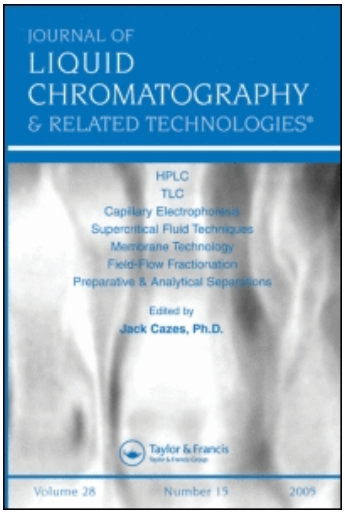


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# Fatty Acids, Triacylglycerols, and Sterols in Neem Oil (*Azadirachta Indica A. Juss*) as Determined by a Combination of Chromatographic and Spectral Techniques

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**Abstract:** Quantitative thin-layer chromatography in silver ion and reversed phase modes, gas chromatography, gas chromatography–mass spectrometry, and $^{13}C$ nuclear magnetic resonance spectrometry were employed to determine the lipid composition in neem (*Azadirachta indica* A. Juss) kernel oil. Thirteen fatty acids and 25 triacylglycerols species were identified and quantified. The seven main triacylglycerols species were found in almost equal amounts in the range 7–10%. Saturated fatty moieties occupied positions 1- and 3- in the triacylglycerol molecule, oleic acid was almost equally distributed, and linoleic acid predominantly occupied position 2. Sitosterol, stigmasterol, campesterol, and fucosterol were the main components in free sterol and sterol ester fractions.

**Keywords:** Gas chromatography, Thin-layer Chromatography, NMR, Fatty acids, Triacylglycerols, Neem oil

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## INTRODUCTION

The neem tree, *Azadirachta indica* A. Juss, is known for centuries for its natural pest control properties. The plant has been used in traditional and comprehensive medicine for various types of diseases and, at present, is a popular and widely advertised component in herbal based cosmetics.[1–4] More than 300 compounds have been characterized from neem seeds and one third of these bear the anti-pest activity. These represent a series of compounds that are biogenetically derived from tetracyclic terpenes (tetranortriterpenoids).[5] The very strong activity and high efficiency of these compounds as pesticides provoked the search of other biologically active components in different parts of the neem tree.

On this basis, the attention focused on the second important product of the neem tree, the glyceride oil, is minimal. Some data on fatty acid composition were reported as early as 1936.[6] Only four fatty acids were identified and their proportions were summarized in the major book by Eckey.[2] Understandably, these data cannot be considered comprehensive since the analytical technique employed was of limited ability. Newer studies employing gas chromatography hardly added any new information about the fatty acid profile increasing the fatty acid components to six.[7–11] There is no information whatsoever about the triacylglycerols and sterol composition of the oil.

The aim of this work was to give more detailed information on the fatty acid profile, and to determine the triacylglycerol and sterol composition of a commercial sample of neem kernel oil by employing various up-to-date chromatographic and spectral techniques, with the hope that this valuable source of glyceride oil will find a practical application.



## EXPERIMENTAL

### Chemicals and Materials

All reagents and solvents were analytical grade. Petroleum ether was b.p. 40–60°C fraction, diethyl ether was peroxide free, and chloroform was extensively washed to remove the stabilizing alcohol, dried over $CaCl_2$ and distilled. Dimethyldichlorosilane (DMDS) was purchased from Fluka (Buchs, Switzerland); Kieselguhr G, Silica gel G, bromine, and sulphuryl chloride were obtained from Merck (Darmstadt, Germany).

A reference mixture of lipid classes was prepared by admixing equal aliquots of 100 mg/mL solutions of docosane, cholesteryl oleate, methyl oleate, oleyl alcohol, cholesterol, 1,3-diolein, 1-monoolein-rac-glycerol, L-$\alpha$ dioleylphosphatidyl-choline (all purchased from Sigma-Aldrich Chemie GmbH, Germany), and purified (preparative silica gel thin-layer chromatography (TLC), see below) triacylglycerol (TAG) fraction from sunflower oil in dichloroethane. Mixtures of purified TAG fractions of lard and

sunflower oil, and pure TAG fractions with known compositions from tangerine oil[12] and from linseed oil[13] were used as reference TAG mixtures employed to identify the TAG components in neem oil.

