

Contribution to the Varietal Delimitation of *Irvingia gabonensis* and *Irvingia wombulu* Chemical Composition Variability of Fats Extracted from Kernels

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Abstract: *Irvingia gabonensis* was long considered to comprise two varieties, one sweet and the other bitter. These have since been classified as distinct species, sweet *I. gabonensis* and the bitter *I. wombulu*, essentially on morphological and organoleptic criteria. Chemical analysis of the oil extracted from the nut and the processed results of multivariate statistical analysis, i.e., Principal Components Analysis (PCA) and Ascending Hierarchical Clustering (AHC) provides a more objective basis for this varietal delimitation. The first principal plane F1F2 in the PCA constructed with four variables (total oil and C14:0, C12:0 and C16:0 contents) gave a very clear-cut separation of the two species. Even within one species the oils obtained by different extraction processes presented a certain dispersion, reflecting some variability in their fatty acid composition. However, this variability was slight compared with the differences between the two species. AHC performed on the same variables gave two groups that coincided exactly with the two species. However, from a more practical standpoint, the simple criterion we recommend is the ratio (%C14:0)/(%C12:0). This value is about 1 for *I. wombulu* (0.8-0.9) and appreciably higher than 1 for *I. gabonensis* (1.4-2.8). The chemical composition of the unsaponifiable fraction allowed this varietal delimitation to be further refined.

Key words: *Irvingia gabonensis*, *Irvingia wombulu*, oil content, fatty acids, triacylglycerols, unsaponifiable, multivariate statistics, Congo basin

INTRODUCTION

It was long thought that *Irvingia gabonensis* had two varieties, one sweet, the other bitter. Since the last botanical revision of the Irvingiaceae by Harris (1996), these two varieties have been considered as distinct species. The sweet variety has kept the name *I. gabonensis* while the bitter variety is now called *I. wombulu* (Leakey *et al.*, 2000). Although, the chemical composition of the fat extracted from the nut of *I. gabonensis* has been addressed, albeit sparsely (Joseph, 1995; Silou *et al.*, 2004; Matos *et al.*, 2009), no such specific research on *I. wombulu* has to the knowledge been reported. We sought information to document this interspecific delimitation which to date has been based solely on morphological data (shape and size of the nut) and organoleptic considerations (sweet or bitter taste of the pulp).

MATERIALS AND METHODS

Fat extraction

Soxhlet method: The dried and crushed kernels were introduced into a Soxhlet extractor. After 5 h extraction with hexane as solvent, the extract was dried with sodium sulphate. The solvent was evaporated in a rotary vacuum evaporator and the solvent traces were eliminated by drying oil in an oven at 103°C for 6 h.

Bligh and dyer method (1959): Dried and crushed kernels were homogenised with chloroform. The addition of the mixture of methanol/water (1:1, v/v) lead to two phases: aqueous phase (methanol-water) and organic phase (chloroform).

Fat was recovered from the organic phase by evaporating off the solvent and residual solvent was removed by drying in an oven at 103°C for 6 h.

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Folch method (Folch *et al.*, 1957): Dried and crushed kernels were homogenised with Chloroform/methanol (2:1, v/v). The addition of NaCl solution (20%) lead to two phases. Fat was recovered from the organic phase by evaporating off the solvent and residual solvent was removed by drying in an oven at 103°C for 6 h.

Determination of fatty acid composition: After the trans esterification of fatty acids by sodium methylate 1 N in methanol at ambient temperature during 2 min and under agitation, the methyl ester fat were analysed on a Hewlett-Packard gas chromatograph model 5890, equipped with a FAME column (100 m×0.25 mm, 0.25 µm), programming from 140°C (5 min)-240°C at 4°C min⁻¹, 15 min hold.

Hydrogen as carrier gas (0.7 mL min⁻¹); injection in split mode (1:40); injector and detector temperature, 280 and 300°C, respectively. FAMES were identified by comparison of retention times with authentic standards and quantification was performed by internal normalisation.

