lipidology

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Lipid Profile of Fresh and Aged *Wollemia nobilis* Seeds: Omega-3 Epoxylipid in Older Stored Seeds

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Abstract: Wollemi pine, *Wollemia nobilis* W. G. Jones, K. D. Hill & J. M. Allan (*Araucariaceae*) was discovered in a remote canyon 150 km north-west of Sydney, Australia. As fewer than 100 adult trees of this plant survive in the wild, efforts to conserve this species have included seed storage. Fresh and stored seeds were analysed for yield and composition of the seed oil. The seed kernels, from both fresh and stored seed, were rich in oil with contents of 42% and 48%, respectively. The fatty acid profile of Wollemi pine seed oil was determined by GC-MS analyses of fatty acid methyl ester derivatives. Oleic acid makes up 32% of the fatty acid profile, while the major polyunsaturated fatty acid is linoleic acid (25%). Most of the detectable omega-3 fatty acid content of the oil is α-linolenic acid (3%). The seed oil has a high content of C20 to C24 fatty acids (25%) consisting of long-chain saturated fatty acids (19%). The polyunsaturated C20 omega-6 fatty acid content consists of eicosadienoic acid, dihomo-γ-linolenic acid, and arachidonic acid (total 4%). ^{1}H NMR analyses of the intact oil showed that the lipids were largely in the form of triglycerides with a degree of unsaturation of 1.5 double bond equivalents per fatty acid residue. In artificially aged or stored seeds, minor additional ^{1}H NMR spectral signals were attributed to an omega-3 epoxylipid, tentatively identified as *cis*-15,16-epoxy-9*Z*,12*Z*-octadecadienoic acid or ester derivative. Other minor signals were characteristic of a hydroxy or a hydroperoxy *E*,*Z* diene containing fatty acid. These products are typically formed by metabolic lipid oxidation of fatty acids. The content of the omega-3 epoxylipid, determined by the ^{1}H NMR method, varied with storage conditions and duration from less than 0.1% to a maximum of 3.3%.

Keywords: lipids; fatty acids; omega-3 epoxylipid; oxylipins; *Wollemia nobilis*; seed oil; stored seeds

Citation: Ng, M.C.H.; Tran, V.H.; Duke, R.K.; Offord, C.A.; Meagher, P.F.; Cui, P.H.; Duke, C.C. Lipid Profile of Fresh and Aged *Wollemia nobilis* Seeds: Omega-3 Epoxylipid in Older Stored Seeds. *Lipidology* **2024**, *1*, 92–104. https://doi.org/10.3390/lipidology1020007

Academic Editor: Nicola Ferri

Received: 17 July 2024
Revised: 25 August 2024
Accepted: 3 September 2024
Published: 25 September 2024

1. Introduction

Wollemi pine (*Wollemia nobilis* W. G. Jones, K. D. Hill & J. M. Allan) is a monotypic species of the gymnosperm family *Araucariaceae* [1]. Once thought to be extinct, this tall monoecious tree (Figure S1) was discovered in 1994 by David Noble in a remote part of Wollemi National Park in the Blue Mountains, about 150 km north-west of Sydney, New South Wales, Australia [1]. With a wild population of fewer than 100 mature trees, it is one of the most endangered tree species in the world. Comparison of the new living Wollemi Pine with the Australian Araucarian Cretaceous fossils indicated that the plant in this genus thrived in this period 60–70 million years ago [2]. Wollemi pine has a low genetic diversity [3], representing a living monotypic genus of the *Araucariaceae* family with two extant sister genera, *Agathis* and *Araucaria* [2,4].

Since its discovery, Wollemi pine has been subjected to extensive botanical investigation comparing anatomical and morphological features [2] as well as chemical composition [5–7] with other members of the *Araucariaceae* family. The chemical composition of Wollemi pine

epicuticular leaf waxes is n-alkanes (30.5%), secondary alcohols (49.9%), and alkane diols (9.2%) as well as a small amount of a mixture of fatty acids, primary alcohols, ketones, aldehydes, and alkyl esters [5]. A significant amount of nonacosan-10-ol was found in the epicuticular leaf wax of Wollemi pine [5]. In another study of the *Araucariaceae* family [7], analysis of steam-volatile leaf essential oil showed that the major constituent in Wollemi pine leaf is (+)16-kaurene (60%), which is the highest amount found among the members of the *Araucariaceae* family.

Although Wollemi pine seed (WPS, Figure S2) had not been previously investigated, the lipid content and fatty acid profile of the closest living relative, *Agathis robusta*, an Australian gymnosperm (*Araucariaceae*), have been determined. Using a combination of chromatographic and spectrometric techniques, it was shown that a small amount of long-chain polyunsaturated essential fatty acids, including arachidonic (ARA) and eicosapentaenoic (EPA) acids, were present in the seeds of *Agathis robusta* [8]. According to the authors, it was the first time that long-chain polyunsaturated essential fatty acids had been identified in the seed oil of a higher plant.

Many plants accumulate unusual fatty acids from oxidation of polyunsaturated essential fatty acids [9–11] by lipoxygenases [12,13]. These oxygenated fatty acids, collectively called oxylipins, occur naturally as oxidative bio-transformation metabolites in mammals [14,15] as well as in plants [16,17]. Naturally occurring oxylipins, including the well-known phytohormone jasmonic acid, are a large family that is predominantly made up of a mixture of molecules with hydroxyl-, epoxy-, oxo-, or keto-fatty acid or aldehyde functional groups. The molecules from this family may perform various significant physiological functions through anti-inflammatory or cytotoxic activities. A randomised crossover study in asthmatics and healthy individuals showed that the oxylipins may have anti-inflammatory effects [18].

