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Note

Two-dimensional thin-layer chromatography technique for use in lipid analysis

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Two-dimensional thin-layer chromatography (TLC) is a technique which has had limited application except in certain fields such as amino acids, carbohydrates and phospholipids[1,2]. While many reported applications rely on the use of two different solvent systems acting on the same support system, combinations of normal, reversed and silver nitrate coated phases have also been reported. Thus Bergel'son *et al.*[3] separated isomers of unsaturated fatty acids, first on silica gel coated with dodecane, and then, after coating the plate with silver nitrate, in the other direction on the silver nitrate coated silica gel. Kaufman and co-workers[4,5] separated mixtures of lipids first by class of compound on silica gel, then coated the plate with dodecane or paraffin, and separated individual classes by carbon number in the second direction. More recently, in a rather novel procedure, mercuric adducts were separated by degree of unsaturation in one direction, the adducts decomposed, the plate impregnated with paraffin, and partition chromatography carried out in the second direction[6].

The impregnation procedures used in previous methods are somewhat time consuming, and not completely reproducible. A recent development has overcome some of these problems. Multi-K CS5 plates consist of a strip of octadecyl-bonded silica gel along one side of a commercial silica gel plate. Thus, separations can be carried out by reversed phase, followed by normal adsorption chromatography in the other direction. This system has been used to confirm the presence of morphine in urine, to fingerprint lubricating oils, and to identify and quantify sulphonamides[7].

It was considered possible that a modification to these plates would enable oils and fats to be characterised. By initial separation on the reversed-phase strip, impregnation with silver nitrate and development in the second direction, a fingerprinting of the triglycerides of the oil could be obtained, and a semi-quantitative estimation of individual triglycerides made. A method was therefore developed, the effect of possible interferring substances investigated, and various oils separated using the system.

EXPERIMENTAL

Reagents

Multi-K CS5 plates were obtained from Whatman, marker triglycerides from

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Sigma, and other chemicals from BDH. Ethanol-free chloroform was obtained either by washing four times with 0.2 volumes of water, drying and distilling, or by standing over 10X molecular sieve for 10–12 days, and distilling.

Method

A 10-μl volume of a solution containing 20 μg/μl of sample and 5 μg/μl of marker triglycerides (triarachidin and trimyristolein) was spotted onto the reversed-phase band of the plate. The plate was developed three times along the reversed-phase band with dry acetone–acetonitrile (4:1, v/v). After the third development, solvent residues were removed from the plate. This was than plunged rapidly into a 10% (w/v) solution of silver nitrate in water–ethanol (50:50), removing after 20–30 sec, with excess solution removed by dragging a folded tissue lightly across the plate. After activation of the plate at 100°C for 1 h, it was developed once at right angles to the previous direction of development, using dried toluene–ethanol-free chloroform (1:1, v/v). Spots were visualised using the two-stage development technique of Dallas and Padley[8], except that plates, instead of being immersed, were sprayed with the two reagents [ammonium hydrogen sulphate solution (5%, w/v), then molybdophosphoric acid solution (7.5%, w/v) both in water–ethanol (50:50)]. Plates were dried at 100°C for 15 min after the first spraying, and again after the second, before finally developing the colour at 180°C for at least 40 min.

In order to identify the triglycerides, it was necessary to construct a grid, best drawn on a photocopy of the plate, taking care to mark minor components visible on the plate but not on the photocopy. The grid was drawn as a 10 by 10 rectangle with trimyristolein at the origin, and triarachidin at the corner diagonally opposite. The coordinates of individual triglycerides are defined as two digit numbers (ignoring decimal point), quoting the x coordinate first, *i.e.* the centre of the grid would be 50/50.

RESULTS AND DISCUSSION

Development of method

Various solvent systems were investigated for the reversed-phase separation used above. For oils and fats containing largely triglyceride, of carbon number 48 and above, the triple development with acetone–acetonitrile (4:1) was found to give the most satisfactory separations. When fats such as palm kernel or coconut oil, containing mainly glycerides of carbon number below 48, were studied, however, a mixture of acetone–acetonitrile (3:2) was found to be most satisfactory.