Determination of TAG profiles: Triacylglycerol (TAG) profile was obtained by High Performance Liquid Chromatography (Heron *et al.*, 2007). The chromatographic system consisted of a model LC-6A Pump (Shimadzu, Kyoto, Japan) Rheodyne loop a model 7125 injector valve with a 10 µL loop (Rheodyne, Cotati, CA, the United States) and a model Sedex 75 Light-scattering detector (Sedere, Alfortville, France) The nebulizing gas was air set 3,5 bar, the evaporation temperature was set 37°C and the gain (PM) at 11. The column temperature was controlled using an igloo-cil column oven (Cluzeau, Sainte-Foy-la-Grande, France) and set at 30°C evaporative detector with diffusion of light Sedere Sedex 75).

TAG were separated at 20°C using a Kromacil C18 (5 µm), 250×4.6 mm column (Part Number KR5C18-25QS mfg # 806610) and was eluted from the column with a mixture of acetonitrile/dichlorom ethane (70/30-40/60). Chromatograms were recorded using an Azur (v 3.0) data acquisition software (Datalys, Saint Martin d'Herès, France).

Determination of unsaponifiable fraction composition (norme NF T 60-205-2): The unsaponifiable fraction of the fat was analysed on a Agilent gas chromatograph Model 7890, coupled to a Agilent MS model 5975C, equipped with a DB5 MS column (20 m×0.18 mm, 0.18 µm) programming from 50°C (3.2 min)-320°C at 8°C min⁻¹, 3 min hold. Helium as carrier gas (1.0 mL min⁻¹); injection split mode (1:150); injector and

detector temperature, 320 and 280°C, respectively. The MS working in electron impact mode at 70 eV; ion source temperature, 230°C; mass spectra data were acquired in the scan mode in m/z range 33-450.

Thermal behaviour: The thermal property of the fat samples was investigated by differential scanning calorimetry using a Perkin-Elmer Diamond DSC (Norwalk, USA). The instrument was calibrated using indium and zinc. The purge gas used was 99.99% nitrogen with a flow rate of 100 mL min⁻¹ and a pressure of 20 psi. Sample weights ranged from 5-7 mg and were subjected to the following temperature program: Frozen oil sample was heated at 50°C in an oven until completely melted. Fat sample was placed in an aluminium volatile pan and was cooled to -50°C and held for 2 min, it was then heated from -50 to 50°C at the rate of 5°C min⁻¹ (normal rate) (Che Man and Swe, 1995) and held -50°C isothermally for 2 min and cooled from 50-50°C at the rate of 5°C min⁻¹. The heating and cooling thermograms for the normal and the fast (hyper DSC) scan rates were recorded and the peak and offset temperatures were tabulated. These values provide information on the temperature at which the melting process starts, the temperature at which most of the TAG have melted and the complete melting temperature of the oil, respectively.

Statistics: Statistics were processed using the XLSTAT 2000.6 software which is a Microsoft Excel add-in (www.xlstat.com).

RESULTS AND DISCUSSION

Fat content and fatty acid composition: Three methods of extraction of fat using organic solvents was used: the standard Soxhlet method (SXL), the method of Bligh and Dyer (BD) and the method of Folch simplified (FL) to determine the fat contents of the two species with a view to seeking similarities and differences to help achieve a better delimitation of these species (Table 1).

With the Soxhlet method, 16 samples of *I. gabonensis* (hereafter Ig) and 3 of *I. wombulu* (hereafter Iw) were extracted with hexane. The values of fat content obtained indicated a high degree of homogeneity of the plant matter used: the standard deviations for the three extraction runs for each sample were extremely low (0.05-0.80) for oil contents of generally >50%.

The extraction of fat from a given sample is thus a perfectly reproducible operation: the oil is distributed evenly throughout the powder obtained by milling the nut. The fat content of 16 samples of Ig ranged from 56-69% for Ig against about 68% for Iw. The Iw nuts

Table 1: Fat content and Fatty acid composition of Ig and Iw fats extracted by different methods

