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HIGH FIELD ^{13}C - NMR SPECTROSCOPIC ANALYSIS OF THE TRIACYLGLYCEROLS OF *ADENOPUS BREVIFLORUS* SEEDS OIL

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High resolution carbon - 13 NMR (gated decoupled) spectra of the carbonyl, saturated and olefinic carbons in *Adenopus breviflorus* seeds oil have been used for direct determination of the acyl composition and acyl positional distribution on the glycerol backbone. The spectra revealed the presence of saturated, oleic and linoleic fatty acids. Semi quantitative analysis using the integrals of the allylic carbons signals gave the percentage composition of the oil as saturated 25.00%, oleic 14.00% and linoleic 60.90%. These percentage compositions were confirmed by gas chromatography. The spectra further revealed that while the saturated fatty acids are distributed between the 1,3 (α) and 2 (β) glyceridic positions, oleic acid is attached only at the (α) glyceridic position while linoleic acid is attached mostly at the (β) glyceridic position.

***Key words*:** ^{13}C-NMR, *Adenopus breviflorus*, Linoleic fatty acids, Gas chromotography, Triacylglycerols.

Introduction

Most seed oils are composed of triacylglycerols which contain an array of fatty acids, saturated as well as unsaturated and distributed among the three positions of the glycerol backbone. In defining the acyl positional distribution between the α - (i.e. the 1 and 3 positions of the glycerol) and β - (i.e. the 2 position of glycerol), carbon - 13 NMR has been found most useful. There have been also some efforts in the past (Ng 1984; Gunstone 1993; Lie Ken Jie *et al* 1996), where ^{13}C - NMR was used to identify, confirm or evaluate the fatty acids composition of different seeds oil. These reports indicated that except for lack of differentiation of the saturated fatty acids, the ^{13}C - NMR technique provided the same information as the time consuming, conventional gas chromatographic technique for establishing fatty acid composition of oils and the tedious enzymatic hydrolysis for identifying the positional distribution of the oils acyl groups.

Adenopus breviflorus (Cucurbitaceae) grows in the wild in Savanah forest of Southern Nigeria. It has about 55-60% oil (Esuoso and Bayer 1998). Oderinde (1990) and Oshodi (1996) reported the fatty acids composition of the *Adenopus breviflorus* seeds oil. We have characterized the oil and indicated some possible uses of the seeds oil (Akintayo and Bayer 2002a). In an earlier investigation, we have tried to identify *Adenopus breviflorus* seeds oil by ^{1}H-NMR spectroscopy (Akintayo and Bayer 2002b). In continuation of our efforts on the systematic studies of the lesser known and under-utilised tropical seeds oils, the present effort aims at the ^{13}C-NMR spectroscopic analysis of *Adenopus breviflorus* seed oil to *(i)* confirm the presence of the reported fatty acids, *(ii)* identify and semi-quantiate the fatty acids and most importantly *(iii)* determine the fatty acids distribution on the glycerol backbone. The quantitative integrity of the NMR derived fatty acid composition is verified by gas chromatographic analysis of the oil.

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Experimental

Adenopus breviflorus (ADB) seeds were purchased from some markets in Ibadan, Akure and Ado-Ekiti in the south - western part of Nigeria. The seeds were screened, washed and dried in the oven (103°C) and the oils extracted with hexane for 20 h by Soxhlet method. The extracts were desolventised under reduced pressure in a rotavapour.

The ^{13}C - NMR of the samples dissolved in deuteriated chloroform were recorded on the BRUKER AMX -400(BRUKER Instruments, Inc. Karlsruhe, Germany) Fourier transforms spectrometer operating at 100.6MHz. The gated decoupling pulse sequence was used with the following parameters. Number of scans 512, acquisition time 1.3665sec, pulse width 10.3μsec, delay time 1.0 sec. Free induction decay (FID) was transformed and zero filled to 300K to give a digital resolution of 0.366Hz /point.