In plants, oxylipins have also been found to participate in a variety of important physiological processes, such as cell signalling [19] and defense in response to attack from microbes or pathogens [20]. Accumulation of oxylipins has been shown to increase antifungal and antimicrobial activity [21], enhance resistance and control of disease [22,23] and protect against the action of the rice blast fungus [24]. Oxylipins have been found to accumulate on prolonged storage of seeds and potatoes [25,26]. The oxygenated acids were identified as hydroxy acids and mono-epoxides with conjugated double bonds, and their levels were shown to increase gradually during the storage of *Cichorium intybus* L., *Crepis thomsonii* Babc., *Crepis vesicaria* L., and sunflower seeds [25,26].

Age-dependent loss of viability for wheat and barley seeds has been associated with increased lipid oxidation and hydrolysis [27]; however, oxylipins were not specifically identified by the analytical method used. The occurrence of oxylipins in plants varies considerably; thus, convenient and versatile methods for the detection and identification of the fatty acid profile are required. The classical method involves transesterification of the triacylglycerol with subsequent separation, identification, and quantification by gas chromatography. Compared with the classical method, ^{1}H NMR spectroscopic analysis is a simple, direct, effective, and rapid method without the risk of forming artefacts due to chemical modification [28]. It is a widely used method to analyse edible oil samples without chemical modification. Based on the assignment of spectral signals from ^{1}H NMR analysis of the intact oil to different types of protons and their integration, quantitative structural information is determined, such as the degree of unsaturation, proportions of omega-3, omega-6, and omega-9/saturated fatty acid acyl chains, as well as the proportion of acyl chains to glyceryl moieties [29–32].

To our knowledge, the composition of the seed-fixed oil of Wollemi pine has not been previously investigated. Understanding the factors involved in seed longevity during storage, such as lipid peroxidation, is important for long-term seed banking efforts [33], particularly for critically threatened species such as Wollemi pine. This paper reports the fatty acid profile of the Wollemi pine seed oil and the effect of the storage time on the lipid composition employing chromatographic and spectrometric techniques.

2. Materials and Methods

2.1. Seed Collection and Storage Treatments

Seed cones of Wollemi pine that were just beginning to turn brown were collected under a NSW National Parks and Wildlife permit from a wild population by helicopter during the maturation period or from two cultivated trees at the Australian Botanic Garden Mount Annan (January to March). Viable seeds were sorted from the cones as they dried in the laboratory from green to brown. Fresh seeds were kept in room conditions until sent for analysis. Due to the conservation needs of this endangered species, seed availability of fresh and stored samples was very low at the time of this work; hence, seeds for analysis were derived from a number of sources, i.e., they were either 'fresh' or 'stored' in short-term (5 °C) or long-term (−20 °C) seed bank conditions, after drying [34] or in 'aging' conditions, high humidity and temperature [35], for varying periods of time:

1. Fresh;
2. 5 °C, 84 months;
3. −20 °C, 84 months;
4. 60% RH and 45 °C, 12 months;
5. 60% RH and 45 °C, 49 months.

The drying of seeds was conducted by placing seeds over silica gel until they equilibrated to between 3–7% moisture content (MC) (around seven days). Seeds were then packed into aluminium foil packets and hermetically sealed to maintain the MC and were placed into a refrigerator (5 °C) or freezer (−20 °C).

Other seeds were placed into 'aging conditions' sealed boxes with high humidity (60% RH provided by LiCl saturated salt solution in bottom of box) and 45 °C provided by an oven [34]. A sample of fresh seeds, and seeds stored for six months and seven years at the two temperatures, were sown onto wet filter paper in petri dishes (17 seeds per storage treatment/time) and incubated at 26 °C until they germinated or were deemed unviable (soft and mushy) [36].

2.2. Reagents and Materials

Analytical-grade hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and ethanol (EtOH) were supplied by Asia Pacific Specialty Chemicals Ltd. (Botany Bay, NSW, Australia). and Sigma Aldrich Pty Ltd. (North Ryde, NSW, Australia). Magnesium (Mg) was supplied by Fluka Chemika. Sodium (Na) metal in liquid paraffin was supplied by BDH Chemicals Australia Pty Ltd. (Bayswater, VIC, Australia). The solvent used for ^{1}H NMR was deuterochloroform ($CDCl_3$) containing 0.03% tetramethylsilane (TMS), supplied by Sigma Aldrich Pty Ltd. (North Ryde, NSW, Australia). The rotary evaporator for removal of solvent under reduced pressure was a Büchi Rotovap R-114 with Waterbath B-480 (Flawil, Switzerland). A Büchi diaphragm vacuum pump V-700 system with a vacuum controller V-850 was used with the rotary evaporator.

Anhydrous methanol was freshly distilled over magnesium methoxide under a nitrogen gas atmosphere [37].

All the standards used for the GC-MS were provided by Chromalytic Technology (Restek, VIC, Australia; 99% purity specific for GLC). They were FAME#6 Mix (Cat#35015); FAME#7 Mix (Cat#35016); FAME#8 Mix (Cat#35017); FAME#12 (Cat#35021); FAME#13 Mix (Cat#35034) and Food Industry FAME mix (Cat#35077), which contains methyl butanoate (C4:0), caproate (C6:0), heptanoate (C7:0), caprylate (C8:0), nonanoate (C9:0), caprate (C10:0), undecanoate (C11:0), laurate (C12:0), tridecanoate (C13:0), myristate (C14:0), myristelaidate (C14:1, *cis*-9), pentadecanoate (C15:0), *cis*-10-pentadecenate (C15:1, *cis*-10), palmitate (C16:0), palmitoleate (C16:1, *cis*-9), heptadecanoate (C17:0), *cis*-10-heptadecenate (C17:1, *cis*-10), stearate (C18:0), oleate (C18:1, *cis*-9), elaidate (C18:1, *trans*-9), linoleate (C18:2, all-*cis*-9,12), linolelaidate (C18:2, all-*trans*-9,12), γ-linolenate (CD18:3, all-*cis*-6,9,12), α-linolenate (C18:3, all-*cis*-9,12,15), nonadecanoate (C19:0), arachidate (C20:0), eicosenoate (C20:1, *cis*-11), eicosadienoate (C20:2, all-*cis*-11,14), dihomo-γ-linolenate (C20:3, all-*cis*-8,11,14), eicosatrienate (C20:3, all-*cis*-11,14,17), arachidonate (C20:4, all-*cis*-5,8,11,14), eicosapentaenate (C20:5, all-*cis*-5,8,11,14,17), heneicosanoate (C21:0), behenate (C22:0), erucate (C22:1, *cis*-13),

docosadienoate (C22:2, all-*cis*-13,16), docosahexaenate (C22:6, all-*cis*-4,7,10,13,16,19), tricosanoanate (C23:0), lignocerate (C:24:0) and nervonate (C24:1, *cis*-15).

