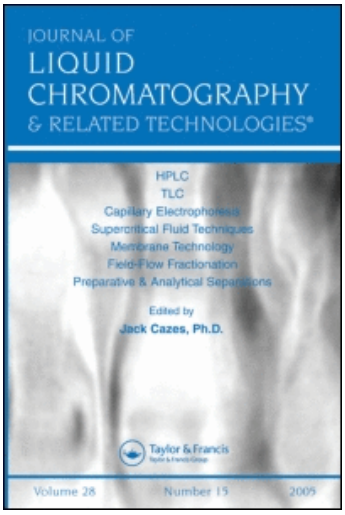

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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF TRIACYLGLYCEROLS. PRINCIPLES AND APPLICATION

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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF TRIACYLGLYCEROLS. PRINCIPLES AND APPLICATION

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ABSTRACT

The efficiency of silver ion and reversed-phase thin layer chromatography in the analysis of triacylglycerols is discussed in this paper. The experimental conditions that allowed for the conversion of these techniques into full-scale quantitative analytical methods are presented and details of the respective protocols are given. Examples are presented that demonstrate the achievements of thin-layer chromatography in elucidating the triacylglycerol structure of natural and modified lipid samples.

INTRODUCTION

Triacylglycerols (TAG) are often described as having the most complex composition of any natural products of comparable molecular weight.[1] The three fatty acid residues differ in chain-length and unsaturation and can combine in the glycerol molecule to give, at least theoretically, 3^n different species, where n is the number of the fatty acids present.

Determination of fatty acid composition is mandatory in the analysis of TAG. It supports, to a great extent, the evaluation of the possible TAG composition but is, in most cases, insufficient. The present status of many aspects of lipid chemistry demands information about the intact TAG molecular species.

Chromatography is the backbone of lipid analysis and, in particular, of TAG analysis.[1-10] Different chromatographic techniques have been in vogue in different periods of time. It is now widely accepted that there is no single chromatographic method able to resolve the complex structure of natural TAG mixtures.[4,7,8]

For many years, thin-layer chromatography (TLC) has been the basic separation method used by lipid chemists for the analysis of TAG. It has been employed in three modifications: (i) preparative silica gel TLC which separates lipid classes according to their overall polarity and is still in use to isolate and purify TAG fractions of natural lipid mixtures; (ii) silver ion TLC (Ag-TLC) which separates TAG into classes according to the overall number of double bonds in the acyl residues; (iii) reversed-phase TLC (RP-TLC) which separates TAG classes into groups according to their overall polarity. By applying Ag-TLC and later Ag-TLC/RP-TLC in a complementary mode, a major contribution has been made in elucidating the TAG structure of various natural fats and oils of different origins.[2-7] Additionally, basic relationships between the TAG structure and migration in the respective TLC environment have been established. Later, these were also found to apply in high-performance liquid chromatography modifications.[7,8]

The achievements of TLC in lipid analysis have been brought to the attention of the chromatographic community, being reviewed by Sherma and Fried in their volumes on TLC,[9,10] and by Christie in his edited series Advances in Lipid Methodology (see the volumes in refs. 7 and 8, for example).

A boundary that required considerable effort to be crossed was the conversion of Ag- and RP-TLC into full-scale quantitative methods that allowed for detailed determination of natural TAG mixtures with a broad range of unsaturation. Species resolved by TLC are usually quantified directly on the plate by scanning densitometry using instruments specially designed for the purpose. The utilization of TLC-densitometry for TAG determination requires well resolved distinctive, and more importantly, evenly stained zones on a clear, high contrast background.

The principles that enabled the conversion of Ag- and RP-TLC from qualitative or semi-quantitative into reliable quantitative methods is presented and critically evaluated in this review. These techniques have been in use by the author and her colleagues for about 20 years and several examples of their

practice are discussed herein with the hope that they will show clearly that detailed analysis of complex TAG mixtures can be achieved with simple TLC techniques.

SILVER ION-TLC

Resolution of TAG Classes

Silver ion chromatography is representative of adsorption solid-liquid separation techniques and is unique in that it separates TAG based on a single property, unsaturation, due to the formation of weak charge-transfer complexes between double bonds and silver ions. In general, responsible for the separation is the overall number of double bonds in the acyl chains. A TAG mixture which comprises the common range of C_{14}-C_{18} acyl residues with up to three double bonds in a single chain can be resolved into 20 unsaturation classes. The components migrate in the order (of increased retention):

SSS<SSM<SMM<SSD<MMM<SMD<MMD<SDD<SST<MDD<SMT<MMT <DDD<SDT<MDT<DDT<STT<MTT<DTT<TTT,

where S, M, D and T denote saturated-, monoenoic-, dienoic- and trienoic- acyl residues, respectively, but do not indicate the position of this residue in the molecule. As a rule, in the case of TAG classes with equal overall number of double bonds, those will be retained stronger in which the double bonds are grouped in a single acyl moiety, as in MMM and SMD, for example.[11] Depending on the nature of the mobile phase and on the specific quantitative proportions of adjacent zones, some changes in the migration order of TAG that contain trienoic acyl moiety may occur.[7,12]

In 1962, Barett, Dallas, and Padley described the first application of Ag-TLC to the analysis of TAG.[13] Most of the work done afterwards followed the general patterns suggested by these authors. Technically, Ag-TLC is performed by first preparing a plate (glass) of standard dimensions covered with ca. 0.2 mm thick silica gel G layer that contains silver ions in the form of silver nitrate (most often, although other Ag salts have also been examined). Silver ions are incorporated into the layer by adding the salt to the slurry or, better yet, by impregnation of the layer via spraying with or by dipping into a methanolic solution of the salt. Silver content of 10 to 30% has been considered essential for good resolution.[7] Development proceeds in covered tanks after preliminary saturation of the atmosphere with vapors of the mobile phase. The saturation is considered to shorten the development time and to improve the resolution. Multiple development is often applied with the same purpose. Usually, it starts with the most polar mobile phase and proceeds with phases of gradually

decreasing polarity. Highly unsaturated TAG classes are separated first and do not move further with the subsequent development when more saturated components are separated (see ref. 7 for more details).

The analytical Ag-TLC as developed and used in our laboratory[14-16] has some features that differ substantially from the generally accepted pattern, with an aim to provide resolution and layer-background of suitable quality for unequivocal densitometric quantification. These are as follows:

- low percent silver nitrate in the layer (0.5% to 2.0% methanolic silver nitrate solution for impregnation);

- development in open cylindrical tanks;

- a carefully chosen charring procedure to visualize the separated components, which includes treatment of the plate, subsequently, with bromine and sulphuryl chloride vapors.

A systematic examination of analytical Ag-TLC revealed that the high percent of silver nitrate in the layer, used at present by many lipid chemists, has no substantial enhancing effect on the TAG resolution when increased above 2%. In contrast, while resolution does not improve, the plates become very difficult to handle, mainly because they become strongly light-sensitive. Usually, the background darkens rapidly, losing the contrast required for reliable densitometric quantification. As will be shown later, excellent resolution can be achieved with only 0.5% silver nitrate in the impregnating solution. This fact reveals that silver ions are essential but not the single factor to affect the resolution and should be considered in combination with all other chromatographic conditions.