## Preparation of the Neem Oil Sample

Approximately 198 g neem seeds were decorticated manually to obtain 99 g kernels, which were then spread over filter paper and left to air dry for about 96 h to a constant weight. The dry kernels were ground in a coffee mill and portions of the material were used for further investigation. The oil was extracted by an 8 h Soxhlet extraction with petroleum ether. To determine the oil content, two separate samples of milled kernels, 55 g and 30 g, were treated in the same way.



## Extraction of Lipids

The procedure described by Christie[14] utilizing extraction with isopropanol as a first step was applied. After evaporation of the solvents, the residue was dissolved in dichloroethane to give 100 mg/mL stock solution of total lipids. An aliquot was taken to give 5 mg/mL solution in dichloroethane, which was used to identify the lipid classes.

## Identification of the Main Lipid Classes by Analytical Silica Gel TLC

The lipid class composition was determined by analytical silica gel TLC. For this purpose, an aliquot of the 5 mg/mL lipid solution (sample size of about 50–100 μg) in dichloroethane was applied on a 19 cm × 4 cm glass plate (ca. 0.2 mm thick silica gel G layer), and a reference lipid mixture (20 μL of 10 mg/mL solution in dichloromethane) was applied alongside the plate. After a single development with ca. 4 mL petroleum ether-acetone, 100:8 (by volume), the lipid zones were detected by spraying with 50% ethanolic sulphuric acid and heating at 200°C, on a metal plate with temperature control.

## Isolation, Purification and Quantification of Lipid Classes by Preparative Silica Gel TLC

Aliquots of the 100 mg/mL stock solution (sample size of 100 mg, precisely measured) were applied on 20 cm × 20 cm glass plates (ca. 1 mm thick silica gel G layer) and developed with petroleum ether-acetone, 100:8 (by volume). The separated zones were detected by spraying the edges of each plate with

2′,7′-dichlorofluorescein for visualization under UV. Partial acylglycerols, free fatty acids, and polar lipids, that migrated below the sterols in this system, were collected jointly, and the components were separated on other preparative silica gel G plates with mobile phase petroleum ether-acetone-acetic acid, 70:30:1 (by volume). The corresponding zones from all plates were scrapped, transferred to small glass columns, and eluted with diethyl ether. The solvent was evaporated under a stream of nitrogen and the residue was weighted in small glass containers (of known weight) to a constant weight.

## Analysis of Triacylglycerols (TAG)

### Quantitative Ag-TLC

The procedure is described in details elsewhere.[15] Briefly, TAG classes differing in unsaturation were separated on 19 cm × 4 cm glass plates, coated with ca. 0.2 mm silica gel G layer, and impregnated by dipping into a 0.5% methanolic solution of silver nitrate. The sample size and the mobile phase composition depended on the required separation and are shown in Table 1. Continuous ascending development with the specified volume of the mobile phase in open cylindrical tanks (24 cm × 5 cm i.d.) was performed. The plates were then dried (1 hour at 110°C), and treated consecutively with bromine and sulphuryl chloride vapours (30 min each, in



***Table 1.*** Chromatographic conditions for the separation of TAG classes by silver ion TLC

| | |
|---|---|
| Separation of $S_3$, $S_2M$ and $SM_2$ | |
| Sample (μg) | 50 |
| Mobile phase (v/v) | PE:A 100:4 |
| Vol. (mL) | 6 |
| Separation of TAGs from $SM_2$ to SDT | |
| Sample (μg) | 30–35 |
| Mobile phase (v/v) | PE:A 100:4 |
| Vol. (mL) | 6 |
| Separation of TAGs from $M_2D$ to $D_2T$ | |
| Sample (μg) | 25–30 |
| Mobile phase (v/v/v) | Hx:A:EtOH 100:4:2 |
| Vol. (mL) | 6 |

S—saturated; M—monoenoic; D—dienoic; T—trienoic fatty acyl residues; PE—petroleum ether (b.p. 40–60°C); A—acetone; Hx—hexane; EtOH—ethanol.

closed tanks and in a fume-cupboard) to ensure quantitative charring (at 180–200°C on a metal plate) of the separated TAG classes.

