Journal of Chromatography, 519 (1990) 349–358
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 651

Dynamic impregnation of silica stationary phases for the argentation chromatography of lipids

K. AITZETMÜLLER* and LIRENY A. GUARALDO GONCALVES[a]
Institute of Chemistry and Physics, Federal Centre for Lipid Research, Piusallee 68-76, Münster (F.R.G.)
(First received February 19th, 1990; revised manuscript received May 9th, 1990)

ABSTRACT

Homogeneous silver-impregnated thin-layer chromatographic (TLC) plates were obtained in a dynamic manner by chromatographing an acetonitrile solution of silver nitrate on ready-made silica TLC plates. Plates obtained in this manner are very reproducible. Separations according to the number of double bonds in the individual triglycerides on dynamically impregnated plates are equal to or better than those obtained on silver nitrate TLC plates prepared in the conventional manner. The plates are easier to handle, and savings in silver nitrate result in comparison with the dipping procedure. It is possible to impregnate a plate simultaneously with both phloxin and silver in one run, the immobilized phloxin then serving as a fluorescence detection agent for a subsequent triglyceride separation and for semi-quantitative densitometry. Silica high-performance liquid chromatographic columns can also be impregnated with silver nitrate in a similar dynamic manner.

INTRODUCTION

Argentation thin-layer chromatography (TLC) is a well established and important method for the separation of unsaturated lipid species such as triglycerides, wax esters, fatty acid methyl esters and steroids [1–10]. There are hundreds of papers describing this technique, its selectivity and its applications with lipids, and a number of reviews have appeared [3].

Usually, the silver nitrate ($AgNO_3$)-containing silica plates used for this technique are prepared in the laboratory by spreading an aqueous (or aqueous–alcoholic) slurry of silica on glass plates [1–5]. The $AgNO_3$ is dissolved in the solvent mixture used to prepare the slurry, and the amount of $AgNO_3$ used is calculated as, *e.g.*, 5, 10 or 15% by weight based on the weight of silica used. Plates prepared in this way are ready for use after drying and activation.

As $AgNO_3$–silica plates prepared manually in this traditional way are often less than ideal with respect to homogeneity, resolving power and mechanical stability, various other methods of impregnating the more homogeneous and standardized commercially available ready-made pre-coated silica TLC plates have been sought.

[a] Present address: Universidade Estadual de Campinas–F.E.A., Caixa Postal 6091, 13.083 Campinas SP, Brasil.

0021-9673/90/$03.50

It has been reported [6–10] that good-quality $AgNO_3$ silica TLC plates can be produced from commercially available ready-made TLC plates simply by dipping the whole plate in a solution of $AgNO_3$ in water, ethanol, methanol or acetonitrile. The plate is dipped into the solution for about 20–100 s, then quickly removed, allowed to dry and activated. A similar procedure was used in the authors' laboratory during the 1970s [11].

Another procedure is to spray pre-coated silica plates evenly with a solution of $AgNO_3$ in water or in other solvents [12,13].

In previous work, the best results were achieved by dipping pre-coated Merck TLC plates into a 10–20% solution of $AgNO_3$ in acetonitrile for about 30 s. In our hands, plates produced in this way gave better separation results than manually prepared plates [11]. However, the plates still exhibited a number of minor disadvantages, so that other ways of preparing silver-containing plates were sought.

Another impregnation procedure has been in use for some years that avoids most of these disadvantages, and it is described in detail in this paper.

EXPERIMENTAL

Dynamic impregnation of TLC plates

Ready-made TLC plates are impregnated with $AgNO_3$ in a dynamic and reproducible way as follows. We use a TLC tank containing a small separate rectangular vessel (*ca.* 1 cm × 21 cm × 1 cm), the latter containing a 10–20% solution of $AgNO_3$ in acetonitrile (Fig. 1). (The tank may contain a piece of cardboard in another separate chamber or vessel with pure acetonitrile to saturate the tank volume

