Quantification of Nitro-toxins in Karaka (*Corynocarpus laevigatus*) Drupes

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Abstract

The karaka (*Corynocarpus laevigatus*) is a tree native to New Zealand (NZ), found throughout the North and South Islands. Traditionally, many parts of the karaka were used by Māori, however the parts of the karaka of most interest are the berry and nut as they were valuable food sources, in particular the nut. Karaka was known to be a toxin-bearing food, however implementation of a traditional baking and soaking process meant that the nuts were left in states fit for consumption, more often than not. The nuts also had the benefit of being able to be stored for future use when other food sources were short in supply. Karaka was readily consumed up until the 1950s, after this consumption rates decreased rapidly, after the toxic effects were associated with the incorrect preparation of the nuts.

Studies on karaka have revealed that the toxicity of the nuts primarily arises from various nitropropanoyl glucopyranoses (NPGs), twelve of which have been detected in karaka. The quantity of these NPGs has been infrequently studied and it is often only one of the NPGs-karakin which is quantified, although it is believed the toxicity in nuts arises collectively from all of the NPGs. As a result, there is the need to be able to quantify the NPGs in karaka and look at potential toxin removal techniques. If toxin removal can be accomplished, there is the potential for karaka to be a marketable 'traditional' or 'Kiwi' food item. Additionally, the toxins removed as a by-product may prove to have their own potential commercial applications, for example as insecticides or repellents.

Since all the NPGs are assumed to be toxic, a method was developed to quantify the total toxicity in the nuts. The method involves the release of NPA *via* acid hydrolysis of the nitropropanoyl ester groups of the NPGs. The NPA is then able to be measured and subsequently quantified using HPLC. The method incorporates a correction factor (CF) that was determined to give the original NPA content at a time of zero (NPA_0), accounting for the unavoidable loss of NPA that arises from the hydrolysis method.

The average quantity of NPA in karaka nuts was found to range from 50.25 to 138.62 g kg^{-1} (dry weight) which is equivalent to 5.0 to 13.9%. These are much higher percentages than any previously reported because the method used in this study measured total NPA. Earlier methods measured a limited range of NPGs (often a single NPG) unhydrolysed and could have missed NPA arising from other compounds that contribute to total toxicity. Additionally, the concentrations of NPA in karaka nuts were influenced by a number of factors including intra and inter tree variation, storage conditions and ripeness. Although quantification was focused on determining NPA content in nuts, a test conducted also showed NPA to be present in increasingly lower levels in the berry flesh, shell and pellicle respectively.

Nuts were subjected to various potential industrial processing techniques, treatment times and conditions, in order to determine their efficacy for toxin removal. Treatments included boiling, microwave cooking, soxhlet extraction, oven roasting, autoclaving and cold-water treatments. The efficiency of each treatment varied considerably, with both heat and water proving to be beneficial in toxin removal. However, treatments involving water were found to be more effective than heat treatments alone. Out of all treatment types, times and conditions tested, none were found to leave nuts with a NPA concentration lower than the estimated safe level of daily consumption for a 70 kg adult (< 1.75 mg). Additionally, only some of the treatments resulted in nuts being left in visually appealing states. At present, toxin removal by a single process in order to render karaka nuts safe to consume appears to be an impractical activity. However, if treatment protocols were combined and modified, there is the potential for the development of an effective detoxification process. Furthermore, tests on the treatment solutions revealed that NPA can be obtained in solution as a by-product of nut treatment; with cold-water proving to be more effective than treatments that also used heat. Obtaining NPA as a by-product of toxin removal in nuts appears to be a feasible option for a natural source of NPA.

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α	alpha (anomeric configuration)
β	beta (anomeric configuration)
AA	acetic acid
Au	absorbance units
CCC	counter current chromatography
CF	correction factor
DL	detection limit
GC	gas chromatography
HPLC	high performance liquid chromatography
LD	lethal dose
LOQ	limit of quantification
MA	malonic acid
MLD	minimum lethal dose
mg NO_2 g^{-1}	milligrams of nitrogen dioxide per gram
NMR	nuclear magnetic resonance
NP	nitropropanoyl aglycone ($COCH_2CH_2NO_2$)
NPA	nitropropanoic acid
NPG	nitropropanoyl glucopyranose
NZ	New Zealand
PC	plate chromatography
TLC	thin layer chromatography
UV	ultra violet light
w	weight

1 Introduction

1.1 The karaka tree

1.1.1 The genus *Corynocarpus* and *Corynocarpus laevigatus* (karaka)

Corynocarpus is the only genus of the Corynocarpaceae family. The genus has six species; *C. australasicus, C. cribbianus, C. dissimilis, C. rupestris, C. similis, C. laevigatus*, which grow on various islands in the Western Pacific Ocean, including New Caledonia, Papa New Guinea, Australia and New Zealand (NZ).[1,2]
C. laevigatus is native to NZ and is more commonly known as the karaka tree. It can be found throughout NZ in both coastal and lowland forests of the North and South Islands, as well as in the Chatham and Kermadec Islands.[3] In the Chatham Islands, the karaka is often referred to by the name 'Kopi'.[4] There are a large number of trees found throughout NZ, in the form of both deliberately planted and 'wild' trees and groves.[5] The trees are plentiful in the North Island but are not so prevalent in the South Island. Their location often relates to areas historically occupied by Māori settlers, especially in and around old pā sites.[6,7]

1.1.2 Karaka tree description

The karaka tree is an evergreen, which has large glossy dark green leaves and grows up to 18 metres in height (**Figure 1.1**).[3,8] The trees are most distinctive for bearing plentiful quantities of bright orange fruit (berries or drupes) and will bear fruit from as young as 5-6 years old.[3,5] Karaka drupes grow in clusters which ripen from green to bright orange (**Figures 1.2** and **1.3**). The tree flowers from late winter through spring, and fruits from mid-summer through to autumn.[9] In NZ, the season for berry yield is December through to March. Trees produce an average of 20-30 kg of berries per tree and up to 50 kg for individual trees.[5] The fruit is variable in size and can range from 2-5 cm in length.[8,10] The part of the karaka drupe that is of most nutritional interest is not the berry but the elliptically shaped kernel/nut which is enclosed inside a fibrous coat under the berry flesh (**Figure 1.4**).[3,11,12]

Figure 1.1 Karaka tree in fruit, located on the corner of Victoria and Bridge Streets, Hamilton.*

Figure 1.2 Clusters of karaka drupes at early stages of ripening, most drupes are still green.

*Image obtained from and printed with the permission of D Klinac.

Figure 1.3 Clusters of karaka drupes which are almost ripe.

Figure 1.4 Karaka nut with pellicle, enclosed in its' fibrous shell, with the berry flesh removed.

1.1.3 Historical uses of karaka leaves, wood, berries and nuts

Historically, there are reports of various parts of the karaka tree being utilised. The trees were often used as boundary markers and some hollow trees were even used as burial places. The leaves were used to dress wounds, the wood for making paddles and most importantly the berries and nuts were used as a food source.[5,12] Karaka nuts are known to have been second only in importance to the kumara tuber, meaning that the nuts were a staple part of the Māori diet.[13] The nuts were a highly valuable food source as they were able to be stored and eaten during winter when other food was in short supply.[5,12] The nuts were also important in traditional ceremonies, banquets, funerals and in formal exchanges between tribes.[5] Many reports show that karaka nuts were readily consumed up until the 1950s. However, the popularity of karaka nuts as a part of the staple diet for the Māori diminished greatly after the 1950s. The main reason for this was the realisation that there were often detrimental and on occasion fatal toxic effects on the consumers, especially if the nuts were prepared incorrectly. Additionally, there was easier access to alternative food sources (without associated toxicity).

1.1.4 Historical preparation of karaka nuts

The traditional preparation of karaka nuts involved baking the fruit in earth ovens (‘hangi’ / underground pits) for several hours. The fruit was then placed in woven baskets laced up and placed in a nearby river or stream over rocks and left for a period of time (usually a few days).[5,11,14-16] This process was accepted as the best way of loosening the flesh and removing some of the ‘bitter’ taste often associated with the nuts (possibly the flavour of the nitro-toxins). Afterwards, the nuts were dried in the sun and stored in baskets for future use.[5,11,14-16] The Māori employed two distinct processes, baking and soaking in their preparation of the nuts. It has never been established whether both processes are needed to remove the toxic properties of the nut and, if not, which is most important.[14]

1.2 Why the karaka nut is of interest

Nutritional composition tests show that karaka was a valuable addition to the Māori diet. The berries contain the sugars sucrose and glucose, the fatty acids stearic and oleic acid, and six of the eight essential amino acids (leucine, methionine, phenylalanine, threonine, tryptophan and valine), while the treated nuts have a food value resembling that of oatmeal.[5,11]

Table 1.1 Treated* karaka nut percentage composition[17]

Composition of treated karaka nuts	
Carbohydrate	58%
Fat	15.5%
Water	11.9%
Protein	10.9%

*Treated nuts were steamed (7 h) followed by soaking (3 wk) before being sun dried (72 h) as by McCurdy. [17]

Tests on the karaka nut have allowed for valuable comparisons to be made with other tree nuts. The results of the tests have shown that karaka nuts are low in starch but high in protein compared to chestnuts; have relatively high levels of fatty acids (with higher levels being reported only in walnuts); high dietary fibre and a higher energy content than other tree nuts.[5] In addition, AsureQuality Ltd. analysed karaka nuts and reported that they are gluten free. This makes karaka nuts suitable for people with coeliac disease.[5]

As a result, there are many potential commercial applications for karaka, especially in the food industry, including dried karaka nuts and berries, health bars, beers and liqueurs, karaka flour and gluten-free bakery products.[5] However, before commercial applications of any kind can be considered, the toxicity and removal of toxins from karaka nuts needs to be investigated.

There are also a range of potential spin-off applications for the toxic elements, such as karaka derived insecticides and repellents.[5]

1.2.1 Benefits of nuts in the human diet

Around the world, nuts are a commonly consumed food item, with individual intakes varying. Some of the nuts readily consumed by individuals include peanuts, cashews, pine nuts, pistachios, walnuts, Brazil nuts, macadamia nuts, hazel nuts and more.[18] Nuts are known to be high energy foods (23.4 to 26.8 kJ g^{-1}) with high levels of natural fats (45 - 75% weight), most of which are the more desirable unsaturated fats. In addition, nuts are a good source of protein, fibre, vitamins (Vitamins B6 and E, niacin and folic acid) and minerals (copper, magnesium and potassium) as well as containing various phytosterols and polyphenols.[18-20] Health professionals have long recommended nuts in a healthy diet, mainly owing to the variety of benefits nuts have been associated with inclusive of antioxidant, anti-inflammatory, anticancer, cardio-protective and anti-diabetic benefits as well as other functional properties.[18-20] If karaka nuts can be shown to provide these health benefits, it adds to the reasons why toxin removal needs to be investigated. Not only would they be marketable as a 'Kiwi' or traditional NZ food, but also in the health industry. However, the additional health properties of karaka nuts will need investigation in the future, once toxin quantification and removal is achieved.

1.3 Occurrence of nitro-toxins in plants

Worldwide, thousands of species of plants are known to contain nitro-compounds. A classic example is the species *Astragalus* of the Leguminosae family. More than 450 species of *Astragalus* are known to contain nitro-compounds, as either nitroproanoic acid (NPA) and its' derivatives or nitropropanol and its' derivatives. It is interesting to note that a species that produces NPA and its derivatives will not produce nitropropanol and its derivatives and vice versa.[21,22] Other legumes such as *Corinilla, Indigoferra,* and *Lotus* have been found to synthesise NPA. NPA is also reported to be synthesised by members of the Malpighiaceae, Violaceae and Corynocarpaceae families, the latter is the family to which karaka belongs.[21] The foliage of many of these species is consumed by various livestock including sheep and cows. Most species are not consumed by humans with the exception of *Corynocarpus,* but some are used for medicinal purposes. For information on the biosynthesis of NPA in plants see **Section 1.4.3.1**.

1.4 Nitro-compounds of potential toxicity in the karaka nut

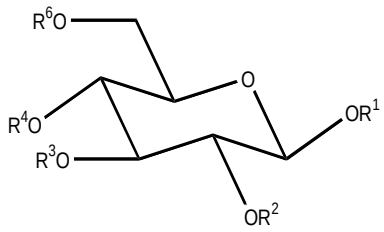
Karaka nuts are known to contain various toxic nitropropanoyl glucopyranoses (NPGs).[23,24] NPGs are glucose esters of nitropropanoic acid (NPA).[25] It is NPGs that are believed to be responsible for the toxicity and 'bitter' taste associated with incorrectly prepared karaka nuts. However, it is unknown which of the NPGs found in karaka nuts have the greatest toxicities or in what concentrations they occur in karaka nuts.[23] It is also unknown whether it is the pure NPGs or chemically modified products, such as those formed by hydrolysis (including NPA) that are responsible for the toxicity.[26] More information on NPA itself can be found in **Section 1.4.3**.

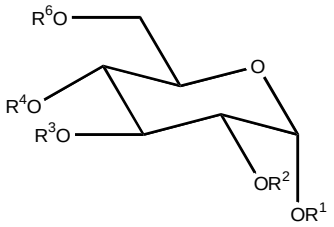
1.4.1 The NPGs found in karaka nuts

In 1871, Skey[14] isolated and characterised one of the chemicals believed to be responsible for the bitterness of karaka nuts as a glucoside which was subsequently named 'karakin'. The chemistry of this glucoside was further investigated by Easterfield and Aston,[6,27] Carrie[28] and Carter.[29,30] Carter established that the molecular formula was $C_{15}H_{21}O_{15}N_3$, and suggested that the structure was 1,4,6-tris-(β-nitropropanoyl)-D-glucopyranose. However, the correct structure was later elucidated as 1,2,6-tris-(β-nitropropanoyl)-D-glucopyranose (**1**).[24,31]A subsequent investigation of karaka nuts by Moyer *et al.*,[24] found karakin to be accompanied by four additional esters of 3-nitropropanoic acid with glucose; cibarian (**2**), coronarian (**3**), corynocarpin (**4**) and corollin (**5**). The occurrence of a further six unidentified nitro-compounds was also noted.[24] In 1993, Majak and Benn successfully used thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) analyses to identify the six compounds as: 3,4,6-tris-O-(3-nitropropanoyl)-α-D-glucopyranose (**6**), 6-O-(3-nitropropanoyl)-α/β-D-glucopyranose (**7a,b**), 1,3,6-tris-O-(3-nitropropanoyl)-β-D-glucopyranose (**8)**, 1,2,3,6-tetrakis-O-(3-nitropropanoyl)-β-D-glucopyranose (**9**), 1,3,4,6-tetrakis-O-(3-nitropropanoyl)-β-D-glucopyranose (**10**) and1,2,4,6-tetrakis-O-(3-nitropropanoyl)-β-D-glucopyranose (hiptagin, (**11)**).[23]

The structures of all known NPGs found in karaka nuts are shown in **Figure 1.5**. Theoretically, all of the NPGs should contribute to the toxicity, however it is

unknown what concentrations of each are toxic, and in what concentration each of the NPGs occur. Hence, there is a need for further investigation and the quantification of these compounds.





