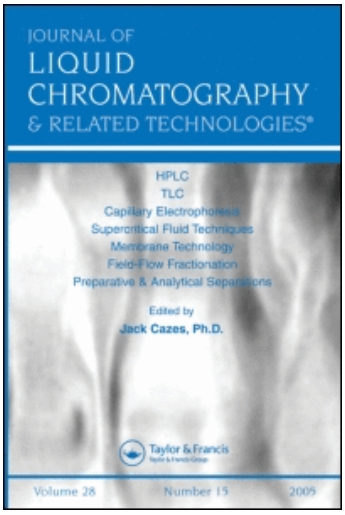

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DENSITOMETRY OF LIPIDS WITH IODINE STAINING ON HPTLC

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ABSTRACT

Scanning densitometers, equipped with the flying spot or meander principle of measurement were used to study the partition of iodine vapors to various lipid classes, such as phospholipids and triacylglycerols. The purpose was to use the densitometer for *in situ* quantitation of spots on thin layer plates. It was found that the soft visualization procedure of iodine vapors could be applied to the densitometric evaluation.

INTRODUCTION

Detection of lipids on TLC. Numerous methods for detection and charring of lipids, separated into spots on thin-layer chromatographic (TLC) plates have been described. Common methods to accomplish this is by spraying the plate with, or immersing it in a suitable detection reagent [1-5]. In the first case, it is necessary to spray uniformly in order to achieve accurate quantitative results. In the second case, the solvent must be chosen with care so that no components dissolve in the reagent.

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The spots may also become less concentrated, due to diffusion caused by the wetting of the adsorbent layer.

The softest method for visualization of spots on thin layer chromatograms is the use of vapor. Iodine vapors have been used as a qualitative detection reagent [6-11] for several decades. By exposing a developed plate to iodine vapors in a sealed tank under controlled temperature and time of exposure, the separated substances appear as brownish spots. However, this procedure has not been suitable for quantitative analysis because iodine evaporation starts immediately after the TLC-plate has been removed from the vapor chamber. Attempts have been made to hinder the evaporation by immediate spraying with acetic acid [12]. The limitation of such a procedure is the remaining dependence on uniform spraying. It has been claimed that iodine is added across the double bonds of hydrocarbon chains, and thereby staining unsaturated lipids more intensely than saturated [13].

Scanning modes. The new generation of scanning densitometers, equipped with a point scanning function as well as a regular linear scanning mode have improved the accurancy of *in situ* quantitation of spots on thin layer plates. In these point scanners, the light beam size is small compared to the magnitude of the sample spot and it performs a movement in two dimensions over a lane. The net result is that the spot is repeatedly sampled along its profile as it is scanned. For the compact spots observed in high performance thin layer chromatography (HPTLC), only a few passages of the beam through the profile of the spot are possible [14-15]. At the turning points automatic zeroing is

optional. In cases of inhomogenous concentration profiles of spots, quantitation using point scanning functions is more correct than when using the linear scanning mode. Some modern instruments have an automatic tracing function as well, which permits scanning of sloping lanes. With the use of such a method, tracing within the spot predicts the deviation of the entire lane.

Point scanning is in various respects superior to linear scanning, but the instrumentation needed is complex and requires sophisticated data processing [16-26]. A drawback of point scanning in general, is that it is more time-consuming than conventional linear scanning, due to the transversal movement of the light beam. Two variations of point scanning are flying-spot (Shimadzu instrumentation) and meander scan (Desaga instrumentation) [27].

In order to establish if iodine vapor can be used for quantitative purposes with scanning densitometry, the factors affecting the partition of iodine to lipids were explored. Studies were performed on various lipid classes, such as phospholipids and triacylglycerols.

MATERIAL AND METHODS

Equipment. In this study High-Performance Thin Layer Chromatography plates (HPTLC-plates, silica 60 G, 10 x 10 cm; Merck, Darmstadt, FRG) were used. To obtain high reproduciblity in the application of samples a semi-automatic device was used (TLC-Spotter PS 01; Desaga, Heidelberg, FRG) [28]. This device was equipped with a 1 ul Hamilton Series 7000 syringe (Nr. 7101;

Hamilton Bonaduz AG, Bonaduz, Switzerland). By this procedure no disturbance of the silica layer occured.

