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Aging Effects on Macadamia Nut Oil Studied by Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

Nicholas W. Proschogo,*[,†,‡] Peter L. Albertson,[§,∥] Johanna Bursle,[§] Cameron A. McConchie,[§,⊥] Athol G. Turner,[†] and Gary D. Willett[†]

[†]School of Chemistry and [‡]Centre for Vascular Research, University of New South Wales, Sydney NSW 2052, Australia

[§]CSIRO Plant Industry, St Lucia QLD 4067, Australia

[∥]Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Brisbane QLD 4001, Australia

[⊥]Department of Resources, Northern Territory Government, GPO Box 3000, Darwin NT 0801, Australia

ABSTRACT: High-resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry is successfully used in the detailed molecular analysis of aged macadamia nut oils. The results are consistent with peroxide values, the current industry measure for rancidity, and provide detailed molecular information on the oxidative and hydrolytic degeneration of such oils. Mass analysis of macadamia oil samples stored for extended periods at 6 °C revealed that oils obtained by the cold press method are more susceptible to aging than those obtained using modified Soxhlet or accelerated solvent extraction methods.

KEYWORDS: *Fourier transform ion cyclotron resonance mass spectrometry, macadamia oil, free fatty acid, triacylglyceride, oil oxidation, oil rancidity, electrospray ionization*

INTRODUCTION

Rancidity affects the nutritional quality of food products and therefore can have a marked effect on our health.[1] Maintaining the nutritional quality of food for the maximum amount of time is extremely important to all food industries. All plant oils have the potential to become rancid, although certain plant oils appear to be more stable because of natural preservatives in the oil. The stability of plant oils is often related to the amount of unsaturated free fatty acid or acylglyceride compounds present in the oil,[2] and oils can become rancid via oxidative, enzymatic,[3] and hydrolytic[4] pathways.[5] Therefore it is often difficult to assign which parent molecule an intermediate oxidation product derives from due to the many different reaction pathways possible[5] and the variation in reaction rates due to the components in the oil.[6]

Lipid oxidation has been recognized as the predominant reaction in plant oils causing deteriorative changes in their chemical, sensory, and nutritional properties.[7] The reactions of oxygen with either the free fatty acids or the fatty acid side chains on the acylglyceride represent the major mechanism of lipid oxidation in such oils and are often involved in a free radical chain reaction.[3] The process first entails the initiation reaction, which is often a hydrogen abstraction from the lipid[3,8,9] at the allylic carbon position which will change the methylene interrupted system to a conjugated diene if it is from a polyunsaturated fatty acid.[8,10] This step is often light activated, and the rate of oxidation is related to the period of exposure to light.[2]

The second step in this mechanism is a propagation step in which a molecule of triplet oxygen reacts with the lipid radical to form a lipid hydroperoxide.[3,8,9,11,12] This reaction can occur at the last carbon before the double bond system.[6,9] The amount of such hydroperoxides that are present in the plant oil is often used as a measure of oil rancidity.[3,10,11,13,14]

Because hydroperoxide species are labile,[11,15] they readily convert into a wide variety of volatile and nonvolatile secondary oxidation products including alcohols, aldehydes, alkyl formates, ketones, epoxides, and hydrocarbons,[3,5,9−11,16−18] and in the presence of a catalyst (for example a transition metal ion), they will produce lipid peroxy and lipid alkoxy radicals.[3,11]

The termination step in this mechanism is the reaction of the radical species to form polymers[3,6,9] such as a dimer formed from two triolein monomers joined by an oxygen bridge.[19]

Important enzymes that oxidize plant oils are cyclooxygenase[8,10,18] and lipoxygenase.[3,8,10,15,18,20] The latter enzyme is an oxidoreductase that catalyzes the conversion of polyunsaturated fatty acid based acylglycerides with a 1,4-pentadiene substructure to hydroperoxides. The hydroperoxides are formed by different strands of lipoxygenase at specific carbon positions,[9,10,14,21] and the enzyme can also be involved in the removal of a hydrogen atom from methylene interrupted groups.[10]