β-Glucopyranosides

1. $R^1=R^2=R^6=$ NP, $R^3=R^4=H$

2. $R^1=R^6=$ NP, $R^2=R^3=R^4=H$

4. $R^1=R^4=R^6=$ NP, $R^2=R^3=H$

7a. $R^6=$ NP, $R^1=R^2=R^3=R^4=H$

8. $R^1=R^3=R^6=$ NP, $R^2=R^4=H$

9. $R^1=R^2=R^3=R^6=$ NP, $R^4=H$

10. $R^1=R^3=R^4=R^6=$ NP, $R^2=H$

11. $R^1=R^2=R^4=R^6=$ NP, $R^3=H$

α-Glucopyranosides

3. $R^2=R^6=$ NP, $R^1=R^3=R^4=H$

5. $R^2=R^3=R^6=$ NP, $R^1=R^4=H$

6. $R^3=R^4=R^6=$ NP, $R^1=R^2=H$

7b. $R^6=$ NP, $R^1=R^2=R^3=R^4=H$

NP = $COCH_2CH_2NO_2$

Figure 1.5 Nitropropanoyl glucopyranosides (NPGs) found in karaka nuts.

1.4.2 NPGs found in other sources

The isolation of karakin (**1**) from *C. laevigatus* is the first known isolation from a natural source of a compound with an aliphatic nitro component.[32] The isolation of karakin has subsequently been followed by isolations of other NPGs over the last century and a half, from *Corynocarpus* as well as other plant genera (**Table 1.2**). Often, when esters of NPA are isolated, karakin has been reported as the major nitro-compound (NPG) present.

NPGs found in other genera include: 4,6-di-*O*-(3-nitropropanoyl)-α/β-D-glucopyranose[33,34], 2,3,6-tri-*O*-(3-nitropropanoyl)-α-D-glucopyranose (coronillin)[25] and 2,3,4,6-tetra-*O*-(3-nitropropanoyl)-α-D-glucopyranose.[34,35] Although NPGs are usually referred to as glucose esters of nitropropanoyl, the term endecaphyllin is sometimes used. One of the endecaphyllins from species of *Indigofera* is identical to karakin.[26,36] Other endecaphyllins identified in *Indigofera* research also appear to be identical with other NPGs described here.[34-36]

1.4.3 Nitropropanoic acid (NPA)

Nitropropanoic acid (NPA) or 3-nitropropanoic acid (IUPAC) is known by various common names, including β-nitropropionic acid and hiptagenic acid.[15] NPA is toxic to animals and has been isolated from various species of plants and microorganisms. In plants, it is more commonly found as a derivative such as a glucose ester (NPG) than as the free acid.[15] NPGs such as karakin are known to hydrolyse rapidly in mammalian digestive systems to release NPA.[21] In non-ruminants such as humans, NPGs are metabolised by esterase enzymes in the upper digestive tract, releasing NPA which is rapidly absorbed into the bloodstream. In contrast, ruminants are protected from such an increase in NPA concentrations in the blood, by the action of microbial enzymes in the rumen.[21,32] The mode of action of NPA upon absorption is the non-competitive inhibition of succinate dehydrogenase, a mitochondrial membrane enzyme. As a result, mitochondria are no longer able to oxidise succinate to fumarate, resulting in changes to brain tissue that resemble the effects of Huntington's disease, a neurodegenerative disease.[15]

1.4.3.1 Biosynthesis of NPA/NPGs in plants and fungi

NPA is a toxic metabolite produced by both plants and fungi. It appears the biosynthesis routes in plants and fungi are different. This is illustrated by an isotopic-labelled feeding study on *Indigofera* that found malonate and hydroxymalonate to have a precursory relationship to NPA.[37] In contrast, biosynthesis studies on NPA from the fungus, *Penicillium atrovenetum* showed that the carbon skeleton is derived from L-aspartate.[38] The biosynthetic pathway to form NPA is believed to occur from the various precursors as described above however, currently the mechanism for esterifying NPA to a glucose molecule and forming the various NPGs appears to be unidentified.[39] In addition, the correlation between NPA and enzyme concentrations in various types of plant tissue remains to be determined. As yet, it is also unknown if NPA and NPGs when synthesised in one tissue can be trans-located to other parts of the plant for storage.[39]

Table 1.2 NPGs found in *Corynocarpus* and in other genera.

Compound	Position of NPAs and anomeric configuration[a]	Common name	Structural data	Genera
1	1,2,6-β	Karakin	MS[40] NMR[40] synthesis[31]	*Astragalus*[40], *Coronilla*[25,41], *Hippocrepis*[42], *Indigofera*[31,34], *Lotus*[43], *Corynocarpus*[23,28-31]
2	1,6-β	Cibarian	MS[33] NMR[33]	*Astragalus*[33,40], *Coronilla*[25,41], *Hippocrepis*[42], *Lotus*[44], *Corynocarpus*[23,24]
3	2,6-α	Coronarian	NMR[25]	*Coronilla*[25], *Indigofera*[33,35], *Lotus*[44], *Corynocarpus*[23,24]
4	1,4,6-β	Corynocarpin	NMR[24]	*Corynocarpus*[23,24]
5	2,3,6-α	Corollin	NMR[25]	*Coronilla*[25], *Corynocarpus*[24]
6	3,4,6-α	-	NMR[23]	*Indigofera*[34], *Corynocarpus*[23]
7a	6-β	-	MS[41] NMR[23,41]	*Coronilla*[41], *Astragalus*[40], *Hippocrepis*[42], *Corynocarpus*[23], *Indigofera*[35]
7b	6-α	-	MS[41] NMR[23,41]	*Coronilla*[41], *Astragalus*[40], *Hippocrepis*[42], *Corynocarpus*[23], *Indigofera*[35]
8	1,3,6-β	-	NMR [23,45]	*Corynocarpus*[23]
9	1,2,3,6-β	-	NMR[23]	*Corynocarpus*[23]
10	1,3,4,6-β	-	NMR[23]	*Corynocarpus*[23]
11	1,2,4,6-β	Hiptagin	NMR[36,44] synthesis[36]	*Astragalus*[40], *Heteropteris*[46], *Lotus*[44], *Hiptage*[36], *Indigofera*[36,47] *Corynocarpus*[23]

a. All compounds are nitropropanoyl derivatives of D-glucopyranose.
Note: The table includes some, not necessarily all genera in which NPGs are found in.

1.5 Toxicology

A number of experiments in NZ to investigate the toxicity of karaka extracts and NPA have been reported. Most of the reports of toxicity are of NPA poisoning rather than from NPGs. This is most likely due to the fact that NPGs are much harder to isolate and detect than NPA, especially considering the analytical techniques available at the time when much of the research was conducted.

There is little in the literature regarding reasons as to why the toxicity of NPGs have not been readily studied. One possible reason is that they appear to have low to sparing solubilities in water. The low solubility of the NPG karakin has been reported in work carried out by Bell,[11] Easterfield and Aston,[6] Carter[30] as well as Hutchins *et al*.[48] In addition, Hutchins *et al.,* stated that the NPGs coronarian and cibarian also had low solubilities in water. These findings are at odds with the fact that NPGs such as karakin are the glucose esters of NPA and glucose groups are generally known to make substances more soluble in water. However, the influence of the alkyl chains might be enough to reduce the H-bonding which normally helps sugars to solubilise. The greater the number of alkyl chains, the lower the solubility one would expect. Alternatively, because researchers used NPGs extracted from a natural source and purified by crystallisation, the low solubility observed may be due to the fact that sometimes crystals can be hard to solubilise.

Although NPA toxicity has been shown to reduce over time, it remains in nuts for considerable time periods. For example, a study conducted by Bell[11] found that some extracts of karakin from karaka nuts were found to remain toxic even after a year. These findings confirm the Māori's belief that the fruit contained some sort of toxin.

Other studies have shown that lethal doses are dependent on the way in which toxins are introduced to animals and whether or not the animal is a ruminant (**Section 1.4.3**). An example of this is that the lethal dose of NPA in sheep has been found to be 52 mg kg^{-1} if introduced intravenously, while it is more than doubled (118 mg kg^{-1}) if administered orally.[21,32]

1.5.1 Variation in karakin content with age

Easterfield and Aston[6] reported that the concentration of karakin diminishes as fruit ages; 0.3% karakin was recorded in fresh nuts; 0.1% after three months and after 12 months the nuts were still bitter but only a small amount of karakin could be detected. These results were obtained by an alcohol extraction and direct crystallisation. Since the percentage calculation is based on weight, it is likely that there is an underestimation of the actual levels. The observed decline in toxin concentration over time may have been due to a number of factors. Storage conditions are the most likely factor influencing the decline in the karakin content, although these were not considered in the study. Without knowing what sort of conditions the nuts were stored under and without further investigation no conclusions can be made, other than that with increased storage time karakin content was found to diminish. Whether the toxins diminish to non-toxic levels with storage is still in need of investigation, especially considering that Bell[11] obtained results indicating that some 20-year old berries were still toxic.

1.5.2 Observed effects of poisoning on humans from karaka consumption

Some of the symptoms associated with poisoning through eating raw or incorrectly prepared nuts were violent spasms, convulsions of the whole body, hot flushes and protrusion of the eyes and tongues.[13,14] Frequently, victims were buried in sand up to their heads to reduce the effects of distorting limbs.[13,14] Often, unless speedily attended to, poisoning by karaka proved to be fatal. Despite this, there are reports of Māori enjoying the taste and valuing karaka as part of their diet until around the 1950s.

1.5.3 Observed effects of poisoning on animals from karaka consumption

There are widely differing reports on the extent to which animals are affected by consuming karaka nuts and which particular animals are most sensitive to the toxins. This is most likely due to the quantity and differing toxicity of nuts consumed, but may also arise due to the differing metabolic or digestive pathways to which the toxins are subjected in various animals (as discussed in **Section 1.4.3**). Both formal and informal observations including sheep vomiting, pigs and cattle losing power over their hind limbs and dairy cows' milk drying up, have all been reported after consumption of the nuts and/or foliage of karaka trees.[11,49-50] There is also a case in which a kiwi is believed to have been poisoned by consuming karaka nuts.[5] Historically, there is a report that the nectar of the karaka tree was toxic to honey-bees.[51] However, in recent years, many of these effects have only been observed infrequently despite the widespread distribution of karaka trees, even in urban areas.[5] This is probably related to the dietary changes of humans and the implementation of fencing as opposed to free grazing for livestock, over the last 60 or so years.

1.5.4 Toxic consumption levels in various animals

Various species of animal have been used to investigate the toxicity of nitro-compounds. Poisoning has been experimentally demonstrated in mice, rats, chickens, pigs, rabbits and pigeons.[11,15,21] A toxicity study using karakin and NPA showed that a lethal dose of karakin is 100 mg kg^{-1} for pigeons. The minimum lethal dose (MLD) of NPA was 60-70 mg kg^{-1} (with a range of 38-80 mg kg^{-1}) for pigeons and 100 mg kg^{-1} (with range of 65-120 mg kg^{-1}) for rats.[11] Another study concurs with these results, reporting an LD_{50} for rats and mice between 60-120 mg kg^{-1} depending on the mode of introduction.[15] In addition, a concentration of karakin extract that was toxic to pigeons, was found to retain toxicity even after a year of storage, however the storage conditions of the extract are unstated.

1.5.5 NPA exposure and toxicity to humans

NPA is known to be produced by common moulds such as *Aspergillus, Penicillium* and *Arthrinium. Aspergillus* is widely used as an economic mould in the production of foods. As a result, NPA has been found in trace amounts in various routinely consumed foods including cheese, soybean, peanuts, potatoes and bananas. Thus, there is a known lengthy and widespread exposure of humans to low levels of NPA.[15]

Japan is a population that has well known exposure to NPA through food products such as soy sauce and miso, with daily intakes estimated to be as much as 5.5 mg day^{-1} (0.079 mg $kg^{-1}day^{-1}$ for a 70 kg adult).[15] Despite the common consumption of foods containing NPA producing moulds, human poisonings are rare and often confined to specific circumstances, for example the accidental poisonings in China in the 1970-1990s which arose from the sale of mouldy sugar cane. This should have been avoided if good manufacturing practices were in place.[15]

Owing to the toxicity and lack of antidote, no tolerance studies of NPA or NPGs have ever been trialled on humans. However, one study predicted a safe average daily intake of NPA could be 0.025 mg kg^{-1} day^{-1} or 1.75 mg day^{-1} for a 70 kg adult.[15] This value was based on the 'no observed adverse effect limit' (NOAEL) of a chronic rodent study with a safety factor of 100.[15] This value falls well below what is estimated as the average daily consumption by the Japanese. Thus, one could consider this to be well on the 'safe' side of estimation. However, without further research, this cannot be concluded as a definitive safe level.

1.5.6 NPA toxicity versus NPG toxicity

Few studies have done research whereby comparisons of NPA and NPG toxicities have been made. In studies that have made a comparison, it appears that free NPA is less toxic than the glucose esters (NPGs). One particular study conducted by Greeenwood[43] on the metabolism of karakin by larvae of grass grubs, found that the esters themselves appear more toxic than the equivalent amount of NPA

(accounting for the fact that one mole of karakin is capable of producing three moles of NPA). The differing toxicities may reflect the different subjects of the studies and the way in which the substances are absorbed in these different species (see **Section 1.4.3**). Currently, there are no studies indicating whether it is NPA or NPGs that are most toxic to humans.

