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

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**Abstract**

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 composition were confirmed by gas chromatography. The spectra further revealed that while the saturated fatty acids are distributed between the 1,3 ($\alpha$) and 2 ($\beta$) glyceridic positions, oleic acid is attached only at the $\alpha$ glyceridic positions while linoleic acid is attached mostly at the ($\beta$) glyceridic position.

## 1. 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 $\alpha$-(i.e. the 1- and 3- positions of the glycerol) and $\beta$-(i.e. the 2-position of glycerol), carbon-13 NMR has been found most useful (Wollenberg, 1990). There have also been some efforts in the past (Ng, 1984; Gunstone, 1993; Lie Ken Jie and Pasha, 1996) where $^{13}C$ NMR was used to identify , confirm or evaluate the fatty acids composition of different seed oils. 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* (Cucurbitacea) 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, 2002a). In an earlier investigation, we have tried to identify *Adenopus breviflorus* seeds oil from other seed oils using the peak area ratio $(-CH_2-)/(=CCH_2C=)$ derived from their $^1H$ NMR spectroscopy (Akintayo, 2002b) as a qualitative index. 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.

## 2. 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 20hr by Sohxlet method. The extracts were desolventised under reduced pressure on a rotavapour.

The extracted oil was purified. 2g of extracted oil was percolated through a silica gel (15g) column with a mixture of petroleum ether (b.p. 40-60°C) and diethyl ether (95:5, v/v, 150ml). The eluate was evaporated under reduced pressure to 5ml portion and this portion further concentrated by a gentle blow of nitrogen gas to yield a mixture of triacylglycerols (1.72g).

The $^{13}C$ NMR of the sample dissolved in deuteriated Chloroform were recorded on the BRUKER AMX-400 (BRUKER Instruments, Inc. Karlsruhe, Germany) Fourier transform 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 1.0sec. Free induction decay (FID) was transformed and zero filled to 300K to give a digital resolution of 0.366Hz/point.

Fatty acid methyl ester (FAME) of the oil was prepared as follows: approximately 2mg crude seeds oil was transferred into a 5-10ml 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 10µl acetic acid was added. The equation of the transmethylation is shown above.

The clear supernatant was used for gas chromatographic analysis. 0.2µl of the FAMES was injected into Hewlett-Packard 5890 GC (Hewlett-Packard Co, Palo Albo CA). The column was HPUltra Performance coated with crosslinked 5% Phenol +95% Polysiloxane, 30x0.25nm, 0.2µ coating thickness. Temperature programming was as follows: Initial

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**Table 1**: $^{13}C$ NMR chemical shifts of *Adenopus breviflorus* seed oil

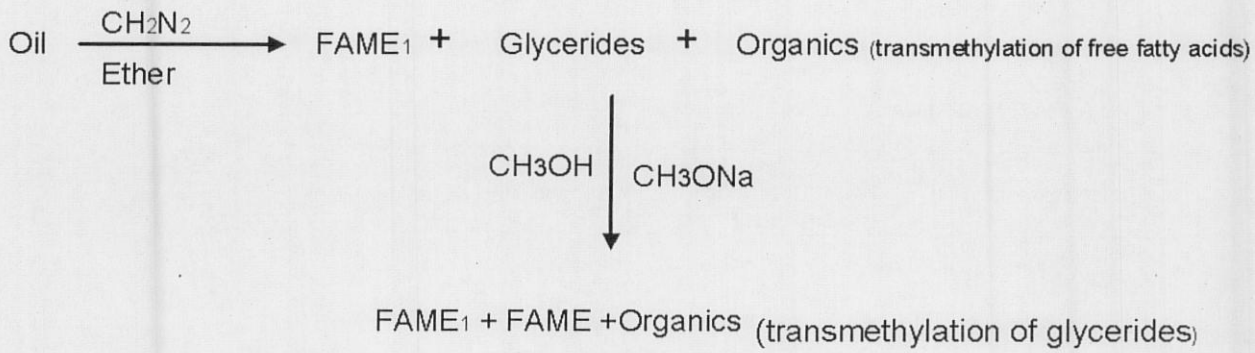
| $^{13}C$ NMR Shift of ADB | $^{13}C$ NMR shift of standard esters* | Assignments |
|---|---|---|
| 173.3188 | 173.312 | C-1, Sat |
| 173.2752 | 173.171 | C-1, L(α) |
| 172.8606 | 172.771 | C-1, L(ß) |
| 130.2467 | 130.182 | C-13, L |
| 130.0357 | 130.029 | C-10, O |
| 130.0067 | 129.980 | C-9, L |
| 129.7375 | 129.720 | C-9, O |
| 128.1083 | 128.114 | C-10, L(ß) |
| 128.0938 | 128.095 | C-10, L(α) |
| 127.9265 | 127.932 | C-12, L(α) |
| 127.9120 | 127.920 | C-12, L(ß) |
| 34.2180 | 34.242 | C-2, Sat(ß) |
| 34.1307 | 34.190 | C-2 L(ß) |
| 34.0798 | 34.041 | C-2, O(α) |
| 34.0507 | 34.074 | C-2, Sat(α) |
| 24.9082 | 24.896 | C-3, L(α) |
| 24.8718 | 24.860 | C-3, L(ß) |
| 27.2575 | 27.254 | C-11, O |
| 27.2356 | 27.226 | C-14, L |
| 27.2065 | 27.202 | C-8, O |
| 25.6573 | 25.655 | C-11, L |
| 31.9632 | 31.962 | ω3, Sat |
| 31.9414 | 31.956 | ω3, O |
| 31.5632 | 31.557 | ω3, L |
| 22.7335 | 22.733 | ω2, Sat |
| 22.7189 | 22.726 | ω2, O |
| 22.6098 | 22.610 | ω2, L |

