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IN SITU QUANTITATION OF LIPIDS BY THIN-LAYER CHROMATOGRAPHY USING REFLECTANCE DENSITOMETRY

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SUMMARY

A method for the quantitation of phosphatidylcholine, lysophosphatidylcholine, palmitic acid and propyl palmitate involving *in situ* reflectance densitometry is described. The logarithm of the weight of lipid in a spot and the peak area derived from a plot of reflected light intensity across the spot are shown to be linearly related over a wide range. The correlation between elution distance and the slope of the regression line, together with its implications for the commonly used linear relationship between spot loading and peak area squared are examined. The logarithmic relationship is compared with a general power law.

INTRODUCTION

Various methods for the *in situ* quantitation of lipids have been reported[1–8]. Most of these involve charring with sulphuric acid mixtures followed by densitometry, although Fewster *et al.*[2] used a copper acetate reagent and Nicolosi *et al.*[5] a rhodamine 6G spray followed by fluorescence measurements. Adams and Sallee[7] used an ammonium bisulphate charring reagent followed by scanning with a dual-beam spectrodensitometer in the transmittance mode. However, Frei[8] found that reflectance measurements using clean "backing plates" to simulate infinite layer thickness gave superior accuracy.

Many workers using reflectance densitometry for the quantitative assessment of chromatograms have assumed that the square of the peak area is proportional to the amount of material in the spot. Frei[8] has pointed out that this is a highly simplified version of the Kubelka–Munk equations[9,10] dealing with radiative transfer in highly scattering media. A number of workers, however, have found this relationship to be inadequate. For example, Touchstone *et al.*[11] have found the relationship valid only at low spot loadings (2 μg or less). Other workers have found that two distinct straight-line relationships occur at high and low loadings[7] or that distinct non-linearity occurs at higher loadings[3].

It is probable that some of this apparent conflict may be attributed to the

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differing experimental techniques involved. Thus most workers (*e.g.*, Nutter and Privett[3], Biezenski *et al.*[4], Privett *et al.*[1], Fewster *et al.*[2], Adams and Sallee[7] and Goldman and Goodall[12,13]) measured the intensity of transmitted light. Frei, however, appears to have shown[8] that measurements of reflected light are superior, pointing out that Goldman and Goodall's experiments[12] were unfairly biased in favour of transmittance measurements. In addition many workers (*e.g.*, refs. 1–3, 5, 7, 8, 12 and 13) used circular spots of material where the absorbance varies greatly from the centre to the edge. As Goldman and Goodall have pointed out[12], the Kubelka–Munk equations are not valid under such conditions, particularly when a spot or slit of light of greater diameter or length than the diameter of the spot is used. This appears to be the method employed by many workers (*e.g.*, refs. 1 and 2), although frequently the relevant details are not stated (*e.g.*, refs. 3, 5, 7 and 11).

Some workers have attempted to ensure uniform absorbance by using "streaks" rather than spots (*e.g.*, refs. 3, 4 and 11). When scanned with a slit of light slightly shorter than the streak, this criterion is approximately fulfilled but it is difficult to produce streaks of a uniform and reproducible form. In addition, the present author has noticed that the illumination provided by the Zeiss Mark I chromatogram spectrophotometer is by no means constant over the length of the slit, particularly for slits of more than 1 cm in length.

The present work incorporated some of the best points of these workers' methods, whilst proposing that the logarithm of peak area should be taken to be a linear function of the logarithm of spot loading. This relationship appears to apply to a greater range of spot loadings than other simplified relationships.

EXPERIMENTAL

Reagents and materials

Synthetic *rac*-1,2-diacylglycerol 3-phosphorylcholine (PC) (product no. L-8878) and 1-acyl-*sn*-glycerol 3-phosphorylcholine (LPC) prepared by the action of phospholipase A_2 on egg yolk lecithin[14] were purchased from the Sigma (St. Louis, MO, U.S.A.). The latter material travelled as a single spot at the 200-μg level in silica gel thin-layer chromatography (TLC), whilst the former was found to be contaminated with about 0.5 mole-% of LPC, presumably as a result of a chromatographic method of purification[15–17]. As this contamination could be allowed for in calculations, this material was also used without further purification. Palmitic acid (PA) was purchased from Hopkins and Williams (Chadwell Heath, Great Britain). Propyl palmitate (PP) was prepared from the parent acid as follows.

