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PHOTODENSITOMETRY IN THE THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF NEUTRAL LIPIDS

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SUMMARY

A procedure for photodensitometric quantitation of thin-layer chromatograms is described which can give a complete analysis of a mixture of neutral lipids in a single chromatogram without the necessity for reference mixtures.

A variety of methods is available for the quantitation of lipids separated by thin-layer chromatography[1,2]. Potentially, the most rapid and precise analyses would be obtained by photodensitometry of the chromatograms after the organic materials have been charred, particularly as described by investigators at the Hormel Institute[3–8]. However, several factors complicate the procedure so that it becomes a tedious and painstaking operation. Usually, several chromatograms are necessary to produce an analysis, and there is a constant need for comparison with reference mixtures. Ideally, it would be possible to obtain a complete analysis of a lipid mixture with one uninterrupted scan over a single thin-layer chromatogram. This ideal has now been attained by careful consideration of the principles which affect the photodensitometry of thin-layer chromatograms.

GENERAL PRINCIPLES

Photodensitometry implies measurement of the proportion of the incident light which is transmitted by a semi-opaque material. The method is relatively insensitive when the proportion of light transmitted falls below 5%, so that it is usual to work with optical densities below 1.3. It is obvious that for a spot on a chromatogram this limitation should apply not to the reading obtained on the photodensitometer, but to the actual density over all parts of the spot.

Provided one is working within the practical limits of optical density, spot size should be inversely proportional to spot density for any given quantity of substance. This will be true if the photodensitometer is in each case measuring only that light which is passing through the spot, and not light which is passing through blank areas

of the plate, although it is probably acceptable if the same proportion of the incident light passes through blank areas in each case. BLANK *et al.* recommended a slit 25% longer than the diameter of the spot[7]. This presumably meant that the slit had to be adjusted for each spot on a chromatogram, since the spots vary in size according to both quantity of substance and distance of migration. It would certainly mean that the maximum optical density recorded would be about 0.7, since even with a totally-absorbing spot there would still be at least 20% transmission between the edges of the spot and the ends of the slit. Under these circumstances it will not be noticed when density of a spot is beyond the measurable limit, in which case the readings would bear little relationship to density. However, with a fixed slit length the readings would be proportional to spot area, and since spot area is in turn proportional to the quantity of organic compound in the spot, at any particular R_F value, the readings may still be an effective means of quantitation. When the densitometer is thus used as a planimeter, the variation of spot size with R_F value, and other factors, necessitates the constant use of reference materials. Moreover, a reference compound is required for each class of compound in the chromatogram[9]. As many as seven chromatograms may have to be scanned to produce one analysis[10].

When the density over all or a major proportion of a spot is greater than the practical limit, further increase in density will be largely undetected. This will mean, firstly, that differences in charring yield will be eliminated. This may explain why many investigators have not detected differences in carbon yield between compounds of widely differing carbon content. Secondly, the inverse relationship between spot size and spot density will be lost, giving rise to the commonly-observed effect of increased "degree of charring" with increased distance of travel on the chromatogram. Both of these effects are apparent in the results reported by LOUIS-FERDINAND *et al.*[9].

In addition to the effect of variation in spot size, a number of factors will affect the density of the spot produced by a given amount of a compound after charring. The principal of these will be the charring reaction itself, which is achieved by a succession of oxidations and dehydrations to produce something approaching elemental carbon. There is the possibility of great variation in the efficiency of this conversion with any one compound, but the present stage of development indicates that conditions can be found under which yields are reproducible.

Basically, the proportion of carbon which a compound contains (the so-called "carbon-density") will govern the maximum yield of elemental carbon it can produce on charring. In any technique which involves spot density this factor must be taken into account, but it is one which involves a constant mathematical relationship.

Of more concern are factors which can vary for any particular compound. For instance, compounds which are not readily attacked by the charring agent are capable of evaporating from the thin-layer plate before they can be converted to carbon, although the excessive temperature of 360° has been cited[6] for instances where this has occurred. To avoid such evaporation it is common practice to include a powerful oxidizing agent, such as potassium dichromate, in the primary charring agent, which is usually sulphuric acid. It was recognized by PRIVETT *et al.*[8] that the dichromate reagent is capable of oxidizing organic compounds to carbon dioxide, with a consequent decrease in the yield of carbon, but there is no report that this possibility was investigated.