* Established data as reported by Lie Ken Jie,*et. al.* (1992, 1993, 1995).

**Table 2**: 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*) Percentage Fatty acid composition as reported by Oderinde (1990)
(*b*) Percentage Fatty acid composition as reported by Oshodi (1996).
(*c*) Percentage Fatty acid composition as obtained in the present effort byGas Chromatographic method
(*) Percentage Fatty acid composition reported together as total saturated.




temperature, 160°C for 2 min, temperature increased at 2.5°C/min up to 300°C and maintained at this final temperature for 5min. Injector and dectector temperature were 280°C and 340°C respectively.

### 3. 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 given below.

Table 1 presents the important signals in the $^{13}C$ NMR spectra of ADB oil (Fig. 1) along with those of established data (Lie Ken Jie *et.al.*, 1992, 1993, 1995) and their assignments.

The high resolution $^{13}C$ NMR spectrum of the carbonyl carbons of the triglycerides of ADB is presented in Figure 2 and it shows three signals at 173.3188ppm, 173.2752ppm and 172.8606ppm. Ng (1983) has reported that the carbonyl carbons of saturated chains appear as the highest frequency peak in the NMR spectrum (at approximate δ of 173.3). The highest chemical shift in the spectrum of ADB oil 173.3188ppm can therefore be assigned to carbonyl carbon of Sat in α position. Ng (1983) has also 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±0.002ppm while L differs by 0.041±0.002ppm).

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, 173.2752ppm differs from the 173.3188ppm signal by *ca* 0.043ppm. Referring to Ng (1983) and Lie Ken Jie *et.al.* (1992, 1993, 1995), the pair of signals 173.2752ppm/ 172.8606ppm 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 reports by Ng (1983) and Shiao and Shiao (1989) have shown that resonances of saturated fatty acids were not resolved in the carbonyl region.

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

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

where the superscripts stand for carbon numbers.

The $^{13}C$ NMR signal profiles in the upfield region (20-36ppm) of the ADB oil (Figure 3) 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 carbon shift region (*ca* 34ppm) and (ii) the C-3 (*ca* 24ppm), allylic (25-27ppm), C-17(*ca* 22ppm) and C-16 (*ca* 31ppm) carbon shift region.