Hydrolytic pathways are associated with the cleavage of the fatty acid from the acylglyceride to form glycerides and free fatty acids.[4,16] The extent of these processes is often measured by the amount of free fatty acid present in the plant oil. These reactions are significant as the free fatty acids are more

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susceptible to the free radical oxidation reactions[21,22] compared to the fatty acid side chain of the acylglyceride. As macadamia kernels are dried to approx 1.5% moisture content during processing, they have extremely low water activity which inhibits hydrolytic and enzymatic reactivity.[16,22]

The composition of macadamia nuts is primarily oil, which contributes to approximately 65−80% of the nut weight,[16,23] with the remainder as proteins and sugars,[16,23] with trace amounts of α-tocotrienol,[16] tocopherols,[24,25] phenolic compounds,[25] waxes, and sterols.[23] The composition of the macadamia nut oil is mostly triacylglycerides, accounting for over 70% of the lipid fraction,[22,23,26,27] although diacylglycerides, monoacylglycerides, and free fatty acids are also observed.[4] Macadamia nut oil has the highest proportion of monounsaturated fatty acid based molecules (oleic acid (~60%) and palmitoleic acid (~20%))[4,16] when compared to other vegetable oils.[24,28]

Gas chromatography (GC) is one of the earliest methods used to separate and analyze plant oils.[27,28] The major step with GC is that it requires the transesterification of the acylglycerols prior to analysis to obtain the volatility necessary for GC analysis.[1,4,17,21,29−35] This derivatization step masks the identification of the source of a fatty acid methyl ester in the parent triacylglyceride. Furthermore, functional groups attached to fatty acid based molecules may also react; for example hydroperoxides are converted into hydroxides.[13] Three advantages of using GC are that there are several mass spectrum databases available[4,34,36] for the common components of plant oils, it is possible to quantify results,[31] and it is straightforward to couple to mass spectrometry detectors. Recently, GC has also been used in the analysis of hydrocarbons in the oil[37] and for the analysis of other volatile products from the oil due to oxidation.[16]

Plant oils have also been investigated by high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry with the separation of acylglycerides and oxygenated acylglycerides studied in reverse phase[13,27,38−48] and normal phase HPLC.[5,27,45]

Traditionally electron impact GC mass spectrometry has been the favored technique to study the components of plant oils. Recently, newer methods of ionization including matrix assisted laser desorption ionization,[29,31,49−54] electrospray ionization (ESI),[4,5,10,32,36,44,45,53,55,56] and atmospheric pressure chemical ionization[8,42,43,45,47] have been used on time-of-flight and ion cyclotron resonance (ICR) mass analyzers.[4,45,49,55]

ESI-FTICRMS is a technique that shows great promise in providing some direct identification of the individual components of a complex oil mixture. The high resolution (>50 000) and high mass accuracy (<3 ppm) associated with Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) and the observation of adduct parent molecular ions have provided some direct identification of the individual components of a complex plant oil mixture,[4,56,57] while this work applies this technique to the study of rancidity in plant oils.

MATERIALS AND METHODS

Sample Preparation. Nuts of HAES 814 were harvested from commercial orchards in Bundaberg, commercially dehusked (Alstonville Steel, Alstonville, NSW, Australia), and dried in a heat pump drier (Australian Heat Pump Systems Pty Ltd., Gold Coast, Queensland, Australia) at 30 °C. The nut in shell (NIS) was dried down to 1.5% kernel moisture (wet basis) before being cracked by machine. NIS was cracked using an Armanasco (Brisbane, Queensland) mechanical cracker and hand sorted into kernel wholes or fragments before being graded into size classes 15−21 mm. In this work the 19 mm kernels were used. Kernels were stored at 4 °C in nitrogen flushed, foil lined satchels until processed or subjected to aging treatments.

