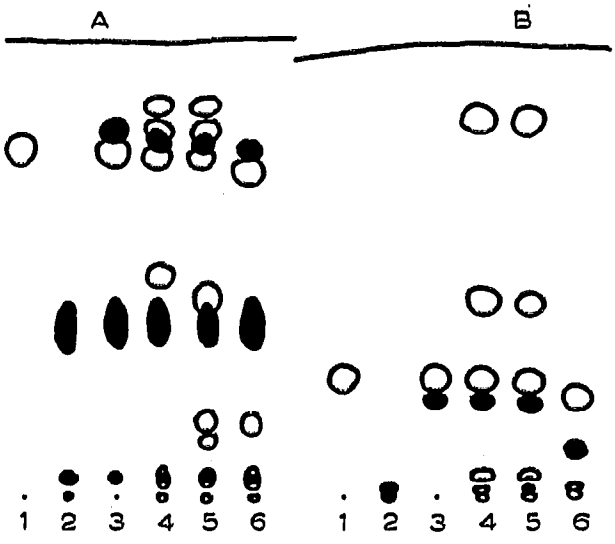

Fig. 3.

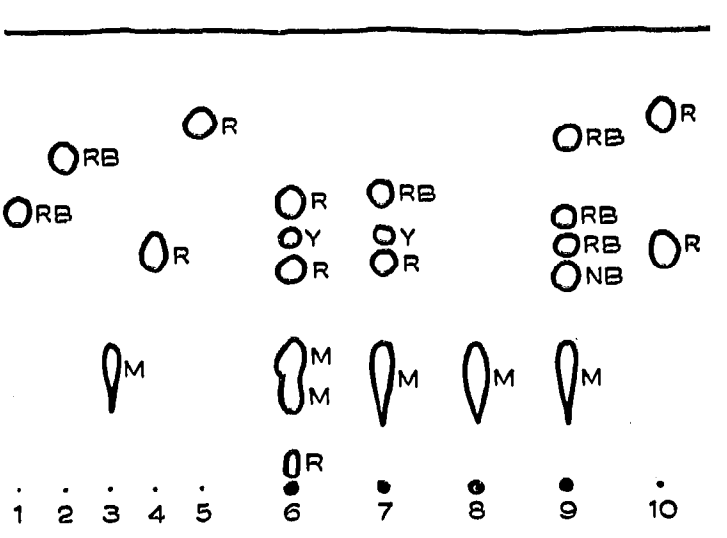

Fig. 4.

Fig. 3. Detection of carbonyl compounds in waxes. 1 = Reference *n*-alkyl ketone, 10 μg; 2 = 2,4-DNPH; 3 = reference *n*-alkyl ketone, 10 μg, +2,4-DNPH; 4 = *Brassica oleracea*, 50 μg, +2,4-DNPH; 5 = *Clarkia elegans*, 50 μg, +2,4-DNPH; 6 = sugar cane, 50 μg, +2,4-DNPH. A = System III; B = System I. Black spots appear yellow in daylight; remainder appear on spraying with Rhodamine 6 G, 0.05 % aqueous.

Fig. 4. Detection of sterols and triterpenoids in waxes using System IV. Loading: 10 μg of reference compounds, approx. 50 μg of waxes. 1 = Lupeol, β-amyrin; 2 = lupeyl acetate, β-amyrenyl acetate; 3 = ursolic acid; 4 = cholesterol, β-sitosterol; 5 = cholesteryl stearate, cholesteryl palmitate; 6 = *Eucalyptus gunnii*; 7 = *Eucalyptus globulus*; 8 = *Pyrus malus*; 9 = *Rhododendron ponticum*; 10 = human ear wax. Detection: chlorosulphonic acid 1 vol.; glacial acetic acid 2 vols. and heating to 120° for 10–15 min. Initial colours produced on heating. M = Mauve; R = red; RB = reddish brown; NB = navy blue; Y = yellow.

The vanillin reagent was found to be a semi-specific reagent, unsaturated compounds, hydroxy compounds in general, sterols and triterpenoids in addition to alcohols and ketones giving blue colours. The results are included in Table IV.