#### *C-2 carbon shift region (ca 34ppm)*

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

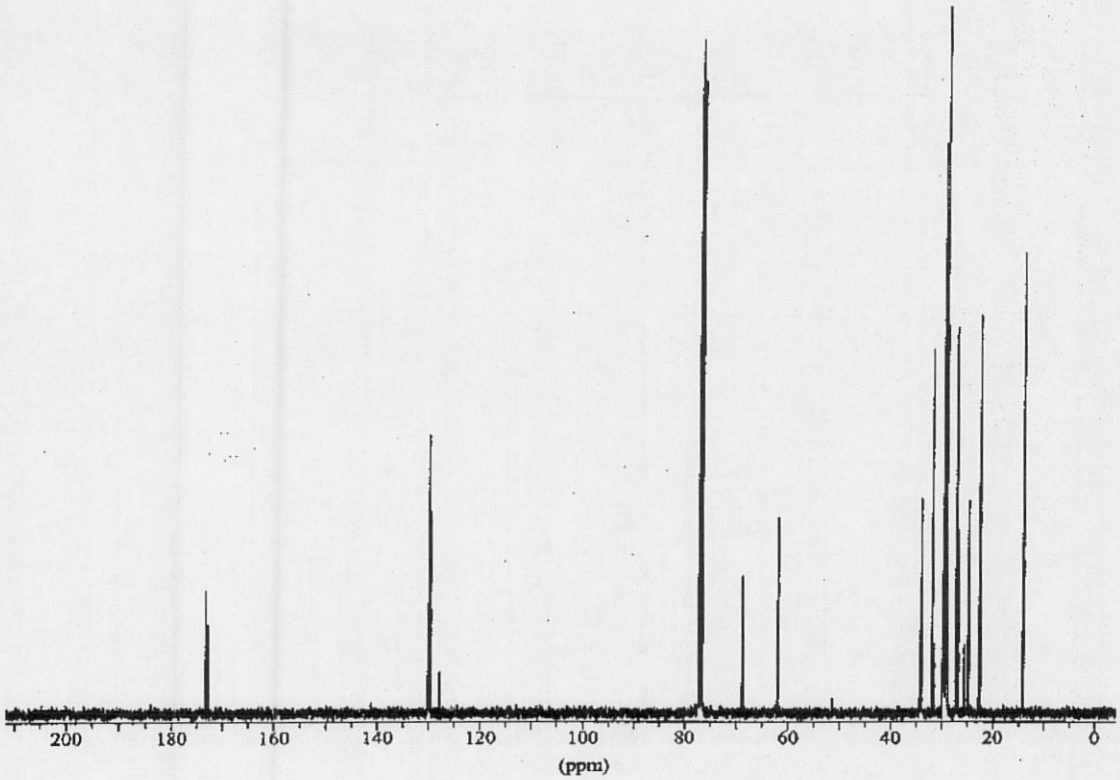



**Figure 1**: Proton decoupled high resolution $^{13}C$ NMR (100.6MHz) spectrum of the Triacylglycerols in *Adenopus breviflorus* seeds oil