The foregoing principles have been taken into account in devising the present

procedure for quantitative thin-layer chromatography. In particular, the quantities of lipid in each chromatogram were limited to that which would produce spots of measurable optical density. This factor also minimized spot size, and the maximum size of spots was further defined by the use of narrow lanes on the chromatographic plates. The slit length was made slightly less than the width of the lanes, and with this arrangement there was an almost constant relationship between slit length and spot width. The charring procedure was carried out in a manner calculated to avoid both over-oxidation and evaporation. Development was performed in such a way as to resolve the mixtures of lipids on a single chromatogram.

Application of the technique to some representative mixtures of lipids indicates that it can achieve the major objectives of convenience and accuracy. It must be emphasized that the present application is confined to determination of the relative amounts of constituents in a mixture rather than the absolute amounts of lipid applied to the plates.

EXPERIMENTAL

Preparation of the thin-layer plates

The 20 × 20 cm glass plates were spread with a 0.25 mm layer of Silica Gel G (E. Merck and Co.) using a Quickfit-Reeve Angel plate-leveller and spreader. The layers were dried at 120° for 3 h and then stored in glass tanks. Before use the plates were developed overnight in ether to remove contaminants. The adsorbant layer was then divided into vertical lanes 7 mm wide. Immediately before application of the samples the plates were re-activated by heating at 130° for 30 min.

Development of the chromatograms

For a simple mixture of lipids, development in a single solvent mixture was usually adequate. For a more complex mixture of lipids however, a system of multiple development was employed. This consisted of:

(1) development with hexane to a line 1 cm below the top of the plate;

(2) after drying at room temperature for 10 min the plate was developed to the line with benzene;

(3) after drying again the plate was developed to the 10 cm level in a mixture of hexane–ether–acetic acid (70:30:1).

Multiple development techniques in which the more polar solvent systems precede the less polar should be avoided because the more mobile components form seriously-distorted spots under such conditions.

Charring of the chromatograms

The developed chromatogram was normally sprayed with 50% sulphuric acid, using a pressure pack can fitted with a polypropylene spray head and a glass reagent jar (Crown Industrial Products, Hebron, Ill.). The acid was sprayed as evenly as possible until the first appearance of fine spots of dampness on the silica gel. Actual wetness of the layer was rigorously avoided. The plate was then set upon a cold aluminium plate (8 × 8 × 0.25 in.) lying on a 6 × 6 in. hot plate (Autemp Heater, Fisher Scientific). Heating was commenced by turning the hot plate control to 5.5. The temperature rose from 20° to a maximum of 220° during a period of approximately

30 min. Under these conditions even saturated lipids began to char at about 180°, and by the time full temperature was reached, maximum darkening was achieved and most of the sulphuric acid had evaporated. A further 10 min heating was allowed for complete vaporization of the acid.

Scanning the chromatograms

The photodensitometer (Photovolt Corp., New York, Model 52-C) was fitted with a thin-layer chromatography search unit, a Varicord Recorder (Model 42 B) and an Integraph integrator (Model 49). A substage slit was installed which limited the incident light to a beam of 1 × 4 mm. A mask over the photocell was provided with a 0.02 × 4 mm slit.

The dimensions of the lanes on the thin-layer plate allowed it to be aligned on the stage of the search unit so that the beam of incident light passed symmetrically through each spot of the series of spots produced by each lipid sample. In this way it was possible to scan each lane in a single uninterrupted run.

Analysis of synthetic mixtures

A standard solution of each individual pure lipid (Applied Science Labs., Inc.) was prepared, and appropriate mixtures of these lipids were then obtained by combination of accurately-measured volumes of the standard solutions. The resulting mixtures contained between 0.3 and 3 μg per ml of each lipid, and usually between 1 and 2 μl of a solution was applied to a lane of the plate. The chromatograms were then developed, charred, and scanned on the densitometer as described. Normally, it was possible to calculate percentage composition directly from the integrator output on the recorder chart. Where variations in the baseline interfered with accurate