### Preparative Ag-TLC

Preparative Ag-TLC was carried out as described.[16] TAG classes were separated on 20 cm × 20 cm glass plates (ca. 1 mm thick silica gel G layer, which contained 2% silver nitrate) using the following mobile phases: chloroform-acetone, 100:1.4 (by volume) for $S_2M$ and $SM_2$ classes, chloroform-acetone, 100:3 (by volume) for $S_2D$ and SMD classes, and chloroform-methanol, 100:3.5 (by volume) for $SD_2$, $S_2T$, and SMT classes (S, saturated-, M, monounsaturated-, D, diunsaturated- and T, triunsaturated acyl residues). Plates were sprayed with 2′,7′-dichlorofluorescein and TAG zones were visualized under UV light, scrapped off, and eluted with diethyl ether. The purity and identity of each zone were checked by analytical Ag-TLC, after cochromatographing with the reference TAG mixture and the source oil applied alongside. The solvent was removed under nitrogen and samples were redissolved in hexane to give 1 mg/mL solution.

### Quantitative RP-TLC

The procedure described by Chobanov et al.[17] was used. In brief, 19 cm × 4 cm glass plates, covered with ca. 0.2 mm thick Kieselguhr G layer, were first treated for 6 hours with vapours of DMDS and, then, washed by a single elution with methanol. A 5–10 μL aliquot of the 1 mg/mL TAGs hexane solution was applied on the plate and developed twice in a closed cylindrical tank (dimensions as above), each time with fresh 3 mL of the mobile phase to a solvent front movement of 17 cm. A three component mobile phase (acetone/acetonitrile/water) was used with acetone/acetonitrile ratio kept constant (7:3, by volume). The mobile phase compositions employed, depended on the degree of unsaturation of the TAG class and are presented in Table 2. Plates were dried (at 110°C for 1 h) and separated species were visualized by spraying with 50% ethanolic sulphuric acid and heating at 200–220°C for about 5 min, over a temperature controlled metal plate.

### Quantification by Densitometry

The densities of the charred spots were measured by a CS-930 densitometer (Shimadzu Corporation, Kyoto, Japan), equipped with DR-2 Shimadzu integrator, in the zigzag reflection mode at 450 nm. Beam-slit was varied from 0.4 × 0.4 mm to 1.2 × 1.2 mm, and the stage step varied depending on the separation achieved. The quantity of each spot was presented as relative area percent, as derived from the integrator. Two sets of densitometric results were obtained: Ag-TLC provided the quantitative data for the

***Table 2.*** Water proportion in the mobile phase acetone/acetonitrile/water, 70:30:W (v:v:v), for the separation of TAG classes into molecular species by RP-TLC

| TG class[a] | TG species[b] | PN[c] | Water proportion, W, 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 |

[a]For the abbreviations see the footnote to Table 1.

[b]The order of designation does not indicate positional isomers, P—palmitic; St—stearic; A—arachidic; B—bechenic; O—oleic; L—linoleic; Ln—linolenic fatty acyl residues.

[c]Partition number, PN = CN-2NDB (CN, number of carbon atoms, NDB, number of double bonds).



TAG classes differing in unsaturation, and RP-TLC for the TAG species differing in chain length within a given class. The standard deviation of Ag-TLC in the present analysis (three separate TLC runs) did not exceed 1% abs.

## 4,4-Dimethyloxazoline (DMOX) Derivatives of Fatty Acids (FA)

DMOX derivatives were produced following the procedure described by Christie.[18] Briefly, 0.25 g of 2-amino-2-methyl-1-propanol were added to the free fatty acids (up to 2 mg) in a test tube, which was flushed with nitrogen, stoppered, and placed in a silicon oil bath at 190°C for 2 h. The mixture was then cooled, dichloromethane (1 mL) and hexane (5 mL) were added, and the solution was washed twice with 2 mL of water. It was then passed through a small column (Pasteur pipette) with anhydrous sodium sulphate. The completeness of reaction was checked by silica gel TLC with a mobile phase of 3 mL chloroform-methanol, 100:3 (by volume) to a front of 10 cm. A standard mixture with free fatty acids was applied alongside as a reference substance. DMOX derivatives ($R_f = 0.4$) were detected by treating the plate successively with bromine and sulphuryl chloride vapours and heating at 180°C on a metal plate. The solvent was evaporated under stream of nitrogen and dissolved in hexane, to give a 20 mg/mL working solution for analysis by gas chromatography-mass spectrometry (GC-MS).

