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Simple method of gradient elution in thin-layer chromatography of lipids

A number of techniques for gradient thin-layer chromatography (TLC) which are particularly useful for separating lipid mixtures having a wide range of polarity have been described[1,2]. This communication describes a simplified procedure for gradient elution which requires only the addition of a glass trough to the usual TLC equipment.

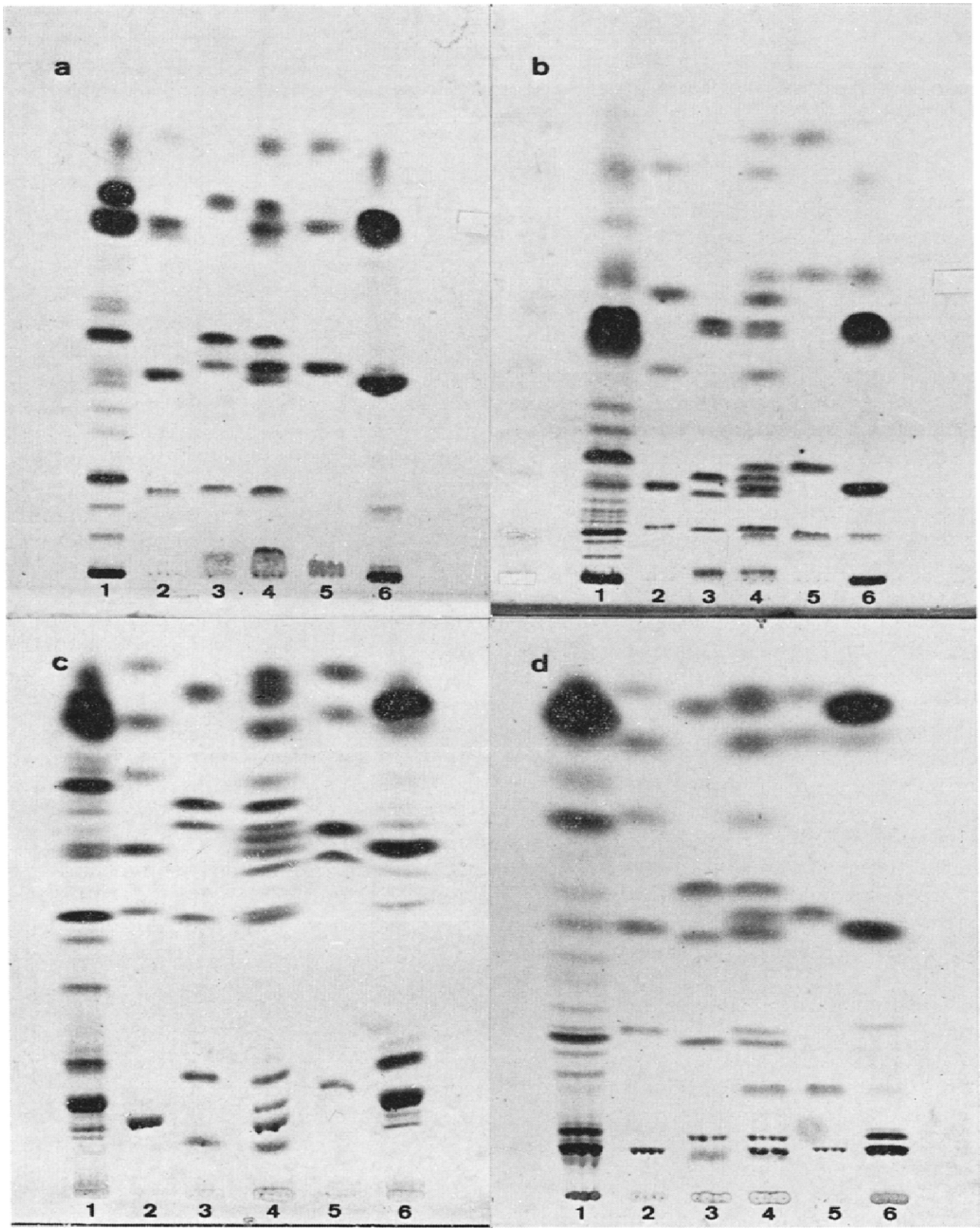
A tank and its filter paper liner are first saturated with a volatile non-polar solvent (the "atmospheric" solvent). The thin-layer plate is activated by heating, cooled to room temperature, and the samples applied. An excess of solvent is shaken onto the liner to help prevent desaturation while the tank is open. The trough is quickly placed on the bottom of the tank near the back. A relatively polar solvent ("trough" solvent) is added to the trough and the plate is placed in it. The trough solvent evaporates as it moves up the plate and is gradually replaced by atmospheric solvent. Both evaporation and influx are necessary to produce gradients sufficiently steep to be useful for most lipid analyses. The lipids to be separated are initially compressed into a thin band. As the polarity of the developing solvent mixture decreases, the more polar components lag behind.