2.3. Oil Extraction and Isolation

Generally, each seed was processed and analysed for seed oil lipid and fatty acid profiles separately. The outer coat of the weighed seed was removed by peeling to give the kernel that was weighed, then crushed and finely ground in a mortar and pestle. To extract the oil from the ground kernel, CH_2Cl_2 (1 mL) was added to the mortar and pestle. The mixture was filtered through a bed of diatomaceous earth (512 Medium, calcined, Fluka) on top of a small wad of cotton wool in a Pasteur pipette (40 mm × 5 mm i.d.) using reduced pressure to increase the rate of filtration. Repeatedly, CH_2Cl_2 (3 × 1 mL) was added and washed through the Pasteur pipette. The combined filtrate, CH_2Cl_2, was removed under reduced pressure by a rotary evaporator on a 40 °C water bath for 20 min to give a clear, pale yellowish fluid oil that was weighed, labelled, and covered with aluminium foil. For individual seed weights, and kernel and seed oil, weights and yields, see Tables S1 and S2. All the samples were then stored in a refrigerator for further analysis. Chemical characterisation of each seed was performed within 1 day after oil extraction to avoid the degradation of the heat and oxygen-sensitive fatty acids in the lipid samples. Furthermore, samples were protected from exposure to light and stored under N_2 to reduce oxygen exposure.

2.4. Fatty Acid Methyl Ester Preparation

Transesterification of the oil to give fatty acid methyl ester (FAME) derivatives was carried out using sodium methoxide solution, which was freshly prepared by reaction of sodium metal (8–10 mg) with anhydrous methanol (15 mL). This solution was added under nitrogen to the oil sample (8–12 mg), and a homogeneous solution resulted. The reaction mixture was stirred under N_2 at 40–45 °C on an oil bath for one and a half hours. Aqueous 10% sodium chloride (5 mL) and CH_2Cl_2 (5 mL) were added to the oil and mixed. The CH_2Cl_2 layer was separated and washed with aqueous 10% sodium chloride (3 × 3 mL) and dried with anhydrous magnesium sulphate, filtered, and concentrated under reduced pressure. Complete removal of the solvent residue was achieved under a stream of dry nitrogen gas [37].

2.5. GC-MS Analysis

The seed oil samples were transesterified and analysed as FAME for their fatty acid profiles using a Thermo Finnigan PolarisQ gas chromatography, equipped with an ion Trap MS-MS System. A fused-silica capillary column (Phenomenex® ZB-WAX, 30 m length × 0.25 mm i.d. × 0.25 µm film thickness, Phenomenex, Torrance, CA, USA) was used for analytical separation. Helium carrier gas was operated at a flow rate of 1 mL/min, and methane (CH_4) was used as a reagent gas for chemical ionisation. The injector was held at a constant temperature of 220 °C. The oven was temperature programmed from 100 to 250 °C, at a rate of 8 °C/min, and maintained at 250 °C for 20 min. FAME samples (1–2 µL) with the concentration between 0.05–0.1 mg/mL were injected into the system. The mass spectrometer was operated in full scan mode in the range of m/z 100–500. The contents of individual fatty acid methyl esters were determined by the percentages of ion counts of individual FAME to the total full scan ion counts in each sample. A comparison of the retention times of the samples with those of the authentic standard mixture (Restek, VIC, Australia; 99% purity specific for GC), run on the same column under the same condition, was made to facilitate the identification (Table S3).

2.6. ^{1}H NMR Analysis

The intact oil content under ambient conditions was also characterised by ^{1}H NMR spectroscopy analysis (300 MHz) carried out by a Gemini 300 spectrometer (Varian, Palo Alto, CA, USA) in the Sydney Pharmacy School, The University of Sydney. NMR analyses of the samples (10–20 mg) were carried out in deuterated chloroform (~0.6 mL). Chemical shifts (ppm) were determined relative to internal $CDCl_3$ (^{1}H, 7.24, ^{13}C, 77.0) or tetramethylsilane $(CH_3)_4Si$ or TMS (^{1}H, δ0.0) as reference peaks for each spectrum. The relative compositions

of the average degree of saturation, unsaturation, double bond equivalent, and CH_3/glycerol ratio (3 = 3 fatty acids per 1 glycerol), together with the percentage of omega-3, omega-6, and omega-9 plus saturated fatty acids were determined from the integration or the peak height of the signals (ACD Lab 12.0 software), (Tables S4 and S5).

2.7. Statistical Analysis

Experiments for the determination of the GC-MS analysis of fatty acid composition were carried out once for each seed, and results are expressed as mean values ± SD. NMR and MS results are expressed as values for single runs. The quantitative data were analysed using PASW Statistics 18 software (IBM SPSS Inc., Chicago, IL, USA). A statistically significant difference was assumed at $p < 0.05$.

3. Results and Discussion

A summary of Wollemi pine seed oil weights after extraction of seed kernels with dichloromethane is shown in Table 1.

Table 1. A summary of Wollemi Pine seeds and oil yielded in this study (n = 61).