## Gas Chromatography-Mass Spectrometry of Fatty Acids DMOX Derivatives and Sterols

GC-MS analyses were performed on a HP 6890-Plus gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), coupled to an HP 5793 mass selective detector (Agilent Technologies, Santa Clara, CA, USA). The latter operated in electron impact mode (70 eV) with source temperature of 230°C and quadrupole temperature of 150°C; scanning from m/z 45 to m/z 750. Helium was the carrier gas at a constant flow rate of 0.8 mL/min; split 20:1.

A 30 m × 0.25 mm (I.D.) CPWax 52 capillary column (Varian Chrompack International B.V., Middelburg, Netherlands) was employed for the FA DMOX derivatives. The oven was programmed from 165°C to 240°C at 4°C/min and held at this temperature for 15 min. Intact free sterols and sterols derived from the sterol esters were separated and identified on a SPB-50 column (Supelco Inc., Bellefonte, PA, USA), 25 m × 0.25 mm, with the oven programmed from 285°C, held at this temperature for 3 min, and increased to 295°C with 2°C/min, and held at this temperature for 30 min.



## Gas Chromatography of Fatty Acids Methyl Esters (FAME) and Sterols

FAME from sterol esters (SteE), TAG, partial acylglycerols (PA), and polar lipids (PL) were prepared according to Ref. [19]. Free fatty acids were methylated according to Christie,[20] with 1% methanolic sulphuric acid for 2 h at 50°C. A Hewlett Packard model 5890 (Hewlett Packard GmbH, Austria) gas chromatograph was used with a 30 m × 0.25 mm (I.D.) capillary INNOWax column (cross-linked PEG, Hewlett Packard GmbH, Austria). The column temperature was programmed from 165°C (held for 3 min) to 200°C at 4°C/min, and held at this temperature for 20 min; injector and detector temperatures were 260°C. Nitrogen was the carrier gas, flow, 0.8 mL/min; split 80:1.

The proportion of free sterols and of those derived from the sterol esters were determined on 30 m × 0.25 mm (I.D.) SPB-50™ capillary column (Supelco Inc., Bellefonte, PA, USA) under the following conditions: temperature gradient from 230°C to 270°C at 4°C/min, and held at this temperature for 10 min, injector temperature was 300°C, and detector temperature was 320°C. Nitrogen was the carrier gas, flow, 0.8 mL/min; spilt 80:1.

Results were presented as a mean area percent (as derived from the integrator) of three separate injections ± Standard Deviation (S.D.).

## Positional Distribution of Acyl Residues in TAG Molecules

High-resolution $^{13}C$ NMR spectroscopy on a Bruker DRX-250 spectrometer was applied to determine the positional distribution of the unsaturated acyl

residues in the TAG molecules. The method developed by Simova et al.[21] was employed.

## RESULTS AND DISCUSSION

### Lipid Class Composition

The sample was found to contain 43% oil, which was very close to the 45% found previously,[8] and represented almost pure glyceride oil with components like sterol esters (SteE), free sterols (Ste), partial acylglycerols (PA, i.e., monoacylglycerols and diacylglycerols), free fatty acids (FFA), and polar lipids (PL) not exceeding 2% each (Table 3).



### Fatty Acid Composition

The FA composition of total lipids and that of SteE, TAG, PA, FFA, and PL lipid classes are shown in Table 4. Thirteen FA's were identified by applying GC-MS of their DMOX derivatives (Table 4). In contrast to other kernel oils,[22,23] neem oil is characterized with an unusually high content of stearic acid (18:0, 16% in total lipids), almost equal to that of palmitic acid (16:0, 17%), while oleic acid (9–18:1, 40%) and linoleic acid (9,12–18:2, 21%) were the main components. The oil also contained minor amounts of a series of other saturated (14:0, 20:0, 22:0, and 24:0) and monoenoic (9–16:1, 11–18:1, 9–20:1, and 13–22:1) fatty acids. Under the chromatographic conditions employed, *cis*-vaccenic acid, 11–18:1, which often accompanies oleic acid in many seed oils[24] was clearly separated from oleic acid and was correctly quantified. The neem oil sample contained less than 1% linolenic acid (9,12,15–18:3). As is seen from Table 4, FA's were specifically distributed between the lipid classes, the only common feature being that 16:0, 18:0, 9–18:1, and 9,12–18:2 were major components in each class. The

***Table 3.*** The major lipid classes in neem kernel oil (wt. %)

| Lipid class | Present work |
|---|---|
| Sterol Esters | $0.6 \pm 0.1^a$ |
| Triacylglycerols | $94.5 \pm 2.1$ |
| Free sterols | $1.5 \pm 0.2$ |
| Free Fatty Acids | $0.8 \pm 0.1$ |
| Partial Acylglycerols | $0.4 \pm 0.1$ |
| Polar Lipids | $0.6 \pm 0.2$ |

[a]Mean $\pm$ S.D. (S.D., Standard Deviation), n = 4.

