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Fatty Acids Profile of *Alphitonia neocaledonica* and *Grevillea exul* var. *rubiginosa* Seed Oils, Occurrence of an ω5 Series

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Abstract This is the first report of the chemical composition of *Alphitonia neocaledonica* (AN) and *Grevillea exul* var. *rubiginosa* (GER) seed oils. Using retention indices and gas chromatography–mass spectrometry, an unusual family of unsaturated ω5-fatty acids has been identified. These include 14:1, 16:1, 18:1 and 20:1. Identification of the unsaturated fatty acids was confirmed by formation of DMOX derivatives which gave characteristic and easily interpreted mass spectra. DMDS adducts were used to identify the positions of double bonds in the monounsaturated fatty acids. The major fatty acids were 16:1ω5 (45.6%) and 18:1ω9 (20.9%) for GER and 18:2 (23.6%) and 18:3 (20.4%) for AN. The total ω5-monoenes were 63.4 and 21.5% for GER and AN, respectively. The seed oils of AN and GER can be considered as a good source of ω5-monoenes, especially for GER. The occurrence of the ω5-monoenes in *Alphitonia neocaledonica* can at present be considered as an exception within the Rhamnaceae family. Except for *Ziziphus jujuba* var. *inermis*, no species of this family has been described with a broad profile of ω5-monoene fatty acids.

Keywords *Alphitonia neocaledonica* · *Grevillea exul* var. *rubiginosa* · Seed · New Caledonia · ω5-Monoene fatty acid · GC–MS

Introduction

New Caledonia is well known for its high biodiversity with a global endemicity of 74.3%. This high rate of endemicity is due to several factors: the late separation of New Caledonia from Australia, the climatic variability due to the island's topography and the multiplicity of geological substrates with one-third of the total area covered by ultramafic rocks [1]. Erosion and alteration of these rocks have led to the enrichment of the soil with heavy metals and its impoverishment in essential nutrients. This has led to the development of a unique flora which can reach up to 90% endemicity on this type of soil. Two New Caledonian endemic plant species, that grow on this ultramafic soil, were investigated in order to characterise their seeds. The two species, *Alphitonia neocaledonica* (Schltr.) Guillaumin (AN) and *Grevillea exul* var. *rubiginosa* (Brongn. & Gris) Virot (GER), which belong to the Rhamnaceae and Proteaceae families, respectively, are of interest because of their use in revegetation of serpentine soils exploited by nickel mining.

The oil i.e., neutral lipid fraction and water content of the seeds has been investigated. Fatty acids composition was determinated using gas chromatography and gas chromatography coupled with mass spectrometry.

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Experimental Procedures

Raw Materials

As many seeds as possible were collected from different mother plants (about 10 plants) localised in a restricted area of 50 × 50 m i.e., one population in the south of New Caledonia during December 2005 and at January 2004 for *Alphitonia neocaledonica* and *Grevillea exul* var. *rubiginosa*, respectively. However, sufficient seed was only available to give a sample of each species for analysis.

Oil and Moisture Content

For the oil i.e., neutral lipid fraction extraction, one seed lot was dried and ground into fined powder and 10 g were extracted with *n*-hexane in a Soxhlet apparatus. Moisture content was determined by drying 4 × 100 mg of seeds using the low temperature constant oven method of 103 °C for 17 h [2]. The seed moisture content was used to convert the oil and percentage of individual fatty acids present to a dry weight basis.

Preparation of Fatty Acid Methyl Ester (FAME) Derivatives [3]

Fatty acid methyl esters were prepared by refluxing 1 g of oil with 10 mL sodium methanolate (4 g sodium in 100 mL methanol), for 1 h. After addition of 10 mL water, FAME derivatives were extracted twice with 10 mL *n*-pentane. The organic layer was washed with water then dried over magnesium sulphate, filtered and concentrated using a rotary evaporator. 449.3 and 235.7 mg of FAME derivatives were obtained for AN and GER, respectively.