1.5.7 Organic acid degradation

In karaka nuts, hydrolysis of the nitropropanoyl groups from the glucoside of the NPGs, results in the release of nitro-substituted propanoic acid molecules-NPA (**Figure 1.6**). All carboxylic acids can be degraded under a variety of conditions including heat (thermolytic), pressure, photolytic, microbial (anaerobic and aerobic) and more.[52-54] Processing conditions are likely to include heat and pressure. In the case of consumption by ruminants, microbial action may be important but the latter is outside the scope of this thesis.

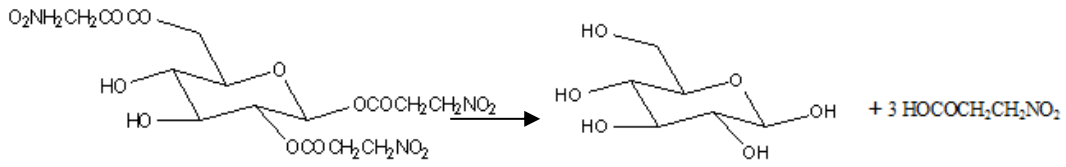


Figure 1.6 Hydrolysis of the nitropropanoyl groups of the glucoside karakin; hydrolysis yields one mole of glucose and three moles of nitropropanoic acid.

Decomposition of NPGs and NPA

The main routes of NPG and NPA decomposition known to date involve heat and/or hydrolysis reactions.

1.5.7.1 Hydrolysis of nitropropanoyl glucopyranoses

The NPG karakin has been reported by Bell[11] to be sparingly soluble in cold water, but hydrolyses almost completely to NPA when boiled in water for more than six hours.[26,30] The hydrolysis of karakin yields glucose and NPA[29] (**Figure 1.6**). NPA itself is hydrolysed to give hydroxylamine and malonic (MA) which subsequently degrades to yield acetic (AA) acid and carbon dioxide, **Equation 1.1.**[29]

Hydrolysis of karakin by Carrie[28] whereby 0.5 g of karakin was sealed for two weeks in a container with 2 mL of HCl and 8 mL of acetone, revealed that one mole of karakin yields three mole of NPA and one mole of glucose. This indicates that full hydrolysis of karakin is possible and hydrolysis of the other NPGs would also be expected to occur in a similar fashion.

$$NO_2CH_2CH_2CO_2H \rightarrow OHNHCOCH_2CO_2H \rightarrow NH_2OH + CO_2HCH_2CO_2H \rightarrow MeCO_2H + CO_2 \quad (1.1)$$

1.5.7.2 Decomposition of NPGs and NPA using heat and pressure

NPA has been reported to be completely decomposed via the route in **Equation 1.1**, when heated to temperatures of 100 ºC.[55] Autoclaving plants (*Indigofera*) for one hour at 100 ºC at 15 lb/in^2 of pressure has also been shown to decompose the acid almost completely, to a level whereby the toxicity of NPA was eliminated (based on a rodent feeding trial).[55]

These findings, along with Skey's[14] report that heating nuts at 212 ºC for four hours or soaking the nuts for two days results in loss of bitterness to taste, appear consistent with the historical Māori preparation of karaka nuts which generally yielded nuts that were safe for consumption.

1.6 Techniques used for extracting nitro-compounds from plant material

Extractions of nitro-compounds from plants have all been performed in a similar manner. Plant material was crushed or ground, followed by an initial extraction using an organic solvent such as acetone. This was followed by washing with a non-polar solvent such as hexane to remove fatty acids. Following this, an optional extraction into solvent such as ethyl acetate is performed before being concentrated. The concentrate is then ready for separation and characterisation. One of the most refined methods for extracting nitro-compounds from karaka nuts was developed by Majak and Benn.[23] Nitro-compounds were extracted using acetone as the solvent; the concentrate was suspended in water and washed with

hexane prior to further concentration. The final concentrate was dissolved in propanone and separated on silica gel columns using a gradient elution scheme of $CHCl_3$-Me_2CO (1:9-9:1), whereby 500 mL of solvent was used for every 10% increase of propanone.

1.7 Techniques used for isolating and characterising NPGs

NPGs have been identified in various species of plants using a variety of methods. Common techniques involve an initial separation followed by isolation. Isolation is usually performed by either direct crystallisation or by chromatography, usually plate (PC) or thin layer (TLC) and on occasion counter current chromatography (CCC). During the isolation, reagents such as Griess-Illovsay reagent or *para*-nitroaniline sprays have been used successfully to identify the presence of nitro-compounds.[23,40,56] Identification of NPGs can be a difficult task. The isolation and extraction of many of these compounds were carried out in the early to mid-1900s, hence the readily available isolation techniques were TLC and PC. Today more efficient and effective techniques such as HPLC would be utilised. Techniques such as NMR have proven to be the most effective for characterisation, with the NMR spectra of many NPGs available in literature. Although the resolution of NMR instruments at the time was lower than the present day, the structural elucidation remains valid. Other techniques such as IR and GC with chemical ionisation have also been used to identify some of the NPGs.

1.8 Quantification of nitro-compounds

1.8.1 Quantification of NPGs

Quantification of NPGs has been restricted to a few cases. Percentage concentration of individual NPGs has been calculated but only by weight (through direct crystallisation) or through reactions that have yielded NO_2 to be measured.[24] Techniques to simultaneously quantify multiple NPGs appear to have not been studied to date.

The quantity of toxins found in karaka berries or nuts has been investigated infrequently. It must be noted that when quoting NPG values found in karaka nuts

or berries, one must be very careful. The literature is flawed because often the concentrations of NPGs, such as karakin, are quoted as a concentration arising from the berry, when in fact it has arisen from the nut itself. The ambiguity surrounding some of the quoted values allows for some misinterpretation. Illustrations of this include when Carter[30] states 'karakin, a crystalline glucoside extracted from the berries of the karaka tree has been the subject of previous investigation', implying the karakin is found in the flesh of the berry. However, if one looks at the literature references from which this information was obtained- Aston and Easterfield[27] and Carrie,[28] it can be seen that the karakin was not extracted from the berries but the nuts. In addition, Raistrick and Stossl[57] make a statement that could be misinterpreted as it misrepresents the original research: 'Carrie investigated the known glycoside karakin from the berries of the NZ karaka tree...', again the karakin was obtained from the nuts. Furthermore, when talking about karakin levels, Bell[11] states 'McChesney[58] found 0.08% in whole ripe berries from Paekakariki compared with 0.025% in whole green berries from Dunedin'. If one looks at the original research, it is found that these values arise from the nuts not the berry flesh and again ambiguity prevails. Thus, when quoting NPG or NPA values, it is vitally important to make it explicit as to what part of the karaka drupe the concentration is attributed (e.g., berry flesh, nut, shell or pellicle), in order to avoid misinterpretation.

It has been reported that the concentration of karakin found in nuts varies with location and ripeness, it appears that climate, season, soil, storage conditions and other factors may also play a part in the toxin content of the fruit and nuts. An illustration of this is that McChesney[58] found the concentration of karakin to vary depending on ripeness and location; with 0.08% in nuts from whole ripe drupes (from Paekakariki), while nuts from unripe drupes (from Dunedin) were found to contain 0.025% karakin. However, one must be careful interpreting this result, as it is unknown which of the two variables, ripeness or location has the most significant effect on the differences in concentrations observed. In addition, storage effected a change in concentration of karakin; 0.3% karakin in fresh nuts was found to diminish to 0.1% over a storage period of three months.[6] These values were obtained from research where percentages are based on direct crystallisation. The yields reported for extraction are likely to be lower than in a

direct assay. In addition, researchers often only isolated one of the NPGs (karakin), consequently disregarding the contribution of the other presumably toxic compounds.

1.8.2 Quantification of NPA

Several approaches for determining NPA levels have been trialled. The three main methods are thin-layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). Of the three, HPLC has been used most recently as a wide variety of substances can be identified, no derivatisation is required as for GC and it has greater sensitivity than TLC. Initially, quantification of NPA content in plants was performed *via* colorimetric techniques. However, these techniques have disadvantages which led to development of alternative methods, such as the pH dependent spectrophotometric method used by Matsumoto *et al*.[59] A few studies have also implemented the use of HPLC in order to isolate and quantify nitro-compound content. The most successful study by Harlow *et al.*,[40] utilised a micro-Porasil column with different solvent systems (unspecified) and UV detection at 254 nm. Other HPLC analyses that were NPA specific use a single wavelength (210 nm).[15]

Quantities of NPA detected in various plant species, differ considerably (a range of < 1 to 26,000 ppm), where this range is equivalent to < 1 - 26 mg $N0_2$ g^{-1} plant material. This is significant as 1 - 84 mg $N0_2$ g^{-1} of NPA can be fatal to grazing animals.[22] Although NPA content has been determined in a variety of plant extracts, there have been no quantitative studies of NPA content in karaka nuts.

1.9 Research objectives

The primary aim of this research was to develop a method that will allow for the rapid determination of toxin (NPG) content in karaka nuts; based on the concentrations of NPA arising from the hydrolysis of these compounds in karaka. This will allow for the relative determination of the amount of toxins in karaka nuts. The method will also be applied to the investigation of various detoxification treatments of karaka nuts to determine their efficacy.

2 Methods and materials

2.1 Materials

2.1.1 Chemicals

Analytical grade sulfuric acid (95 - 98%) and acetic acid (AA, 99.7%) were sourced from Ajax Finechem Pty Ltd. Malonic acid (MA, 99%) and 3- nitropropionic acid (NPA, > 97%) were obtained from Sigma Aldrich. Trifluoroacetic acid (TFA, > 98%) was sourced from Merck. MilliQ water was collected from a Barnstead E-Pure water filter at a resistivity ~17.9 MΩ.

2.1.2 Karaka drupe and nut samples

The trees for berry (drupe) and nut collection were chosen for locality and ease of accessibility with assistance from a local NZ Treecrop member. There were three main trees from which drupes in various stages of ripening were collected; two on Victoria Street, Hamilton; one by the road (A) and one just off the road by a path (B). The third tree on River Road, Hamilton, had other trees including karaka planted around it (C). The collection dates, stage of ripening, quantities and location of all samples collected were recorded, **Table 2.1**. An additional single sample was collected from a tree at Whatawhata (D), which is known to be over one hundred years old. Upon collection, the drupes and nuts were stored in air tight zip lock bags and stored in a freezer at -17 ºC, until analysis.

Table 2.1 Location, date and collection details of karaka samples

Sample ID #	Tree	Date of drupe/nut collection	Amount of drupes/nuts collected	State of drupes/nuts upon collection
1	C	10/1/12	1 bag of drupes	Unripe, green drupes
2	A	10/1/12	~40 drupes	Unripe, green drupes
3	A	24/1/12	~30-40 drupes	Half ripe, orange/green drupes
4	B	24/1/12	1/3 bag of nuts	Collected off the ground, had little to no berry flesh
5	C	24/1/12	½ bag drupes	Half ripe, orange/green drupes
6	C	24/1/12	½ bag of nuts	Collected off the ground, had little to no berry flesh
7	C	24/1/12	½ bag of nuts	Collected off the ground, had little to no berry flesh. Stored outside exposed to weather conditions (sun and light rain) for 7 weeks, before being frozen (12/3/12)
8	A	24/1/12	~40 nuts	Collected off the ground, had little to no berry flesh. Stored outside exposed to weather conditions (sun and light rain) for 7 weeks, before being frozen (12/3/12)
9	C	6/2/12	1 bag of drupes	Ripe orange drupes
10	C	6/2/12	~50 nuts	Collected off the ground, had no berry flesh
11	A	8/2/12	1 bag of drupes	Ripe orange drupes
12	B	8/2/12	1/3 bag of drupes	Ripe orange drupes
13	B	8/2/12	~60 nuts	Collected off the ground, had no berry flesh
14	C	8/2/12	1 bag of drupes	Ripe orange drupes
15	C	8/2/12	~40 nuts	Collected off the ground, had no berry flesh
16	D	13/2/12	1 &1/3 bag of drupes	Ripe orange drupes

Note: 1 full bag of drupes is approximately ~ 120

2.2 General methods

2.2.1 High performance liquid chromatography (HPLC)

2.2.1.1 HPLC configuration

HPLC was carried out using a Waters pump (model 515), a Waters photodiode array (PDA) detector (model 2996), a Rheodyne 20 µL injector loop and a BIO-RAD Micro-Guard® cation H guard cartridge. Separation was performed on a BIO-RAD Aminex® HPX-87H ion exclusion column (300 mm length, 7.8 mm internal diameter), eluted isocratically at 0.5 mL min^{-1} with 0.005 M sulfuric acid as the mobile phase. The total run time was 51 minutes for detection of NPA, MA and AA in nuts because of two small late-eluting unidentified peaks. This was reduced to 35 minutes for detection of NPA only and the two peaks previously mentioned then carried over to the beginning of the subsequent chromatogram but did not interfere. Detection was carried out monitoring wavelengths between 190 and 400 nm. The chromatogram at 210 nm was extracted and integrated using Empower Pro® software. All HPLC analyses were carried out under these conditions unless otherwise stated.

2.2.1.2 Detection of compounds

Standards of 3-nitropropionic acid, malonic acid and acetic acid (NPA, MA and AA) were analysed by HPLC and were found to elute at ~ 23.2, ~ 12.1 and ~ 18.1 minutes respectively. In addition, an aliquot of solution from a nut that was heated (100 ºC for 180 min) in sulfuric acid (1 M[a]) was analysed. Equal aliquots of this sample were then spiked, one with MA, one with NPA and another with AA. These were also analysed and confirmed the elution times of NPA, MA and AA as being identical with the standards. There were no observed shifts in compound elution times due to matrix effects. The UV spectra of a chromatogram extracted at a wavelength of 210 nm, for NPA, MA and AA can be seen in **Section 4.1**.

[a] Actual concentration was 0.9875 mol L^{-1}

2.2.1.2.1 Calibration of nitropropanoic acid (NPA)

Standards of 0.003, 0.115, 0.544, 1.077 and 1.466 mg mL^{-1} NPA were made in sulfuric acid (1 M[a]). The NPA standards were then analysed by HPLC.

2.2.1.2.2 Calibration of malonic acid (MA)

Standards of 0.08, 0.11, 0.31, 0.53 and 0.81 mg mL^{-1} MA were made in the same way as the NPA standards and analysed by HPLC.

