Journal of Chromatography, 446 (1988) 283–291
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1338

SEPARATION OF TRIGLYCERIDE GROUPS BY REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY ON SILANIZED KIESELGUHR

B. NIKOLOVA-DAMYANOVA* and B. AMIDZHIN
Department of Lipid Chemistry, Institute of Organic Chemistry, Bulgarian Academy of Sciences, 1040 Sofia (Bulgaria)

SUMMARY

The resolution of triglyceride mixtures on a Kieselguhr G layer silanized with dimethyldichlorosilane and the mobile phase acetone–acetonitrile–water under different chromatographic conditions has been investigated. The influence of the water content on the separation has been studied. Each triglyceride mixture needs a mobile phase with a definite polarity, which depends on the partition numbers of the triglyceride components. Since a linear correlation between the partition number and the water content was observed, prediction of the mobile phase composition for any triglyceride mixture is possible, provided the partition numbers of the components are known. The chromatographic conditions found can be applied for resolution of triglyceride groups with parition numbers ranging from 30 to 52, in different triglyceride mixtures.

INTRODUCTION

Progress in triglyceride analysis has been achieved mostly with the combination of the two main chromatographic techniques: silver nitrate chromatography, in which the triglycerides (TGs) are separated according to their degree of unsaturation, and liquid–liquid partition chromatography, in which resolution is based on the partition of TGs between two phases with different polarities[1,2]. Reversed-phase thin-layer chromatography (RP-TLC) is one of the simplest and easiest modifications of partition chromatography to perform. In RP-TLC the lipophilic stationary phase is held on a layer of an inert support. This chromatographic technique, first introduced into TG analysis by Kaufmann *et al.*[3], has been successfully used to resolve model and natural TG mixtures according to their partition number (PN): $PN = CN - 2m$, where CN is the carbon number and m is the number of double bonds in the triglyceride molecule[1]. The best resolutions have been achieved by using stationary phases formed by long-chain hydrocarbons[1] or liquid paraffin[4,5]. However, with these highly effective phases, the use of chromogenic reagents for detection and quantitation of TGs is most difficult. Quantitation proceeds usually by indirect gas chromatographic[4–6] or spectrophotometric[7] methods being laborious and time-consuming. Nowadays, densitometry is the method of choice for quantitation of compounds separated

0021-9673/88/$03.50

by TLC. Since charring is most often used for the direct densitometric determination of TGs it is impossible to use long-chain hydrocarbons and liquid paraffin as the stationary phases. Thus our aim was to find a RP chromatographic system which would combine good selectivity with the possibility of direct densitometric quantitation.

In 1967 Ord and Bamford[8] separated model TG mixtures on a methyl-substituted lipophilic phase, formed by silanization of a silica gel layer with dimethyldichlorosilane (DMCS). Recently Chobanov[9] demonstrated that this stationary phase allows charring of TGs. Thus, when sufficient selectivity for natural TG mixtures can be achieved, this RP-TLC system should combine the technical advantages of the chemically bonded phases with the possibility for direct densitometric determination.

We have studied the resolution power of silanized Kieselguhr and silica gel layers using the mobile phase acetone–acetonitrile–water for the most common natural TG mixtures. We tried to optimize the chromatographic conditions and to rationalize the choice of a selective mobile phase in order to develop a procedure for TG analysis via RP-TLC.

EXPERIMENTAL

Materials

All solvents were reagent grade and were purified by distillation. Kieselguhr G (Merck, Darmstadt), silica gel G (Merck, Darmstadt) and their 8:2 and 6:4 mixtures were used as the supports. Kieselguhr needed a preliminary three-fold washing with chloroform–methanol (1:1, v/v) at a weight:volume ratio of 1:2. It was then air-dried overnight and additionally for 2 h at 110°C. Dimethyldichlorosilane (Fluka) was used as the silanization agent. The model TG mixtures used were triglyceride classes differing in unsaturation, isolated by preparative silver nitrate TLC from the purified TGs (preparative silica gel TLC) of commercial oils and fats (Table I). The component TG groups were identified on the basis of the fatty acid composition of the corresponding source and compared to the data of Kaufmann and Wessels[4,5]. The following TG mixtures were also used for testing and as illustrations: the S_3 class (trisaturated TGs) from coconut oil, the SM_2 class from peanut oil, the SMD class from lard and purified TGs from sunflower oil. Coconut oil, peanut oil and lard were also of commercial origin.