The kernel was incubated in darkness in a plastic container at 40 °C for 12 weeks and a further 2 weeks at 50 °C. Subsamples of the kernel were removed at various stages of the incubation (aging) period at times 0, 12, and 14 weeks as subsamples X, Y, and Z respectively. The samples were vacuum packed with an oxygen scavenger to minimize further aging and stored at 2 °C until the oil was extracted from the nuts. Each of the oils from the subsamples X, Y, and Z were extracted via a cold press, modified Soxhlet (FexIKA (IKA-Werke GmbH, Staufen, Germany), and accelerated solvent extraction (ASE 300 Dionex Corporation, Sunnyvale, CA, USA) at 40 and 100 °C as discussed below. All twelve oil subsamples were tested for their peroxide value and split into two sets, stored for six months at 6 and −20 °C respectively. Consumers detected all aging treatments by sensory evaluation.[58]

Cold Oil Press Method. The whole kernel was placed into an aluminum sample tube. Folded filter paper (Whatman 1) was positioned over the upper surface of the kernel and the tube placed into the press. The manually operated level was lowered onto the kernel/paper, and when force was applied, the oil was squeezed out into the sample tube. The oil flowed into a collection vessel via a drainage duct. The process was repeated until 1−2 mL of oil had been collected. Samples were then centrifuged in a microfuge at maximum speed for 10 min, and the upper fraction was transferred to a clean tube and stored at −20 °C.

FexIKA Method. The kernel was shaved (thin sections hand sliced with scalpel blade) and defatted using a FexIKA 200 solvent extraction system (IKA-Werke GmbG and Co. KG, Staufen, Germany), using petroleum ether over fifteen boiling−condensing cycles for 6 h (20 min per cycle) at 100 °C. Samples were then stored at −20 °C.

ASE (Accelerated Solvent Extraction System). Pooled kernel[3] was finely diced for each sample. Approx. 4 g of tissue was transferred into each sample vial and the oil extracted in petroleum ether at 40 or 100 °C using the Accelerated Solvent Extraction (ASE) system (Dionex Corporation, Sunnyvale, CA, USA). The extraction procedure involved 3 cycles of 5 min each at 1500 PSI. The petroleum ether/oil fraction collected was then heated to 40 °C on a hot plate within an operating fume hood to remove residual solvent. Samples were then stored at −20 °C.

All twelve subsamples stored at 6 °C and −20 °C were prepared for positive ion and negative ion ESI-FTICRMS analysis by transferring 1 μL of the oil sample into IPA (1 mL), vortex shaking (5 min), and centrifuging (5 min) to assist dissolution. 0.1 mL of this solution was transferred to a solution containing 12-hydroxyoctadecanoic acid in IPA (0.7 mL, 2.9×10^{-6} M) and H_2O (0.3 mL), vortex shaken (5 min), and centrifuged (5 min). For negative ion mass spectrometry ammonia solution (2 M, 0.23 mL) was added. The subsamples stored at 6 °C were also diluted 10-fold prior to negative ion mass spectrometry analysis.

Peroxide Value. Oil (1.000 g ± 0.01 g) was added to an acetic acid/chloroform mixture (6 mL, 3:2) and shaken. Saturated KI solution (100 μL) was added to the mixture for placing in the dark (5 min). Distilled water (6 mL) was added and the mixture shaken to give two phases. This mixture was titrated with sodium thiosulfate (0.1 M) until the yellow color had faded. Starch solution (1% w/v) was added and titration continued.

All twelve subsamples stored at 6 °C were prepared for both positive ion and negative ion ESI-FTICRMS by transferring the oil sample (0.5 mL) into MeOH (0.5 mL), vortex shaking (4 min), centrifuging (4 min), and storing at 0 °C for 3 days. 0.1 mL of the supernatant was then transferred to a solution of 12-hydroxyoctadecanoic acid in MeOH (1.00 mL, 6.3×10^{-6} M), vortex shaken (5 min), and centrifuged (5 min).

