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EFFECT OF SOLVENTS ON THE RESOLUTION OF NEUTRAL LIPIDS ON CHROMARODS*

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SUMMARY

The chromatographic behavior of neutral lipids on chromarods, used for the Iatroscan TH-10 analyzer, was evaluated and compared to that observed in adsorption thin-layer chromatography on silica gel. Various proportions of hexane, diethyl ether and formic acid were used in the developing solvent to determine changes in R_F values of the neutral lipid classes. In addition, acetic and propionic acids were investigated as substitutes for formic acid in the developing solvent. A knowledge of the chromatographic behavior of the neutral lipid classes, with systematic changes in the concentration of individual solvents in the developing solvent mixture, allows maximum resolution to be obtained. The R_F values of some lipid components change with extensive use of the chromarods (25 to 30 uses); however, the life of the chromarods can be extended by changing the proportion of the solvent components in accordance with the chromatographic characteristics determined in this study.

INTRODUCTION

The Iatroscan[1] is an instrument that combines the resolution capabilities of thin-layer chromatography (TLC) with the possibility of quantitation by using a flame-ionization detector. The application of this method is still in the experimental stages, and new developing solvents are continually reported.

The apparent similarity between the use of chromarods and adsorption TLC on plates coated with silica gel has tempted many workers to apply solvent systems successfully developed for TLC on silica gel directly to chromarods. However, it is evident from recently published data on the resolution of neutral lipids with chromarods, that the effects of solvent systems are quite different[2-6] from those applicable in adsorption TLC. The solvent systems containing hexane, diethyl ether and organic acid are either low in acid[2,3] or have no acid at all[4], giving the same separation sequence as with TLC, or are low in diethyl ether[5], which results in an inversion of the

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triglyceride (TG) and free fatty acid (FFA) sequence obtained in adsorption TLC. A separation of neutral lipids has also been reported in which a chlorinated solvent system is used[6].

In this communication, the effects of various solvents and solvent mixtures on the resolution of the neutral lipid classes will be examined.

EXPERIMENTAL

Instrument and operating conditions

The Iatroscan TH-10 TLC Analyzer, Mark II (Iatron Labs., Tokyo, Japan; Canadian distributor: Technical Marketing Associates, Mississauga, Canada) used, was equipped with a flame-ionization detector and an electronic stepping integrator. The flame-ionization detector was operated with a hydrogen flow-rate of 160 ml/min and an air flow-rate of 2 l/min. The scanning speed was 0.42 cm/sec. A two-pen linear recorder (Fisher Recordall, Model 5000) was used at 10 mV full-scale deflection and a chart speed of 0.47 cm/sec.

Procedure

The chromarods (type S; mean thickness of sintered coating of active silica gel adsorbent 75 μm) were cleaned and placed overnight in a 9 N H_2SO_4 solution, rinsed with distilled water, then dried at 110°C for 10 min and scanned twice in the Iatroscan before use. A mixture containing equal amounts (by weight) of cholesterol ester (CE), methyl ester (ME), triglyceride (TG), free fatty acid (FFA) and cholesterol (C) was purchased from Nu Check Prep (Elysian, MN, U.S.A.); oleic acid was the fatty acid used in this standard mixture. A heptane solution was prepared containing 30 μg/μl of the total mixture (or 6 μg/μl of each of the five compounds). It was later necessary to spike a portion of the standard solution with approximately double the amount of TG, and to prepare simpler mixtures, to differentiate between the TG, ME and FFA peaks in many instances. The chromarods, held in an appropriate frame, were spotted with the standard solution (1 μl) and placed in glass tanks lined with filter paper. A variety of solvent mixtures was used, and development was for *ca.* 11 cm on the chromarods. The rods were then dried at 110°C for 5 min, transferred into the Iatroscan and scanned. All R_F values quoted are the means of 10 rods developed simultaneously.

Developing solvents

All solvent mixtures used for development were prepared by mixing X volumes of diethyl ether with 100 − X volumes of hexane. The organic acids (formic, acetic or propionic) were added to the 100 ml of solution containing hexane and diethyl ether in amounts of 0.04 to 1 ml. All solvents were of reagent grade and were distilled in glass before use.