The application of silver nitrate to the plate by dipping was preferred to application by elution up the plate, as it appeared to give a more even distribution of silver, and was quicker. The solvent system of the subsequent separation had previously been used many times for similar uni-dimensional separation systems[8].

The optimum sample loading for good separation was found to be 200 μg. This could lead to difficulties in detection of minor components of triglyceride mixtures and necessitated use of the most sensitive method of detection. Thus the ammonium hydrogen sulphate–molybdophosphoric acid spray was used, though this did, on occasions, give low results for saturated triglycerides. In the unusual cases where large proportions of trisaturated triglycerides are present, a longer period at

180°C is recommended, preferably at least 1.5 h to ensure maximum charring of these spots. Even then, it is possible that trisaturated components may be underestimated.

Possible interference from non-triglycerides

Because it was thought that the method might be useful for examination of fats as extracted from, for example, microbial sources, the effect of other components likely to be present was investigated. Using the same experimental conditions as those described above, spots containing the following were run through the system: (1) free fatty acid mixture, stearic acid–linoleic acid (1:1); (2) diglyceride mixture, 1-palmitoyl-3-stearoyl glycerol–1-stearoyl 3-oleoyl glycerol–dioleoyl glycerol (1:1:1); (3) monoglyceride mixture, monopalmitoyl glycerol–monooleyl glycerol (1:1); (4) unsaponifiable matter extracted form microbial oil from *Candida* 107; (5) phospholipid mixture, phosphatidyl choline–phosphatidyl ethanolamine (1:1).

The R_F values of the components of the above mixtures are given in Table I. As can be seen, few travel far in the adsorption phase, and comparison with triglycerides of known composition indicates that, for most fats of normal composition, these compounds are unlikely to interfere with triglyceride spots. The only exception might be fats containing short to medium length triglycerides, where high levels of diglycerides might interfere.

TABLE I

R_F VALUES OF POSSIBLE INTERFERING COMPOUNDS IN SYSTEM

Compound	*R_F values*	
	Reversed phase	*Silver nitrate phase**
Stearic acid	0.98	0.10
Linoleic acid	0.95	0.02
1-Palmitoyl-3-stearoyl glycerol	0.93	0.00
1-Oleoyl-3-stearoyl glycerol	0.88	0.06
1,3-Dioleoyl glycerol	0.75	0.02
1-Palmitoyl glycerol	0.44	0.04
1-Oleoyl glycerol	0.88	0.00
Unsaponifiable matter	Various spots	No spot
Phosphatidyl choline	Spots remain at origin	
Phosphatidyl ethanolamine	Spots remain at origin	

* R_F from interface of reversed phase to adsorption phase.

Markers for grid

A grid with two marker compounds was found necessary because of variations in absolute R_F values. A number of marker compounds were considered. Triarachadonin was of low oxidative stability, tribehenin had low solubility in suitable solvents at ambient temperatures, and trilaurin was less sensitive to charring reagents, and occasionally was difficult to detect. A compromise was reached, whereby trimyristolein and triarachidin, absolute R_F values (0.94, 0.02) and (0.00, 0.81) were chosen. It is possible that for fats with high levels of partial glycerides, the trimyristolein spot could be misidentified, and therefore care should be taken in these cases. Triarachidin

had an R_F value of 0.00 on the reversed-phase portion of plate, and attempts to elute triarachidin by using other solvent systems such as acetonitrile–tetrahydrofuran–hexane (57.9:31.9:10.2), as has been used in high-performance liquid chromatography when similar problems arose[9], were unsuccessful. Few natural fats have appreciable concentrations of these triglycerides, and so this is not normally a problem. A typical separation of a cocoa butter substitute fat, showing the grid, is given in Fig. 1.