Boron trifluoride–propanol complex was first prepared by repeated evaporation of boron trifluoride–methanol complex (BDH, Poole, Great Britain) with *n*-propanol (BDH, redistilled at 97.0–97.2°C) under reduced pressure. This process was carried out three times, the dry residues were taken up in propanol and palmitic acid added so that the propanol remained in excess. The reaction mixture was warmed for about 30 min at 70°C on a water-bath and the resulting mixture partitioned between water and light petroleum. This latter fraction was evaporated to dryness and the resulting pale yellow oil purified on a silica gel column eluted with light petroleum–diethyl ether (10:1). The absorbance of the eluate was monitored in a 1-cm flow-through cell at 1750 cm^{-1} with an SP 200G IR spectrophotometer, and the first absorbing frac-

tion was collected. The final product was a white waxy solid with a melting point of 25°C. At a loading of 100 μg it travelled as a single spot in silica gel TLC (eluent as above) distinctly from a methyl ester, similarly prepared, which had a lower R_F value. The IR spectrum was consistent with the propyl ester of a saturated fatty acid, showing no evidence of O–H stretching absorption.

Chromatography

For the graphs in Fig. 2 three dilution series were prepared as follows. A 99.2-mg amount of PC was dissolved in chloroform and made up to 10 cm^3, 6 cm^3 of this solution were diluted to 10 cm^3 and the procedure was repeated with the new solution until, in all, nine different concentrations had been prepared. Similarly, 27.9 mg of LPC were made up to 10 cm^3 in chloroform–light petroleum (1:1), 5 cm^3 of the solution were made up to 10 cm^3, and so on as above until six different solutions had been prepared. Propyl palmitate and palmitic acid were used as a 9:1 (w/w) mixture; 50 mg were dissolved in 100 cm^3 of light petroleum (b.p. 90–100°C) and aliquots of 5, 4, 3, 2, 1.5, 1, 0.7 and 0.5 cm^3 were placed in 10-cm^3 flasks, evaporated to dryness under reduced pressure and taken up in 0.5 cm^3 of light petroleum (b.p. 90–100°C).

For the data in Table II the procedure was slightly different. Six reference solutions were prepared, three at a high concentration of PC and a low concentration of LPC and three at a low concentration of PC and a high concentration of LPC. Either 2 cm^3 of an approximately 0.1% (w/v) solution of PC in *n*-propanol and 0.2 cm^3 of 0.05% LPC in the same solvent or 0.2 cm^3 of the PC solution and 2 cm^3 of the LPC solution were mixed, evaporated to dryness under reduced pressure and taken up in 0.5 cm^3 of chloroform–light petroleum–methanol (25:25:2).

In either instance, 20-μl aliquots of these solutions were spotted 2 cm from the bottom of pre-coated 20 × 20 cm silica gel TLC plates (either E. Merck, Darmstadt, G.F.R., or Supelco, Bellefonte, PA, U.S.A.) using a piece of apparatus, which although very simple is worth describing because of its convenience of use and the very even spot size and shape that result. The latter factors are known to reduce errors[18].

It was constructed by bending the last 3 mm of a 20-μl Microcap micropipette

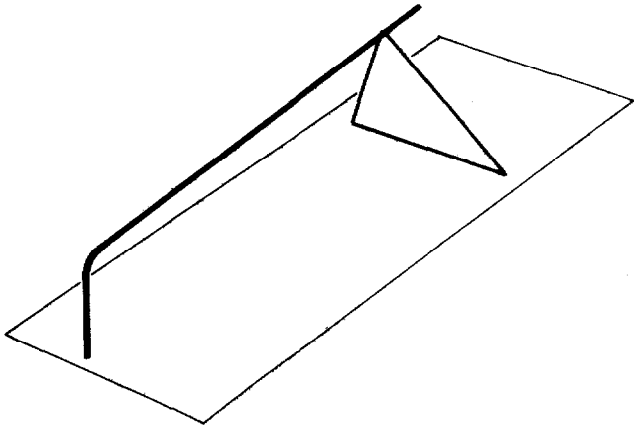

Fig. 1. Modified Microcap micropipette used for sample application. Perspective drawing with the supporting triangle of plastic at the rear.