The Liebermann-Burchard and chlorosulphonic acid reagents were found to

TABLE III

COLOUR REACTIONS OF SOME TRITERPENOIDS AND STEROLS WITH THE LIEBERMANN–BURCHARD AND CHLOROSULPHONIC ACID SPRAY REAGENTS

	Initial colour	*On standing*	*U.V. light*
Lupeol	Red-brown	Mauve	Mauve
Lupeyl acetate	Red-brown	Mauve	Mauve
β-Amyrin	Red-brown	Mauve	Mauve
β-Amyrenyl acetate	Red-brown	Mauve	Mauve
Sterols	Red	Black → green	Red
Sterol esters	Red	Black → green	Red
Ursolic acid	Purple	Violet-blue	Yellow

detect both sterols and triterpenoids. The colours produced by both reagents on heating were similar. Esters gave similar colours to their parent compounds. The colours produced, however, changed on standing a short time but the fluorescence under long wave U.V. light remained constant. The results obtained with the triterpenoids and sterols examined are shown in Table III, and results obtained in wax analysis are shown in Fig. 4.

For the detection of acids Universal indicator was found to be suitable. Acids appeared as red spots against an orange background. The colour reactions adapted from paper chromatography by KAUFMANN and coworkers[23] were both difficult to perform and time consuming. Bromothymol blue was found to be a general reagent.

The start line reactions for –OH and –COOH groups were most useful for the

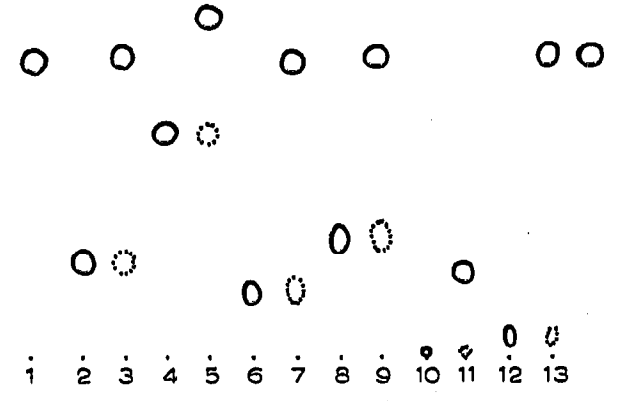

Fig. 5.

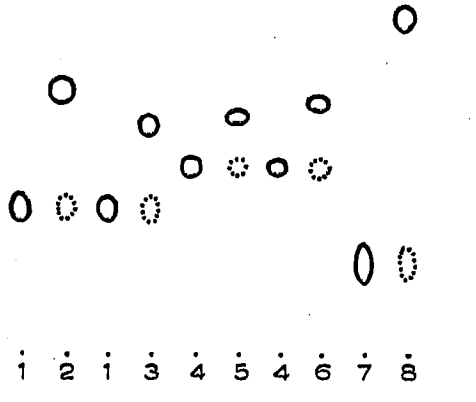

Fig. 6.

Fig. 5. Detection of hydroxy and carboxyl compounds by reaction on the start lines with acetyl chloride and boron trifluoride methanol complex using System III. Load of compounds 10 μg. 1 = Reference *n*-fatty acid methyl ester; 2 = *n*-primary alcohol; 3 = *n*-primary alcohol + CH_3COCl; 4 = *n*-secondary alcohol; 5 = *n*-secondary alcohol + CH_3COCl; 6 = sterol; 7 = sterol + CH_3COCl; 8 = lupeol; 9 = lupeol + CH_3COCl; 10 = ursolic acid; 11 = ursolic acid + BF_3; 12 = *n*-fatty acid; 13 = *n*-fatty acid + BF_3. Detection: Rhodamine 6 G, 0.05% aqueous.

Fig. 6. Detection of hydroxy and carboxyl compounds by reaction on the start lines with acetyl chloride and boron trifluoride methanol complex using System IV. Load of compounds 10 μg. 1 = *n*-ω-Hydroxy acid; 2 = *n*-ω-hydroxy acid + CH_3COCl; 3 = *n*-ω-hydroxy acid + BF_3; 4 = *n*-hydroxy acid; 5 = *n*-hydroxy acid + CH_3COCl; 6 = *n*-hydroxy acid + BF_3; 7 = *n*-α,ω-diol; 8 = *n*-α,ω-diol + CH_3COCl. Detection: Rhodamine 6 G, 0.05% aqueous.

analysis of single compounds. The derivatives formed were characteristic of the class of compound. Where both -OH and -COOH groups were present in the same compound, two derivatives were formed, one with each reagent. The reactions were also successful with sterols and triterpenoids, the derivatives giving also a positive reaction with chlorosulphonic or Liebermann-Burchard reagents. The results of such tests are shown in Figs. 5 and 6.