The development in open cylindrical tanks increases, substantially, the efficiency of resolution. Under this condition, the entire volume of the mobile phase is allowed to pass through the plate. Development is carried out, therefore, in a non-equilibrated system in which, inevitably, the composition of the adsorbed solvents and of the vapors change alongside the height of the plate. However, the resulting effect is similar to those achieved by gradient development in the horizontal sandwich chamber designed by Dzido and Soczewinski,[17] i.e., a highly efficient resolution is achieved despite the limited migration distance. A new parameter to conduct the system appears and that is the mobile phase volume. In addition, chromatography in open tanks does not exclude the use of multiple development. So far, two-fold development is sufficient even for difficult to resolve TAG with the first phase being of higher polarity and smaller volume than the second. No intermediate drying of the plate is required.

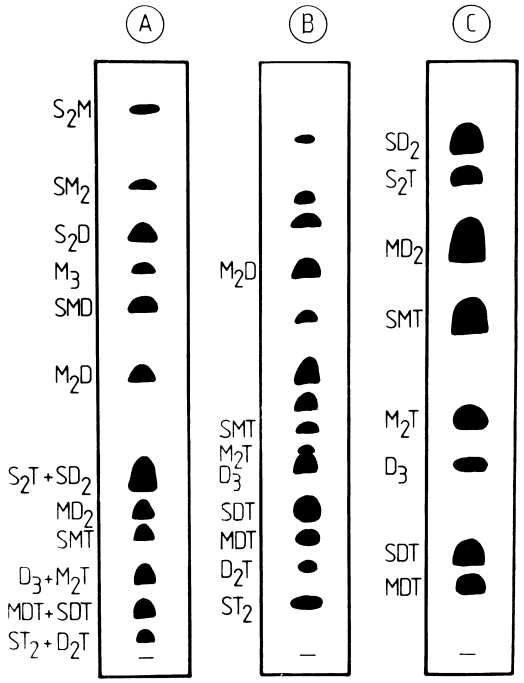

Figure 1. Separation of triacylglycerols from orange seed oil by Ag-TLC. Experimental conditions as given in the Ag-TLC protocol. Plate A, mobile phase 8 mL petroleum ether-acetone, 100:6, by volume, sample size: 25 μg; plate B, mobile phase 5 mL petroleum ether-acetone, 100:8, by volume, followed by 8 mL petroleum ether-acetone, 100:5, by volume, sample size: 50 μg; plate C, mobile phase 4 mL petroleum ether-acetone, 100:7, by volume, followed by 15 mL petroleum ether-acetone, 100:4, by volume, sample size: 15 μg. S, saturated, M, monoenoic, D, dienoic and T, trienoic fatty acid residues respectively. (Reproduced by the kind permission of The Oily Press).

Obviously, most important for the resolution is the choice of the mobile phase. As will be shown below, separation of simpler TAG mixtures of low or moderate unsaturation (which means also a limited variety of acyl residues and limited number of TAG) can be performed by using a binary mixture of light petroleum ether and acetone in different proportions and volumes. Another strategy is required, however, for the resolution of TAG that contain trienoic acyl residues. Obviously, adequate resolution can not be expected in case of more than 8-10 TAG classes to be separated in the limited migration distance (20 cm) of the standard TLC plate. The solution found is demonstrated in Fig. 1, with the separation of orange seed oil that contains about 6% linolenic acid (cis9, cis12, cis15-18:3) and, respectively, TAG that have a linolenic acyl

moiety are formed.[15] Sixteen TAG classes in total are present in this oil. As seen from the scheme, the sample was applied on three different plates, which were developed separately with three different mobile phases. On each plate, a different task of TAG analysis was solved.

Plate A gave the general picture of the composition and a single mobile phase of 8 mL of light petroleum ether-acetone100:6 (v/v) was used for the separation. As seen, some of the highly unsaturated TAG formed mixed zones.

On plate B, most of these zones were partially resolved by two-fold development, first with mobile phase of 5 mL light petroleum ether-acetone 100:8 (v/v), followed by 8 mL light petroleum ether-acetone, 100:5 (v/v).

On plate C, where the aim was to clearly resolve SST from the adjacent SDD and MDD TAG, the first mobile phase was 4 mL of light petroleum ether-acetone, 100:7 (v/v) and the second 15 mL light-petroleum ether-acetone 100:4 (v/v). Note, also, that different sample aliquots were applied to each plate depending on the task: 25 μg on plate A, 50 μg on plate B, and only 15 μg on plate C. Thus, it was possible to resolve and to identify the 16 TAG classes in the orange seed oil. More examples will be given below to demonstrate that, by using all elements of the chromatographic system in a proper way, it is possible to improve, by far, the resolution power of Ag-TLC.

The author is convinced, also, that the geometry of the developing chamber (dimensions 22 cm x 4.5 cm I.D) has a certain role in the resolutions achieved. All efforts to obtain similar separations as those shown in Fig.1, in standard Desaga chambers, failed so far. Experiments have shown, also, that the 4-5 cm excess in height of the cylindrical chamber has, also, a positive effect, probably acting as a "chimney" for the mobile phase vapors.

Lastly, separated TAG classes are visualised by an approach that allows for a densitometric quantification of reliable accuracy and reproducibility. It has been suggested that an even action of the charring reagents could be achieved by treating the plate with the vapors of the reagent, instead of spraying.[14] Sulphuryl chloride was chosen for the purpose with good results. A preliminary treatment of the plate with bromine vapors was also introduced. The reason for that choice can be found in the first communications on densitometric quantification of TAG,[18] where a destruction of TAG with loss of substance during the heating stage has been reported. For the correct quantification of components, introduction of correction coefficients was required, therefore, and their values increased with increasing unsaturation. Chobanov and colleagues assumed that a treatment with bromine vapors would result in bromination of the double bonds, thus preventing the cleavage during the heating.[14] This procedure is still in use, although later, while examining the densitometric quantification of

unsaturated fatty acid derivatives on a Ag-TLC plate, the author found[19] that the main effect of bromine was mostly to enhance the carbonization of the long chain and/or saturated species, acting as a synergist to the sulphuryl chloride, i.e., bromine acts as an oxidizing agent. In addition, it has been assumed that bromine reacted with silver ions in the layer to form a stable, white salt. After heating, the plates remained with almost white background, i.e., a good contrast between background and charred zones was achieved, which facilitated the densitometric quantification.

Technical problems, such as preparation of the slurry and of the layer, and the sample application had been solved by using simple, laboratory made devices, designed by the late Dr Chobanov, former head of the laboratory.[20,21] The author is well aware that the lipid community dislike using home-made plates. Our experience, however, reveals that relevant resolution of TAG cannot be achieved on pre-coated plates, mainly because the layer density is too high to allow effective development in open tanks.

The reader will note, in the protocol, the strange dimensions of the TLC plates we are using. These originate from the dimensions of the plate-holder of our first densitometer, a former East Germany production (see ref. 14 for more details). The same resolution is achieved on 5 x 20 cm plates, provided the layer is home-made.

Among the drawbacks that should be considered first, and may be most important, is that the analysis requires almost constant participation of the analyst, considerable attention, and certain technical skills. Also, the separation is substantially affected by the laboratory environment (temperature and humidity) and requires periodical adjustment of the mobile phase, i.e., in general the reproducibility of resolution is relatively low.