TABLE I

COMPOSITION OF THE MODEL TRIGLYCERIDE MIXTURES

S = Saturated; M = monoenoic; D = dienoic fatty acids; St = stearate; P = palmitate; O = oleate; L = linoleate. The order of designation does not indicate positional isomers.

TG class	*TG group*	*PN*	*Source*
S_2M	St_2O, StPO, P_2O	52, 50, 48	Cocao butter
SM_2	StO_2, PO_2	50, 48	Olive oil
S_2D	St_2L, StPL, P_2L	50, 48, 46	Sunflower oil
SMD	StOL, POL	48, 46	Sunflower oil
SD_2	StL_2, PL_2	46, 44	Sunflower oil

Methods

Preparative silica gel TLC was carried out on 20 cm × 20 cm glass plates coated with a 1-mm thick layer of silica gel G and with a mobile phase of light petroleum (b.p. 40–60°C)–acetone (100:12). Preparative silver nitrate TLC was carried out on 20 cm × 20 cm glass plates coated with a 1-mm thick silica gel G layer containing 5% (w/w) silver nitrate. A modification of a method proposed by Chobanov *et al.*[10] was used. The plates were developed overnight in an open Desaga chamber with a definite volume of a chloroform–diisopropyl ether–acetone solvent mixture. The whole volume of the mobile phase was allowed to pass through the layer. The chromatographic conditions depend on the sample type, and on the amount and the unsaturation of the corresponding TG classes. The conditions used in this work are presented in Table II. The purity of each class was checked by analytical silver nitrate TLC[11] using a suitable reference mixture.

The equipment and the working techniques introduced by Chobanov[12] and Chobanov and Tarandjiska[13] for analytical silver nitrate TLC were used here for RP-TLC. Laboratory-made 12 cm × 4 cm and 19 cm × 4 cm glass plates were covered with *ca.* 0.2 mm of the inert support, air-dried for 24 h, followed by 1 h at 110°C. They were then cooled and stored until silanization in a desiccator over phosphorus pentoxide. Silanization of the layer was carried out by placing the plates for a certain period of time in a closed chamber over DMCS vapours. The plates were then washed by elution with methanol and were dried for 1 h at 110°C. The plates were then stored in closed glass boxes, without any precautions. Samples of 5–10 μl of a 0.1% TG mixture solution in chloroform were applied as a 4-mm long band on the plate. The plates were developed in 16 cm × 4.5 cm I.D. or 22 cm × 4.5 cm I.D. closed cylindrical glass jars with 3 ml mobile phase to a solvent front of 10 and 17 cm, respectively. When developing twice, after the first development the plate was dried for 10 min at 110°C, cooled and developed for a second time with a fresh volume (3 ml) of the same mobile phase.

TABLE II

CHROMATOGRAPHIC CONDITIONS FOR THE ISOLATION OF PURE TRIGLYCERIDE CLASSES BY PREPARATIVE SILVER NITRATE TLC

Sample of 40–80 mg purified TGs of the corresponding oil or fat per plate. The TG classes are as in Table I.

TG classes	*Mobile phase*	
	*Component ratio (v/v/v)**	*Total volume (ml)***
S_3, S_2M***	100:0.7:0.25	80
S_3, S_2M	100:1.5:0.5	80
S_2M, SM_2, S_2D, M_3	100:4.0:1.2	80
SM_2, S_2D, M_3, SMD	100:6.0:1.5	100
M_3, SMD, M_2D, SD_2	100:8.0:2.5	100

* Chloroform–diisopropyl ether–acetone. The chloroform is purified from the stabilizing alcohol and freshly distilled.

