Qualitative and Quantitative Analysis of Fatty Acid Composition of Parkia Biglobbossa Seed Oil Using Gas Chromatographic and High Resolution Nuclear Magnetic Resonance Spectroscopic Methods

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Abstract
Gas chromatography (GC) and high resolution nuclear magnetic resonance (NMR) spectroscopic techniques were used to determine the qualitative and quantitative fatty acid composition of *Parkia biglobbossa* seed oil. The results obtained from GC, ^{13}C NMR and ^{1}H NMR methods were compared using statistical analysis of one way analysis of variance and there was no significant difference in all the percentages obtained for both saturated and unsaturated fatty acid (P> 0.05). The result showed that ^{1}H NMR, ^{13}C NMR and GC techniques could be used for determination of the saturated and unsaturated fatty acid composition of *P. biglobbossa*. NMR may therefore be more suitable because it is reliable, less time consuming and not laborious compared to other classical methods.
Keywords: *Parkia biglobbossa* oil, Gas chromatography, Nuclear Magnetic Resonance, Fatty acid composition.

1. Introduction

Most seed oils compose of triacylglycerols which contain an array of fatty acids both saturated and unsaturated. To determine these fatty acids composition, gas chromatographic (GC) technique has commonly been used (Bagci, 2007; Bagci and Özçelik (2009); Bhide *et al*., 2013).This method has a wide application in the determination of the triacylglycerol and thus fatty acid composition of seed oils (Dijkstra *et al*., 2007). Determination of fatty acid composition by GC involves conversion of the lipids into methyl esters before analysis. This is however a destructive method because it involves hydrolysis of triacylglycerols and methylation of the free fatty acids prior to their chromatographic analysis (Mavromoustakos *et al.*, 1997). It is also labour intensive and time consuming.

Spectroscopic methods combined with computer aided statistical and mathematical procedures are some of the emerging analytical techniques in determining the fatty acid composition of seed oils. Nuclear magnetic resonance (NMR) spectroscopy has been used and found useful in determining the properties of oils of vegetable origin (Vlahov, 1996). It has been found useful in determining organic structures in complex matrices such as foods, pharmaceutical and biological samples (Beyer *et al*., 2010; Martinez *et al*., 2003).

The use of ^{1}H NMR method allows a rapid, simultaneous, non invasive and non destructive study of oil composition and also provides information about the acyl distribution and the acyl positional distributions of the triacylglycerols (Guillen and Ruiz, 2001; Guillen and Ruiz, 2003a, b; Lie and Mustafa, 1997). ^{13}C NMR spectroscopy has also been used as a technique that allows the determination of the more abundant fatty acid composition and positional distribution of the acyl groups within a short time. Several authors have used these three techniques for determination of fatty acid composition of seed oils separately.

Wollemberg (1990) used ^{13}C NMR to carry out the positional analysis of fatty acids in triacylglycerols of vegetable oils. Akintayo *et al*. (2004) also used ^{13}C NMR to determine the fatty acid composition of *Jatropha curcas* oil. Salinero *et al*. (2012) used ^{1}H NMR to analyse the triacylglyceride composition of cold – pressed oil from *Camellia japonica*. Miyake *et al*. (1998) used both ^{1}H NMR and ^{13}C NMR to determine the unsaturated fatty acid composition of some vegetable oils.

The aim of this work is to use these three techniques: GC, ^{1}HNMR and ^{13}C NMR to determine the percentage composition of saturated and unsaturated fatty acid of oil extracted from *Parkia biglobbossa* and to further carry out a statistical comparison of the results to know if there is any significant difference between the results obtained from the three methods.

Parkia biglobbossa belongs to the family *Fabaceae*. It bears fruits and the matured and ripe pods have edible pulp. The seeds are processed into a local product (Iru in Yoruba) which is normally used as a local condiment among the Yorubas in Nigeria (Akintayo, 2003). Chemical analysis shows it to be rich in lipid, protein, soluble sugars and ascorbic acid and therefore it can be a source of these nutrients when taken (Dawodu, 2009).