Advantages are the low consumption of solvents of no special purity grade (a distillation is sufficient in most cases), of adsorbent, and of silver salt. Besides, Ag-TLC is an excellent tool for easy identification of TAG classes in different samples. Each examined sample of known composition might be used as a reference for newly analysed TAG mixtures.

As can be seen in ref. 15, a mixture of sunflower oil and lard in equal amounts was used to identify most of the TAG classes in orange oil. If the sample and the reference are applied close enough on the plate, identical TAG classes form joint zones. We often now use orange and tangerine[16] seed oils as reference for identification of TAG that contain moderate amounts of 18:3. The above is true provided the fatty acid composition varies in the relatively narrow limits of C_{14}-C_{18} chains.

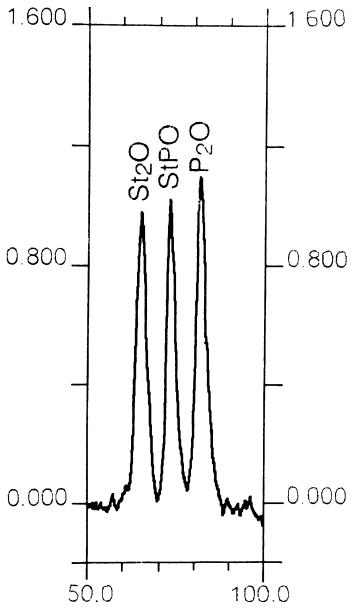

Figure 2. Separation of standard mixture of StStO, PStO, and PPO, 2.4 µg each by RP-TLC. Experimental conditions as given in the RP-TLC protocol. Mobile phase of 2 x 3 ml acetone-acetonitrile-water, 70:30:12, by volume. St, stearic, P, palmitic, O, oleic acid residues, respectively. (Reproduced by the kind permission of Journal of Planar Chromatography).

The Analytical Protocol

Glass plates, 4 cm x 19 cm, are covered with ca 0.2 mm thick silica gel G 60 layers (Merck, Darmstadt, Germany) and left for a week to dry in the open air. The plates are kept in a covered place without any precautions. For the analysis, they are impregnated by dipping in 0.5% methanolic solution of silver nitrate, left for 1h to dry in the air and for 5 min at 110°C in an oven. After cooling, an estimated sample aliquot (of 0.2%-0.3% working solution of the sample in hexane) is applied as a 4 mm long band 1.5-2.0 cm above the narrow edge of the plate. Development is performed overnight, in 24 cm x 5 cm (I.D.) cylindrical tanks, with estimated composition and volume of the mobile phase. The plates are then dried for 1 h at 110°C in an oven and treated consecutively with bromine and sulphuryl chloride vapors (30 min each, in closed tanks, fume-cupboard). The separated zones are carbonized by heating for 4-5 min at 180-200°C on a temperature-controlled metal plate.

REVERSED PHASE TLC

Resolution of TAG Groups

RP-TLC is representative of distribution separation techniques and is based on the distribution of TAG between a non-polar stationary phase and a relatively polar mobile phase. TAG are, therefore, separated according to their overall polarity, expressed by the Partition Number (PN). PN relates the migration of a component to the total number of carbon atoms, CN (in the acyl residues only) and the total number of double bonds, n[22]:

$$PN = CN - 2n$$

The higher the PN, the stronger is the TAG retained in the non-polar layer (the migration distance is shorter).

In TAG analysis, RP-TLC was introduced by Kaufmann and co-workers.[23,24] The best resolution was achieved by using a stationary phase of long- chain hydrocarbons[2] or liquid paraffin[25,26] applied by impregnation on a kieselguhr G support. Despite the clear resolution achieved by Kaufmann and colleagues, it was obvious that separation of TAG based on their PN was less informative if applied to the total sample. TAG components of different unsaturation would combine to give a limited number of mixed zones, thus providing a limited information on the TAG composition of the sample.

In order to increase the information value of the method, Kaufmann and Wessels suggested to use RP-TLC in combination with preparative Ag-TLC.[25,26] The idea was to first fractionate TAG mixture into classes according to unsaturation and to subject each class to RP-TLC and elucidate the composition according to the PN of the components. Evidently, the PN of TAG in the common plant oils, which contain, normally, only one major monoenoic fatty acid, the oleic (cis-9-18:1) and only one dienoic fatty acid, the linoleic (cis-9,cis-12-18:2) will be determined by the chain length of the saturated fatty acids. The approach was successfully applied to a number of seed oils[25-27] with the best achievement being the analysis of palm-kernel oil performed by Wessels in 1973.[28]

Visualization was difficult and different staining reagents such as rhodamine B,[23] iodine vapors,[23] iodine-α-cyclodextrine,[25,26] and phospho-molybdic acid[27] have been tested. None of these was suitable for densitometric quantification and indirect laborious and time-consuming procedures like scraping, methylation, and GC of methyl esters[4] or spectrophotometry[28] were utilized.

Table 1

Water Proportion in the Mobile Phase Acetone-Acetonitrile-Water, 70:30:X, for the Separation of Triacylglycerol Classes of Common Plant Oils into Molecular Species by RP-TLC

TG Class[a]	TG Species[b]	PN[c]	X, Water Proportion, by Volume
S_2M	PPO, PStO, StStO	48, 50, 52	12
SM_2	POO, StOO	48, 50	14
S_2D	PPL, PStL, StStL	46, 48, 50	12
SMD	POL, StOL	46, 48	18
SD_2	PLL, StLL	44, 46	20
SMT	POLn, StOLn, AOLn	44, 46, 48	16
SDT	PLLn, StLLn, ALLn, BLLn	42, 44, 46, 48	18
ST_2	PLnLn, StLnLn	40, 42	21

[a] S, saturated; M, monoenoic; D, dienoic; T, trienoic fatty acid residue; [b] The order of designation does not indicate positional isomers, P - palmitic; St - stearic; A - arachidic; B - behenic; O - oleic; L - linoleic; Ln - linolenic fatty acid moieties; [c] Partition number PN = C - 2n; C - number of carbon atoms, n - number of double bonds.

Independently, Ord and Bamford[29] demonstrated successful resolution of reference TAG on silica gel layer treated with dimethyldichlorosilane (DMCS) to give a lipophilic C_1 layer. Later, Chobanov[30] demonstrated that this layer could be treated with charring reagents so that the applied TAG could be visualized by carbonization via heating, while the background remained intact.

All these achievements were combined and modified, after careful systematic examination by Nikolova-Damyanova and Amidzhin, to give an analytical RP-TLC procedure, complementary to Ag-TLC as suggested by Kaufmann et al., that combines the good resolution of longer-chain, non-polar phases with simpler visualization approach suitable for densitometric quantification.[31,32]

The main features are:

- the C_1 lipophilic stationary phase produced by silanization with DMCS;

- the three component mobile phase of acetone-acetonitrile-water, with constant acetone-acetonitrile ratio, 70:30, and water proportion varied depending on the TAG class to be resolved;

- two-fold development with small volumes of the mobile phase in closed cylindrical tanks (the same as described in the previous section), without preliminary saturation of the atmosphere.