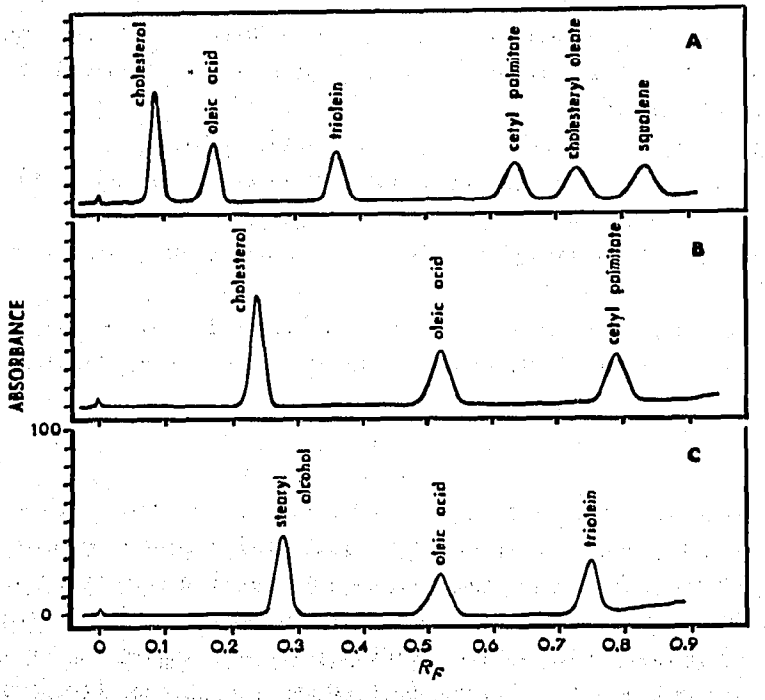

Fig. 1. Photodensitometer records of the thin-layer chromatograms of some synthetic mixtures of lipids. (A) Resolution obtained in the treble-development system. (B, C) Resolution in hexane–ether–acetic acid (70:30:1).

integration of a peak, its area was determined by triangulation and converted to integrator units by means of a factor determined experimentally. The number of integrator units for each peak was then adjusted by a factor derived by dividing the molecular weight of the respective molecule by the sum of the atomic weights of the carbon atoms in the molecule. The proportion of each constituent was then calculated from the adjusted integrator units.

RESULTS

Fig. 1A is a representative example of the densitometer output in scanning a chromatogram of a mixture containing equal weights of the various lipids. The resolution in this instance was obtained with the multiple development technique. It is apparent that the carbon yield for cholesterol was far greater, in relation to the other lipids, than would be expected on the basis of its carbon density. It was therefore necessary to determine whether this was due to chemical structure or to the position of cholesterol in the chromatograms, where it was the constituent closest to the origin. Mixtures containing cholesterol were chromatographed in the hexane–ether–acetic acid solvent system, which in this instance was allowed to migrate to the top of the plates, so that the cholesterol travelled a greater distance than usual, as shown in Fig. 1B. This did not affect the relative carbon yield. To determine whether it was the steroidal structure or the hydroxyl function which was responsible for the high carbon yield, a reference mixture containing a non-steroidal fatty alcohol, stearyl alcohol, was chromatographed, and a high carbon yield was obtained from this compound also (Fig. 1C). Finally, a mixture containing equal weights of cholesterol and stearyl alcohol was chromatographed, and the carbon yields for the two alcohols were virtually identical.

It can, therefore, be accepted that the hydroxyl function causes a greatly

TABLE I

ANALYSIS BY THIN-LAYER CHROMATOGRAPHY OF REFERENCE MIXTURES USING CORRECTION FACTORS FOR THE CARBON CONTENT OF THE RESPECTIVE MOLECULES AND FOR THE HYDROXYL GROUP EFFECT

7 mm lane width and 0.02 × 4 mm slit.

Constituents	R_F *value*[a]	*Composition (weight %)*			
		Sample 1		*Sample 2*	
		Found[b]	*Known*	*Found*[c]	*Known*
Cholesterol	0.09	16.6 ± 0.68	16.6	2.3 ± 0.34	2.5
Oleic acid	0.19	17.2 ± 0.53	16.6	26.4 ± 1.34	25.0
Triolein	0.36	17.3 ± 0.38	16.6	30.4 ± 1.51	30.0
Cetyl palmitate	0.63	17.5 ± 0.90	16.6	24.0 ± 1.59	25.0
Cholesteryl oleate	0.72	15.2 ± 0.90	16.6	2.4 ± 0.28	2.5
Squalene	0.82	16.1 ± 0.89	16.6	14.6 ± 1.23	15.0

[a] Distance travelled/height of plate in the treble development system.

[b] Mean and standard deviation for eight consecutive chromatograms on the same thin-layer plate.

[c] Mean and standard deviation for fifteen consecutive chromatograms on the same thin-layer plate.