***Table 4.*** Distribution of fatty acids between the lipid classes of neem oil[a]

| Fatty acids | Total lipids | Sterol esters | TAG | Partial acyl-glycerols | Free fatty acids | Polar lipids |
|---|---|---|---|---|---|---|
| 14:0 | Traces | 0.2 ± 0.1 | Traces | 0.1 ± 0.05 | 0.4 ± 0.1 | 0.3 ± 0.1 |
| 16:0 | 17.6 ± 0.5 | 6.5 ± 0.2 | 17.1 ± 0.2 | 15.3 ± 0.2 | 22.0 ± 0.3 | 21.7 ± 1.1 |
| 9–16:1 | 0.1 ± 0.05 | 0.5 ± 0.1 | 0.1 ± 0.05 | 0.1 ± 0.05 | Traces | 0.5 ± 0.1 |
| 18:0 | 16.1 ± 0.2 | 10.7 ± 0.2 | 16.1 ± 0.1 | 12.7 ± 0.2 | 13.6 ± 0.1 | 18.1 ± 0.3 |
| 9–18:1 | 40.1 ± 0.2 | 23.3 ± 0.1 | 40.0 ± 0.1 | 47.5 ± 0.1 | 36.1 ± 0.2 | 37.8 ± 0.3 |
| 11–18:1 | 2.0 ± 0.2 | 1.5 ± 0.1 | 2.3 ± 0.1 | 2.0 ± 0.1 | 1.5 ± 0.1 | 2.0 ± 0.1 |
| 9,12–18:2 | 21.3 ± 0.3 | 38.2 ± 0.1 | 21.5 ± 0.3 | 20.4 ± 0.2 | 23.7 ± 0.8 | 15.5 ± 0.7 |
| 9,12,15–18:3 | 0.8 ± 0.1 | 3.0 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.1 | 1.7 ± 0.2 | 0.5 ± 0.1 |
| 20:0 | 1.3 ± 0.1 | 4.9 ± 0.1 | 1.3 ± 0.1 | 0.7 ± 0.1 | 1.0 ± 0.2 | 1.7 ± 0.1 |
| 9–20:1 | 0.1 ± 0.02 | 0.4 ± 0.06 | 0.1 ± 0.05 | 0.2 ± 0.05 | Traces | 0.2 ± 0.05 |
| 22:0 | 0.3 ± 0.1 | 3.0 ± 0.1 | 0.2 ± 0.01 | 0.2 ± 0.1 | n.d. | 0.7 ± 0.3 |
| 13–22:1 | Traces | n.d. | Traces | 0.1 ± 0.01 | n.d. | 0.2 ± 0.1 |
| 24:0 | 0.2 ± 0.1 | 8.0 ± 0.2 | 0.1 ± 0.01 | 0.1 ± 0.01 | n.d | 0.7 ± 0.1 |
| U/S | 1.8 | 2.0 | 1.9 | 2.7 | 1.7 | 1.4 |

[a]Mean area % ± S.D., n = 3, U, unsaturated, S, saturated acyl residues.

specificity in the FA distribution is illustrated by the unsaturation of each lipid class, represented by the U/S (U, unsaturated, S, saturated fatty acids) ratio (Table 4). Thus, PA is the most unsaturated (U/S = 2.7) and PL is the most saturated (U/S = 1.4) lipid class. Expectedly, the FA composition of total lipids resembled that of TAG.

