JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

^{13}C NMR Characterization of Triacylglycerols of *Moringa oleifera* Seed Oil: An "Oleic-Vaccenic Acid" Oil

GIOVANNA VLAHOV,*,† PAUL KIPRONO CHEPKWONY,‡ AND PAUL K. NDALUT‡

Istituto Sperimentale per la Elaiotecnica, Contrada Fonte Umano, 65013 Città S. Angelo (Pescara), Italy, and Department of Chemistry, Moi University, P.O. Box 1125, Eldoret, Kenya

The composition of acyl chains and their positions in the triacylglycerols of the oil extracted from seeds of *Moringa oleifera* were studied by ^{13}C NMR spectroscopy. The unsaturated chains of *M. oleifera* seed oil were found to comprise only mono-unsaturated fatty acids and, in particular, two ω-9 mono-unsaturated acids, (*cis*-9-octadecenoic (oleic acid) and *cis*-11-eicosenoic acids) and one ω-7 mono-unsaturated acid (*cis*-11-octadecenoic acid (vaccenic acid)). The mono-unsaturated fatty acids were detected as separated resonances in the spectral regions where the carbonyl and olefinic carbons resonate according to the 1,3- and 2-positions on the glycerol backbone. The unambiguous detection of vaccenic acid was also achieved through the resonance of the ω-3 carbon. The ^{13}C NMR methodology enabled the simultaneous detection of oleate, vaccenate, and eicosenoate chains according to their positions on the glycerol backbone (1,3- and 2-positions) through the carboxyl, olefinic, and methylene envelope carbons of the triacylglycerol acyl chains.

KEYWORDS: Carbon-13 NMR; *Moringa oleifera* oil; oleic acid; vaccenic acid; triacylglycerols

INTRODUCTION

Moringa oleifera Lam. belongs to the genus Moringaceae. It is a shrub or small deciduous tree reaching 2.5−10 m in height. This species originally comes from North India but is widely cultivated. Leaves are used as vegetables, and the roots are used as a substitute for horseradish. In some places the species is naturalized (*1*). Fruits are brown and 10−50 by 1.5−2.6 cm; seeds are 1.5−3.9 cm long.

Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy has been successfully employed to study the triacylglycerols of edible vegetable oils (*2*), the cyclopropenoid triacylglycerols of the seed oil from *Sterculia foetida* (*3*), and the triacylglycerols of the cotyledon oil from *Afzelia cuanzensis*, containing the crepenynate and dehydrocrepenynate chains (*4*). Olive oil triacylglycerols have also been investigated by ^{13}C NMR (*5*, *6*).

This study has determined by ^{13}C NMR spectroscopy of triacylglycerols of the oils extracted from *M. oleifera* seeds their fatty acid composition and their distribution among the glycerol positions. No previous studies have been reported on the triacylglycerol structures of the oils extracted from the seeds of *M. oleifera*, and in particular on the fatty acid distribution between the *sn*-1(3)- and *sn*-2- glycerol positions along with the chain specificity at the *sn*-2 position.

* Corresponding author. Tel +39 (0) 85 95212; Fax +39 (0) 85 959518; E-mail vlahov@unich.it.
† Istituto Sperimentale per la Elaiotecnica.
‡ Moi University.

MATERIALS AND METHODS

Materials. *Moringa oleifera* seeds were collected from Siaya District, Kenya, courtesy of Dr. Stephen Ruigin of the International Centre for Research in Agroforestry (ICRAF).

Oil Extraction. A 500-g sample of *Moringa oleifera* dry seeds was ground into fine powder and cold-extracted with hexane for 48 h. The solvent was evaporated under reduced pressure to afford a light yellow oil.

Determination of the Fatty Acid Composition. Fatty acid composition was determined by gas−liquid chromatography. The methyl esters of fatty acids were prepared from oil by ambient temperature transmethylation with sodium methoxide (*7*). A HRGC Mega 2 gas chromatograph (GC) (Carlo Erba Instruments, Milano, Italy) equipped with an on-column mode injection system, flame ionization detector, and a SP-2380 fused silica capillary column (60 × 0.32 mm i.d., 0.15-μm film thickness (Supelco, Sigma Aldrich S.r.l., Milano, Italy)), was used to analyze the fatty acid methyl esters. The GC conditions were as follows: initial oven temperature (120 °C), heated at 30 °C/min to 165 °C, then at 5 °C/min to a final temperature of 200 °C; detector port temperature, 260 °C; carrier gas, hydrogen at a flow rate of 1.0 mL/min. The fatty acids were identified by comparing the retention times of methyl esters of *M. oleifera* oil with standard mixtures of fatty acid methyl esters.