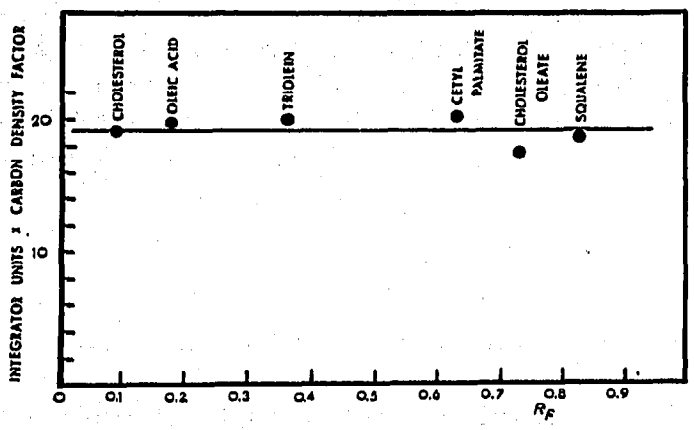

Fig. 2. Demonstration of the independence of the carbon yield from the distance of migration.

enhanced carbon yield compared with non-hydroxylated compounds, and it is necessary to allow for this effect in calculating the composition of mixtures containing free alcohols. The appropriate factor was derived experimentally from the relationship between the average of the peak areas for the non-hydroxylic compounds, adjusted for carbon content of the respective molecules, and the area of the cholesterol peak, also adjusted for carbon content. This showed that the area of the peak for an alcohol must be reduced by the factor 0.66, and this has been applied in the calculation of the results reported here.

Typical results are shown in Table I, which were obtained with a 7 mm lane width and 4 mm slit length. Fig. 2 illustrates the remarkable constancy of the carbon yield over the full extent of the chromatograms and for all of the lipids chromatographed, provided only that the correction factor for the high yield from free alcohols is applied and that the calculations are based on the proportion of carbon in the original molecules. Because of the constancy of the carbon yield it is possible to construct a graph of peak area against the weight of carbon for each compound chromatographed. This produces a straight line passing through the origin, as in Fig. 3.

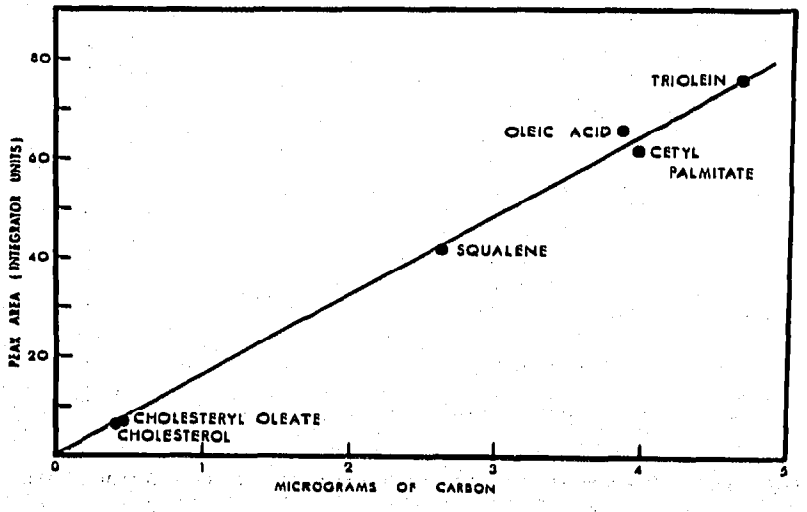

Fig. 3. Relationship between the weight of lipid carbon applied to a chromatoplate and the area of the peak produced in the photodensitometer scan.

TABLE II

EFFECT OF A WIDER LANE AND A LONGER SLIT ON THE ANALYSIS OF THE REFERENCE MIXTURES
14 mm lane width and 0.02 × 7 mm slit.

Constituents	R_F *value*	*Composition (weight %)*			
		Sample 1		*Sample 2*	
		Found[a]	*Known*	*Found*[b]	*Known*
Cholesterol	0.09	5.4 ± 0.30	16.6	2.3 ± 0.41	2.5
Oleic acid	0.20	13.0 ± 0.62	16.6	17.0 ± 1.51	25.0
Triolein	0.35	14.8 ± 0.56	16.6	20.8 ± 1.43	30.0
Cetyl palmitate	0.65	27.2 ± 1.10	16.6	34.5 ± 1.56	25.0
Cholesteryl oleate	0.76	20.3 ± 0.69	16.6	3.1 ± 0.36	2.5
Squalene	0.85	19.2 ± 0.69	16.6	22.4 ± 2.39	15.0

[a] Mean and standard deviation for eleven consecutive chromatograms on one thin-layer plate ruled with 14 mm lanes and scanned with a 7 mm slit length.
[b] Mean and standard deviation for twelve consecutive chromatograms on one thin-layer plate.

