The Use of Iodine Staining for the Quantitative Analysis of Lipids Separated by Thin Layer Chromatography

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The method described for quantitative estimation of lipids separated on thin layer chromatography plates exploits the observation that most lipids can be stained by iodine vapor and that, in "controlled" conditions, the intensity of this staining is proportional to the actual amount of lipid in the spot. The method consists of i) exposing the developed plate to iodine vapor; ii) spraying it with a suitable solvent to prevent halogen evaporation; iii) collecting the stained lipids by scraping the spots off the plate; and iv) determining by a rate-sensing method the absorbed iodine. The final determination is performed by measuring spectrophotometrically (at 410 nm) the rate of decolorization of a solution of Ce(IV) by As(III) in strong acidic conditions. The reaction rate, which is positively related to the concentration of iodine, is derived from the slope of the absorbance change plotted vs time.

Providing that standards and samples are stained simultaneously, a quantitative estimation of lipid components of a mixture is possible in a reasonable time with excellent accuracy and reproducibility. In our hands, the method has been successfully applied to several common phospholipids, long chain fatty acids, cholesterol and deoxycholate, and triacylglycerols, in the range of 5–60 μg.

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Quantitation of lipid spots on thin layer chromatography (TLC) plates involves two steps: visualization of lipids and quantitative (chemical or densitometric) analysis.

One of the most widely used visualization procedures is the exposure of the developed plate to iodine vapor (1). Due to the hydrophobic character of both the halogen and lipids, samples adsorb iodine and readily appear on plates as brownish spots.

Early observations and our present experience (1,2) indicated that molecules as phospholipids, long chain fatty acids, triacylglycerols and cholesterol derivatives adsorb iodine proportionally to the amount of lipid material (up to 60 μg) on the TLC plate. This is true, however, only if iodine evaporation from the plate is immediately blocked and the exposure of plates is not exceedingly long. Under these conditions, a staining procedure widely used for qualitative purposes may become useful also for quantitative determinations.

It must be pointed out that the method described here allows a significant time savings, since it requires less than 30 min of effective work and uses common laboratory equipment and reagents.

This method has not been tailored for routine chromatographic measurements or for very specialized laboratories devoted to lipid chemistry. Rather, this procedure is proposed for investigators who might have occasional need of a rapid and suitable quantitative analysis of lipids.

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MATERIALS AND METHODS

Egg yolk sphingomyelin (containing primarily palmitic acid), cardiolipin (pig heart), phosphatidylinositol (containing primarily linoleic and palmitic acids), dipalmitoylphosphatidylserine, linoleic, oleic and elaidic acids, cholesterol and sodium deoxycholate were purchased from Sigma (St. Louis, Missouri); triolein (glycerol trioleate) was from BDH (Deventer, The Netherlands). All these substances were used without further purification and were solubilized or diluted in chloroform or chloroform/methanol (2:2, v/v) to give final concentrations (gravimetrically assessed) of 2.5 mg/ml.

Precoated silica gel 60 TLC plates with or without fluorescent indicator (0.2 mm thick) were obtained from Merck (Darmstadt, Federal Republic of Germany). The spotting solvent was chloroform or chloroform/methanol (2:1, v/v).

Equipment consisted of an Eppendorf microfuge, a chromatographic tank (22 × 10 × 22 cm) and a Beckman UV 5230 spectrophotometer with a built-in strip-chart recorder. Readings were taken at 410 nm. Normally, full scale was set at O.D. 2 and the chart speed at 2 inches/min.

For the arsenious acid reagent, sodium arsenite (Baker, Poole, England) was dissolved (with heating and stirring) in 0.15 M sulfuric acid to give a final concentration of 0.01 M. After cooling, the solution was divided into small aliquots and stored in the refrigerator. These solutions are stable for long periods. Before analysis the arsenite reagent was diluted 20-fold with 0.15 M sulfuric acid; this solution is hereafter referred to as solution A.

For the ceric-ammonium sulfate reagent, ceric sulfate and ammonium sulfate (Baker) were dissolved (with heating and stirring) together in 2.8 M sulfuric acid to give final concentrations of 0.05 and 0.15 M, respectively. The solution was centrifuged and the clear deep yellow supernatant decanted and divided into small aliquots. These solutions (referred to as solution B) are stable for several months at room temperature.