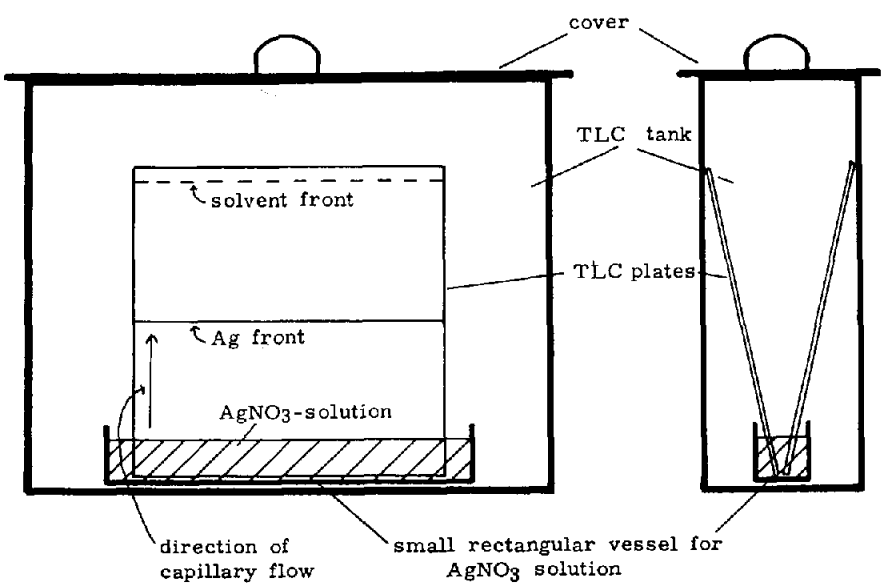

Fig. 1. Schematic diagram of equipment used for dynamic impregnation of commercial ready-made silica TLC plates.

with acetonitrile vapour. However, we prefer to impregnate the plates without chamber saturation.) The ready-made silica TLC plate is then placed in the small vessel containing the silver solution, which is allowed to rise by capillary forces as in ascending chromatography, displacing the air in the silica layer as it moves. Some time after the acetonitrile solvent front has reached the top of the plate, the plate is removed from the tank and allowed to dry. Because of the toxicity of acetonitrile vapour, the whole operation is carried out under a hood.

Silver is adsorbed from the acetonitrile solution by the silica on the plate and therefore the silver does not move all the way with the solvent front (Fig. 1). Plates prepared in this dynamic way will therefore contain $AgNO_3$ only on the lower two thirds to three quarters of the plate, and there is a separate "silver front" which is usually clearly visible. Although gloves must still be used when handling acetonitrile, the dry plates can safely be handled without gloves if the operator takes care to touch the plates only at the silver-free top.

In normal use, TLC plates are often cleaned or pre-washed (*e.g.*, by ascending chloroform–methanol) before applying the samples, to remove dirt, lipids and other organic material adsorbed from the air and packaging materials. With this type of dynamic impregnation, pre-washing of the plates is often not necessary because organic material is displaced by the acetonitrile solvent front. After detection, this displaced material is often seen near the solvent front.

Silver determination

For the determination of the silver distribution on the plates, impregnated plates were divided into narrow horizontal bands and the silica was scraped off and analysed for its silver content by atomic absorption spectrometry and/or potentiometric titration.

Thin-layer chromatography

TLC of triglyceridic fats was carried out as usual. Many solvent systems have been described, and for the best results in double-bond (DB) separations of triglycerides mobile phases containing benzene or toluene have often been used in the past [1–5]. Chlorinated solvents such as chloroform or dichloromethane and diethyl ether can also be used, often to achieve special effects, and always in mixtures with an excess of aliphatic hydrocarbon (hexane, heptane, isooctane or light petroleum). In this work, good results were obtained using hexane–toluene–diethyl ether (42:50:8, v/v/v) as the mobile phase for separations of triglycerides with 0–3 *cis* double bonds. For the more highly unsaturated triglycerides, more polar mobile phases, including mixtures containing increasing proportions of chloroform and/or ethanol, were used.

Spotting of the samples is best done in the form of narrow linear bands of length 2–10 cm using automatic equipment (Linomat IV, Camag). The amounts of sample used range from a few micrograms per spot up to 50 mg per plate. Bands of separated triglycerides can be made visible either by charring or by spraying with a fluorescent indicator, *e.g.*, 2′,7′-dichlorofluorescein [1–5,14] or phloxin [15,16] (see below). For the identification of the separated zones, palm oil or an interesterification mixture of triolein and tripalmitin was often used and was spotted on one side of the plate. The migration distances or R_F values of their major triglycerides (PPP, POP, PPO, POO and OOO) are then compared with those of the sample triglycerides. One has to take

into account, however, that overloading of major triglycerides, particularly in semi-preparative applications, may move that particular band higher up the plate.