^{13}C NMR Spectroscopy. The oil samples (30 mg) were dissolved in 0.5 mL of $CDCl_3$. The spectra were run on a Unity Inova Narrow Bore 500 MHz spectrometer (Varian NMR Instruments, Palo Alto, CA). The probe temperature was 27 °C.

The ^{13}C proton-decoupled (Waltz-16) spectra were measured using the DEPT pulse sequence (distortionless enhancement by polarization transfer) here below reported:

10.1021/jf011054a CCC: $22.00
Published on Web 02/02/2002

^{1}H: d1−pp−τ−pp−2pp−pp−τ−θ−τ decouple

^{13}C: pw pw−2pw−pw acquisition

where pp is the 90° pulse supplied from decoupler, pw is the 90° pulse on the ^{13}C nucleus, and the θ pulse of 45° ensures equal excitation of all protonated carbons. The scalar C−H coupling constant of 144 Hz, which is the average value of one-bond coupling constants experimentally determined (*6*), has been used to optimize the τ delay set at $^{1}/_{2} \times J_{C,H}$.

Free induction decays (FIDs) have been collected with 128 K data points zero-filled to 512 K to yield a digital resolution of 0.09 Hz/point, using a 2.9 s acquisition time and a 10 s relaxation delay, which was set at four times the value of the longest T_1 for the proton connected to the carbon nuclei, to avoid signal saturation (*6*). The FIDs were multiplied by a resolution enhancement function to improve resolution and obtain a signal-to-noise ratio high enough to ensure integration accuracy for rigorous quantitative spectrum measurement.

The proton-decoupled spectra of carbonyl carbons were measured for quantitative purposes with full NOE enhancement (*8*) over the frequency range of carbonyl resonances using 64 K data points zero-filled to 128 K to obtain a digital resolution of 0.03 Hz/point. FIDs were acquired with a 19.5 s acquisition time and a 16 s relaxation delay. It was set at four times the longest T_1 longitudinal relaxation time (4 s) of carbonyl carbons which was determined by the inversion−recovery pulse sequence. The FIDs were processed for high-resolution spectra by applying a resolution enhancement of 0.2 Hz and a Gauss apodization of 2 s.

RESULTS AND DISCUSSION

The ^{13}C NMR spectrum at natural abundance of the oil extracted from the seed fruits of *M. oleifera* showed the signals of triacylglycerols which represent the most abundant fraction (97−98%) of vegetable oils. The chemical shifts of both the fatty acid and glycerol moieties of triacylglycerols of *M. oleifera* oil are listed in **Table 1**.

The spectral region of the carbon-13 spectrum of *M. oleifera* oil in the range where the carbonyl carbons resonate (172.8−173.4 ppm), is shown in **Figure 1**. The carbonyl carbon resonance at 173.336 ppm was assigned to saturated chains at 1(3)-positions (*2*, *9*). No resolution of saturated chains on the basis of carbon number is achievable by NMR spectroscopy. The signals at 173.303 and 172.891 ppm, assigned to the oleate chain at 1(3)- and 2-positions, respectively, were associated with the oleate resonances appearing in the unsaturated carbon region of the carbon-13 spectrum of *M. oleifera* oil at 129.655 (1,3-) and 129.629 ppm (2-) for C-9, and for C-10 at 129.959 (1,3-) and 129.972 ppm (2-) (**Figure 2**).

The chemical shift difference between the carbonyl resonances at the 1,3- (173.322 ppm) and 2-positions (172.909 ppm), which is close (0.413 ppm) to the shift difference consistently detected in all the acyl chains (*9*), suggested that the carbonyl resonances belong to the same chain.

The C═O resonances were associated, on the basis of relative integrals, to the double bond carbons appearing at 129.777 (1,3-) and 129.760 ppm (2) for C_n and for C_{n+1} at 129.877 (1,3-) and 129.885 (2-), whereas the carbonyl carbons correlated with the unsaturated carbons resonating at 129.773 and 129.881 ppm, were not detected.