Commercial TLC plates are available with or without fluorescent indicators and may contain several other substances as binders or salts. Because it cannot be assumed that all these substances do not affect the reactions described below, plates, different in type and source, should not be considered necessarily interchangeable.

Preliminary experiments should be performed to assess the possibility of using the various types of commercially available plates. Most of our studies were performed on Merck Silica gel 60 plates (20 × 20 cm) with or without fluorescent indicator. However, a few experiments carried out with similar plates from different commercial sources gave essentially the same results.

Lipids were dissolved in suitable solvents (chloroform or chloroform/methanol). Amounts ranging from 5 to 60 μg were spotted on TLC plates with the aid of a small glass syringe with a Teflon tip fitted with a micrometric device (Radiometer SBU1A). Any device for precise and reproducible delivery of solutions on plates may be used.

Iodine crystals were placed in a round plastic dessiccator and allowed to vaporize at room temperature until the jar was saturated with iodine vapor. The plate was quickly put in the dessiccator horizontally with its four corners based on the inner round edge of the container. The gel side was toward the transparent cover of the jar to allow a rapid inspection of the staining.

Several durations of exposure of the plates to iodine vapors were tried, but 90 sec was found to be satisfactory for most of the tested substances. Some lipids (e.g. phosphatidylinositol, phosphatidylethanolamine), however, required a longer exposure (120 sec), especially at minimal concentrations of lipids. The lipids appear as brown spots on a white background (after longer exposure the background tends to become yellowish).

Densitometric measurements were performed using a Shimadzu CS 920 densitometer.

The principle of the assay method has been discussed extensively in a previous paper (3). In brief, the assay is based on the ability of iodine to act as catalyst in the ceric arsenite reaction (4). Since the formation of the reaction product (CeIII) results in a dramatic decrease in the absorption of the solution at 410 nm, the kinetics of this process may be easily measured by monitoring the rate of decolorization. The change in absorption at 410 nm follows zero order kinetics and may be conveniently monitored spectrophotometrically (the use of a strip-chart recorder is advisable). The initial velocity (initial reaction rate) is proportional to iodine concentration, which is, in turn, proportional to the amount of lipid in the spot. The lipid concentration in an unknown sample may be easily estimated by comparison with a standard curve prepared simultaneously with the same material. In a typical assay, a mixture of lipids (the amount of each component should not exceed 60 μg) is spotted on the left corner (ca. 2 cm from the bottom) of a chromatographic plate and developed as needed. At the end of the run, the plate is removed from the tank and let to dry at room temperature. With the aid of a micrometric device, scalar volumes of standards are regularly placed on the large area of the plate that has not been used for the chromatographic separation. After drying, the plate is moved into a jar containing iodine crystals and stained by exposure (normally 90 sec) to iodine vapor (Fig. 1). The plate is removed and immediately sprayed with glacial acetic acid. All the round brown areas are marked with a glass tip, carefully scraped off the plate and transferred into plastic Eppendorf vials (1.5 ml). After addition of 1 ml of 1.8 M sulfuric acid, the samples are vigorously shaken on a vortex (1-2 min) and, finally, centrifuged on a microfuge (5 min). Blanks consisting of background areas (having approximately the size of the broader sample spot) are similarly treated. The supernatants (0.8 ml) are collected in small capped tubes, where they can be stored for 48 hr or more. In fact, iodine determinations performed at various times on the same samples gave essentially similar results after a few minutes to up to 60 hr (Fig. 2). To perform the final iodine quantitation, the samples (e.g., 50 μl) are diluted fivefold with 1 M sulfuric acid. This dilution diminishes the chances of accidental transfer of silica gel particles in the optical cuvette, thus preventing possible artifactual interferences. Furthermore, if the lipid amount in the sample is rather small, the dilution step may be skipped or proportionally reduced. Two-hundred-and-fifty μl of this diluted solution is transferred directly to a cuvette (plastic disposable cuvettes are convenient to use).

