Journal of Chromatography, 243 (1982) 329–338
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,858

SEPARATION OF LIPID CLASSES BY THIN-LAYER CHROMATOGRAPHY ON UREA–SILICA GEL PLATES

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(First received January 20th, 1982; revised manuscript received March 1st, 1982)

SUMMARY

A method is described for separating free fatty alcohols from sterols, or wax esters from sterol esters, by chromatography on silica gel thin-layer plates impregnated with urea, using butyl acetate as developing solvent. Molecules containing unbranched hydrocarbon chain form urea adducts and are retarded relative to sterols or sterol esters (including esters containing long-chain fatty acids). A procedure is also given for visualising spots by charring on the plate. The separation of wax esters from sterol esters in the principal ester fraction of rabbit meibomian lipid is described, as well as a limited separation of branched-chain from straight-chain fatty acids.

INTRODUCTION

There are a number of simple methods for separating sterols from other classes of lipid, and since mammalian tissue lipids contain few alcohols apart from sterols, these methods are generally adequate. However, the lipids of the skin surface, and of specialised or modified sebaceous glands such as the meibomian glands of the eyelid, contain significant amounts of wax esters, as well as sterol esters and free sterols. Following alkaline hydrolysis of such lipid samples, the unsaponifiable fraction contains a mixture of fatty alcohols and sterols and (in skin surface lipids) squalene; further analysis of these, especially by gas–liquid chromatography (GLC) is complicated by the presence of large peaks of sterols and squalene which can obscure long-chain alcohol peaks in the same region of the trace. This is generally not serious with skin alcohols, which have no large proportion of very long-chain alcohols[1], as the chromatographic conditions can be manipulated so that squalene and sterol peaks elute after fatty alcohols. However, meibomian lipid from the cow[2,3], man[3–5] and rabbit[6,7] contains significant amounts of alcohols in the C_{24}–C_{31} region which can be obscured by sterol peaks. To avoid multiple GLC runs it is convenient to be able to separate the sterols from the fatty alcohols beforehand. This can be done in some cases by separating wax esters from sterol esters before hydrolysis by a thin-layer chromatographic (TLC) method using magnesium oxide[8,9], but success depends to a large extent on the grade of magnesium oxide used, and these methods have not

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proved reliable in this laboratory. A more recent method uses magnesium hydroxide plates[10]; the merits of this method relative to the urea–silica gel method will be discussed.

The TLC system reported here uses silica gel plates impregnated with urea, on which urea-adduct formation by normal or terminally-branched fatty alcohols causes a considerable reduction in their R_F values compared to sterols. The same effect is seen with fatty acids, and the technique has also proved valuable in separating sterol esters from wax esters, and in effecting a partial separation of straight-chain from branched-chain fatty acids or alcohols.

EXPERIMENTAL

Materials

Reagents. All solvents used were reagent grade, redistilled before use. Merck silica gel in several grades, and plaster of Paris ($CaSO_4 \cdot \frac{1}{2} H_2O$) and AnalaR grade urea, were obtained from BDH (Poole, Great Britain).

Lipid standards. Fatty acids and alcohols and glycerides were obtained from Sigma (Poole, Great Britain), and standard mixtures of fatty acid methyl esters from Applied Sciences Labs. (State College, PA, U.S.A.). A C_8–C_{21} fatty acid methyl ester mixture was prepared by mixing roughly equal amounts of the standard ester mixtures K101 (C_8–C_{20}, even-carbon), branched-chain mix L (*iso*-C_{14}–*anteiso*-C_{17}, odd- and even-carbon) and branched-chain mix 1 (*iso*-C_{18}–*anteiso*-C_{21}, odd- and even-carbon), plus small amounts of normal C_{15}, C_{17} and C_{19} fatty acid methyl esters.

Meibomian lipid samples. Rabbit meibomian secretion was obtained by dissecting the strip of meibomian glands from fresh rabbit eyelids and crushing in diethyl ether–hexane (1:1) to extract the lipid. This material contains free cholesterol from the surrounding tissues which is not normally present in the glandular secretion.

