Journal of Chromatography, 624 (1992) 11–19
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 035

Review

Advances in planar chromatography for the separation of food lipids

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ABSTRACT

A survey of the advances in planar chromatography for the separation of food lipids is presented. Techniques of planar chromatography [densitometry, preparative thin-layer chromatography (TLC) and TLC with flame ionization detection, etc.] together with applications from the areas of dairy, marine and plant lipids are discussed. Additives such as lecithins (emulsifying agents) are also considered.

CONTENTS

1. INTRODUCTION

Chromatography performed in open systems, such as on thin-layer chromatograms or on Chromarods, represents a dynamic and versatile alternative route to column liquid chromatography for analyses for food lipids. The advances in this technique over about the last 5 years in the separation of food lipids is discussed in this review.

Planar chromatography [1] on a silica matrix has traditionally been the method of choice for the separation of lipophilic substances. Since its appearance in the late 1950s it has been the most practical method capable of distinguishing between lipid classes [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), etc.]. Lipid analysis prior to planar chromatography was an imprecise and tedious task, performed by methods such as iodine value determination (measure of unsaturation) and elemental analysis for nitrogen, phosphorus or sulphur.

During the past decade it has been gratifying to see that planar chromatography, *i.e.*, thin-layer chromatography (TLC), has maintained its position

0021-9673/92/$15.00

as one of the major separation techniques in the area of lipid analysis [2,3]. The improvements in TLC scanners, equipped with, *e.g.*, zig-zag scanning functions, for *in situ* evaluation of planar chromatograms has led to enhanced accuracy in quantification and ease of operation (computer control). Sample requirements for quantification in TLC have been reviewed by Poole *et al.* [4].

As a consequence of the above, quantitative planar chromatography can no longer be claimed to represent an inexpensive analytical technique. Reproducible sampling devices and computer-controlled *in situ* scanners place TLC in the same cost range as high-performance liquid chromatography.

It is beyond the scope of this review to discuss the benefits of planar chromatography in comparison with other analytical techniques, or the latest instrumental developments in the field of TLC which have been extensively reviewed by Erdelmeier and König [5].

Analyses for lipids in foods by planar chromatography serve mainly to determine the lipid class composition [6]. Lipid classes can be divided into two sub-groups [7]: the complex, polar lipid classes and the simple, neutral lipids. The former yield at least three different substances on hydrolysis, such as free fatty acids, glycerol and phosphorylcholine from PC. The latter yield no more than two different groups on hydrolysis, *e.g.*, free fatty acids (FFA) and glycerol from triacylglycerols. The polar lipids are ubiquitous structural components in the membranes of all plant and animal tissues and they can also serve important functions as multi-purpose food additives.

Phospholipids contribute to the flavour and taste of cereals, legumes and meats. As natural phospholipids contain mono-, di- and polyunsaturated fatty acyl chain, they are susceptible to oxidative deterioration which might result in phospholipid residues with off-flavors. Hydrolytic enzyme systems present in foods, *i.e.*, in the cellular membranes, will degrade phospholipids, producing free fatty acids and monoacyl phospholipid residues [8]. The level of free fatty acids, easily monitored by TLC, can be used as a measure of food deterioration.

Fish meats and oils, rich in polyunsaturates, are especially susceptible to auto-oxidative molecular degradation, which eventually leads to relatively low-molecular-mass carbonyl compounds with unpleasant smells. Lipid deterioration can be measured by monitoring aldehydic peroxidation (secondary oxidation products) with TLC in conjunction with densitometry [9].

2. FUNDAMENTALS OF LIPID ANALYSIS BY TLC

The fatty acid composition (O-acyl or N-acyl lipophilic chains) of polar and neutral lipids is usually determined (in the form of methyl ester derivatives) by gas chromatography (GC) and the cascade of molecular species, within defined underivatized lipid classes [neutral (NL) and polar lipids (PL)], is generally monitored by reversed-phase high-performance liquid chromatography (HPLC) or by high-temperature GC (only NL) between 280 and 360°C. Thus, planar chromatography is mainly utilized in normal-phase (silica matrix) applications and in group separations according to degree of unsaturation (silica matrix, impregnated with silver ions), *i.e.*, argentation chromatography. Both of these methods have also been used in preparative applications. A chromatographic description (R_F values) of some food lipids is given in Table 1.

