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Note

Resolution of phospholipids on copper(II) sulphate-impregnated Chromarods*

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It was reported recently by Kaimal and Shantha[1] that impregnating the Chromarods used in the Iatroscan instrument with copper(II) sulphate resulted in a flame ionization detection (FID) response that was a function of the mass of a component rather than its structure. This finding was confirmed when differences in the FID response of triglycerides (TGs) differing in chain length and unsaturation were removed by impregnating the Chromarods with copper(II) sulphate[2]. Furthermore, it was confirmed[2] that copper(II) sulphate impregnation improved baseline stability and rod-to-rod variation[1]. However, what was not apparent to these authors[1] was that copper(II) sulphate impregnation altered the chromatographic behaviour of lipids on the Chromarods. The migration of unsaturated neutral lipids was markedly reduced[2]. This study was undertaken to evaluate copper(II) sulphate-impregnated Chromarods for the analysis of phospholipids. It was found that addition of small amounts of formic acid was essential to restore the resolution of complex phospholipids on Chromarods similar to that reported for unimpregnated Chromarods.

EXPERIMENTAL

The Iatroscan TH-10, Mark II (Iatron Labs., Tokyo, Japan) was obtained from Technical Marketing Associates (Mississauga, Canada). The Chromarods were burned using a scan speed of 0.42 cm/s (gear No. 40). Peaks were integrated with a Hewlett-Packard (Cupertino, CA, U.S.A.) 3350A laboratory automation system with reintegration capacity. Details regarding the operating conditions were described previously[2,3].

The Chromarods (type S) were acid washed [sulphuric acid–perchloric acid (1:1)], rinsed with distilled water and burned three times before use. Impregnating the Chromarods with copper(II) sulphate was accomplished by immersing the rods in 5% $CuSO_4 \cdot 5\ H_2O$ solution for 30 min, followed by drying at 110°C for 30 min, and two complete burns[1].

Pure lipids were obtained from Nu-Chek-Prep (Elysian, MN, U.S.A.). Total lipids from rat hearts and calf serum were available in the laboratory.

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RESULTS AND DISCUSSION

A mixture of methyl ester (ME) (17:0) and three pure TGs (16:1 $n-7$, 22:1 $n-9$ and 22:6 $n-3$), in the ratio of 3:1:1:1 (w/w), were shown to resolve on type S Chromarods without copper(II) sulphate[4]. The R_F values of the TGs increased with increasing chain length and increasing unsaturation of the fatty acids (R_F of 22:6 $n-3$ > 22:1 $n-9$ > 16:1 $n-7$). When this mixture was applied onto copper(II) sulphate-impregnated Chromarods, using the same solvent system 1,2-dichloroethane–chloroform–formic acid (92:8:0.1)[5], the ME and TG peaks partially overlapped. An overlap of ME and TG peaks was also observed with the solvent used by Kaimal and Shantha[1], *n*-hexane–chloroform–isopropanol–formic acid (89:11:0.55:0.055). The solvent hexane–diethyl ether–formic acid (95:5:1), which maximises the separation of ME and TGs[6,7], resolved this mixture. However, the migration order changed to R_F 22:1 $n-9$ > 16:1 $n-7$ > 22:6 $n-3$ (see Fig. 1 in ref. 2). Copper(II) sulphate impregnation reduced the migration of unsaturated fatty acids, presumably by formation of metal (Cu) complexes with double bonds.

Depending on the relative concentration and fatty acid composition of the different lipid classes in a biological sample, the previously used solvent systems[1,5,6,7] could remain effective in giving good resolution of the neutral lipids with copper(II) sulphate-impregnated Chromarods. For example, in Fig. 1A, calf serum total lipids were separated using 1,2-dichloroethane–chloroform–formic acid (92:8:0.1). Methyl heptadecanoate was added as an internal standard to the serum lipids, and the sample was spiked with a small amount of free heptadecanoic acid to clearly identify free fatty acids (FFAs). Only traces of FFAs were observed in the original calf serum.