Samples	Fat content (%)	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	R*
Soxhlet (SXL)									
Ig 1	58.67 (0.05)	1.08	36.09	49.63	5.14	0.67	1.69	0.56	1.4
Ig 2	59.79 (0.31)	-	27.56	45.16	5.43	0.76	1.44	-	1.6
Ig 3	57.45 (0.52)	0.61	29.25	54.49	5.78	0.67	0.91	-	1.9
Ig 5	57.41 (0.45)	0.51	31.69	59.12	6.07	0.84	1.38	0.38	1.9
Ig 6	57.38 (0.50)	-	23.32	65.65	7.91	0.92	2.20	-	2.8
Ig 7	56.33 (0.27)	0.57	31.57	58.97	5.90	0.71	1.70	0.57	1.9
Ig 8	56.53 (0.35)	0.82	35.84	56.73	5.34	0.65	0.62	-	1.6
Ig 9	57.11 (0.18)	0.57	29.42	61.19	6.62	0.96	-	-	2.1
Ig 10	56.87 (0.40)	0.85	36.43	55.12	5.39	0.95	1.26	-	1.5
Ig 11	57.31 (0.75)	0.63	32.71	59.35	6.05	0.68	0.59	-	1.8
Ig 12	67.91 (0.82)	0.77	34.81	56.73	5.69	0.75	1.02	0.44	1.6
Ig 13	67.70 (0.22)	1.02	36.28	54.39	5.53	0.68	1.46	-	1.5
Ig 14	67.93 (0.12)	1.06	36.40	53.59	5.66	0.79	1.95	0.54	1.5
Ig 15	67.81 (0.05)	1.03	37.09	54.06	5.43	0.67	1.37	0.34	1.5
Ig 16	66.74 (0.23)	1.18	39.34	51.96	5.01	0.65	1.40	-	1.3
Iw 17	67.96 (0.33)	2.05	48.54	43.43	3.37	0.59	1.71	0.31	0.9
Iw 18	68.38 (0.21)	2.37	49.84	41.52	3.00	0.66	2.17	0.44	0.8
Iw 19	68.69 (0.19)	2.38	50.00	41.49	2.97	0.65	2.16	0.34	0.8
Bligh and Dyer (BD)									
Ig 12	58.02 (0.49)	0.87	35.80	55.70	5.54	0.69	1.38	-	1.6
Ig 13	57.27 (0.28)	0.84	35.53	56.53	5.62	0.68	1.19	-	1.6
Ig 14	58.70 (0.31)	1.04	36.44	53.95	5.72	0.79	2.03	-	1.5
Ig 15	57.48 (0.40)	0.99	37.55	54.09	5.36	0.66	1.33	-	1.4
Iw 17	58.70 (0.05)	2.01	48.99	43.57	3.28	0.56	1.56	-	0.9
Iw 18	62.07 (0.16)	2.60	51.55	40.65	2.91	2.28	-	-	0.8
Iw 19	62.95 (0.08)	2.34	50.68	41.70	2.85	0.59	1.82	-	0.8
Folch (FL)									
Ig 12	61.23 (0.53)	0.78	34.73	56.71	5.77	0.73	1.26	-	1.6
Ig 13	60.25 (0.33)	0.86	35.80	55.65	5.52	0.67	1.22	0.24	1.6
Ig 14	61.61 (0.18)	0.95	35.02	54.51	5.93	0.84	2.14	0.59	1.6
Ig 15	60.08 (0.24)	1.03	38.19	53.54	5.26	0.63	1.32	-	1.4
Iw 17	64.59 (0.50)	2.07	49.11	43.33	3.29	0.57	1.61	-	0.9
Iw 18	65.02 (0.30)	2.18	48.71	42.61	3.24	0.73	2.52	-	0.9
Iw 19	65.36 (0.10)	2.42	50.85	41.23	2.85	0.61	2.03	-	0.8

*R = [%C14:0]/[%C12:0]

studied were also slightly more oil-rich than those of Ig. This difference was confirmed by the other two extraction methods: The fat extracted was made up essentially of two saturated fatty acids: lauric acid (C12:0) and myristic acid (C14:0).

Palmitic acid (C16:0) came third with contents ranging between 3 and 8% followed by oleic acid (C18:1, 1-2%). Capric acid (C10:0), stearic acid (C18:0) and linolenic acid (C18:2) were present at levels <1%.

Hexenoic acid (C6:1) was found in trace amounts (0.00-0.03%) in some samples. Going from Ig to Iw, the order of the first two major constituents was reversed: C14:0 changed places with C12:0. The following two profiles were thus identified:

- For Ig: %C14:0>%C12:0>%C16:0
- For Iw: %C12:0>%C14:0>%C16:0

These two profiles were the same whichever extraction method was used: The calculation of R = [(%C14:0)/(%C12:0)] gave values ranging from 1.4-2.8 for Ig and from 0.8-0.9 for Iw.