Quantification of separated lipids in planar chromatography is performed either by *in situ* measurements (densitometry [10], radiochromatography [11]) or by tedious gravimetric methods which

TABLE 1

R_F VALUES OF SELECTED FOOD LIPIDS ON TLC IN VARIOUS SOLVENTS (AFTER REF. 40)

Solvents: 1 = chloroform–methanol–water (65:25:4); 2 = chloroform–methanol–acetic acid–water (50:30:8:4); 3 = chloroform–methanol–28% (w/v) ammonia solution (65:25:5).

Lipid component	$R_F \times 100$ in solvents		
	1	2	3
PI	23	47	11
PC	33	31	33
PE	62	83	41
PS	15	55	5
PA	74	–	5
SPH	16	18	22
DGDG[a]	62	–	–
MGDG[b]	77	–	–

[a] Digalactosyl diglyceride.
[b] Monogalactosyl diglyceride.

include scraping off the spots and subsequent extraction of substances from the silica matrix. Flame ionization detection (FID) measurements can be used in confunction with chromatography on silica-coated quartz rods, *i.e.*, Chromarods. This methodology is called TLC–FID, or simply Iatroscan after the instrument generally utilized (Iatron, Japan). Detector linearity with TLC–FID methods is better than that obtained in densitometric measurements, which is the major justification for this principle of planar chromatography.

3. DENSITOMETRY

Charring of spots on thin-layer chromatograms by, *e.g.*, sulphuric acid and heat has traditionally been the route to detection and is still widely used. However, this procedure can generate a high background signal when used in conjunction with scanning densitometry, partly because extraneous particles (dust) present in the air adhere to the silica surface of the plate, becoming charred together with the separated compounds, Further, this method is destructive and separated/purified lipids cannot be recovered.

Another way to detect phospholipid classes on planar chromatograms for further densitometric evaluation is to use a lipophilic fluorescent reagent in the mobile phase [12]. By this elegant procedure phospholipid amounts down to 0.29 μg could be detected and measured.

The problem of revealing lipid spots on TLC plates has been addressed by Olsson *et al.* [13]. The utility of iodine vapour for detection and subsequent densitometry was investigated. It was shown that iodine vapour partitioned reversibly to all lipid samples rested, even fully hydrogenated fish oil and hydrogenated soybean PC. The results indicated that the partition of iodine to lipid spots on the TLC plates was due to several factors, of which unsaturation seemed to be the most influential. Other factors were the nature of the acyl carrier and the average chain length of the acyl group. Further, the rate of elimination of iodine from spots appeared to be mostly dependent on the lipid class, rather than the degree of unsaturation. It was found that 15–20% of the iodine remained in natural soybean PC 7 days after removal of the plate from the iodine chamber.

It might be speculated that this procedure could be utilized in quantitative analysis, even though a steady state in the elimination of iodine does not seem to occur.

3.1. Two-dimensional TLC

Two-dimensional planar chromatography represents an efficient utilization of TLC plates in terms of resolution of complex mixtures found in foods and food additives. Two-dimensional chromatograms contain a large amount of information and, used in conjunction with a reproducible integration system, this information can be made available. The drawbacks of the technique are that the sample capacity is limited to one spot per plate and that quantitative *in situ* evaluation is cumbersome.

In a paper by Lam and Sequera [14], polar lipid classes [PC, PE, PI and phosphatidic acid (PA)] of soybean were model substances in the exploration of quantitative two-dimensional (2D) TLC, utilizing a Shimadzu CS 9000 U scanner in conjunction with QuantaScan 2D Analysis software. The results clearly showed that 2D quantification could only be used when the data were normalized to an internal standard. Even if the variations in the normalized 2D determinations were worse than those obtained in the regular densitometric evaluation of linear lanes of spots, it was a major accomplishment compared with, *e.g.*, gravimetric evaluation, which otherwise would have had to be employed.