Preparation of Dimethyl disulfide (DMDS) Derivatives from FAMEs of AN [3]

FAMEs (104 mg) dissolved in 5 mL heptane left to react with 5 mL DMDS and 1 mL iodine solution (1.2 mg diiodine in 20 µL diethylether) for 24 h at room temperature. The mixture was then diluted with 10 mL heptane and the iodine removed by shaking with aqueous sodium thiosulphate (5%). A quantity of 67.5 mg of DMDS derivatives were obtained from *Alphitonia neocaledonica* FAMEs.

Preparation of the 4,4-Dimethyl oxazoline (DMOX) Derivatives from AN Free Fatty Acids [3]

Free fatty acids (FA) were obtained from neutral lipid fraction (274.4 mg) by saponification with 100 mL of an ethanolic solution of potassium hydroxide (2 N). After refluxing for 1 h, 100 mL water was added. The aqueous phase was extracted with 3 × 40 mL pentane. Organic layers, which contained the unsaponifiable matter, were washed with water then dried over magnesium sulphate. 20.1 mg of unsaponifiable material were obtained. The aqueous phase was then acidified with HCl 2 N and extracted with 3 × 50 mL chloroform to give 199.1 mg FA.

50 mg of *Alphitonia neocaledonica* FA in 1 mL methylene chloride were added to 60 mg of dicyclohexylcarbodiimide in 1 mL methylene chloride and stirred for 10 min with magnetic stirrer. Then 60 mg of 2-amino-2-methyl-1-propanol was added. The mixture was stirred for 4 h at room temperature. The excess reactant was then evaporated. Diethylether and 500 µL $SOCl_2$ were added over 30 min at 0 °C with the solution being continuously stirred. The solution was then stirred for another 30 min at room temperature. Then, 7 mL of water was added and the solution was then washed with sodium carbonate. Finally 11.9 mg of DMOX derivatives was obtained.

Gas Chromatography (GC)

GC analyses were performed for both species using a Varian 3800 gas chromatograph fitted with a MEGA 10 capillary column (30 m, 0.32 mm i.d.; 0.25 µm film thickness), with a flame ionisation detector. Samples were injected into an oven at a temperature initially set at 150 °C but raised at 2 °C min^{-1} to 220 °C. Detector and inlet temperatures were 240 and 230 °C, respectively. Helium was used as the carrier gas at an inner pressure of 0.4 Bar. For a complete separation of the 18:1ω9 and 18:1ω7 and 18:3ω3 and 20:1ω9 a DB-23 capillary column was also used (30 m, 0.25 mm i.d.; 0.25 µm film thickness), with a flame ionisation detector. Samples were injected into an oven temperature initially set at 50 °C but raised at 10 °C min^{-1} to 180 °C, stay at this temperature until 5 min before raising at 3 °C min^{-1} to 240 °C then kept at this temperature for 10 min giving a total of 48 min for the run. Detector and inlet temperatures were 255 and 250 °C, respectively. Hydrogen was used as the carrier gas at an inner pressure of 0.53 Bar.

The fatty acid composition was expressed as the relative proportion of all the integrated FAMEs.

GC–Mass Spectrometry (GC–MS)

GC–MS analyses were carried out using a HP 5890 gas chromatograph equipped with a DB-5MS capillary column [60 m × 0.25 mm × 0.25 µm (J&W Scientific Inc.)] coupled to a Quadripolar HP 5989 A spectrometer operating in electron ionisation mode (EI 70 eV), with a transfer line at 295 °C and source temperature of 240 °C.

Samples were injected into methylene chloride. The oven temperature program was as follows: 1 min at 30 °C, from 30 to 70 °C at 50 °C min^{-1}, then from 70 to 120 °C at 10 °C min^{-1}, 120 to 290 °C at 2 °C min^{-1}, then 40 min at 290 °C. The inlet temperature was 295 °C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}, 17.8 psi.

FTIR Analysis

Spectra were recorded from 4,000 to 650 cm^{-1}, with 4 cm^{-1} resolution and 100 scans on a "Nicolet Avatar" spectrometer, equipped with a DGTS detector, an Ever-Glo source and a KBr/Germanium beam splitter. Samples were deposited, without preparation. Air was taken as reference for the background spectrum.