Thin-layer chromatography

Early attempts to use silica gel G containing 13% gypsum as binder, alone with urea, or to impregnate coated plates by soaking or spraying with urea solution, were largely unsuccessful. The urea layer was friable and hygroscopic, and tended to swell and flake off the glass plate unless more binder was included. More reliable plates were achieved using silica gel HR (without binder), plaster of Paris and urea. The silica gel and binder were weighed out dry and mixed thoroughly, and the urea was added as a 0.5 g/ml solution, with enough additional water to give a pourable slurry. At this stage ammonium sulphate (5% by weight of the total) was also added as a 0.5 g/ml solution to allow spot visualisation by charring.

Glass plates 20 × 20 cm were spread with a layer 0.3 mm thick, using a Shandon leveller–spreader. Plates were spread with layers containing various proportions of urea, silica gel or binder, to assess the contributions of each. After spreading, the plates were allowed to stand for about 15 min until the gel layer had become firm, then dried in the oven at 70°C for 30 min, and finally activated at 105°C for one hour. Plates were used immediately after cooling to room temperature in a desiccator.

Thin-layer separations and analysis of products

Samples were spotted on the plate in amounts from 5 to 20 µg in hexane

solution, and the spots dried in a stream of warm air. Initial experiments suggested trichlorethylene–diethyl ether (1:1, v/v) as a suitable developing solvent system[5], but dry *n*-butyl acetate has since been found preferable. The plates were developed in a tank lined with filter paper and equilibrated with the solvent vapour. After development, the plate was dried with warm air until no solvent smell remained, then heated in an oven at 150°C for about 20 min to decompose the urea. The temperature was then slowly raised up to about 250°C to decompose the ammonium sulphate to give free sulphuric acid[11]. Heating was continued until the charred spots had reached an adequate density. The presence of ammonium sulphate during plate development has not been found to affect chromatographic separations.

On preparative plates, a marker lane was spotted on one side, and the sample streaked across the remainder of the plate. After development, the preparative area was scraped off in 2 cm bands parallel to the origin, and the remaining marker lane was then charred. This procedure was used as application of indicator reagents by spraying or soaking often caused damage to the rather friable gel layer.

In samples scraped from the plate, the urea complex was disrupted by addition of 3 ml ethanol, and the lipid extracted with 3 × 1 ml of hexane, with the addition of a little water to achieve a two-phase system. Silica gel, binder, urea and most of the ethanol remained in the lower aqueous phase. Ethanol was used, instead of water alone, to precipitate the solids cleanly, rather than forming a flocculent and sticky mass capable of sequestering much of the lipid, as sometimes happened if water was added first. Recovery of a number of lipid classes by this method varied widely over the range 50–90%; this could probably be considerably improved with a more thorough extraction step.

The isolated lipid was evaporated to dryness under a stream of nitrogen and analysed by GLC in a Pye 204 instrument with flame ionisation detector. Alcohols and sterols were acetylated by warming with acetic anhydride in dry pyridine for 15 min, then evaporating to dryness under nitrogen and redissolving in hexane. Esters were injected directly without derivative formation. The column used was 1.5 m × 4 mm packed with 3% OV-1 on 100–120 mesh Chromosorb W, and the oven was programmed from 140 to 350°C at 8°C/min, with a nitrogen carrier gas flow-rate of 40 ml/min.

RESULTS

Separation of fatty alcohols and sterols

Hexadecanol and cholesterol were chosen as the type compounds since the method was developed originally as a means of separating the principal non-saponifiable components of skin surface lipid and related sebaceous materials, in which the predominant fatty alcohols were C_{14} or longer.