This ratio can be taken as an indicator to differentiate between the two species: Principal Components Analysis

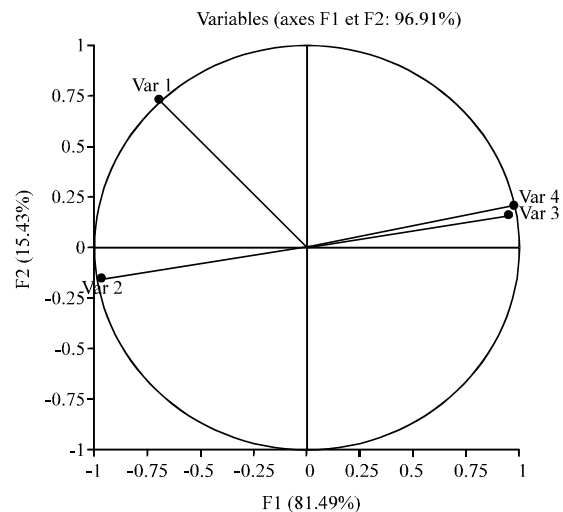


Fig. 1: Correlation by ACP of 4 variables (%fat; %C2:0, %C14:0, %C16:0 of Ig and Iw fats) for the first two principal components F1F2

(PCA) constructed with four variables (total fat content and percentages of C12:0, C14:0 and C16:0) gave the correlation circle shown in Fig. 1. Researchers see that the

Table 2: Correlation matrix by ACP of 4 variables (%fat; %C2:0; %C14:0; %C16:0 of Ig and Iw fats) for the first two principal components F1F2

Variables	Var1	Var2	Var3	Var4
Var1	1.000	0.552	-0.544	-0.519
Var2	0.552	1.000	-0.887	-0.968
Var3	-0.544	-0.887	1.000	0.957
Var4	-0.519	-0.968	0.957	1.000

Var1 = %fat. Var2: %C14:0; Var3 = %C12:0; Var4 = %C16:0

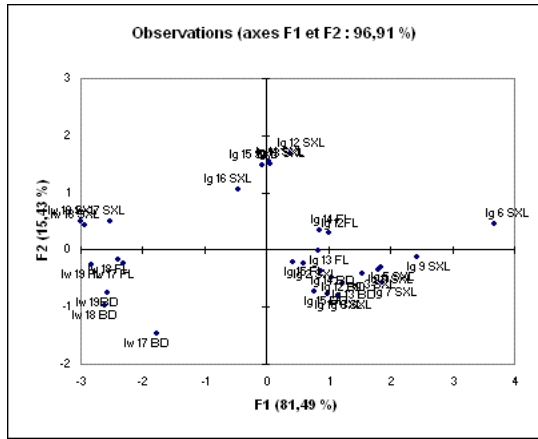


Fig. 2: Representation by PCA of individuals (Ig and Iw fats) in the first principal plane F1F2

first principal plane contains almost all the information on the variability (96.91%) and that all the variables are correctly represented. Axis F1 contains all the information on the fatty acid composition and the information on the oil content is distributed on the two axes F1 and F2. The C14:0 and C16:0 contents are correlated and are anticorrelated with that of C12:0 (Table 2).

The composition seems to vary independently of the oil content: The representation of individuals on the F1F2 plane shows a clear separation of the two species with Iw to the left of F2 and Ig to the right (Fig. 2).

The more homogeneous samples of Iw occupy a smaller surface area irrespective of the extraction method used but BD and FL are closer together and shift away from SXL.

The samples of Ig are a little more dispersed but here again BD and FL are closer together and form a single block distant from SXL. Ascending Hierarchical Clustering (AHC) confirms the distinction between the two new species of *Irvingia* (Table 3 and Fig. 3) and provide a physical and chemical justification for the separation due to Harris (1996).