Methods. The plates were developed in a saturated normal-chamber (N-chamber) and the eluents consisted of; chloroform / methanol / acetic acid / water (68 : 22 : 6 : 4, (v/v/v/v) for the phospholipids [system 1] and petroleum ether (b.p. 60-70 ^{o}C) / diethyl ether / acetic acid (79 : 20 : 1, v/v/v) for the triacylglycerols [system 2]. After development the plates were dried and transferred to a sealed vapor chamber, containing a few iodine crystals (0.5 g). The atmosphere in the sealed tank had been equilibrated over the crystals for several hours at ambient temperature. The lipid substances were exposed to the vapors for 10 minutes at room temperature, after which they appeared as brownish spots. To minimize the background noise caused by adsorption and overloading of the spots, the plates were left out for at least 2 minutes to allow excess iodine to evaporate.

Scanners. For quantitative evaluation of the spots, the plates were analyzed with a scanning densitometer in the absorbance reflectance mode. The wavelength was set at 360 nm. The instrument used was the Densitometer CD 60 (Desaga, Heidelberg, FRG). The point scanning function of the densitometer was used throughout this study. However, its advantages were not especially needed in these cases, because the spots were scanned at a 90 degree angle to the direction of developement. Therefore, no tailing affects due to the partial separation of molecular species within the spot representing a specific lipid class was registered.

Lipid standards. The phospholipid standards 1,2-dilinoleyl-*sn*-glycero-3-phosphocholine (di-18:2-PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0-18:1-PC) were purchased from Sigma (St. Louis, MO, U.S.A.). These standards required a purification step by preparative TLC, using developing system 1. The products were checked for purity by HPTLC and found pure when exposed to a) iodine vapor and b) a charring procedure using a 3% (w/v) cupric acetate in 8% (v/v) orthophosphoric acid spray reagent (180 ^{o}C).

CPL-phosphocholine from soybean (Chromatographically Purified Lipids) and fully hydrogenated CPL-phosphocholin of soybean orgin were obtained through Larodan (Malmö, Sweden).

The triacylglycerol (TG) standards; trilinoleoylglycerol (tri-18:2), tripalmitoylglycerol (tri-16:0), trilauroylglycerol (tri-12:0) and tricaproylglycerol (tri-10:0) were purchased from Sigma. The trilinoleoylglycerol standard required a purification step by preparative TLC, using developing system 2. The trilinoleoylglycerol product was checked for purity by HPTLC, utilizing iodine vapor and a spray consisting of a 0.1% (w/v) solution of 2',7'-dichlorfluorescein in 95% ethanol.

CPL-fish oil and fully hydrogenated fish oil from the same batch of raw material, were obtained through Larodan.

EXPERIMENTAL

Equimolar amounts (13 nano-moles) of PC from soybean, fully hydrogenated PC from soybean, 16:0-18:1-PC and di-18:2-PC were spotted on the same plate, which was then developed in system 1 for 3 minutes. After exposure to iodine vapors the plate was