at 90° to the remaining longer portion and then glueing the end of this longer part to the apex of a small triangle of plastic which acted as a support during spotting. This is shown as a perspective outline drawing in Fig. 1. The pipette could be filled completely by capillarity (which sometimes causes problems with the unmodified variety for solvents of low surface tension) and emptied gradually and completely when the end of the short portion was contacted gently against the adsorbent layer. The longer portion would be approximately horizontal at this stage. As the apparatus is extremely light, and stands on its own during spotting, there was no disturbance to the adsorbent layer on draining, as is common with other methods. Although the pipette no longer contains exactly 20 μl after bending, this does not cause problems since in quantitative work both reference and test solutions can be spotted with the same pipette. For this reason, the spot loadings referred to in this work have a relative, rather than absolute, significance, but are not expected to differ appreciably from the true values. The pipette was always rinsed twice with the solution to be spotted before applying a third aliquot to the thin layer.

After spotting, plates were dried in the cold air-stream from a fan-heater (Belling, 3 kW). Hot air has been found to cause irreversible binding of some lipids to the adsorbent[19]. The plates were eluted with the appropriate solvent system in rectangular glass tanks, lined with filter-paper, which had been equilibrated for at least 2 h. The eluent for PC and LPC was chloroform–methanol–water (65:25:5), for PA, light petroleum (b.p. 40–60°C)–diethyl ether–acetic acid (70:30:1) and for PP the same solvents as for the PA but in the proportions 90:10:1. When the solvent front had risen to the top of the plate, the plate was removed from the tank and dried in a current of hot air from the fan-heater. It was then sprayed until uniformly translucent with 20% ammonium sulphate solution, dried again with hot air, laid on a thick aluminium sheet (25 × 28 × 1.25 cm) and placed in a pre-heated oven. To develop PC and LPC spots the plate was heated for 1 h with an oven temperature of 210°C whilst PA and PP were developed for 30 min at 250°C. These temperatures and times were found to give the best contrast between spots and background after many different combinations had been tried.

After being withdrawn from the oven and allowed to cool, the plates were scanned with a Zeiss Mark 1 chromatogram spectrophotometer in the reflectance mode at a wavelength of 450 nm. This wavelength was found to give the best contrast between charred and uncharred areas. Two clean silica gel plates were placed underneath the test plate to simulate an infinite layer thickness[8]. The slit width was set at 0.2 mm whilst its length was adjusted so as to accommodate comfortably the largest spot of a set. The instrument was connected to a pen recorder to display changes in the reflected light intensity as peaks. The chart speed and scanning speed were selected so as to ensure a peak area that could easily be measured by planimetry whilst the response speed of the chart recorder was not exceeded. These settings were not altered during the scanning of a given set of spots.

RESULTS AND DISCUSSION

Preliminary results indicated strongly that scanning in the direction of elution was to be preferred. A comparison of seven 19.4-μg spots of PC on the same plate gave a coefficient of variation for peak area of 3.8% for scanning in the elution

direction and 10% in a perpendicular direction. It is possible that the somewhat elongated shape of the spots was significant here. A more surprising finding was that for thin glass chromatoplates (Merck) there was a distinct advantage in scanning from the reverse side (through the glass). Thus seven identical LPC spots on the same plate gave a coefficient of variation of 2% when scanned through the glass, but 4% from the other side. This effect did not occur with the Supelco plates, where the glass was about twice as thick (*ca.* 2 mm). The explanation was probably that the exposed side of the adsorbent layer tended to pick up specks of dust and smoke particles during the experiment. These discoloured the exposed face, in a random fashion, more than that facing the glass and would therefore have a greater effect on the relfectance when the plates were scanned from the front. In addition, the adsorbent surface on the glass side was considerably smoother. The effect was not observed for the thicker plates because the spectrophotometer became much less accurate when the measuring head was more than about 1 mm from the adsorbent layer. These procedures were therefore adopted.