2. Materials and Methods

Parkia biglobbossa seeds were purchased from local markets in Ado – Ekiti, Ekiti State, Nigeria. The seeds were dehulled, washed and sun dried. The oils were extracted with hexane for 5h by soxhlet method and was concentrated using rotary evaporator.

50mg of the extracted fat content of the samples was saponified for 5 min at 95ºC with 3.4ml of 0.5M

KOH in dry methanol. The mixture was neutralized by using 0.7M HCl. 3ml of 14% boron triflouride in methanol was added and the mixture was heated for 5 min at 90ºC to achieve complete methylation process. The fatty acid methyl esters were extracted with redistilled n-hexane. The content was concentrated for gas chromatography analysis and 1μl was injected into the injection port of GC. The GC conditions of analysis are as follows: HP 6890 powered with HP ChemStation Rev. A09.01 [1206] software, split ratio: 20:1, carrier gas: Nitrogen, inlet temperature 250ºC, column type: HPINNOWax, Oven program: initial temperature at 60^{0}C, first Ramping at 12^{0}C/min for 20min, maintained for 2min, second ramping at15ºC/min for 30 min maintained for 8 min, detector: free induction decay (FID), detector temperature: 320ºC

The ^{1}H NMR and ^{13}C analysis were performed on a Bruker AM – 600MHz (Bruker Instruments, Inc., Karlsruhe Germany) Fourier transform spectrometer. Triplicate samples of the oil were dissolved in 1 ml of deuterated chloroform CDCl$_3$ and introduced into a 10mm NMR tube. The gated decoupling pulse sequence was used with the following parameters: Number of scans 512, acquisition time 1.366s, pulse width 10.3s, delay time 1.0s, free induction decay (FID) is transformed and zero filled to 300ºK. The chemical shifts are reported in ppm, and the area of the signals was determined by using the equipment software and the integrations were carried out three times to obtain average values.

Statistical Analysis was carried out using analysis of variance (ANOVA). Chi Test was used to compare mean variance. Significance was accepted at 5% level of probability.

3. Results and Discussion

The gas chromatography spectrum of *P. biglobbossa* is shown in Fig. I. The percentage composition of each fatty acid from the GC analysis is 21.56%, 37.48%, 3.12%, 37.05% for oleic, linoleic, linolenic and saturated fatty acids respectively. The fatty acid composition is comparable with 25%, 50%, 7%, 15% for oleic, linoleic, linolenic and saturated fatty acid composition respectively obtained for soybean by Haiyan *et al.* (2006). It is also comparable with the report of Olanipekun et al. (2012) on unfermented Bambara nut with fatty acid composition of 8.77% oleic, 42.34% linoleic, 1.56% linolenic, 40.09% saturated fatty acid. These observations may be due to the fact that the three seeds belong to the same family thus making them to have similar fatty acid composition. This is also an indication that their oils are safe for consumption and can be used in place of one another for the same purposes.
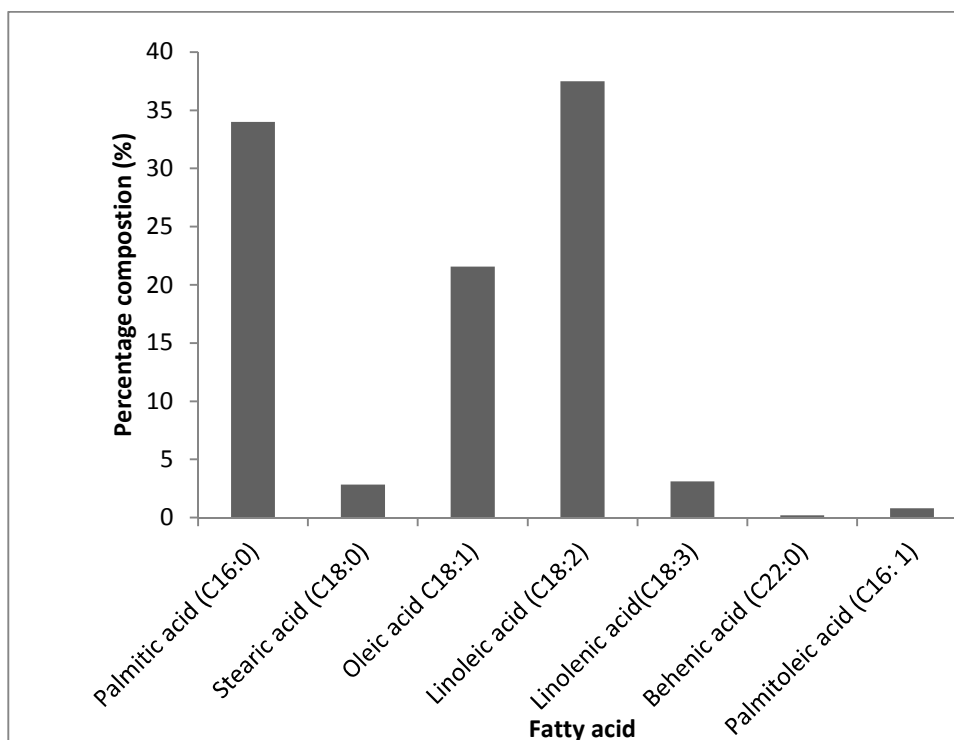