## TAG Composition

Table 5 presents the TAG composition as determined by a combination of analytical Ag-TLC, preparative Ag-TLC, and RP-TLC. As has been shown previously,[25] this combination provides results comparable to the detailed TAG analysis achieved by the complementary application of Ag-HPLC and RP-HPLC.[26] The analytical Ag-TLC was the core stage for the quantification and much care was paid to produce clear resolution and reproducible densitometric quantification of TAG classes differing in unsaturation. Three plates and two mobile phases were employed for a single analysis, conditions being given in Experimental. The first plate was used to determine the correct proportion of the most saturated TAG classes, $S_2M$, $SM_2$, $M_3$, and $S_2D$. The total TAG composition (according to the unsaturation) was determined on a second plate. The proportions of TAG classes with 4 and more double bonds were determined on a third plate with appropriate mobile phase. These classes were separated using appropriate quantities to obtain satisfactory densitometric determination. $D_3$ class was used as an internal standard being clearly separated from the neighbouring TAG classes on both plates. Preparative Ag-TLC was then applied allowing isolation of substantial amounts of TAG for subjection to RP-TLC. It was possible, therefore, to correctly determine the species in TAG classes that were minor components in the oil.

The RP-TLC differentiates TAG species differing in partition number (PN), where PN = CN-2NDB (CN, number of carbon atoms, NDB, number of double bonds). The technique has two major limitations at present: (i) no conditions were found to separate the components of trisaturated TAG class and (ii) it is not possible to separate species differing in the chain length of the monounsaturated acyl residues (i.e., 16:1 from 18:1), probably because of the significant difference in their amounts (0.1% and 42%, respectively). Despite the limitations, 25 molecular species were determined in neem oil, the main being PStO, StOO, POO, OOO, POL, StOL, OOL (St, stearic-, P, palmitic-, O, oleic- and L, linoleic acyl residues). These species were present in almost equal amounts and comprised about 60% of the total TAG content. In Table 5, TAG's are ordered according to the elution order in the given TLC technique, starting from the least strongly retained placed on the top of the respective column. Acyl residues of all four major FA's were presented and, in agreement with the general rules postulated by Litchfield,[27] mixed acid TAG dominated with OOO being the single monoacid TAG. TAG

*Table 5.* Composition of neem oil TAG classes[a]

| TAG classes (number of double bonds) | TAG molecular species[b] | |
|---|---|---|
| $S_2M$ (1) | PPO | 4.3 ± 0.2 |
| | PStO | 7.6 ± 0.1 |
| | PAO + StStO | 4.7 ± 0.1 |
| | PAO + StAO | 0.8 ± 0.1 |
| $SM_2$ (2) | POO | 9.8 ± 0.1 |
| | StOO | 10.6 ± 0.1 |
| | AOO | 0.1 ± 0.05 |
| $M_3$ (3) | OOO | 8.0 ± 0.2 |
| $S_2D$ (2) | PPL | 3.4 ± 0.1 |
| | PStL | 5.5 ± 0.1 |
| | PAL + StStL | 3.4 ± 0.1 |
| | StAL | 0.8 ± 0.1 |
| SMD (3) | POL | 9.1 ± 0.2 |
| | StOL | 8.2 ± 0.1 |
| | AOL | 0.7 ± 0.1 |
| $M_2D$ (4) | OOL | 7.8 ± 0.2 |
| $SD_2$ (4) + $S_2T$ (3) | PLL | 4.0 ± 0.2 |
| | StLL | 3.1 ± 0.1 |
| | ALL | 0.3 ± 0.1 |
| $MD_2$ (5) | OLL | 4.0 ± 0.2 |
| SMT (4) | POLn | 0.1 ± 0.05 |
| | StOLn | 0.1 ± 0.05 |
| $D_3$ (6) | LLL + OOLn | 2.6 ± 0.2 |
| SDT (5) | StLLn | 1.0 ± 0.2 |
| $D_2T$ (7) | LLLn | 0.3 ± 0.1 |

[a]For the abbreviations see the footnotes to Tables 1 and 2.

[b]Relative area %, mean of three separate Ag-TLC and three separate RP-TLC determinations. For details see Experimental.



containing 22:0 and 24:0 were not detected, probably because the content was below the detection limits of the chosen technique.

The quantitative results for TAG composition were verified by the good fit between the FA proportions as calculated from TAG and as determined by GC, Table 6.

