Cod Lipids, Solvent Systems and the Effect of Fatty Acid Chain Length and Unsaturation on Lipid Class Analysis by Iatroscan TLC-FID

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The chromatographic behavior of molecular species of free fatty acids, triglycerides, sterol esters and wax esters on Chromarods-SII was investigated in four developing solvent systems of different polarities. In accordance with previous reports it was observed that molecular species within a lipid class are partially separated according to the chain length and degree of unsaturation of the acyl groups. The separation is more affected by the degree of unsaturation than the chain length, especially in nonpolar solvent systems. In polar solvent systems the separation within a lipid class is less efficient; a slight separation according to the chain length was observed, and the degree of unsaturation had little or no influence. The partial separation of molecular species within a class leads to the superimposing of certain lipid classes, for example glyceryl ethers and highly unsaturated fatty acids of marine origin. This poses a potential problem in identification of Iatroscan peaks. However, with totally hydrogenated marine lipid samples a complete separation of the lipid classes was achieved when developed in a nonpolar solvent system. It is proposed that at least two kinds of authentic standards varying in the degree of unsaturation and chain length should be used for the identification of the peaks of natural lipid samples of unknown composition, and that total hydrogenation be applied to improve separations and ensure sample stability, and probably to improve quantitation accuracy.

The Iatroscan thin layer chromatography-flame ionization detector system (TLC-FID) is now generally accepted as an effective, simple and rapid method for the separation and quantitative analysis of lipid classes (1-15), yet apparently straightforward analyses by techniques not previously applied to a particular sample often reveal unexpected problems requiring extensive reinvestigation of the whole analytical system. Cod flesh lipids had been thoroughly examined by silicic acid column chromatography (16,17) and it was unexpected when a new investigation using the Chromarod apparently indicated a substantial diacyl glyceryl ether component. We have confirmed that novel diacyl glyceryl ethers are in fact present, although at a very low level (18). The component tentatively identified as diacyl glyceryl ether in the solvent system used migrated between the free fatty acid identified by co-chromatography with a palmitic acid standard, and the anticipated triglyceride, but was in fact a highly unsaturated free fatty acid. Recently, it has been reported that the chromatographic behavior of the molecular species of triglycerides (TG) and phosphatidylcholine (PC) on Chromarods-S, with development in 1,2-dichloroethane/chloroform/acetic acid (92/8/0.1, v/v/v), was influenced by the degree of unsaturation and chain length of the fatty acids esterified on the glyceryl moieties (19). The R_f values of the TG and PC classes on the rods increased both with increasing chain length and greater unsaturation of the fatty acid moieties. The increase of the R_f value was influenced more by the chain length than the degree of unsaturation of the constituent fatty acids in TG and PC (19). It is known that the separation of lipid classes from others, as well as the separation among the molecular species within a lipid class, varies with the polarity of the developing solvent system (20).

In the present investigation, we have extended the study of Kramer et al. (19) by evaluating the chromatographic behavior of the common "neutral" lipid classes, fatty acids (FFA), triglycerides (TG), sterol esters (SE) and wax esters (WE) in four different solvent systems. The four solvent systems selected are representative of the most common solvent systems used by the majority of Iatroscan TLC-FID users for lipid class separation (21).

Experimental materials. Authentic standards of free fatty acids, triglycerides, sterol esters, glyceryl ethers and wax esters, of > 99% purity, were purchased from Serdary Research Labs., Inc. (London, Ontario, Canada), Supelco Canada Ltd. (Oakville, Ontario, Canada), Analabs, Inc. (North Haven, Connecticut) and Nu-Chek-Prep., Inc. (Elysian, Minnesota). All solvents were redistilled in glass under nitrogen before use.

A mixture of highly unsaturated wax esters was synthesized by alcoholysis between fatty acid ethyl esters and a fatty alcohol (22). The fatty acid ethyl esters (0.12 g) enriched in n-3 polyunsaturated fatty acids (isolated from menhaden oil) and oleyl alcohol (0.10 g) were heated at 105 C for 1 hr in the presence of sodium methylate catalyst (10 mg). Water was added to the tube, and the reaction product was extracted in the usual manner with diethyl ether. The highly unsaturated wax ester thus obtained gave a single peak on the Iatroscan TLC-FID chromatogram.

