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Note

Correction for deviation from the Lambert-Beer law in the quantitation of thin-layer chromatograms by photodensitometry

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In a previous communication[1] it was shown that quantitative thin-layer chromatography (TLC) of lipids can be carried out so as to produce a linear relationship between the amounts of lipid and the photodensitometric peak areas obtained on scanning the acid-charred chromatograms. Furthermore, a single straight line represented this relationship for a variety of lipids provided that the results were expressed in terms of the weight of carbon contained in each quantity of lipid. Under these conditions, quantitative analysis of lipid mixtures was carried out without the use of reference compounds or the construction of standard curves. However, this was true only if the chromatographic adsorbent was first scored into narrow (6–7 mm) lanes so as to prevent the expansion of spot width with distance of migration during development or with the amount of lipid present in a spot. This allowed for a constant relationship between spot width and the length of the densitometer light beam.

In the previous study the densitometric peak areas obtained by triangulation, involving the usual (height $\times$ width) calculation, were linearly related to the quantity of lipid chromatographed. This could imply that the system of transmission photodensitometry obeyed the Lambert-Beer law, such that the optical density was linearly related to the amount of material (carbon) encountered by the light beam. However, the favorable correlation actually resulted from automatic correction for non-linearity of optical density provided by the strip-chart recorder employed (Varicord Model 42B; Photovolt, N.Y., U.S.A.). This consisted of an adjustable, quasiexpontial recorder response, instead of a truly linear response, relative to optical density. Photodensitometers from other manufacturers usually have a fixed response which is calibrated to be linear with optical density, so that deviations from the Lambert-Beer law are troublesome, especially as most modern instruments are useable over much wider ranges of optical density.

Several previous investigators have devised mathematical techniques for the correction of deviations in optical density encountered in thin-layer densitometry. Goldman and Goodall[2,3] used the theory of Kubelka and Munk[4] to calculate the nature and extent of deviations from the Lambert-Beer law which are to be expected from the light-scattering nature of chromatographic thin-layers. They then devised empirically an equation approximating the Kubelka-Munk expression and found that the still-complex calculation provided linear analyses over a limited range of sample quantity. Treiber[5] showed that neither the Lambert-Beer law nor the simulation

of the Kubelka-Monk expression gave linear results but that a more complex expression derived from both gave very much better results.

The present study provides a very much simpler correction factor for use with linearly-calibrated photodensitometers in carrying out quantitative TLC.

EXPERIMENTAL

Chromatographic plates

The 20 × 20 cm glass plates were spread with a 0.25 mm layer of silica gel G (E. Merck, Darmstadt, G.F.R.) using a Quickfit-Reeve Angel plate leveller and spreader. The layers were dried at 120° for 2 h, cooled, and then cleansed by development in chloroform–methanol (2:1). Before use the adsorbent layer was ruled into vertical lanes 6 mm wide.

Standard chromatograms

Solutions of pure docosanol, cholesteryl oleate, oleic acid and triolein (Applied Science Labs., State College, Pa., U.S.A.) of known concentration in toluene were combined and then diluted to produce a series of solutions in which 4 μl aliquots contained 1, 2, 4, 8, 12, 16, 24 and 32 μg of each lipid. Three such aliquots from each solution were applied to three separate lanes on each chromatographic plate, using a capillary pipet. The chromatograms were then developed to 19 cm in hexane–ether–acetic acid (80:20:1). After drying, each chromatographic plate was sprayed with 50% sulfuric acid and placed on an aluminum slab (20 × 20 × 0.65 cm) lying on a cold electric hotplate (Model HP-A1915B; Thermolyne, Dubuque, Iowa, U.S.A.). The hotplate was adjusted to a setting ("500") found to eventually produce a temperature of 220°, and then switched on. After 50 min, when the maximum degree of charring had been obtained and all of the sulfuric acid had been driven off, the system was allowed to cool to room temperature.

Photodensitometry

The light beam in the densitometer (Clifford Model 445; Corning Medical Products, Medfield, Mass., U.S.A.) was adjusted to illuminate the chromatograms from below with a rectangle of light 1 mm wide and just long enough (5 mm) to extend across the chromatographic lanes. The instrument was trimmed electronically so that when a photographic step tablet (Kodak No. 2) was laid on a blank, adsorbent-coated chromatographic plate and scanned, the recorder response was linear with optical density over the full range of the optical standard (0–3 O.D.). When chromatograms were subsequently scanned, the "zero" and "span" adjustments were set so that when the darkest spot on a plate was scanned the peak produced by the recorder just occupied the full width of the strip chart. The full series of chromatograms on the plate was then scanned without changing these parameters. This procedure was facilitated by an automatic plate scanner provided with the instrument which allowed all of the chromatographic lanes to be scanned in succession without intervention.

Each of the peaks on the recorder output was triangulated and the height (h) and width (w) measurements of the triangles were used for calculations either according to the conventional formula $hw/2$ or variations in which the height parameter was adjusted exponentially, as in $h^x w/2$. The adjusted peak areas obtained in this

way were plotted against the weight of carbon calculated to be present in the amounts of each lipid applied to the chromatograms.

RESULTS AND DISCUSSION

Table I shows the height and width of the triangles constructed around each of the chromatographic peaks on one of the sets of densitometer scans. Fig. 1 shows that when the areas of the triangles were obtained by the conventional calculation and then plotted against the quantities of lipid carbon applied to the chromatograms, the results deviated severely from linearity and each lipid produced a distinctly separate curve. The deviations were such as to indicate that the optical density (peak height) of the chromatographic spots increased at a rate that was less than linear relative to the increasing weights of lipid carbon. It was found by empirical means that the correlations became linear when the triangulation height of each peak was raised to the power 1.4 before calculation of the areas of the triangles, as shown in Fig. 2.

