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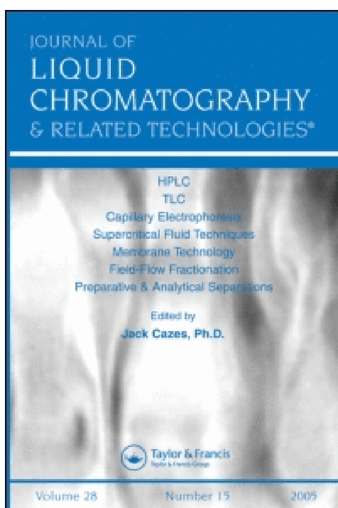

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Saha, S. K. and Das, S. K.(1996) 'A Simple Densitometric Method for Estimation of Polar and Non-Polar Lipids by Thin Layer Chromatography with Iodine Vapor Visualization', Journal of Liquid Chromatography & Related Technologies, 19: 19, 3125 — 3134
To link to this Article: DOI: 10.1080/10826079608015812
URL: http://dx.doi.org/10.1080/10826079608015812

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J. LIQ. CHROM. & REL. TECHNOL., 19(19), 3125–3134 (1996)

A SIMPLE DENSITOMETRIC METHOD FOR ESTIMATION OF POLAR AND NON-POLAR LIPIDS BY THIN LAYER CHROMATOGRAPHY WITH IODINE VAPOR VISUALIZATION

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ABSTRACT

A simple reflectance densitometric method for quantitation of polar and non-polar lipids was developed, by thin layer chromatography (TLC) or high performance TLC. The solvent systems used were a mixture of n-hexane:diethylether:glacial acetic acid (80: 20: 1, V/V) for non-polar lipids and chloroform: methanol:water (65:25:4, V/V) for polar lipids. After removal of solvents, lipid fractions were visualized by exposing the plate to iodine vapor. The plate was then covered with a glass plate and scanned at 365 nm. Different lipid fractions were quantitated by using appropriate reference standards. Iodine color is stable for at least three hours; the integrated area values of the lipid components are linear with their concentrations with a variation of 2 to 4 percent. The method of quantitation being nondestructive, the TLC plates may be used for further studies.

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I NTRODUCTION

Lipids include a variety of compounds, ranging from simple glycerides to complicated gangliosides. Thus, separation, identification and quantitation of these compounds, when isolated from natural sources, is difficult. With modern chromatographic techniques, lipids may be separated into different classes and these individual lipid classes may further be fractionated into different subclasses and into individual components.

The number of fractions that can be obtained, thus, is quite substantial and the task involved in estimation of these individual components by classical methods is enormous. Phospholipids are generally estimated by measuring phosphorous content,[1] sterols and their esters by measuring sterol[2] and glycerides by IR-spectroscopy or chromic acid reduction[3] method, etc.

Estimation of amino groups, carbohydrates, amino sugars, sphingosine, etc. are also used to quantitate different types of lipids. However, these methods are manipulatively difficult and are generally used after separation of the components by chromatography.

Incomplete separation and poor recovery often leads to erroneous results. Thus, *in situ* quantitation by densitometry, after fractionation of the lipid components by TLC, was attempted by a number of laboratories.[4-13] Most of these methods involve charring with sulfuric acid, followed by reflection[4-7] or transmission[8-12] densitometry.

Scanning in fluorescence mode[4] was also tried. However, these methods were useful only over a narrow concentration range, due to non validity[7-16] or oversimplification[6] of the Kubelka-Munk equation.[13,14] Diffusion or washing out of the sample during reaction with chromogenic reagents also interferes with assay by densitometry.[15]

Visualization by iodine vapor is widely used for detection of lipids fractionated by thin layer chromatography.[15,17] Since iodine is physically absorbed by lipids and remains as a "solution",[17] Kubelka-Munk theory is applicable for these spots. However, this method could not be used for quantitation of lipids because iodine starts evaporating from the plate as soon as the plate is taken out of the iodine chamber. Spraying the plate with acetic acid, starch, cyclodextrin, etc., to stabilize the iodine color was attempted by different workers with limited success.[18-20] Also, these treatments may render the plate unsuitable for scanning densitometry.[15]

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We have found that, if the chromatogram is covered with a glass plate immediately after exposure to iodine vapor, evaporation of iodine is delayed considerably and the plate can be scanned comfortably. We have standardized this method for quantitative analysis of lipids by scanning densitometry using a dual beam zig zag scanning densitometer. The results are described below.