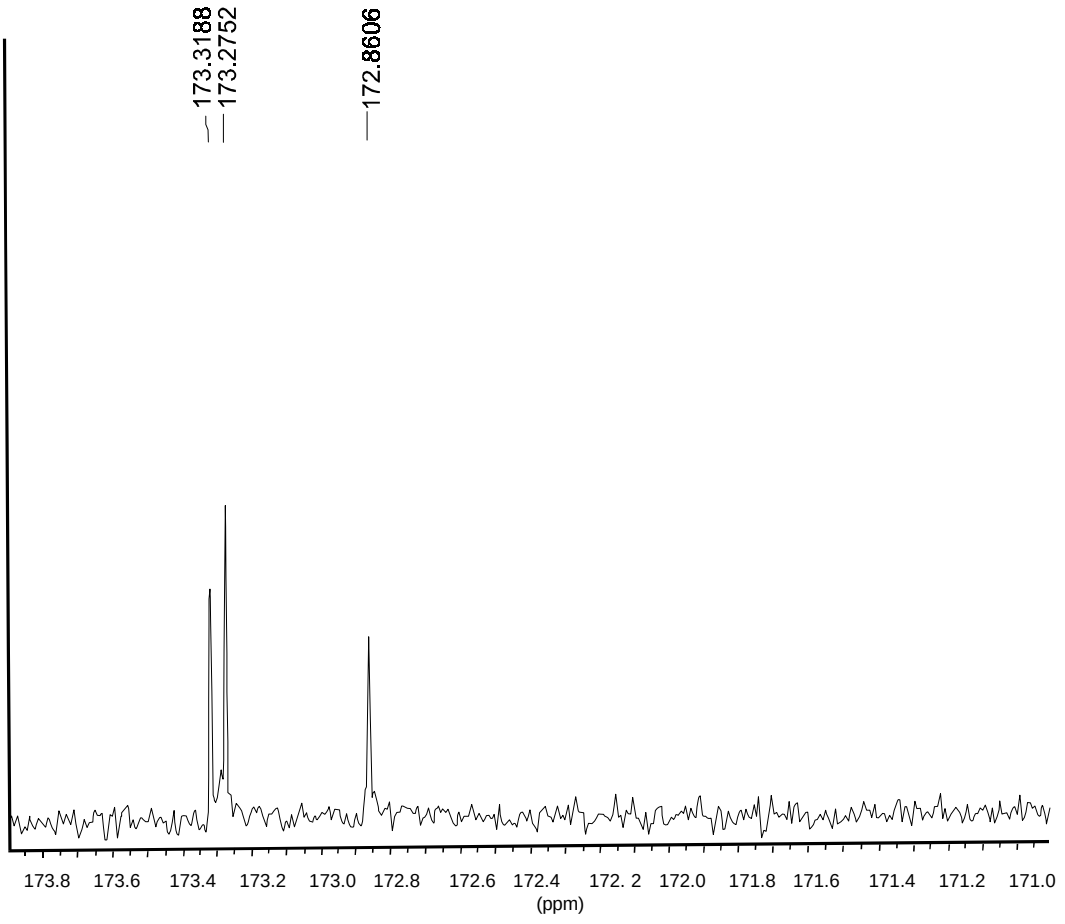

Fig 1. Proton-decoupled high resolution ^{13}C -NMR (100.6 MHz) of the carbonyl carbons of the triacylglycerols in *Adenopus breviflorus* seeds oil.

Fatty acid methyl esters (FAMES) of the oil was prepared as follows: Approximately 2mg crude seeds oil was transferred into a 5 - 10 ml glass vial and 1ml of diazomethane-ether solution added. The mixture was shaken thoroughly and allowed to stand for 1 min. Then 16µl of 3.33M CH_3ONa / CH_3OH solution was added, mixture shaken and allowed to stand for 10 min after which 10 µl acetic acid was added. The clear supernatant was used for Gas chromatographic analysis . 0.2 µl of the FAMES was injected into Hewlett-Packard 5890 GC (Hewlett - Packard Co, Palo Albo CA). The column was HP Ultra Performance coated with crosslinked 5% Phenol + 95% polysiloxane, 30 x 0.25nm, 0.2µ coating thickness. Temperature programming was as follows: Initial temperature,160˚C for 2 min, temperature increased at 2.5˚C / min up to 300˚C and maintained at this final temperature for 5 min. Injector and dectector temperature were 280˚C and 340˚C, respectively.