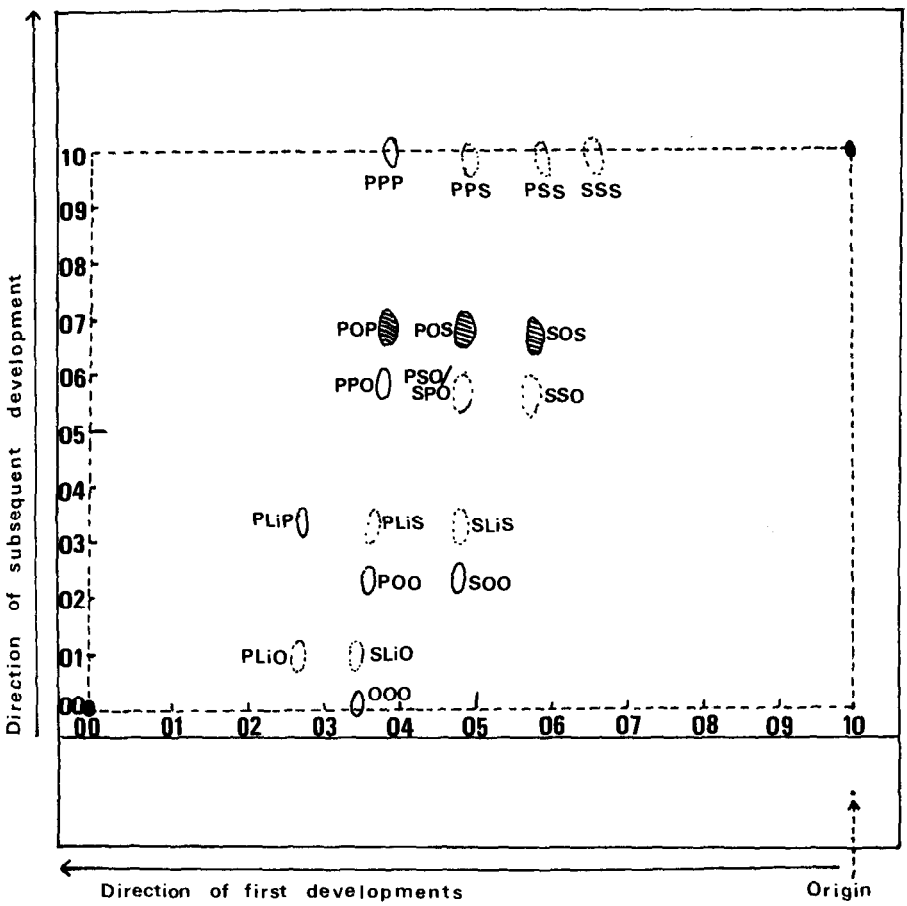

Fig. 1. A typical separation of a cocoa butter substitute fat (Coberine) on a Multi-K TLC plate.

Application to sample of fats

Three samples of fats were examined by the method, palm oil, a microbial oil extracted from *Candida* 107, and Coberine (a commercially available cocoa butter substitute obtained by blending of vegetable fat fractions). In order to determine reproducibility, the Coberine sample was run three times, twice simultaneously in the same tank, and once separately.

The results are given in Table II. As can be seen, retention values are not very reproducible when TLC is carried out at different times, but reproducible when car-

TABLE II

GRID REFERENCES OF MAJOR TRIGLYCERIDES OF FATS EXAMINED

ND = None detected; P = palmitic acid; S = stearic acid; O = Oleic acid; Li = Linoleic acid.

Sample	*Triglyceride*								
	Run	*POP*	*POS*	*SOS*	*PPO*	*POO*	*OOO*	*PLiP*	*PLiO*
Coberine	1*	26/52	33/51	40/53	ND	ND	ND	ND	ND
	2*	27/52	33/51	40/53	ND	ND	ND	ND	ND
	3	34/58	40/58	47/58	ND	ND	ND	ND	ND
Palm oil	–	36/63	41/60	ND	35/49	33/16	31/02	27/28	26/05
Microbial oil	–	34/49	38/48	ND	ND	33/11	30/02	27/19	25/03

* Run simultaneously.

ried out simultaneously in the same tank. The reason for the large variation in the reversed-phase development would probably be that the small differences in R_F which do occur are amplified when development is carried out three times. In order that misidentification of spots does not occur, a plate spotted with a standard fat, or triglyceride mixture, should be run simultaneously to the sample. An alternative would be to carry out a gas-liquid chromatographic analysis of triglycerides by carbon number.

CONCLUSIONS

The method, as developed, separated individual triglycerides well. Variations in retention indices necessitated the simultaneous development of a plate spotted with a fat of known composition. This method provides a useful rapid semi-quantitative tool for the chemist involved in triglyceride analysis, and, with different solvents, possibly in other fields also.

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