The replacement of the long-chain hydrocarbons was essential, as it allowed the use of charring for a staining procedure and gave a perspective for densitometric quantification. The weak C_1 stationary phase required correspondingly modified mobile phase. Water and formic acid were tested as modifiers to the acetone-acetonitrile and, since both led to practically the same resolution, water was chosen.[31] When studying the effect of water content on the resolution of reference TAG classes (SSM, SMM, SSD, SMD, SDD, isolated from seed oils), it appeared that each TAG class requires a specified water proportion in the mobile phase for maximal resolution. When plotting the mean PN of these classes (arithmetical mean of the PNs of the component TAG species in a given class, see Table 1) against the water proportion at maximal resolution, a straight line, was obtained with parameters: A = 82.778, B = -1.505 and r = 0.9998.[31]

It was found that the relationship holds in a broader PN interval than those used to construct the graph.[31] The graph allowed to easily estimate the water proportion suitable for a given TAG class, provided the fatty acid composition of the sample is known, which is normally the case in the practice. Table 1 shows the water proportions required for the RP-TLC resolution of the most abundant plant TAG classes.

Fig. 2 demonstrates the separation of a reference mixture of StStO, StPO and PPO TAG. We were not able to separate trisaturated TAG mixtures that contain 16:0, 18:0 and longer-chain fatty acids. Differentiation between species that contain 18:1 and 16:1 fatty acids was not possible either.

All parameters of the chromatographic system were carefully examined, optimized, and standardized. It was found that preliminary saturation of the developing tanks had a pronounced negative effect on the resolution. The same effect was observed when the mobile phase volume exceeded 6 mL. Also, Kieselguhr G layers provided better shaped zones, than did silica gel G layer.[31]

The capacity of the C_1 stationary phase is relatively low; thus, the sample size should be respectively small, 10 μg at the maximum. The same glass plates and all the supporting devices designed for the Ag-TLC were also useful for RP-TLC. Any well closed chamber was suitable to perform the silanization

procedure. It is, indeed, laborious but, once a large number of plates is prepared, they can be kept at least 6 months without any precaution and without losing resolution power.

The separation is not affected by the laboratory environment and is reproducible.

Analytical Protocol

Glass plates, 4 cm x 19 cm, are covered with ca. 0.2 mm thick kieselguhr G (Merck, Darmstadt, Germany) layers, air-dried for 24 h, followed by 1 h at 110°C. They are then cooled and stored in a desiccator over phosphorous pentoxide. Silanization is carried out by placing the plates for 6 h in a closed chamber over DMCS vapors in a fume cupboard. The plates are then washed by elution with methanol and were dried again at 110°C.

Aliquots of 5µL-10µL of 0.1% sample solution in chloroform are applied as a 4 mm long band on the plates. They are then developed twice in closed cylindrical tanks each time with fresh 3 mL of a mobile phase with a composition as shown in Table 1, to a front of 17 cm. Plates are dried for 10 min at 110°C after the first development. Once the development is finished, the plates are dried for 1h at 110°C (oven), sprayed with 50% sulphuric acid in ethanol, and heated at 200-220°C on a temperature controlled metal plate.

DENSITOMETRIC QUANTIFICATION

As already discussed in the above sections, there are two crucial moments in the TLC analysis of TAG: a relevant resolution of the components, and a relevant staining of the separated components. Obviously, since TLC is the separation method, densitometry is the natural choice for quantification. The procedure has been introduced in TAG analysis by Dallas and Padley in 1977 for Ag-TLC[33] and by Nikolova-Damyanova and Amidzhin in 1988 for RP-TLC.[31,34] When it comes to quantification of lipids, all separation techniques face the same problem: the lack of a chromogenic groups. This is, therefore, why we considered that staining by carbonization is as good as any other staining procedure tested so far with TAG. Charring is sensitive and is not affected by the concentration of the charring reagent or by the duration of treatment and is not time-dependent.

The charring procedures utilized for Ag-TLC and RP-TLC are described in full above. They are much alike and have been standardised, to a great extent, to give evenly carbonized zones on a white background. Obviously, the weak

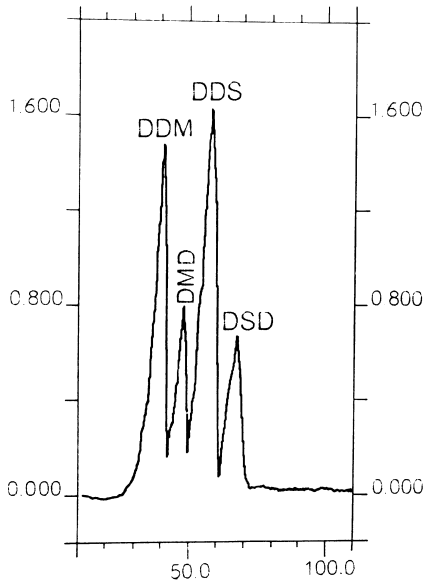

Figure 3. Separation of DSD, DDS, DMD, and DDM TAG species in randomised sunflower oil by Ag-TLC. Plate is impregnated with 2% methanolic silver nitrate and developed with 6 mL of 2.5% methanol in chloroform followed by 15 mL of 1.5% methanol in chloroform. Other experimental conditions as given in the Ag-TLC protocol. S, saturated, M, monoenoic and D, dienoic fatty acid residues, respectively. (Reproduced by the kind permission of Journal of Liquid Chromatography).

point was the home-made thin-layer. In order to fight back the skepticism, evidence had to be given which show that the layer, the impregnation, and the TAG structure do not hamper the densitometric quantification. Therefore, the quantification stage was given careful examination.[12,34]

Using SSS, MMM, DDD, and TTT as reference TAG for Ag-TLC and StStO, stop, and PPO as reference TAG for RP-TLC in different concentrations (in μg per spot), the response of scanning densitometry was examined. Zig-zag measurements, in transmittance and reflectance mode, at a wave-length of 430 nm were performed on a Shimadzu CS-930 instrument equipped with a Shimadzu DR-2 electronic integrator. The concentration range over which the transmittance mode measurements were linearly dependent on concentration was 0.2 to 10 μg per spot for Ag-TLC and 0.2 to 2.5 μg per spot for RP-TLC. For reflectance measurements, the corresponding ranges were 5 to 20 μg and 0.5 to 3.5 μg per spot. It was recommended to express results as normalized area percents, i.e., as derived form the integrator. It was confirmed that the relative

area percents represent the true sample composition with a relative standard deviation and relative error not exceeding 9% rel., irrespective of the mode of scanning.

Measurements in the reflectance mode are preferable, as they are less influenced by the thin-layer irregularities. Geometry of the beam should be selected in accordance with the resolution achieved. The author uses, usually, a beam of either 1.2 mm x 1.2 mm or 0.4 mm x 0.4 mm dimensions.

A danger of overloading always exists because of the great variety of TAG proportions in natural samples. A single overloaded zone will confuse the entire determination. Therefore, a comparison between the fatty acid composition as determined directly by gas chromatography with those calculated from the TAG data has been always considered mandatory. When the analyst follows, carefully, the respective protocols, a good agreement between the two sets of results is usually obtained, and this will be demonstrated in the following section.

The author is convinced, therefore, that densitometric quantification of TAG after charring is in no way inferior to other methods used for the same purpose with TLC and HPLC.

