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A simple method for determination of phosphate from thin-layer chromatographic plates

In assays of phospholipids from thin-layer chromatographic (TLC) plates, the number of samples that have to be handled is often great. For this reason, semi-automatic and automatic assay procedures have been introduced[1,2]. Several conventional methods have also been published in which the phosphate assay is carried out on the TLC scrapings without previous elution of the spots[3–8]. It has been customary to add the two basic reagents, ammonium molybdate and the reducing agent, to the reaction mixture separately. In our method, the reagents are premixed to make the method more rapid and flexible.

Reagents

(A) 10 *N* H_2SO_4; (B) 30 % H_2O_2; and (C) 1 part of 5 % ammonium molybdate, 1 part of Fiske–Subbarow reagent, prepared as by BARTLETT[9] and 10 parts of distilled water, mixed just before the initation of the reaction; reagent C cannot be stored.

Procedure

The TLC spots are scraped into the centrifuge tubes and 0.5–2.0 ml of A is added, depending on the size of the spot and the estimated phosphate content. The tubes are heated for 2 h at 180°, then cooled, and two drops of B are added. The samples are again heated for 2 h at 180°. After cooling, 3–12 ml of C are added, the volume and the corresponding dilution depending on the amount of acid initially added to the tubes. All pipettings are carried out with automatic pipettes. The contents of the tubes are rapidly mixed with a Rotamixer®, heated for 7 min in a boiling water bath, centrifuged and measured at 820 nm in standard 3-ml cuvettes. Reagent blanks and blank silica gel scrapings are assayed simultaneously.

Results and discussion

Acid concentration. The troublesome elution of the spots is avoided by ashing the TLC spots directly with the gel. As both the quantity of the individual phospholipid fractions and the size of the spots are variables, the amount of sulphuric acid needed, varies correspondingly. We have used 0.5 ml of 10 *N* acid for small spots (*e.g.* phosphatidic acid and sphingomyelin) and 1.0–2.0 ml for large spots (*e.g.* phosphatidylethanolamine and phosphatidylcholine). Reagent C is added in proportion to the amount of acid to allow proper dilution of the larger spots. Somewhat different values for a suitable acid concentration have been published. According to BARTLETT[9], the acid concentration should be 0.6–1.8 *N*. PARKER[7] used 0.5 ml of concentrated H_2SO_4 per 10 ml of reaction mixture, the acid concentration thus being approximately 1.8 *N*. RHEE AND DUGAN[10] examined the significance of the acidity, using concentrated H_2SO_4, 70 % $HClO_4$ and a mixture of these acids. Their results indicate that the correct acid concentration should be 0.25–0.5 ml of concentrated H_2SO_4 per 10 ml of the reaction mixture, corresponding to 0.9–1.8 *N* acid. In our procedure, the acidity is approximately 1.7 *N*, a value which

is at the upper limit. This is advisable, however, since a certain minimum volume of the acid is needed to wet the gel which is to undergo combustion.

Premixing of the reagents. In almost all methods for the measurement of orthophosphate by the formation of the ammonium molybdate complex, ammonium molybdate and the reducing agent are added separately to the reaction mixture. Only CHEN *et al.*[11], using ascorbic acid as a reducing agent, made a reaction mixture before the initation of the reaction, but with their procedure a period of 1.5–2 h at 37° is required for the development of the colour. With our method, a reaction mixture containing ammonium molybdate and 1-amino-2-naphthol-4-sulphonic acid is prepared before the assay, and it has been found that the same results are achieved by this modification as when the reagents are added in sequence. In this procedure, one pipetting phase is omitted, with saving of time and reduction of pipetting errors. It was estimated from several experiments that the standard error was reduced by 20–25 %, in different series of experiments.

Sensitivity of the assay. The practical lower limit is about 0.2 μg of PO_4-P per sample, and about 0.45 μg of PO_4-P is required to produce a change of 0.100 in the optical density. For other purposes, however, it would be easy to develop an ultramicro modification.

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