Triacylglycerol (TAG) profiles of the oils studied: The study of 16 fat samples extracted by different methods showed the stability of the TAG profiles characteristic of each of the two species (Fig. 4 and 5). Researchers found

Table 3: Data of the distribution of individuals (Ig and Iw fats extracted by different solvents) by Ascending Hierarchical Clustering (AHC)

Class 1 (n = 22)	Class 2 (n = 9)
Ig 2 SXL	Iw 17 SXL
Ig 3 SXL	Iw 18 SXL
Ig 5 SXL	Iw 19 SXL
Ig 6 SXL	Iw 17 BD
Ig 7 SXL	Iw 18 BD
Ig 8 SXL	Iw 19 BD
Ig 9 SXL	Iw 17 FL
Ig 10 SXL	Iw 18 FL
Ig 11 SXL	Iw 19 FL
Ig 12 SXL	-
Ig 13 SXL	-
Ig 14 SXL	-
Ig 15 SXL	-
Ig 16 SXL	-
Ig 12 BD	-
Ig 13 BD	-
Ig 14 BD	-
Ig 15 BD	-
Ig 12 FL	-
Ig 13 FL	-
Ig 14 FL	-
Ig 15 FL	-

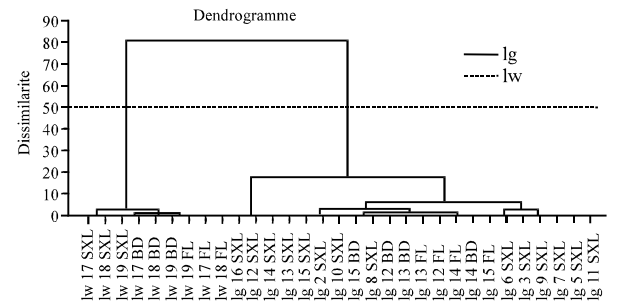


Fig. 3: Distribution of individuals (Ig and Iw fats extracted by different methods) by Ascending Hierarchical Clustering (AHC)

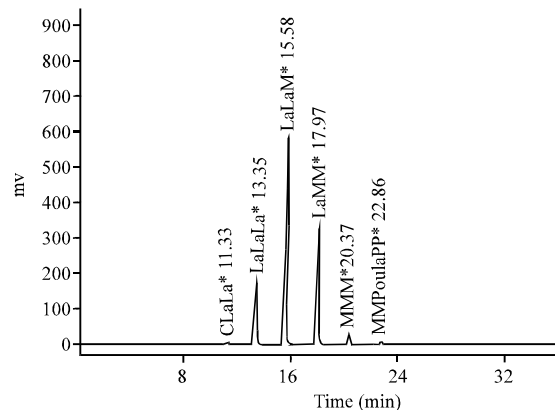


Fig. 4: Triacylglycerol profile of *Irvingia gabonensis* fat (HPLC)

that LaMM, the main TAG of Ig was largely predominant; it was followed by LaLaM which was closely followed by MMM. LaLaLa came fourth, in only trace amounts. The following typical profile was thus found:

% LaMM>% LaLaM>% MMM>%LaLaLa

In the oil of Iw, C12:0 replaced C14:0 as the predominant constituent. This reversal was reflected in a twofold reversal of the TAG pattern, one in the mixed TAGs LaMM and LaLaM and the other in the simple TAGs LaLaLa and MMM. The following profile was found:

%LaLaM>%LaMM>% LaLaLa>%MMM

Composition of the unsaponifiable fraction: In an exploratory study we sought compounds that could discriminate between the two species in the unsaponifiable fraction of the fats. The Table 4 and Fig. 6 shows the results obtained for the fats of Ig from Congo, Cameroon, Gabon and Nigeria compared with those of three samples of oil from Congolese Iw. Nearly half of the substances identified occurred in both species at widely different levels namely squalene, beta-sitosterol, stigmasterol, delta-5-ergosterol, bis(p-octylphenyl)amine and 2,2'-methylene-bis 6-(1,1-dimethylethyl)-4-methylphenol.

While two sterols made up the two prime major constituents of the unsaponifiable fraction of Ig namely beta-sitosterol (32-36%) and stigmasterol(1-18%), these were present in Iw in lower amounts, respectively 9-13 and 2-4%. The most abundant constituent of Iw which is still being identified, accounted for about 20% of the oil, followed by two other substances that were absent in the Ig oil namely isobutyl phthalate and stigmasta-3,5-dien-7-one with levels in the range 7-10%. We can conclude that the fat of Ig characteristically contains much beta-sitosterol and stigmasterol and no isobutyl phthalate or stigmasta-3,5-dien-7-one. Sample 5, presented as Iw, proved closer to Ig than to Iw by the chemical composition of its non-saponifiable fraction. Determination of chemical composition may thus correct errors in the identification of plant material.