Mass Analysis. The mass spectrometry experiments were performed on a BRUKER BioApex-IIe FT-ICR mass spectrometer equipped with a 7 T magnet using an off axis ANALYTICA ESI

source. All samples were injected into the ESI source with a flow rate of 120 μL h^{-1} using a Cole-Palmer 74900 syringe pump. Ions were introduced into the FTICRMS from a grounded spray needle under a potential field of 4 kV. Nitrogen was used both as a sheath gas and as a spray drying gas (250 °C) at flow rates of 15 and 1.5 L h^{-1} respectively. The Pt coated quartz capillary exit voltage was set to 50 V and the skimmer voltage to 5 V. Broadband data acquisition (typically 1 M spectra) was performed using Xmass 6.0.2 software running on a Windows 2000 operating system. Positive mode internal calibration (m/z 279−908) was performed using palmitic acid, oleic acid, 12-hydroxyoleic acid, palmitolyl oleoyl glyceride, dioleoyl glyceride, dipalmitolyl oleoyl glyceride, palmitolyl dioleoyl glyceride, and trioleoyl glycerides. Negative mode internal calibration (m/z 253−902) was performed using palmitoleate, oleate, 12-hydroxyoleate, palmitolyl oleoyl glyceride, dioleoyl glyceride, hydroxyl palmitolyl dioleoyl glyceride, and hydroxyl trioleoyl glycerides if these species were present in the sample. The individual compounds above were standardized using external calibration with NaI assignments made based on mass accuracy <5 ppm.

All subsamples that were prepared in the same conditions were run on the same day and duplicated the following day.

RESULTS AND DISCUSSION

Identification of Oxidation Products. This study applying the ESI-FTICRMS reveals that oxygenated compounds other than peroxides are also related to rancidity. However these compounds would not normally be accounted for in the measurement of peroxide values, routinely used by industry to determine rancidity. The mass spectrometry technique also provides information on the composition of the macadamia nut oils including relative concentration profiles for the free fatty acids and acylglycerols, with variations occurring with different oil extraction methods and aging treatments. Table 1 shows the peroxide values of the 12 oil subsamples that were analyzed. The results from Table 1 show that the incubation of the macadamia nuts has resulted in an increase of peroxide value as expected and also that different extraction methods may cause variations to the composition of hydroperoxides and peroxides present in the oil.

Table 1. Measured Peroxide Values for Macadamia Nut Oil Samples Extracted Using Four Different Extraction Methods

	peroxide value		
extraction method	0[a]	12[a]	14[a]
cold press	2.3	2.3	11.6
FexIKA	1.3	1.4	8.3
accelerated solvent extraction			
at 40 °C	1.3	1.5	9.8
at 100 °C	1.3	1.5	10.0

[a]Time kernels were heated/weeks.

Positive Ion ESI-FTICRMS of Neat Oils. We identified the presence of free fatty acids in the oils, e.g., oleic acid. Further, we also observed ions associated with acylglycerides with a number of them contain several additional oxygen atoms, which may be in the form of peroxy, hydroperoxy, epoxy, oxo (ketone or aldehyde functionalities), and/or hydroxy substituents, e.g., glycerol C18:1 C18:1 HO-C16:0, which are present in larger amounts in oils undergoing longer aging treatments. We also assigned a number of unusual free fatty acids and mono- and diacylglycerides that contain an odd number of carbon atoms, e.g. glycerol C18:0 C18:0 HO-C19:0. It was found that the results from the FexIKA and ASE experiments presented were very similar, so only those of the FexIKA extractions are displayed to show the general trends observed.

The oils stored at 6 °C displayed a high concentration of oleic acid with few oxygenated components for both positive ion and negative ion neat oils and methanol extractions. Figure 1 shows the positive ion ESI-FTICR mass spectra of neat oils from (a) cold pressed and not accelerated aging, (b) cold pressed and accelerated in aging for 14 weeks, and (c) FexIKA extracted and accelerated in aging for 14 weeks with all samples stored at 6 °C for 6 months. From Figure 1 it is observed that a high concentration of oleic acid with some oxygenated components appeared only for the oil samples which were not artificially oxidized, suggesting that enzymatic activity has occurred. Since the high intensity of the assigned sodiated oleic acid peak is present in the sample which has the least amount of aging, it is likely that chemical reactions have taken place as the oil has continued to degenerate. This was also confirmed when using different sample preparation techniques.