## Distribution of FA in TAG Molecule

The distribution of FA in TAG has very rarely been the focus of lipid studies, as the classic methodology for stereospecific analysis is very laborious and time consuming.[14] Approaches using chromatography[28,29] or NMR[30]

***Table 6.*** Comparison between the fatty acid compositions of neem TAGs (a) determined directly by GC and (b) calculated from TAG data[a]

| Fatty Acids | (a) | (b) |
|---|---|---|
| 16:0 | 17.4 | 17.1 |
| 18:0 | 16.1 | 17.2 |
| 18:1 | 42.3 | 40.0 |
| 18:2 | 21.5 | 23.6 |
| 18:3 | 0.7 | 0.5 |
| 20:0 | 1.3 | 0.8 |

[a]Saturated fatty acids under 0.2% and monoenoic fatty acids under 1.0% could not be determined by the RP-TLC procedure used.



have been developed, and these produce similar information distinguishing FA in positions 1,3- (or $\alpha$-) from position 2- (or $\beta$-) in the TAG molecule. The results provided by the $^{13}C$ NMR by applying the approach described in[21] were as follows: 100% of the saturated FA were placed in positions 1- and 3- of the TAG molecule, oleic acid was almost equally distributed between the positions (51% in positions 1,3- and 49% in position 2), and linoleic acid predominantly occupied position 2 (67%), while 33% were found in positions 1,3-. The FA distribution in the TAG molecule in neem oil was, therefore, typical for higher plants.[27]

## Sterol Composition

Composition of free sterols and sterols derived from sterol esters is presented in Table 7. In each sterol class, the identified components

***Table 7.*** Main sterols (rel. area %)[a]

| Compound | Free sterols | Sterols derived from sterol esters |
|---|---|---|
| Campesterol | $8.3 \pm 0.2$[b] | $10.4 \pm 0.2$[b] |
| Stigmasterol | $16.8 \pm 0.2$ | $6.7 \pm 0.1$ |
| Sitosterol | $70.1 \pm 0.3$ | $66.2 \pm 0.5$ |
| Fucosterol | $4.1 \pm 0.1$ | $13.7 \pm 0.2$ |
| Unidentified | 0.7 | 2.3 |

[a]Normalized.
[b]Mean $\pm$ S.D., n = 3.

comprised over 95% of the total content and the same four sterols were present. Free sterols and sterol esters differ only by the proportions of the individual components. As with all high terrestrial plants, sitosterol is the main component, stigmasterol follows in free sterols, while fucosterol is the second major component in the sterol esters fraction.

## CONCLUSION

The neem kernel oil content has a specific FA and TAG composition. The oil is characterized by a relatively high general content of saturated FA, half of which is due to the unusually high amount of stearic acid. The seven main TAG groups were present in almost equal moderate amounts in the range of 7% to 10%. Due to the bitter elements, the oil is considered not suitable for nutrition[8] but could be a promising raw material for tailor made fats and oils.



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## REFERENCES

1. Roy, A.C.; Dutts, S. Composition of Neem Oil, so Called Margosic Acid. J. Soc. Chem. Ind. London **1929**, *48*, 333T–335T.
2. Eckey, E.W. *Vegetable Fats and Oils*; Reinhold Publishing Corporation: New York, 1954; 559–561.
3. Schumutterer, H.L. *The Neem Tree*; VCH Publishers: Berlin, 1995; 188.
4. Singh, R.P.; Saxena, R.C. *Azadirachta indica A. Juss*; P.B. Science Publishers Inc.: Enfield, 1999; 322.
5. Kumar, C.S.; Srinivas, M.; Yakkundi, S. Limonoids from the seeds *Azadirachta indica*. Phytochemistry **1996**, *43*, 451–455.
6. Child, R.; Ramanathan, S. The Fatty Acids of Margosa Oil. J. Soc. Chem. Ind. London **1936**, *55*, 124T.
7. Skellon, J.H.; Thorburn, S.; Chatterjee, S.N. The Fatty Acids of Neem Oil and their Reduction Products. J. Sci. Food Agric. **1962**, *13*, 639–643.
8. Rukmini, C. Chemical and Nutritional Evaluation of Neem Oil. Food Chem. **1987**, *26*, 119–124.