Thermograms of fats obtained by differential scanning calorimetry:

The fats obtained from the two species studies were solid at ordinary temperature. Researchers used Differential Scanning Calorimetry (DSC) to seek indicators that could distinguish between them. The thermograms obtained are shown in Fig. 6 and the corresponding parameter values are shown in Table 5.

These assignments were made on the basis of the diagram proposed by Berger and Akehurst (1996) drawn up after crossing the results of TLC and GC fractioning and the DSC of the identified fractions and the total oils of palm, cottonseed and soy. It assigns the melting point range 20-50°C to the TAGs of unsaturation 000 and the range -20-20°C to TAGs of unsaturation 100.

Close inspection of the data in the table reveals that the oil extracted from Ig by BD method which probably contained less minor constituents, presents only the peak that appears at about 40°C and which corresponds to the highest enthalpy (>100) whereas the two largely predominant saturated TAGs did not change significantly from one method to another. This peak is therefore, the

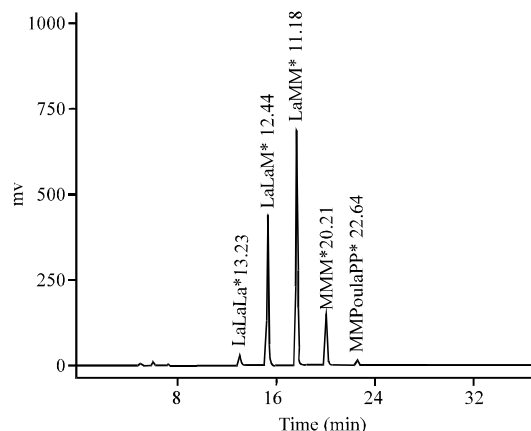


Fig. 5: Triacylglycerol profile of *Irvingia wombulu* fat (HPLC)

Table 4: Composition of the unsaponifiable fraction of Ig and Iw fats

Composition	Sample 1 Ig (Gabon1)	Sample 2 Ig (Congo)	Sample 3 IG (Cameroun)	Sample 4 Ig (Nigeria)	Sample 5 Iw (Congo1)	Sample 6 Iw (Congo2)	Sample 7 Iw (Congo3)
Squalene	2.46	9.85	7.45	12.74	6.61	1.05	0.82
Beta-sitosterol	32.62	36.26	35.82	35.02	34.66	9.98	12.74
Stigmasterol	16.73	17.62	18.13	16.08	14.03	2.78	3.92
γ-tocopherol	-	-	2.05	2.11	-	-	-
Δ5 Ergosterol	5.90	8.53	9.48	7.71	5.30	1.38	1.77
Fucosterol	-	-	6.01	5.75	3.07	-	-
Cycloartenol	-	-	2.46	1.87	1.95	-	-
cyclolaudenol	-	-	1.65	2.12	2.44	-	-
Isobutyl phtalate	-	-	-	-	-	9.23	10.53
A	-	-	-	-	-	7.61	9.04
B	2.07	0.44	0.52	0.45	0.74	1.67	1.49
C	4.45	4.93	2.02	-	-	0.96	1.09
D (NI)	-	-	-	-	-	23.48	22.68

A: stigmasta-3,5-dien-7-one; B: bis (p-octylphenyl) amine; C: Phenol.2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl]; D (NI: Non Identifie)