Results and Discussion

In this discussion we abbreviate saturated acyl groups as Sat., oleate [18:1 (9*Z*)] as O and linoleate [18:2 (9*Z*,12*Z*)] as L (where the first number in bracket denotes the number of carbon atoms in fatty acid chain, the second number denotes the number of double bonds, the other numbers denote the position of double bonds and *Z* stands for the *Z* configuration of the corresponding double bond). The structures of oleate and linoleate and the respective carbon numbers used throughout this discussion are as follows:

Oleate - $^{18}CH_3$, $^{17}CH_2$, $^{16}CH_2$, $^{15}CH_2$, $^{14}CH_2$, $^{13}CH_2$, $^{12}CH_2$, $^{11}CH_2$, $^{10}CH = {}^{9}CH$, $^{8}CH_2$, $^{7}CH_2$, $^{6}CH_2$, $^{5}CH_2$, $^{4}CH_2$, $^{3}CH_2$, $^{2}CH_2$, $^{1}CO\bar{O}$

Linoleate - $^{18}CH_3$, $^{17}CH_2$, $^{16}CH_2$, $^{15}CH_2$, $^{14}CH_2$, $^{13}CH = {}^{12}CH$, $^{11}CH_2$, $^{10}CH = {}^{9}CH$, $^{8}CH_2$, $^{7}CH_2$, $^{6}CH_2$, $^{5}CH_2$, $^{4}CH_2$, $^{3}CH_2$, $^{2}CH_2$, $^{1}CO\bar{O}$

where the superscripts stand for carbon numbers.

The high resolution ^{13}C - NMR spectrum of the carbonyl carbons of the triglycerides of ADB is presented in Fig 1 and it shows three signals at 173.3188 ppm, 173.2752 ppm and 172.8606 ppm. Referring to established data (Lie Ken Jie *et al* 1992; Lie Ken Jie and Cheng 1993; Lie Ken Jie and Lam 1995) two of the signals could be paired, 173.2752 / 172.8606 with a

