A rapid chemical method for quantification of lipids separated by thin-layer chromatography

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SUMMARY Lipids, separated by thin-layer chromatography, are oxidized in an acid dichromate solution. The reduction in absorbance at 350 mμ is proportional to the amount of lipid. One reagent suffices to quantify all lipid classes to a lower limit of 15 μg of lipid.

Although a wide variety of techniques for separating small amounts of lipids by thin-layer chromatography (TLC) have been reported, most of the chemical methods proposed to determine the small amounts of isolated lipids are modifications of methods that have relatively specific reactions with particular lipid compounds (1–4). Such specific chemical reactions are unnecessary after the lipid compounds have been separated by chromatography. A relatively simple, general chemical reaction for the quantification of all lipid classes has been proposed by Bragdon (5), based upon the ability of lipids to reduce an acid dichromate solution. Since the amount of dichromate that is reduced varies with different lipid classes, corrections must be applied. Because the resulting solution of potassium chromium sulfate has a relatively low absorbance at 580 mμ, this colorimetric method has the disadvantage of being relatively insensitive for small amounts of lipids.

The rapid separation of lipid classes by TLC neatly resolves the problems arising from the lack of specificity in the dichromate method; indeed, when the lipids are separated, this lack of specificity becomes an advantage in that all the lipids may be determined with a single reagent. Since silica gel does not react with the concentrated sulfuric acid–dichromate solution, extraction of the lipid from the silica gel is obviated. The disadvantage of the relative insensitivity of the method is overcome by determining the amount of oxidized form ($Cr_2O_7^{=}$) remaining, rather than the amount of reduced form (Cr^{3+}) produced (6). The sensitivity is increased by a factor of about 40.

For the acid dichromate reagent, 2.5 g of $K_2Cr_2O_7$ is dissolved in 1 liter 36 N H_2SO_4. This concentration allows for adequate reduction by small amounts of lipid (0.5 ml of reagent) while providing sufficient dichromate in a manageable volume (6–9 ml of reagent) to oxidize larger amounts of lipid. Lipids are separated on silica gel by standard techniques (7). Wide variations have been found in the amounts of reducing impurities in various brands of silica gel; recent lots of Anasil[1] and Silica Gel G[2] have been found to be relatively free of these impurities. Solvents used are petroleum ether–ethyl ether–acetic acid; they should be tested for the presence after evaporation of any significant amounts of dichromate-reducing impurities. Redistillation is necessary if significant residue remains. The following lipid standards were used in this study: cholesterol, cholesteryl stearate, tripalmitin, palmitic acid, and lecithin.[3]

[1] Analytical Engineering Laboratories, Inc., Hamden, Conn.
[2] Research Specialities Co., Richmond, Calif.
[3] Nutritional Biochemical Corporation, Cleveland, Ohio.

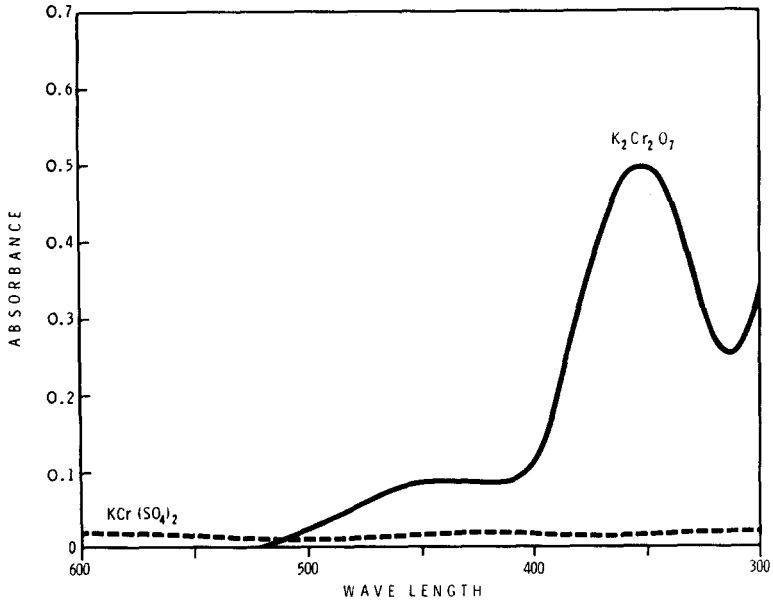

FIG. 1. Spectra of equimolar solutions of $K_2Cr_2O_7$ and $KCr(SO_4)_2$. Spectra of 0.17×10^{-3} M acid solutions were recorded in a Spectronic 505 Ratio Recording spectrophotometer with a 1.0 cm light path.