TABLE I

PEAK DIMENSIONS OBTAINED IN TRIANGULATION OF THE DENSITOMETER SCANS OF THE THIN-LAYER CHROMATOGRAMS ON ONE PLATE

Amount of lipid applied (μg)	*Peak dimensions (mm)*							
	Docosanol		*Oleic acid*		*Triolein*		*Cholesteryl oleate*	
	Height	*Width*	*Height*	*Width*	*Height*	*Width*	*Height*	*Width*
	8.0	2.6	4.0	3.9	4.0	4.0	5.0	4.1
1	8.0	2.7	4.0	4.2	4.5	3.9	5.0	4.2
	8.0	2.6	4.0	4.3	4.0	4.2	5.0	4.3
	14.5	2.7	8.0	4.2	8.0	4.1	9.0	4.2
2	16.0	2.6	8.0	4.3	8.0	4.2	9.0	4.2
	14.0	2.9	8.0	4.3	8.0	4.1	9.0	4.1
	24.0	2.8	14.0	4.3	14.5	4.2	16.0	4.4
4	24.0	2.8	15.0	4.3	14.0	4.3	16.0	4.3
	23.0	3.0	14.0	4.4	14.5	4.1	15.0	4.4
	34.5	3.2	23.0	4.8	24.0	4.5	26.5	4.7
8	35.5	3.3	23.0	5.1	24.0	4.6	26.0	4.7
	34.5	3.2	24.0	4.9	24.0	4.4	26.0	4.5
	44.0	3.4	29.0	5.2	31.0	4.6	33.0	4.8
12	43.0	3.4	29.0	5.3	31.0	4.6	33.0	4.7
	44.0	3.4	29.0	5.3	31.0	4.6	33.5	4.7
	50.5	3.5	35.0	5.6	37.0	4.7	40.0	5.0
16	50.5	3.5	35.5	5.6	38.5	4.7	40.5	5.0
	51.0	3.5	35.5	5.6	38.0	4.7	41.5	4.7
	63.0	4.1	43.5	6.4	49.0	5.0	54.0	5.2
24	62.0	4.1	45.0	6.4	50.5	4.8	55.0	5.1
	63.0	4.0	47.0	6.4	50.5	5.0	53.0	5.3
	69.0	4.1	51.5	6.7	57.5	5.2	62.0	5.2
32	69.0	4.2	51.5	6.7	58.5	5.1	63.0	5.3
	66.0	4.3	51.0	6.8	57.5	5.2	60.0	5.6

Furthermore, the results for all of the compounds then fell on a single straight line, even when the amounts of each lipid chromatographed were so excessive (24 and 32 μg) as to severely distort the shapes of the spots which they gave rise to after charring. In the normal practise of the technique described here, not more than 10 μg of any lipid would be applied to one of the chromatographic lanes.

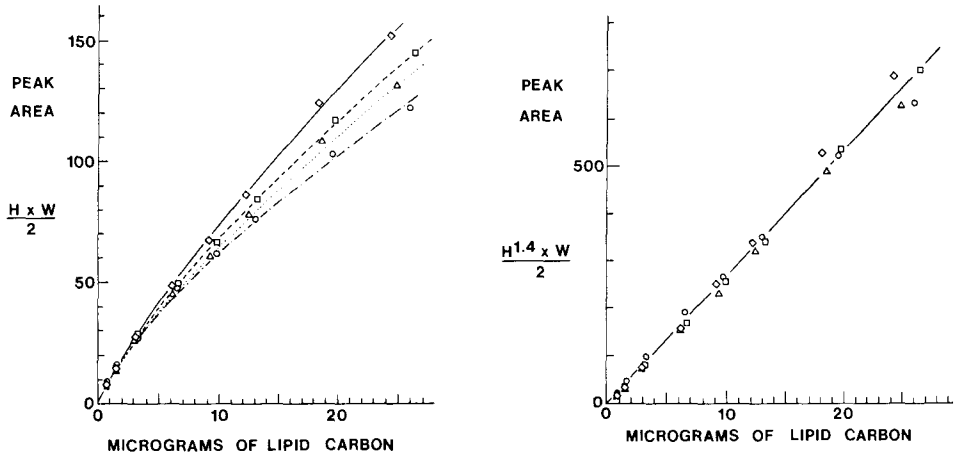

Fig. 1. Relationship between the weights of lipid carbon applied to chromatograms and the areas of the photodensitometer peaks obtained by the normal triangulation procedure for: ◇, oleic acid; □, cholesteryl oleate; △, triolein; ○, docosanol.

Fig. 2. Relationship between the weights of lipid carbon applied to chromatograms and the areas of the photodensitometer peaks obtained by the modified triangulation procedure for: ◇, oleic acid; □, cholesteryl oleate; △, triolein; ○, docosanol.

The present results confirm the previous report[1] that conditions can be found under which quantitative TLC can be performed without the continual use of reference compounds. It is apparent that accurate photodensitometric quantitation depends upon either an appropriate correction factor (mathematical or electronic) for deviation from the Lambert-Beer law or extensive use of reference compounds for the construction of a standard curve for each lipid to be assayed. The inherent non-linearity of the photodensitometric assay of thin-layer chromatograms appears often to have been overlooked, perhaps because the instrument (Photovolt) most commonly used by developers of the procedure provided an electronic correction.

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