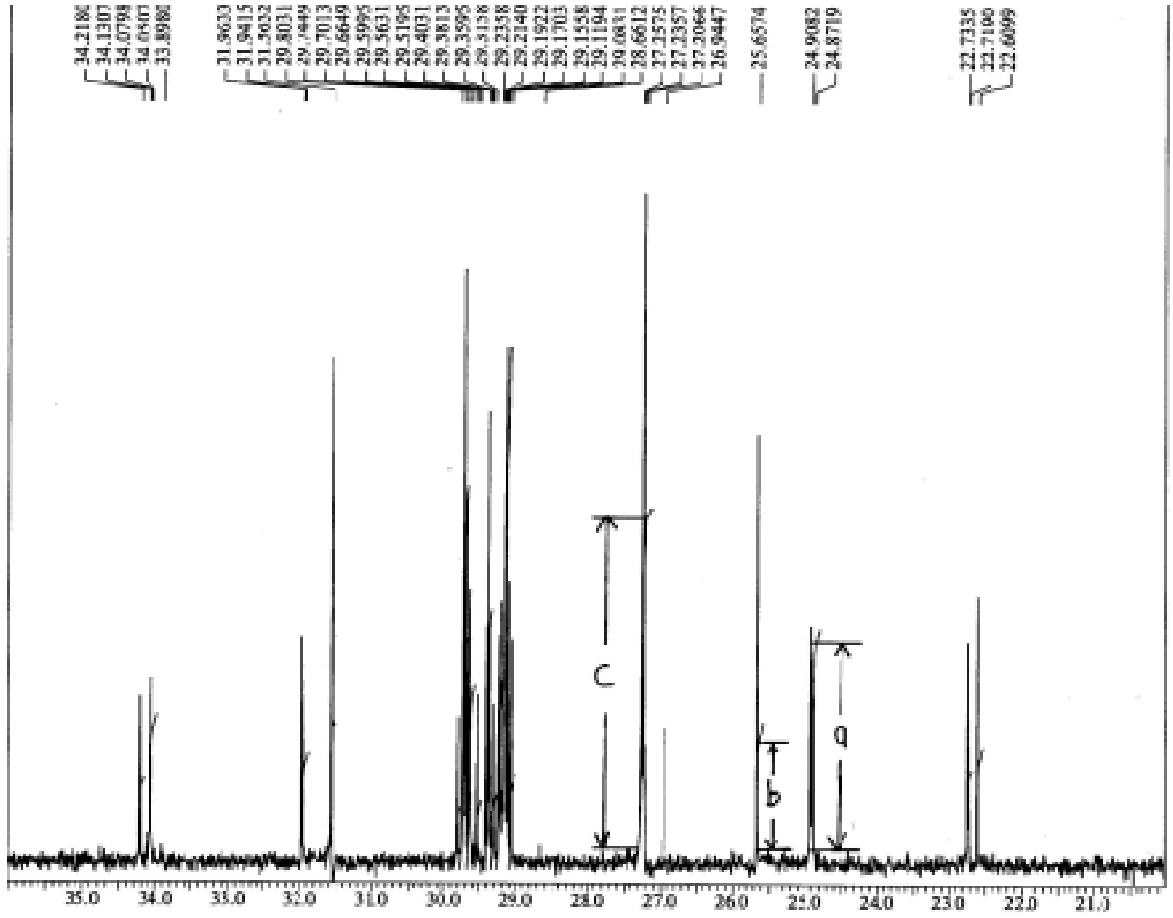

Fig 2. Proton-decoupled ^{13}C - NMR (100.6MHz) of the saturated carbons of the fatty acid chains in *Adenopus breviflorus* seeds oil. The integral value 'a' is for the peak at *ca* 24ppm,'b' is for the peak at *ca* 25 ppm and 'c' is for the peak at *ca* 27 ppm.

chemical shift difference of *ca* 0.415. The highest chemical shift in the spectrum 173.3188 ppm can be assigned to carbonyl carbon of Sat. in α position.

Ng (1983) has shown that C-1 of O and L attached to either of the 1,3 glyceridic carbons (i.e. at α position) occur at a slightly lower field to that of Sat. occupying the same position (O differs by 0.029 ± 0.002 ppm while L differs by 0.041 ± 0.002 ppm).

Rather than relying solely on chemical shift values, we have also made use of the difference values to ascertain the type of the ester and their positions on the glycerol backbone throughout this discussion. The higher value of the pair of signals, 173.3018 ppm differs from the 173.3188 ppm signal by *ca* 0.0043 ppm. Referring to Ng (1983), the pair of signals 173.2752 ppm/172.8606 ppm could, therefore, be assigned to L in α and β positions. Signals observed in the carbonyl region of this oil indicate the presence of Sat. and L. Earlier report by Ng (1983) has shown that resonances of saturated fatty acids were not resolved in the carbonyl region.

The ^{13}C - NMR signal profiles in the upfield region (20 - 36 ppm) of the ADB oil (Fig 2) were also found to be very characteristic and could be used for identification of the acyl groups and their positional distribution on glycerol backbone. There are two sub-regions in the spectra that are useful for these purposes (i) the C - 2 carbon shift region (*ca* 34 ppm) and (ii) the C - 3 (*ca* 24 ppm), allylic (25 - 27 ppm), C - 17 (*ca* 22 ppm) and C - 16 (*ca* 31ppm) carbon shift region.

C-2 carbon shift region (ca 34 ppm). Four signals 34.2180 ppm, 34.1307 ppm, 34.0798 ppm and 34.0507 ppm appear in this region. Two of the signals 34.2180 ppm/34.0507 ppm could be paired (shift difference of 0.167 ppm). These shifts are assigned to the C-2 carbon atoms of Sat. in the α and β positions. The 34.1307 ppm is assigned to L in β glyceridic position and the 34.0798 ppm assigned to O in α glyceridic position. These assignments were based on established data, (Lie Ken Jie *et al* 1992; Lie Ken Jie and Cheng 1993; Lie Ken Jie and Lam 1995).