Thin-layer chromatography

High-performance Whatman TLC plates (Type HP-K; 10 × 10 cm) coated with silica gel were used. These plates are coated with a special silica gel of particle size 5 μm. The developing solvents were the same as used for the chromarods. To visualize the compounds, the TLC plates were sprayed with H_2SO_4–methanol (1:1) and heated.

RESULTS AND DISCUSSION

Results using thin-layer chromatography

In order to understand the chromatographic behavior of neutral lipids on chromarods, a study of the behavior of these lipid classes in adsorption TLC was useful. The well-known sequence of resolution in adsorption TLC on silica gel with mobile phases containing various proportions of hexane, diethyl ether and formic acid is shown in Table I. The mobility of CE, ME, TG and C was increased by increasing the proportion of the polar solvents (diethyl ether and formic acid). The effect of formic acid was not evident when the ratio of hexane to diethyl ether was 85:15, presumably because of the small change in the total volume of polar solvents. On the other hand, the migration of FFA in adsorption TLC was dependent on the organic acid, provided that more than 5 % of diethyl ether was present in the developing solvent. The FFA did not migrate from the origin when the developing solvent was hexane–diethyl ether (95:5), even with the addition of 1 ml of formic acid (i.e., hexane–diethyl ether–formic acid, 95:5:1). Similarly, a developing solvent containing hexane–diethyl ether, (85:15) did not move the FFA from the origin (not shown). However, the presence of 0.1 or 1 ml of formic acid in this hexane–diethyl ether mixture significantly increased the mobility of the FFA (Table I).

TABLE I

THE R_F VALUES OF NEUTRAL LIPIDS ON HIGH-PERFORMANCE TLC PLATES (TYPE HP-K)

Component	R_F value			
	Hexane–diethyl ether–formic acid			
	95:5:0.1	85:15:0.1	95:5:1	85:15:1
Cholesterol ester	0.44	0.73	0.58	0.71
Methyl ester	0.26	0.53	0.36	0.51
Triglyceride	0.05	0.32	0.09	0.31
Free fatty acid	0	0.09	0	0.17
Cholesterol	0.01	0.04	0.03	0.05

Results using chromarods: comparison with thin-layer chromatography conditions

It was evident from the results with chromarods (Fig. 1) that the developing solvent ideal for adsorption TLC (hexane–diethyl ether–formic acid, 85:15:1) gave no resolution of ME, TG and FFA (Fig. 1–5). It was therefore necessary to prepare several simpler lipid mixtures and to "spike" the five-component standard mixture with TG in order to resolve these complex chromatograms. In order to achieve a separation sequence similar to that observed in adsorption TLC, the formic acid content had to be lowered to 0.04 ml (Fig. 1–6). Another resolution of these lipid classes was reported by Sipos and Ackman[5], who used a developing solvent containing small amounts of diethyl ether (hexane–diethyl ether–formic acid, 97:3:1). As shown in Fig. 1-1, such a developing solvent results in inversion of the FFA and TG peaks as compared with adsorption TLC on silica gel.