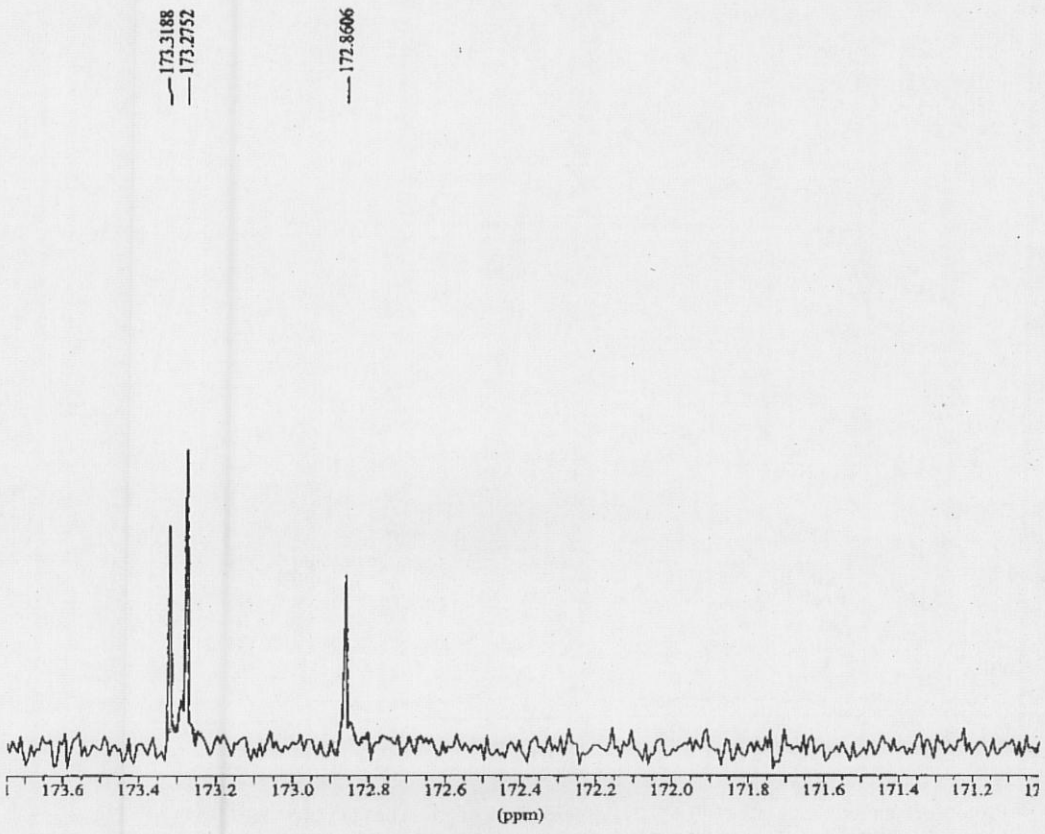



Figure 2: Proton-decoupled high resolution $^{13}C$ NMR (100.6MHz) of the carbonyl carbons of the triacylglycerols in *Adenopus breviflorus* seeds oil

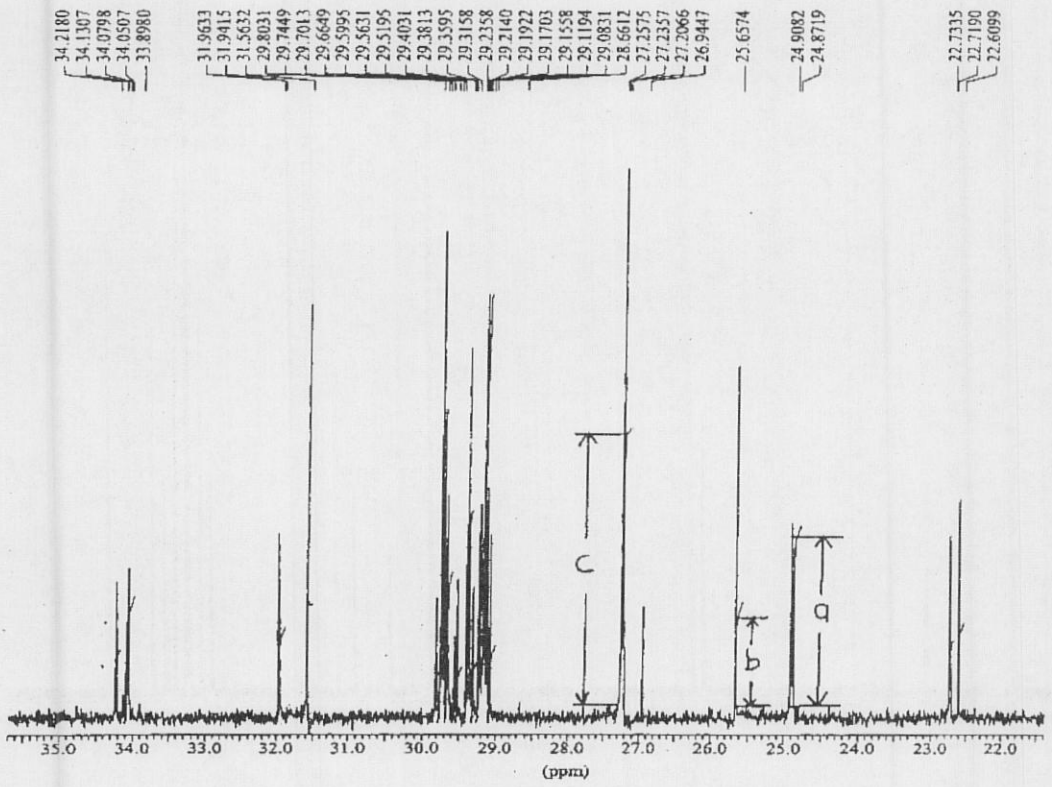



**Figure 3**: Proton-decoupled $^{13}C$ NMR (100.6MHz) of the saturated carbons of the fatty acid chains in *Adenopus breviflorus* seeds oil. The integral value *'a'* is for the peak at *ca* 24ppm, *'b'* is for the peak at *ca* 25ppm and *'c'* is for the peak at *ca* 27ppm.