Dynamic impregnation with both silver and phloxin

Phloxin (C.I. 45405) or Phloxine B (C.I. 45410) can also be used as an indirect fluorescence detection agent for lipids on silver-impregnated plates. Hammond [15,16] preferred phloxin for densitometric scans. We have now found that it is possible to impregnate ready-made commercial TLC plates simultaneously with both silver and phloxin in one ascending run. For this, we use a solution of 10% $AgNO_3$ plus 0.08% phloxin in acetonitrile.

After spotting triglyceride samples, silver/phloxin-impregnated plates are developed normally. The solvents regularly used for triglyceride argentation TLC do not move phloxin, and the separated triglyceride bands are immediately visible under UV light, after removing the plate and air-drying it under a hood. The technique described here for phloxin does not work with 2',7'-dichlorofluorescein. The phloxin present also permits indirect fluorescence densitometry of triglycerides [15–17].

In situ impregnation of high-performance liquid chromatographic (HPLC) columns

For the impregnation of silica HPLC columns with silver, we use the following procedure. A conventional silica HPLC column is mounted and heptane is pumped through it continuously. A large, 2-ml loop valve (Specac, Glasgow, U.K.) is mounted between pump and the injector, using wide-bore capillaries. The 2-ml loop is filled with a 10% solution of $AgNO_3$ in acetonitrile and this is injected onto the column as a 2-ml plug of immiscible acetonitrile solution within a stream of flowing heptane. The acetonitrile is pumped through the column, which is then rinsed by pumping heptane for another 15–30 min. The whole operation may be repeated. After this, the column must be reactivated by pumping heptane–diethyl ether mixtures until all the adsorbed acetonitrile is removed and the retention and separation of a test mixture (preferably oleic and elaidic acid methyl esters) is stable.

RESULTS AND DISCUSSION

Dynamically impregnated TLC plates

Among the disadvantages of the earlier dipping procedures were the following: (a) air was often trapped in the silica layer during the dipping process, leading to unequal wetting in parts of the plate; (b) the dipping, run-off and drying process could lead to inhomogeneous deposition of $AgNO_3$, including crystallization effects; (c) the dipping as such was not easy, as vessels of suitable size and material (to withstand both the solvents and the $AgNO_3$) were difficult to find, contamination occurred all over the hood or bench and gloves had to be used all the time to avoid contact with $AgNO_3$ whenever the plate was touched; and (d) relatively large volumes of solvent and large amounts of expensive $AgNO_3$ had to be used or were wasted.

With the dynamic procedure described here, it was of course of primary interest whether or not an even distribution of silver along the plate could be achieved by ascending impregnation. This could be expected if the solvent used does not readily displace silver from the active sites on the silica, so that an eventual saturation of active sites with silver would occur. Previous attempts with ascending impregnation using

aqueous solutions of $AgNO_3$ [14] have met with limited success; at least the technique has been neglected after these initial experiments.

The amount of silver on the silica TLC plates was usually *ca.* 11–13% of the weight of the silica when a 15% solution of $AgNO_3$ in acetonitrile was used for ascending impregnation without chamber saturation. Fig. 2 shows the distribution of silver (as percentage of silver calculated on the silica) as determined by potentiometric titration. For this, zones were removed at various intervals from the plates in the analysis of two different plates. The results indicate that silver is indeed fairly evenly and reproducibly distributed, although the level decreases slightly towards the silver front. This fairly even distribution of silver is found because adsorption with saturation of active sites occurs, because there are no air pockets (air is displaced) and because there is no run-off problem (the plate is equally wet, except for the lowest 1 cm in the $AgNO_3$ solution) when the plate is removed from the tank.

The impregnation process takes longer than the dipping or spraying procedures (*ca.* 30–60 min per plate), but is easier to carry out and requires much smaller volumes of $AgNO_3$ solution. Waste of expensive $AgNO_3$ and contamination of the workplace with silver is minimized. The plates can be stored dry in the dark and their mechanical stability is excellent. Results achieved with plates of this type are at least as good as those obtained with plates impregnated by dipping, and usually much better than those with laboratory-made plates prepared by spreading a slurry on glass. It should be kept in mind, however, that the silver ends at two thirds to three quarters of the height of the plate and that above the "silver front" no further separation due to the presence of silver can take place.