C-3, allylic, C-17 and C-16 carbon shift region. The two signals in the C-3 region (*ca* 24 ppm) 24.9082 ppm and

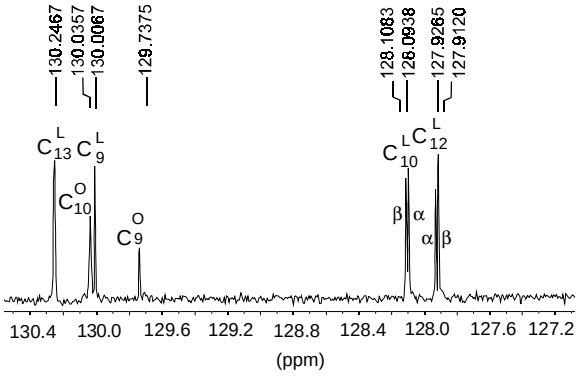

Fig 3. Proton-decoupled ^{13}C - NMR (100.6MHz) of the olefinic carbons of the triacylglycerols of *Adenopus breviflorus* seeds oil. In the assignment of the peaks, the superscripts of symbol C are defined as follows, O for oleic and L for linoleic. The subscripts of symbol C represents the specified carbon in the fatty acid chain.

Table 1
Fatty acid composition of *Adenopus breviflorus* seed oil

Fatty acids	a (%)	b (%)	c (%)	^{13}C NMR
Palmitic	10.10	10.10	10.84	*
Stearic	2.50	9.90	14.06	*
Oleic	24.56	19.40	13.84	14.10
Linoleic	62.86	60.70	61.26	60.90
Saturated	12.60	19.90	24.90	25.00
Unsaturated	87.42	80.10	75.10	75.00

a, % Fatty acid composition as reported by Oderinde (1990); b, % Fatty acid composition as reported by Oshodi (1996); c, % Fatty acid composition as obtained in the present effort by GC method; *, % Fatty acid composition reported together as total saturated.

24.8718 ppm can be paired having a chemical shift difference ($\Delta\delta$) of 0.036 ppm. Referring to established data, this pair of signals are assigned to C-3 of L distributed in the α and β glyceridic positions. No signal is found in the region *ca* 32 ppm, hence the presence of *trans* ethylenic system in the seeds oil can be ruled out.

Ten signals appear in the region (20 - 27 ppm). The signal at 27.2575 ppm is due to C-11 carbon atom of O, the 27.2356 ppm signal is due to C-14 carbon atom of L, the 27.2065 ppm is due to C-8 carbon atom of O and L and the 25.6573 ppm signal is due to C-11 of L. The relative intensities of the allylic methylene protons are distinct and the signals profile and intensity could serve as fingerprint for the identification of the oil.

Lie Ken Jie and Lam (1995) have observed a de-shielding order for the shifts of C-16 carbon nuclei as follows, Sat. (31.976 ppm) > O (31.954 ppm) > L (31.567 ppm). This trend was also

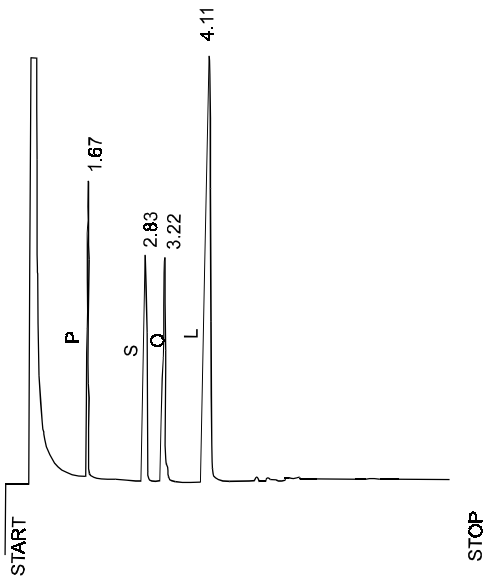

Fig 4. GC Chromatogram of *Adenopus breviflorus* seeds oil. The numbers are retention times. The symbols are: P for Palmitic acid, S for stearic acid O for oleic acid and L for linoleic acid.

observed by the same authors for C-17 carbon nuclei. The spectra of ADB also shows this de-shielding effect, so the signals at 31.9632 ppm, 31.9414 ppm and 31.5632 ppm are assigned to the shift of C-16 carbon nuclei of Sat., O and L respectively present in the ADB oil. In the same manner, the 22.7335 ppm, 22.7189 ppm and 22.6098 ppm are assigned to the shift of C-17 carbons of Sat., O and L respectively.