A systematic approach was therefore undertaken to elucidate the chromatographic behavior of these lipid classes with change of diethyl ether content, keeping the
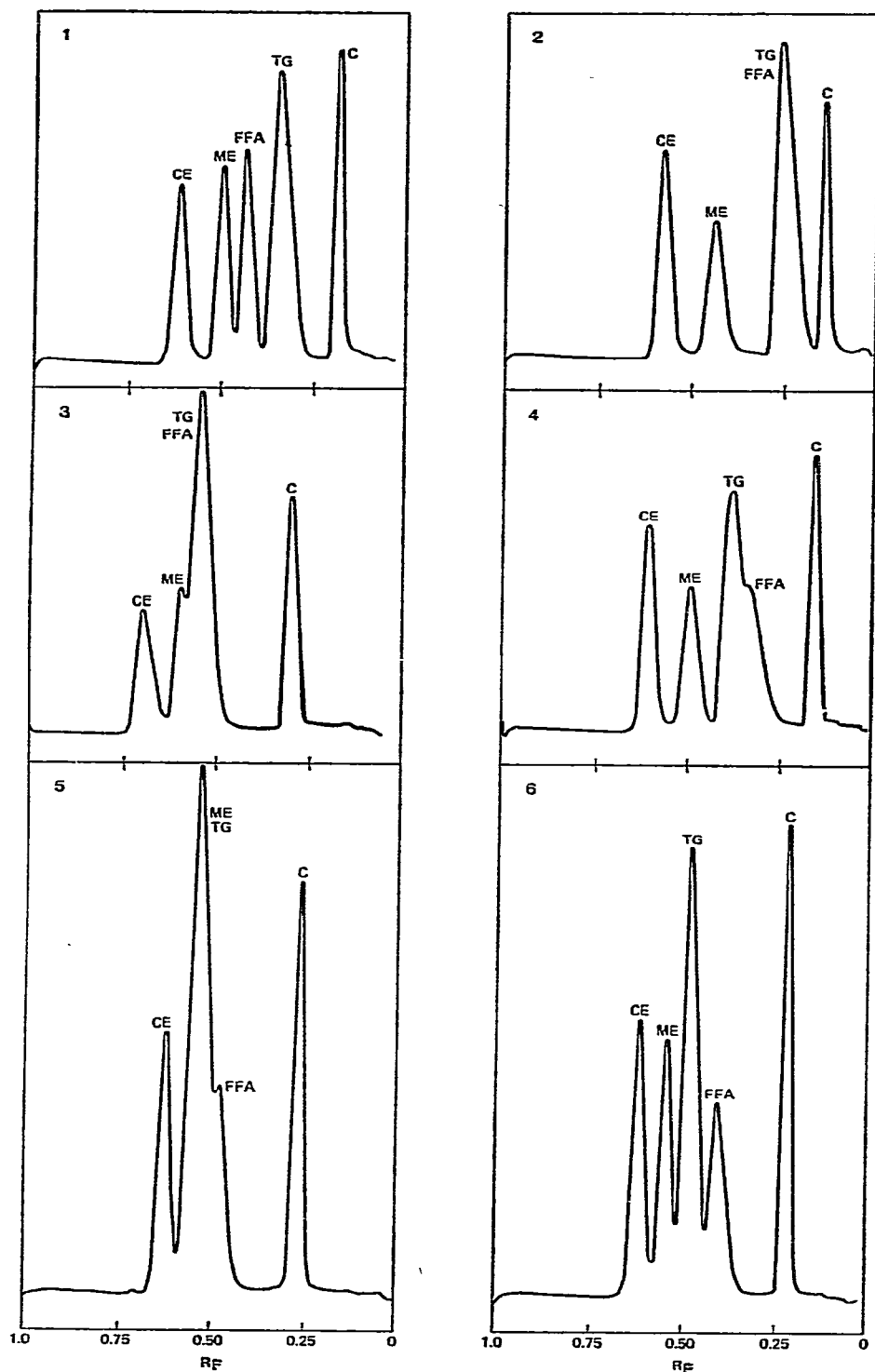
Fig. 1. Chromatograms showing the separation of neutral lipid classes on chromarods with solvents containing hexane–diethyl ether–formic acid in the proportions 95:5:1 (1); 95:5:0.04 (2); 90:10:1 (3); 90:10:0.04 (4); 85:15:1 (5); 85:15:0.04 (6); R_F values are indicated. CE = cholesterol ester; ME = methyl ester; TG = triglyceride; FFA = free fatty acid; C = cholesterol.
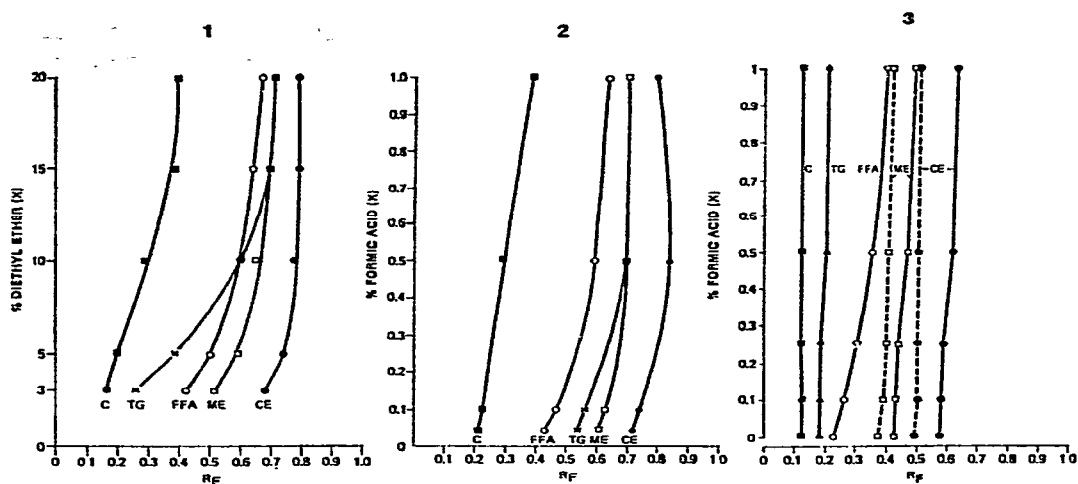
Fig. 2. R_F values of cholesterol (C), free fatty acid (FFA), triglyceride (TG), methyl ester (ME) and cholesterol ester (CE) plotted at various concentrations of hexane, diethyl ether and formic acid in the developing solvent. In Fig. 2-1, the concentration of diethyl ether is decreased, keeping the level of formic acid constant (hexane–diethyl ether–formic acid, 100−X:X:1). In Figs. 2-2 and 2-3, the concentration of formic acid is decreased, keeping the proportions of hexane and diethyl ether constant (85:15 in Fig. 2-2, and 97:3 in Fig. 2-3). The broken lines in Fig. 2-3 are the R_F values of ME and CE on chromarods used at least 25 to 30 times; the R_F values of C, TG and FFA on the used chromarods were the same as on new chromarods.