Extracts containing mixtures of lipids (8) are applied to a 20 cm silica gel plate that has been divided into a number of lanes (2–3 cm lanes have been found to be satisfactory). To each of the last two lanes a solution containing known amounts of standard lipids is applied. This solution containing the known lipids should be made up to approximate the quantities of lipids in the unknowns being chromatographed.

After development, the plate is air-dried for 10 min. The plate is then covered with a sheet of aluminum foil so that only one of the standard lanes remains exposed. Exposure to iodine vapor for about 5 min reveals the positions of the separated lipids. Corresponding areas in the unexposed lanes are outlined and the plates are heated at 90–100° for 30 min to evaporate traces of solvent. The silica gel in the outlined areas is transferred to small (5–10 ml) stoppered test tubes, to which the dichromate reagent has been added. The volume of dichromate reagent used will vary with the range of lipid to be determined: 1 ml for 20–125 μg lipid, 3 ml for 60–400 μg lipid, 6–9 ml for larger amounts. A silica gel blank containing an area of silica gel equal to those bearing lipid is also placed in dichromate. The stoppered tubes are shaken so that the silica gel forms a fine suspension and placed in a water bath at 100° for 45 min. The tubes are shaken two or three times during the heating period. The tubes are cooled and centrifuged for 15 min at high speed. A 0.5 ml aliquot from each clear supernatant solution is pipetted into colorimetric cuvettes, and 20 ml of water is added; the contents of the tubes are thoroughly mixed, and absorbance is determined at 350 mμ against a zero setting of equimolar $KCr(SO_4)_2$ (5). Since a totally reduced dichromate solution, i.e., an equimolar solution of $KCr(SO_4)_2$, will give a reading of 0.01–0.02 against a water blank, water can be used to set the zero absorbance with very little error. It is essential that the same volume of reagent be used for the silica gel blank and the unknown.

The calculation of the unknown is routine:

$$\frac{\text{Absorbance of silica gel blank} - \text{absorbance of unknown}}{\text{Absorbance of silica gel blank} - \text{absorbance of standard}} = \frac{\mu\text{g lipid in unknown}}{\mu\text{g lipid in standard}}$$

In a preliminary study the absorbance of equimolar amounts of potassium dichromate and potassium chromium sulfate in acid were scanned in a Spectronic 505 Ratio Recording spectrophotometer (Fig. 1). The absorbance of an equimolar amount of potassium dichromate at 350 mμ is approximately 40–50 times the absorbance of a similar amount of potassium chromium sulfate at 580–600 mμ. Standard solutions of dichromate were prepared by dilution of the dichromate reagent with water to the range of 8–60 μg/ml. The absorbance of these solutions, plotted against dichromate concentration, was found to be linear over this range as determined in a Beckman B spectrophotometer with standard 19 x 105 mm round cuvettes.

Aliquots of standard solutions of tripalmitin, cholesterol, cholesteryl stearate, lecithin, and palmitic acid, ranging in content from 50 to 300 μg of each lipid were developed