Figure I: Fatty acid composition of *Parkia biglobbossa* using GC

The ^{1}H NMR spectrum is shown in Figure II. The proton resonances of the major triacylglycerols (TAG) present in the oil were assigned according to literature (Hatzakis *et al*., 2011; Salinero *et al*., 2012; Vlahov, 1999) and are shown in Table I.
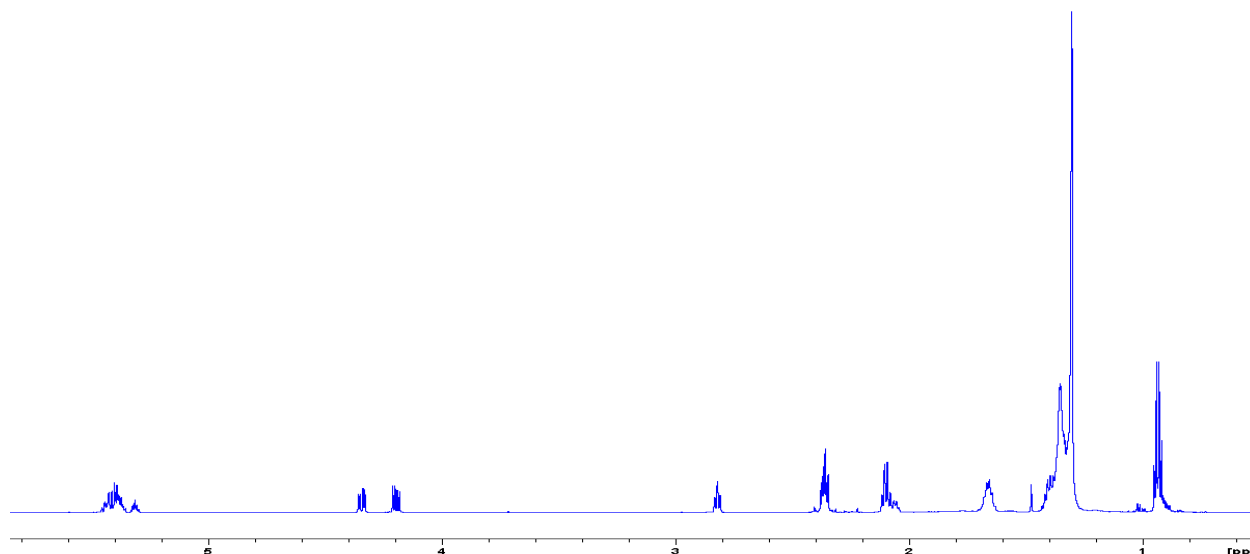
Figure II: ^{1}H NMR of *Parkia biglobbossa*

The olefinic protons –CH=CH – of unsaturated fatty acids resonate at 5.4 – 5.5ppm. The tertiary proton of glyceryl molecule was found at 5.3ppm. H1 & H2 protons of diacylglycerols (DAG) were found at 4.3ppm while the protons of triacylglycerol were found at 4.2ppm. The terminal methyl protons of saturated and unsaturated chains appear at 0.8ppm while methyl protons of polyunsaturated chains are shifted to 1.0ppm. The signals at 2.0 – 2.1ppm and 2.7 – 2.9 ppm indicated those of monounsaturated and polyunsaturated acyl chains.