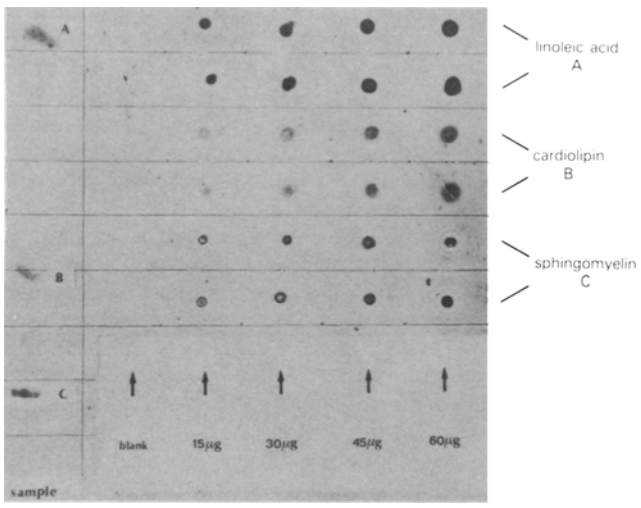

FIG. 1. An example of quantitative assay of a simple lipid mixture. Left side: linoleic acid, 25 μg (A, top), pig heart cardiolipin, 25 μg (B, middle) and egg yolk sphingomyelin (containing primarily palmitic acid), 30 μg (C, bottom), after their chromatographic separation on a silica gel 60 plate (chloroform/methanol/water [67:25:3, v/v/v]). Right side: Scalar amounts of each of the above substances. Arrows indicate the relative quantities. Standards have been loaded on the gel immediately after the sample separation and before the exposure to iodine vapor. By this procedure, staining of samples and standards is accomplished simultaneously under identical conditions.

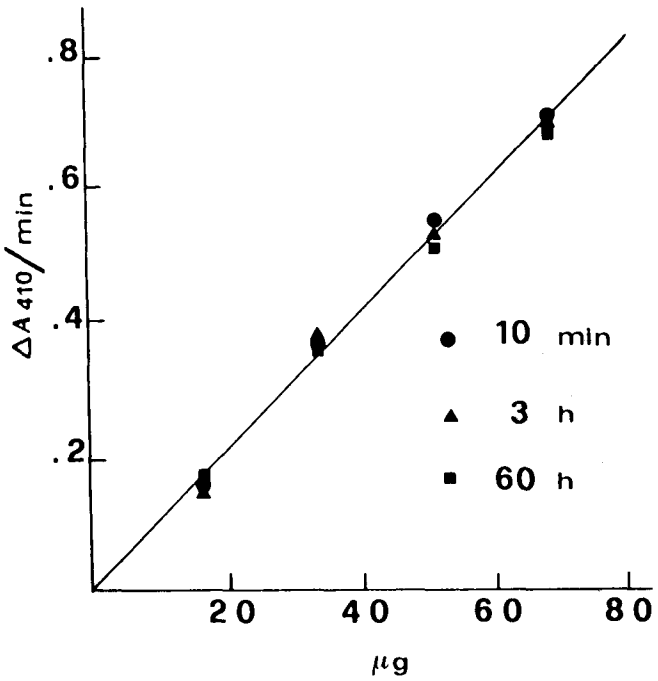

FIG. 2. Acetic acid prevents evaporation of iodine from stained plates. Quantitative determinations of dipalmitoyllecithin performed at various intervals of time (10 min–60 hr) after spraying the iodine-stained plate. Essentially the same reaction rate is observed in all determinations.