Results and Discussion

Moisture contents have been evaluated at 4.02 and 7.26% for *Alphitonia neocaledonica* (AN) and *Grevillea exul* var. *rubiginosa* (GER), respectively. Seed storage reserves of both species were found to be high in oil, i.e., a neutral lipid fraction of 19.25 and 25.60% of the dry matter of the seed for AN and GER, respectively (Table 1) and can be considered as oily seeds [4–10]. AN seed oil content (19.25%) is higher than that observed in the Australian native *A. excelsa* (11.8%) [4]; but was within the range reported for other Rhamnaceae [5, 6]: *Berchemia discolor* (11%); *Rhamnus frangula* (41.9%); *Ziziphus mauritiana* (29.3%). Similarly the GER seed oil content, at 25.6%, is within the range reported in the literature for other Proteaceae [5, 7–9]: *Stirlingia sinuatus* (9.7%); *Grevillea robusta* (13.8%); *G. leucopteris* (31%); *Gevuina avellana* (46%).

Fatty acid identifications were done first using retention indices and co-elution with authentic samples (Supelco 37 Component FAME Mix). For GER in particular, identification was also made by comparison with AN profile. The infra red spectra of the two seed oils were examined in order to detect the presence of *trans*-acids or free fatty acids and no absorption was detected. Considering *Alphitonia neocaledonica*, in order to confirm the presence of unsaturated fatty acid isomers, Dimethyl oxazoline (DMOX) and Dimethyl disulfide (DMDS) derivatives [11] were prepared and analysed by GC–MS. DMDS and DMOX characteristic fragments used for the identification of the monoene and polyene positions are presented in Table 2. The ω5-monoenes were characterised from the mass spectrum of the fatty acid DMDS adducts that gave the expected ω fragment at *m/z* 117 (145 and 173 for ω7- and ω9-monoene, respectively). The ester group also lost MeOH to provide a prominent secondary fragment ion (δ-MeOH). There is a high percentage of unsaturated fatty acids in both species (AN: 79.9%; GER: 87.1%) leading to a relatively low saturated to unsaturated ratio (AN: 0.20; GER: 0.07) (Table 3).

In the case of AN, the ratio appears to be in accordance with the others species of Rhamnaceae but for GER, the ratio value is one of the smallest with *Gevuina avellana* (0.07%) actually found in the Proteaceae family (Table 4). For AN, the fatty acids most represented are linoleic (LA) (23.6%), α-linolenic (ALA) (20.4%) and oleic acids (12.7%). The high content in essential fatty acids confers a potential nutritional value to this oil especially since the relative proportion of unsaturated fatty acids is high (79.9%) and the two major fatty acids of *Alphitonia neocaledonica* oil are the precursors of the omega 3 and omega 6 fatty acid family, respectively. According to Dubois et al. [12], AN oil belongs to the PUFA class

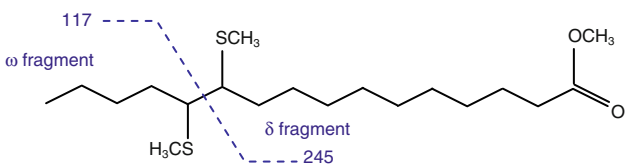
Table 1 Oil and water content of *Alphitonia neocaledonica* (AN) and *Grevillea exul* var. *rubiginosa* (GER) seeds

Species	Oil[a]	Water[a]
AN	19.25	4.02 ± 0.2
GER	25.60	7.26 ± 0.8

[a] Percentage from dry material

Table 2 Characteristic fragments of the *Alphitonia neocaledonica* (AN) DMDS and DMOX derivatives

Fatty acid	DMDS				DMOX
	MW	δ	δ-MeOH	ω	
14:1ω5	334	217	185	**117**	
16:1ω7	362	217	185	145	
16:1ω5	362	245	213	**117**	307, 236
18:1ω9	390	217	185	173	335, 208
18:1ω7	390	245	213	145	
18:1ω5	390	273	241	**117**	335, 264
20:1ω11	418	217	185	201	
20:1ω9	418	245	213	173	
20:1ω5	418	301	269	**117**	
18:2ω6					333, 208, 248
18:3ω3					331, 208, 248, 288