Table I: ^{1}H NMR chemical shifts of *Parkia biglobbossa* oil

Signals	Chemical shift (ppm)	Protons	Multiplicity	Compound
1.	0.8	- CH$_2$ –CH$_3$	T	Terminal methyl chains, S,O
2	1.0	=CH– CH$_3$	D	Terminal methyl chains Ln, L chains
3.	1.4 – 1.2	-(CH$_2$)n	D	All acyl chains
4.	1.8 – 1.6	-CH$_2$ –CH$_2$COO	M	All acyl chains
5.	2.1 – 2.0	-CH$_2$CH=CH-	M	O, L, Ln chains
6.	2.4 – 2.3	-CH$_2$COO	M	All acyl chains of TAG
7.	2.9 – 2.7	-CH=CHCH$_2$CH=CH-	M	L, Ln Chains
8.	4.2	COOCH$_2$CHCOO-	DD	Glycerol of TAG
9.	4.3	OHCH$_2$CHCOOCH$_2$COO	DD	Glycerol of 1,2- DAG
10.	5.3	COOCH$_2$CHOCOCH$_2$COO	M	Glycerol of TAG
11.	5.5 – 5.4	-CH$_2$-CH=CHCOO-	M	All acyl chains

S – Saturated, O – oleyl, Ln – linolenyl, L – Linoleyl, TAG – Triacylglycerol, 1,2 - DAG – 1,2- Diacylglycerol

The qualitative analysis as presented in Table I showed the presence of oleic, linoleic, linolenic and saturated fatty acid in the sample. To determine the quantity of these fatty acids in the oil sample, some selected peaks were measured and integrated as shown in Figure III.
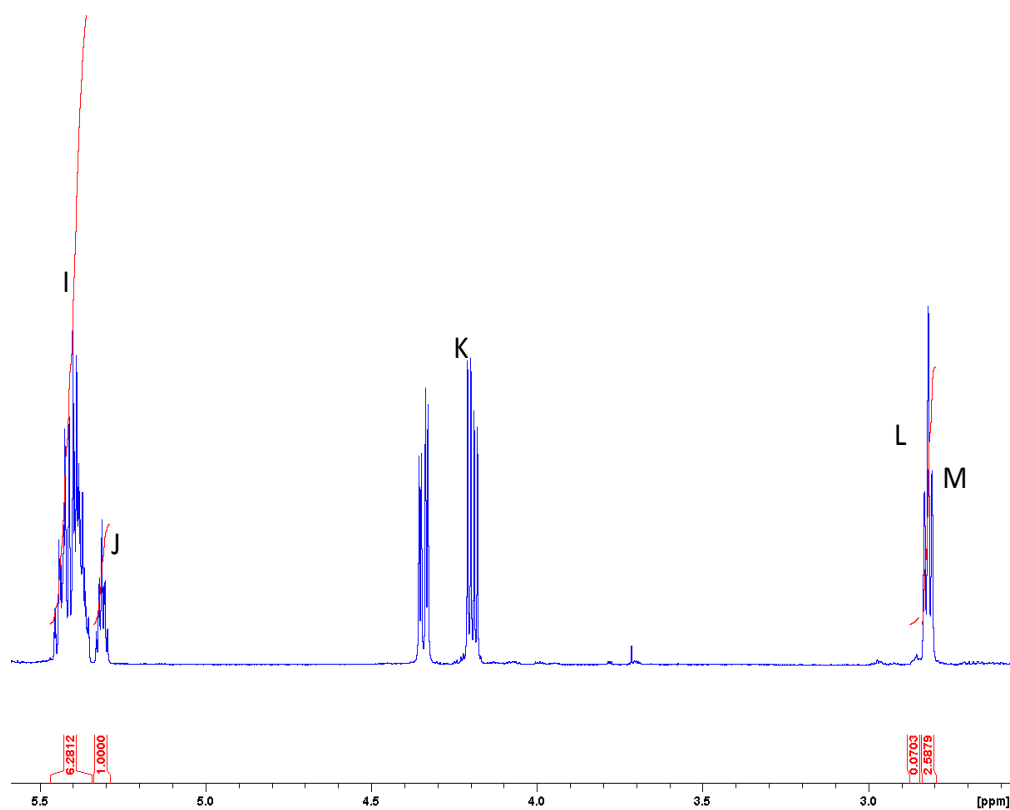
Figure III: Integrated ^{1}H NMR spectra of *Parkia biglobbossa*