MATERIALS

Apparatus

a) Spectrophotometer: Shimadzu UV-VIS double beam recording spectrophotometer, model UV-240 with OPI-4.

b) TLC Scanner: Shimadzu dual beam zigzag scanner, model CS-930.

c) Sample applicator: Camag Nanomat III with 100 nL nanopipette, 1 μL micropipette and holder and 20 μL micropipette.

d) Table centrifuge: Model Remi-8RC (Swing head, 5000 rpm).

e) TLC plate: (i) 20x20 cm silicagel 60 F-254 precoated plate with zone concentrating layers (E. Merck). Activated by heating at 110°C) for 1 hr before use. (ii) 20x20 cm laboratory coated plates with 0.4 mm thick layer of silicagel, E. Merck (60G:60HF_{254} :: 10:3,W/W) containing a 5cm wide sample concentrating zone of Kieselguhr G. Activated as above.

f) HPTLC Plate: 10x10 cm silicagel 60F-254 plates (Glass, E. Merck). Activated as above.

g) Developing Tank: Camag twin trough developing chamber.

Reagents

Phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, triolein and ergosterol were purchased from Sigma Chemicals, USA and squalene was a product of Fluka, AZ, Switzerland. The solvents used were of Lichrosolve (E. Merck) grade. Potassium dichromate (Analar) and ammonium molybdate were products of BDH (India) and ascorbic acid (GR) was from Sarabhai(M), India. Ground nut oil (arachis oil), cod liver oil and soyabean oil were purchased from a local market.

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Standard Preparation

For non polar lipids (a) triolein (20 mg.) ergosterol (4 mg) and squalene (2 mg) were dissolved in 10 mL of n-hexane. Further dilutions of solution (a) were prepared by taking (b) 3 mL, (c) 2 mL and (d) 1 mL of (a), respectively, and making up the volume to 4 mL with n-hexane.

For polar lipids (e) 4 mg each of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol were dissolved in 10 mL of chloroform. Further dilutions were prepared by taking (f) 3 mL, (g) 2 mL and (h) 1 mL of (e) and making up the volume to 4 mL with chloroform.

When not in use, the standard solutions were preserved at -20°C under nitrogen.

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Sample Solution

About 100 mg each of ground nut oil, cod liver oil and soyabean oil were accurately weighed and taken in separate Teflon®-lined screw capped graduated test tubes and dissolved in 10 mL of n-hexane. The lipid solutions were preserved under nitrogen at -20°C when not in use.

Mobile Phase

(1) Non polar lipids: A mixture of n-hexane:diethylether:acetic acid. (80:20:1, V/V).
(2) Polar lipids : A mixture of chloroform:methanol:water, (65:25:4, V/V).

PROCEDURE

For assay by TLC-scanning densitometry, 1 μL each of test solutions and standard solution (c or g) were applied as separate, compact spots 10 mm apart on an imaginary line 15 mm (on the sample concentration zone) from the bottom of the plate. The plate was developed up to 15 cm in usual way in a filter paper lined tank, previously saturated with mobile phase 1 (non-polar lipids) or 2 (polar lipids).