SELECTED APPLICATIONS

Quantitative Ag-TLC, applied as a single method, is by far more informative than is RP-TLC. It has been successfully employed in this laboratory to determine the TAG structure of a number of natural fats and oils: sunflower and olive oils,[14] orange,[15] grapefruit, tangerine, lemon,[16] tomato, pepper, and grape seed oils,[35] papaya,[36] wheat germ oil,[37] cocoa butter, lard, tallow,[15] mink fat.[38]

Another area that Ag-TLC has shown substantial efficiency is the resolution of positionally isomeric TAG, Fig. 3,[39] and of TAG that contained configurationally isomeric acyl residues, Fig. 4.[40] We were especially interested in the possibility to resolve positionally isomeric TAG, assuming that, in some cases, it would help to avoid the laborious stereospecific analysis. Species such as the pairs SMS-SSM, MMS-MSM, and SDS-SSD were resolved better than previously reported,[18,27] while the resolution of DSD-DDS and DMD-DDM shown in Fig. 3 has also been reported. Plates were impregnated with 1% methanolic silver nitrate for SMS-SSM, MMS-MSM, and SDS-SSD, and with a 2% solution for DSD-DDS and DMD-DDM TAG. Mobile phases were 0.5% to 2.5% methanol in chloroform. The resolution allowed reliable quantification of the isomeric TAG.

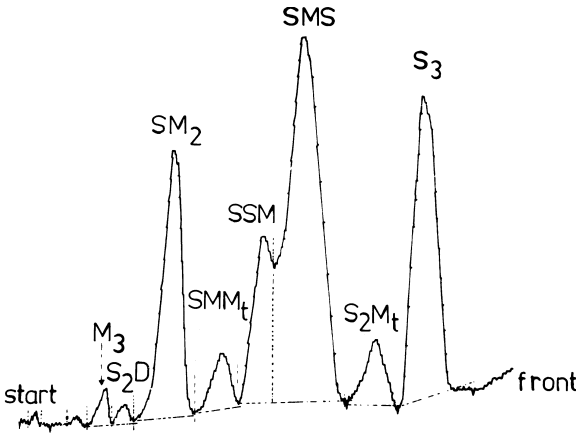

Figure 4. TAG profile in plasticiser from fur leather waste on Ag-TLC. Experimental conditions as given in the Ag-TLC protocol. Sample size, 25 μg; mobile phase 8 mL of petroleum ether-acetone, 100:3, by volume. S, saturated, M, monoenoic, M_t, trans-monoenoic, D, dienoic fatty acid residues. (Reproduced by the kind permission of the authors and Journal of the Society of Leather Technologists and Chemists).

More detailed information about the TAG structure of natural lipid samples has been obtained by using Ag-TLC and RP-TLC in complementary mode, with preparative Ag-TLC as intermediate stage. Preparative Ag-TLC is described in detail elsewhere[12,31,32,41] and will be not discussed here. Figure 5 demonstrates one of the first attempts to apply Ag-TLC and RP-TLC in succession, the analysis of peanut oil.[41] The oil is characterised by the presence of small amounts of long-chain saturated fatty acids: arachidic, 20:0 (0.9 %), behenic, 22:0 (3.1%) and lignoceric, 24:0 (1.0%). Fig. 5A shows the Ag-TLC separation of all 9 (of 10 theoretically possible according to the fatty acid composition) TAG classes. In Fig. 5B, are shown the densitograms of the RP-TLC separation of the five peanut TAG classes that contain saturated acyl residues (after isolation by preparative Ag-TLC form the total mixture). 23 individual TAG species and 6 two-component mixtures were quantified in the peanut oil. The same approach was applied to the analysis of sunflower[32] and olive[41] oils.

A more serious challenge was the analysis of plant oils of higher unsaturation, i.e., oils that contain moderate to high amounts of oleic, linoleic, and linolenic acid. While such oils did not raise any difficulties for the RP-TLC, they were a serious task for the Ag-TLC. Fig. 6 demonstrates the approach used to separate and quantify all 11 TAG classes present in corn oil with major fatty acids oleic (ca. 30%) and linoleic (ca. 55%).[42] It is similar to that shown above for orange seed oil, i.e., three separate developments on three different plates

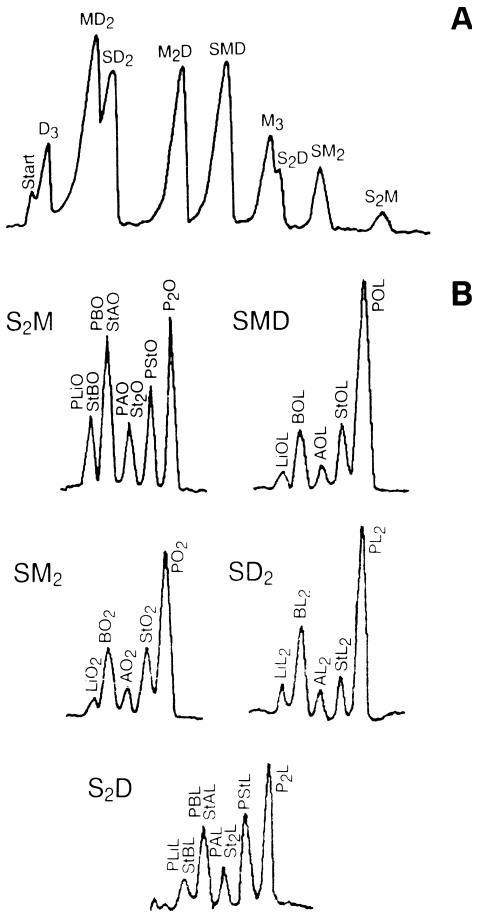

Figure 5. Separation of peanut oil TAG by complementary Ag-TLC and RP-TLC. A, separation of TAG classes by Ag-TLC; mobile phase is 7 mL hexane-acetone, 100:4.5, by volume; experimental conditions as given in the Ag-TLC protocol. B, separation of TAG classes by RP-TLC; mobile phases: acetone-acetonitrile-water, 70:30:10, by volume, for SSM, SMM and SSD; 70:30:12 for SMD and 70:30:16 for SDD TAG classes; two-fold development; other experimental conditions as given in the RP-TLC protocol. S, saturated, M, monoenoic, D, dienoic, T, trienoic, St, stearic, P, palmitic, A, arachidic, Li, lignoceric, O, oleic, L, linoleic fatty acid residues respectively. (Reproduced by the kind permission of La Rivista Italiana delle Sostanze Grasse).