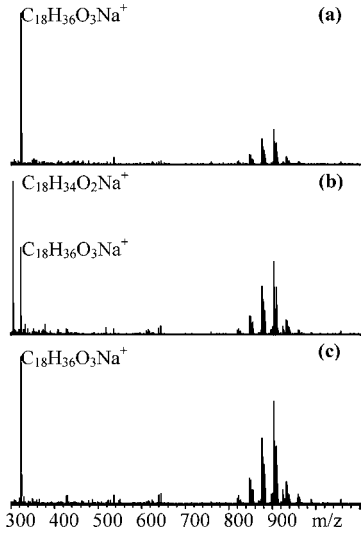

Figure 1. Positive ion FTICR mass spectra of neat oils from (a) cold pressed and not accelerated aging, (b) cold pressed and accelerated in aging for 14 weeks, and (c) FexIKA extracted and accelerated in aging for 14 weeks. Samples were stored at 6 °C for 6 months. In each spectrum, peak heights are scaled relative to the highest-magnitude peak.

Negative Ion ESI-FTICRMS of Neat Oils. Figure 2 show the negative ion ESI FTICR mass spectra of neat oils from (a) cold pressed and not accelerated aging, (b) cold pressed and accelerated in aging for 14 weeks, and (c) FexIKA extracted and accelerated in aging for 14 weeks with all samples stored at 6 °C for 6 months. From the negative ion spectra in Figure 2, the neutral triacylglycerides are not observed due to their lack of an acidic proton thus disabling it from forming a negative ion in the conditions under which ESI operates. The overall observations to those in the positive ion spectra are mirrored in the negative ion spectra with a high concentration of oleic acid observed which may have resulted in ion interactions in the ICR detector resulting in signal suppression of any minor components. With the information from Figure 1 and Figure 2, it is likely that enzymatic activity has occurred, as the mass spectra do not show a large proportion of oxygenated components but do show a very high concentration of oleic acid. It is important to note that if this experiment had been conducted using traditional GC−MS techniques, this observa-

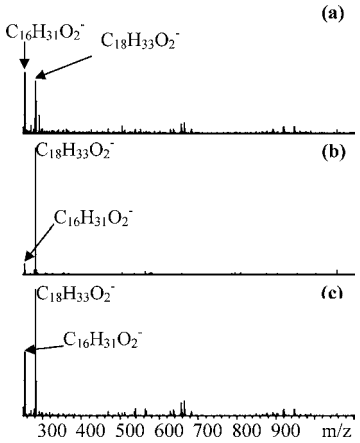

Figure 2. Negative ion FTICR mass spectra of neat oils from (a) cold pressed and not accelerated aging, (b) cold pressed and accelerated in aging for 14 weeks, and (c) FexIKA extracted and accelerated in aging for 14 weeks. Samples were stored at 6 °C for 6 months.

tion would not have been possible. This is because in GC−MS the method involves modifying the oil through transesterification prior to analysis and, since the oil comprises mostly triacylglycerides, the methyl esters from the triacylglycerides would have dominated the products observed. This demonstrates a significant advantage of using an analytical technique that examines the oil components in their pristine state.

The influence of the mode of extraction of the oil from the nut showed that the cold pressed extracted oils showed higher levels of free fatty acids when compared to the FexIKA and ASE methods. This suggests that enzyme reactions may have occurred in the cold pressed oil and supports the earlier hypothesis regarding the irregularities found in the peroxide values.