Wollemi Pine Seeds		Weight (mg)			wt% of Oil	
		Whole Seed	Kernel	Oil Obtained	In Whole Seed	In Kernel
Fresh seeds (n = 27)	Range	6.7–44.4	1.1–37.2	2.2–14.4	27.1–41.1	34.7–48.6
	Avg ± SD	23.8 ± 9.5	18.9 ± 8.4	7.5 ± 2.9	33.4 ± 3.9	42.2 ± 4.0
Stored seeds (n = 34)	Range	4.7–42.8	0.8–32.1	2.0–16.4	20.8–41.7	32.7–50.9
	Avg ± SD	26.6 ± 11.6	19.9 ± 9.8	10.5 ± 3.8	34.2 ± 5.9	48.0 ± 11.9

The fatty acid profile of the Wollemi pine seed oil was determined by GC-MS analysis of fatty acid methyl esters formed by transesterification of the seed oil. The unmodified seed oil was analysed by ^{1}H NMR to estimate the degree of unsaturation, the triacylglyceride content, the molar proportions of omega-3, omega-6, and omega-9/saturated fatty acids, and the presence of any atypical seed oil proton signals.

3.1. Wollemi Pine Seed Fatty Acid Composition Determined by GC-MS Analysis

A simple direct alkaline-catalysed transesterification in methanol was used to convert the WPS oil to fatty acid methyl esters (FAMEs) in high yield. The fatty acids in WPS were identified by comparing the retention times of their methyl esters with a mixture of authentic standards. A representative GC-MS chromatogram of FAME of fresh WPS (USTD ID: WPS-5) is shown (Figure 1).

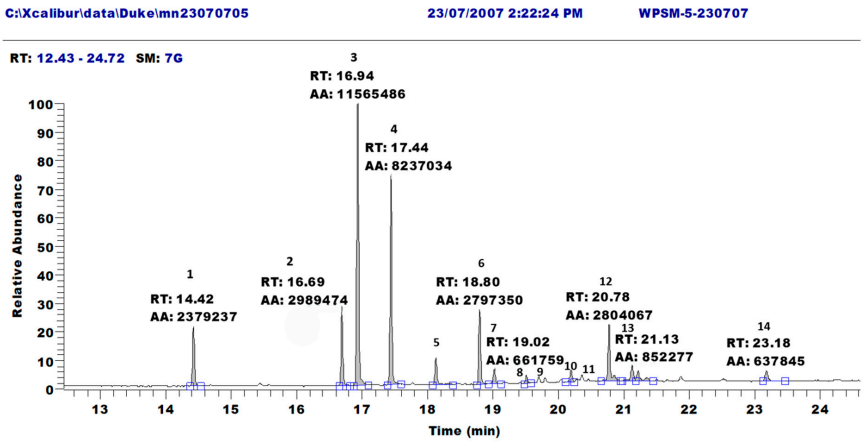

Figure 1. GC-MS chromatogram of FAMEs prepared from the representative fresh WPS oil. 1: palmitic acid; 2: steric acid; 3: oleic acid; 4: linoleic acid; 5: α-linolenic acid; 6: arachidic acid; 7: gondoic acid; 8: eicosadienoic acid; 9: dihomo-γ-linolenic acid; 10: heneicosanoic acid; 11: arachidonic acid; 12: behenic acid; 13: tricosanoic acid; 14: lignoceric acid.

The percentage of the individual fatty acids identified, groups of fatty acids with the same chain length, the saturated and unsaturated fatty acids (omega series) are shown in Table 2.

Table 2. (**a**). Individual fatty acids in fresh Wollemi pine seeds (n = 11) were determined and quantified from the corresponding fatty acid methyl ester by GC-MS. The amount of the individual fatty acid was expressed as the percentage of the total fatty acids (100%). (**b**). Composition of fatty acids shown in groups with the same chain lengths in fresh Wollemi pine seeds as determined by GC-MS (n = 11). (**c**). Saturated and unsaturated fatty acids in fresh Wollemi pine seeds as determined by GC-MS (n = 11).

(a)	
Fatty Acid [a]	**% of the Total FAME (Mean ± SD)**
C16:0, palmitic acid	6.7 ± 0.3
C18:0, stearic acid	8.1 ± 1.3
C18:1 (n-9), oleic acid	32.2 ± 1.9
C18:2 (n-6), linoleic acid	24.6 ± 1.7
C18:3 (n-3), α-linolenic acid	3.1 ± 0.2
C20:0, arachidic acid	7.6 ± 0.4
C20:1 (n-9), gondoic acid	2.0 ± 0.2
C20:2 (n-6), eicosadienoic acid	1.3 ± 0.2
C20:3 (n-6), dihomo-γ-linolenic acid	0.9 ± 0.1
C20:4 (n-6), arachidonic acid	1.8 ± 0.8
C21:0, heneicosanoic acid	0.7 ± 0.1
C22:0, behenic acid	7.5 ± 1.0
C23:0, tricosanoic acid	1.1 ± 0.8
C24:0, lignoceric acid	2.4 ± 1.4
(b)	
Fatty Acids	**% of the Total FAME (Mean ± SD)**
C16 fatty acids	6.7 ± 0.3
C18 fatty acids	68.0 ± 2.6
C20 fatty acids	13.7 ± 0.8
C21 fatty acids	0.7 ± 0.1
C22 fatty acids	7.5 ± 1.0
C23 fatty acids	1.1 ± 0.8
C24 fatty acids	2.4 ± 1.4
(c)	
Fatty Acids	**% of the Total FAME (Mean ± SD)**
Saturated fatty acids (S)	34.1 ± 2.8
Unsaturated fatty acids (U)	65.9 ± 2.8
PUFA	31.7 ± 3.0
ω3 fatty acids	4.0 ± 0.3
ω6 fatty acids	27.7 ± 2.7
ω9 fatty acids	34.2 ± 2.1
Ratio (U/S)	1.9

[a] Corresponds to the peaks found in the chromatogram shown in Figure 1.