Two examples each of neutral and total lipid separations are shown in Fig. 1 and a reasonably complete list of trough solvents potentially useful with this method is presented in Table I. Benzene, cyclohexane, and alkanes containing five to eight carbon atoms are good atmospheric solvents. Almost any combination of polar solvent in the trough and non-polar solvent in the atmosphere will produce a suitable gradient for neutral lipids. Also useful are trough solvents made by mixing two or more polar solvents, or a polar solvent and a non-polar solvent. Systems for the fractionation of total lipids are more difficult to devise (see note 4 below). One approach is the addition of about 0.5 volumes of methanol–formic acid–water (3:2:1) to one volume of moderately polar trough solvent, and about 0.3 volumes of methylene chloride, chloroform, or diethyl ether to one volume of atmospheric solvent. This gradient method has also been combined successfully with argentation TLC and reversed-phase TLC. In reversed-phase TLC the trough contains the less polar solvent. A system which has been used successfully is a stationary phase of 5 % paraffin oil on Kieselguhr G, a methanol atmosphere, and butanone–acetonitrile (2:1) as the trough solvent.

The factors affecting (or effecting) separation interact in a complex fashion. Although the method is best described as gradient elution, in certain aspects it resembles polyzonal, flux gradient, and vapor-programmed TLC. Moreover, unlike other gradient elution methods, the polarity decreases with time and the composition of the solvent at the bottom of the plate is essentially constant. The method is not suitable for column chromatography, and the theoretical analyses of gradient elution by NIEDERWIESER AND HONEGGER[1] and SNYDER AND SAUNDERS[2] are not entirely applicable.

The following comments are intended as practical aids:

(1) The trough solvent must be volatile enough to evaporate from the plate, but not so volatile as to evaporate completely from the trough during chromatography.


a
1 2 3 4 5 6
b
1 2 3 4 5 6
c
1 2 3 4 5 6
d
1 2 3 4 5 6

In use, its composition may change slightly, but adjustments in trough volume or geometry are seldom necessary.

(2) Temperature is important because of its effect on volatility, and must be reasonably well controlled. Each trough solvent is best suited to a restricted temperature range, *e.g.*, diethyl ether to -5 to $+5°$.

(3) In a series of atmospheric solvents of similar polarity (*e.g.*, hydrocarbons), increased volatility flattens the gradient. Perhaps the major factor is decreased fractionation of the developing solvent by the adsorbant; with higher-boiling hydrocarbons (*e.g.*, heptane) a region of very steep gradient appears somewhere on the plate, resulting in a non-polar developing solvent above this region (see Fig. 2). This region is undoubtedly homologous to the secondary front(s) observed in polyzonal development, and the effect is important for many separations achieved with the present method. Defects in the plate and sometimes sample composition can affect solvent fractionation, leading to irregular lipid fractionation. For instance, in Fig. 1c the "hump" evident in column 5 appears to be due to the presence of stearic acid.

(4) The greater the difference in polarity between atmospheric and trough solvents, the greater the gradient. However, if gradients are desired that are steep enough to chromatograph both phospholipids and neutral lipids, the miscibility of the two solvent mixtures must be considered. The production of two phases at some time during the run is not always a problem. Indeed, a second phase does appear just above the origin with the system shown in Fig. 1c. The second phase can be eliminated by decreasing the proportions of the two most polar components of the trough solvent, but the region of very steep gradient in the middle of the plate also disappears. The separation of lipids of intermediate polarity is thus enhanced at the expense of both the neutral and polar lipid groups. The separation resembles that shown in Fig. 1d, which exhibits a single phase throughout. Again, decreased solvent fractionation is probably responsible.