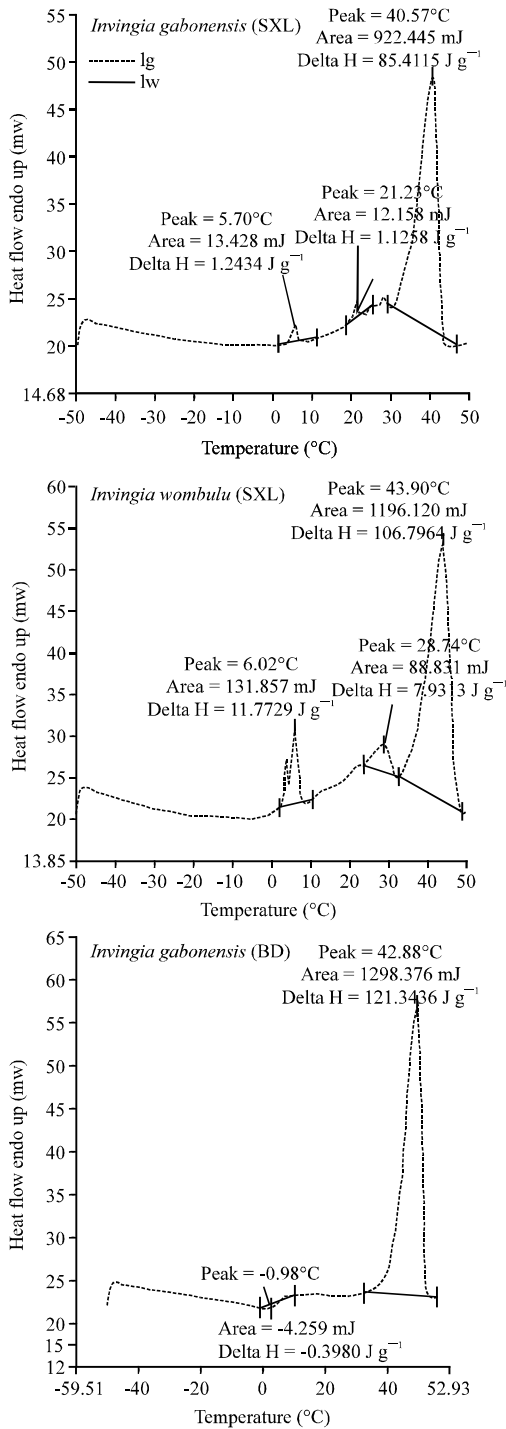


Fig. 6: Thermograms of Ig et Iw fats recorded by Differential Scanning Calorimetry (DSC)

one that corresponds to the melting of the two largely predominant mixed TAGs LaMM and LaLaM By difference, we assigned the second peak between 2 and 30°C to the other remaining saturated constituents, the simple TAGs LaLaLA and MMM.

Table 5: Data of thermal behaviour of Ig and Iw fats by Differential Scanning Calorimetry (DSC)

Parameters	Ig (SXL)	Iw (SXL)	Ig (BD)	Attribution	
				Insaturation	TAG
T ₁ (°C)	5.70	6.02	- 0.98	100	-
ΔH ₁	1.24	11.77	- 0.40		
T ₂ (°C)	21.23	28.74	-	000	LaLaLa/MMM
ΔH ₂	1.13	7.93	-		
T ₃ (°C)	40.57	43.90	42.88		
ΔH ₃	85.41	106.80	121.34	000	LaMM/LaLaM

CONCLUSION

The results of the chemical analysis of the fat extracted from the kernels of Ig and Iw subjected to multivariate statistical analysis (PCA and AHC) supports the separation of the two former varieties of Ig into the two clearly distinct species Ig and Iw.

Their fat content is similar; Iw contains slightly more than Ig: PCA constructed with four variables (oil content and content of C14:0, C12:0 and C16:0) showed two totally separate species in the first principal plane F1F2. This result was confirmed by AHC. However, we found the ratio R = [(%C14:0)/(%C12:0)] to be a sure discriminating indicator for these two species. Its value is about 1 for Iw (0.8-0.9) but generally much >1 for Ig (1.4-2.8).

In addition, in preliminary research, marked differences in composition of the non-saponifiable fractions of the oils from the two species were evidenced. The fat of Ig was 3 and 6 times richer in the common sterols beta-sitosterol and stigmasterol than that of Iw while the oil of Iw contained appreciable amounts of isobutyl phthalate and stigmasta-3,5-dien-7-one which were not found in the oil of Ig. The most abundant constituent of the oil from Iw, accounting for >20% of the total, is still being identified. Hence, the study of the chemical composition of both the glyceride fraction and the unsaponifiable fraction of oils can help to delimit different species of Irvingiaceae.

ACKNOWLEDGEMENTS

This research was carried out as part of the RAMSES EDULINK project: researchers thank the European Union, Blaise Pascal University (Clermont-Ferrand, France), University of Orsay (LETIAM, IUT, Paris, France) and Marien Ngouabi University (Brazzaville, Congo).

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