The chemical shift differences between the double bond carbons C_n and C_{n+1} were found to be 0.10 for the chain resonating at 129.777 (1,3)- and 129.877 ppm (1,3-), and 0.11 for the chain signals at 129.773 (2-) and 129.881 (2-) ppm. Because the shift differences are almost equal, the two chains are likely to have the same number of C−C bonds separating the ester chain end and the double bond, in agreement with the σ-inductive theory of transmission of the dipolar effect of a C═O bond upon a C═C bond operating through C−C bonds in monoethenoid acids (*10*). The theory allows the shift differences of a double bond pair to be predicted as a function of the number of C−C bonds intervening between the dipolar group and the C═C bond. The shift difference between the double bond carbons of eicosenoate chain was predicted to be 0.1 ppm. As a consequence, the vaccenate and eicosenoate chains which are both "Δ^{11}" chains (double bond at C-11), were studied. Carbon-13 spectra of model triacylglycerol mixtures from triolein (C18:1, Δ^{9}*cis*, ω9), trieicosenoin (C20:1, Δ^{11}*cis*, ω9), and trivaccenin (C18:1, Δ^{11}*cis*, ω7) were measured ("Δ" indicates the double bond position from the ester chain end, "ω" indicates the position from the methyl chain end).

The carboxyl carbons of eicosenoate and vaccenate moieties resonated as singlets at 173.322 and 172.909 ppm for 1,3- and 2-position chains, respectively. However, the double bond carbons of eicosenoate and vaccenate moieties appeared as separated resonances in correspondence to 1(3)- and 2-position chains (**Figure 2**). They were found to resonate at the same frequencies detected in the *M. oleifera* oil spectrum, except for the olefinic carbons C-11 (129.754 ppm) and C-12 (129.889 ppm) of the eicosenoate chain at the 2-position which was not revealed.

Table 1. ^{13}C NMR Chemical Shifts of Saturated (n:0), 9-octadecenoate, 11-octadecenoate, and 11-eicosenoate Chains at 1(3)- and 2- Glycerol Positions (125 MHz, $CDCl_3$, TMS as Internal Standard) of Triacylglycerols of *M. oleifera* Oil

carbon	position	n:0	18:1 9c	18:1 11c	20:1 11c
		fatty acid moiety			
1	1(3)	173.336	173.303	173.322	173.322
	2	-	172.891	172.909	172.909
2	1(3)	34.020	33.999	34.011	34.011
	2	-	34.164	34.181	34.181
3	1(3)	24.873	24.851	24.862	24.862
	2	-	24.887	24.902	24.902
4	1(3)	-	-	-	-
	2	-	29.061	-	-
5	1(3)	29.294	29.190	-	-
	2	-	29.210	-	-
6	1(3)	29.502	-	-	-
	2	-	-	-	-
7	1(3)	-	-	-	-
	2	-	-	-	-
8	1(3)	-	27.173	-	-
	2	-	27.173	-	-
9	1(3)	-	129.655	-	-
	2	-	129.629	-	-
10	1(3)	-	129.959	27.207	27.216
	2	-	129.972	27.207	27.216
11	1(3)		27.226	129.777	129.773
	2		27.226	129.760	-
12	1(3)	-	29.785	129.877	129.881
	2	-	29.785	129.885	-
13	1(3)	-	29.346	27.230	27.216
	2	-	29.346	27.230	27.216
14	1(3)	-	29.553	-	-
	2	-	29.553	-	-
15	1(3)	29.392	29.346	-	-
	2	-	29.346	-	-
16	1(3), 2	31.953	31.932	31.812	-
17	1(3), 2	22.703	22.693	22.669	-
18	1(3), 2	14.084	14.080	14.072	31.932
19	1(3), 2	-	-	-	22.693
20	1(3), 2	-	-	-	14.080
		glycerol moiety			
Gl	1(3)	62.069			
	2	68.926			