2.2.1.2.3 Calibration of acetic acid (AA)

Four different concentrations of AA standards, 0.0641, 0.2704, 0.6566 and 1.281 mg mL^{-1} were made in the same way as the NPA standards and analysed by HPLC.

2.3 Extraction of NPGs

2.3.1 Initial preparation of karaka nuts

The karaka nuts were collected as drupes (sometimes without berry flesh) and stored whole in the freezer at -17 ºC. Before any extraction procedure was carried out, the berry flesh was removed, the shell cut open using secateurs and the nut and pellicle removed. The pellicle was peeled off the nut and discarded; the nut was then either prepared for analysis or used in toxin removal treatments.

2.3.2 Hydrolysis procedure used in method development

Nuts were freeze dried overnight and ground until as homogeneous as possible. A homogenate of ~ 1 g was weighed out accurately (5 d.p.) and sulfuric acid (1 M[a], 10 mL) was added. The sample was mixed on a stir-plate (speed 8, 10 min) before being heated (100 ºC, 1 h). The solution was centrifuged (3000 rpm, 1 min) and the supernatant was removed into a vial to be analysed. A sub-sample of 100 µL was diluted to 500 µL using MilliQ water (1:4) and analysed by HPLC.

[a] Actual concentration was 0.9875 mol L^{-1}

2.4 Kinetic trial methods

2.4.1 The degradation of nitropropanoic acid to malonic acid

Four different concentrations (0.5024, 0.5026, 0.2700 and 0.8140 mg mL^{-1}) were made up in sulfuric acid (1 M[a]). Aliquots (14) of equal volumes (3 mL) were placed in individual vials for each concentration and heated in a heating block (100 ºC) for the desired times (t = 0 to 180 min). Each sample was analysed by HPLC using the method outlined in **Section 2.2.1.1**.

2.4.2 The degradation of malonic acid to acetic acid

Three different concentrations (0.1132, 0.511 and 1.0652 mg mL^{-1}) of MA were prepared and treated in the same way as the NPA (**Section 2.4.1**).

2.4.3 The pH dependence of nitropropanoic acid degradation to malonic acid

Three solutions of ~ 0.5 mg mL^{-1} NPA were made using 1 M, 0.1 M sulfuric acid and 1 M TFA as the solvents (Trials 1, 5 and 6 respectively). Aliquots of equal volume (3 mL) were placed in 14 different vials and heated at a temperature of 100 ºC for the desired times (t = 0 to 180 min). Each sample was diluted, 1:4 with deionised water and analysed by HPLC using the method outlined in **Section 2.2.1.1**.

2.4.4 The activation energy of nitropropanoic acid degrading to malonic acid

A solution of NPA (0.389 mg mL^{-1}) was made by weighing out the desired amount of NPA using sulfuric acid (1 M[a]) as the solvent. Three millilitre aliquots of this solution were placed in 42 different vials, 14 vials for each of the three different heating temperatures (60, 100 and 120 ºC). Each batch of vials (14) were heated for various times (t = 0 to 180 min) and the vials were left to cool to room temperature, shaken and then analysed by HPLC using the method outlined in **Section 2.2.1.1**.

[a] Actual concentration was 0.9875 mol L^{-1}

2.4.5 The activation energy of malonic acid degrading to acetic acid

A MA solution of 0.2391 mg mL^{-1} was prepared and analysed in the same way as the NPA (**Section 2.4.4**) with different heating temperatures of 100, 110 and 120 ºC, because temperatures below 100 ºC proved to be too low to show measurable degradation of MA to AA.

2.5 Quantification of NPA concentration in individual nuts

As previously discussed, the toxicity of karaka nuts arises from the release of NPA through the hydrolysis of nitropropanoyl glucopyranoses (NPGs). Karaka nuts contain various NPGs (12 different ones) (**Figure 1.5**), each with varying numbers of nitropropanoic ester groups (between 1 and 4) attached to a glucose molecule that can be hydrolysed to release acid (NPA) which is measured. The quantity of each NPG in individual nuts is unknown and also expected to vary, however full hydrolysis of NPGs is expected. This was previously reported in a study of karakin which was found to yield three moles of NPA per mole of karakin corresponding to the three nitropropanoic ester groups attached to the glucose.[28] Hence, the method was designed to hydrolyse all esters, thus the NPA concentration is equivalent to the total number of nitropropanoic groups afforded by all NPGs found in the nuts. Henceforth, when NPA concentration from karaka is quoted, it is actually the equivalent concentration of NPA bound in the glycosides inclusive of any free NPA found in nuts, because the method does not distinguish between the two.

2.5.1 Final preparation and hydrolysis method for releasing NPA from NPGs in karaka, allowing for quantification as NPA equivalence

Nuts were freeze dried overnight and ground until as homogeneous as possible. A homogenate of ~ 1 g was weighed out accurately (5 d.p.) and sulfuric acid (1 M[a], 10 mL) was added. The sample was mixed (stir-plate, speed 8, 10 min) before being heated (100 ºC, 1 h). The solution was filtered using filter paper (Analytix No. 2) and analysed by HPLC.

[a] Actual concentration was 0.9875 mol L^{-1}

2.5.2 Quantification of NPA in individual karaka nuts

Ten individual nuts were randomly selected from each of the 16 different samples (#1-16), and prepared as in **Section 2.5.1** and analysed by HPLC.

The calculation of original NPA content (NPA_0) in mg (**Equation 2.1**) and mg g^{-1} or g kg^{-1} (**Equation 2.2**) was performed using the following formulae:

$$\mathrm{NPA_0(mg)} = \frac{NPA\ peak\ area}{Slope\ of\ NPA\ calibration\ curve} \times\ volume\ of\ solution\ \times CF^{a} \tag{2.1}$$

$$\mathrm{NPA_0\ (mg\ g^{-1})} = \mathrm{NPA_0\ (g\ kg^{-1})} = \frac{\text{Answer Eqn 2.1}}{Weight\ of\ nut\ used\ (g)} \tag{2.2}$$

2.5.3 Quantification of the nitropropanoic acid concentration in the left and right halves of individual nuts

Five of the 16 samples were randomly selected (#'s 2, 6, 11, 12, 14) and an individual nut from each of these was randomly selected and cut vertically in half (**Figure 2.1**). One half was assigned right (R), while the other was assigned left (L). Both halves were then prepared separately as in **Section 2.5.1**, and analysed by HPLC.

The percentage differences between left and right halves ($\%D_{L/R}$), of the nuts were calculated (**Equation 2.3**).

$$\%D_{\frac{L}{R}}(w/w) = \left[\frac{\text{Largest value (L or R)– smallest value (L or R)}}{\text{smallest value}}\right] \times 100 \tag{2.3}$$

2.5.4 Quantification of the nitropropanoic acid concentration in the top and bottom halves of individual nuts

Individual nuts, one from each of the 16 samples were randomly selected and cut horizontally in half (**Figure 2.1**). The half that was closest to the stalk was

[a] Correction factor as determined as 1.23 in Section 4.3.2.1.

labelled top (T), while the other half was labelled bottom (B). Both halves were then prepared separately as in **Section 2.5.1** and analysed by HPLC.

The percentage difference between tops and bottoms ($\%D_{T/B}$) of the nuts were calculated **Equation 2.4**.

$$\%D_{\frac{T}{B}}(w/w) = \left[\frac{\text{Largest value (T or B) – smallest value (T or B)/}}{\text{smallest value}}\right] \times 100 \qquad (2.4)$$

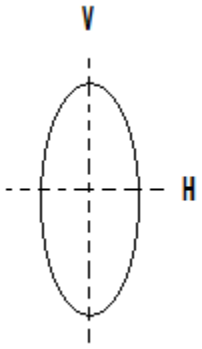


Figure 2.1 Cross section depiction of vertical and horizontal cutting of nuts; H= horizontal cut, V= vertical cut.

2.5.5 Quantification of parts of a karaka drupe; berry flesh, shell, pellicle and nut

Two whole drupes were randomly selected from each of the samples #1 and #9 (1 being green unripe drupes, while 9 was ripe drupes). The berry flesh was removed, the shell cut off and the pellicle (skin) removed, leaving all four separate individual parts. The parts were freeze dried overnight prior to being weighed and prepared for analysis. After freeze drying, the nut was ground into a homogeneous powder, while the berry flesh and shell were cut into small pieces (< $5mm^3$) and the pellicle was left as is. In the case of the nut and berry flesh ~ 0.1 g was used, while for the pellicle and shell the whole sample was used. Once weighed (5 d.p.), all parts were prepared using the same method used for karaka nut preparation (**Section 2.5.1**). All samples were analysed by HPLC and processed using the equations in **Section 2.5.2**.

2.6 Quantification of moisture content in individual nuts

The individual weights of 10 nuts for each of the 16 samples were recorded prior to and after freeze drying. This allowed for the moisture content of individual nuts to be calculated (**Equation 2.5**).

$$\text{Moisture \% in a nut } (w/w) = \left(\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}}\right) \text{x } 100 \qquad (2.5)$$

2.7 Treatment methods

2.7.1 Autoclaving

Nuts were randomly selected from sample 9 and cut vertically in half. One half of each nut was prepared as in **Section 2.5.1**, while the other half was autoclaved (121 ºC, 16 psi) prior to preparation for HPLC analysis. Three nuts for each of the five different autoclaving times were tested; 2, 5, 20, 60 and 120 minutes. The autoclaved nuts were rinsed three times with distilled water (50 mL) directly after removal from the autoclave and the water was discarded and the nuts were prepared as in **Section 2.5.1**. All samples were then analysed by HPLC and processed using equations in **Section 2.5.2**.

In addition to testing nuts, twelve drupes were randomly selected from sample 9; three drupes each for 2, 5, 20 and 60 minutes of autoclaving respectively. Once removed from the autoclave, the drupes were rinsed three times with water (50 mL) and the water discarded. The drupes were cut open and the nuts were extracted from the shell (ensuring to remove the pellicles), following this the nuts were prepared as in **Section 2.5.1,** and analysed by HPLC. The data was processed using the equations in **Section 2.5.2**.

2.7.2 Soxhlet extraction

Four individual nuts were randomly selected from sample 9. The nuts were cut vertically in half, one half was prepared as in **Section 2.5.1**, while soxhlet extraction (17 hours in water) was performed on the other half after it had been

cut into small pieces (~ 3 mm^3). All samples and solutions were analysed by HPLC and processed using the equations in **Section 2.5.2**.

2.7.3 Hydrolysis of solutions in treatment processes

In treatment processes where a set volume of water was used, the solution was retained and analysed for NPA content. Solvent solutions were filtered (Analytix No. 2 filter paper) and analysed by HPLC (unhydrolysed). In addition, a sub-sample of 2.0 mL was diluted with an equal volume of sulfuric acid (2 M[a]) and heated (100 °C, 1 h), the same conditions as previously described for hydrolysing NPGs in nuts (**Section 2.5.1)**. This hydrolysed solution was analysed by HPLC and compared with the unhydrolysed solution.

2.7.4 Boiling

Nuts were randomly selected from sample 9 and cut vertically in half. One half of each nut was prepared for HPLC as in **Section 2.5.1**, while the other half was boiled (100 ºC) in a beaker of water (150 mL), prior to preparation for HPLC analysis. Each beaker had a watch-glass placed on top to avoid evaporation. After boiling, the volume was topped up to 150 mL (to account for any evaporation that did occur). Three individual nuts for each of the four different boiling times; 0.5, 1, 3 and 6 hours were tested. All samples were analysed by HPLC, including the solutions, both hydrolysed and unhydrolysed (**Section 2.7.3**).

2.7.5 Oven roasting

Nuts were randomly selected from sample 9 and cut vertically in half. One half of each nut was prepared as in **Section 2.5.1**, while the other half was oven roasted at a set temperature (80, 120 or 175 °C) and time period (10, 20 or 30 min). Three individual nuts were tested for each of the three different temperatures and time periods. All samples were analysed by HPLC.

[a] Actual concentration was 1.975 mol L^{-1}

2.7.6 Microwave cooking

Nuts were randomly selected from sample 9 and cut vertically in half. One half was prepared for HPLC as in **Section 2.5.1**, while the other half was cooked in a beaker of water (100 mL) in a microwave (850 Watts), prior to preparation for HPLC analysis. Three individual nuts were tested for each of the three microwave cooking times of 1, 3 and 5 minutes. All samples were analysed by HPLC, including the solutions, both hydrolysed and unhydrolysed (**Section 2.7.3**).

2.7.7 Cold-water treatments

2.7.7.1 Cold-water soaking

Nuts were randomly selected from sample 9 and cut vertically in half. One half was prepared as in **Section 2.5.1**, while the other half was placed in a covered beaker of water (250 mL) for the desired time period. Three individual nuts were tested for both time periods of 4 and 14 days. All samples were analysed by HPLC, including the solutions both hydrolysed and unhydrolysed (**Section 2.7.3**).

2.7.7.2 Constant cold-water flow

Nuts were randomly selected from sample 9 and cut vertically in half. One half was prepared as in **Section 2.5.1**, while the other half was treated in a 'cold-water flow column' (**Figure 2.2**) for the desired time period. The cold-water flow column was a glass column with glass wool in the neck to prevent loss of the nut if it degraded into smaller pieces. It had a constant dripping in-flow of water from a tap. The inflow rate of water was equivalent to the outflow of water, ensuring that there was a constant volume of water in the column while the nuts were being soaked. Three individual nuts were tested for the two different time periods of 4 and 14 days. All samples were analysed by HPLC, including the solutions both hydrolysed and unhydrolysed (**Section 2.7.3**).

Figure 2.2 Cold-water flow column set-up: The water drips in from a tap at the top and flows out the bottom to a drain, at a constant rate. The column has been treating nuts for 4 days, hence the observed colour change in the glass wool to an orange/brown, believed to be due to the loss of toxins from the nuts.

3 Method Development

3.1 High performance liquid chromatography (HPLC)

3.1.1 HPLC configuration

Details of the HPLC configuration can be found in **Section 2.2.1**.