Attempts to resolve the total rat heart phospholipids on copper(II) sulphate-impregnated Chromarods failed (Fig. 2A), using the solvent chloroform–methanol–water (68.5:29:2.5) which gave good separation of the phospholipids on unimpregnated Chromarods[3,8,9]. There was evidence of band spreading, particularly with diphosphatidylglycerol (DPG) and phosphatidylethanolamine (PE), which was

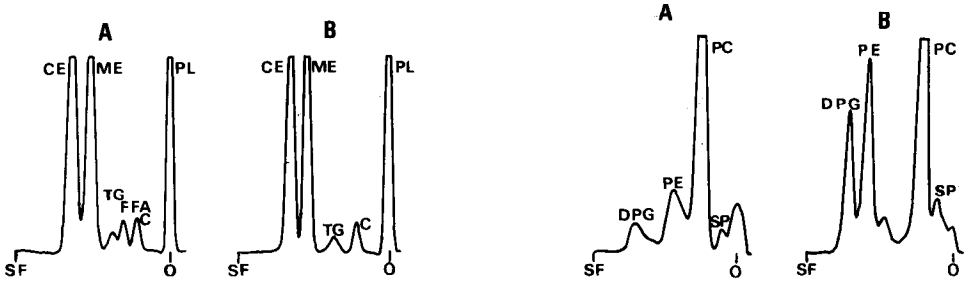

Fig. 1. Separation of calf serum lipids on copper(II) sulphate-impregnated Chromarods with (A) and without (B) 0.1% formic acid in the developing solvent of 1,2-dichloroethane–chloroform (92:8). CE = Cholesteryl ester; ME = methyl ester (methyl heptadecanoate); TG = triglyceride; FFA = free fatty acid (heptadecanoic acid); C = cholesterol; PL = phospholipid; O = origin; SF = solvent front.

Fig. 2. Separation of rat heart phospholipids on copper(II) sulphate-impregnated Chromarods without (A) and with (B) 0.5% formic acid in the developing solvent of chloroform–methanol–water (68.5:29:2.5). DPG = Diphosphatidylglycerol; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SP = spingomyelin; O = origin; SF = solvent front.

confirmed by analyzing isolated DPG and PE from rat heart lipids on impregnated Chromarods. This might be expected, since these two lipids classes contain particularly wide ranges in fatty acid chain length and unsaturation. Changing the ratio of chloroform, methanol and/or water failed to improve the separation. Addition of small mounts of formic acid (0.5%) restored the resolution of the phospholipids in a single development (Fig. 2B) to that previously observed with a double development on unimpregnated type S Chromarods. There was no evidence of spreading and tailing, and the isolated DPG and PE gave sharp peaks. The large peak at the origin also disappeared.

The evidence indicates that the quantitative analysis of lipids by the Iatroscan method is greatly improved by the use of copper(II) sulphate impregnation of the Chromarods[1,2]. The results of this study indicate that small amounts of formic acid were required in the developing solvent to achieve satisfactory resolution of the phospholipids. The first few developments after a copper(II) sulphate impregnation might require 1% formic acid in the developing solvent. The phenomenon of band spreading due to the impregnation had not been observed for the neutral lipids, mainly because all commonly used developing solvents for neutral lipids already contain small amounts of formic acid[1,5,6,7]. Removing the formic acid from the solvent system in Fig. 1A did not cause noticeable band spreading of cholesteryl ester (CE) and TG, but the identification of FFAs was lost (Fig. 1B). For this reason formic acid should be retained in the developing solvents of neutral lipids.

REFERENCES

1 T. N. B. Kaimal and N. C. Shantha, *J. Chromatogr.*, 288 (1984) 177.
2 J. K. G. Kramer, B. K. Thompson and E. R. Farnworth, *J. Chromatogr.*, 355 (1986) in press.
3 J. K. G. Kramer, E. R. Farnworth and B. K. Thompson, *Lipids*, 20 (1985) 536.
4 J. K. G. Kramer, R. C. Fouchard and E. R. Farnworth, *Lipids*, 20 (1985) 617.
5 W. W. Christie and M. L. Hunter, *J. Chromatogr.*, 171 (1979) 517.
6 J. C. Sipos and R. G. Ackman, *J. Chromatogr. Sci.*, 16 (1978) 443.
7 J. K. G. Kramer, R. C. Fouchard and E. R. Farnworth, *J. Chromatogr.*, 198 (1980) 279.
8 J. K. G. Kramer, *Lipids*, 15 (1980) 651.
9 S. M. Innis and M. T. Clandinin, *J. Chromatogr.*, 205 (1981) 490.