Effect of Additional Heating of Oils on Oxidation. Figure 3 shows the oils stored at 6 and −20 °C with both

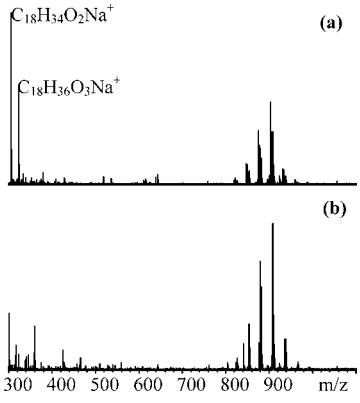

Figure 3. Positive ion FTICR mass spectra of neat oil for cold pressed accelerated in aging for 14 weeks and stored for 6 months at (a) 6 °C and (b) −20 °C.

primary and secondary oxidation products from the oil without separation or derivatization of the components in the sample. It is important to note from Figure 3 that when this oil was stored at 6 °C a larger proportion of oleic acid was observed compared to when the oil was stored at −20 °C while no other free fatty acids are observed. This observation is more noticeable when the oil was analyzed using negative ion ESI-FTICRMS where in Figure 4 it shows that levels of oleic acid are high enough to

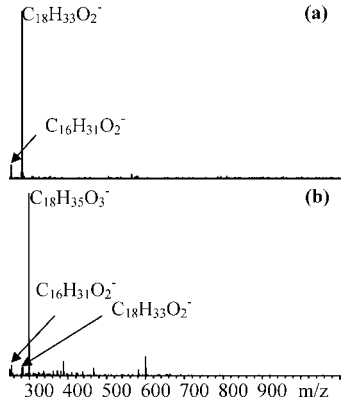

Figure 4. Negative ion FTICR mass spectra of neat oil for cold pressed accelerated in aging for 14 weeks and stored for 6 months at (a) 6 °C and (b) −20 °C.

cause the signal suppression of the internal standard in the oil stored at 6 °C whereas the intensity of oleic acid in the oil stored at −20 °C was small compared to the internal standard. This suggests that while the oil was stored at 6 °C, it is still possible for enzymatic reactions to occur while those samples stored as low as −20 °C show little reaction to enzymatic activity. Although 6 °C is a low temperature and the enzyme activity is extremely low compared to the activity at room temperature, the long duration (6 months) may be sufficient to cause significant changes in chemical composition. This effect could be reduced by either (1) applying extraction methods involving heat, which damage protein structure and therefore further decrease enzymatic activity, giving subsequent damage to proteins, (2) reducing the storage temperature, or (3) reducing the lag periods between extraction and assay. Since these above results suggest that reactions are occurring in the oil, this has relevant implications on the extraction and subsequent analysis of the oil, particularly when applying the cold press extraction methodology. The oil analysis should be performed immediately after extraction from the nut, otherwise storage should be well below 6 °C to prevent further reactions in the oil, which can lead to increased rancidity.

Positive Ion and Negative Ion ESI-FTICRMS of Methanol Extracted Oils. Since it appears that subtle changes to the storage temperature can allow the aging effects of macadamia nut oil to be observed, a variety of extraction methods of the oil from the nut were compared using ESI-FTICRMS. Although these observations can be observed in the neat oil results in Figures 1 and 2, it was found that better results were obtained when the oil underwent a methanol extraction before analysis by ESI-FTICRMS as it allows for the triacylglycerides to precipitate out of the oil and effectively concentrates the solution before analysis. Figure 5 shows the positive ion ESI FTICR mass spectra of methanol extracted oils from (a) cold pressed and not accelerated aging, (b) cold pressed and accelerated in aging for 14 weeks, and (c) FexIKA extracted and accelerated in aging for 14 weeks with the samples stored at 6 °C for 6 months.