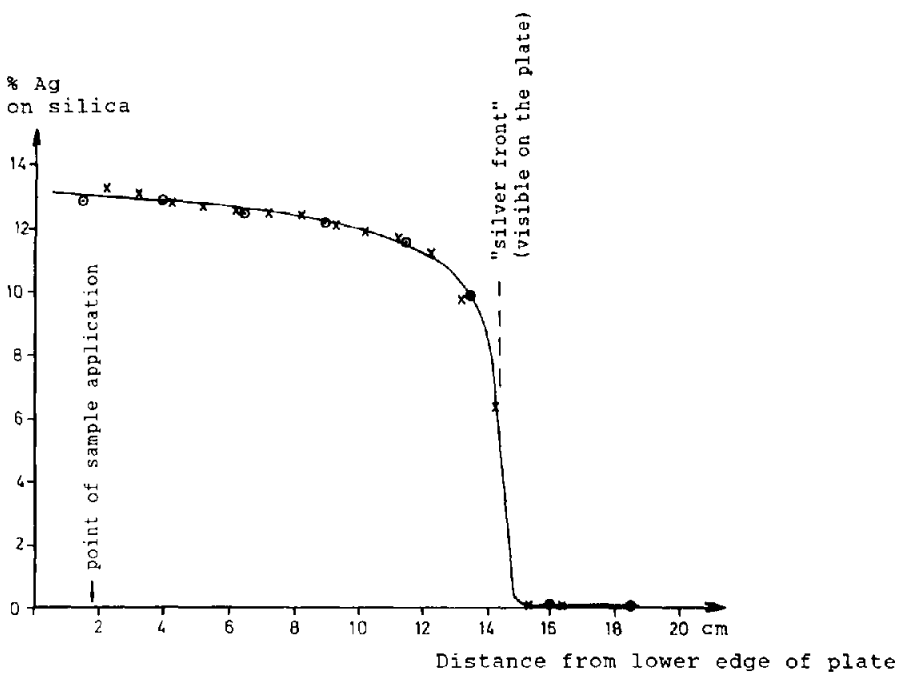

Fig. 2. Curve showing distribution of silver in two dynamically impregnated plates (○, plate 1; ×, plate 2) and relation of silver front to solvent front, as determined by experiment.

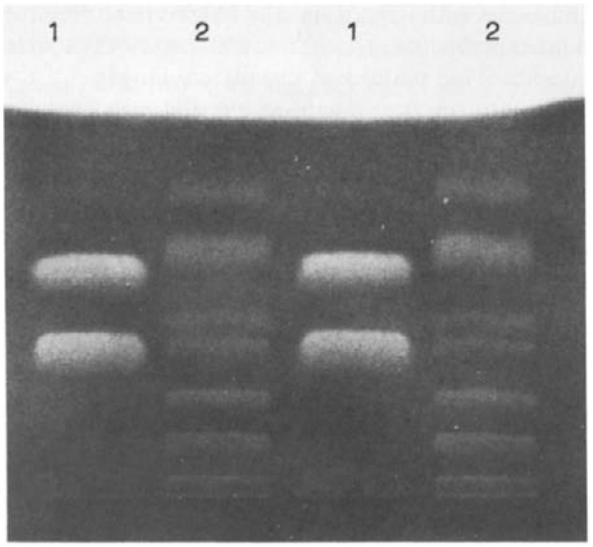

Fig. 3. Separations of elaidic (top) and oleic acid methyl esters (lane 1) and of a triglyceride test mixture (lane 2). The triglyceride test mixture consisted essentially of (from the top) SSS + PPP; PEP + PPE; SOS + POP; SSO + PPO; EEE; SOO + POO; and OOO (S = stearic, P = palmitic, O = oleic and E = elaidic acid residues). Total lipid applied: 100 μg per 3-cm band. Solvent: toluene–hexane (85:15, v/v), developed to the top of the plate. Detection with 2',7'-dichlorofluorescein.