3.2. Wollemi Pine Seed Fatty Acid Composition Determined by ^{1}H NMR Analysis

The ^{1}H NMR chemical shift of the terminal methyl group is determined by the location of the double bonds and substituents in the fatty acid chain. The chemical shift of a terminal methyl group increases with increasing proximity to a double bond and electronegativity of a substituent. The signal at δ0.89 is a combined signal of three triplets produced by the overlap of three different terminal methyl groups from the total omega-6,

total omega-9, and saturated fatty acids. The area under these methyl triplets represents relative proportions of omega-3, omega-6, and omega-9/saturated fatty acids in the mixture. However, overlapping resonances for omega-6 and omega-9/saturated fatty acids triplet signals prevent direct integration of the individual triplets. The centre peak height is proportional to the area of a triplet, thus representing the proportion of the respective fatty acid in a mixture. As the centre peak of the triplets at δ0.89 (total omega-6) and δ0.88 (total omega-9/saturated) is largely free of overlap, their height can also be used to estimate the relative amount of the respective fatty acids in a mixture (Figure 2).

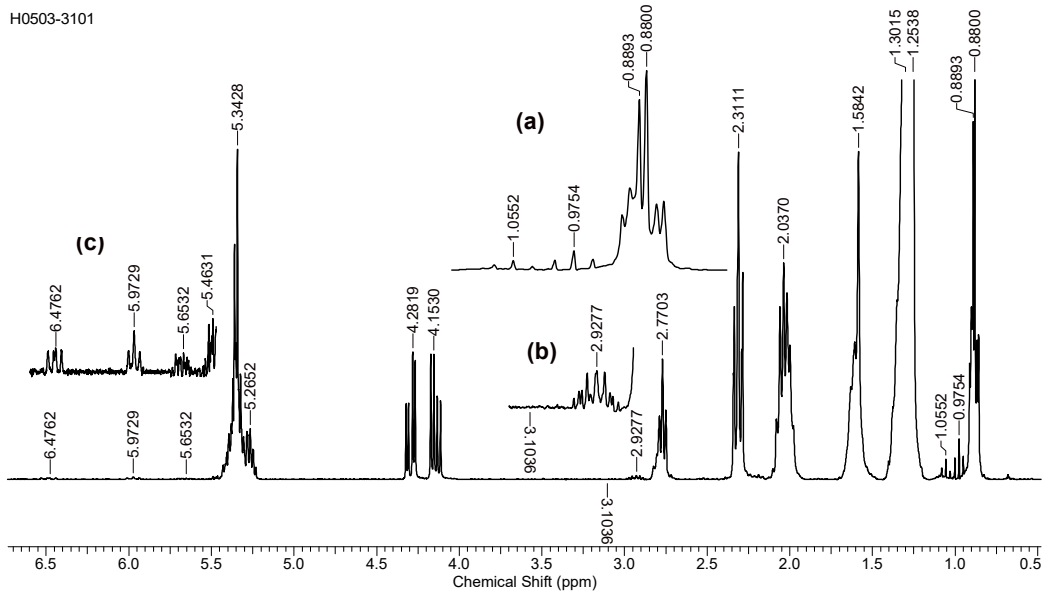

Figure 2. ^{1}H NMR spectra of intact oil from a stored Wollemi pine seed representative (Seed No. H0503-3101). (a) The signals of the terminal methyl group of the omega-3 epoxide (δ1.06), omega-3 fatty acid (δ0.98), omega-6 fatty acid (δ0.89), and omega-9, plus saturated fatty acid (δ0.88). (b) The signal of the 3-membered ring H of omega-3 epoxide (δ2.93). (c) The signals of the *E,Z* conjugated diene system of an oxylipin (δ6.48, δ5.97, δ5.65, and δ5.46).

Whilst the relative amount of fatty acids can be calculated from the methyl triplets (δ0.88 to δ1.06) in the ^{1}H NMR spectrum, the remaining part of the spectrum confirms the resonances typical of these fatty acid triacylglyceride mixtures. These resonances include the methylene α and β to the carbonyl (δ1.61 and δ2.31), the allylic methylene (adjacent to a single double bond, δ2.04), the doubly allylic methylene (adjacent to two double bonds, δ2.77), the 1- and 3-glyceryl methylene (δ4.15 and δ4.28), the overlapping 2-glyceryl methine (δ5.34), and the olefinic protons in the fatty chains (δ5.27). In the ^{1}H NMR analyses of Wollemi pine seed oil samples, an atypical deshielded methyl triplet signal was observed at 1.06 ppm. ^{1}H NMR spectra of omega-3 epoxylipids isolated from the oil of stored seeds and prepared by synthesis [38] showed a similar methyl triplet signal at 1.06 ppm. In this study, the signal centred at 1.06 ppm was too weak for accurate integration; therefore, the ratio of the omega-3 epoxylipid 1.06 ppm centre peak height to the α-linolenic acid 0.970 ppm centre peak was used to estimate the amount of omega-3 epoxylipid in the intact oil.

Both GC-MS and ^{1}H NMR methods show that the most abundant fatty acids in the oil are omega-9 and saturated fatty acids, followed by omega-6 fatty acids, then omega-3 fatty acids (Table 3).

Although the two methods yielded different results, a broad similarity in the abundance of each type of fatty acid in the seed oil was established using the independent analytical methods. Due to the overlap of ^{1}H NMR signals of lipids, greater precision and accuracy are expected for the determination of the fatty acid profile by the GC-MS method, where fatty acid methyl esters are chromatographically separated and selectively measured by mass spectrometry.

Table 3. Total ω series fatty acids in Fresh WPS as determined by GC-MS and proton NMR.