concentration of formic acid constant (Fig. 2-1). Conversely, the hexane–diethyl ether ratio was kept constant at 85:15 (Fig. 2-2) or 97:3 (Fig. 2-3), and the concentration of formic acid was changed.

Effect of diethyl ether

It is evident from Fig. 2-1 that a reduction of the diethyl ether content in the developing solvent retarded the relative mobility of TG more so than that of C, ME and CE; this effect was also noted for adsorption TLC. However, the relative mobility of FFA on chromarods paralleled that observed for C, ME and CE, which was totally different from the behavior of FFA in adsorption TLC. This difference in the relative mobility of FFA and TG therefore permitted separation of all five components at low concentrations of diethyl ether. The effect was an inversion of the TG and FFA resolution sequence on chromarods at hexane–diethyl ether–formic acid ratios of 95:5:1 and 97:3:1 compared with that observed in adsorption TLC. From Fig. 1-3, it can be seen that the developing solvent hexane–diethyl ether–formic acid (90:10:1) does not separate the TG and FFA peaks, which is contrary to the results of Tanaka et al.[7,8].

Effect of formic acid

As stated before, the developing solvent hexane–diethyl ether–formic acid (85:15:1) did not separate TG and ME on chromarods, and FFA was poorly resolved (Fig. 1-5). A reduction in the formic acid content lowered the relative mobility of both TG and FFA more so than that of ME, thus permitting separation of all five components (Fig. 2-2). A developing solvent of hexane–diethyl ether (85:15), with no formic

acid, gave the same separation sequence as did hexane–diethyl ether–formic acid (85:15:0.04) (see Fig. 1-6), but the FFA peak was slightly broader when the former developing solvent was used (results not shown). A broad FFA peak is evident from the results of Vandamme et al.[4]. The addition of small amounts of formic acid is therefore recommended in order to obtain a sharper FFA peak and a greater separation between FFA and C. As demonstrated by van Tornout et al.[2], the presence of a small amount of formic acid (light petroleum (b.p. 60–80°C)–diethyl ether–formic acid, 85:15:0.1) allowed the use of a long-chain alcohol as internal standard, which migrated between FFA and C.

In Fig. 2-3, the effect is shown of decreasing the amount of formic acid in the developing solvent hexane–diethyl ether (97:3) on the chromatographic behavior of the five lipid classes (solid lines only). The sequence of resolution of the lipid classes remained the same, but a significant decrease was observed in the relative migration of FFA.