The separation of hexadecanol and cholesterol on a plate with a 0.3 mm gel layer containing 25% urea is shown in Fig. 1. The amount of cholesterol spotted in each lane was 5 µg, while the hexadecanol (applied as 2 mg/ml solution in hexane) varied from 2 µg to 64 µg, with each lane containing twice as much as the lane to its left, to show the effect of overloading of the adduct-forming capacity of the urea. The principal region of adduct formation is above the origin, even at low alcohol loading, but complete separation is maintained up to 64 µg. A further doubling of the amount

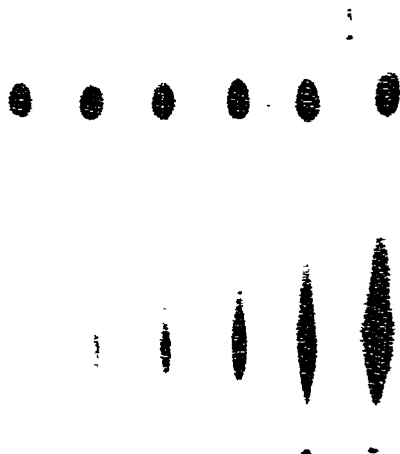

Fig. 1. Separation of hexadecanol and cholesterol on silica gel plate with 25% urea, showing the effect of increasing loadings of hexadecanol. All lanes contain 5 μg cholesterol and (from left) 2, 4, 8, 16, 32 and 64 μg hexadecanol.

of hexadecanol would, however, probably cause some overlap with the cholesterol spot. This effect is more marked on thinner layers, and layers up to 0.4 mm thick have been used to allow separation of larger amounts of lipid without overloading. No appreciable difference in R_F was seen between cholesterol and any other sterols.

Variation of thin-layer composition

Trial plates containing less than about 15% of binder were found to be too friable for use. Even with 15–20% binder, there was a tendency for the gel to break up at the lower edge of the plate where it dipped into the developing solvent, and this gave an irregular solvent front. A very hard surface was achieved with 40% binder but the chromatographic efficiency and speed of running of the plate were both reduced. A good compromise was achieved with 25% binder.

The proportion of urea was also varied from 0 to 30%, and the separation of cholesterol from hexadecanol observed in each case. The spot loading was 5 μg of each compound. In the absence of urea, both components appeared in a diffuse spot at R_F 0.46–0.61. Some separation was apparent at 10% urea, with cholesterol at R_F 0.70–0.75 and hexadecanol giving a broad spot at R_F 0.31–0.49. At both 20% and 30% urea, complete separation was achieved, with cholesterol at R_F 0.70–0.75 and hexadecanol rather closer to the origin but still in a broad spot at R_F 0.15–0.35. All subsequent plates contained 25% urea.

Separation of other lipid classes

The experiments with hexadecanol and cholesterol showed that a complete separation could be achieved based on the ability of the urea-loaded gel to form adducts with molecules containing an appreciable section of saturated paraffinic chain. Further experiments were made to identify both the types of lipid which would

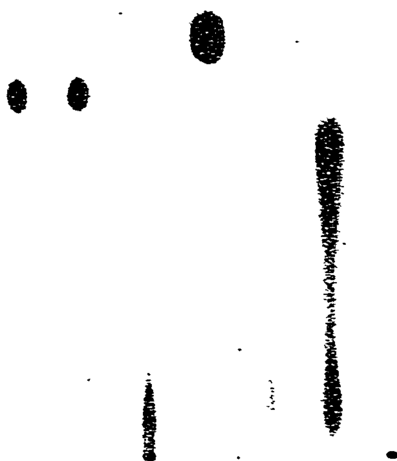

Fig. 2. Separation of lipid classes on silica gel with 25% urea. From left, 10 μg each of (1) cholesterol, (2) hexadecanol, (3) cholesterol and hexadecanol, (4) paraffin wax, (5) squalene, (6) *n*-tetracosane, (7) *n*-dodecanoic acid, (8) *n*-tetracosanoic acid.

or would not form adducts, and to indicate the effective range of chain length necessary for retardation of mobility by adduct formation.

Plates 0.3 mm thick containing 25% urea were run with samples of paraffin wax (approximate chain length range C_{21}–C_{40}), squalene, *n*-tetracosane, dodecanoic acid and tetracosanoic acid, in addition to cholesterol and hexadecanol as before (Fig. 2). Squalene formed a rather diffuse spot above the position of cholesterol (R_F 0.83–0.94); this reflects both the absence of any adduct-forming ability because of the branched and unsaturated nature of the molecule, and its greater chromatographic velocity relative to cholesterol on silica gel layers. Tetracosane readily formed adducts because of its long straight saturated chain, so remained near the origin, as did tetracosanoic acid. With dodecanoic acid, however, the formation of adducts retained some of the sample close to the origin, but the binding was sufficiently unstable for a large streak to be formed which almost reached the cholesterol region. The size of this streak might be reduced by lighter loading or thicker gel layer, but it seems that the practical lower limit of chain length for good separations under these TLC conditions is about C_{12}–C_{14}.