Lipids of cod (*Gadus morhua*) flesh were extracted by the method of Bligh and Dyer (23). A neutral or "non-polar" lipid fraction, including FFA and a little phospholipid, was separated from the total lipid according to the procedure of Galanos and Kapoulas (24). A portion of this neutral lipid fraction was hydrogenated as a stirred solution in chloroform in the presence of platinum oxide (Adam's catalyst) with hydrogen gas at atmospheric pressure for 1 hr.

METHODS

The Chromarods-SII (Iatron Laboratories, Tokyo, Japan) were thoroughly cleaned before use by soaking in 50% nitric acid overnight, and were then rinsed with running tap water, distilled water and acetone in that order. The Chromarods were dried in an oven at 115 C for 30 min, and then scanned twice through the Iatroscan FID immediately before sample application.

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Blended chloroform solutions were prepared containing 6 to 10 μg/μl of the total standard mixture (3 to 5 g/μl of one standard material). In the case of cod lipids, the sample concentration was ~20 μg/μl. The chloroform solution of the sample was spotted on the Chromarods-SII as 1 μl with a single spotting action using Drummond Microcap disposable pipets (Acadian Instrument Ltd., Etobicoke, Ontario, Canada). The rods, after spotting, were placed in a constant humidity tank over saturated sodium chloride solution for 10 min and were then transferred immediately to the developing tank. Development was carried out in the four different types of solvent systems for the times noted:

No.	Solvent system	Developing time (min)
I	Hexane:diethyl ether: formic acid (97:3:1)	40
II	Hexane:diethyl ether:formic acid (85:15:0.04)	40
III	Benzene:chloroform:formic acid (70:30:1)	40
IV	Dichloroethane:chloroform:acetic acid (92:8:0.1)	30

The solvents were evaporated from the rods in an oven at 115 C for 3 min. The Chromarods were then scanned on the Iatroscan TH-10 analyzer MK III (Iatron Laboratories, Tokyo, Japan). The air and hydrogen flow rates for the Iatroscan TH-10 analyzer were 2,000 ml/min and 160 ml/min, respectively, and the scan speed was set at 40 sec/scan. The peak areas were recorded on a SP 4200 Computing Integrator (Spectra-Physics, San Jose, California).

RESULTS AND DISCUSSION

It has been observed by Kramer et al. (20) that when the solvent system is of low polarity, FFA as a class interact strongly with the silica of Chromarods-SII, and that the polarity of the solvent system is particularly influential in the chromatographic behavior of lipid classes. Accordingly, the investigation of the influence of the degree of unsaturation and chain length on the separations among FFA, as well as within the classes TG and SE, was extended to other common developing solvent systems of different polarities. The results are summarized in Table 1. The descending order of polarities of the four solvent systems used in the present study, estimated from the value of the solvent strength parameter (25) of each solvent component, is solvent IV > solvent III > solvent II > solvent I.

Chromatographic behavior of FFA. The data presented in Table 1 clearly show that the degree of unsaturation and chain length of the fatty acids affect the migrations, especially in weakly polar solvent systems. As the solvent polarity was increased from I to IV, all the fatty acids migrated rapidly as expected, giving higher R_f values, but the separation between individual FFA was poor. With more polar solvents the FFA emerged as one peak, except for the pair 14:0 and 22:0. Peak splitting between these two acids was always observed irrespective of the type of solvent. The results in Table 1 suggest that in polar solvent systems a slight separation among FFA species could be expected according to the chain length, rather than through the degree of unsaturation which had no influence at all.

TABLE 1

Interaction of Fatty Acid Structures with Solvent System Polarities in Separations within FFA, TG, SE and WE on Chromarods S-II

	Solvent system			
Pair of standard	I	II	III	IV
Free fatty acids				
18:0/18:1	−	−	−	−
18:0/18:2	+	+	−	−
18:0/18:3	++	+	−	−
22:0/22:1	−	−	−	−
22:0/20:3	++	+	−	−
22:0/20:4	+++	+	−	−
22:0/22:6	+++	+	−	−
14:0/22:0	±	±	±	±
16:0/22:6	+++	+	−	−
18:3/22:6	+	+	−	−
Triglycerides				
TG-12:0/TG-16:0	++	+	±	++
TG-18:0/TG-18:3	++	++	−	−
TG-16:0/TG-18:3	+++	++	−	−
Sterol esters				
SE-16:0/SE-20:0	−	−	−	−
SE-20:0/SE-20:4	++	−	−	−
SE-16:0/SE-20:4	++	−	−	−
Wax esters				
16:0-16:0/n-3 PUFA-18:1	−	−	−	−

−, no separation.
±, shoulder separation with peak broadened.
+, slight separation with apex splitting.
++, partial separation.
+++, base line separation.