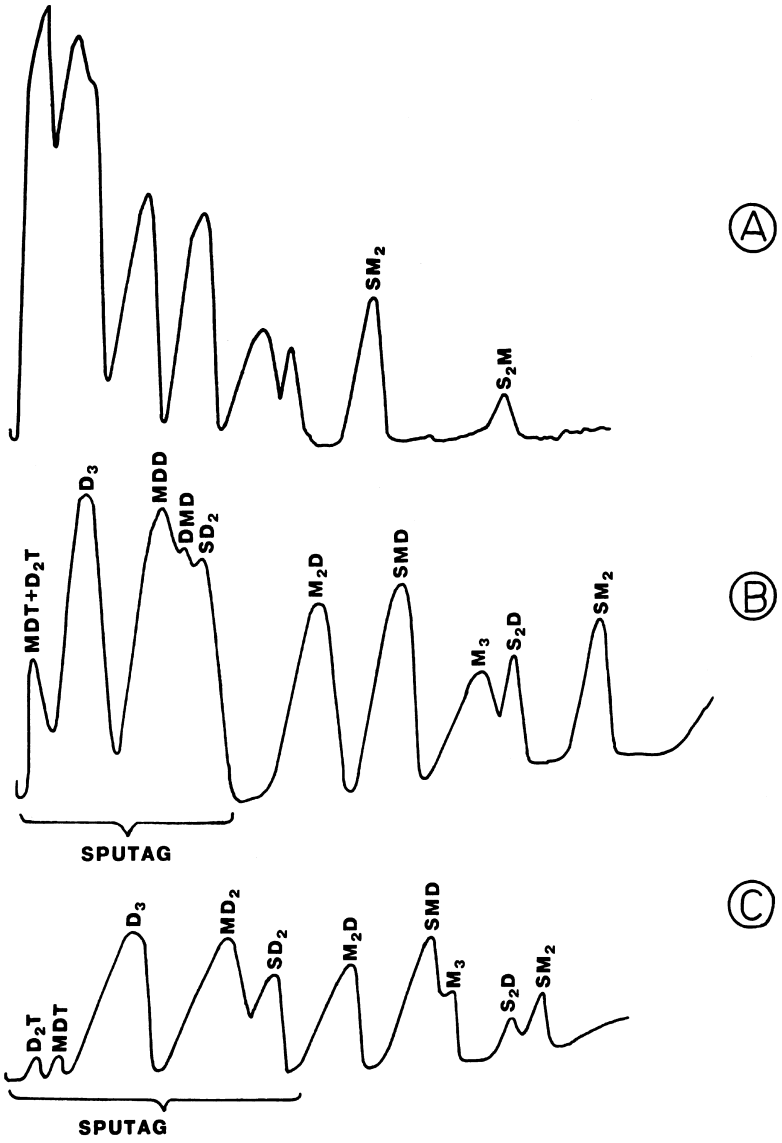

Figure 6. Separation of corn oil TAG by Ag-TLC. Experimental conditions as given in the Ag-TLC protocol. Plate A, mobile phase 7 mL petroleum ether-acetone, 100:5, by volume, sample size of 35-40 μg; plate B, mobile phase 5 mL petroleum ether-acetone, 100:8, by volume followed by 6 mL petroleum ether-acetone, 100:5, by volume, sample size of 30-35 μg; plate C, the mobile phases as for plate B, sample size 25-30 μg. S, saturated, M, monoenoic, D, dienoic, T, trienoic fatty acid residues, respectively.

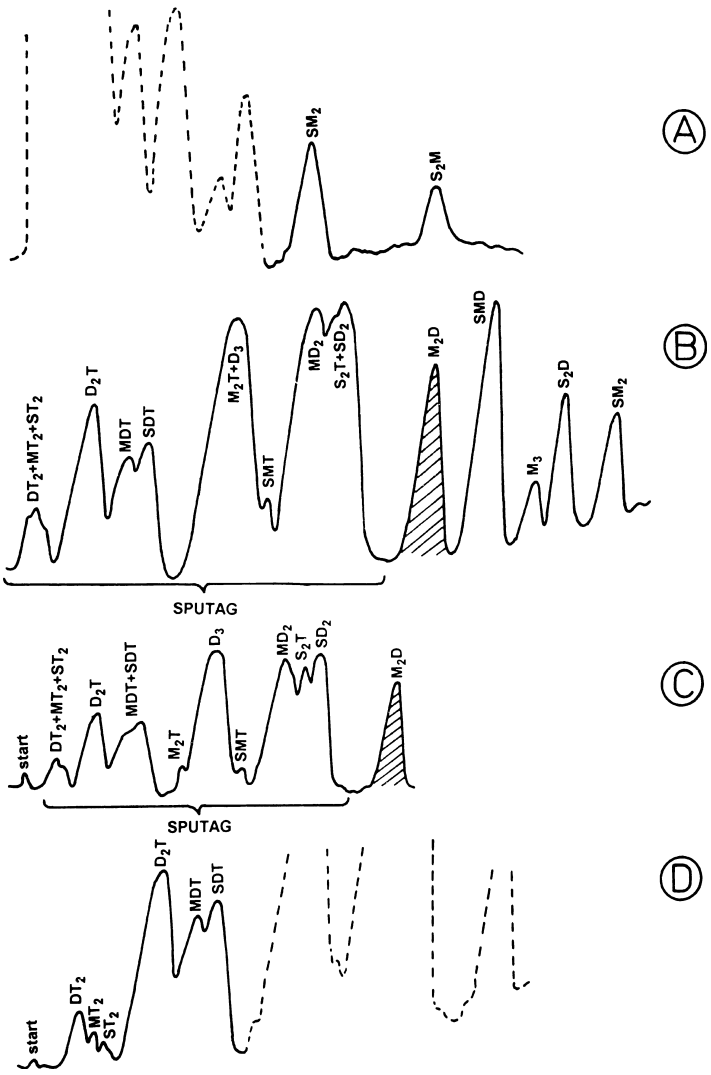

Figure 7. Separation of soybean oil TAG by Ag-TLC. Experimental conditions as given in the Ag-TLC protocol. Plate A, mobile phase 6 mL petroleum ether-acetone, 100:5, by volume, sample size 50 μg; plate B, mobile phase 12 mL hexane-acetone-ethanol, 100:8:1, sample size 30-35 μg; plate C mobile phase 8 mL hexane-acetone-ethanol, 100:6:1, by volume sample size 20-25 μg, plate D mobile phase 8 mL hexane-acetone-ethanol, 100:6:2, by volume, sample size 35-40 μg. S, saturated, M, monoenoic, D, dienoic, T, trienoic fatty acid residues, respectively. (Reproduced by the kind permission of Journal of Science Food and Agriculture).

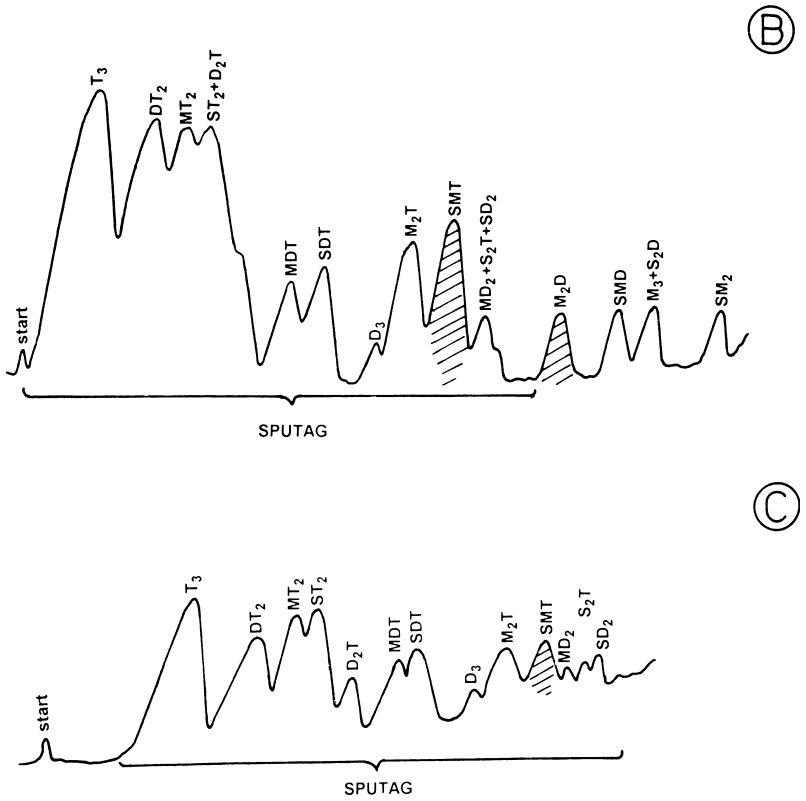

Figure 8. Separation of linseed oil TAG by Ag-TLC. Experimental conditions as given in the Ag-TLC protocol. Plate B, mobile phase 10 mL hexane-acetone, 100:12, by volume, sample size 30-35 μg; plate C, mobile phase 12 mL hexane-acetone-ethanol, 100:8:2, by volume, sample size 20 μg. S, saturated, M, monoenoic, D, dienoic, T, trienoic fatty acid residues, respectively. (Reproduced by the kind permission of Journal of Science Food and Agriculture).