'I' is the vinylic hydrogen (Hv); it has a characteristic chemical shift and could be used to determine the ratio of saturated to unsaturated esters. 'J' is the tertiary hydrogen in the glycerin moiety (Hg) and could be used to determine the ratio of saturated to unsaturated esters because there is only one hydrogen for each triacylglycerol molecule. 'K' represents methylene protons in the glyceryl group. 'L' the protons of the bis – allylic of polyunsaturated acyl chains (Ht) and this could be used to determine the nature of the unsaturated components. 'M' is the protons of diunsaturated acyl (Hd) chains and this could be used to determine the nature of diunsaturated fatty acid. The structure of the major TAGs present in the oil is presented in Figure IV.
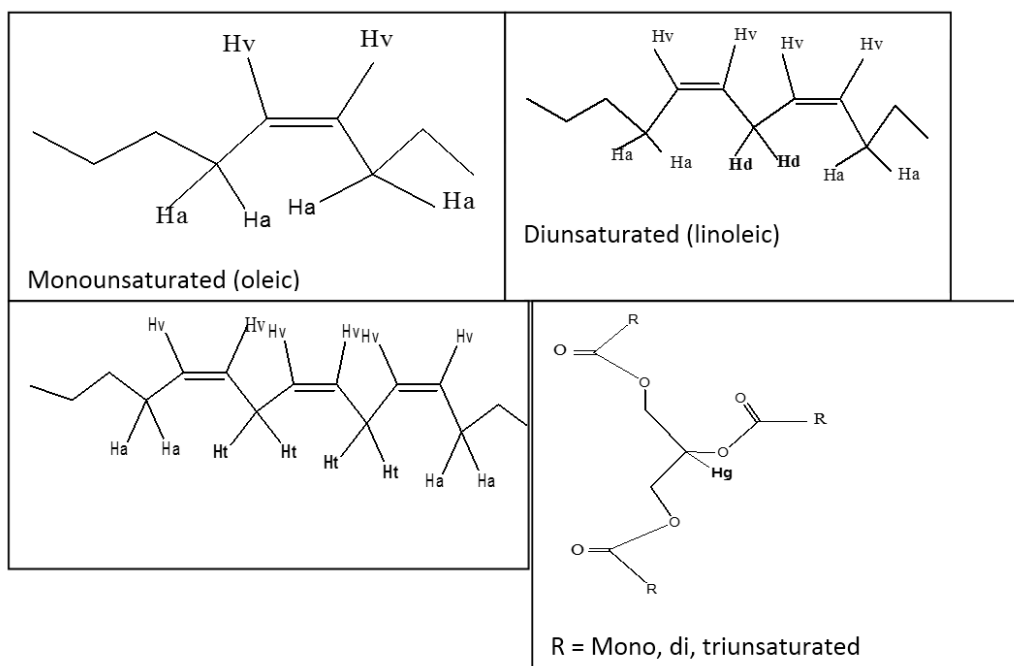
Figure IV: Chemical structures of main triacylglycerols in oils

The signal from tertiary glycerin hydrogen and the vinylic ones were integrated separately. The measured integrals are shown in Fig. III; Hv = 6.28, Hd = 2.59, Ht = 0.07, Hg = 1.00. The percentage composition of each fatty acid was calculated by solving the equations below.

Integral of vinylic (oleic) hydrogens (Hv) = 2I+4M + 6L ---------- (I)

Integral of linoleic bisallylic hydrogens (Hd) = 2M------------------ (II)

Integral of linolenic bisallylic hydrogen (Ht) = 4L ------------------ (III)

I+J+L+M = 3-- (IV)

The quantitative value of each fatty acid using ^{1}H NMR is: oleic (16.56 %), linoleic (43.17%), linolenic (0.58%), saturated (39.67%).