Provided the esterified fatty acid chain or chains are long enough, the presence of ester linkages, carboxyl or free hydroxyl groups does not lead to greater chromatographic velocities. Thus mono-, di- and triglycerides also form adducts and remain fairly close to the origin (not shown).

In all the cases shown, the separation of cholesterol from hexadecanol was used as an indicator of the condition of the plate; adduct formation was found to be sensitive to traces of water in the gel layer or in the developing solvent.

Separation of branched and straight chain fatty acids

It has been known for some time that at least a partial separation can be

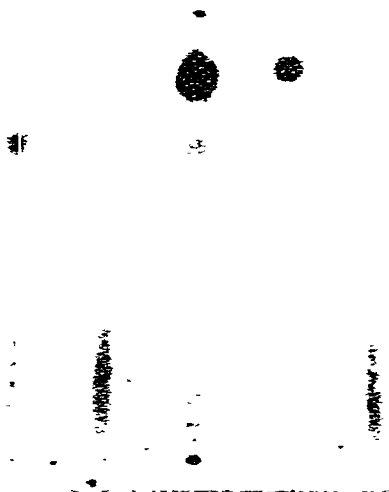

Fig. 3. Separation of esters on silica gel with 25% urea. From left, (1) cholesterol and hexadecanol, (2) C_8–C_{21} mixture of branched-chain and straight-chain fatty acid methyl esters, (3) rabbit meibomian secretion, (4) cholesteryl palmitate, (5) hexadecyl palmitate.

achieved of straight-chain from branched-chain fatty acids, either by passing the acid mixture through a column packed with urea[12] or by elution from a urea–Celite column containing the acid mixture[13], or by selective partitioning from solution[14–16]. Fatty acid separations on urea–TLC have also been reported[13,17]. Lane 2 of Fig. 3 shows the streak formed by a mixture of saturated fatty acid methyl esters of chain length C_8–C_{21}, containing *normal* even-carbon chains, *iso*-methyl branched even-carbon chains and *anteiso*-methyl branched odd-carbon chains. The spot loading was relatively low, so the extended streak seen for dodecanoic acid in Fig. 2 is not seen in this case. Similar samples were applied to another plate, and horizontal bands 2 cm wide were scraped off and the lipid extracted. Fig. 4 shows GLC traces of the original mixture and the lipid extracts from bands 2 and 4 (*i.e.* 2–4 cm and 6–8 cm, respectively, above the origin). As expected, band 2 still contains a high proportion of straight-chain acids, but of this class C_8 is missing, and the relative amounts of C_{10}, C_{12} and C_{14} (slightly) are reduced. This indicates that the chain-length limit of C_{12-14} for TLC–adduct formation still holds; these acids have therefore travelled further up the plate than longer chains. Of the branched-chain acids, only *anteiso*-C_{15} is substantially reduced relative to its *normal* isomer; although the branched/normal ratio is less for all carbon numbers, increasing chain length, and hence more tenacious adduct formation, means that there is very little change in the proportions of later peaks. The GLC trace for band 4 shows a number of differences. Of the straight chains, C_{12} is now larger, showing that it is streaked a considerable distance up the plate, and a little C_{14} is seen, but C_{15}, C_{16} and C_{17} appear only as shoulders on the peaks of the corresponding isomeric branched acids, while C_{18} and longer chains are absent. All the other visible peaks are branched acids.