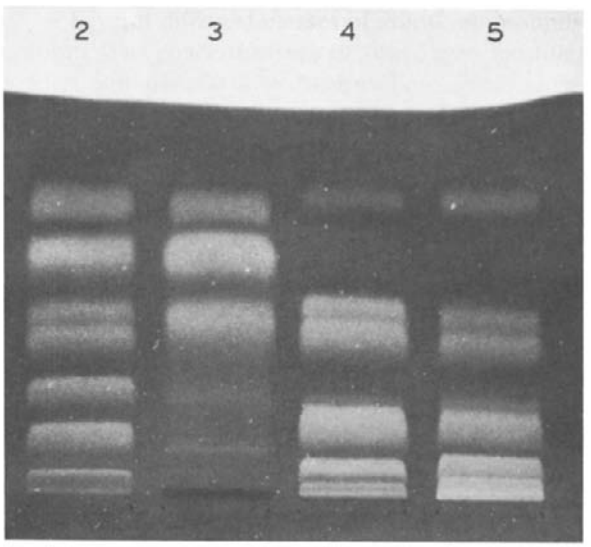

Fig. 4. Separation of various triglyceride mixtures and fat compositions. Experimental details as in Fig. 3. Lanes: 2 = as lane 2 in Fig. 3; 3 = elaidinized fraction of a palm oil; 4 = lower melting fraction of an interesterified mixture (*ca.* 2:1) of olive oil and hardened sunflower oil; 5 = interesterified mixture (*ca.* 3:1) of rapeseed oil and hardened sunflower oil.

With a number of mobile phases, the movement of triglyceride bands on the silver-containing part of the plate is faster than that on the silver-free part of the plate near the top. In some benzene- or toluene-based mobile phases [18], triglycerides will be retarded or may even stop moving when they reach the silver-free part of the plate, so that the best procedures for triglyceride separations are those where saturated triglycerides such as tristearin or tripalmitin, as the fastest moving triglyceride in most mixtures, migrate to a distance just below the "silver front". The difference in mobility between silver-containing and silver-free areas of the plate is less pronounced when more polar mobile phase modifiers are used, such as diethyl ether, acetone or tetrahydrofuran in hexane. Thus, with these partially impregnated plates and with a proper choice of solvents, special effects can be achieved [18]. The use of partially impregnated plates for two-dimensional TLC has been described [14].

The "silver front" can be seen easily with the naked eye on both wet and dry plates. After spraying with lipid reagents such as 2′,7′-dichlorofluorescein, the area above the "silver front" appears yellow and the silver-containing area of the plate is reddish pink. Figs. 3 and 4 show examples of triglyceride separations by degree of unsaturation.

