The quantitative determination of phospholipids by direct photodensitometry of thin-layer chromatograms

The quantitative determination of phospholipids separated by thin-layer chromatography (TLC) is based on determination of phosphorus in the isolated fractions, after elution and mineralization[1–3], or after a direct mineralization in the presence of the adsorbent[4–7]. All these recovery methods include removal and transference of the corresponding zones of the adsorbent to test tubes for further analysis. This is a long and complicated procedure, during which there is a risk of losing some of the substance[6]. We came to this conclusion during the investigation of phospholipids in different regions of the brain[8]; the main difficulty in these experiments was the time taken for quantitation of many fractions in a number of tissue samples. On the other hand, the methods of direct spot analysis, by photometry of the chromatoplates, which offer many advantages in such investigations, are not in general use.

PRIVETT *et al.*[9,10] developed a method for the quantitative analysis of lipids, based on the densitometry of the spots produced by charring the fractions after spraying the plates with 50% aqueous H_2SO_4 and heating. The method has been applied to lipid analysis in animal[11] and vegetable[12] tissues. Under these charring conditions, equal quantities of different lipids give spots of unequal density value and the areas under the densitometric peaks vary, so that the area under the peak expressed as a percentage of the total area registered does not represent exactly the content of the corresponding fraction in a lipid mixture[11]. Although BLANK *et al.*[13] later considerably resolved this problem by changing the charring conditions, the analysis of polar lipids is not as satisfactory as that of the neutral lipids. Errors occur particularly when minor phospholipid fractions have to be determined in the proximity of a number of nonphospholipid fractions, as is often the case in TLC of lipid extracts from animal tissues.

It seemed that in the quantitative evaluation of phospholipids photometry of the spots depending on the phosphorus content would be a convenient way of avoiding the difficulty mentioned above. An attempt has been made to adapt a well known colorimetric reaction, *viz.* the reduction of phosphomolybdic acid for a photodensitometric determination on the chromatoplate.

Experimental

The standard mixture of phospholipids. Phosphatidylethanolamine (isolated from mouse brain, purified by TLC), phosphatidylcholine and sphingomyelin (Koch–Light Laboratories, Colnbrook, England) were dissolved in chloroform–methanol, 1:1, v/v (1 ml = 60 μg P) and stored at —15°.

Thin-layer chromatography. The glass plates (3.5 × 20 cm) were coated with a layer (600 μ) of Silica Gel G (E. Merck). The density of the chromatoplates was controlled photometrically: only the plates having a uniform density were used. The plates were activated at 110° for 30 min immediately before use.

The phospholipids (50 μl of standard mixture, equivalent to 3 μg P) were applied 2.5 cm from the edge of the plate.

The plates were developed with chloroform–methanol–water (70:30:5, v/v)[14], in jars (2 l) previously saturated with the vapors of the solvent system, until the solvent front had advanced 15 cm from the starting line.

After briefly drying with a hot-air blower, the spots were made visible by exposure to iodine vapor and delimited by two channels scratched in the layer (15 mm apart) parallel to the length of the plate. The chromatoplates were finally left 30 min in an oven at 110° for complete drying.

Mineralization and chromogenic reaction. The plates were sprayed lightly with the following mixture: 1 ml 5% ammonium molybdate, 3 ml concentrated HNO_3, 16 ml 65 % perchloric acid. With an atomizer, which gives a suitable spray (Pulverisateur Vaast, Unis, France) and at a distance of 30 cm, about 10 ml of the reagent were required for one plate. After spraying, the chromatoplates were covered with glass plates and heated for 1 h in an oven at a temperature rising from 120° to 160° during the first 30 min. During the last 10 min the plates were uncovered to remove a part of the acid.

After cooling, the plates were sprayed with a freshly prepared reagent of the following composition: 2 ml 5 % ammonium molybdate, 3 ml 65 % perchloric acid, 5 ml 1 % ascorbic acid (ascorbic acid is added last). Under the conditions mentioned above, about 15 ml of the reagent were required to obtain a "just wet" state of the layer. As in the case of the first reagent, even spraying and the volume of the reagent applied to the layer have great influence on the chromogenic reaction and the appearance of the color spots. The chromatoplates were covered with the glass plates and kept for 1 h at 50°. Blue spots on a slightly pale blue background were formed.

Photodensitometry. Before measurement, the adsorbent was made translucent by spraying with paraffin–ether (1:1, v/v)[15]. The Jouan photometer (Jouan, Paris), used in paper electrophoresis, was used without modification, except that the chromatoplate was covered with a metal plate (3.5 × 20 cm) which has a slit (1 × 15 cm). This plate is placed on the layer so that the spots are framed by the slit.

The instrument was adjusted to 100 % transmission when the light beam passes through the adsorbent only. The areas under the densitometric curves were determined with a planimeter.

Results and discussion

The results obtained by three separate determinations of the same standard mixture of phospholipids are given in Table I.

The percentage composition, calculated from the ratio between the areas under the peaks and the total, agree well with the known composition expressed as percentage phosphorus, confirming that the size and intensity of the spots are proportional

TABLE I

ANALYSIS OF A STANDARD MIXTURE OF THREE REFERENCE PHOSPHOLIPIDS BY A DIRECT TLC–PHOTODENSITOMETRY METHOD

Compound	*Percentage composition*				
	*Determination**				*Theoretical***
	1	*2*	*3*	*Average*	
Phosphatidylethanolamine	51.6	52.8	53.6	52.7	51.8
Phosphatidylcholine	30.6	31.4	30.3	30.8	31.0
Sphingomyelin	17.8	15.7	16.1	16.5	17.2

* Values given represent area under peak as percentage of total area.
** Values given represent P as percentage of total P.

to the amount of phosphorus in the phospholipid fractions. Previous investigations[16] have shown that the sensitivity and range of the method permit the analysis of phospholipids in tissue extracts.

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The separation of collagen molecular subunits by gradient elution chromatography

PIEZ and co-workers[1–3] have developed a simple linear gradient technique using acetate buffers for the elution of the α-, β- and γ-subunits of heat-denatured collagen or parent gelatin from CM-cellulose. Experiments have shown that their method does not give a complete picture of the subunit structure, particularly with acid-soluble collagen. In many cases incomplete resolution has also been reported (see for example TRISTRAM, WORRALL AND STEER[4]). Further, the column effluent from the chromatographic separation is monitored either by chemical analysis or by optical absorption in the region of 230 mμ. In the latter case the use of acetate buffers can cause difficulties, as these have a high absorption at this wavelength, and any small changes in acetate concentration can effect the stability of the baseline. The separation and properties of these subunits are most important in determining collagen structure, therefore, the gradient elution system reported here could have important applications.