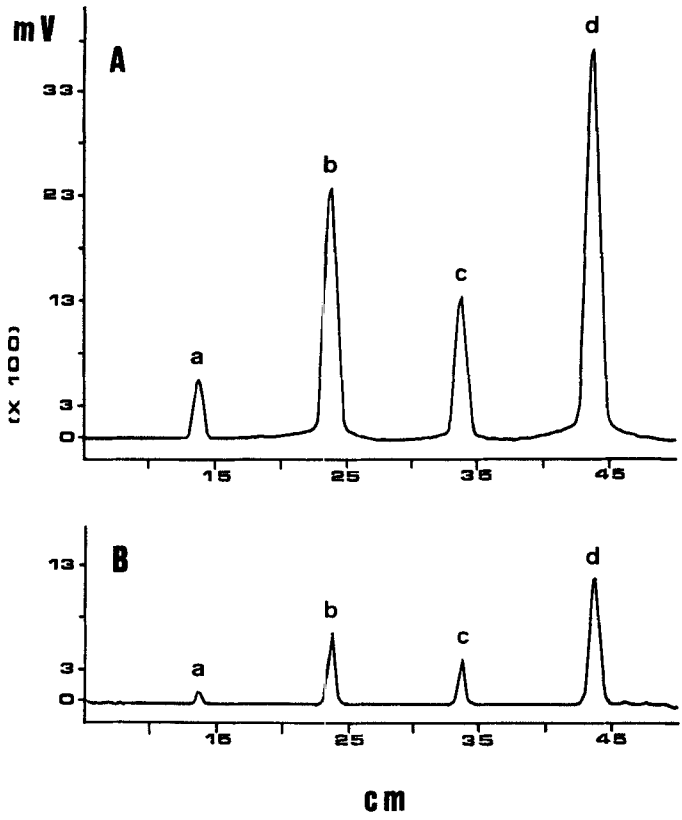

Fig. 1. Chromatogram of hydrogenated soybean PC (a), soybean PC (b), 16:0-18:1-PC (c) and di-18:2-PC (d), 13 nano-moles each. Solvent system; Chloroform / methanol / acetic acid / water (68 : 22 : 6 : 4, v/v/v/v). Scanned at 90 degree angle to the direction of development; 10 minutes (A) and 7 days (B) after iodine exposure.

scanned (Fig. 1A). The plate was left in a sealed container for one week after which it was scanned again (Fig. 1B). This procedure was then repeated twice. Photodesitometric detection was conducted 10 minutes and one week after each exposure. The detector response values of the entire series are shown in table

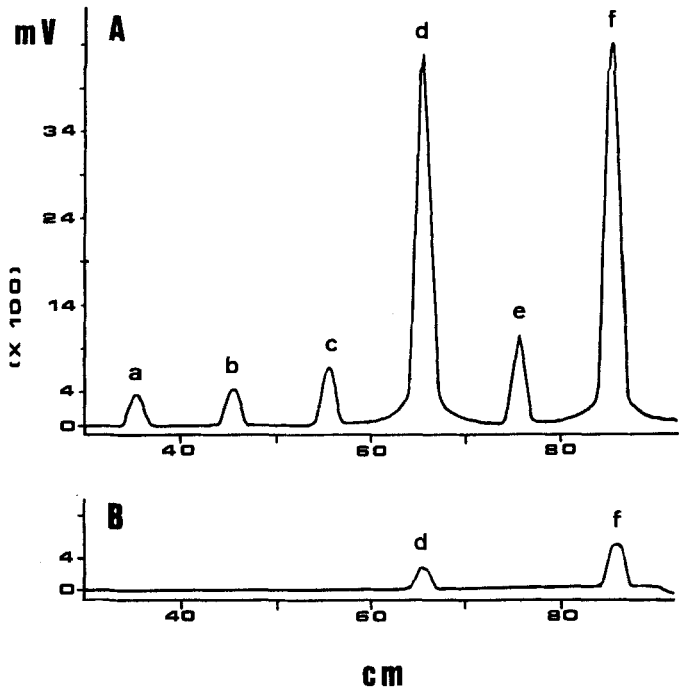

Fig. 2. Chromatogram of tri-10:0 (a), tri-12:0 (b), tri-16:0 (c), tri-18:2 (d), hydrogenated fish oil (e) and fish oil (f), 13 nano-moles each. Solvent system; Petroleum ether (b.p. 60-70 °C) / diethyl ether / acetic acid (79 : 20 : 1, v/v/v). Scanned at 90 degree angle to the direction of development; 10 minutes (A) and 7 days (B) after iodine exposure.

1. 80 to 85% of the iodine initially absorbed by the spots was eliminated 7 days after exposure. Equal amounts (13 nano-moles) of the different triacylglycerols were spotted on a HPTLC-plate. The plate was developed, using system 2, for 3 minutes after which it was exposed to iodine vapors and scanned in the densitometer (Fig. 2A). After one week in a sealed container, the plate was scanned again (Fig. 2B). The previous procedure (Fig.

TABLE 1

Area response values of phosphatidylcholin

	S - PC[a] hydrogenated	S - PC[a]	PC[b] 16:0, 18:1	PC Di-18:2
		Iodine exposure no. 1		
Day 1[c]	510	2835	1512	5103
Day 7	72	485	292	1097
		Iodine exposure no. 2		
Day 1[c]	516	2790	1491	5035
Day 7	69	481	297	997
		Iodine exposure no. 3		
Day 1[c]	501	2710	1499	5011
Day 7	72	477	287	997

[a]Phosphatidylcholine from soy bean.

[b]1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospatidylcholine.

[c]Measured 10 min. after removal from the iodine chamber.

1, Table 1) was adopted to this series of triacylglycerols. Only the spots containing unsaturated triglycerides *ie.* fish oil and trilinoleylglycerol, were detected 7 days after iodine exposure. The detector response values of the three week series are shown in table 2.