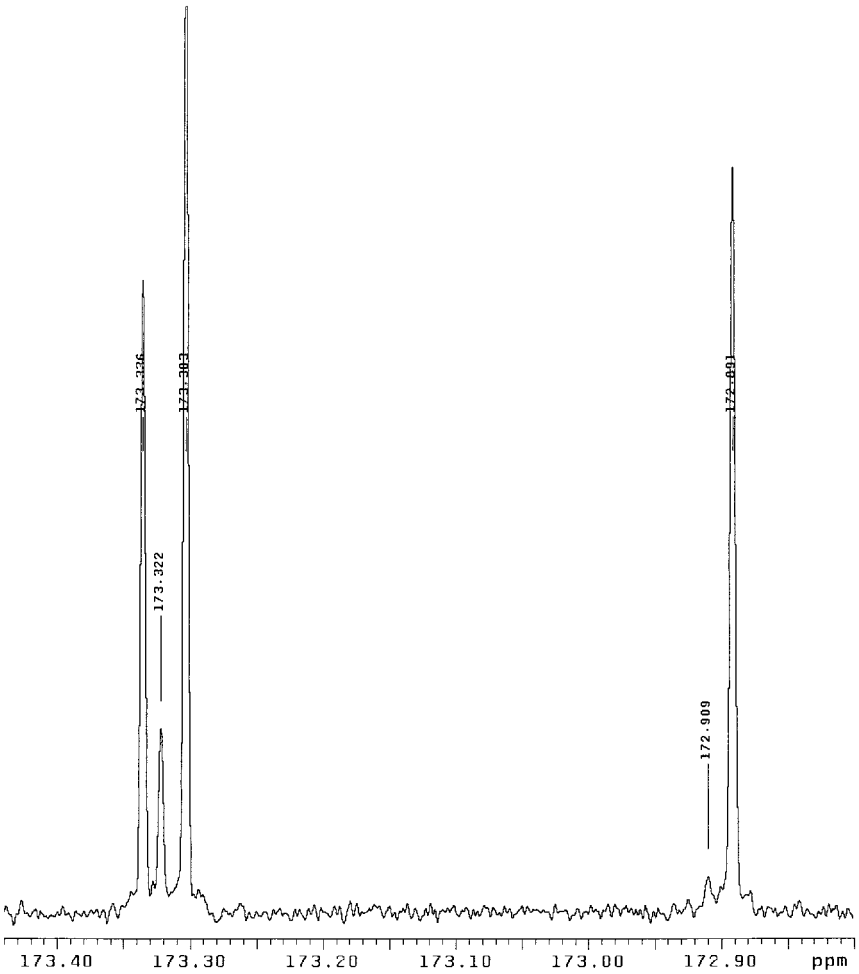

Figure 1. Carbonyl carbon region of the 500 MHz ^{13}C NMR spectrum of *Moringa oleifera* oil. The resonances of saturated (173.336 ppm), vaccenate (173.322–172.909 ppm), and oleate (173.303–172.891 ppm) chains esterified at 1(3)- and 2-glycerol positions, respectively, are indicated.

Examination of the frequency regions where the methyl (ω1) and the methylene carbons (ω2, ω3) resonate, showed that the resonances of ω1, ω2, and ω3 carbons of oleate and eicosenoate chains overlapped at 14.080, 22.693, and 31.932 ppm, respectively, whereas the ω1, ω2, and ω3 carbons of oleate and vaccenate chains each resonate as two peaks, separated by 0.008, 0.024, and 0.12 ppm, respectively. This result can be explained by considering that the ω1 to ω3 carbons have the same chemical environment in oleate and eicosenoate chains because both the chains are ω9 chains (*11*), unlike the ω7 vaccenate chain whose resonances were constantly shifted toward lower frequency from the ω9 oleate chain.

The same shift differences were found in the *M. oleifera* oil spectrum, between the oleate chemical shifts (14.080, 22.693, and 31.932) and the resonances at 14.072, 22.669, and 31.812, which were definitively assigned to vaccenate chains. It is noteworthy that the ω3 carbon enables the unambiguous detection of vaccenic acid; however, the positional distribution of vaccenic acid cannot be defined because this resonance is not split according to the chain position on the glycerol backbone (**Figure 3**).

The C-2 and C-3 signals in the acyl chain moieties of trieicosenoin and trivaccenin overlapped (**Table 1**) as expected for two chains where the same number of C−C bonds separate the ester chain end carbons from the double bond. On the other hand, they were resolved from the C-2 and C-3 carbons of the oleate chain which are two C−C bonds closer to the double bond. However, the detection of C-2 and C-3 carbons of the vaccenate chain in the *M. oleifera* oil spectrum was difficult because of the presence of C-2 and C-3 carbons of the saturated chain resonating at 34.020 and 24.873 ppm, respectively. The C-2 and C-3 resonances of unsaturated chains are differentiated according to 1,3- and 2- positions on the glycerol backbone, the latter being shifted at higher frequency (*5*), unlike saturated chains which enter only the 1,3- glycerol positions.