Structure of the 16:1 ω5 DMDS derivative

Table 3 Fatty acid composition of *Alphitonia neocaledonica* (AN) and *Grevillea exul* var. *rubiginosa* (GER) seed oil[a]

ECL[b]	Family *Genus and species*	Rhamnaceae *Alphitonia neocaledonica*	Proteaceae *Grevillea exul* var. *rubiginosa*
Saturated fatty acid			
14	14:0	0.07	Tr
16	16:0	10.00	1.37
18	18:0	4.21	0.75
20	20:0	0.51	0.59
22	22:0	0.65	1.35
24	24:0	0.36	1.75
Unsaturated fatty acid			
14.79	14:1Δ9 (ω5)	0.16	8.50
16.57	16:1Δ9	0.25	Tr
16.76	16:1Δ11 (ω5)	10.65	45.58
18.52	18:1Δ9	12.66[c]	20.94
18.54	18:1Δ11	1.23[c]	–
18.75	18:1Δ13 (ω5)	10.49	9.31
–	20:1Δ9	ni[d]	–
20.47	20:1Δ11	0.21[c]	Tr
20.72	20:1Δ15 (ω5)	0.24	–
19.37	18:2Δ9,12	23.62	2.11
20.47	18:3Δ9,12,15	20.38	0.68
Total			
Saturated		15.80	5.81
Unsaturated		79.89	87.12
ω5		21.54	63.39

[a] Relative GC percentage obtained on MEGA10 capillary column

[b] Equivalent chain length on MEGA 10 column

[c] 18:1Δ9 and 18:1Δ11 are coeluted on the MEGA 10 column (total 13.83%) but the relative GC percentage can be obtained by considering the ratio 18:1w7/18:1w9 (9/91) on DB-23 capillary column

[d] Not integrated

Tr Traces

characterised by a major quantity of polyunsaturated fatty acid (44%) and to the LA + MUFA subclasses (Table 4). This classification is due to a large amount of linoleic acid (23.62%) and monounsaturated fatty acid (35.89%) in its seed oil. Moreover, AN oil appears to be an interesting nutritional source. Indeed, α-linolenic acid content (20.38%) in AN seed oil is higher than in soybean seed oil (7.8%), and ω-6/ω-3 ratio is close to 1, providing a good equilibrium between these two essential fatty acids. C16:1 was also present in a considerable quantity (10.9%) since its concentration rarely exceeds 10% in most seed oils and animal fats [13].

In the case of GER, the unusual C16:1ω5 predominates accounting for 45.58% of the total fatty acids, followed by oleic acid (20.94%) and the two others unusual monounsaturated fatty acids C18:1ω5 (9.31%) and C14:1ω5 (8.50%). The monounsaturated fatty acid amount of 84.33% classifies GER seed oil in the MUFA class and subclass [14]. From a nutritional point of view, GER oil could be interesting because of its ratio ω-6/ω-3 and because of its low amount of saturated fatty acids (5.8%) similar to *Gevuina avellana* seed oil. The high content of the unusual C16:1ω5 (45.58%) and the lack of information on the dietary effects of consuming such high concentrations of this fatty acid means there is a need for caution, however, *G. avellana* nuts (22–24% of C16:1ω5) have been eaten for a long time in South America [9].