were performed. On the first plate, A, a large sample-size was applied in order to determine, correctly, the ratio between the minor SSM and SMM components. On the second plate, B, some of the main TAG classes were clearly separated but SDD, MDD, DDD, and the minor components MDT and DDT were only partially resolved. These five TAG classes, denoted in Fig. 6 as SPUTAG (Sum of the Polyunsaturated TAG), were much better resolved on a third plate, C. The same sequence of two mobile phases was used as for plate B, but the sample size was lower. In the plates B and C, the MMD class which was well separated from the adjacent components was used as internal standard. The

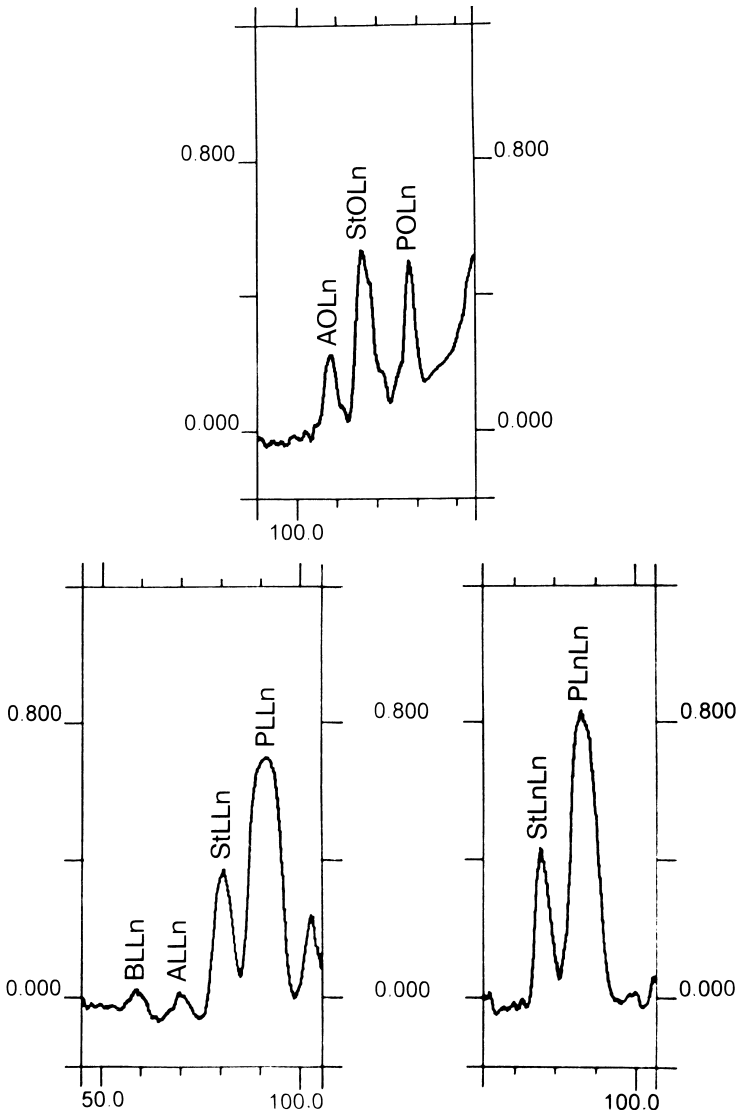

Figure 9. RP-TLC profile of SMT and SDT classes of soybean oil and STT class of linseed oil. Experimental conditions as given in the RP-TLC protocol. Mobile phases as shown in Table 1. St, stearic, P, palmitic, A, arachidic, Li, lignoceric, O, oleic, L, linoleic, Ln, linolenic fatty acid residues, respectively.

whole sample was applied on each plate. The analysis of corn oil is a good example of the role of the sample size in TLC. Evidently, it was not possible to clearly resolve and accurately quantify under the same conditions TAG classes differing so much in their proportion as were SSM (ca. 1%), MDD (27%) and DDD (19.0%). It was obvious that, for an accurate densitometric measurement of SSM, a reasonably large sample size was required. However, this would cause substantial overload of MDD and eventually of DDD zones; hence the different plates and different sample sizes. Special care was paid also, to prevent the overload of the major components in plate C. Quantification was considered correct only when the SPUTAG/MMD ratio remained constant.

In general, the same approach was applied to resolve the highly unsaturated soy bean and linseed TAG.[12] Soya oil contains not only a high percent of linolenic acid (56%), but also a substantial percent of linolenic acid (ca. 7%). Linseed oil is a rich source of linolenic acid (ca 53% of the total fatty acid content). These oils, therefore, contain a large amount of trienoic TAG. Thus, for the adequate resolution of all 18 TAG classes in soy been oil, four plates, four different mobile phases, and four different sample sizes were required (Fig. 7). The resolution of linseed oil was performed on three different plates with three mobile phases and three sample sizes.

Plates B and C are shown only in Fig. 8, since plate A was used to examine and confirm the absence of SSS and SSM TAG in the oil by applying 100 μg of the sample on the plate. Evidently, the resolution of these highly unsaturated oils was efficient and partial separation of the pairs SDD+SST; MMT+DDD; SDT+MDT and SST+MTT (considered to be critical pairs) was achieved that allowed for the first time to report their separate quantification.

TAG classes that contain saturated fatty acid residues were subjected to RP-TLC. Fig. 9 shows a typical densitometric profile of some of the trienoic classes, (see Table 1 for the mobile phases employed).

As a result, 32 molecular TAG species were determined in soy been oil and 26 TAG species - in linseed oil. As it has been shown in the paper,[12] by using Ag-TLC/RP-TLC, more TAG species have been separated, identified, and quantified in soya oil than previously reported by others who used RP-HPLC as a single separation method.

A single RP-HPLC separation evidently missed most of the minor TAG species because they form unresolved mixed peaks with some of the major TAG. The author is convinced, therefore, that for a relevant analytical determination of TAG composition, the successive application of silver ion - and reversed phase chromatography is essential, irrespective of the techniques used.