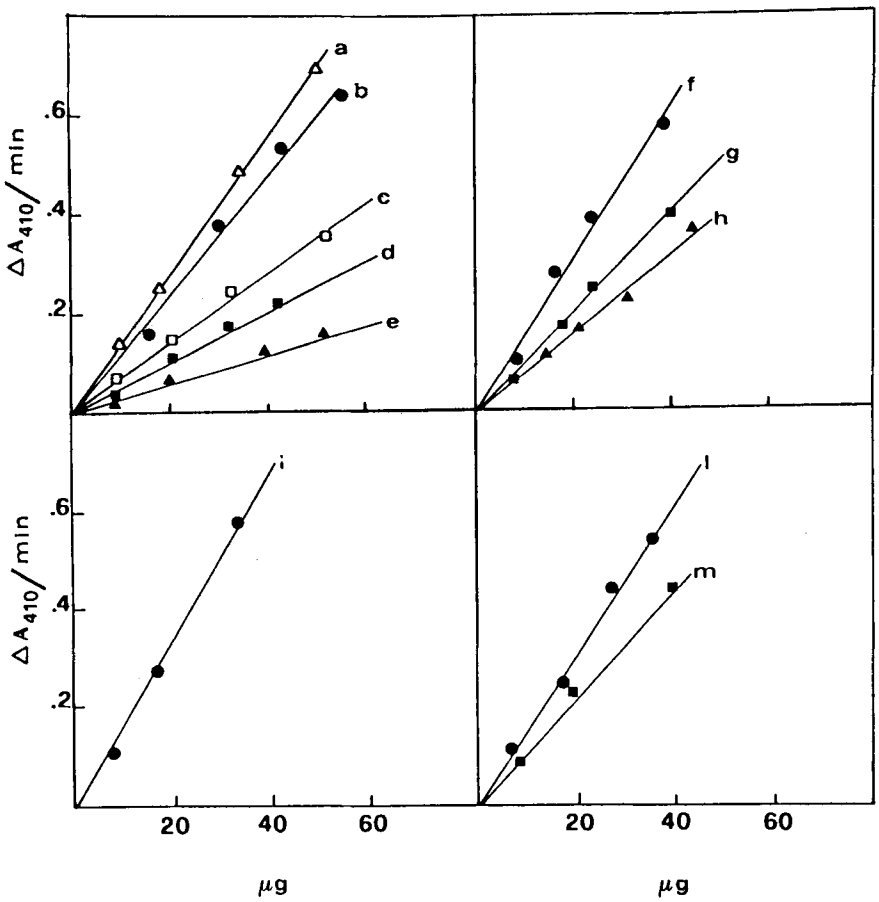

FIG. 3. Standard curves (in the range 5–60 μg) obtained with various lipids: a, egg yolk sphingomyelin (containing primarily palmitic acid); b, cardiolipin (from pig heart); c, phosphatidylinositol (containing primarily linoleic and palmitic acids); d, dipalmitoyllecithin; e, dipalmitoylphosphatidylserine; f, linoleic acid; g, oleic acid; h, elaidic acid; i, triolein (glycerol trioleate); l, sodium deoxycholate; m, cholesterol.

Six-hundred μl of solution A (arsenite) and 60 μl of solution B (ceric-ammonium sulfate) are then sequentially poured in the same cuvette. After rapid mixing by inversion, the cuvette is placed in the spectrophotometer, set at 410 nm for reading. The calibration curve is obtained by plotting the initial rate (i.e., the slope of the change in absorbance vs time) expressed as ΔA/min vs the lipid (iodine) content of standards. The actual amount of lipid in a mixture is readily obtained by reading the values off this curve, the amount corresponding to the measured rate (ΔA/min).

Figure 3 shows several standard curves obtained using different types of lipid molecules. It seems clear that the response is correlated to lipid saturation (linoleic acid > oleic acid > elaidic acid). Specific details are given in the figure legend.

Precision of the assay was evaluated as between-day or day-to-day precision using lipid extracts from three amniotic fluids containing increasing amounts of lecithin (palmitoyl lecithin was used as standard) for a period of 15 days (Table 1). The procedure for lipid extraction and separation was that of Gluck et al. (5).

TABLE 1

Precision of Assay

	μg of lecithin		
Sample	1	2	3
Precision between day	18.0 ± 1.2	31.1 ± 1.8	46.2 ± 1.7
Precision day to day	17.2 ± 1.4	31.5 ± 2.0	46.9 ± 2.4

Precision has been assessed over a period of 15 days. Samples 1, 2 and 3 are three amniotic fluids containing three different levels of lecithin. The extraction of lecithin and its separation has been performed according to Gluck et al. (5).