It is also interesting to highlight the high quantity of ω5 fatty acids in both AN and GER (AN: 21.5%, GER: 63.4%), with a predominance of the C16:1ω5 representing 49.4 and 71.9% of the total ω5 monoenes, respectively (Table 3). The ω5 fatty acids rarely occur in plants. The broad spectrum of ω5 monoenes could be due to the presence in the seed of either a highly active ω5 desaturase [10] or an elongase responsible for a considerable chain elongation [13]. The unusual C16:1ω5 fatty acid has only been reported in some Proteaceae and Primulaceae [10, 13, 14]. The presence of ω5 fatty acids in Rhamnaceae has been only reported in the fruit pulp of *Ziziphus jujuba* var. *inermis* but never with this broad spectrum. Moreover, it has never been detected in Rhamnaceae seeds. The GER C16:1ω5 value (45.6%) seems to be the highest reported up till now, the nearest being *Kermadecia sinuata* (40.3%) [14]. This latter one is also endemic to New Caledonia and belongs to the same family, but grows in a different type of soil i.e., sedimentary substrate and different climatic conditions i.e., humid forest. It thus appears that the occurrence of this fatty acid is not related to the environment but

Table 4 Characteristics of some species seed oils belonging to Rhamnaceae and Proteaceae families

Species	Oil content (%)	Unsaturated (unsat) fatty acids (%)	Saturated (sat) fatty acids (%)	Sat/unsat ratio	MUFA[a]	PUFA[b]	ω-6/ω-3 ratio
Rhamnaceae							
Alphitonia neocaledonica	19.25	79.89	15.80	0.20	35.89	44	1.16
Alphitonia excelsa [3]	11.2	80.10	19.70	0.25	30.00	50.1	4.69
Ventilago calyculata [4]	40.0	81.20	18.70	0.23	63.10	18.1	0.33
Berchemia discolor [4]	11.0	70.00	30.00	0.43	52.00	18	17
Paliurus aculeatus [4]	–	82.40	16.60	0.20	39.40	43	85
Emenospermapancheranum [4]	13.2	85.40	14.50	0.17	23.40	62	8.84
Ziziphus mauritiana [4]	29.3	79.50	18.90	0.24	67.10	12.4	–
Proteaceae							
Grevillea exul var. *rubiginosa*	25.6	87.12	5.81	0.07	84.33	2.79	3.1
Grevillea decora [4]	–	78.90	8.70	0.11	77.60	1.30	5.5
Grevillea robusta [13]	13.3	84.70	14.30	0.17	83.40	1.30	3.3
Grevillea banksii [12]	–	86.80	13.20	0.15	84.20	2.60	–
Kermadecia sinuata [12]	40.3	84.20	15.80	0.18	81.60	2.60	–
Beauprea balansae [12]	–	66.70	33.30	0.50	39.50	27.2	–
Beauprea neglecta [12]	51.7	81.40	18.60	0.23	53.9	27.5	–
Gevuina avellana [8]	46.0	93.20	6.50	0.07	87.50	5.70	5.4
Stirlingia sinuatus [3]	9.7	87.00	12.40	0.14	85.20	1.80	8.0
Stirlingia simplex [3]	5.1	90.00	10.00	0.11	87.20	2.80	8.3
Stirlingia tenuifolia [3]	1.6	87.20	12.90	0.15	75.90	11.30	–

[a] Monounsaturated fatty acids

[b] Polyunsaturated fatty acids

is strictly due to a family feature. Indeed, Vickery [13] has identified its presence in numerous Proteaceae species including three endemic to New Caledonia, *K. sinuata*, *Beauprea balansae*, *B. neglecta*, the latter two growing on ultramafic soils.

Conclusion

To conclude, the patterns of occurrence and concentrations of the fatty acids contained in their seed oil suggest they could be a good source of ω5 monoenes, especially GER. The fatty acid profile of GER is in accordance with those reported by Vickery [13] for the genus *Grevillea*, i.e., the occurrence of long chain saturated fatty acids (C22:0, C24:0) and low amounts of palmitic and linoleic acid amounts. However, GER does not contain the C22:1 and C24:1 fatty acids found in other *Grevillea* species. *Alphitonia neocaledonica* can at the moment be considered to be an exception within the Rhamnaceae family. Our next objectives will be to compare these profiles with other New Caledonian *Grevillea* and *Alphitonia* species that inhabit the same dry environment and exclusively grow on ultramafic rocks [15]. Also, New Caledonian Proteaceae and Rhamnaceae species will be studied in relation to their environment (soil, climatic conditions…).

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