Table 2

Comparison of Fatty Acid Compositions of Selected Plant Oils as Determined Directly by GLC (A) with Those Calculated from the TAG Quantitative Data (B)[a]

Fatty Acids[b]	Peanut Oil		Corn Oil		Soybean Oil		Linseed Oil	
	A	B	A	B	A	B	A	B
14:0	---	---	<0.1	---	0.1	---	---	---
16:0	9.0	9.4	10.8	9.9	11.2	11.7	5.7	7.9
16:1	---	---	<0.1	---	<0.1	---	---	---
18:0	2.6	2.1	1.5	1.9	3.6	4.5	3.0	3.3
18:1	38.6	37.6	29.0	31.3	20.5	20.3	20.3	19.6
18:2	45.6	44.8	57.5	55.4	56.4	54.3	17.3	16.0
18:3	---	---	0.6	0.9	7.5	8.6	53.6	53.0
20:0	0.8	0.9	0.2	0.5	0.2	0.2	0.1	0.1
20:1	---	0.4	0.1	---	0.1	---	---	---
22:0	2.2	3.1	<0.1	---	0.3	0.2	---	---
24:0	0.8	1.1	---	---	---	---	---	---

[a] Saturated fatty acids under 0.2% and monoenoic fatty acids under 1.0% could not be determined by the RP-TLC procedure used;
[b] Fatty acid notation: carbon number:number of double bonds.

As stated in the previous section, quantitative densitometric results were always validated by comparing the fatty acid composition as determined by GC with those calculated from the respective TAG composition. Table 2 shows the results obtained for the samples used as examples in this section.

The good agreement is, in the authors' opinion, evidence for the high accuracy of the analytical approach presented here.

CONCLUSION

In 1990, W. W. Christie, in his regular column in Lipid Technology,[43] entitled his article, “Has Thin-Layer Chromatography Had Its Day?” giving the answer that “traditional TLC is not dead, but alive and well, and likely to be around for some time to come.” The author believes that the discussion and the results presented here are a vivid confirmation of these words.

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REFERENCES

1. A. Kuksis, L. Marai, J. J. Myher, J. Chromatogr., **273**, 43-66 (1983).

2. C. Litchfield, **Analysis of Triglycerides**, Academic Press, New York, 1972.

3. L. J. Morris, J. Lipid Res., **7**, 717-737 (1962).

4. W. W. Chrisrie, **Lipid Analysis**, Pergamon Press, Oxford, 1982.

5. G. Dobson, W. W. Christie, B. Nikolova-Damyanova, J. Chromatogr., B, **671**, 197-222 (1995).

6. V. Ruiz-Gurierrez, L. J. R. Barron, J. Chromatogr., B, **671**, 133-168 (1995).

7. B. Nikolova-Damyanova, "Silver Ion Chromatography and Lipids," in **Advances in Lipid Methodology-One**, W. W. Christie, ed., The Oily Press, Ayr, 1992, pp. 181-237.

8. B. Nikolova-Damyanova, "Reversed-Phase High-Performance Liquid Chromatography: General Principles and Application to the Analysis of Fatty Acids and Triacylglycerols," in **Advances in Lipid Methodology-Four**, The Oily Press, Dundee, 1997, pp. 193-251.

9. B. Fried, J. Sherma, "Lipids," in **Thin Layer Chromatography**, J. Cazes, ed., Marcel Dekker, New York, 1994, pp. 215-285.

10. B. Fried, "Lipids," in **Handbook of Thin-Layer Chromatography**, J. Sherma, B. Fried, eds., Marcel Dekker New York, 1991, pp. 593-625.

11. F. D. Gunstone, F. B. Padley, J. Am. Oil Chem. Soc., **42**, 957-961 (1965).

12. R. B. Tarandjiiska, I. N. Marekov, B. M. Nikolova-Damyanova, B. S. Amidzhin, J. Sci. Food Agric., **72**, 403-410 (1996).

13. C. B. Barrett, M. S. J. Dallas, F. B. Padley, Chem. Ind. (London), 1052 (1962).

14. D. Chobanov, R. Tarandjiska, R. Chobanova, J. Am. Oil Chem. Soc., **53**, 48-51 (1976).

15. R. Tarandjiiska, H. Nguyen, Riv. Ital. Sost. Grasse, **65**, 489-492 (1988).

16. R. Tarandjiiska, H. Nguyen, Riv. Ital. Sost. Grasse, **66**, 99-102 (1989).

17. T. H. Dzido, E. J. Soczewinski, J. Chromatogr., **516**, 461-466 (1990).

18. C. B. Barrett, M. S. J. Dallas, F. B. Padley, J. Am. Oil Chem. Soc., **40**, 580-584 (1963).

19. B. Nikolova-Damyanova, S. Momchilova, unpublished results.

20. D. Chobanov, Lab. Pract., **26**, 481 (1977).

21. D. Chobanov, R. Tarandjiiska, Chem. Commun., **15**, 45-53 (1982).

22. H. K. Mangold, B. G. Lamp., H. Schlenk, J. Am Oil Chem. Soc., **77**, 6070 (1955).

23. H. P. Kaufmann, Z. Makus, Fette Seifen Anstrichmittel, **62**, 1040-1045 (1960).

24. H. P. Kaufmann, Z. Makus, B. Das, Fette Seifen Anstrichmittel, **63**, 807-811 (1961).

25. H. P. Kaufmann, H. Wessels, Fette Seifen Anstrichmittel, **66**, 81-86 (1964).

26. H. P. Kaufmann, H. Wessels, Fette Seifen Anstrichmittel, **68**, 249-255 (1966).

27. H. Wessels, N. S. Rajagopal, Fette Seifen Anstrichmittel, **71**, 543-552 (1969).

28. H. Wessels, Fette Seifen Anstrichmittel, **75**, 478-483 (1973).

29. W. O. Ord, P. C. Bamford, Chem. Ind. (London), 277-278 (1967).

30. D. Chobanov, personal communucation.

31. B. Nikolova-Damyanova, B. Amidzhin, J. Chromatogr., **446**, 283-291 (1988).

32. B. Amidzhin, B. Nikolova-Damyanova, J. Chromatogr., **446**, 259-266 (1988).

33. F. B. Dallas, F. B. Padley, Lebensm. Wis. u. Technol., **10**, 328-331 (1977).

34. B. Nikolova-Damyanova, B. Amidzhin, J. Planar Chromatogr., **4**, 397-401 (1991).

35. R. Tarandjiiska, H. Nguyen, V. Lichev, Riv. Ital. Sost. Grasse, **68**, 309-312 (1991).

36. H. Nguyen, R. Tarandjiiska, Fat Sci. Technol., **97**, 20-23 (1995).

37. B. Ivanova, R. Tarandjiska, D. Chobanov, A. Popov, Rev. Fr. Corps Grass, **24**, 439-440 (1977).

38. D. Chobanov, A. Popov, R. Tarandjiska, B. Ivanova, Rev. Fr. Corps Grass, **23**, 671-673 (1976).

39. B. Nikolova-Damyanova, D. Chobanov, S. Dimov, J. Liquid Chromatogr., **15**, 3997-4008 (1993).

40. M. Velcheva, D. Bileva, D. Ivanova, R. Tarandjiiska, J. Soc. Leath. Technol. & Chem., **76**, 51-53 (1992).

41. D. Chobanov, B. Amidzhin, B. Nikolova-Damyanova, Riv. Ital. Sost. Grasse, **68**, 357-362 (1991).

42. R. Tarandiiska, I. Marekov, B. Nikolova-Damyanova, B. Amidzhin, J. Liq. Chromatogr., **18**, 859-872 (1995).

43. W. W. Christie, Lipid Technology, **2**, 22-23 (1990).

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