Quantitative densitometric evaluation of 2D planar chromatograms seems to require considerable further development before it could be considered a convenient and reliable method. With the recent developments in computer-controlled image analysis, such tools should soon become available.

3.2. Optimization

Optimization of chromatographic performance has traditionally been performed on one variable at a time (stationary phase, ionic strength, pH, etc.), *i.e.*, with univariate methods. As essentially all variables interact in the complex chromatographic process, univariate methodology often fails to locate true optimum conditions (optimum spot resolution, optimum response, etc.) [15]. Optimization based on multivariate methods [16] can handle related variables in a rational and, thereby, time-efficient way. Optimization in planar chromatography has been reviewed by Siouffi [17].

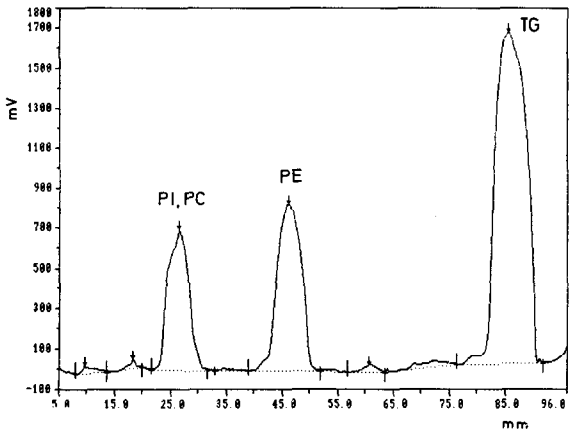

Fig. 1. Densitogram of a standard mixture (PI, PE, PC and TG) developed with original system; chloroform–methanol–acetic acid–water (68:22:6:4, v/v). (Ref. ref. 19, with permission).

Emulsifying agents, such as lecithins [18], are used as additives by the food industry in low-fat spreads and ice cream. An optimized TLC method was developed by Olsson *et al.* [19] for the analysis of the major lipid classes of natural lecithins, utilizing factorial design and multivariate optimization methods. An optimum separation, within the chosen experimental domain, between PI, PC, PE and the triacylglycerols (TGs) was obtained in 21 experiments. The results of this investigation are illustrated in Figs. 1 and 2, where the optimized TLC system (Fig. 2) is shown in comparison with the non-optimized original system (Fig. 1).

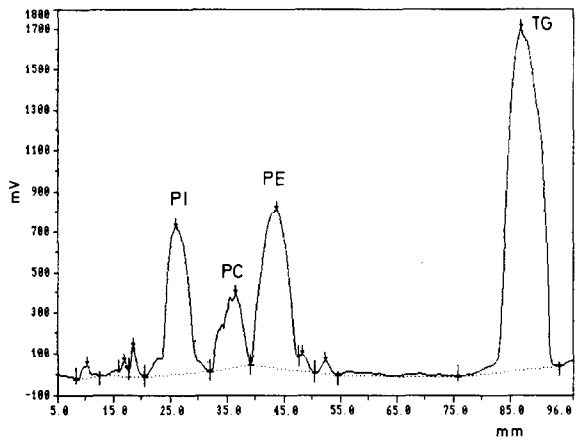

Fig. 2. Densitogram of a standard mixture (PI, PE, PD and TG) developed with optimized system: chloroform–methanol–1-butanol–25% (w/v) aqueous ammonia–ethyl acetate–0.25% (w/v) Ca^{2+} (80.2:44.1:4.9:5:5:6, v/v). (Ref. ref. 19, with permission).

4. PREPARATIVE TLC

Ready-to-use preparative TLC plates (20 × 20 cm, layer thickness 0.5 mm) have loading capacities (for, *e.g.*, crude lecithins) of *ca.* 30–35 mg, when applied as a band. This generally allows enough material, such as PE and PC, to be recovered for further analytical evaluation in a single TLC run.

4.1. Argentation chromatography

Ruminant milk fat consists of a complex mixture of TG molecular species, composed of fatty acids, ranging from C_2 to C_{24}, with odd-numbered, branched-chain and *cis–trans* isomers. This complexity presents a major analytical challenge.