Typical results for PC, LPC, PA and PP, are displayed in Fig. 2. Good linearity was observed over the range 3–200 μg per spot for PC, in particular, although the relationship was found to break down at higher and lower loadings. Non-linearity was also observed for LPC at loadings below 1 μg per spot. The greater error observed with this lipd can be ascribed to the low R_F value[11]. The other lipids were not examined outside the ranges displayed in the graph. The results of linear regression analyses of the results are given in Table I. Spot loading has been taken to be the dependent variable, as similar calibration lines were to be used for the determination of the concentration of solutions from measured peak areas[20].

The results show that the commonly used relationship in which the spot loading is taken to be proportional to the square of the peak area is clearly invalid under the conditions of the experiment for PC, LPC, and PA, as it would imply a slope equal to 2. Only the regression for PP showed a value for the slope that did not differ significantly from this value; for all except this lipid the relevant *t*-statistics are highly significant.

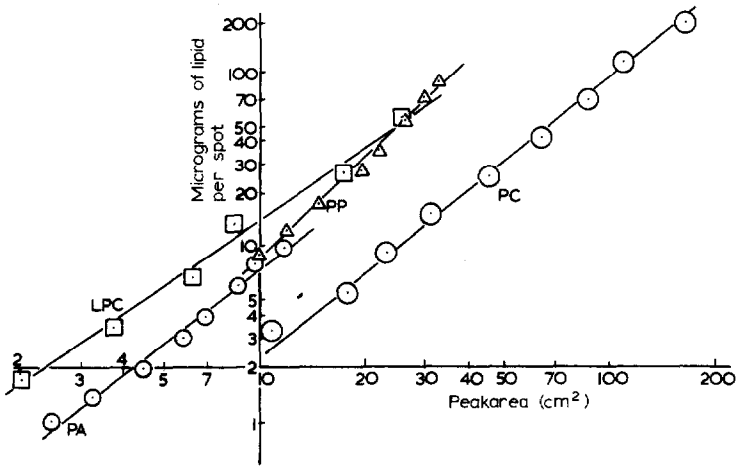

Fig. 2. Relationship between spot loading and peak area for different lipids.

TABLE I

REGRESSION OF ln (SPOT LOADING) ON ln (PEAK AREA)

The number of degrees of freedom may be inconsistent with the number of points on the graph, as different spots with the same loading are represented by a single mean value of peak area on the graph.

Parameter	*Lipid*			
	Lyso-phosphatidyl-choline	*Phosphatidyl-choline*	*Palmitic acid*	*Propyl palmitate*
Slope (slope 1)	1.369	1.620	1.539	1.913
Intercept	−0.475	−2.890	−1.528	−2.242
Correlation coefficient	0.991	0.997	0.993	0.996
Residual error in ln(spot loading)	0.172	0.106	0.108	0.085
Percentage error in predicted spot loading	17	11	11	9
Approximate R_F value	0.1	0.3	0.3	0.5
Standard error in slope	0.0579	0.0367	0.0759	0.0744
Degrees of Freedom	10	11	6	6
Two-tailed t-statistic for slope = 2	10.9	10.3	6.1	1.18

It may also be seen that the value of the slope tends to increase with the R_F value, with PP showing both highest R_F and the value of slope closest to 2. However, during these experiments, conditions such as spotting solvent, eluent and development were not kept constant and therefore, in order to investigate the tendency further, a large number of regression lines were examined where these parameters were not varied.

Chromatoplates that had been spotted with reference solutions of LPC and PC during kinetic investigations[20] were examined and the slope of the regression line (hereafter referred to as slope 1) and the distance from the base line to the final spot position (elution distance) were noted (Table II). The R_F values were approximately equal to elution distance divided by 18.

An analysis of the regression of slope 1 on elution distance is given in Table III. From the calculated two-tailed t-statistics it is immediately apparent that both the slopes of these regression lines (hereafter referred to as slope 2) and the intercepts are highly significant at the $P = 0.01$ level[21]. Whilst the values of slope 2 differ between the lipids ($t_{46} = 2.67$, which is very close to the $P = 0.01$ level of significance), the intercepts do not ($t_{46} = 0.78$). This latter observation implies that the values of slope 1 would be the same for zero elution distance.