3.1.1.1 HPLC run-time

The HPLC run time was 51 minutes to ensure that all peaks of interest acetic acid, malonic acid and nitropropanoic acids (eluted at ~ 18.1, ~ 12.1 and ~ 23.2 minutes, respectively) could be seen and where possible integrated and quantified. There were still some other very small peaks that eluted after 23 minutes, the last peak of which eluted ~ 49 minutes. Initially, the run-time was 51 minutes as it was undesirable to have carry-over, however after all kinetic trials were conducted, the run time was shortened to 44 minutes. This meant that the small peak that eluted ~ 49 minutes now eluted before the solvent peak at 7 minutes and did not interfere with the following sample. The run time was further shortened to 35 minutes, whereby peaks after 35 minutes eluted well before 20 minutes of the following run and therefore well clear of the NPA peak being measured at ~ 23.2 minutes. The modifications to run time were made to increase the efficiency of HPLC analysis. In future, the HPLC conditions could be modified in other ways, for example by implementing a gradient elution scheme to speed up the elution time of the small peaks that elute after the NPA peak.

3.1.2 UV extraction wavelength

Previous research on NPA used UV detection at single wavelengths of 254 nm or 210 nm.[15,40] Standards of NPA, MA and AA were analysed using variable wavelength detection between 190 and 400 nm. Chromatograms were extracted at wavelengths of 210 and 254 nm. The wavelength, 210 nm was the closest wavelength to λ_{max}, thus 210 nm was used as the extraction wavelength in all HPLC detection (a representative chromatogram extracted at 210 nm, can be seen in **Section 4.1**).

3.1.3 Mobile phase concentration

Mobile phase concentration can have significant effects on peak shape, resolution and peak elution time.[60] As a result, different concentrations (0.002, 0.005, 0.008 and 0.011 M) of mobile phase (sulfuric acid, H_2SO_4) were trialled when the column was installed. There was found to be no significant difference (< 1%) in the peak area integration with change in concentration of mobile phase. In addition, there was only a minimal change in elution times across the different concentrations. Therefore, 0.005 M sulfuric acid was chosen as the mobile phase because of the convenience of dilution.

3.1.4 Detection limits

A common method used to determine detection limits in HPLC was utilised. Serial dilution of a known concentration standard was carried out until aliquots of three and ten times the height of baseline noise (detection limit (DL) and limits of quantification (LOQ) respectively) were reached.[61,62] A baseline noise of 0.0001 absorbance units (Au), corresponding with the baseline obtained when analysing a nut, was used to determine detection limits (DL) and limits of quantification (LOQ), since it is higher than the baseline of the sulfuric acid matrix. Therefore, DL and LOQ were defined when standards had peak heights of ~0.003 and 0.01 Au respectively.

3.1.4.1 Detection limit of nitropropanoic acid

Aliquots of a NPA standard (0.159 mg mL^{-1}) were diluted by varying degrees, until a peak height of ~ 0.003 Au was obtained. This point was reached when NPA peak area was ~ 96000, which means the DL of NPA is 1.40×10^{-3} mg mL^{-1}.

3.1.4.2 Limit of quantification for nitropropanoic acid

Aliquots of the NPA standard (0.159 mg mL^{-1}) were diluted and analysed, until a peak height of ~ 0.01 Au was obtained. The LOQ for NPA was found to be 2.98×10^{-3} mg mL^{-1} (peak area of ~203,000).

3.1.4.3 Detection limit of malonic acid

Aliquots of a standard of MA (0.315 mg mL^{-1}) were diluted until a peak height of ~ 0.003 Au was obtained. The detection limit of MA was determined to be 0.03 mg mL^{-1} (peak area ~ 66,000).

3.1.4.4 Limit of quantification for malonic acid

Aliquots of an MA standard (0.14 mg mL^{-1}) were diluted and analysed, until a peak height of ~ 0.01 Au was obtained. The LOQ for MA was found to be 0.08 mg mL^{-1} (peak area of ~ 177,000).

3.2 Hydrolysis of nitropropanoyl glucopyranoses to nitropropanoic acid

3.2.1 Initial trial to ensure nitropropanoyl glucopyranoses could be hydrolysed to nitropropanoic acid

An initial trial was conducted whereby, nuts (5) were blended, a sub sample of homogenate (~ 1 g) was weighed and sulfuric acid (10 mL, 1 M[a]) was added. The sample was mixed on a stir-plate (speed 8, 10 min) before being heated (100 ºC, 1 h). Following this, the solution was filtered (Whatman filter paper, No.2) and analysed by HPLC. The resulting chromatogram had a peak that corresponded with the retention time and UV spectrum of NPA.

3.2.1.1 Selecting a hydrolysis acid concentration

Stronger solvents are generally more effective at hydrolysis. Different concentrations of sulfuric acid were tested, to determine the optimum concentration of hydrolysis solvent. Nuts (20) were blended and a homogenate (~ 1 g) was weighed into five different vials. Sulfuric acid (10 mL) of various concentrations (0, 0.5, 1.0, 1.5 and 2.0 M) was added and the solutions were stirred (speed 8, 10 min) prior to being heated (100 ºC, 1 h). After heating, the samples were washed three times with equivalent volumes of chloroform to remove any unwanted fats and oils, before a sub-sample of 100 µL was diluted

[a] Actual concentration was 0.9875 mol L^{-1}

1:4 with MilliQ water and analysed by HPLC (**Figures 3.1 and 3.2**; for raw data see **Appendix B1**).

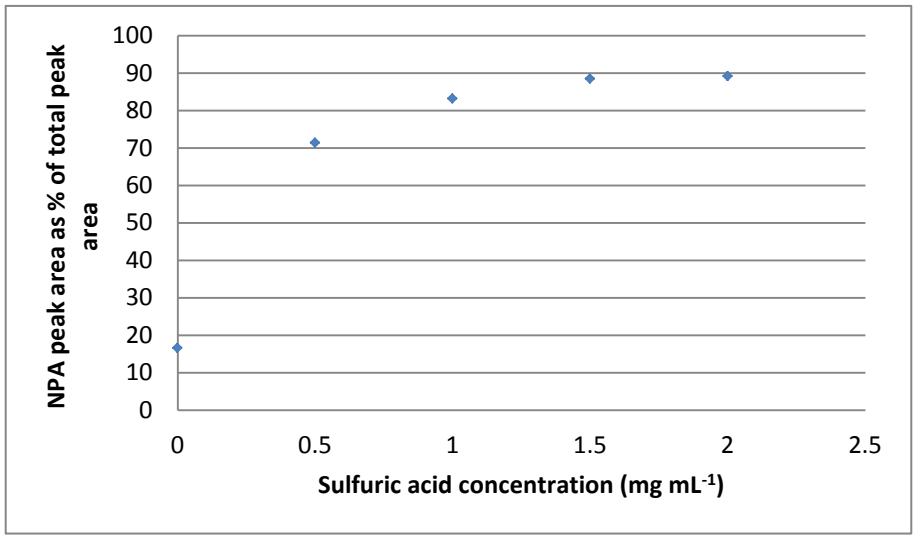


Figure 3.1 Nitropropanoic acid peak area as a percentage of total peak area, where total peak area is the peak area observed for all peaks in the sample chromatogram.

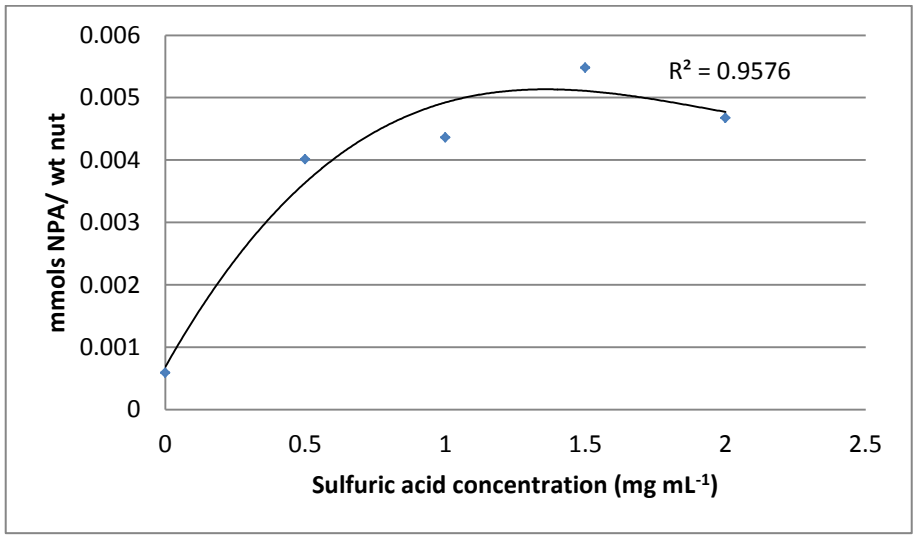


Figure 3.2 NPA concentration in mmols per weight (g) of nut versus acid concentration.

The results in **Figure 3.1** show that after the sulfuric acid concentration exceeds 1 M, there is only a minimal change in peak area percentage with increased concentration. In **Figure 3.2** when NPA concentration is measured in relation to the weight of nut homogenate in the sample and a smooth fitting line of best-fit is drawn, a maximum is apparent at ~ 1 M. Sulfuric acid concentrations in excess of

1 M show the mmols of NPA per weight of nut decreasing. This trend coincides with the observation that there are peaks corresponding to both NPA and MA in the chromatograms obtained with sulfuric acid concentrations of 1.5 M and 2 M. However, the MA peak is very small and is hidden by two neighbouring peaks that overlap near the baseline. The small and hidden MA peak proved to be a problem as MA was only detected in low quantities. Despite the low levels of MA, these observations are consistent with the assumption in **Equation 1.1** that NPA degrades to release MA which further degrades to yield AA. As a result, a hydrolysis solvent of 1 M sulfuric acid was used for all further tests.

3.2.2 Optimisation of other hydrolysis conditions

The choice of solvent, solvent concentration, heating time and heating temperature were further optimised to determine the ideal hydrolysis conditions.

Karaka nuts (40) were blended together and sub-samples of homogenate (~ 1 g) were weighed into 13 different vials for each of the six trials (A-F). Following this, the appropriate solvent (10 mL, 1 M[a]) was added, the solutions were stirred (speed 8, 10 min) before being heated at set temperatures between 0-180 minutes (13 vials, every 10 min first hour, every 15 min in hour two and every 20 min in the third hour). The solvent and heating conditions of each trial are outlined in **Table 3.1**. The samples were then cooled, before being diluted (100 µL sample + 400 µL water) and analysed by HPLC (**Figures 3.3**; for raw data see **Appendix B2)**.

Table 3.1 Heating temperatures and acids used in trials to determine the optimum hydrolysis conditions.

Trial	Hydrolysis solvent	Heating temperature (ºC)
A	H_2SO_4	60
B	TFA	60
C	TFA	100
D	H_2SO_4	100
E	TFA	120
F	H_2SO_4	120

[a] Actual concentration was 0.9875 mol L^{-1}

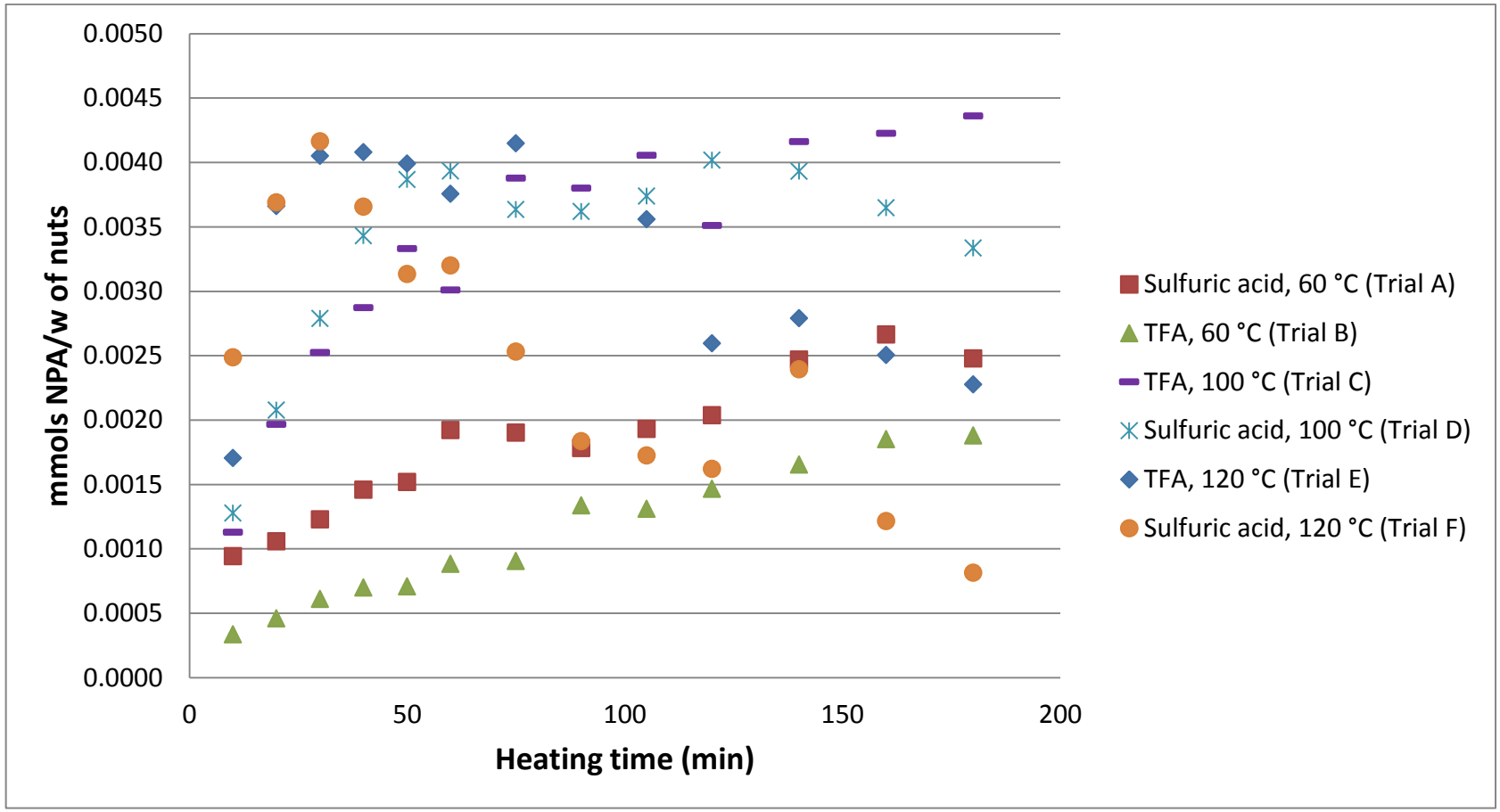


Figure 3.3 Comparison plot of NPA released in mmols of NPA per grams of nut used, for 1 M TFA (Trial B 60 °C, Trial C 100 °C and Trial E 120 °C) and sulfuric acid (Trial A 60 °C, Trial D 100 °C and Trial F 120 °C) at various heating temperatures over 180 minutes.