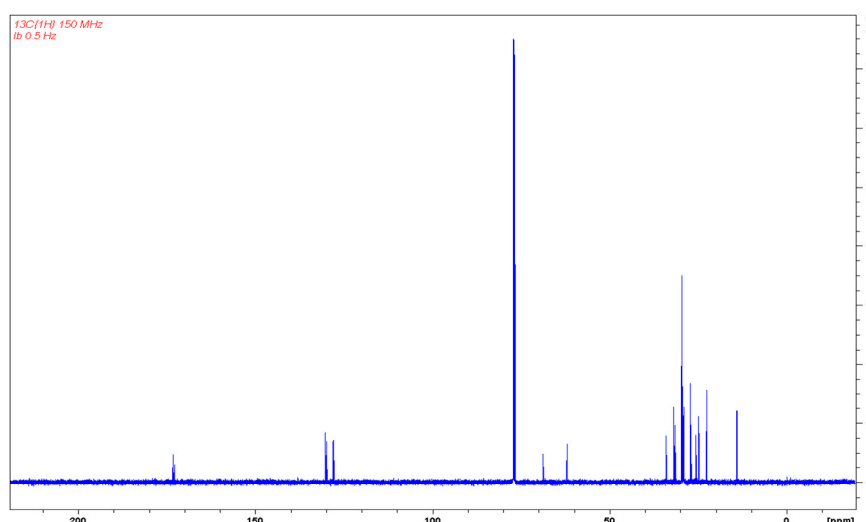The ^{13}C NMR spectrum of *P. biglobbossa* is shown in Figure V and the chemical shift assignments in Table II.

Figure V: ^{13}C NMR spectrum of *Parkia biglobbossa* seed oil

The chemical shifts were assigned according to literature data (Gunstone, 2014; Vlahov, 1999) and presented in Table II. Like some other vegetable oil, ^{13}C NMR spectrum of *P.biglobbossa* showed signals at the carbonyl region (172 – 174ppm), olefinic carbon region (124 – 134 ppm), glycerol carbon region (60 -72ppm), methylenic and methyl carbon region (10 – 35ppm). The signals at 173.27 and 172.85ppm with chemical shift

difference of 0.42ppm are those of saturated 1,3 TAG and 2- positions of oleyl, linoleyl. The signals at the olefinic carbon regions confirmed the presence of unsaturation in the oil. Linoleyl and linolenyl chains can easily be detected only in this region.

The signals at 130.25ppm indicated those of linolenyl on carbon – 11 while the signals at 130.00ppm, 128.11ppm, 128.09ppm, 127.91ppm indicated those of linoleyl at positions 9, 10 and 12 respectively as shown in Table II. These signals indicated that linoleyl is more abundant in the oil. The presence of oleyl was shown by the signals at 130.03ppm and 129.71ppm.

The glycerol carbons of mono-, di-, and triacylglycerols resonate in the spectral region 60 – 72ppm. The signals at 68.91 and 62.11ppm indicated those of triacylglycerol in the *Parkia biglobossa* seed oil.

Three signals appear in the C-2 carbon shift region. The signals at 34.22ppm and 34.05ppm can be paired with chemical shift difference of 0.17ppm. The shifts are assigned to Carbon -2 atom of 1,3- diacylglycerol in the oleyl, linoleyl and linolenyl and all acyl chains. The 34.08ppm signal is assigned to Carbon -2 of linolenyl while the peak at 31.95ppm is assigned to ω_2 of oleic in the trans isomer.

The C-16 carbons appear at chemical shifts 31.92 and 31.54ppm with saturated, oleyl, linoleyl chains resonating in this region while C-15 – C-8 resonate between 29.78ppm – 25.65ppm, within each set of signals, saturated, oleyl, linoleyl and linolenyl resonates from higher to lower frequency. The signals at 24.89ppm and 24.86ppm can be paired (chemical shift = 0.03ppm) and these are assigned to C-3 of oleyl, linoleyl and linolenyl. Also, ω_2 and ω_1 carbon regions were found at 22.71ppm – 14.08ppm. ω_2 of oleyl and linoleyl resonated at 22.71 and 22.59ppm respectively and ω_1 of oleyl and saturated with linoleyl at 14.13ppm and 14.08ppm respectively. These signals were assigned according to literature (Gunstone, 2014; Vlahov, 1999).