Myher *et al.* [20] investigated a (25-year-old) molecular distillate of bobine butter oil (stored at 4 or -2°C), which was compared with a regular milk fat triacylglycerol fraction, enriched in C_4 and C_6 acids. The triacylglycerols of the distillate were purified by preparative TLC on silica gel. The TGs were then submitted to preparative argentation TLC (silica gel, impregnated with 15% $AgNO_3$), using chloroform as the developing solvent. The argentation TLC procedure resulted in seven bands, separated according to chain length, degree of unsaturation and the geometrical configuration of the double bonds. Each of the seven bands was recovered from the plate and submitted to polar-phase gas chromatography in conjunction with mass spectrometry (GC–MS). By utilizing GC–MS the molecular species pattern of a number of triacylglycerols in the TLC fractions was determined. The results clearly showed the complexity of the material and indicated that prefractionation was essential in order to obtain a reasonable resolution by GC.

It is possible that the food chemist would have benefitted more from the results of this investigation if it had been performed on a more readily obtainable material.

Argentation TLC was also used by McDonald *et al.* [21] to separate methyl esters of partially hydrogenated soybean oil. The aim was to identify *trans*-diene isomers formed during partial hardening of vegetable oils. In this instance, safflower oil was also studied.

Preparative TLC plates, coated with silica gel G (impregnated with 20% silver nitrate) were used to separate the methyl esters of the above-mentioned soybean oil. With chloroform as the developing solvent, six bands were recovered from the plates, of which each of the four most retained contained *trans*-diene isomers. The formation of *trans*-dienes during hydrogenation was confirmed by GC of the fatty acid methyl esters and by ^{13}C NMR spectrometry. It was established that over 20% *trans*-dienes were formed during the hardening procedure of soybean oil.

All papers that address the topic of *trans*-fatty acid analysis are of importance [22].

Kallio *et al.* [23] used preparative argentation TLC to fractionate the triacylglycerols of Baltic herring (*Clupea harengus membras*) flesh into eight bands, mainly according to degree of unsaturation. For this purpose, silica gel 60 G plates, impregnated with 8% $AgNO_3$ was used. The total lipid content of the flesh was 5.2% (w/w) and the proportion of neutral lipids thereof was 69.1%. The TGs comprised 61.5% of the neutral lipids. The intact TGs of the four most unsaturated TLC bands were scraped off the plate and subsequently analysed by capillary supercritical fluid chromatography (SFC) with FID. The acylcarbon numbers of the TGs were thus determined with reference to authentic TG standards. Also, identification of methyl ester derivatives of the triacylglycerol fatty acids was also performed by GC–MS.

4.2. Normal-phase applications

The lipid composition of fat globule membranes from butter milk and butter serum of Murrah buffalo and Red Dane and Sahiwal cross-breed cow milk was determined by Sharma *et al.* [24], utilizing TLC (silica gel G) for the class separation, iodine vapour for detection and gravimetric methods of evaluation. The fatty acid composition of total lipid was determined as methyl esters by GC. The lipid composition of the ruminant milk fat investigated is shown in Table 2. In this context it is of interest that animal milk sphingomyelins (SPH) contain remarkable levels of C22:0, C23:0 and C24:0 N-acylated fatty acids [25], which in bovine milk can be as high as 21–25% each [26]. Fatty acids from sphingomyelins are derivatized to methyl esters by acidic methanolysis.

The paper cited [24] unfortunately did not include a chromatogram showing the separation achieved. It is usually difficult to resolve phosphatidylinositol from sphingomyelin; the latter also often has a tendency to split into two peaks in normal-phase chromatography.

Preparative centrifugal accelerated thin-layer chromatography (CA-TLC), utilizing the Chromatotron, Model 7924T, was used by Bergheim *et al.* [27] to fractionate 300- and 500-mg (load limit) batches of crude soybean lecithin and egg yolk lecithin into lipid classes. The lipid class compositions were determined gravimetrically from the collected fractions ($n = 3$) and their fatty acid compositions were analysed as methyl ester derivatives by GC.