RESULTS AND CONCLUSIONS

Iodine vapor partitions to all investegated lipids regardless the degree of unsaturation. This means that even fully

TABLE 2

Area response values of triacylglycerols

	Fish oil	Fish oil hydrogenated	Tri-18:2	Tri-16:0	Tri-12:0	Tri-10:0
			Iodine exposure no. 1			
Day 1[a]	8726	1562	9035	1158	769	662
Day 7	312	-	114	-	-	-
			Iodine exposure no. 2			
Day 1[a]	8701	1601	8994	1123	777	668
Day 7	301	-	104	-	-	-
			Iodine exposure no. 3			
Day 1[a]	8731	1551	8900	1163	765	651
Day 7	701	-	104	-	-	-

[a]Measured 10 min. after removal from the iodine chamber.

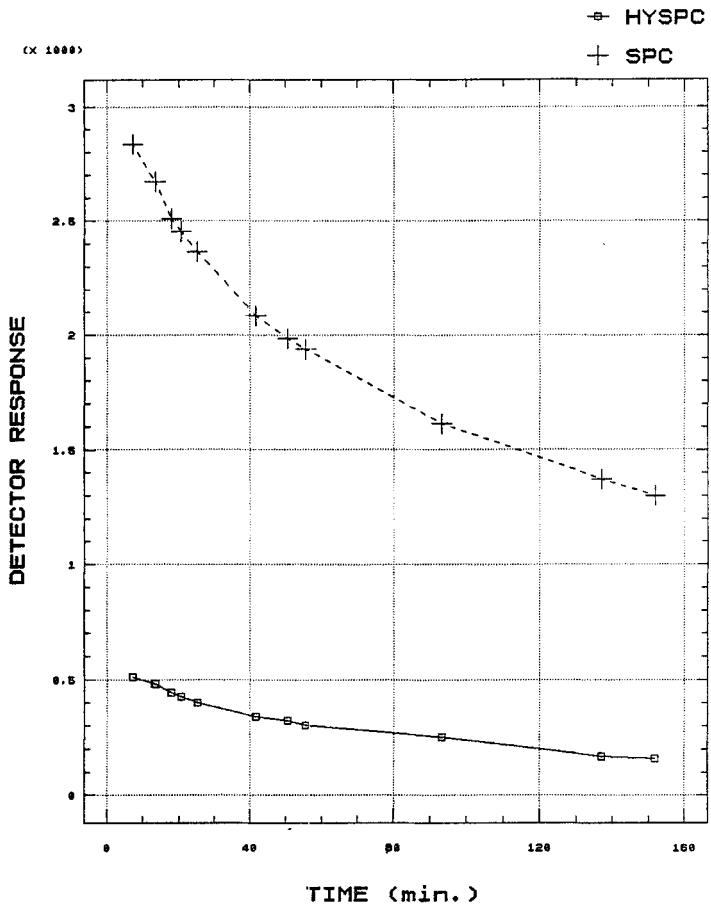

Fig. 3. Example of the decline in detector response over time. HYSPC; hydrogenated soybean PC. SPC; soybean PC.

saturated compounds such as hydrogenated PC (Table 1) and trilaurin (Table 2) can be detected by this procedure. The presence of double bonds in the acyl moities enhance the response compared to the saturated species. Soybean PC, fish oil and their respective hydrogenated equivalences may serve as examples. In

both cases the unsaturated compound has 5-6 times higher response than the saturated one.

The elimination rate of iodine from the spots seem to be dependent on the class of lipids rather than the degree of unsaturation. Thus, after seven days 15-20 % of the iodine still remains in all PC:s (Table 1). After the same time a smaller residue of 1-3 % could be detected in the TG:s, which means that it is detectable only in the polyunsaturated spots corresponding to fish oil and trilinolenoylglycerol (Table 2).

The obtained results indicate that the partition of iodine to the spots is due to several factors of which the degree of unsaturation seems to be the most important. Other factors are the nature of the acyl carrier, *i.e.* the particular lipid class, and the average length of the acyl groups.

It is clear from our results that unsaturation is not required to obtain iodine response, it is also clear that the elimination rate of iodine from the spots is independent of the number of double bonds. This means that this mode of detection is general and that it can be utilized for different applications in the lipid field.

The mechanisms determining iodine partition to lipids on HPTLC-plates merit further investegation.

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