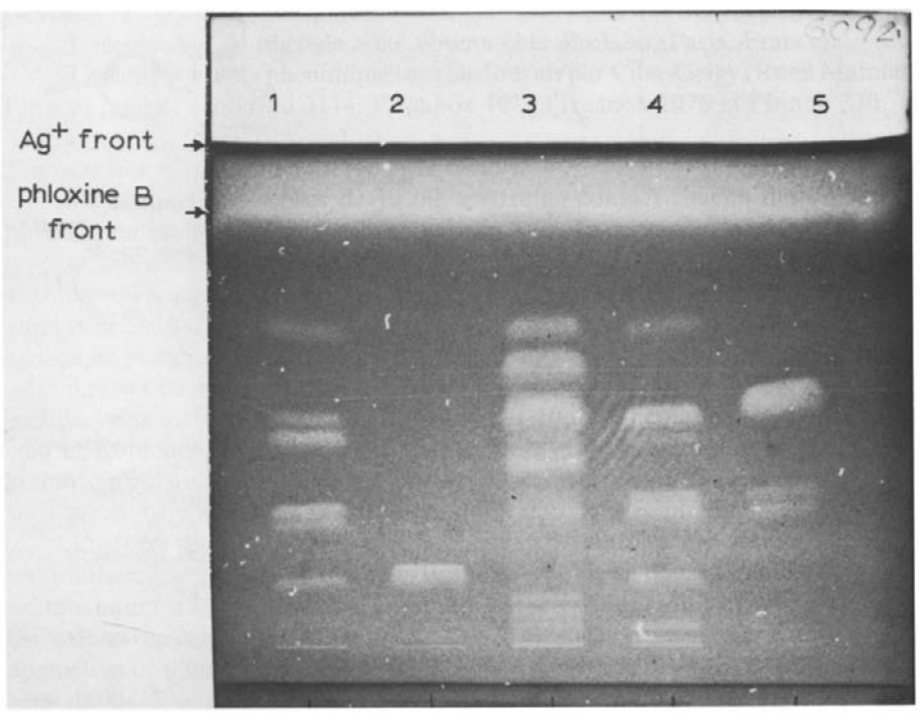

Fig. 5. Silver phloxin plate, impregnated by ascending chromatography with a solution of 10% $AgNO_3$ plus 0.08% phloxin in acetonitrile. Mobile phase for the triglyceride separation: hexane–toluene–diethyl ether (42:50:8, v/v/v); solvent migration until the silver front. Both the "silver front" and the "phloxin front" are clearly visible. Lanes: 1 = interesterified mixture of triolein and tripalmitin; 2 = triolein; 3 = partially hardened palm oil; 4 = commercial palm oil; 5 = cocoa butter. Total lipid applied: 100 μg per 2-cm Linomat band.

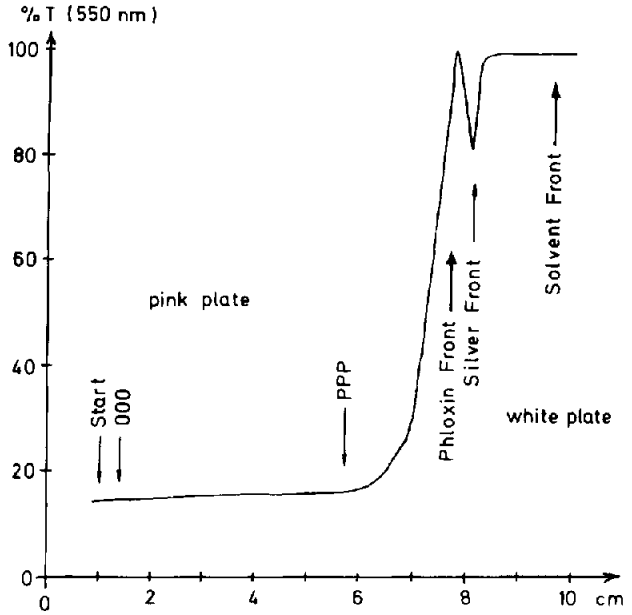

Fig. 6. Dynamic simultaneous impregnation of a TLC plate with silver and phloxin in one run. Phloxin distribution across the plate, as measured by reflectance densitometry in visible light at 550 nm. There is a "phloxin front" visible just below the silver front.

Plates impregnated with silver and phloxin

Impregnation with $AgNO_3$ and phloxin has the added advantage that no separate spraying procedure is needed and that the phloxin is evenly distributed across the lower half of the plate (as is the silver).

With dynamically (silver–phloxin)-impregnated plates, a clearly visible "phloxin front" is found about 1 cm behind the "silver front", which in turn is a few centimetres below the solvent front (Fig. 5). The phloxin distribution on the plate can be measured in UV or visible light, and indeed it can be continuously measured by scanning a lipid-free lane of the plate using visible light reflectance densitometry (Fig. 6). The phloxin distribution across the plate shows a pattern similar to the silver distribution.

Semi-quantitative work using densitometry of induced fluorescence on plates of this type will be described in a subsequent paper [17].

First results with dynamically impregnated silver HPLC columns

Commercial ready-made silica HPLC columns usually have much higher plate numbers than laboratory-made $AgNO_3$ silica columns, where the $AgNO_3$ is first deposited on the silica and then a slurry of $AgNO_3$–silica is pumped into the empty column. If "dynamic impregnation" works equally well on columns as on plates, then it should be possible to prepare $AgNO_3$-containing columns of good quality in simple, clean operation.