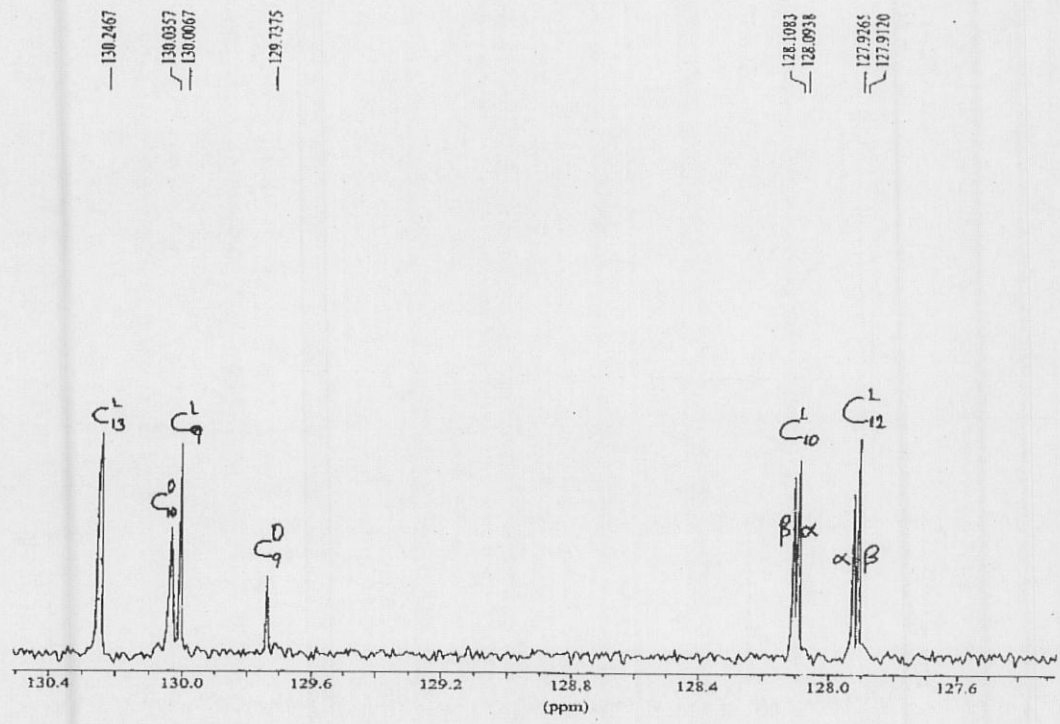



**Figure 4**: Proton-decoupled $^{13}C$ NMR (100.6MHz) of the olefinic carbons of the triacylglycerols of *Adenopus breviflorus* seeds oil. In the assignment of the peaks, the superscripts of symbol *C* are defined as follows, *O* for oleic and *L* for linoleic. The subscripts of symbol *C* represents the specified carbon in the fatty acid chain.