Thus, two effects can be distinguished: short-chain acids (or their methyl esters) run higher than long-chain acids, irrespective of branching; and straight chains stay closer to the origin than branched chains of similar length, although there

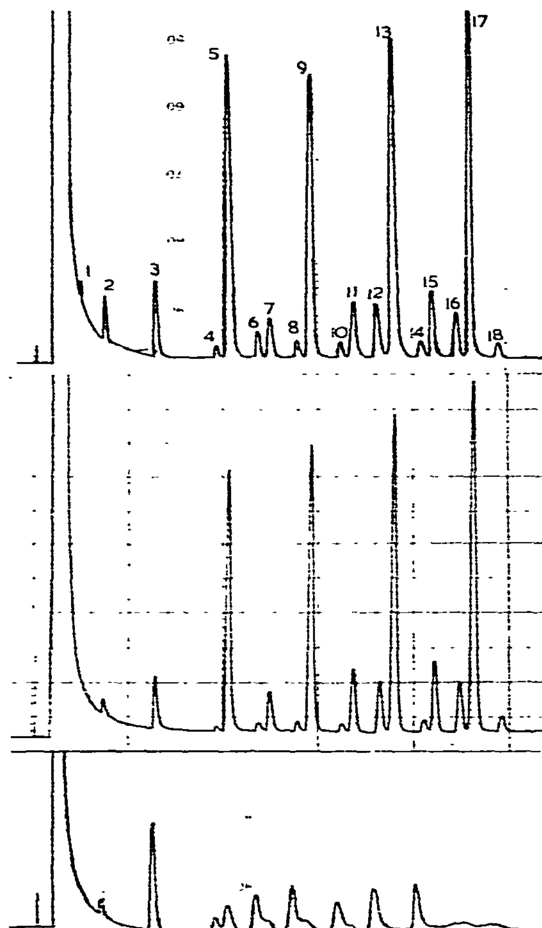

Fig. 4. GLC traces for branched-chain and straight-chain fatty acid methyl ester mixtures separated on silica gel with 25% urea. Top, sample prior to separation; middle, band 2–4 cm from origin; bottom, band 6–8 cm from origin. The chains are: 1 = *n*-C_8; 2 = *n*-C_{10}; 3 = *n*-C_{12}; 4 = *iso*-C_{14}; 5 = *n*-C_{14}; 6 = *anteiso*-C_{15}; 7 = *n*-C_{15}; 8 = *iso*-C_{16}; 9 = *n*-C_{16}; 10 = *anteiso*-C_{17}; 11 = *n*-C_{17}; 12 = *iso*-C_{18}; 13 = *n*-C_{18}; 14 = *anteiso*-C_{19}; 15 = *n*-C_{19}; 16 = *iso*-C_{20}; 17 = *n*-C_{20}; 18 = *anteiso*-C_{21}.

is no complete separation of the two classes in any of the fractions. This technique, either by TLC or adduct formation from solution, can be used to indicate branching in complex mixtures of fatty acids or fatty alcohols[18].

Separation of wax esters and sterol esters

Since Nicolaides and Foster[15] had found that wax esters and sterol esters of human skin surface lipid could be separated by urea adduct formation from solution, the same type of separation was tried by the urea–TLC method. Lanes 4–6 of Fig. 3 show that an excellent separation is achieved between hexadecyl palmitate and cholesteryl palmitate. The limitations of this separation were found to be similar to those of the free alcohols: in the wax ester, a total carbon number of at least 12–14 was necessary, and in this length range either the acid or the alcohol moiety needed to be 10–12 units long. No retardation of cholesteryl esters was seen even when the acyl moiety was as long as 22 carbons.