The chemical shifts of allylic carbons C-8, C-11 of the oleate chain and C-10, C-13 of vaccenate and eicosenoate chains centered at 27.21 ppm confirmed the cis configuration of the C 9,10 and C 11,12 double bonds (*3*).

The methylene carbons C-4 to C-7 and C-12 to C-15 of the oleate chain were assigned according to both peak integration and measurements of T_1 relaxation times, which in long chain fatty acids increase regularly from the glycerol backbone up to the methyl chain end (*3*).

The study of ^{13}C chemical shifts of *M. oleifera* spectra proved that the simultaneous detection of oleate, vaccenate, and eicosenoate chains is achievable by high-resolution ^{13}C nuclear magnetic resonance spectroscopy. The olefinic carbons of oleate, vaccenate, and eicosenoate chains can be measured as separate resonances according to their positions on the glycerol backbone, and the presence of a vaccenate chain is definitively confirmed by the ω3 carbon resonance, thus providing a tool for carrying out the positional analysis of the *cis*-vaccenate and *cis*-eicosenoate chains, which has been a difficult and unresolved task (*12*).

The acyl chains composition and their distribution between the 1(3)- and 2- positions of triacylglycerols of *M. oleifera* oil

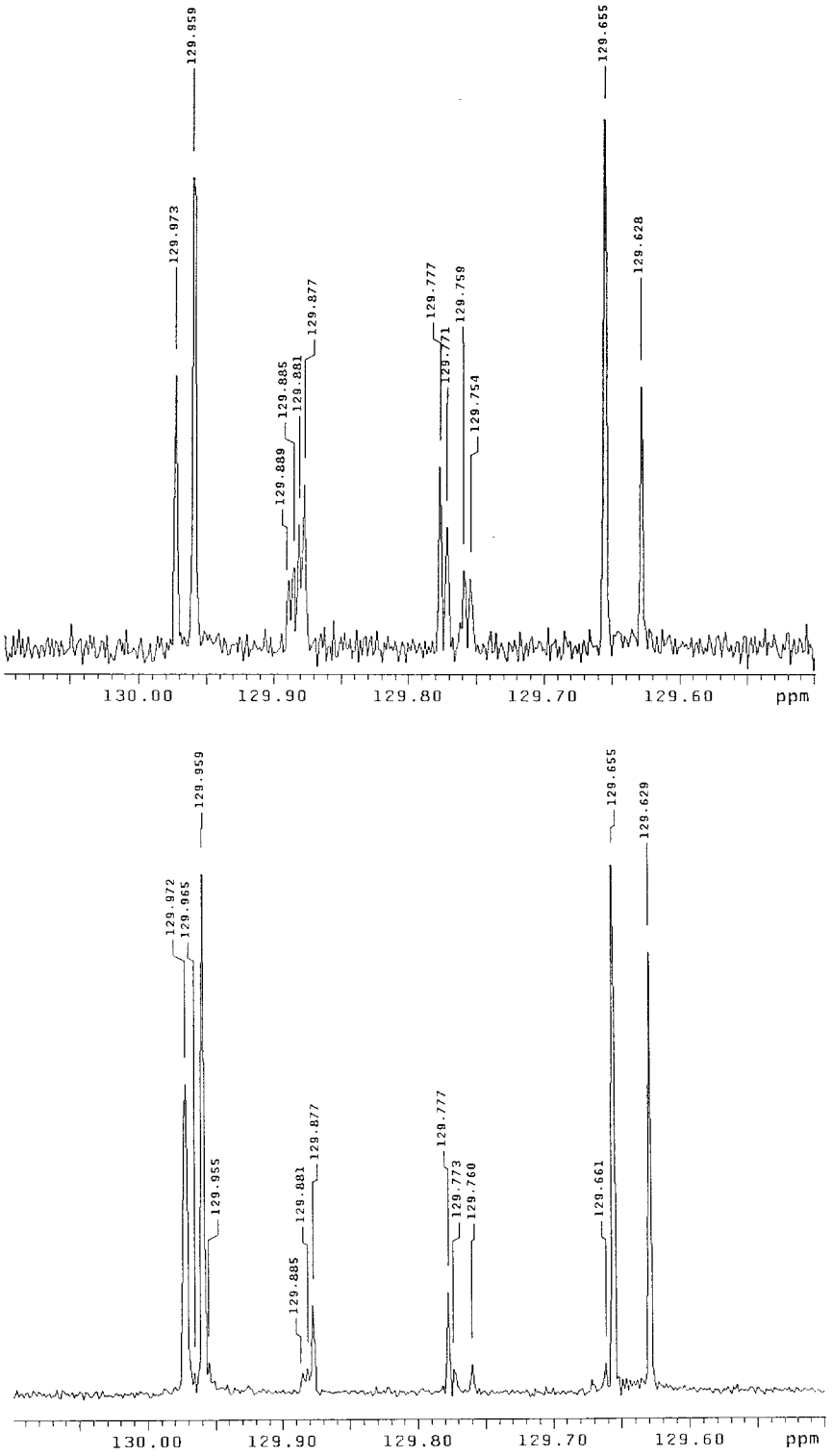