For HPTLC, 100 nL of the sample was spotted 5 mm apart on a line 10 mm from the bottom of the plate and the plate was developed up to 5 cm. After development, the plate was dried in a current of warm air from a hair drier for

10 minutes. The different lipid spots were visualized by exposing the plates to iodine vapor in an iodine chamber (at 30°C) for 2 minutes. The plate was immediately covered with a scratch-free clear glass plate about 1 mm thick and of same size (a used precoated glass plate from which adsorbernts were carefully removed and was thoroughly cleaned, may be used). The edges of the sandwiched plates were sealed with an adhesive tape and the plate was scanned in the densitometer using reflection mode at the scanning wavelength of 365 nm, background correction wavelength of 650 nm, slit 0.4 mm x 0.4 mm (0.05 mm x 0.4 mm for HPTLC) in zig zag mode with auto zero mode on. Other parameters were set as given in the operation manual of the instrument. The amounts of the different lipid components were estimated from the area values obtained.

For assay by spectrophotometry after TLC , laboratory drawn plates (B) were used. The standard and the samples (20 μL) were applied as separate 10 mm-wide bands, 15 mm apart, and developed with solvent system 1 or 2, as the case may be. The different lipid components were visualized by exposing the plate briefly (30 sec) to iodine vapor, marked with a needle and iodine was allowed to evaporate. Adsorbent containing the different components were scraped into separate, scrupulously cleaned 15 mL glass centrifuge tubes. Glycerides and squalene were estimated by a chromic acid reduction method[3] using 0.25% potassium dichromate in 36N sulfuric acid. Sterols were estimated by a ferric chloride colorimetric method[2] and phospholipids were estimated by measuring inorganic phosphorus[1] after digesting the adsorbent containing the lipid fraction with perchloric acid. Suitable silicagel blanks were used in all cases.

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To study the linearity of the integrated area values (from densitometer) with concentration of the different lipid fractions, standard solutions b, c, and d or e, f and g were chromatographed as described above and respective area values were determined.

RESULTS AND DISCUSSION

Absorption spectra of the lipid fractions (triglycerides), visualized by iodine vapor, showed an absorption maximum at about 365 nm. At 650 nm, the absorption was negligible (data not shown). Thus, we used 365 nm as scanning wavelength. Scanning at 650 nm may be used to compensate the noise caused by irregularities of the adsorbent layer when laboratory drawn plates are used. Precoated plates used by us did not require any such correction.

Table 1

Linearity of Concentrations of Different Lipids with Integrated Area Value Obtained by Densitometry after TLC and HPTLC

Lipid	Amt. Appl. (µg)	Integrated Area* Average	SD	'r'	Amt. Appl. (µg)	Integrated Area* Average	SD	'r'
Triglycerides	5	319686	24021	0.9991	0.5	9300	676	0.9992
	10	617990	27804		1.0	19403	1151	
	15	934956	37106		1.5	27901	2271	
Steroids	1	30576	3090	0.9973	0.1	2049	163	0.9944
	2	60481	1613		0.2	4127	158	
	3	91920	1650		0.3	6114	260	
Squalene	-	-	-	-	0.05	2296	95	0.9853
	-	-	-		0.1	4485	225	
	-	-	-		0.15	6657	502	
Phosphatidyl Inositol	2	20274	628	0.9980	0.2	2745	123	0.9983
	3	30681	570		0.3	4126	78	
	4	40828	732		0.4	5568	81	
Phosphatidyl Choline	2	54286	550	0.9995	0.2	12817	52	0.9919
	3	81430	670		0.3	17162	149	
	4	109169	1184		0.4	23168	913	
Phosphatidyl Ethanolamine	2	26406	543	0.9967	0.2	3523	59	0.9941
	3	40642	564		0.3	5023	69	
	4	52333	1028		0.4	7008	228	

* Average of 4 determinations.

When iodine vapor is used to visualize lipids on TLC plates, the background may also absorb iodine. This happens, generally, due to incomplete removal of developing solvents or contaminations of the plate by, e.g., laboratory fumes or simply by passive absorption iodine by silicagel. The colored background may cause a non-linear shift in baseline and error may occur in the integrated peak areas. In most of the cases, washing the plate by developing with methanol before activation and thorough drying of the plate after development reduces the background color. However, use of an auto zero system (background correction) also helps to rectify these problems.