Effect of extensive use of chromarods

The results in Fig. 1 and Fig. 2 (solid lines) were obtained from new chromarods. However, after about 25 to 30 developments on the chromarods, the TG and FFA peaks could no longer be resolved by hexane–diethyl ether–formic acid (85:15: 0.04). A switch to the developing solvent hexane–diethyl ether–formic acid (97:3:1) to take advantage of the TG and FFA inversion proved unsuccessful, because the FFA and ME now migrated together (Fig. 2-3). A detailed study of these older chromarods was undertaken as shown in Fig. 2-3 (broken lines) to see whether or not the FFA and ME could again be separated by taking advantage of the relatively greater decrease of the R_F value of FFA compared with ME. The results are superimposed in Fig. 2-3; the R_F values of C, TG and FFA had not changed from new to old chromarods, but the R_F values of ME and CE were greatly reduced (broken lines). By reducing the formic acid content in the developing solvent containing hexane–diethyl ether (97:3), an effective resolution of these neutral lipid classes on the chromarods could be maintained beyond the 20–25 developments previously reported[5]. In fact, through knowledge of the chromatographic behavior of these lipid classes and the effects of various solvents, we have been able successfully to complete over 50 separations.

Effect of other organic acids

The effect of using organic acids other than formic acid was also investigated. Substitution of acetic or propionic acid for formic acid in the developing solvent hexane–diethyl ether–formic acid (85:15:0.1) did not improve the resolution of the five-component mixture (Table II). In fact, when acetic acid was used, the TG and FFA peaks were incompletely resolved. The R_F values of all components, except C, were increased with increasing chain-length of the organic acid.

Evaluation of the chromatographic behavior of the chromarods

With a knowledge of the chromatographic behavior of the common neutral lipid components and the effects of developing solvents, the investigator can select the most appropriate conditions for a given sample and extend the life expectancy of the chromarods. Further, conditions may be selected to provide separations when certain internal standards are used. For example, van Tornout et al.[2] recently recom-

TABLE II

THE R_F VALUES OF NEUTRAL LIPIDS ON CHROMARODS

The developing solvent was hexane–diethyl ether–organic acid (85:15:0.1).

Component	R_F value		
	Formic acid	Acetic acid	Propionic acid
Cholesterol ester	0.74	0.79	0.86
Methyl ester	0.63	0.67	0.74
Triglyceride	0.56	0.56	0.64
Free fatty acid	0.47	0.50	0.55
Cholesterol	0.23	0.20	0.23

mended the use of a long-chain alcohol as an internal standard. The use of the developing solvent that inverted the TG and FFA peaks (hexane–diethyl ether–formic acid, 97:3:1[5]) is not recommended, as it resulted in poor separation of TG and the long-chain alcohol. However, the use of small amounts of formic acid is recommended; this gives a sharper FFA peak and greater separation between FFA and C. The use of propionic acid instead of formic acid has the added advantage of giving still greater separation between C and FFA (Table II).

The chromatographic behavior of FFA on chromarods was markedly different from that in adsorption TLC. On chromarods, FFA migrated with an R_F value of 0.4 without an organic acid (Fig. 2-2) or a high ether content (Fig. 2-1) in the developing solvent; even with hexane–diethyl ether (97:3), the R_F value of FFA was still 0.25 (Fig. 2-3). This is totally unlike the chromatographic behavior of FFA in adsorption TLC. Further, the R_F values of the other lipid classes were also much greater on chromarods than on silica gel layers when identical solvents were used. This suggests that forces other than those operative in adsorption chromatography operate on chromarods. Presumably, capillary action (or other forces) could be involved, which acts independently of the developing solvent.

The present study clearly indicates that resolution of components and the effects and influences of developing solvents on chromarods are very different from those in adsorption TLC, despite the fact that silica gel is used in both techniques. A better understanding of the nature of separation on chromarods is therefore essential. Procedures involving separation on chromarods and flame-ionization detection have in the past 5 years been shown to have advantages and potential as a complementary technique in lipid research.

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