Fresh WPS (% in Total FAs)	GC-MS (Ion Current %)	^{1}H-NMR (mol %)
Total ω3 fatty acids	4.0 ± 0.3	6.9 ± 1.0
Total ω6 fatty acids	27.7 ± 2.7	43.5 ± 2.1
Total ω9 + saturated fatty acids	68.3 ± 4.9	49.6 ± 2.5

3.3. Wollemi Pine Seed Oil Content and Fatty Acid Composition

This study, for the first time, reports the yield and composition of fresh and aged Wollemi pine seed oil. The Wollemi pine seeds (WPS) used in this study were analysed over a period of four years as the seeds became available. The combined collection of 61 seeds in total was made up of 27 fresh and 34 stored seeds. Seven seeds (1 fresh and 6 stored) were excluded from the study because of insufficient quantity of oil for fatty acid profile analysis. The weight of the whole seed, kernel, and oil yield are presented (Table 1). A wide variation in the weight for fresh (6.7–44.4 mg) and stored (4.7–42.8 mg) seeds as well as fresh (1.1–37.2 mg) and stored (0.8–32.1 mg) kernels was observed. The weight of the oil in the fresh and stored kernels was 2.2–14.4 mg and 2.0–16.4 mg, respectively, and corresponds to 34.7 to 48.6% and 32.7 to 50.9% of the weight of the fresh and stored kernel, respectively. The seed kernels, from both fresh and stored seed, were rich in oil with average contents of 42% and 48%, respectively. Storage does not appear to have a significant effect on the amount of oil in the seeds.

The fatty acid profile of WPS oil was determined by a GC-MS analyses of fatty acid methyl ester derivatives. In terms of the carbon chain length of the fatty acids, the amounts of C16 fatty acid (~7%) and C18 (~68%) are typical amounts present in many seed oils. However, the content of C20 fatty acid (~14%) and C21 to C24 fatty acid (~12%) is relatively high. Oleic acid makes up 32% of the fatty acid profile, while the major polyunsaturated fatty acid is linoleic acid (~25%). Most of the detectable omega-3 fatty acid content of the oil is α-linolenic acid (3%), not unusual for a seed oil. However, the seed oil has relatively high contents of C20 omega-6 fatty acids (~4%) and C20 to C24 long chain saturated fatty acids (~19%). About 66% of the total fatty acids are made up of unsaturated fatty acids, comprising largely of omega-9 (~34%) and omega-6 (~29%) fatty acids.

The fatty acid composition of Wollemi pine seed oil is similar to that reported for *Agathis robusta*, the Wollemi pine's sister genus from the gymnosperm family *Araucariaceae* [8]. *Agathis robusta* seed oil is made up with 40% saturated fatty acids comprising long and very long hydrocarbon chains (16:0 to 24:0, mostly are even-numbered) and 60% unsaturated fatty acids. It is noteworthy that the amount of α-linolenic (18:3ω3) acid (3.1%), arachidonic (20:4ω6) acid (1.8%), and dihomo-γ-linolenic (20:3ω3) acid (0.9%) was found to be higher in WPS than *A. robusta* seed. No significant eicosapentaenoic (20:5ω3) acid was detected in WPS, but a trace amount was found in *A. robusta* (0.05%).

A higher value of total omega-3 and omega-6 fatty acids, but a lower value of total omega-9 and saturated fatty acids in the total lipid content of fresh WPS was found using ^{1}H NMR compared with GC-MS. Artefacts formed in the derivatisation step in the formation of FAMEs and thermal decomposition in the GC column can undermine the accuracy and precision of fatty acid composition determined by the classical characterisation of seed oil by GC. The ^{1}H NMR analysis, on the other hand, does not require pre-treatment, thereby enabling analyses of intact lipid. Furthermore, quantitative analysis of intact lipid from seed oil by ^{1}H NMR has been reported [28–32].

3.4. Characterisation of the Omega-3 Epoxylipid Detected in Stored WPS

The intact oil content of fresh (n = 26) and stored (n = 28) WPS were analysed by ^{1}H NMR. Nine major signals were found in the ^{1}H NMR spectra of fresh and stored seeds, and five additional signals in stored seeds (Figure S3). These signals are associated with the hydrogen atoms of the acyl groups [29,32]. A representative ^{1}H NMR spectrum of intact oil from one of the stored seeds is shown in Figure 2. The protons of the terminal methyl

group of an omega-3 epoxylipid have characteristic signals at 1.06 ppm in the ^{1}H NMR spectrum, which enables the proportion of this type of oxylipin to be determined in the intact oil.

The H-15 signal at 3.1 ppm observed for *cis*-15,16-epoxy-13-hydroxy-9*Z*,11*E*-octadecadienoic acid [39] was absent from all samples of WPS oil, indicating that this omega-3 fatty acid was not present in significant quantity in any samples. Close examination of the ^{1}H NMR spectra of samples with relatively large amounts of omega-3 epoxylipid as evidenced from the methyl triplet signal at δ1.06 showed other signals centred at δ2.95, δ2.91, δ2.41, and δ2.22. These signals showed very similar chemical shifts, coupling patterns, and relative intensities to the H-15, H-16, H-14a, and H-14b signals, respectively, observed in a referenced ^{1}H NMR spectrum observed for methyl cis-15,16-epoxy-9Z,12Z-octadecadienoate [38] (Figure S4). As the WPS oil omega-3 fatty acid content appears to be almost entirely in the form of α-linolenic acid, thus additional signals observed at δ2.95, δ2.91, δ2.41, δ2.22, and δ1.06 are postulated to be due to the 15,16-epoxide derivative of α-linolenic acid (Figure 3). In samples containing omega-3 epoxylipids, additional signals characteristic of a conjugated *Z*,*E*-diene system of oxylipins were observed at δ 5.46 (dd, J = 14.5, 6.8 Hz), δ 5.65 (ddd, J = 15.2, 6.9, 2.7 Hz), δ5.97 br t (J = 10.9 Hz), δ6.48 ddd (J = 14.7, 11.1, 1.3 Hz) corresponding to the H-9, H-12, H-10, and H-11, respectively. This indicates the presence of other oxylipin fatty acids such as 13(S)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid or 13(S)-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (Figure 3).