Figure 2. Olefinic carbon region of the 500 MHz ^{13}C NMR spectrum of *Moringa oleifera* oil (bottom trace) and of a standard mixture of triolein, trieicosenoin, and trivaccenin triglycerides (upper trace). The resonances of C-9 and C-10 of oleate chain at 1(3)-positions (129.655 and 129.959 ppm), and 2-position (129.629 and 129.972 ppm) are indicated. The signals of C-11 and C-12 of vaccenate chain at 1(3)-positions (129.777 and 129.877 ppm) and 2-position (129.760 and 129.885 ppm), and of eicosenoate chain at 1(3)-positions (129.773 and 129.881 ppm) and 2-position (129.754 and 129.889 ppm), are shown. The resonances of oleate and vaccenate chains at 1(3)- and 2-glycerol positions are detected in the *M. oleifera* oil, whereas the eisosenoate chain is detected only at 1(3)-positions.

were calculated on the basis of the integrals of olefinic carbon resonances and corrected for the saturated chain by means of C-1 resonances. The results reported in **Table 2** show that the oil is composed of the major chains, saturated (23.8%) and oleate (67.2%) chains, and of the minor chains, *cis*-vaccenate (7.2%) and eicosenoate (1.8%). The linoleate chain was not detected, thus confirming the peculiarity of this oil as compared to the largest group of vegetable oils, the "oleic−linoleic acid" group characterized by saturated (≤ 20% of the total), oleic, and linoleic acids (*13*). The fatty acid composition of *M. oleifera*

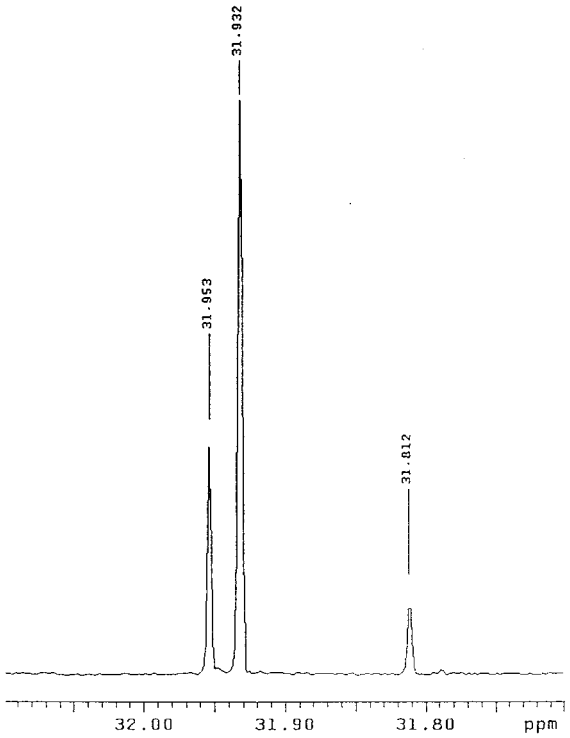

Figure 3. ω3 Carbon region of the 500 MHz ^{13}C NMR spectrum of *M. oleifera* oil. The resonances of saturated (31.953 ppm), oleate (31.932 ppm), and vaccenate (31.812 ppm) chains are indicated.