(5) The mesh of the adsorbant affects the rate of development, and therefore the steepness of the gradient. Silica Gel G (Merck or MN) on 200-mm plates will produce approximately the same total gradient with a certain solvent system as does the finer mesh Adsorbosil 3 on 100-mm plates.

(6) Increasing the length of run by using a longer plate generally gives improved resolution only for lipids that chromatograph high on the shorter plates. However,

Fig. 1. Gradient elution chromatography of neutral lipids (a and b), and total lipids (c and d). Solvent systems are: (a) ethyl acetate in benzene; (b) diethyl ether in hexane–methylene chloride (10:1); (c) ethylene chloride–dimethoxyethane–methanol–formic acid–water (5:5:5:1:1) in heptane–methylene chloride (3:1); (d) hexane–2-propanol–water (5:7:1) in hexane. Plate b was chromatographed at 5°, the rest at room temperature (20–24°). Samples for a and b are: (1) total lipid extract of female *Ascaris lumbricoides*; the complexity of the neutral lipids of this nematode is due mostly to a large number of closely related glycoside esters; (2) in descending order, the compounds chromatographed are: cholesterol palmitate, cholesterol acetate, α-tocopherol, cholesterol, and 1-octadecylglyceryl ether; (3) triglyceride (double band of tristearin and tripalmitin, see 1b), 1,3-distearin, 1,2-distearin, 1-monostearin, and glycerol; (4) mixture of 2, 3, and 5; (5) squalene, methyl stearate, octadecanol, and stearic acid; (6) Total lipid extract of rat liver. Samples for c and d are identical except that 2 also contains sphingomyelin (lowest band), 3 contains phosphatidylethanolamine (second lowest band), and 5 contains phosphatidylcholine (lowest band). Plates 80 × 100 mm (projector slide cover glasses) coated with 0.2 mm layers of Adsorbosil 3 were developed to the top (about 20 min) with 2 ml trough solvent and 20 ml atmospheric solvent (tank volume 1 l). Plates were visualized by spraying with conc. sulfuric acid containing 3% conc. formalin, and charring on a 200° hot plate. Adsorbant and standards were purchased from Applied Science Laboratories, Inc., State College, Pa., U.S.A.

TABLE I

TROUGH SOLVENTS FOR USE FROM 0° TO 25°

The criteria for inclusion are volatility and low cost. Solvents are listed in order of their boiling points. Physical constants are those given in the *CRC Handbook of Chemistry and Physics.*

Solvent	*Mol. wt.*	*b.p.*[a]	ε[b]	*Solubility in water*[c] (*g*/100 *g*)	*Notes*[d]
Furan	68	32		"i"	ether
Methyl formate	60	34	8.5	30^{20}	ester
Diethyl ether	74	35	4.3	7.5^{20}	ether
Propene oxide	58	35		65^{30}	epoxide
Methylene chloride	85	40	9.1	2^{20}	alkyl chloride
Dimethoxymethane	76	44		d	acetal
Ethyl formate	74	54			ester
Diethylamine	73	56		∞	secondary amine
Acetone	58	57	21	∞	ketone
Methyl acetate	74	57	8.5	32^{20}	ester
Chloroform	119	61	4.8	1.0^{15}	alkyl chloride
Methanol	32	65	33	∞	alcohol, PLS
Tetrahydrofuran	72	66		∞	ether
Diisopropyl ether	102	68	3.9	$c.2^{20}$	ether, readily forms peroxides
Ethyl acetate	88	77	6.0	8.6^{20}	ester
1,3-Dioxolane	74	78		∞, d	acetal
Ethanol	46	78	26	∞	alcohol
n-Butylamine	73	78		∞	primary amine
2,2-Dimethoxypropane	104	80		d	ketal
Butanone	72	80	19	50^{20}	ketone
Methyl acrylate	86	80			ester, unsaturated
Acetonitrile	41	82	39	∞	nitrile, PLS
2-Propanol	60	82	18	∞	alcohol
tert.-Butanol	74	83		∞	alcohol
Diisopropylamine	101	84		8^{20}	secondary amine
1,2-Dimethoxyethane	90	85		∞	diether
Isopropyl acetate	102	89	5.6	3^{20}	ester
Triethylamine	101	90	3.1	1.5^{20}	tertiary amine