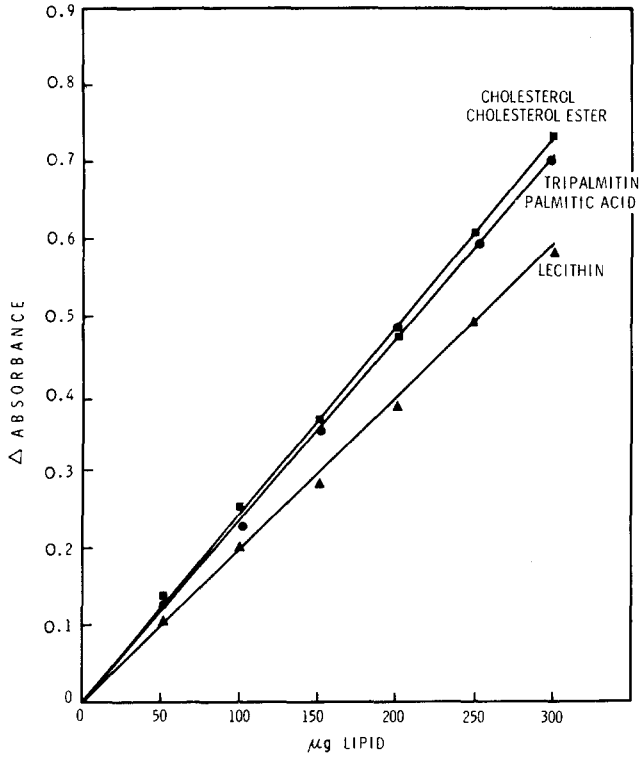

FIG. 2. Acid dichromate reduced by lipid standards. Lipid standards developed on silica gel plates with petroleum ether–ethyl ether–acetic acid 90 : 10 : 1 (v/v/v), reacted with 2 ml of dichromate reagent as described. Change in absorbance of $Cr_2O_7^=$ plotted against amount of lipid.

on silica gel plates and determined as described. Reduction of dichromate (Fig. 2) was found to be linearly proportional to the amount of lipid present over this range. The amount of dichromate reduced by lecithin was less than that reduced by the other lipids and may be partly due to the greater degree of unsaturation of this standard. A standard of linoleic acid, however, gave results similar to those obtained with stearic acid. Although not used in this study, mixtures of naturally occurring lipids of the various classes (quantified by more specific procedures) might in some cases be better standards. Oxidation of nonchromatographed standards gave almost identical results, indicating that most of this departure from the theoretical total oxidation (5) was due to the reagent rather than the presence of silica gel or losses in chromatography.

To determine the optimum time that the tubes should be heated in the boiling-water bath, lipid standards were tested with the dichromate reagent for varying periods of time. Although most of the reduction of dichromate occurred within the first 15 min, a 45 min period of heating was found to give more reproducible results. This was apparently due to the fact that occasionally the silica gel did not completely fragment in the dichromate reagent, and a longer heating interval was necessary to oxidize the lipid.

The reproducibility of the method was checked by determining the lipids present in the liver of a hydrazine-treated rat. The lipid was extracted from approx 100 mg of liver tissue, and the lipids in six aliquots were separated on silica gel plates and quantified as described. The standard deviations varied between ±3 and ±6%. For purposes of comparison, a second series was separated as described but placed in iodine vapor prior to quantification. Although heating the plate in a drying oven greatly reduced the amount of residual iodine there was still a 5–15% increase in the amount of dichromate reduced, as compared with plates not exposed to iodine. This increment was also manifested in the chromatographed standards, however, so that the final calculated results were similar to the values obtained with plates not exposed to iodine.

The amount of lipid that can be determined with this method is quite flexible and depends only on the volume of dichromate reacted with the lipid-containing silica gel. Volumes of dichromate from 0.5 ml up to the capacity of the reaction tube can be used. If reduction of $Cr_2O_7^=$ proceeds to the point that the contents are colorless, the heating in the water bath should be repeated with an additional volume of dichromate reagent. Although the theoretical lower limit of this method is in the range of 1 μg of lipid, it has been found that the limiting factor is not the sensitivity of the reagent but rather the small amounts of dichromate-reducing impurities in the silica gel and in the solvents. By using 0.5 ml of dichromate reagent, the lower limit of this method is approximately 15 μg of lipid. The range as given in the method approximates the range of most spot-locating reagents and has been found adequate for studies on the lipids in 0.5 ml of rat serum or in 50 mg of tissue slices.

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REFERENCES

1. Zöllner, N., and G. Wolfram. *Klin. Wochschr.* **40:** 1098, 1962.
2. Zöllner, N., G. Wolfram, and G. Amin. *Klin. Wochschr.* **40:** 273, 1962.
3. Vioque, E., and R. T. Holman. *J. Am. Oil Chemists' Soc.* **39:** 63, 1962.
4. Doizaki, W. M., and L. Zieve. *Proc. Soc. Exptl. Biol. Med.* **113:** 91, 1963.
5. Bragdon, J. H. *J. Biol. Chem.* **190:** 513, 1951.
6. Rosen, H. *Ann. N. Y. Acad. Sci.* **92:** 414, 1961.
7. Vogel, W. C., W. M. Doizaki, and L. Zieve. *J. Lipid Res.* **3:** 138, 1962.
8. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **226:** 497, 1957.