Table 2. Composition and 2-Positional Specificity of Triacylglycerol Fatty Acids by ^{13}C NMR in $CDCl_3$ at 125 MHz[a]

		Moringa oil		olive oil	
fatty acid		%	2-specificity	%	2-specificity
C n:0		23.8 (1.9)		21.7 (1.8)	
C 18:1	9*cis*	67.2 (1.7)	47.3	58.7 (1.7)	42.0
C 18:1	11*cis*	7.2 (2.7)	25.0	4.7 (3.4)	
C 20:1	11*cis*	1.8 (4.3)		nd	
C 18:2	9,12*cis*	nd		14.9 (3.4)	48.8

[a] Values are means of three replicate determinations. Coefficients of variation are given in parentheses.

in comparison to that of a "high vaccenic" olive oil was also determined by gas−liquid chromatography (**Table 3**). The results were in good agreement with the ^{13}C NMR data, except for the saturated chains which cannot be differentiated by ^{13}C NMR on the basis of the sole chain carbon number (*5*) (they can be found under the label "saturated"), and for the hexadecenoate-9 chain (C16:1, Δ^9*cis*, ω7). The unsaturated C-9 and C-10 carbons of the hexadecenoate-9 chain were difficult to detect. The problems were the "low intensity" resonances of the hexadecenoate-9 chain compared to the "large lines" of the oleate chain, and the carbons of two Δ^9 chains being very close in frequency.

The results confirmed the fatty acid profile of *M. oleifera* var. Mbololo seed oil, except for the detection of *cis*-vaccenic acid (*14*).

The 2-positional specificities of unsaturated chains were calculated by normalizing the 2-position resonance value to both 1,3- and 2-positions for each chain (the saturated chains were found only at the 1,3- positions). The 2-specificity data of *M. oleifera* oil were compared to those of the "high vaccenic" (4.7%) olive oil sample which, unlike *M. oleifera*, also contains linoleic acid (14.9%). The 2-specificity values confirmed that the chain distribution in *Moringa* oil was far from random, as 33.3% of a randomly distributed chain would be at the 2-position. The oleate chain (47.3%) was preferentially acylated at the 2-position, whereas the vaccenate chain (25.0%) was acylated at the 1,3-positions. Trace amounts of eicosenoate chain were determined only at the 1,3-positions and confirmed that the higher unsaturated acids such as eicosenoic generally accompany the saturated acids in the 1,3-positions (*15*).

Table 3. Fatty Acid Composition[a] of Triacylglycerols by GLC

		area %	
fatty acid		Moringa oil	olive oil
C 14:0		0.1 (1.2)	0.02 (1.1)
C 16:0		5.9 (0.5)	17.3 (0.5)
C 16:1	9*cis*	1.8 (0.6)	2.7 (0.6)
C 17:0		0.1 (1.1)	0.1 (1.2)
C 17:1		0.0	0.4 (1.2)
C 18:0		7.2 (1.8)	1.9 (1.5)
C 18:1	9*cis*	66.9 (0.2)	57.9 (0.3)
C 18:1	11*cis*	7.3 (0.5)	4.4 (0.6)
C 18:2	9,12*cis*	0.6 (0.3)	13.9 (0.3)
C 18:3	9,12,15*cis*	0.2 (1.1)	0.8 (1.2)
C 20:0		4.0 (1.5)	0.3 (1.4)
C 20:1	11*cis*	1.8 (1.9)	0.2 (1.8)
C 22:0		4.1 (1.0)	0.08 (1.1)
total saturated		21.4	19.7

[a] Values are means of three replicate determinations. Coefficients of variation are given in parentheses.

The 2-specificities of oleate (42.0%) and linoleate (48.8%) chains in olive oil showed that oleic and linoleic acids compete for the 2-position and linoleic acid is present at this position rather more than oleic acid (*15*). The absence of the linoleate chain in *M. oleifera* oil is likely to make the oleate chain replace the linoleate chain at the 2-position with the consequent increase (47.3%) of the oleate fraction at the 2-position.

Nevertheless, the chain specificities at the 2-position of oleate (42.0%) and linoleate (48.8%) chains in olive oil confirmed the values determined for a wide range of olive oils (39.0% and 50.0%, respectively) and indicated that this ratio could be considered a characteristic of that chain (*16*).

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Received for review August 7, 2001. Revised manuscript received November 16, 2001. Accepted November 16, 2001.

JF011054A