The qualitative analysis of *Parkia biglobossa* seed oil using carbon13 NMR spectroscopy showed the presence of saturated and unsaturated fatty acid in the seed oil.

Table II: ^{13}C NMR chemical shifts of *Parkia biglobbossa*

Signals	Chemical shift (ppm)	Carbons
1	173.31	C_1, S (α)
2	173.27	C_1 (1,3-TAG) S
3	172.85	C_1 (TAG) O,L
4	130.25	C_{11}, Ln
5	130.03	C_{10}, O (α)
6	130.00	C_9 L
7	129.71	C_9, O (β)
8	128.11	C_{10}, L (β)
9	128.09	C_{10}, L (α)
10	127.91	C_{12} L (β)
11	68.91	TAG
12	62.11	TAG
13	34.22	C_2 (MAG, 1,2 - DAG) O, L, Ln
14	34.08	C_2 (all acylglycerols) Ln
15	34.05	C_2 (1,3-DAG) all acyl chains
16	31.95	ω_2, O (E)
17	31.92	C_{16} (all acylglycerols) S
18	31.54	C_{16} (all acylglycerols) O, L
19	29.78	C_{12} (all acylglycerols) O
20	29.65	C_7 (all acylglycerols) O
21	29.55	C_9 (all acylglycerols) O, L
22	29.50	C_6 (all acylglycerols) O, S
23	29.38	C_{13}, C_{15} (all acylglycerols) O
24	29.33	C_6, C_{15} (all acylglycerols) O, Ln
25	29.29	C_6, C_{15} (all acylglycerols) L, Ln
26	29.19	C_{15} (DAG) Z
27	29.14	C_{15}, ω4
28	29.10	C_6 (all acylglycerols)
29	27.25	C_{11}, O
30	27.22	C_{14}, L
31	26.93	C_8, L
32	25.65	C_{11}, L
33	24.89	C_3, O, L, Ln
34	24.86	C_3, O, L, Ln (E)
35	22.71	ω_2, O
36	22.59	ω_2, L
37	14.13	ω_1, O, S (E)
38	14.08	ω_1, L

S – Saturated , O –Oleyl, L –Linoleyl, Ln –Linolenyl , TAG –Triacylglycerols, MAG –Monoacylglycerols, DAG –Diacylglycerols, E – Trans isomer, Z- Cis isomer

To determine these fatty acids quantitatively, the ^{13}C NMR spectrum of the methyl and methylene regions of *P. biglobbossa* were selected and integrated as shown in Figure VI. The peaks were assigned according to Miyake *et al*. (1998).

'a' represents the three α carbonyl methylene carbons; 'b' the ω – 3 carbons of saturated and monounsaturated fatty acids; 'b´' the ω -3 carbons of n- 6 polyunsaturated fatty acids; c the saturated methylene carbons; d the cis allylic carbons; 'e' – divinyl methylene carbons; 'f' the three β – carbonyl methylene carbons; 'g', ω – 2 carbons of saturated, monounsaturated and n – 6 polyunsaturated fatty acids. 'g´' ω – 2 carbons of n-3 polyunsaturated fatty acid.
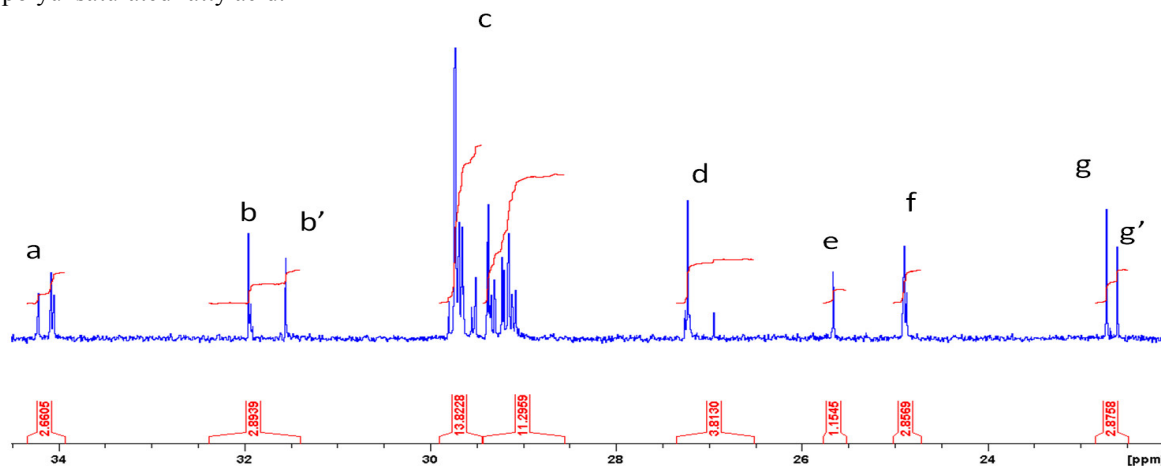