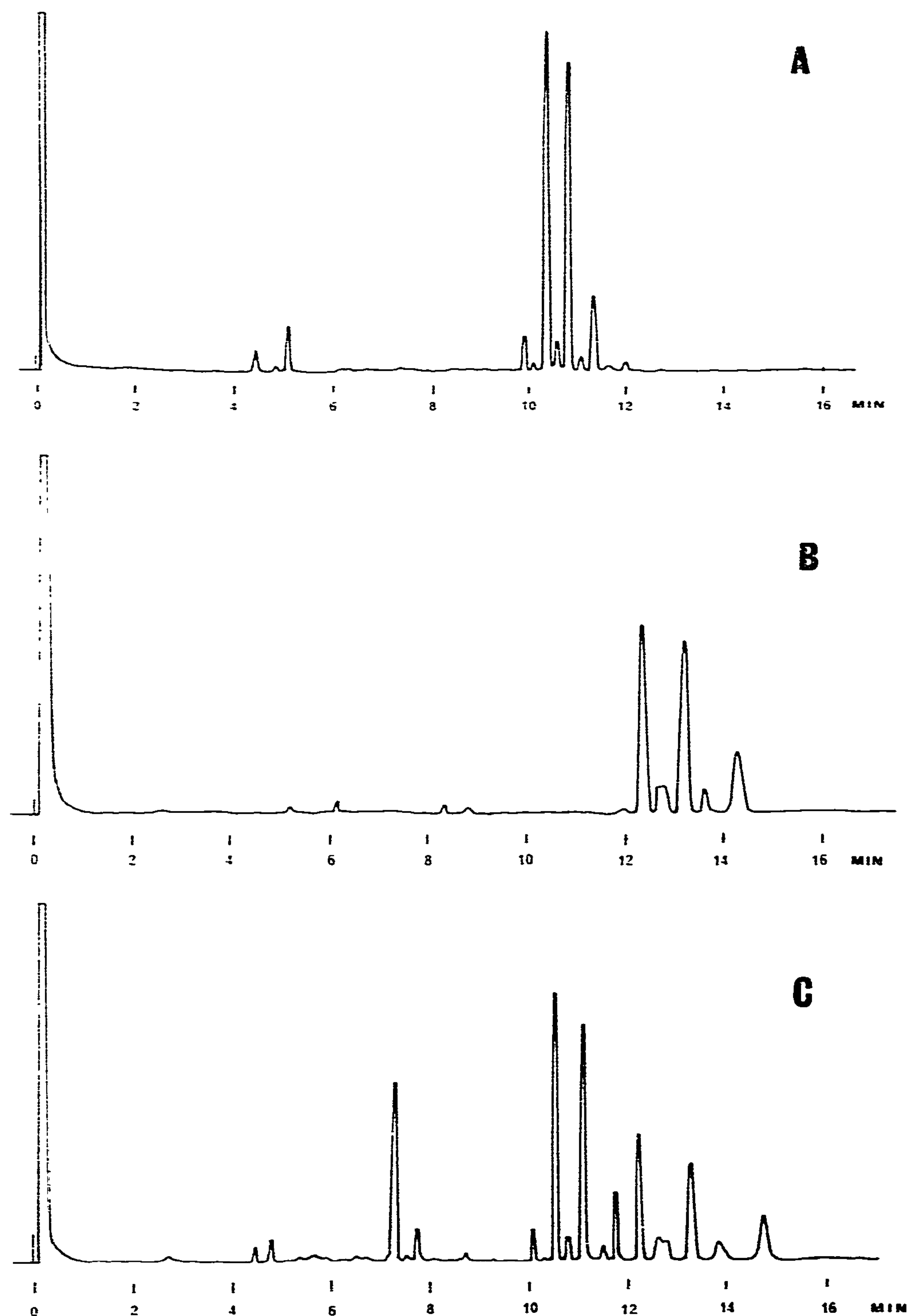

Fig. 5. GLC traces for wax ester and steryl ester fractions of rabbit meibomian secretion, isolated by TLC on silica gel with 25% urea. A, wax esters; B. steryl esters; C. whole material.

An example of the value of this separation is given in Fig. 3. Lane 3 shows the separation of a sample of meibomian lipid from the rabbit. A similar sample was run on another plate and bands scraped off parallel to the origin at levels corresponding to the wax ester and sterol ester standards in lanes 4 and 5 of Fig. 3. After extraction, the lipids were analysed by GLC (Fig. 5). The major peaks found in this secretion have been identified[7] and fall into the two distinct classes of wax esters and sterol esters.