** For two plates.

*** For saturated oils and fats.

The spots were detected by spraying the plate with 25% methanolic sulphuric acid and charring on an heated plate at *ca.* 220°C. The densitograms, shown as an illustration, were obtained on a Shimadzu CS-930 densitometer by zigzag scanning in a transmission mode at 450 nm and a slit 1.2 mm of 1.2 mm.

RESULTS AND DISCUSSION

The TG components in the model TG mixtures used have PNs in the range 44–52. This is the range to which the main TG groups of the most common vegetable and animal oils and fats belong. At the same time, each of these mixtures is a TG class, in which the TG groups have an equal number of double bonds, and the monoenoic and the dienoic fatty acids are represented by oleic and linoleic acids, respectively. It follows, therefore, that the PNs of the TGs in each class are determined only by the different chain lengths of the saturated fatty acids. In the TG classes, chosen here as model mixtures, these are myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids. In this way, no critical pairs are formed in the model mixtures and each zone in the chromatogram corresponds to a definite TG species.

Since in many cases, upon changing the chromatographic conditions, a considerable change in the zone size occurred, with the R_F values remaining unchanged, the resolution of two adjacent zones was expressed as

$$R = \frac{R_{F(A)} - R_{F(B)}}{r_{(A)} + r_{(B)}} \cdot L_0$$

where $r_{(A)}$ and $r_{(B)}$ are the radii of the corresponding zones, and L_0 is the pathlength from the sample origin to the solvent front. Obviously, the two zones will be better separated when the value of R is larger than 1. This way of expressing the resolution proved to be very useful when comparing the different chromatographic conditions.

Choice and influence of the mobile phase

In all cases studied, the stationary phase is formed by methyl groups. Compared to the C_8 and C_{18} hydrocarbon chains of the phases usually employed in RP-TLC, the CH_3 groups are much less lipophilic. It has been shown that, due to their small dimensions, they are distributed more uniformly and densely on the support surface[14]. It was unexpected, however, that this should compensate their weak interaction with the TG molecules. A mobile phase with a correspondingly low solvent strength was needed to achieve the selectivity required. An acetone–acetonitrile mixture[4,5] was used as an initial solvent. Its elution strength was reduced by adding a third highly polar component. Water and formic acid proved to be equally good modifiers and further water was used as a modifier. The effect of its content in the mobile phase, when keeping constant the ratio acetone–acetonitrile (70:30), is shown in Fig. 1. Complete resolution of the components of the model mixtures was achieved in a relatively narrow range of water concentration in the mobile phase. This range depends on the overall polarity of the TG mixture being resolved. The higher the polarity, the wider is the water concentration range, and R_{max}, *i.e.*, the best resolution occurs with more polar mobile phases. This indicated that a definite mobile phase is needed for each TG class, in order to achieve optimum resolution.

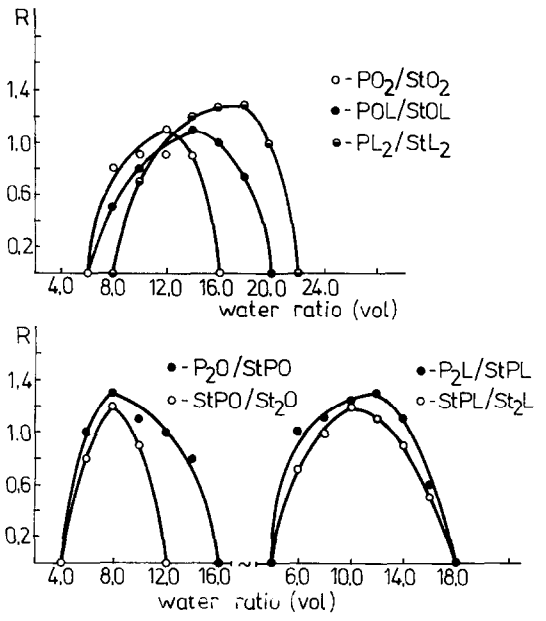

Fig. 1. Effect of the water content in the mobile phase on the resolution of the model TG mixtures. Conditions: 12 cm × 4 cm Kieselguhr G plates; silanization time 4 h and 30 min; mobile phase, acetone–acetonitrile (70:30); one development; solvent front, 10 cm. The TG groups are denoted as in Table I.