The ferric hydroxamate test for esters[24,25] was very sensitive for wax esters, examined as pure compounds and in waxes, when performed as a semi-micro test. However, the test was not successfully modified for the detection of esters on Kieselgel G and Aluminium Oxide G layers; the adsorbents appearing to interfere with the test. Alkyl esters were detected by examining the products of saponification with 2 *N* methanolic KOH, *i.e.* alcohols and acids, by thin layer chromatography using System III. This was also applied to waxes by examining the thin layer patterns before and after saponification. System I was particularly suitable for resolving alkyl esters.

The reactions of the wax constituents studied are summarised in Table IV.

TABLE IV

SUMMARY OF THE REACTIONS OF THE VARIOUS CLASSES OF WAX CONSTITUENT

Class	*Reactions*
n-Alkane	Unreactive, gives an R_F value of 0.9 in all solvent systems including pet. spirit (60–80°)[6].
n-Alkene	Similar to alkane. Positive bromine–fluorescein. Navy blue with vanillin–H_2SO_4.
n-Alkyl ester	Disappearance from TLC pattern on saponification with methanolic KOH.
n-Alkyl ketone	Positive 2,4-DNPH reagent—formation of yellow derivative. Navy blue with vanillin–H_2SO_4.
n-Secondary alcohols	Blue with vanillin–H_2SO_4. Formation of acetate with CH_3COCl.
n-Primary alcohols	Pale blue with vanillin–H_2SO_4. Formation of acetate with CH_3COCl.
n-Fatty acids	Red with Universal indicator. Formation of methyl ester with BF_3–methanol complex.
n-Monounsaturated fatty acids	Similar to fatty acid. Positive bromine–fluorescein. Navy blue with vanillin–H_2SO_4.
n-Hydroxy acids	Blue with vanillin–H_2SO_4. Red with Universal indicator. Formation of methyl ester with BF_3–methanol complex. Formation of acetate with CH_3COCl.
n-α,ω-Diols	Pale blue with vanillin–H_2SO_4. Formation of acetate with CH_3COCl.
Sterols + esters and pentacyclic triterpenoids +esters	Blue-black with vanillin–H_2SO_4. Positive with bromine–fluorescein. Formation of acetate with CH_3COCl. Positive Liebermann-Burchard, chlorosulphonic acid. Acidic triterpenoids form methyl esters with BF_3–methanol complex.

Preparative thin layer

The scaling up of normal thin layer procedures to 1.5 mm thick layers and the use of the solvents previously selected was successful for the isolation of classes of constituents from many natural waxes. These were in a pure form, suitable for further chemical and physical studies, *e.g.*, gas chromatography for homologue content, infra-

red spectroscopy etc. The successful resolution of bands was very much dependent on applying the wax samples as very narrow bands on the start lines. Application of samples as a series of spots on the start line gave very poor separations. The optimum load of wax per plate which could be clearly resolved depended on the thin layer pattern produced in the selected solvent system. If the constituents were well separated a higher load could be applied than if the constituents were only narrowly separated. Loads were normally in the range of 50–100 mg of wax per 20 × 20 cm plate.

The Rhodamine 6 G general reagent was most useful for preparative work, being non-destructive and not interfering with the eventual elution of the fractions from the adsorbent. Preparative plates were sprayed with the reagent as normal and the bands accurately located under long wave U.V. light. The damp bands were then readily removed with a spatula from the plates and carefully dried in an oven. After drying, the adsorbent was packed into a column and the fraction eluted with 50 ml of dry diethyl ether. Rhodamine 6 G was not eluted with the sample provided that adsorbent and eluant were free from water. Recoveries of pure samples of alkyl esters and primary alcohols by this procedure were in the range 95–96 %.

CONCLUSION

Adsorption thin layer chromatography was found to be a valuable technique in the study of long chain aliphatic and cyclic constituents found in natural waxes. Over 60 natural waxes were successfully resolved in the systems designed, and this method is therefore recommended for routine qualitative analysis of waxes. The techniques evolved were also found useful in degradative studies and in synthesis of wax constituents, for purity control to detect foreign classes of compound in samples and in monitoring the fractions collected from column chromatography. On the preparative scale pure fractions can be isolated, from intact waxes, suitable for further analysis. Better separations can be achieved by this method than by column chromatography.

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SUMMARY

The adsorption thin layer chromatography of waxes and 15 classes of reported wax constituent has been studied in detail. The choice of suitable adsorbents, solvent systems and general and specific methods of detection is discussed. A procedure for the isolation of mg fractions from intact waxes by preparative layer chromatography is described.

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