However, although the two sets of results are consistent, with both the mean value of slope 1 between the lipids, and the value of slope 1 for each lipid individually, decreasing as elution distance increases, we see that this trend is at variance with the data in Table I. It thus appears that although elution distance is important in determining slope 1, it is not the only factor, and the conditions of spotting, elution and development are also highly significant. Nevertheless, these results certainly bear out the assertion that the simple square law is not usually adequate for describing the

TABLE II

SLOPE OF THE REGRESSION OF ln(SPOT LOADING) ON ln(PEAK AREA) COMPARED WITH ELUTION DISTANCE FOR PC AND LPC

Phosphatidylcholine (PC)		*Lysophosphatidylcholine (LPC)*	
Slope 1	*Elution distance (cm)*	*Slope 1*	*Elution distance (cm)*
1.4	2.2	1.6	4.5
1.5	1.8	1.7	4.4
1.9	2.1	1.5	4.0
2.0	1.5	1.7	3.8
1.2	1.5	1.7	4.2
1.5	2.1	1.6	5.0
1.4	1.4	1.0	4.5
1.7	1.6	1.1	4.8
1.4	1.6	1.4	4.5
1.7	1.5	1.4	4.2
1.2	1.7	1.5	4.7
1.3	1.5	1.9	4.2
1.6	1.9	1.5	5.2
1.8	1.4	1.6	4.7
1.8	1.4	1.5	4.2
1.8	1.3	1.6	4.3
2.4	1.2	1.6	3.8
2.3	1.1	1.7	3.6
2.2	1.2	1.7	4.1
2.3	1.1	1.8	2.8
2.0	1.4	1.6	4.5
2.1	1.1	1.7	4.0
2.2	0.9	1.9	2.9
2.0	1.0	1.8	3.4
2.3	0.7	1.8	2.8
Mean: 1.60	1.46	1.80	4.15

TABLE III

REGRESSION OF THE SLOPE OF THE CALIBRATION LINES FOR PHOSPHATIDYLCHOLINE AND LYSOPHOSPHATIDYLCHOLINE ON ELUTION DISTANCE

Parameter	*Phosphatidyl-choline*	*Lysophosphatidyl-choline*
Slope (slope 2)	−0.210	−0.655
Standard error in slope	0.063	0.15
Significance of slope t_{23}	3.35	4.24
Intercept	2.47	2.75
Standard error in intercept	0.26	0.23
Significance of intercept, t_{23}	9.38	11.9
Correlation coefficient	0.573	0.663

interdependence of peak area and spot loading. In general, a power law does seem to operate but the index is usually less than 2. Up to an elution distance of about 5 cm (corresponding to an R_F value of about 0.3) the value decreased in the current experiments, but for the more mobile PA and PP an increase was observed. Although the two groups are not directly comparable, as already mentioned, this difference between PA and PP does appear to be significant.

Finally, it is interesting to consider the difference between fitting a linear model:

$$\ln(\text{spot loading}) = a + b \ln(\text{peak area})$$

and a non-linear model:

$$\text{spot loading} = a(\text{peak area})^b$$

At first sight these forms may seem to be equivalent, but in fact they contain different implicit assumptions regarding error distributions (unless special precautions are taken to weight the observations appropriately). Usually, in the absence of evidence to the contrary, the error in the dependent variable is assumed to be constant whether one is considering the logarithm of an experimental parameter or the parameter itself. However, as far as the error in the experimental parameter is concerned, these two situations are very different, as a constant error in the logarithm of the parameter implies a constant *proportional* error in the parameter itself. This may be seen from the relationship

$$\Delta \ln x \approx \frac{\Delta x}{x}$$

where x is the experimental parameter.

In this work the logarithmic relationship, as well as being the easiest to use, as it utilizes the familiar technique of linear regression analysis, has also been found to give a better fit than the power law. When the power law was fitted to the data using a non-linear least-squares regression program written by the author[20], the correlation coefficient for phosphatidylcholine was 0.988 and for lysophosphatidylcholine 0.983. These values are considerably lower than those reported in Table I for the logarithmic relationship, although the data were the same. This strongly suggests that the error of the method increases with the spot loading.

It therefore appears that the logarithmic relationship is more satisfactory than a simple square law for the quantitation of material by *in situ* reflectance densitometry under the conditions described in this paper, while retaining the advantage of simplicity.

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