Initially, we thought that plugging of the column and frits by precipitated $AgNO_3$ from the end zones of the acetonitrile solvent plug would be a problem. However, this was not the case and only once was a narrow-bore capillary between the

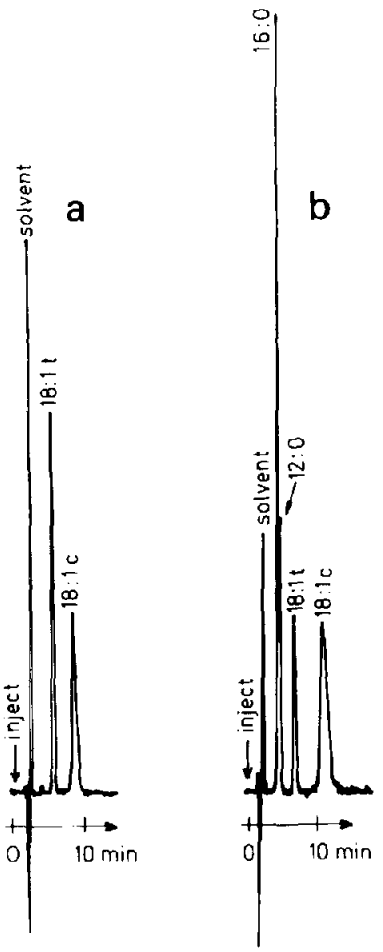

Fig. 7. Separation of saturated and *cis*- and *trans*-monoenoic fatty acid methyl esters on a dynamically impregnated $AgNO_3$–silica HPLC column. Conditions: 150 × 4.6 mm I.D. 5-μm silica column, dynamically impregnated (see text); isocratic mobile phase, (a) 2% diethyl ether in heptane and (b) 1.6% diethyl ether in heptane; flow-rate, 1.5 ml/min; detection, refractive index.

injector and column blocked. (We suggest removing the injector and using wide-bore capillaries only for the impregnation step.)

Initial experiments showed useful *cis–trans* resolution from columns impregnated in this way (Fig. 7). Work is in progress to determine if this technique can be developed further as a useful alternative to the conventional preparation of $AgNO_3$–silica HPLC columns, and to find the optimum conditions for dynamic silver impregnation and column reactivation. In earlier work by others [19] it was found that glass columns filled with a slurry of silica could also be impregnated by percolating $AgNO_3$ solution through the slurry column.

ACKNOWLEDGEMENTS

The authors acknowledge the skilful assistance of Ms. E. Thiele during the early part of the experimental work. L.A.G.G. gratefully acknowledges financial support by DAAD for a 12-month study visit to F.R.G.

REFERENCES

1 C. B. Barrett, M. S. J. Dallas and F. B. Padley, *Chem. Ind. (London)*, (1962) 1050; *J. Am. Oil Chem. Soc.*, 40 (1963) 580.
2 B. de Vries and G. Jurriens, *Fette-Seifen-Anstrichm.*, 65 (1963) 725.
3 L. J. Morris, *J. Lipid Res.*, 7 (1966) 717.
4 L. J. Morris, D. M. Wharry and E. W. Hammond, *J. Chromatogr.*, 31 (1967) 69.
5 H. Wessels, *Fette-Seifen-Anstrichm.*, 75 (1973) 478.
6 H. K. Lichtenthaler, K. Börner and C. Liljenberg, *J. Chromatogr.*, 242 (1982) 196.
7 D. A. Kennerly, *J. Chromatogr.*, 363 (1986) 462.
8 D. Chobanov, R. Tarandjiska and R. Chobanova, *J. Am. Oil Chem. Soc.*, 53 (1976) 48.
9 D. Gegiou and M. Georgouli, *J. Am. Oil Chem. Soc.*, 60 (1983) 833.
10 H. Heckers and F. W. Melcher, *J. Chromatogr.*, 256 (1983) 185.
11 K. Aitzetmüller, unpublished work.
12 L. J. Morris, *Chem. Ind. (London)*, (1962) 1238.
13 M. Inomata, F. Takaku, Y. Nagai and M. Saito, *Anal. Biochem.*, 125 (1982) 197.
14 J. M. Cubero and H. K. Mangold, *Microchem. J.*, 9 (1965) 227.
15 E. W. Hammond, *Chem. Ind. (London)*, (1981) 710.
16 E. W. Hammond, *J. Chromatogr.*, 203 (1981) 397.
17 L. A. Guaraldo Goncalves and K. Aitzetmüller, to be published.
18 D. Thorburn Burns, R. J. Stretton, G. F. Shepherd and M. S. J. Dallas, *J. Chromatogr.*, 44 (1969) 399.
19 R. A. Stein and V. Slawson, *Anal. Chem.*, 40 (1968) 2017.