In the two non-polar solvents, the R_f values of FFA on Chromarods decreased with an increased degree of unsaturation and with a decrease of chain length. FFA differing by one double bond migrated together as a single peak in solvent I, and also in solvent II, although sometimes a slight splitting at the apex was observed. A partial separation was always observed when the components differed by two double bonds, and this separation was more pronounced when the degree of unsaturation differed by three double bonds. For baseline separation, the difference in the number of double bonds between two components should be four or more. The chain length had only a slight influence on the separation of fatty acids when developed in non-polar solvents. The fatty acid pair 14:0 and 22:0, although differing by eight carbon atoms, did not separate adequately from each other. However, the peaks were broadened somewhat.

From the above results it is concluded that in nonpolar solvent systems, the separation within the class of free fatty acids is influenced more by the degree of unsaturation than by the chain length.

Chromatographic behavior of TG. In the nonpolar

solvent system I a standard mixture of authentic trilaurin (TG-12:0) and tripalmitin (TG-16:0) showed a partial separation on the Chromarods-SII, while in the cases of the tristearin (TG-18:0) and trilinolenin (TG-18:3) pair, and the TG-16:0 and TG-18:3 pair, partial or baseline resolutions between each component were observed (Table 1). These observations with TGs indicate that in non-polar solvent systems the resolutions among TGs are influenced both by the chain length and by the degree of unsaturation. The R_f values of TGs on the rods decrease with an increase of the degree of unsaturation and a decrease of chain length. These chromatographic behaviors of TGs parallel those of FFAs mentioned earlier; the resolution between TG is affected more by the degree of unsaturation than by the chain length.

In polar solvent systems, the separation among TGs was governed primarily by the chain length, as demonstrated with TG-12:0 and TG-16:0. The degree of unsaturation had little or no influence. For the saturated TG pair, TG-12:0 and TG-16:0, the resolution was decreased in going from solvent I to III. In solvent III, only a peak apex splitting was observed. However, as the solvent polarity was further increased, from III to IV, a partial separation was observed. The separation between TG-12:0 and TG-16:0 was much better in solvent IV than in solvent I. In all four solvent systems TG-12:0 had a lower R_f value than TG-16:0. The above observations confirm that reported by Kramer et al. (19). They reported observing a partial separation between TG-22:1 and TG-16:1 but not between TG-22:1 and TG-22:6. Further, from our results and also from Kramer's results (19), it can be concluded that in polar solvent systems the highly unsaturated TGs migrate slightly ahead of less unsaturated TGs on the Chromarods-SII. In non-polar solvent systems I and II the completely opposite pattern occurs and the highly unsaturated TGs migrate more slowly than the the less unsaturated.

Chromatographic behavior of SE and WE. A resolution among SE species was observed only with solvent 1 (Table 1). In this solvent system the chain length of SE did not affect the R_f values, but the degree of unsaturation was quite effective; the R_f values of SE decrease with unsaturation and chain length. With the other three solvent systems, neither the degree of unsaturation nor the chain length had any effect on the resolution among the SE species. From these observations, it can be stated that a solvent system of low polarity is required for a separation among the SEs to occur based on the degree of unsaturation.

The effect of the degree of unsaturation and chain length of WE was investigated by using synthesized highly unsaturated WE (n-3 PUFA-18:1 WE) and authentic standard hexadecylhexadecanoate (16:0-16:0 WE) as shown in Table 1. Highly unsaturated WE and 16:0-16:0 WE did not separate from each other in any of the four solvent systems on the Chromarod-SII. However, in a solvent system of hexane/benzene (27:33, v/v), which is less polar than the solvent system I, highly unsaturated WE partially separated from 16:0-16:0 WE and had a lower R_f value. These results suggest that the chain length of WEs does not affect the R_f value on the Chromarods, although the degree of unsaturation is slightly effective in modifying R_f values. More highly unsaturated WE show lower R_f values than less unsaturated WE in the more nonpolar solvent systems.