DISCUSSION

The formation of adducts or inclusion compounds between alkyl chains and urea has been extensively investigated, and comprehensively reviewed by Schlenk[19]. Urea normally forms tetragonal crystals, but can also crystallise in a hexagonal form to produce long "tubes" of internal diameter about 0.55 nm. This is large enough to enclose a straight saturated hydrocarbon chain (Van der Waals diameter about 0.45 nm) but not an unsaturated or branched chain. The section of alkyl chain enclosed must be at least 8 carbon atoms long for stable adduct formation[20], but could consist of two shorter sections end to end, or a region of 8 or more carbon atoms in length between bulkier groups. There is a very rough stoichiometric relationship between the total length of urea "tube" and paraffinic chain present, in that no excess of "tube" is present, although some paraffinic chain may remain uncovered. The mass ratio of urea to fatty acid is generally about 3:1 when adduct crystals are formed from solution[18]. Thus a mixture of straight-chain and branched-chain saturated molecules may be partially but usually not completely separated by urea adduct formation. The multi-ring structure of sterols is too bulky, and that of squalene too highly unsaturated, to allow these compounds to form urea adducts; their R_F values are roughly similar by the TLC method to those observed in the absence of urea. In this respect the urea–TLC technique has an added advantage over crystallisation of adducts from bulk solution, in that some degree of separation can be seen between those lipid classes which are not retarded by urea. Where compounds are retarded, the nature of their polar group (alcohol, carboxyl or ester linkage) is immaterial. The presence of a substantial proportion of urea and ammonium sulphate in the gel does not seriously affect the R_F values of non-adduct components.

Relatively few attempts have been made in the past to develop column or thin-layer chromatographic methods employing urea; these were frequently less successful than solution methods, and generally did not explore classes of lipids other than fatty acids. Cason *et al.*[21] reported that esters of normal fatty acids longer than C_{18} did not form adducts on passing through a urea column, a result directly contrary to experience with the urea-TLC method described here. Coles[13], in addition to describing a successful separation of straight-chain from branched-chain fatty acid methyl esters on a urea–Celite column, reported relatively poor and variable separations on applying the same stationary phase to thin-layer. The TLC and paper chromatography methods of Bhatnagar and Liberti[17] for fatty acids did not incorporate silica gel into the thin layer, and so gave no chromatographic separation of components not forming adducts. All fatty acids longer than C_{16} remained at the origin. This method appears more useful for separation of saturated from unsaturated fatty acids.

The practicable chain-length limit by the present method appears to be about 12–14 carbon atoms. Molecules shorter than this can travel further up the plate, forming an extended streak rather than a discrete spot. This may be due to the choice

of the developing solvent; if chains shorter than 12 carbons are appreciably soluble in butyl acetate, they may be transported up the plate despite their ability to form adducts. When adducts are formed from solution in a liquid which does not dissolve urea, it is necessary to moisten the urea crystals with a small volume of methanol so that the urea can recrystallise around the hydrocarbon chains[12,19]. The very slight solubility of urea in butyl acetate (approx. 12 mg/100 ml at room temperature) may make this additional solvent unnecessary, but at the same time limit the range of separations possible.

As currently used, the method is quite sensitive to traces of water in either the gel or the developing solvent, and occasionally no separation is achieved, with all components in the sample running to about the same point on the plate. To avoid this, it is preferable to use freshly-made plates rather than storing ready-made plates for any length of time, even in a desiccator, and to fill the developing tank with fresh dried solvent for each day's work. It has also been found an advantage to include a lane on the plate in which a standard mixture of cholesterol and hexadecanol is spotted, as a check on the separating power of the plate.

Recently Stewart and Downing[10] published a TLC method for the separation of wax esters and steryl esters of skin surface lipid, using magnesium hydroxide–Celite plates. This gives good separation of the ester classes (R_F approximately 0.85 for wax esters and 0.58 for steryl esters), but a less clear-cut separation than urea–TLC plates for free fatty alcohols and free sterols (R_F approximately 0.35 for hexadecanol and 0.10 for cholesterol). Both free sterols and steryl esters seem to be retarded relative to fatty alcohols and wax esters, possibly by virtue of their "relative molecular flatness" as proposed by Nicolaides[9]. Under optimum conditions the urea–TLC method gives the same degree of separation for free alcohols and sterols as for wax and steryl esters, with the added advantages of a single developing solvent and a means of identifying the branched chains in a mixture by subsequent GLC analysis. The separation of very small amounts of a mixture of lipid classes is also more readily detectable by TLC than by the larger-scale column or solution methods.

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