The egg yolk sample was reported to contain 14.2% PE and 66.8% PC by weight. A slower migrating fraction (fraction 5) than PC was identified as PI (3.6%). This was a surprising observation as PI generally is not present in such amounts in egg lecithin [28]; instead, sphingomyelin, which has similar retention characteristics to PI on silica adsorbents, occurs at that level [29]. The fatty acid composition of fraction 5 was not shown and it might be speculated that this is because the N-acyl group of SPH does not derivatize to fatty acid methyl esters by the alkali methanolysis procedure used. It was also surprising that no docosahexaenoic acid, 22:6 ($n - 3$), was detected in either PE or PC of the egg yolk lecithin. A typical level of this acid is 5%.

A *trans* isomer of oleic acid (18:1) was also suggested in the list of identified fatty acids of the soybean lecithin fractions. It might be speculated that this fatty acid is instead *cis*-vaccenic acid, 18:1 ($n - 7$), which is usually present in minute amounts (*ca.* 0.5–2%) along with the regular oleic acid, 18:1 ($n - 9$), in soybean phospholipids.

The isolated fractions were submitted to ^{1}H and ^{13}C NMR spectrometry, but unfortunately neither spectra nor tables were shown for these unexpected results.

Nevertheless, CA-TLC (also called "rotation planar chromatography") has potential in the analysis or fractionation of food lipids, mainly because it is a forced-flow method which to some extent combines TLC with the flow-controlling capabilities of HPLC [5].

TABLE 3

CHOLESTEROL (INTERNAL STANDARD METHOD) IN SEAFOOD SAMPLES DETERMINED USING IATROSCAN TLC–FID (AFTER REF. 39)

Sample	Cholesterol (mg per 100 g)[a]
Atlantic cod (raw)	15.3 ± 2.40
Atlantic halibut (raw, frozen)	24.8 ± 2.30
Atlantic lobster meat (cooked)	83.6 ± 5.02
Atlantic mussels (cooked)	88.0 ± 12.07
Atlantic shrimp (boiled, shelled)	140.3 ± 19.6

[a] Mean ± S.D. (n = 7–10).

method [38] for the determination of total sterols in natural samples and an Iatroscan TLC–FID method was conducted by Walton *et al.* [39]. The total sterols of the edible portion of boiled shrimp and lobster, cooked mussels and skin free muscle tissue of fresh raw cod and frozen raw halibut, were determined by the two different methods.

The rods were developed in hexane–diethyl ether–formic acid (97:3:1, v/v/v) for 40–45 min, after which all sterols from the food samples appeared as a single peak on the TLC–FID scan, permitting the quantification of total sterol content. The use of an internal standard (5-α-cholestane), which was completely resolved from the total sterol peak, minimized the effect of rod-to-rod variations (cholesterol range 0–20 μg; r = 0.943). Utilizing calibration graphs, estimates of the cholesteral content of the seafood samples were made (Table 3). It was discouraging to find that lobster, mussel and shrimp delicacies are comparatively high-cholestrol hazards!

The TLC–FID method utilizing an internal standard for normalization, developed by Walton *et al.* [39], apparently produces quantitative results in agreement with a comparative GC standard method, hence it can be used for the screening and routine determination of sterols in foods.

6. CONCLUSIONS

A major reason for using planar chromatography for the analysis of food lipids is its flexibility (multi-dimensionality, multi-detectability, etc.) and the ease by which analytical separations are transferred to a preparative scale (*ca.* 30-mg loadings or 300–500-mg loadings in CA-TLC) to generate material for further analytical TLC evaluations, or by techniques other than TLC. One-dimensional TLC is a workhorse for comparative analysis, in quality assurance, process monitoring and quality control. It is the author's experience that the combined use of two different chromatographic techniques (predominatcly TLC and HPLC) greatly enhances the certainty by which retention time-based identifications can be made. Also, the sensitivity of TLC in lipid analysis (in conjunction with suitable detection reagents) is often better than that for the commonly employed evaporative light-scattering detectors used in HPLC.

Open-system chromatography is finding continuous new utilizations for a range of applications of which the analysis of food lipids does not appear to be in a particularly evolutionary phase. It is difficult to see any specific trend as to where researchers currently are focusing their efforts. On the whole, the advances in planar chromatography for the separation of food lipids has more the character of improvements to already existing techniques rather than breakthrough innovative work.