It can be concluded that the effect of the degree of unsaturation of FFA and of acyl group(s) in TG, SE and WE on the R_f value on the Chromarods-SII is revealed more strongly with a decrease in the polarity of the developing solvent. The chain length was less effective in influencing the separation among a lipid class than was the degree of unsaturation when development was in a non-polar solvent system. When developed in polar solvent system, the degree of unsaturation of acyl groups is less effective, but the chain length becomes more important, especially with TG. This observation could be extended to polar lipid classes as well (19).

Effect of solvent system on the separation of lipid classes. Unless understood, the partial separations discussed above for the molecular species of a lipid class superficially pose a major problem in the analysis of lipid class composition of natural lipids by the Iatroscan TLC-FID. The problem is potentially quite severe with marine lipids, because the fatty acid moieties range in chain length from 14 to 24 and have up to six double bonds (19). The most common solvent system that has been used for lipid class composition studies is a nonpolar solvent system such as hexane/diethyl ether/formic acid (97:3:1, v/v/v) (7,9,11,-12,26). The problem associated with this solvent system is illustrated in Figure 1 for a lipid fraction isolated from cod flesh lipids (separated on silicic acid as a class of neutral lipids). In this solvent system, each of the classes FFA and SE gave two peaks (Fig. 1-A). The separation among the individual molecular species of FFA and SE is obviously due to the degree of unsaturation as discussed earlier. The peaks with lower R_f values in FFA and SE were identified as highly unsaturated species. The TG showed a relatively broad single peak, and each ST and PL appeared as a sharp single peak. Under the same experimental conditions, authentic 1-0-palmityl glyceryl ether dipalmitate (GE)

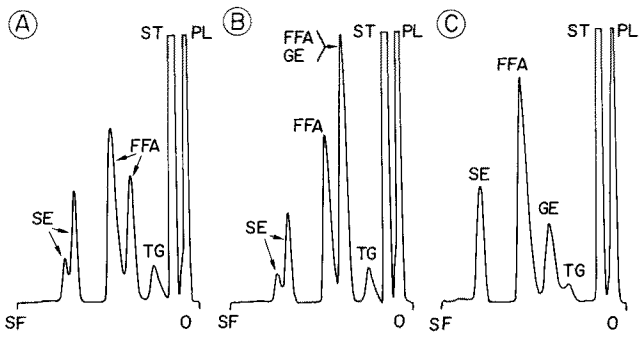

FIG. 1. Iatroscan TLC-FID chromatograms of A, fraction enriched with neutral lipids (NL), isolated from the flesh of cod stored on ice; B, the NL spiked with authentic 1-0-palmityl glyceryl ether dipalmitate (GE), coinciding in position with authentic highly unsaturated acids such as 22:6n-3; C, hydrogenated NL spiked with GE. Development in solvent 1 for 40 min. O, origin; SF, solvent front; FFA, free fatty acid; PL, phospholipids; SE, steryl ester; ST, free sterol; TG, triglyceride.