9. Kaushik, N.; Virt, S. Variation in Fatty Acid Composition of Neem Seeds Collected from the Rajasthan State of India. Biochem. Soc. Trans. **2000**, *28*, 880–882.
10. Kaushik, N. Determination of Azadirachtin and Fatty Acid Methyl Esters of *Azadirachta indica* Seeds by HPLC and GLC. Analyt. Bioanalyt. Chem. **2002**, *374*, 1199–1204.
11. Nandy, S.; Mitra, S.; Mondal, N. In vitro Detection of Neem oil (*Azadirachta indica A. Juss.*) in Seed Derived Callus: An Alternative Source of Future Promise. Plant Cell Biotechnol. Mol. Biol. **2004**, *5*, 161–164.
12. Tarandjiiska, R.; Nguyen, H. Triglyceride Composition of Seed Oils from Vietnamese Citrus Fruits. Riv. Ital. Sost. Grasse **1989**, *66*, 99–102.
13. Tarandjiiska, R.B.; Marekov, I.N.; Nikolova-Damyanova, B.M.; Amidzhin, B.S. Determination of Triacylglycerol Classes and Molecular Species in Seed Oils with High Content of Linoleic and Linolenic Fatty Acids. J. Sci. Food Agric. **1996**, *72*, 403–410.
14. Christie, W.W. *Lipid Analysis*; The Oily Press: Bridgwater, 2003, 373–388.
15. Nikolova-Damyanova, B.; Velikova, R.; Jham, G. Lipid Classes, Fatty Acid Composition and Triacylglycerol Molecular Species in Crude Coffee Beans Harvested in Brazil. Food Res. Intern. **1998**, *31*, 479–486.
16. Nikolova-Damyanova, B.; Amidzhin, B. Separation of Triglyceride Groups by Reversed Phase Thin-Layer Chromatography on Silanized Kieselguhr. J. Chromatogr. **1988**, *446*, 283–291.
17. Chobanov, D.; Amidzhin, B.; Nikolova-Damyanova, B. Direct Densitometric Determination of Triglyceride Groups Separated by Reversed Phase Thin Layer Chromatography. Riv. Ital. Sost. Grasse **1991**, *68*, 357–362.
18. Christie, W.W. Mass-Spectrometry of Fatty Acids with Methylene Interrupted ene-yne System. Chem. Phys. Lipids **1998**, *94*, 35–41.
19. Hartman, L.; Lago, R.C.A. Rapid Preparation of Fatty Acid Methyl Esters from Lipids. Lab. Pract. **1973**, *22*, 475–476.
20. Christie, W.W. *Gas Chromatography and Lipids*; The Oily Press: Ayr, 1989; 68.
21. Simova, S.; Ivanova, G.; Spassov, S.L. Alternative NMR Method for Quantitative Determination of Acyl Positional Distribution in Triacylglycerols and Related Compounds. Chem. Phys. Lipids **2003**, *126*, 167–176.
22. Sheppard, A.J.; Iverson, J.L.; Weihrauch, J.L. Composition of Selected Dietary Fats, Oils, Margarines and Butters. In *Fatty acids and Glycerides*; Kuksis, A., Ed.; Plenum Press: New York, 1978; 341–379.
23. Padley, F.B.; Gunstone, F.D.; Harwood, J.L. Occurrence and Characteristics of Oils and Fats. In *The Lipid Handbook*, 2nd Ed; Gunstone, F.D., Harwood, J.L., Padley, F.B., Eds.; Chapman and Hall: London, 1994; 49–170.
24. Gunstone, F.D.; Herslof, B.G. *A Lipid Glossary*; The Oily Press: Ayr, 1992.
25. Nikolova-Damyanova, B. Quantitative Thin-Layer Chromatography of Triacylglycerols: Principles and Applications, A review. J. Liq. Chromatogr. & Rel. Technol. **1999**, *22*, 1513–1537.
26. Nikolova-Damyanova, B.; Christie, W.W.; Herslof, B. The Structure of the Triglycerides of Meadowfoam Oil. J. Am. Oil Chem. Soc. **1990**, *67*, 503–507.
27. Litchfield, C. *Analysis of Triglycerides*; Academic Press: New York, 1972; 234–265.
28. Christie, W.W.; Nikolova-Damyanova, B.; Laakso, P.; Herslof, B. Stereospecific Analysis of Triacylglycerols via HPLC of Diastereomeric Diacylglycerol Derivatives. J. Am. Oil. Chem. Soc. **1991**, *69*, 695–701.

29. Nikolova-Damyanova, B.; Chobanov, D.; Dimov, S. Separation of Isomeric Triacylglycerols by Silver Ion Thin-Layer Chromatography. J. Liq. Chromatogr. & Rel. Techn. **1993**, *16*, 3997–4008.
30. Gunstone, F.D. High Resolution $^{13}$C NMR Spectroscopy of Lipids. In *Advances in Lipid Methodology – Two*; Christie, W.W., Ed.; The Oily Press: Dundee, 1993; 1–68.

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