Figure VI: ^{13}C NMR spectrum of methyl and methylene regions of *Parkia biglobbossa seed oil*

The fatty acid composition using carbon13 NMR spectroscopy was calculated quantitatively from the integrals of the corresponding peaks using the following equations.

Total area per carbon x = a + f ----------------------- (V)

% oleic acid content = $\frac{d - \frac{1}{2}(g+b)}{x} \times 100$ ---------------- (VI)

% linoleic acid content = $\frac{\frac{1}{2}[(g'+b') - e]}{x} \times 100$ -------- (VII)

% linolenic acid content = $\frac{\frac{1}{2}g - e}{x} \times 100$ ---------- (VIII)

% saturated = $\frac{(g+b) - d}{x} \times 100$ -------------------------- (IX)

The percentages of each fatty acid from the above equations are: 16.82%, 41.82%, 5.14%, 35.46% for oleic, linoleic, linolenic, saturated fatty acid.

Table III shows the fatty acid composition of *P. biglobbossa* using these three different analytical methods.

Table III: Fatty acid composition of *P. biglobbosa* (%)

Fatty acid	GC	^{1}H NMR	^{13}C NMR	*GC
Oleic	21.56	16.56	16.82	14.5
Linoleic	37.48	43.17	41.82	44.5
Linolenic	3.12	0.58	5.14	3.0
Saturated	37.05	39.67	35.46	38

*Values reported by Akintayo (2003)

Statistical analysis (Table IV) showed that there is no significant difference between the four fatty acid compositions of *P. biglobbossa* reported in Table III above, P >0.05 in all. The result obtained from the ^{13}C NMR agrees well with those of ^{1}H NMR and these in turn agree with the results obtained from GC analysis. This further showed that ^{13}C NMR and ^{1}H NMR could be used to determine the fatty acid composition of *P. biglobbossa*.

Table IV: Statistical analysis of the fatty acid composition of *P. biglobbossa*

Fatty acid	GC (%)	^{1}H NMR (%)	^{13}C NMR (%)	*GC (%)	Mean	χ^2	P
Oleic	21.56	16.56	16.82	14.5	17.36	1.541	0.673
Linoleic	37.48	43.17	41.82	44.5	41.74	0.666	0.881
Linolenic	3.12	0.58	5.14	3.0	2.96	3.530	0.317
Saturated	37.05	39.67	35.46	38	37.55	0.248	0.969

*Values reported by Akintayo (2003)

4.   Conclusion

The quantitative and qualitative analysis of fatty acid composition of *P. biglobossa* seed oil using NMR spectroscopic techniques showed the presence of saturated and unsaturated fatty acid in the seed oil. These fatty acids composition were also determined using GC. The statistical analysis showed that there was no significant difference in the result obtained from the two techniques. Therefore any of these two techniques can be used to

determine the fatty acid composition of *P. biglobossa* seed oil, but NMR may be suitable for a busy laboratory because it is less time consuming and not laborious compared to other classical methods.

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