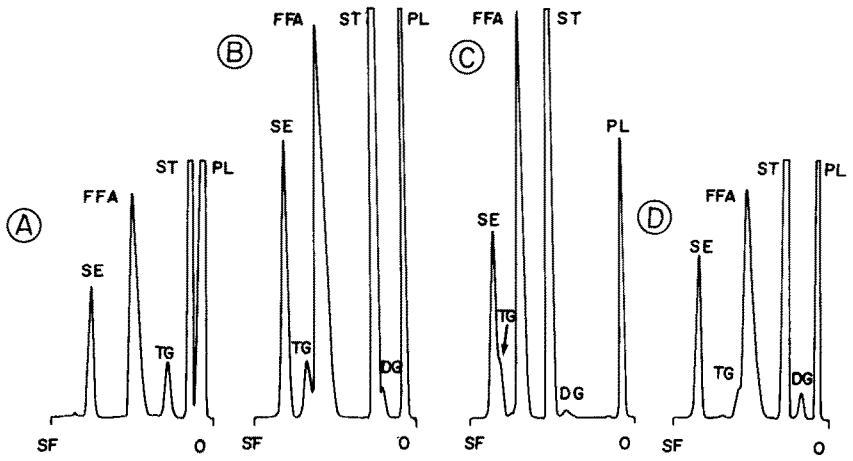

FIG. 2. Iatroscan TLC-FID chromatograms of a hydrogenated lipid fraction, isolated from cod flesh and basically of neutral lipids, in several developing solvent systems. A, solvent I; B, solvent II; C, solvent III; D, solvent IV. Developing time was 40 min in A, B and C, and 30 min in D. DG, diglyceride; all other abbreviations are the same as in Fig. 1.

migrated along with highly unsaturated FFA in one peak (Fig. 1-B); initially, it was thought that this cod flesh lipid contained a large amount of GE. The major fatty acids of cod flesh lipids are (17) 16:0, 18:1, 20:5n-3 and 22:6n-3, thus leading to two naturally occurring FFA peaks and the confusion of one with a glyceryl ether component. Marine glyceryl ethers usually have saturated alkyl chains and less saturated acyl substituents (27-29), so extreme composition differences are not common and natural glyceryl ethers would be expected to give only one peak. Wax esters also pose similar overlapping problems in lipid class analyses by Chromarod (30). Although WE do not separate by degree of unsaturation, the lower R_f peak in SE easily could be confused with WE in less polar solvents. Separation problems, especially among the neutral lipids, are not unique to the hexane/diethyl ether/formic acid (97:3:1, v/v/v) solvent system, but can occur in all the other common solvent systems as well (discussed below).

In order to eliminate this problem of overlapping lipid classes and to simplify the Chromarod analyses, the chromatographic behavior of the fully hydrogenated cod lipid sample was examined on Chromarods-SII in solvent I. Figures 1-C and 2-A illustrate the results. In the hydrogenated sample, each of the lipid classes gave a single sharp peak, which allows accurate quantitative analysis of lipid class composition. The authentic GE spike migrated between FFA and TG (Fig. 1-C), and each FFA and SE emerged as one peak. The WE peak partially separated from SE (results not shown), and the TG peak become much sharper in the hydrogenated sample (Fig. 2-A). We also investigated the separation of the hydrogenated cod flesh lipids in solvent systems II, III and IV. The results are illustrated in Figure 2. TG did not separate from FFA in solvents II and IV and from SE in solvent system III. A small amount of diglyceride (DG) was observed in cod flesh lipid. The DG stayed at the point of application of sample along with PL in solvent I, and it partially overlapped with sterols in solvent II. A complete separation of DG from ST and PL was obtained in the two polar solvent systems. Christie and Hunter (1) have reported, by studying several common authentic standards, that neutral lipid classes are adequately separated by developing in solvent system IV. Because the lipids from natural products almost invariably have a greater variety of fatty acid chain lengths than do commonly used standards, overlaps among lipid classes are possible as demonstrated in Figure 2 for solvent IV, as well as for other solvents.

From the results presented above, it can be concluded that in selecting the most suitable composition of a developing solvent system, some thought should be given to the fatty acid varieties of each of the lipid classes of the sample. Due to the overlapping of lipid classes, ideal identification of peaks using authentic standards should be carried out in at least two solvent systems, with different polarities. Furthermore, for each lipid class at least two kinds of authentic standards, varying in degree of unsaturation and chain length, should be used for identification of a peak. An alternative proposed by Kramer et al. (31) is that mixtures of fatty acids resembling the lipid class to be analyzed should be used.

When working with natural samples, especially those of marine origin, and most especially for the first time, it is better to carry out the peak identification in both unhydrogenated and hydrogenated forms. The hydrogenation process, if carried out promptly on isolated lipids, confers stability against oxidation. This is an important consideration with marine lipids and with other animal organ lipids containing highly unsaturated fatty acids. Autoxidized fatty acids could give false R_f values or lead to polymers remaining at or near the origin. The overall FID program accuracy should also be improved, since most calibration standards are based on saturated fatty acids (12).

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