3.2.2.1 Heating temperature

From **Figure 3.3**, it can be seen that a heating temperature of 60 ºC (Trials A and B) appears to be too low. The amount of NPGs hydrolysed with both TFA and sulfuric acid is considerably lower than at either of the two higher temperatures (100 and 120 ºC). In addition, the amount of NPA detected continues to increase even after the excessively long heating time of 180 minutes. In comparison, a heating temperature of 120 ºC results in the rapid degradation of NPA after heating for 30 and 90 minutes (Trials F and E respectively). Although maximal hydrolysis of NPGs is desired, the related degradation of NPA at this temperature complicates the estimation of original NPA-glycoside content hence 120 ºC was also deemed an undesirable heating temperature. In contrast, trials C and D appear to have steady and only slight fluctuations in NPA content after the point at which maximal hydrolysis occurs. This can be seen by the steady nature of the points plotted around 0.004 mmols of NPA per gram of nuts. Hence, 100 ºC was selected as the optimal heating temperature for the hydrolysis of NPGs to release NPA from the karaka nuts and drupes.

3.2.2.2 Effect of heating time

The maximum amount of NPGs hydrolysed and measured (as NPA) at 100 ºC occurred between 60 to 100 minutes. Therefore, a hydrolysis time of 60 minutes was selected to be used.

3.2.2.3 Effect of choice of acid

The effectiveness of NPG hydrolysis using two different acids, TFA and sulfuric acid, were compared (**Figures 3.3)**. Trials C and D appear to be the optimal conditions and result in the most complete hydrolysis with least NPA degradation. The comparison of trials C and D show that there is little difference as to which acid is most efficient at hydrolysing NPGs to NPA. Both solvents; TFA and sulfuric acid give similar results (at the chosen temperature of 100 ºC) up to 60 minutes, after which there is only small variation in the concentrations of NPGs both acids can hydrolyse. However, TFA does appear to begin to degrade NPA at a point where sulfuric acid is still hydrolysing NPGs slightly. Due to the availability, lower cost and safer handling of sulfuric acid, it was chosen as the hydrolysis solvent.

3.3 NPA degradation

According to literature,[29] NPA is known to degrade to release MA which can also further degrade to AA (**Equation 1.1**). This degradation was observed during initial hydrolysis reactions of NPGs to afford NPA. In these trials, depending on the conditions, certain heating times and/or heating temperatures resulted in reduced NPA concentrations (mmols of NPA/w (g) of nut). Examples of this can be seen in **Figure 3.3**, the most obvious being the degradation of NPA in trial F after 30 minutes.

Since the aim of the method is to determine total NPA concentration in the nuts, one must find a way to account for the degradation that will occur, especially during the hydrolysis method. It could be literally a fatal flaw to under-estimate the NPA content of the nuts due to loss during hydrolysis. There are two ways in which the NPA content can be predicted. The first is to come up with a factor that states an average loss of NPA as a percentage with 1 M sulfuric acid and a heating time of 60 minutes (see **Section 4.3.1**). However, this method is quite crude as it does not account for the fact that at some point in time, there is a simultaneous formation and degradation of MA. The other method involves determining the kinetics of the reaction, in order to predict a more accurate correction factor. Kinetic trials to determine the degradation rates of NPA to MA and MA to AA were performed, in order to determine a factor that would allow for the accurate prediction of the original NPG content from NPA content at time zero, prior to hydrolysis of the NPGs to NPA. This factor also accounts for simultaneous formation and degradation of both NPA and MA that occurs during the hydrolysis stage (see **Section 4.3.1)**.

3.4 Difference in nitropropanoic acid and malonic acid concentrations due to experimental factors

3.4.1 Solubility of nitropropanoic acid and malonic acid in chloroform

The usual preparation of karaka nuts involved an appropriate volume of homogenised nuts mixed with solvent heated at desired temperatures over set time periods. Following this, the filtered extracts were washed three times with equivalent volumes of chloroform. The chloroform washes were incorporated to remove any undesirable fats and oils that may interfere with results. However, it was believed that NPA or MA may actually have considerable solubility in chloroform. Hence, a trial was conducted whereby the solubility of NPA and MA in chloroform was tested.

The test was conducted by weighing out NPA and MA (25.15 and 25.53 mg, respectively) and diluting to a volume of 50 mL using sulfuric acid (1 M[a]). Equal volumes (10 mL) were heated in a heating block for one hour at 100 ºC. One of the samples was analysed by HPLC immediately after heating whilst another was washed three times with equivalent volumes of chloroform (10 mL) and then analysed. The third and fourth samples were adjusted to a pH ~14 with NaOH (2 M). One was then analysed immediately, while the other was washed three times with chloroform prior to HPLC analysis **(**for raw data see **Appendix B3)**.

NPA was found to be highly soluble in chloroform. This was shown by a loss of 18.8 and 42.5% NPA in the solution under usual preparation and pH 14 conditions respectively. In contrast, chloroform washing results in only a 2.1% and 4.0% loss of MA in the solution under usual preparation conditions and at pH 14 respectively. Chloroform washing has a significant impact on the recovery of NPA from the hydrolysis solution and would undoubtedly give false underestimations of NPA content in the karaka nuts. In addition, it was shown that increasing the pH during preparation should be avoided as more basic conditions appear to increase the solubility of both MA and NPA in chloroform. As a result

[a] Actual concentration was 0.9875 mol L^{-1}

of the underestimation of NPA content and thus toxicity, the decision was made to exclude chloroform washes from the preparation method.

3.4.2 The effect of freeze drying the nuts before hydrolysing

Nuts (5) from the Victoria Street tree (sample 11), were de-fleshed, extracted from the shell and cut into small pieces (~ 3 mm^3). Sub-samples of ~ 1 g were weighed into vials for each nut. The exact weights of the nuts prior to and after freeze drying can be seen in **Table 3.2**. Each sample was stirred for 10 minutes (speed 8) in sulfuric acid (1 M, 10 mL) and heated for one hour at 100 ºC. The samples were filtered (Whatman filter paper No.2) and a sub sample of 100 µL was diluted to 1:4 with MilliQ water and analysed by HPLC **(**for raw data see **Appendix B3)**.

As can be seen in **Table 3.2**, freeze drying the nuts reduced the weight of the nuts significantly. On average the five nuts contained 39% moisture. The water content may also be affected by the site at which nuts were sampled. This is evident with variation of moisture content previously reported by McCurdy[17] but is also apparent when nuts are tested throughout this project. As a result, the concentration of NPA per gram of nut to be quoted would be affected if some samples were freeze dried while others were not. This is illustrated by the apparent percentage gain in concentration of NPA per gram of nut as a result of freeze drying which was found to range between 58-71% (**Table 3.2**). Therefore, all results need to be quoted on the same basis of either dry weight or wet weight. For nuts tested, results reported are based on dry weights.

The ~39% moisture content is comparable with previous research that states untreated karaka nuts are ~ 45.8% water (range of 41.8-48.0%), while treated nuts contain ~19% water, according to McCurdy.[17] It is unknown how fresh the previously reported karaka nuts were or what the storage conditions were, hence there is room for considerable differences and no definitive conclusions can be drawn. These differences will also be exacerbated if the nuts have been cooked, for example by boiling, as illustrated by the considerably lower moisture content found in treated nuts by McCurdy. Thus, it is important to include freeze drying as a step in the method. In addition, freeze drying allows for the nuts to be ground into fine powder, increasing the homogeneity of a sample.

Table 3.2 Weights and concentrations of NPA in freeze dried versus fresh karaka nuts.

Nut	Freeze dried Y/N	Wet weight (g)	Freeze dried weight (g)	% loss weight from freeze drying	Concentration mg NPA/ weight nut (g)	Concentration mg NPA/ weight freeze dried nut (g)	Apparent % gain in mg NPA/ weight nut (g) with freeze drying
A_1	Y	1.0325	0.6511	36.9	43.42	68.85	58.58
A_2	Y	1.0207	0.61545	39.7	45.16	74.89	65.85
A_3	Y	1.0060	0.61906	38.4	38.46	62.49	62.50
A_4	Y	1.0093	0.60853	39.7	44.23	73.36	65.86
A_5	Y	1.0301	0.60155	41.6	40.94	70.11	71.24

4 Results

4.1 Calibration curves, chromatogram and UV spectra of standards

The standards of nitropropanoic acid (NPA), malonic acid (MA) and acetic acid (AA) (**Sections 2.2.1.2.1-2.2.1.2.3)** were analysed by HPLC and a plot of peak area versus concentration was generated for each. The plots were linear between 0 and 1.5 mg mL^{-1}, 0 and 0.65 mg mL^{-1} and 0 and 1.28 mg mL^{-1} for NPA, MA and AA respectively. All R^2 values were found to be > 0.99 (**Figure 4.1**; raw data see **Appendix A**).

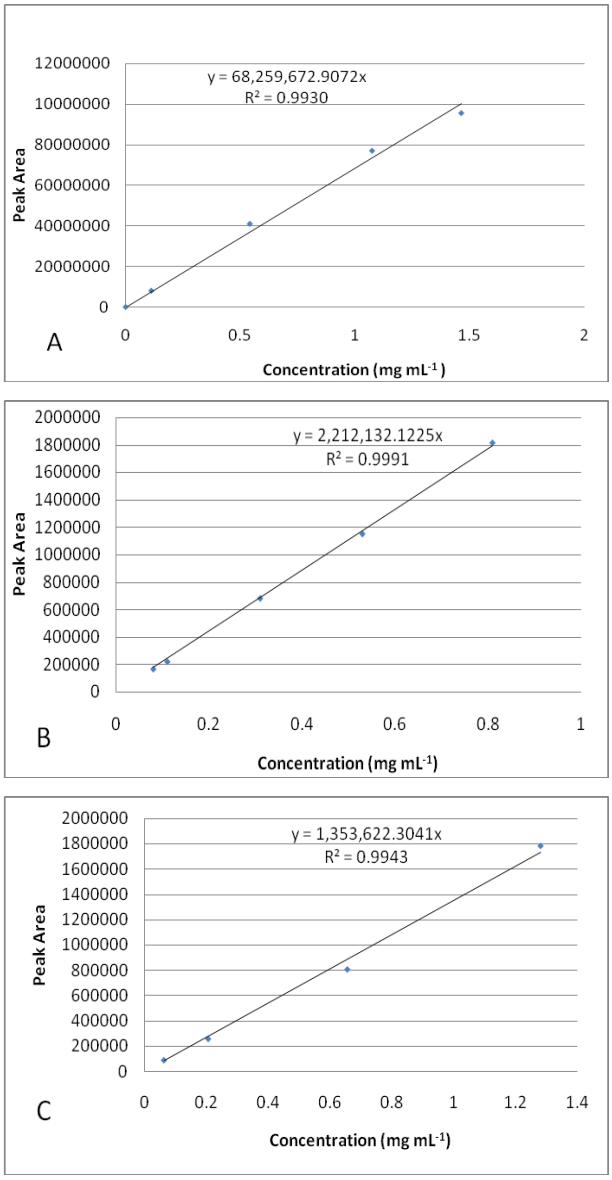


Figure 4.1 Examples of calibration curves for (A) nitropropanoic acid, (B) malonic acid and (C) acetic acid.

As previously stated, chromatograms for standards and samples were extracted at a wavelength of 210 nm. Confirmation of compounds was based on a match of both retention times (~ 23.2, ~ 12.1 and ~ 18.1 minutes for NPA, MA and AA respectively) and UV spectra. Examples of a chromatogram extracted at 210 nm and the UV spectra for all three standards (NPA, MA and AA), can be seen in **Figure 4.2**.

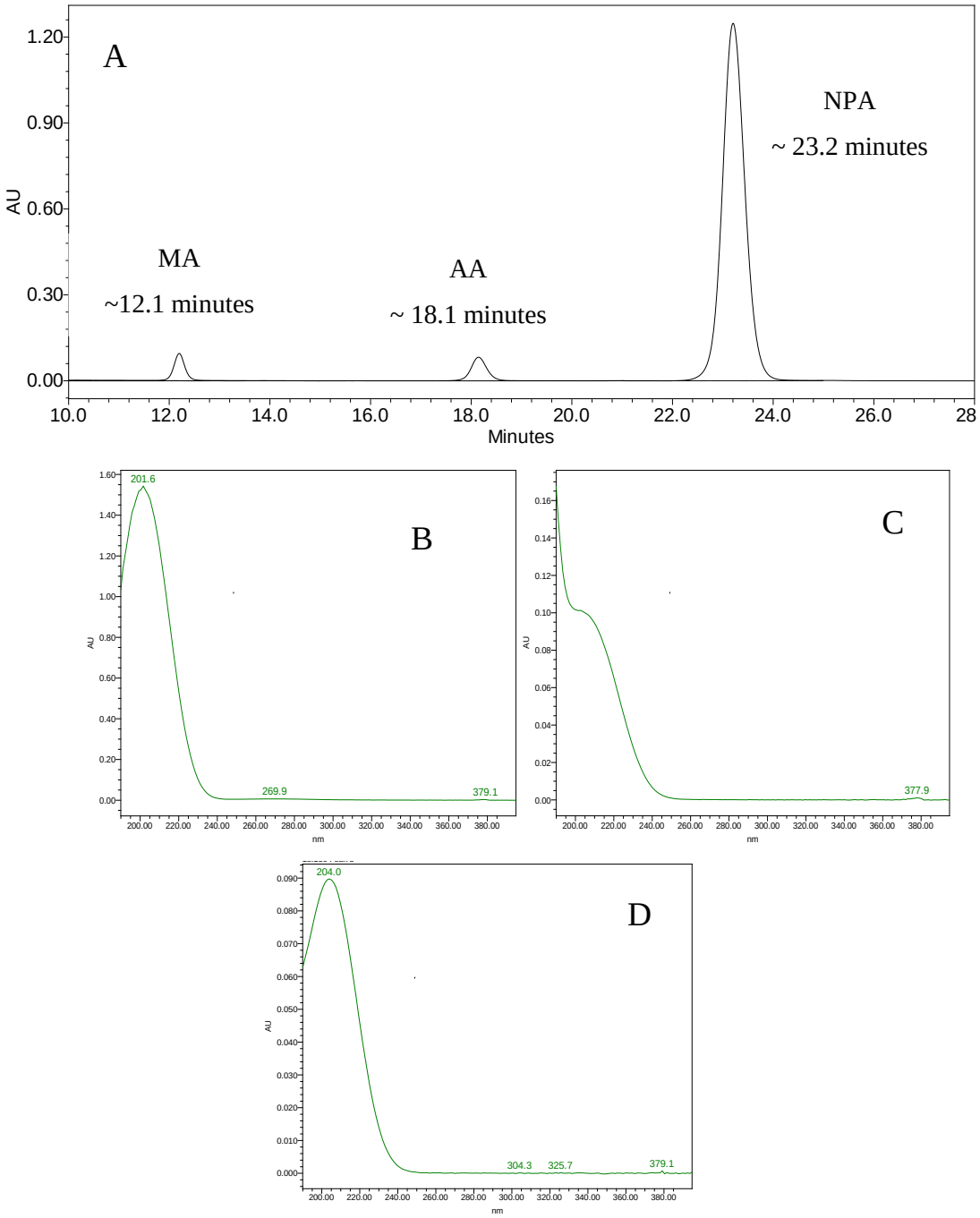


Figure 4.2 Chromatogram (210 nm) of all three standards (NPA, MA and AA) showing retention times (A) and the UV spectra of NPA (B), MA (C) and AA (D).