It can be seen that there are free fatty acids, monoacylglycerides, diacylglycerides, and triacylglycerides present in all of the oils regardless of heat treatment and the mode of extraction of the oil from the nut. What is interesting in this experiment is the presence of oxygenated diacylglycerides and many oxygenated triacylglycerides in the cold pressed sample that did not undergo artificial aging where some of these

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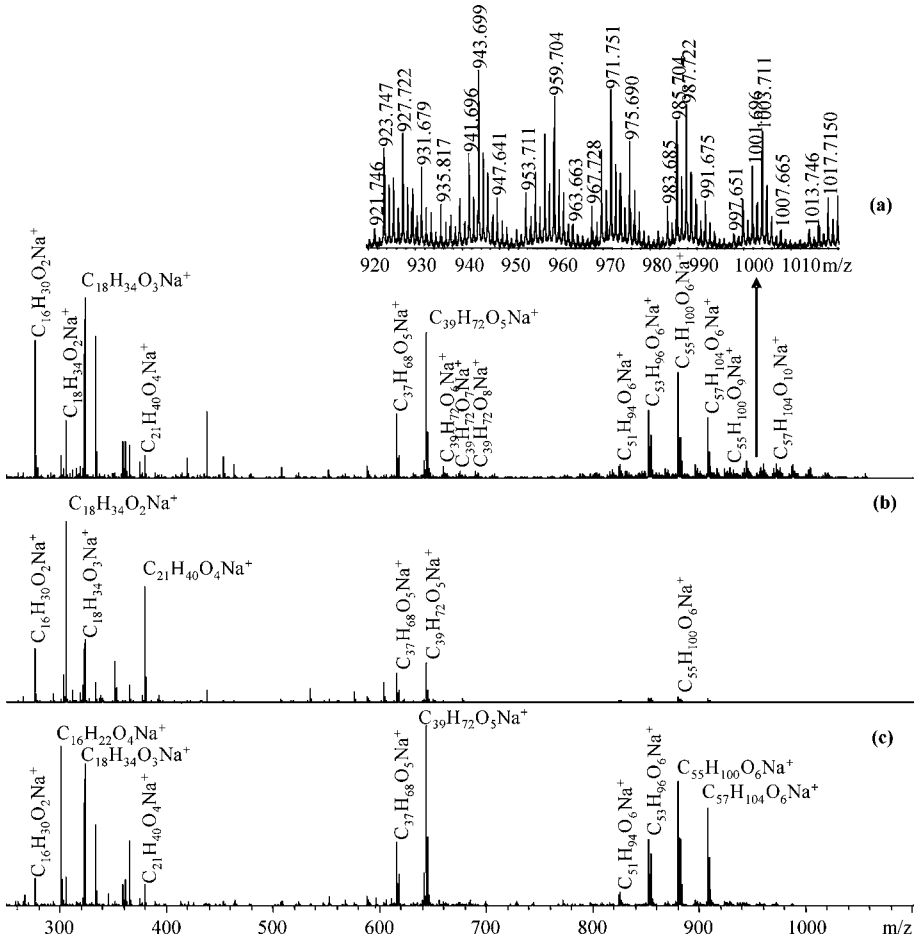

Figure 5. Positive ion FTICR mass spectra of methanol extracted oils from (a) cold pressed and not accelerated aging, (b) cold pressed and accelerated in aging for 14 weeks, and (c) FexIKA extracted and accelerated in aging for 14 weeks. Samples were stored at 6 °C for 6 months.

species contained several additional oxygen atoms. The exact identities of many of these species is ill defined due to overlapping of the ^{13}C isotopes with many of the peaks thus causing irregular peak shapes which can lead to erroneous assignments. Similar results were also observed with methanol extracted species in negative ion ESI-FTICRMS in Figures 6 and 7, although the presence of oxygenated diacylglycerides and oxygenated triacylglycerides was observed at a lower intensity when compared to positive ion ESI-FTICRMS (see Figure 5). How these compounds with extra oxygen atoms can interfere with the peroxide value is not known. It can be seen that these oxygenated species are also present in the other oils (Figures 5b and 5c), but the amount present is lower. The accelerated solvent extraction results were similar to the FexIKA results in both positive ion ESI-FTICRMS and negative ion ESI-FTICRMS (data not shown).