The following solvents may be used at room temperature when mixed with compounds listed above:

Water	18	100	80	∞	excellent solvent for non-lipids, PLS
Formic acid	46	101	48	∞	carboxylic acid, PLS
Nitromethane	61	101	39	10^{20}	lowest boiling nitroalkane, PLS
1,4-Dioxane	88	102	3.0	∞	diether
Pyridine	79	115		∞	tertiary amine
Acetic acid	60	118	6.2	∞	carboxylic acid

[a] Although vapor pressure is the important parameter, boiling point data are readily found for all these solvents. B.p. is misleading only for alcohols, whose volatility it underestimates.

[b] Dielectric constant is a convenient measure of "polarity" (elution power), but weighs hydrogen-bonding properties too lightly. Hence diethyl ether is more polar than methylene chloride. Solubility in water, and comparison of mol. wt. and b.p., afford other estimates of polarity.

[c] i = insoluble; d = decomposes.

[d] PLS = poor lipid solvent: does not by itself dissolve triglycerides sufficiently to prevent severe streaking during TLC.

the relative order of separation of different lipids (*e.g.*, diglycerides and sterols) is not necessarily constant during a run.

(7) Layer thickness exerts considerable effect because solvent efflux and influx vary with surface area, while developing solvent volume varies with layer volume. Thus, thicker layers give shallower gradients. Uniform layers are obviously important unless layer thickness is intentionally varied.

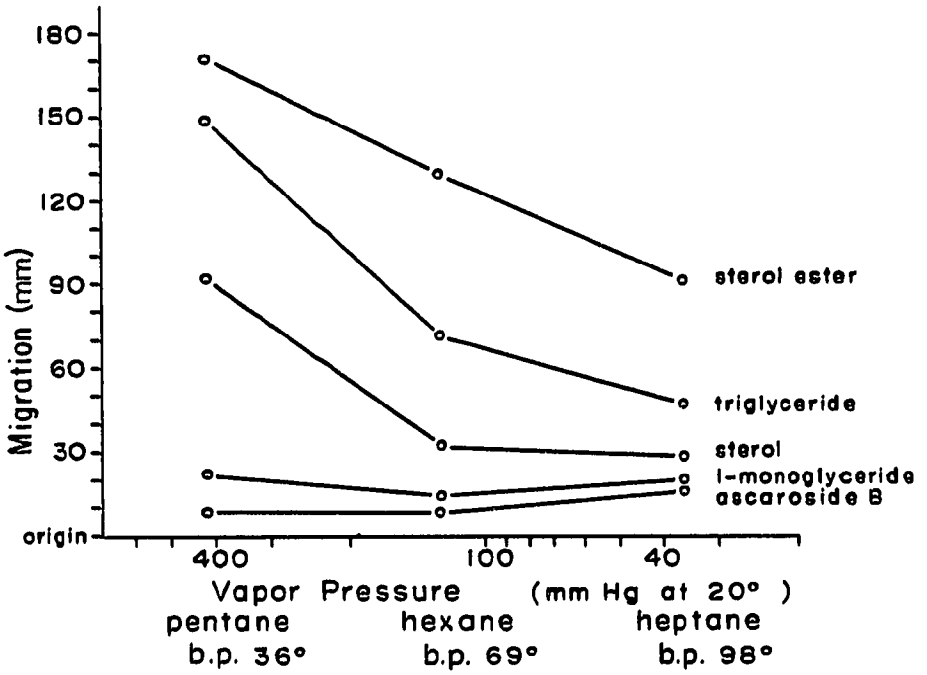


Fig. 2. The relation between the volatility of atmospheric solvent and the migration of lipids. Plates 200 mm long and coated with 0.25-mm layers of Silica Gel G (Merck) were developed to the top with ethyl acetate as trough solvent and the atmospheric solvent indicated.

Despite a large number of interacting variables, the separations are highly reproducible if the parameters are controlled. For some purposes the advantages of a continuous gradient, single development, and simple procedure may outweigh the advantages of direct control over solvent composition in multiple development systems or in continuous gradient systems requiring relatively complex apparatus.

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Department of Zoology, University of Massachusetts, Amherst, Mass. 01002 (*U.S.A.*) GEORGE E. TARR

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