4.2 Kinetic results

4.2.1 Expected kinetics of a sequential reaction

The reaction of NPA degrading to yield MA and upon further degradation to yield AA (**Equation 4.1**) can be considered as a sequential first order reaction.
The reaction can be represented thus:

$$NPA \overset{k_1}{\rightarrow} MA \overset{k_2}{\rightarrow} AA \tag{4.1}$$

If each step of the reaction is assumed to be first order, differential rate expressions for each species can be written as:

$$\frac{d[NPA]}{dt} = -k[NPA] \tag{4.2}$$

$$\frac{d[MA]}{dt} = k_1[NPA]-k_2[MA] \tag{4.3}$$

$$\frac{d[AA]}{dt} =k_2[MA] \tag{4.4}$$

These equations can be used to determine the concentrations of each acid as a function of time. If the assumption is made that at time zero, the only reactant present is NPA, then integration and substitution results in **Equations 4.2** and **4.3** respectively becoming:

$$[NPA] = [NPA_0]e^{-k_1 t} \tag{4.5}$$

$$[MA] = \frac{k_1}{k_2 - k_1}(e^{-k_1 t}e^{-k_2 t})[NPA]_0 \tag{4.6}$$

If another assumption is made that the initial concentration of NPA is equal to the sum of all acid concentrations for t > 0, i.e. $[NPA]_0$= [NPA] + [MA] + [AA], then the expression for NPA becomes:

$$[NPA] = [NPA]_0 - [MA] - [AA] \tag{4.7}.$$

Thus, a final equation for AA can be determined by substituting **Equations 4.5** and **4.6** into **Equation 4.7** to give:

$$[AA] = \left(\frac{k_1 e^{-k_2 t} - k_2 e^{-k_1 t}}{k_2 - k_1} + 1\right)[NPA]_0 \quad (4.8)$$

Rearrangement results in the concentration of NPA at t = 0, being determined as follows:

$$\frac{[AA]}{\left(\frac{k_1 e^{-k_2 t} - k_2 e^{-k_1 t}}{k_2 - k_1} + 1\right)} = [NPA]_0 \quad (4.9)$$

If a concentration profile for the sequential reaction involving NPA, MA and AA was plotted and $k_1 > k_2$ it would be expected that it may look something like **Figure 4.3**.

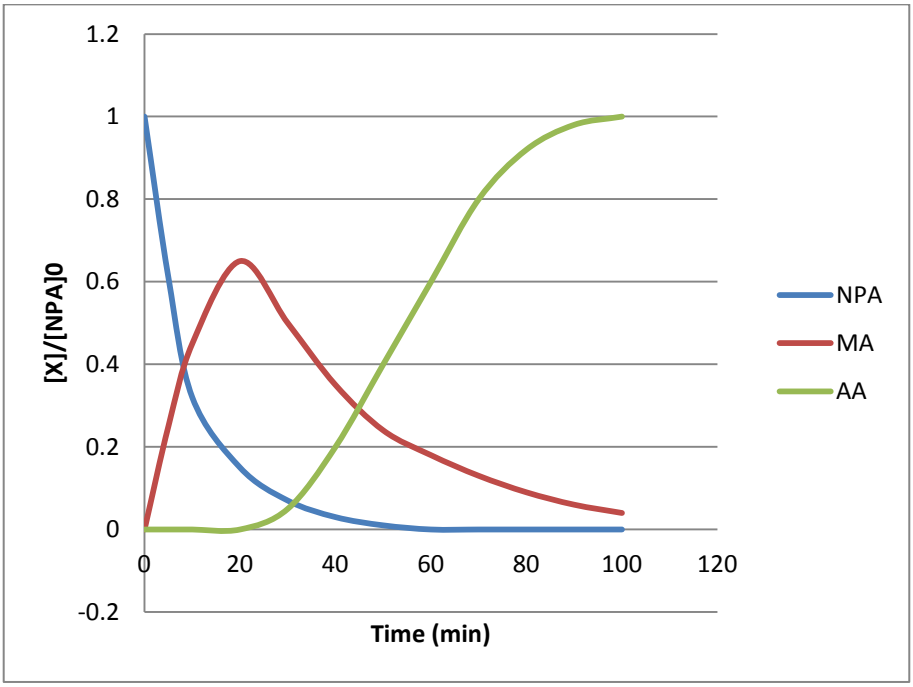


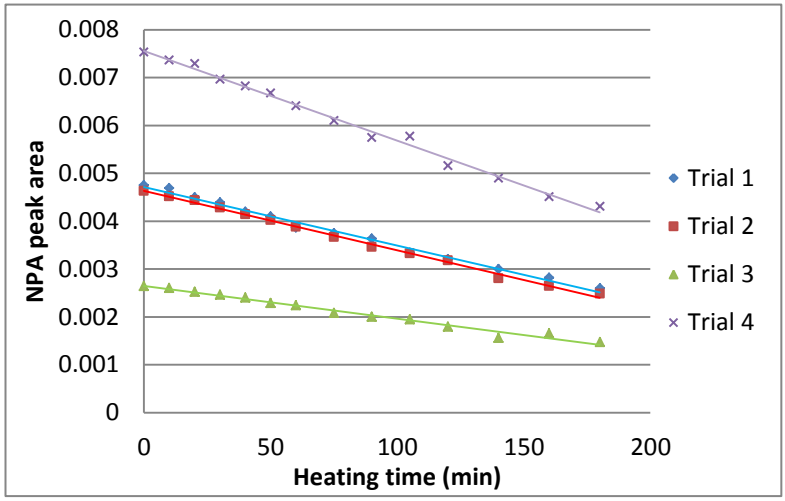
Figure 4.3 Concentration profile for a sequential degradation reaction of NPA.

4.2.2 Experimental kinetics of nitropropanoic acid degrading to malonic acid

4.2.2.1 Nitropropanoic acid degradation in sulfuric acid (1 M)

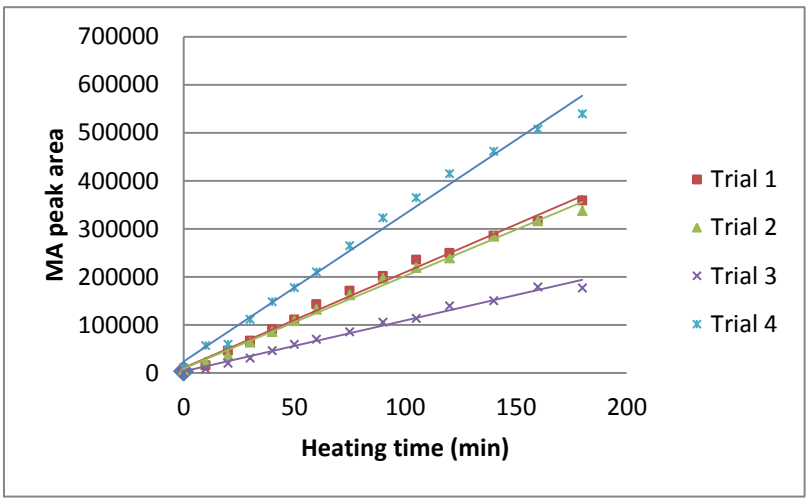
A heating investigation that used four different concentrations of NPA (0.5024, 0.5026, 0.2700 and 0.8140 mg mL^{-1}; trials 1-4 respectively) was carried out as in **Section 2.4.1**. Raw data from the trial can be found in **Appendix C1.**

The results showed that with heating time NPA diminishes, while MA begins forming (**Figures 4.4 and 4.5)**.



Trial	NPA concentration (mg mL^{-1})	Slope	R^2
1	0.5024	-0.000012x + 0.004717	0.992
2	0.5026	-0.000012x + 0.004637	0.996
3	0.2700	-0.000007x + 0.002647	0.981
4	0.8140	-0.000019x + 0.007560	0.993

Figure 4.4 Reduction of NPA peak area versus heating time (min) of NPA.



Trial	NPA concentration (mg mL^{-1})	Slope	R^2
1	0.5024	1,988.16x + 10,840.38	0.993
2	0.5026	1,923.11x + 9,546.35	0.994
3	0.2700	1,059.51x + 3,206.80	0.988
4	0.8140	3,073.52x + 23,852.97	0.991

Figure 4.5 Increase in MA peak area versus heating time (min) of NPA.

The stoichiometry is given by the following:

$$H^+ + H_2O + COOHCH_2CH_2NO_2 \longrightarrow NH_2OH + CH_2(COOH)_2 + H^+$$

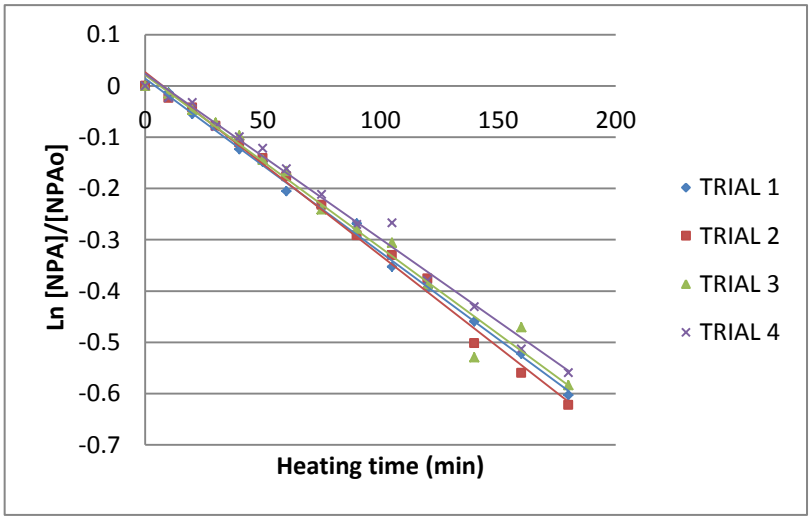
Thus, the reaction is third order, however in sulfuric acid (1 M) the $[H^+]$ is essentially constant which results in a pseudo first order rate constant of k' and the following equation:

$$\frac{-d[NPA]}{dt} = \frac{d[MA]}{dt} = k[NPA][H^+][H_2O] = k'[NPA] \qquad (4.10)$$

The integrated rate equation for the pseudo first order degradation of NPA is

$$-\mathrm{Ln}\frac{\mathrm{NPA}}{\mathrm{NPA_0}} = \mathrm{kt} \qquad (4.11)$$

The NPA data was plotted as Ln [NPA]/[NPA_0] versus heating time **(Figure 4.6).** All concentrations gave linear slopes and similar values for k, indicating that the reaction is indeed pseudo first order for NPA.



Trial	NPA concentration (mg mL^{-1})	Slope	R^2
1	0.5024	-0.003386x	0.997
2	0.5026	-0.003572x	0.994
3	0.2700	-0.003365x	0.979
4	0.8140	-0.003220x	0.990
Average	-----	-0.003386x	0.990

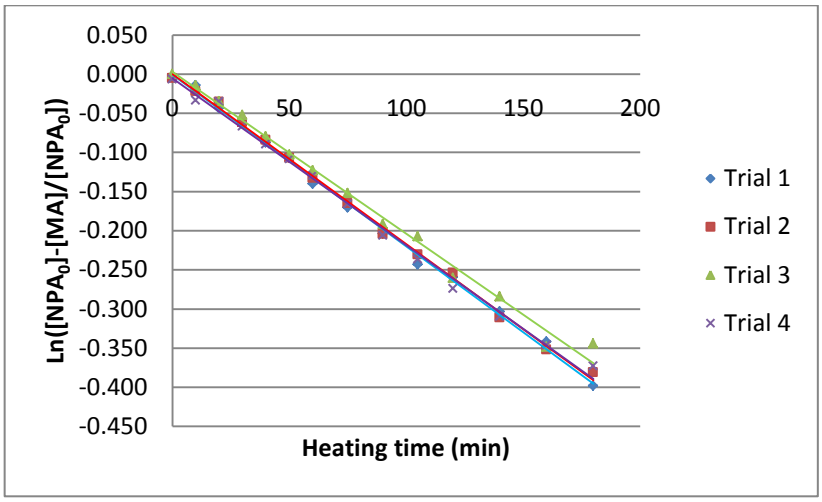
Figure 4.6 NPA plot of Ln [NPA]/[NPA_0] showing relatively similar slopes indicating that the reaction is pseudo first order for NPA.