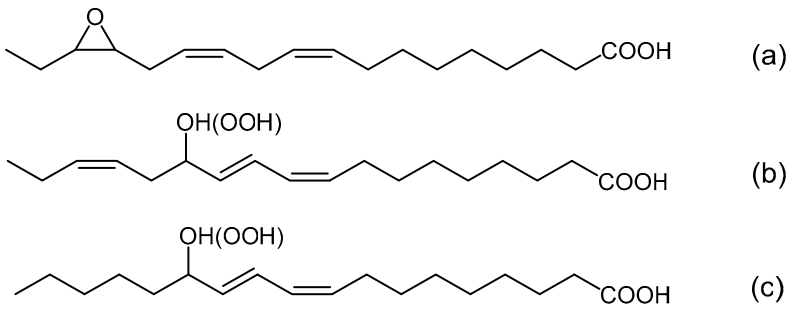

Figure 3. Structure of tentatively identified omega-3 epoxylipid, (**a**) *cis*-15,16-epoxy-9*Z*,12*Z*-octadecadienoate ester or free carboxylic acid; Structures of other postulated oxylipins, (**b**) 13-hydroxy(hydroperoxy)-9*Z*,11*E*,15*Z*-octadecatrienoic acids, or (**c**) 13-hydroxy(hydroperoxy)-9*Z*, *11*E-octadecadienoic acids.

15,16-Epoxy-α-linolenic acid (*cis*-15,16-epoxy-9*Z*,12*Z*-octadecadienoic acid) has been identified as a major epoxy fatty acid in seed oils [40–42]. Characteristic signals for 12,13-epoxy-α-linolenic acid were not detectable in any ^{1}H NMR spectra of intact oil from stored seeds of Wollemi pine indicating that this epoxy fatty acid was not present in a significant amount (<0.04%). Other oxylipins were not individually detectable by this basic NMR method.

3.5. Effect of Storage Conditions on Wollemi Pine Seed Oil

The fatty acid contents of the oil from fresh and stored seeds of Wollemi pine seed, as shown in Table 4, were determined by ^{1}H NMR method.

There is no significant difference in the fatty acid contents in the seed oils from both fresh and stored seeds except for the last condition in which the seeds were subjected to prolonged storage (49 months) at elevated temperature (45 °C) and high humidity (60%). The seed oils had similar average degrees of unsaturation and average proportions of fatty acids of omega-3, omega-6, and omega-9 plus saturated, but the percentage of omega-3 epoxylipid was significantly higher after 7 years of storage (1.97 ± 1.19) in comparison with that stored after 1 year (0.47 ± 0.51) at 4 °C and −18 °C, respectively. Moreover, after 7 years of storage, more omega-3-epoxide was produced at 5 °C, 2.2 ± 1.0 (SD), compared with that at −18 °C, 0.3 ± 0.3 (SD). This indicates that the conditions for producing omega-3

epoxylipid are not only related to the storage time but also to the temperature during storage. Omega-3 epoxylipid levels were also increased in accelerated aging conditions for 12 or 49 months. This omega-3 epoxylipid was, however, not detectable in fresh seeds. This finding is consistent with the literature, which reports that oxygenated fatty acids accumulated gradually after prolonged storage of seed oil in a variety of storage conditions [28–31,40,41].

Table 4. Average fatty acid (FA) composition (Mol % weight) (mean ± SD) of oil of Wollemi pine seeds stored in different conditions and lengths of time.

Treatment	Time in Storage (Months)	DB Equiv	CH_3/glyc	% Omega-3	% Omega-6	% Omega-9 + sat	Omega-3 Epoxide
Fresh (n = 26)	0	1.5 ± 0.1	2.8 ± 0.1	7.2 ± 1.0	44.0 ± 2.0	48.8 ± 2.5	<0.1
Stored dry, 5 °C (n = 4)	84	1.4 ± 0.2	2.8 ± 0.1	6.7 ± 1.4	42.2 ± 1.1	49.3 ± 2.5	2.2 ± 1.0
Stored dry, −18 °C (n = 10)	84	1.4 ± 0.1	3.4 ± 1.2	6.8 ± 0.9	42.1 ± 3.8	50.9 ± 3.9	0.3 ± 0.3
'Aging conditions', 60% RH and 45 °C (n = 11)	12	1.6 ± 0.1	2.5 ± 0.4	7.2 ± 1.3	41.3 ± 1.8	51.5 ± 2.0	0.1 ± 0.1
'Aging conditions', 60% RH and 45 °C (n = 6)	49	1.4 ± 0.1	1.6 ± 0.1	8.3 ± 1.3	30.4 ± 2.8	61.1 ± 3.1	0.3 ± 0.1

Apart from their similarity in double bond equivalent (1.5 and 1.6, respectively), other profiles of fresh and stored seeds were significantly different ($p < 0.05$). The stored WPS was found to contain a higher total amount of omega-3, omega-9, plus saturated fatty acids, and a lower amount of CH_3 per glycerol and omega-6 fatty acids. This indicates more degradation of omega-6 fatty acids and esters, and the lower amount of CH_3 groups may be indicative of the formation of mono- and diacylglycerides resulting from the degradative loss of fatty acids. The latter observation was most prominent with the most testing seed storage conditions, RH 60%, 49 months. Under these conditions, the CH_3/glycerol ratio is significantly decreased (1.6 vs. ~3), indicating partial hydrolysis of the triacyglycerides and loss of the free fatty acid.

3.6. Proposed Source of the Oxylipins Found in Stored WPS

Polyunsaturated fatty acids are the primary targets for oxidative reactions that lead to the formation of oxygenated fatty acids, which are collectively called oxylipins. Oxylipins occur naturally and are formed as metabolites in plants [14–17]. It has been reported that oxylipins such as epoxy or hydroxy fatty acid are produced by enzymatic and non-enzymatic processes on prolonged storage of seeds [43,44].