To demonstrate that the narrow lanes were a significant factor in maintaining a constant densitometer response over the length of the chromatograms, series of analyses were conducted in which wider (14 mm) lanes and a 7 mm slit length were employed. The results shown in Table II, which were obtained in this way, bear little relation to the actual composition of the reference mixtures. The mixtures were the same as those which produced the satisfactory results with narrower lanes shown in Table I. In addition to the tendency for a greater densitometer response with increasing R_F values the results obtained with wide lanes varied erratically from one compound to the next. This appeared to be due to differences in the shapes of the spots. An

TABLE III

EFFECT OF POTASSIUM DICHROMATE SPRAY REAGENT ON THE ANALYSIS OF A REFERENCE MIXTURE OF LIPIDS

Constituents	*Composition (weight %)*		
	Found[a]		*Known*
	50% H_2SO_4	*$K_2Cr_2O_7$–70% H_2SO_4 (ref. 8)*	
Cholesterol	2.9 ± 0.37	3.3 ± 0.18	2.5
Oleic acid	24.3 ± 1.20	28.4 ± 1.00	25.0
Triolein	28.8 ± 1.58	29.5 ± 1.14	30.0
Cetyl palmitate	26.0 ± 0.79	26.4 ± 1.80	25.0
Cholesteryl oleate	2.6 ± 0.28	1.9 ± 0.32	2.5
Squalene	15.4 ± 0.62	10.6 ± 0.79	15.0
Total peak area (integrator units)	263 ± 15	170 ± 9	

[a] Mean and standard deviation for six consecutive chromatograms in each series, all on the same thin-layer plate.

elongated spot tended to give a smaller densitometer response than one which was more round. Narrower lanes minimized variation in spot shape as well as in spot size.

Table III compares two series of results derived from analyses carried out on the same thin-layer plate, on which each of the lanes was spotted with the same amount of the reference mixture. One half of the plate had been sprayed with the standard 50% sulphuric acid solution, and the other half with potassium dichromate–70% sulphuric acid reagent. The lanes which had been sprayed with the dichromate reagent produced a very much lower overall response from the densitometer, as shown by the sums of the peak areas. Those compounds which normally showed the more diffuse spots, because of their higher R_F values, showed the greatest decrease in carbon yield.

DISCUSSION

The constant yield obtained over almost the full extent of the chromatograms is in marked contrast to the results of other investigators. The usual results are exemplified by those of LOUIS-FERDINAND *et al.*[9], who reported a continual increase in "degree of charring" with distance of migration. PRIVETT *et al.* avoided this effect to some extent by fixing a constant ratio of slit length to spot diameter[7]. This allowed them a constant carbon yield between R_F 0.4 and 0.8, but since this was too narrow a range in which to resolve most lipid mixtures they required several chromatograms to obtain an analysis. The present additional range of usable R_F values makes it possible to analyze many lipid mixtures in a single chromatogram. This advantage has been gained by working within the practical limits of optical density, by ensuring a constant ratio of slit length to spot diameter, and by observing charring conditions under which compounds of widely differing reactivity give a constant yield of carbon. A further major advantage of the present technique is the capability of conducting up to 25 analyses on one standard size (20 × 20 cm) thin-layer plate.

It has been shown in this investigation that the addition of potassium dichromate to the sulphuric acid spray reagent can result in a decreased yield of carbon in the charred spots. This presumably is the result of part of the organic compounds having been oxidized to carbon dioxide. It was noticed that the greater the R_F value the greater was the decrease in carbon yield when sprayed with the dichromate reagent. This effect is to be expected, since the greater the area covered by a spot the greater is the amount of oxidizing agent to which it is exposed.

Most investigators have demonstrated the reliability of their particular analytical technique by constructing a graph relating the weight of compound to the thin-layer spot area or the photodensitometer peak area. This usually produces a straight line passing through the origin, but with a different slope for each compound and for each chromatographic system in which the compound is developed. In the present technique such deviations have been eliminated, so that a single line is produced for a wide range of simple lipids. The cause of the deviation of free alcohols from this condition is unknown and is under investigation. It would seem probable that similar effects will be found for other classes of compound, but it appears that once a correction factor has been derived it can be applied without further recourse to reference mixtures.

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