We supposed that the relationship between the nature of the model TG mixtures and the mobile phase composition could be used to facilitate the choice of mobile phases for other TG mixtures. We assumed that, as the PN determines the behaviour of a TG group in RP-TLC, the corresponding parameter for a TG mixture would be $\overline{PN}$, where $\overline{PN}$ is the mean arithmetic value of the PNs of the components. If the $\overline{PN}$ of the model mixtures is plotted against the volume content of water in the mobile phase at R_{max}, a straight line is obtained (Fig. 2). Using this dependence, one can predict the most suitable mobile phase composition, provided the PNs of the TG components of the mixture being analysed are known. As is seen from the densitograms, illustrated in Fig. 2, the dependence holds also when the $\overline{PN}$ is larger or smaller than the $\overline{PN}$ of the model mixtures. The extrapolation on both sides is limited by the solubility of all or part of the components of a given mixture in the mobile phase. So we did not succeed in separating the TGs of the S_3 class of the common oils and fats investigated. The main components of this class are TGs with PNs in the range 48–54, containing myristic, palmitic and stearic acids. The reason most probably is the insolubility of these TGs in the mobile phase. On the other hand, as is seen from Fig. 2, the class S_3 from coconut oil is separated, the PNs of the TG components ranging from 30 to 42[15].

Silanization

The quantity of the stationary phase is the other important factor affecting the resolution[1]. In the case studied, an estimate of this quantity is the degree of silanization of the support. At a constant moisture content in the layer, it is defined by

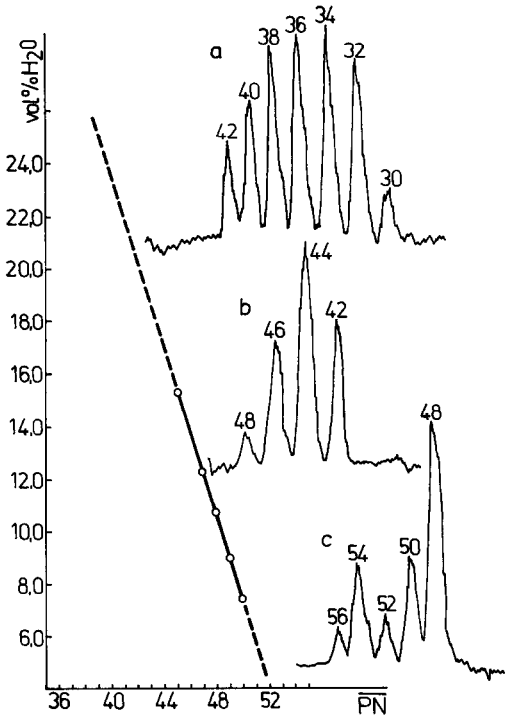

Fig. 2. Plot of the $\overline{PN}$s of the model TG mixtures *vs.* water content in the mobile phase at R_{max}. Examples: (a) S_3 class from coconut oil, $\overline{PN}$ = 3; 30% (v/v) water in the mobile phase; (b) TG of sunflower oil, $\overline{PN}$ = 45; 15% (v/v) of water in the mobile phase; (c) SM_2 class from peanut oil, $\overline{PN}$ = 52; 8% (v/v) of water in the mobile phase.

the silanization time. Fig. 3 shows the influence of the silanization time on the resolution of the SMD class from lard, containing TGs with PNs 48, 46 and 44. The resolution is expressed by the factor R, and for comparison, by the ratios $R_{F(48)}/R_{F(46)}$ and $R_{F(46)}/R_{F(44)}$ as usual. The resolution improves with increasing silanization time from 1 to 9 h. Since this improvement is mainly due to the decrease in the spot sizes, the effect is seen as a change in R, and not in the corresponding R_F ratios. We chose silanization times, between 4 and 6 h. Under these conditions the resolution of adjacent zones is fairly good and the spot size is suitable for densitimetry.

