**METHODS**

Effect of Oxalic Acid Impregnation of Chromarods on the Separation of Phospholipids for Determination by the Iatroscan TLC/FID

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**ABSTRACT**

Phospholipid separations were carried out using Chromarods-SII impregnated with oxalic acid without interfering with Iatroscan TLC/FID detection and measurement. The resolutions were compared with untreated rods. Oxalic acid impregnated Chromarods gave better resolution of phospholipids, and the separations were more reproducible on a day to day basis compared to untreated Chromarods. A concentration of 0.25 M oxalic acid in acetonitrile, with 15 min of impregnation, followed by 60 min of activation at 110 °C, provided the ideal conditions for coating. Three solvent mixtures, viz. CHCl₃:MeOH:H₂O (50:30:8:4, v/v/v/v), CHCl₃:MeOH:H₂O (65:35:4, v/v/v), and CHCl₃:MeOH:28% NH₄OH (70:30:2, v/v/v) were tested as developing solvents. CHCl₃:MeOH:H₂O (65:35:4) was found to be the best solvent system. Double development (initially in acetone, followed by CHCl₃:MeOH:H₂O [65:35:4]) is of minor value in improving separations. All the above solvent systems are capable of separating most of the commonly occurring plant phospholipids, except phosphatidylinositol and phosphatidylserine. Both of these phospholipids eluted together on Chromarods-SII, giving a single peak on the Chromarod, regardless of whether the rods were impregnated with oxalic acid.


**INTRODUCTION**

The use of the Iatroscan-TLC/FID for the measurement of phospholipids separated on Chromarods-S or -SII has been described by several workers (1). Tanaka et al. (2) originally investigated the reliability of Iatroscan in the determination of the composition of the polar lipids. The composition of cardiac phospholipids (3) and the separation of diacyl and plasmalogen phospholipids (4) have been examined using the Chromarod-Iatroscan system. However, there have been problems associated with the separation of certain types of phospholipids. Hiramatsu and Arimori (5), using CHCl₃:MeOH:H₂O (60:30:3.5) containing 500 mg/dl of butylated hydroxy toluene (BHT), found that phosphatidylglycerol and phosphatidylinerine always eluted together on Chromarods and gave a single peak on the Iatroscan. Vandamme et al. (6), using CHCl₃:MeOH:H₂O (80:35:3) separated lysophosphatidylcholine, sphingomyelin, phosphatidylcholine and phosphatidylethanolamine, but were unable to separate cardiolipin, phosphatidylserine and phosphatidylinositol in the same phospholipid mixture. Innis and Clandinin (7) could separate the major phospholipid classes (diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin and lysol-ecithin) by developing the Chromarods in light petroleum-diethyl ether (85:15) and CHCl₃:MeOH:H₂O (80:35:3) and specified the amount of water in the second developing system as a limiting factor for the separation. Instead of pure water, an oxalic acid solution (in water) has been used in the preparation of silica gel G plates for thin-layer chromatography (TLC) by Ponnathan et al. (8) and by Iijima et al. (9) for the separation of phosphatidic acid from the total lipids, using petroleum ether (40-60°C): acetone:formic acid (74:26:0.25) as the solvent system. We have now evaluated this modification of TLC techniques based on a silica gel Chromarod-SII impregnated with oxalic acid. It was found that improved resolution of phospholipids can be achieved with the modified Chromarods.

**EXPERIMENTAL**

Phospholipid standards (phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, cardiolipin, sphingomyelin and phosphatidic acid) were obtained from Serdary Research Labs (London, Ontario, Canada). Phos-
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RESULTS AND DISCUSSION

The decomposition of the formic acid molecule in the normal GLC type FID flame gives little or no signal (10,11). Oxalic acid is essentially 2 molecules of formic acid, and it has been shown that the GLC-FID response of dimethyl oxalate is essentially based solely on the 2 methoxy functions (12). This characteristic of oxalic acid suggested that this strong organic acid could be impregnated in the silica gel of the Chromarods-S or -SII to modify the chromatographic characteristics towards phospholipids without interfering with the Iatroscan FID performance. This has been found to be the case.

The humidity of the atmosphere, the surface condition of the Chromarod and the type of developing solvent system greatly influence the resolution of phospholipids, especially the separations among phosphatidic acid (PA), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) + phosphatidylserine (PS). On some days it was possible to obtain a resolution between PA, PE and PI + PS. However, the same rod, after cleaning and respotting with the same mixture of standard phospholipids, could then occasionally give a very poor separation of these phospholipids in which there was a partial separation between PA and the other phospholipids, with PE, PI and PS eluting together to give one peak. This indicates that the separations are not exactly reproducible on a day to day basis, for the same rod using the same solvent. However, much more reproducible separations could be obtained by impregnating the Chromarods with oxalic acid. The impregnated rods always showed a better resolution than the untreated ones, as illustrated in Figure 1, regardless of the other parameters, viz. concentration of oxalic acid, time of impregnation and developing solvent system. Of the different solvent systems tried, CHCl3:MeOH:H2O (65:35:4) was found to be the best for overall resolution and therefore, in one of the runs with this solvent system 2 more lipid standards, lysophosphatidylethanolamine (LPE) and lyso-phosphatidylcholine (LPC), were added to the "standard mixture of phospholipids" to investigate their behavior. The peak for LPE followed that of PI + PS and the peak for LPC that of PC. These two standards (LPE and LPC) were, however, not included in the "standard mixture" when other developing systems were tried.

With the impregnated rods a distinct separation was always obtained between PA and PE and also between PE and PI + PS. However, there was no separation between PI and PS (shown as PI + PS in figures), both being eluted as a single peak. Apart from the reproducibility of separation, the treated rods produce sharper

The Chromarods were immersed in 30% nitric acid overnight, rinsed with distilled water and activated by passing through the FID of the Iatroscan immediately before use. The cleaned Chromarods were impregnated with oxalic acid by dipping the rods in a solution of the oxalic acid (in acetonitrile) for 15 min (or more in the case of optimization studies), activating them at 110 C for 30 min (or more in the case of optimization studies) and then spotting in the normal way. Standard mixtures of lipids in chloroform were applied (3 µl) by means of disposable pipettes ("Microcaps"—Drummond Scientific Co., Broomall, Pennsylvania) onto the rods. The Chromarods were kept in a saturated sodium chloride humidity tank for 10 min, developed for 40 min in the solvent system, dried at room temperature for 1 min, at 110 C for 2 min, and then scanned in the FID of the Iatroscan.

Standard solutions of samples were prepared by dissolving weighed amounts of standards in chloroform or chloroform:methanol (1 mg/ml). Each standard was stored under an atmosphere of nitrogen at -17 C.

Various concentrations of oxalic acid, different solvent systems and different experimental conditions were taken as parameters to optimize the best possible conditions. The details are given in the Results and Discussion Section.

The decomposition of the formic acid molecule was obtained from Applied Science Labs (Pennsylvania, U.S.A.). According to the suppliers, the phospholipid standards were 96-99%; when the phospholipid standards were examined singly for purity, they produced 1 spot on the TLC plates and gave a single, symmetrical sharp peak on Iatroscan TLC/FID with the different developing systems described in the Results and Discussion Section. Oxalic acid (dihydrate) was Baker Chemical Co. (New Jersey, U.S.A.), ACS reagent grade. All organic solvents were redistilled under nitrogen before use.

The airflow on the Iatroscan TH-10 Analyzer (Iatron Labs, Japan, distributed in Canada by Technical Marketing Associates, world distributor Newman-Howells Assoc., Winchester, United Kingdom) was 2000 ml/min, the hydro-