Quantitative analysis by scanning densitometry is a very convenient method for assay of compounds fractionated by TLC. However, as was stated before, non-uniform density of the sample spot across the measuring beam and a nonlinear relationship between the sample concentration and optical density

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Table 2

Stability of Lipid Iodine Color* on TLC Plates

Lipid Class	Time (minutes) 0	60	120	180
Phosphatidyl Inositol	27984	27350	26990	26961
Phosphatidyl Choline	53389	53041	53002	53106
Phosphatidyl Ethanolamine	19825	19287	19240	19195
Ergosterol	30991	-	31118	30893
Triolein	344108	-	348700	344316

* Measured as Integrated Area

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Table 3

Assay Values[a] of Different Lipid Classes Determined by the Proposed and Conventional Methods

Sample	Lipid Class (b)	Proposed Method TLC Average	SD	HPTLC Average	SD	Conventional (c) Average	SD
Arachis Oil	Sterols	21.9	7.8	21.0	1.0	21.7	1.0
	TG	641.7	14.4	659.1	15.6	652.4	10.9
Cod Liver	Sterols	26.4	0.9	26.6	1.0	27.2	0.9
	TG	818.7	37.2	820.0	25.7	788.7	11.2
Soya Bean Oil	Sterols	24.4	1.5	23.2	1.8	24.3	1.0
	TG	720.8	26.1	722.2	28.6	713.9	19.7
	PI	15.7	0.4	15.5	0.2	16.2	0.4
	PC	68.1	4.4	68.4	2.7	69.8	1.3
	PE	20.2	0.4	20.0	0.3	19.2	0.1

a. In mg/gg oil, average of 4 determinations.
b. TG: Triglyceride; PI: Phosphatidyl Inositol; PC: Phosphatidyl Choline; PE: Phosphatidyl Ethanolamine.
c. See text for procedures.

(obtained in reflectance or transmittance mode) often leads to erroneous results, particularity when area and amount of sample differ from those of the standard. To solve these problems, we scanned the whole spot with a narrow beam of

light and linearized the relationship between reflection absorption and concentration by using a working curve linearizer, programmed by a microcomputer on the basis Kubelka-Monk theory.[15,21,22]

Table 1 shows the relationship between integrated area values and concentrations of different lipid fractions. The area values are linear with amounts of lipids used. Relative percentages of different lipid components, computed directly from the area values for a sample, may differ from those obtained after estimation of individual components using respective standards.

This happens because different components absorb iodine with different intensities. Different migration distances also cause the intensity to vary. However, within same lipid classes, the integrated area values obtained by both methods are comparable.

Intensity of color of lipids visualized by iodine vapor decreases rapidly if proper precaution is not taken. In our method, where the TLC plate is covered with a glass plate and then scanned, the color is stable, at least, for 3 hours (Table 2) whereby quantitation by scanning densitometry is reproducible.

Table 3 shows the content of different lipid components of some naturally occurring lipids determined by the proposed and some conventional methods. It is apparent that results obtained by the proposed densitometric method are essentially the same as those obtained by the conventional methods.

Using appropriate reference standards, the fractionated lipid components can be precisely determined with a CV of about 2 to 4%.

Densitometry is manipulatively much easier and, since same method is used for estimation of all the lipids, estimation of different lipid classes is much more simplified. Use of iodine vapor is a non-destructive method. Thus, after quantitation, we could successfully identify the different lipid components, particularly the phospholipids by using ninhydrine, Dragendorff's and molybdenum blue[19] reagents successively on the same plate.

ACKNOWLEDGEMENT

The authors are grateful to the Director, Central Drugs Laboratory, Calcutta, for providing facilities to carry out this work.

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Received April 1, 1996
Accepted April 25, 1996
Manuscript 4149

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