The support

Owing to its poor adsorption properties Kieselguhr is the support preferred in classical RP-TLC[1,3–5]. When using silanes to obtain lipophilic phases, the support of choice is usually silica gel[1,2]. As has been shown for other cases of RP-TLC[16], in the case of TGs we also found that the nature of the support influences the resolution. All other conditions being equal, the TGs from sunflower oil were better separated on Kieselguhr than on silica gel layers (Fig. 4). The addition of only two parts by weight of silica gel to Kieselguhr leads to a decrease in the R_F values (0.2–0.4 units) and impairs the resolution. A plausible explanation of the influence of the support is the appearance of a mixed retention mechanism in the case of silica gel[16].

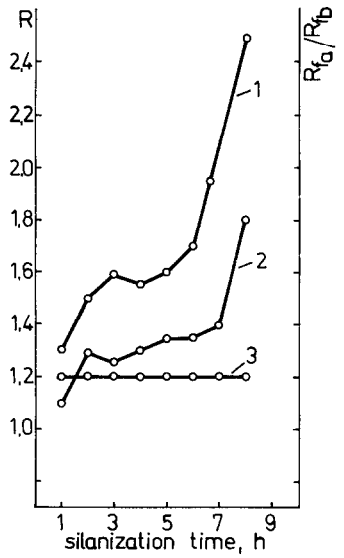

Fig. 3. Effect of silanization time on the resolution. Test TG mixture: SMD class from lard. Resolution of TGs of PN_{44}/PN_{46} (1), PN_{46}/PN_{48} (2), expressed in terms of R; the same separation expressed as ratios $R_{F(44)}/R_{F(46)}$ and $R_{F(46)}/R_{F(48)}$ (3). Conditions: 12 cm × 4 cm Kieselguhr G plates; mobile phase, acetone–acetonitrile–water (70:30:14); one development; solvent front, 10 cm.

Other parameters

The effect of other factors on the resolution is shown on Fig. 5. They are important when using the system studied for analytical purposes. Elongation of the pathlength as well as a second development with the same mobile phase increased the range of water contents in the mobile phase, resulting in good resolution. At the same time, R_{max} occurred in more polar phases. Considering this tendency, the best

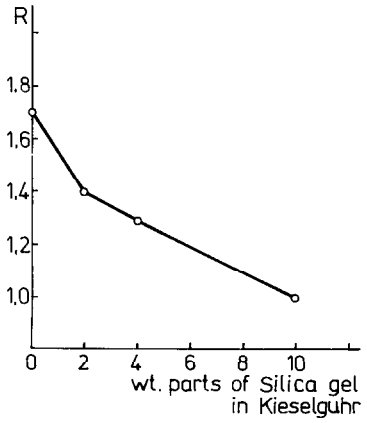

Fig. 4. Effect of the support on the resolution. Test mixture: pure TGs of sunflower oil. Conditions: 19 cm × 4 cm plates; silanization time, 6 h; mobile phase, acetone–acetonitrile–water (70:30:18); solvent front, 17 cm.