The same three amniotic fluids used for determining precision were used for accuracy. It was assessed by comparing our procedure with a well accepted method (6). In this case, the ratio of lecithin to sphingomyelin (μg/μg) was densitometrically measured according to the original

TABLE 2

Accuracy

Sample	1	2	3
R_1 (μg/μg)	1.2	2.3	2.8
R_2 (μg/μg)	1.4	2.5	2.7

R_1 indicates the ratio of lecithin to sphingomyelin in three amniotic fluids determined by means of our method; R_2 is the same ratio densitometrically determined according to Gluck et al. (6).

paper (5) and compared with the ratio obtained by making use of our method (Table 2).

DISCUSSION

A variety of methods for the quantitative estimation of lipids separated by TLC are today available. The lipids are usually visualized and subsequently quantitated by either charring or staining, followed by either direct densitometric (or planimetric) measurement or by scraping the spots off the plate for chemical wet analysis.

Charring simply involves spraying the TLC plate with sulfuric acid or with sulfuric acid containing a strong oxidizing agent, followed by heating at high temperature for various times. Although this method appears to be generally suitable, nevertheless it is known that staining by charring is sensitive to lipid saturation, is influenced by the silica gel and is dependent on the time and temperature of heating. Moreover, when present, the oxidizing agent may affect the result because of the possible loss of carbon by conversion to CO_2.

Dye staining, a nondestructive method, usually allows recovery of material from the plates, so that scraped spots may be used for further analysis. Different types of staining have been proposed over the years. Some have general application (2,7-10), while others make use of more specific reagents and are used when the lipid molecules contain phosphorus, choline, sialic acid or other groups of particular reactivity (11-16). Even in these instances, however, there are frequent problems with background discoloration or fading of spot colors, especially when the spots have to be read by densitometry or directly evaluated by planimetry.

Various other problems have been described after spraying plates with ammonium sulfate, bismuth subnitrates (6,17), etc.

Another widely applied method of staining lipid molecules on TLC chromatographic plates is the exposure of the plate to iodine vapor in a sealed tank. This method is extremely simple, sufficiently sensitive and almost universally applicable. Also in this case, however, staining is dependent on the nature of the lipid molecule (i.e., some molecules stain more than others) and is dependent on the time of exposure to the halogen vapor. Furthermore, once the plate is removed from the container, the brown spots begin to fade within a few minutes and more or less rapidly tend to disappear.

The method presented in this paper makes use of iodine staining by introducing a simple procedure to hinder the evaporation of the halogen. Under controlled conditions and within a relatively wide range of concentrations, it appears that the amount of trapped iodine is linearly related to the amount of adsorbing lipid. The subsequent estimation of iodine is performed by exploiting a rate-sensing method developed in our laboratory a few years ago (3): by this technique we have successfully applied the procedure to various lipid molecules, by making use of appropriate calibration curves, prepared from standards placed on the same plate where the chromatographic separation has taken place.

The method has proved to be largely reliable and reproducible with several test substances, providing that evaporation of iodine from the plates is effectively hindered. After several attempts, we have found that uniform spraying with acetic acid, a solvent in which iodine is highly soluble, is the most effective treatment for this purpose.

The observation that various lipid molecules, even in the same quantity, stain quite differently, does not represent a serious problem. To circumvent this obstacle, in fact, it is possible to reduce up to fivefold the preliminary dilution (see Methods) of the sample before the final spectrophotometric measurement. The assay is performed in a reasonable time, is not particularly cumbersome, does not require special attention or skills and, most important, is highly reproducible. Another important advantage comes from the fact that the assay is a rate-sensing method: the superior accuracy of true kinetic methods over others is generally recognized and, in regard to iodine determination, has been extensively discussed in a previous paper (3).

It must be emphasized that since the response (the change in the absorption of cerium) is correlated with the lipid saturation (see standard curves of Fig. 3), the quantitation of unknown samples is impossible unless the same material is available for calibration curves. However, the linearity of the response observed with synthetic and natural mixtures of lipids indicates the possibility of using such mixtures as standards of a total lipid in tissue or biological fluid extracts, providing that the approximate lipid composition is known.

Bearing in mind such restrictions, the present method may be conveniently used when the occasional need of lipid analysis does not justify the acquisition of dedicated optical devices, such as densitometers, and when turnaround time may be critical for the immediate prosecution of the research work.

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