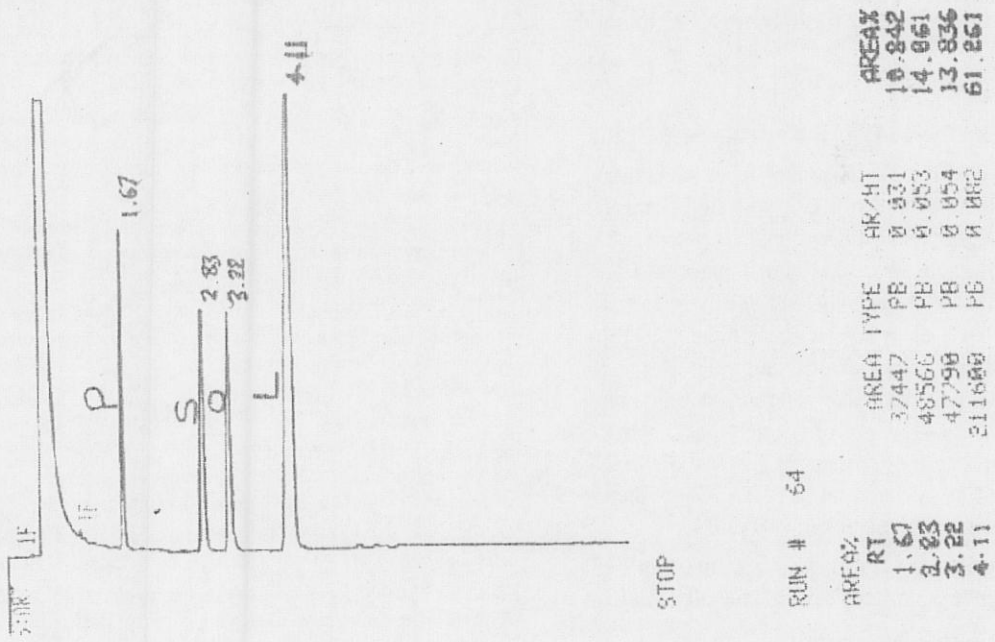



**Figure 5**: *GC* Chromatogram of *Adenopus breviflorus* seeds oil. The numbers are retention times. The symbols are: *P* for Palmitic acid, *S* for stearic acid *O* for oleic acid and *L* for linoleic acid.

***C-3, allylic, C-17 and C-16 carbon shift region.***

The two signals in the C-3 region (*ca* 24ppm) 24.9082ppm and 24.8718ppm can be paired having a chemical shift difference ($\Delta\delta$) of 0.036ppm. Referring to reported data, this pair of signals are assigned to C-3 of L distributed in the $\alpha$ and $\beta$ glyceridic positions. No signal is found in the region *ca* 32ppm, hence the presence of trans ethylenic systems in the seeds oil can be ruled out.

Ten signals appear in the region (20-27ppm). The signal at 27.2575ppm is due to C-11 carbon atom of O, the 27.2356ppm signal is due to C-14 carbon atom of L, the 27.2065ppm is due to C-8 carbon atom of O and L and the 25.6573ppm 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 identification of the oil. Lie Ken Jie and Lam (1995) have observed a deshielding order for the shifts of C-16 carbon nuclei as follows, Sat (31.976ppm) > O (31.954ppm) > L (31.567ppm). This trend was also observed by the same authors for C-17 carbon nuclei. The spectra of ADB also shows this deshielding effect, so the signals at 31.9632ppm, 31.9414ppm and 31.5632ppm 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 22.7335ppm, 22.7189ppm and 22.6098ppm 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 Figure 4.

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 $\beta$ glyceridic position appears at a lower field than that attached at the $\alpha$- position and that the reverse order holds for C-10. These high/low field alterations 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 $\alpha$-positions is 0.30ppm and that between their $\beta$- positions is 0.34ppm. In the L chain $\Delta\delta$ between C-13 and C-9 $\alpha$-positions is 0.20ppm and $\Delta\delta$ between their $\beta$-positions is 0.24ppm while $\Delta\delta$ between C-10 and C-12 $\alpha$-positions is 0.17ppm and their $\beta$ positions is 0.19ppm. Based on these difference values and other established data, the peaks in the olefinic regions are assigned as shown in Figure 3. The spectrum clearly shows the presence of O and L and absence of any triene ester. The intensity of the peaks shows 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 ($\Delta\delta$ = 0.30ppm) points to the fact that O is attached only at the $\alpha$ glyceridic position. The chemical shift difference between the C-13 and C-9 of L ($\Delta\delta$ = 0.24ppm) and the intensities of the pair of peaks observed for the C-10

and C-12 shows that L is mostly attached at the β glyceridic position. These results corroborate 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* 24ppm represents the total number of saturated, monoene and diene chain.The peaks at *ca* 25ppm 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 Figure 3 and the percentage composition of the oil is calculated as:

$$\text{Percentage of } Sat. = [\ (a - 0.5c)/a\ ] \times 100$$

$$\text{Percentage of } O = [\ (0.5c - b)/a\ ] \times 100$$

$$\text{Percentage of } L = [\ b/a\ ] \times 100$$

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 2 along side those obtained by gas chromatography by Oshodi (1997) and Oderinde (1990) and also obtained by GC methods in the present effort. The chromatogram obtained in the present effort is presented in Figure 5. 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 our sample of ADB oil, 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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