Another very characteristic region in the ^{13}C - NMR spectra of oils that defines the acyl composition and positional distribution on glycerol backbone is the olefinic carbon shift region. ^{13}C - NMR spectrum of ADB oil in this region is shown in Fig 3.

Ng (1983) had observed that the chemical shift between a pair of peaks become smaller for the olefinic carbon nearer to the methyl end of the fatty acid chain, i.e. in the O chain, magnitude of the peak separation is in the order C-9 > C-10 > C-12 > C-13. He also observed that in the O chain, the peak for C-9 attached at β glyceridic position appears at a lower field than that attached at the α -position and that the reverse order holds for C - 10. These high / low field alteration in peak position were also observed among the olefinic carbons of L chain. In general, in the O chain, $\Delta\delta$ between C -10 and C-9 α-positions is 0.30 ppm and that between their β- positions is 0.34 ppm. In the L chain, $\Delta\delta$ between C-13 and C - 9 α-positions is 0.20 ppm and $\Delta\delta$ between their α- positions is 0.34 ppm. In the L chain $\Delta\delta$ between C-13 and C-9 β -positions is 0.20 ppm and $\Delta\delta$ between their α-positions is 0.24 ppm while $\Delta\delta$ between C-10 and C- 12 β -positions is 0.17 ppm and their α positions is

0.19 ppm. Based on these difference values and other established data, the peaks in the olefinic regions are assigned as shown in Fig 3. The spectrum clearly shows the presence of O and L and absence of any triene ester. The intensity of the peaks show that L is more abundant than O in ADB oil. The sharpness of the C-9 and C-10 of O clearly indicate that they are single peaks. However, the chemical shift difference ($\Delta\delta$ = 0.30 ppm) points to the fact that O is attached only at the α glyceridic position. The chemical shift difference between the C-13 and C-9 of L ($\Delta\delta$ = 0.24 ppm) and the intensities of the pair of peaks observed for the C-10 and C-12 shows that L is mostly attached at the α glyceridic position. These results corroborates our observations from other regions of the spectra especially the C-3 carbon region which had indicated the distribution of L in the α and β glyceridic positions and the C-2 carbon shift region which had indicated presence of O in α position and L in mainly β position.

*Semi-quantitative analysis of the fatty acid composition.*The results discussed above revealed that ADB oil is composed mainly of Sat., O and L. For oils with non complex composition like this, the peaks at *ca* 24 ppm represents the total number of saturated, monoene and diene chain.The peaks at *ca* 25 ppm belongs to C-11 that is allylic to both double bonds of a *cis-cis* diene (linoleic) such that they represent the total number of diene chains and the peaks at *ca* 27 ppm belong to the two carbons allylic to *cis* double bond i.e. C-8, C-11 of O and C-8, C-14 of L, such that they represent twice the total number of monoene (O) and diene (L) chain (Ng and Ng 1984). The areas of these peaks, therefore permit quantitative analysis of Sat., O and L.

Integrals of these peaks are identified as a, b and c in Fig 2 and the percentage composition of the oil is calculated as:

Percentage of Sat. = [(a - 0.5c) / a] x 100
Percentage of O = [(0.5 c -b) / a] x100
Percentage of L = [b / a] x 100

For the ADB, a = 0.46, b = 0.28 and c = 0.69. The percentage of the acyl composition derived from the NMR spectra is presented in Table 1 along those side obtained by gas chromatography by Oshodi (1996) and Oderinde (1990) and also obtained by GC methods in the present effort. The GC chromatogram obtained in the present effort is presented in Fig 4. The NMR results confirm the GC results that L is the most abundant fatty acid in ADB oil. Our GC results compare very well with our NMR extrapolated results. However, results of other workers differ especially in their O and S contents. These variations may be due to geographical and environmental factors. Going by the agreement between our two results obtained by two independent methods, we can reasonably state that in ADB consumed in the South-western part of Nigeria, percentage saturated fatty acids is ca 25% and unsaturated fatty acids is ca 75% comprising of oleic(ca 14%) and linoleic(ca 61%) acids.

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