In conclusion, it has been established that high resolution ESI-FTICRMS can provide a better technique to analyze aged macadamia nut oils than the current methods used today, which include GC−MS, LC, and simple PV analysis. This has been demonstrated for macadamia nut oils that have been artificially aged and observed to produce results that are consistent with the current industry measures of rancidity. The mass spectrometer also provides very detailed molecular information on the composition of the oil. The application of multivariate analysis to this methodology will be considered in future work.

The storage temperature of the oil samples prior to analysis can have a profound influence on the rancidity obtained. This technique provides the most detailed means of tracing the deterioration of oil but also indicates the role that extraction plays in interpreting these processes. Different methods of oil extraction from the macadamia nut kernel are observed to produce different levels of peroxides in the oil. The cold pressed method exhibited the highest levels, with the FexIKA and ASE producing lower levels. This is due to the FexIKA and ASE methods both involving heat in the extraction process, which would damage proteins and affect enzyme activity. Peroxide levels were observed to change over a six month period when the oil samples were stored at 6 °C. This trend was observed by the FTICRMS experiments with increases in the oleic acid concentration.

This information has wide reaching ramifications for the macadamia nut industry. It suggests that the activity of the enzymes needs to be minimized, especially in the more traditional cold pressed extraction. Also, the kinetics involved in the enzymatic rancidity need to be investigated in order to determine the maximum period permissible between performing the extraction and analyzing the oil prior to significant changes in the oil composition occurring due to enzyme activity. This information also indicates that enzymatic rancidity may play a larger role than free radical oxidation. Future research should be directed to understanding the suite of enzymes present in macadamia nuts to minimize their effect on

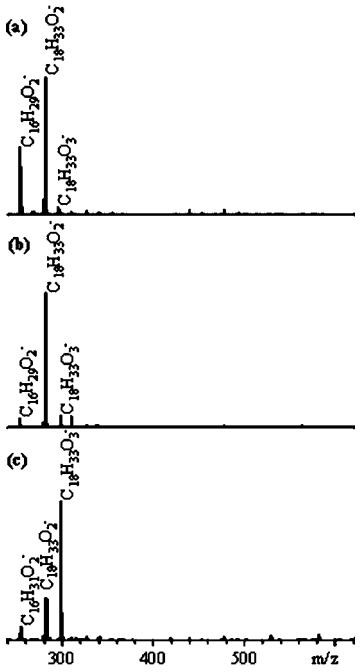

Figure 6. Negative ion FTICR mass spectra of methanol extracted oils from (a) cold pressed and not accelerated aging, (b) cold pressed and accelerated in aging for 14 weeks, and (c) FexIKA extracted and accelerated in aging for 14 weeks. Samples were stored at 6 °C for 6 months.

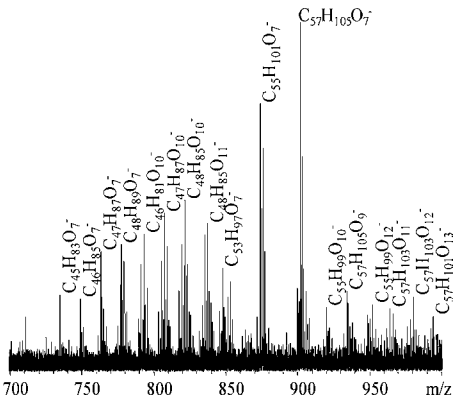

Figure 7. Expansion of Figure 6a of the region m/z 700−1000.

macadamia oil composition and, consequently, kernel and oil quality.

AUTHOR INFORMATION

Corresponding Author

*Tel: +61-2-9351 2738. Fax: +61-2-9351 3329. E-mail: n.proschogo@chem.usyd.edu.au.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ESI, electrospray Ionization; FTICRMS, Fourier transform ion cyclotron resonance mass spectrometry; GC−MS, gas chromatography mass spectrometry; HPLC, high performance liquid chromatography; NIR, near infrared; PV, peroxide value; MeOH, methanol; IPA, isopropanol; NIS, nut in shell

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