In the case of Wollemi pine seed oil omega-3-epoxylipid, it is formed in a relatively large amount and appears to be stored; however, it is possible that it is trapped in the relatively large amount of oil in the seed. A taxol-producing fungus was found in Wollemi pine [45]. Taxol, a well-known and important anticancer drug, was plausibly produced by *Pestalotiopsis guepinii*, an endophytic fungus. Fungal contamination may be one of the factors that trigger the production of oxylipin in stored WPS as part of an anti-fungal and antimicrobial defensive mechanism in the stored seed.

While lipid peroxidation is commonly associated with the deterioration of seed quality of many species during storage [46], it has not been widely explored for other members of the *Araucariaceae*. Elevated oxylipin in stored Wollemi pine seeds may indicate seed viability loss in this species, noting that higher levels were found in seeds stored at the higher temperature for seven years.

The work of Garcia et al. [47] showed that lipid peroxidation was increased in embryonic axes of *Araucaria angustifolia* stored at laboratory temperatures when compared with refrigerator conditions, and this was associated with loss of seed viability over 180 days. In this study, the viability of fresh and low-temperature stored Wollemi pine seeds was similar over time (72% for fresh seeds, 66% for seeds stored at −18 °C for seven years). Seeds stored for seven years at a higher temperature (5 °C), however, showed approximately 50%

lower viability (26%) compared with fresh seeds. Loss of seed viability in this species may be associated with the lipid peroxidation found in this study and requires further investigation.

Given the conservation status of many members of this family, further work on the seed storage behaviour of Wollemi pine and other *Araucariaceae* is warranted.

4. Conclusions

About three-quarters of polyunsaturated fatty acids (PUFA) in the oil content contained 18 and 20 carbon chain lengths, with the C18 being the most abundant (68.0 ± 2.6%). The C18 PUFA consists predominantly of oleic acid (18:1ω9) (32%) and linoleic acid (18:2ω6) (25%). Other PUFA in the oil include linolenic (18:3ω3) (3.1%), gondoic (20:1ω9) (2%), eicosadienoic (20:2ω6) (1.3%), dihomo-γ-linolenic (20:3ω6) (0.9%), and arachidonic (20:4ω6) (1.8%) acids. The PUFA in fresh WPS comprises 34% omega-9, 29% omega-6, and 3% omega-3 fatty acids.

In contrast, the chain length of saturated fatty acids in the oil varies from C16 to C24. These fatty acids are common in plant seed oils, including palmitic (16:0), stearic (18:0), arachidic (20:0), heneicosanoic (21:0), behenic (22:0), tricosanoanoic (23:0), and lignoceric (24:0) acids, totally composed 34.1 ± 5.3% of the seed oil. The content of C20 omega-6 fatty acids, eicosadienoic acid, dihomo-γ-linolenic acid, and arachidonic acid in this study is one of very few reports of significant content of very long-chain essential fatty acids found in a higher plant seed oil.

In this work, the omega-3 epoxylipid detected in stored WPS oil was tentatively identified as *cis*-15,16-epoxy-9*Z*,12*Z*-octadecadienoate ester or free carboxylic acid. Also, the ^{1}H NMR signals observed for a conjugated *E*,*Z*-diene system were consistent with the presence in the stored WPS oil of 13-hydroxy(hydroperoxy)-9*Z*,11*E*,15*Z*-octadecatrienoic acids or 13-hydroxy(hydroperoxy)-9*Z*,11*E*-octadecadienoic acids at levels comparable to those observed for the omega-3 epoxylipid.

While the effect of the conditions and period of storage on the viability of Wollemi pine seeds was not a part of this study due to the low numbers of seeds available, seeds of Wollemi pine are known to be orthodox in their seed-storage behaviour (Offord, unpub.). Orthodox seeds can be dried and frozen without losing significant viability, although the possibility exists that Wollemi pine seeds may be relatively short-lived even at −18 °C.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/lipidology1020007/s1, Figure S1: *Wollemia nobilis* trees; Figure S2: Seeds of *Wollemia nobilis*; Figure S3: Comparison of the ^{1}H NMR spectra of fresh and stored seeds; Figure S4: 300 MHz ^{1}H NMR spectrum of methyl *cis*-15,16-epoxy-9*Z*,12*Z*-octadecadienoate; Table S1: Fresh WPS seed, kernel and oil weights, and whole seed and kernel oil yields; Table S2: Stored WPS seed, kernel and oil weights, and whole seed and kernel oil yields; Table S3: Fatty acid profiles determined by GC-MS analysis of fatty acid methyl esters prepared from WPS oil samples (n = 11); Table S4: Fresh WPS: ^{1}H NMR Analysis for double bond equivalents, omega-3, omega-6, omega-9 (plus saturated) fatty acid content (n = 26); Table S5: Stored WPS: ^{1}H NMR analysis for double bond equivalents, omega-3, omega-6, omega-9 (plus saturated) fatty acid content (n = 31).

Author Contributions: Conceptualisation, R.K.D., C.A.O. and C.C.D.; data curation, C.A.O. and C.C.D.; formal analysis, M.C.H.N. and P.H.C.; investigation, M.C.H.N., V.H.T., R.K.D., C.A.O., P.F.M., P.H.C. and C.C.D.; methodology, M.C.H.N., V.H.T., R.K.D., C.A.O., P.F.M. and P.H.C.; resources, V.H.T., C.A.O., P.F.M. and C.C.D.; software, M.C.H.N.; validation, V.H.T. and P.H.C.; writing—original draft, M.C.H.N. and C.A.O.; writing—review and editing, V.H.T., R.K.D., C.A.O., P.F.M., P.H.C. and C.C.D. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by The University of Sydney Research Maintenance fund (Q0015 00000).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Acknowledgments: We would like to acknowledge Bruce N. Tattam for his assistance with the gas chromatography–mass spectrometry (GC-MS) analysis and Amanda Rollason for her assistance with the seed storage. We are also appreciative of the support of the Wollemi Pine Recovery team and field staff of the NSW National Parks and Wildlife Service for the collection of materials. Karen Sommerville is acknowledged for constructive comments on the manuscript.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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