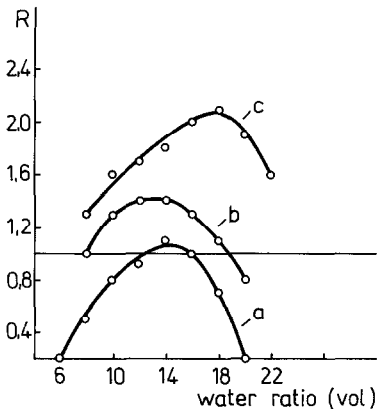

Fig. 5. Effect of development conditions on the resolution. Test mixture: SM_2 class (PO_2/StO_2) from olive oil. Conditions: Kieselguhr G plates; silanization time, 6 h; (a) one development, solvent front 10 cm; (b) one development, solvent front 17 cm; (c) two developments, solvent front 17 cm.

results are expected upon continuous development with a relatively large volume of a comparatively polar mobile phase. Using the SM_2 class from olive oil as a test mixture, we tried the simplest procedure of continuous development, *i.e.*, development in an open tank[11]. Briefly, 5 ml of the mobile phase acetone–acetonitrile–water (70:30:16) were left to pass through the silanized Kieselguhr layer: R_{max} reached 2.5 [upon double development with acetone–acetonitrile–water (70:30:12), R_{max} was 1.3] but this took *ca.* 30 h. Nevertheless, it is expected that, as in adsorption TLC[17], as well as in silver nitrate TLC[11], the resolution would be much better when using continuous development.

The resolution depended strongly on the geometry of the chromatographic chamber, on the saturation of the atmosphere with mobile phase vapours and on the volume of the mobile phase, *i.e.*, on effects that have been observed in adsorption TLC[17]. Previous saturation of the atmosphere in the tank is not only useless, but it should be avoided since it strongly impairs resolution. For the same reason, the volume of the mobile phase should not exceed 6 ml. It must be pointed out that all these effects were observed when using the cylindrical chromatographic jars, described in detail in the Experimental section.

CONCLUSION

The results reported indicate that the stationary phase formed by methyl groups bonded to Kieselguhr G, together with the mobile phase acetone–acetonitrile–water, possesses a sufficiently high resolution power for TGs with PNs in the range 30–52. The resolution attained is of the same order as that achieved with long-chain stationary phases[1,4,5], but has certain technical advantages and hence, is of interest for quantitative RP-TLC of TGs.

ACKNOWLEDGEMENT

This work was supported in part by UNDP Project BUL/77/009.

REFERENCES

1 C. Litchfield, *Analysis of Triglycerides*, Academic Press, New York, 1972.
2 W. W. Christie, *Lipid Analysis*, Pergamon, Oxford, New York, Toronto, Paris, Frankfurt, 2nd ed., 1982
3 H. P. Kaufmann, Z. Makus and B. Das, *Fette, Seifen, Anstrichm.*, 63 (1961) 125.
4 H. P. Kaufmann and H. Wessels, *Fette, Seifen, Anstrichm.*, 66 (1964) 81.
5 H. P. Kaufmann and H. Wessels, *Fette, Seifen, Anstrichm.*, 68 (1966) 249.
6 C. Litchfield, *Lipids*, 3 (1968) 170.
7 H. Wessels, *Fette, Seifen, Anstrichm.*, 75 (1973) 478.
8 W. O. Ord and P. S. Bamford, *Chem. Ind. (London)*, No. 7 (1967) 277.
9 D. Chobanov, personal communication, 1986.
10 D. Chobanov, R. Tarandjiska and M. Topalova, in preparation.
11 D. Chobanov, R. Tarandjiska and R. Chobanova, *J. Am. Oil Chem. Soc.*, 53 (1976) 48.
12 D. Chobanov, *Lab. Pract.*, 26 (1977) 481.
13 D. Chobanov and R. Tarandjiska, *Commun. Dept. Chem., Bulg. Acad. Sci.*, 15 (1982) 45.
14 W. Cheng and M. McCown, *J. Chromatogr.*, 318 (1985) 173.
15 A. El-Hamdy and E. G. Perkins, *J. Am. Oil Chem. Soc.*, 58 (1981) 49.
16 W. F. Van der Giesen and L. H. M. Jansen, *J. Chromatogr.*, 237 (1982) 199.
17 A. Niederwieser and C. C. Honneger, *Adv. Chromatogr. (N.Y.)*, 2 (1966).