7. ACKNOWLEDGEMENTS

The author expresses his gratitude to Drs. P. Kaufmann and B. G. Herslöf, Karlshamns LipidTeknik AB, for helpful discussions.

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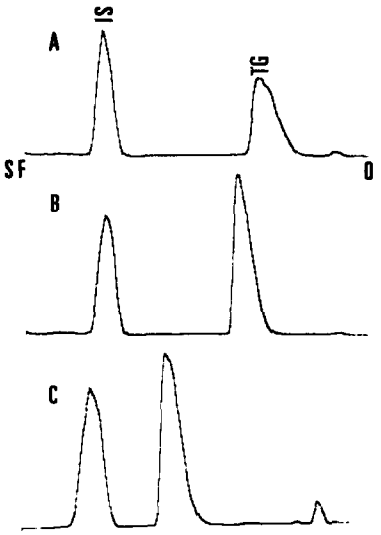

Fig. 3. Iatroscan TLD–FID showing the effect of hydrogenation on peak shape of fish oil TGs on Chromarods SIII. (A) Unhydrogenated. (B) Hydrogenated. Solvent system: hexane–diethyl ether–formic acid (97:3:1, v/v/v). (C) Unhydrogenated. Solvent system; hexane–chloroform (85:15, v/v), the chloroform containing 5% 2-propanol and 0.5% formic acid. IS = internal standard (wax ester); O = origin; SF = solvent front. (From ref. 34, with permission).

In general, the peak shape and the precision of peak areas were better for the rods spotted with hydrogenated lipids than for those containing the natural samples. This was explained by the fact that the number of different fatty acids was greatly reduced after completion of the hydrogenation process of the natural sample and hence the molecular species distribution narrowed down to only the saturates.

It was also interesting that hydrogenation decreased the resolution between PC and SPH, two classes which are structurally related (common polar head group, phosphocholine, and two lipophilic tails). This was due to the fact that the differences between the lipophilic tails of PC and SPH diminished on hydrogenation and hence separation was almost entirely achieved by the influence of the polar head group. Naturally, this was not beneficial to the separation.

A similar approach as that discussed above was successfully addressed by Ohshima and Ackman [35], utilizing hydrogenation of lipids prior to TLC–FID evaluation.

By modifying the construction of the flame ionization detector of the Iatroscan TH-10 Mark II instrument, its sensitivity, linearity and stability were improved [36]. This was done in order to monitor the phospholipid classes of Canola oil, the most widely used edible oil in Canada, during the various stages of oil processing. Przybylski and Eskin [36] adjusted the ion collector to 0.8 mm above the rods and installed a ball electrode, polarized as a detector cylinder inside the ion collector. As a result of these modifications, a tenfold increase in detector response was registered compared with the standard version of the Iatroscan analyser. It is believed that the improved response for phosphatidylserine (PS) and PI is due to better evaporation from the rod and improved combustion in the flame.

It is obvious that the performance of the Iatroscan analyser can be enhanced by technical modifications such as those described by Przybylski and Eskin [36]. Even though the improvement described above was excellent, there might be room left for further optimization of the detection system, possibly with the aid of chemometrics.

A number of factors (sample volatility, amount of substance, fatty acid composition, Chromarod movement rate through the flame, etc.) influence the response of different lipids in the flame ionization detector of an Iatroscan instrument. One way to partly solve these problems is to calibrate the system with standards identical with or similar to the analyte. This was the topic of a study conducted by Whitsett and Kennish [37].

The lipid class composition of Sockeye salmon muscle was determined by TLC–FID after calibration with standards similar to the fish sample in respect of the fatty acid composition. Results were presented for TGs, FFAs, PE and PC. An internal standard (pentacosane) was added to the natural samples which did not fully separate from the triacylglycerols on the Chromarods Type SII. Hence quantification was probably impaired. However, it was confirmed that the calibration sample should bear a close resemblance to the composition of the salmon muscle sample. The reader would also have benefitted from an indication of the variation of the method (for a natural salmon sample) rather than just the sample-to-sample variations listed in the paper.

A comparison between Kovacs *et al.*'s GC–FID

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