Since NPA_0 = NPA + MA, assuming that MA forms nearly instantly as NPA reduces, the following relationship can be derived:

$$-\mathrm{Ln}\frac{[\mathrm{NPA}]}{[\mathrm{NPA_0}]} = -\mathrm{Ln}\frac{[\mathrm{NPA_0}-\mathrm{MA}]}{[\mathrm{NPA_0}]} = \mathrm{k}dt \qquad (4.12)$$

If the only species involved are NPA and MA, the resulting slopes of -Ln[NPA]/[NPA_0] and -Ln[NPA_0-MA]/[NPA_0] should be identical, as both assume the reaction is first order in NPA. The plot of $-\mathrm{Ln}\frac{[\mathrm{NPA_0}-\mathrm{MA}]}{[\mathrm{NPA_0}]}$ can be seen in **Figure 4.7** and shows that the rate constant of NPA degrading to MA is

0.00215 min^{-1}. While the plot of $-\text{Ln}\frac{[NPA]}{[NPA_0]}$ (**Figure 4.6**) shows a rate constant of 0.00338 min^{-1}. The slopes of **Figures 4.6** and **4.7** are not identical, confirming that as predicted MA degrades further to yield acetic acid (AA). The reaction still appears to be pseudo first order as the curves are linear but with different slopes.



Trial	NPA concentration (mg mL^{-1})	Slope	R^2
1	0.5024	-0.002191x	0.998
2	0.5026	-0.002171x	0.998
3	0.2700	-0.002066x	0.992
4	0.8140	-0.002132x	0.996
Average	-----	-0.002140x	0.991

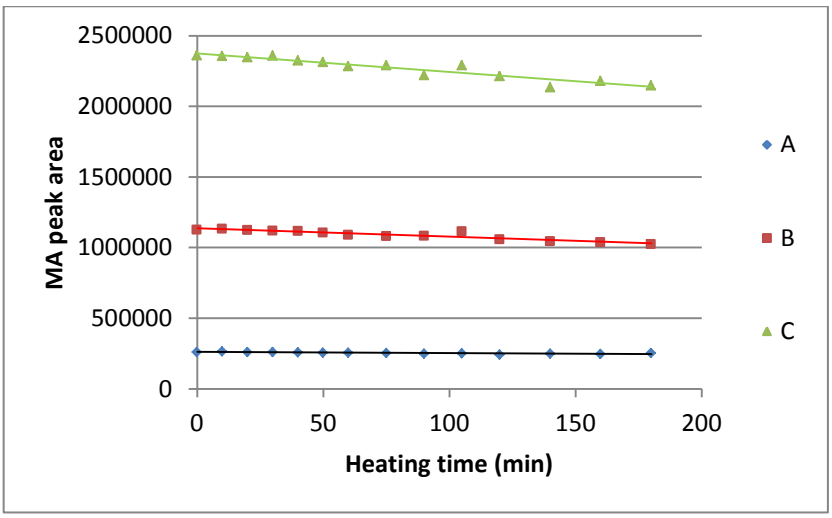
Figure 4.7 NPA degradation plot of Ln([NPA_0]-[MA]/[NPA_0]) versus heating time (min).

4.2.3 Experimental kinetics of MA degrading to AA

4.2.3.1 Malonic acid degradation in sulfuric acid (1 M)

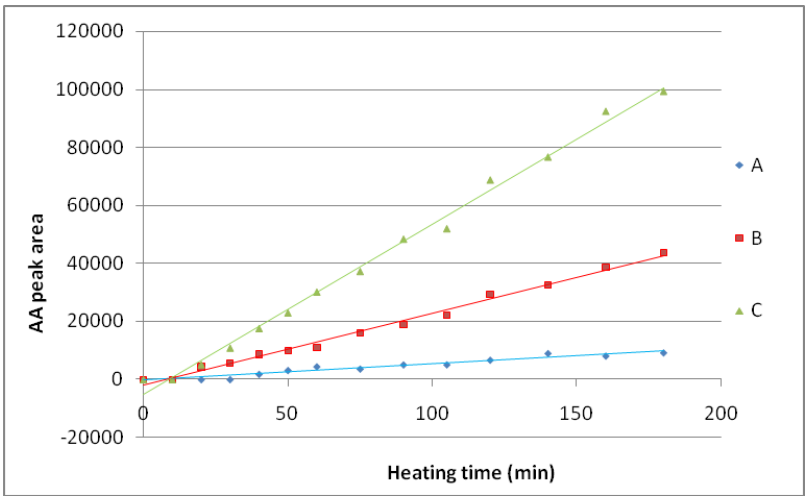
Once MA is formed as a result of NPA degradation, further degradation of MA is predicted to yield AA. Three solutions of MA (0.1132, 0.511 and 1.0652 mg mL^{-1}) were used to test this (Raw data can be found in **Appendix C1**).

The results showed that MA begins degrading and AA appears with increased heating time (**Figures 4.8** and **4.9**).



Trial	MA concentration (mg mL^{-1})	Slope	R^2
A	0.1132	-86.83x + 261,903.32	0.666
B	0.5110	-588.41x + 1,136,195.00	0.867
C	1.0652	-1,314.25x + 2,375,313.96	0.892

Figure 4.8 Reduction of MA peak area with heating time and various concentrations of malonic acid



Trial	MA concentration (mg mL^{-1})	Slope	R^2
A	0.1132	56.47x - 401.52	0.944
B	0.5110	246.23x - 1,844.86	0.992
C	1.0652	587.57x - 5,245.57	0.994

Figure 4.9 Increase in AA peak area, with heating time for various concentrations of malonic acid.

For the reaction of malonic acid degrading to acetic acid:

$$H^+ + H_2O + CH_2(COOH)_2 \longrightarrow CH_2COOH + H^+$$

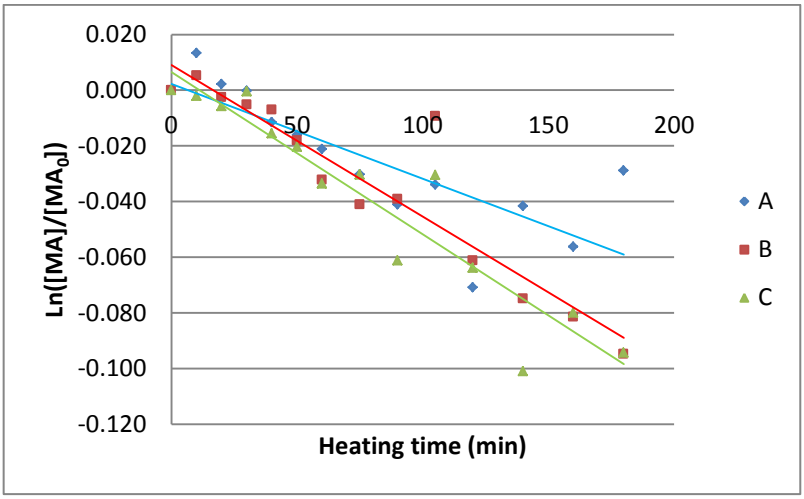
Assuming that $[H^+]$ and $[H_2O]$ are constant, the expected kinetics are:

$$-d[MA]/dt = d[AA]/dt = k[MA][H^+][H_2O] = k'[MA] \qquad (4.13)$$

The integrated rate equation is:

$$-\mathrm{Ln}\frac{\mathrm{MA}}{\mathrm{MA_0}} = \mathrm{kt} \qquad (4.14)$$

This relationship can be seen in **Figure 4.10**, and gives a rate constant of 0.000489 min^{-1} (average) for MA degradation to AA.



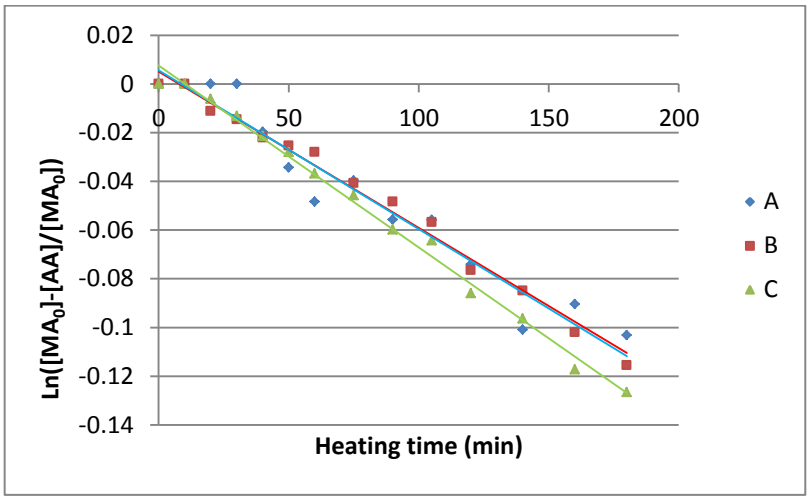
Trial	MA concentration (mg mL^{-1})	Slope	R^2
A	0.1132	-0.000340x	0.661
B	0.5110	-0.000545x	0.868
C	1.0652	-0.000583x	0.890
Average	-----	-0.000489x	0.806

Figure 4.10 MA degradation plot of Ln([MA]/[MA_0]) versus heating time (min).

In addition to MA data, the changes in AA concentrations were also obtained and assuming a relationship of MA_0 = MA + AA occurs with rearrangement and integration, a relationship can be obtained as follows:

$$-\mathrm{Ln}\frac{[\mathrm{MA}]}{[\mathrm{MA_0}]} = -\mathrm{Ln}\frac{[\mathrm{MA_0}-\mathrm{AA}]}{[\mathrm{MA_0}]} = k\mathrm{d}t \qquad (4.15)$$

The plot of $-\mathrm{Ln}\frac{[\mathrm{MA_0}-\mathrm{AA}]}{[\mathrm{MA_0}]} = k\mathrm{d}t$ (**Figure 4.11)** shows the rate constant of MA degradation to AA to be 0.000681 min^{-1} (average).



Trial	MA concentration (mg mL^{-1})	Slope	R^2
A	0.1132	-0.000642x	0.946
B	0.5110	-0.000653x	0.989
C	1.0652	-0.000747x	0.993
Average	-----	-0.000681x	0.976

Figure 4.11 MA degradation plot of Ln([MA_0]-[AA]/[MA_0]) versus heating time (min).

The slopes of Ln([MA_0]-[AA]/[MA_0]) and Ln([MA]/[MA_0]) have a small degree of discrepancy between the two, similar to that observed for NPA degradation to MA. The most likely reason for the discrepancies in slope values for the MA degradation is that the AA is hard to measure as it only forms in very small quantities. In addition, AA itself may be degrading. The slopes from each are still

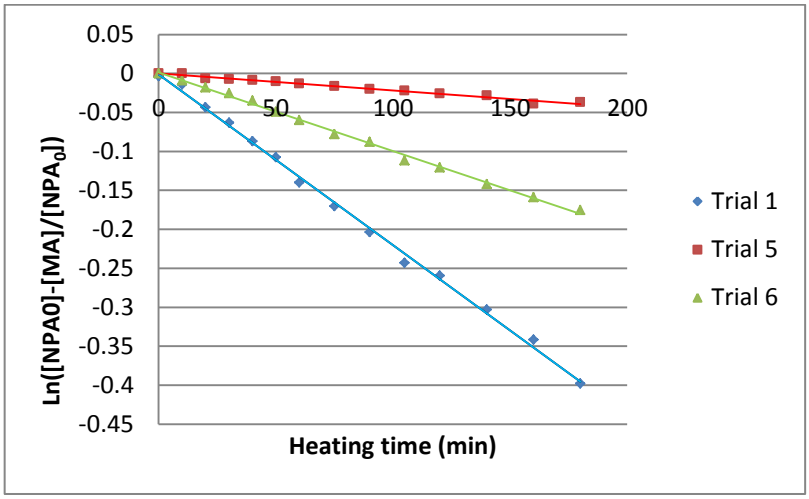
in same order of magnitude and of linear nature indicating that the degradation is pseudo first order for MA to AA.

4.2.4 Comparison on NPA and MA degradation rates

From the above degradation trials, it has been found that the degradation rate constant of NPA to MA is 0.002140 min^{-1}. In comparison, the degradation rate constant of MA to AA was found to be 0.000681 min^{-1}. Thus, in the overall reaction k_2 is significantly smaller than k_1.

4.2.5 Effect of pH on nitropropanoic acid degradation

It is always important to determine how a reaction is dependent upon pH. This is especially important in the hydrolysis of NPGs to NPA, as an acid is used to induce the hydrolysis. In order to determine whether or not the pH has an effect on hydrolysis and degradation, a trial was conducted whereby three solutions of ~ 0.5 mg mL^{-1} NPA were prepared as in **Section 2.4.3** using sulfuric acid 1 M and 0.1 M and 1 M TFA as the solvents (Trials 1, 5 and 6 respectively). The results can be seen in **Figure 4.12** and all raw data can be found in **Appendix C1.**



Trial	NPA concentration (mg mL^{-1})	Solvent	Slope	R^2
1	0.5024	H_2SO_4 (1 M)	-0.002191x	0.997
5	0.5040	H_2SO_4 (0.1 M)	-0.000217x	0.982
6	0.4980	TFA (1 M)	-0.001008x	0.997

Figure 4.12 pH dependence NPA degradation plot of Ln([NPA_0]-[MA]/[NPA_0]) versus heating time (min).

Theoretically, if the reaction has the relationship k' = k[H^+][H_2O], then k' should change when pH changes. If the assumption is made that the reaction is first order in [H^+], then the reactions involving the 1 M and 0.1 M sulfuric acid can be compared, whereby:

$$k_1 = \frac{[k_1']}{[H^+][H_2O]} \tag{4.16}$$

$$k_2 = \frac{[k_2']}{[H^+][H_2O]} \tag{4.17}$$

$$\frac{k_1'}{k_2'} = \frac{[H^+_1]}{[H^+_2]} \tag{4.18}$$

If the data from the 1 M and 0.1 M sulfuric acid solutions (1 = 1 M, 2 = 0.1 M) are substituted into this:

$$\frac{k_1'}{k_2'} = \frac{0.002191\ \text{min}^{-1}}{0.000217\ \text{min}^{-1}} = 10.096 = \frac{[H_2SO_4]_1}{[H_2SO_4]_2} = 1\ \text{M}/0.1\ \text{M} = 10$$

This confirms the reaction is first order in [H^+] as well as in [NPA]. In addition to comparing whether the reaction is first order in H^+, using different sulfuric acids concentrations, TFA was also used in a trial (6). This required the [H^+] of the solution to be calculated and compared to its rate (0.00108 min^{-1}):

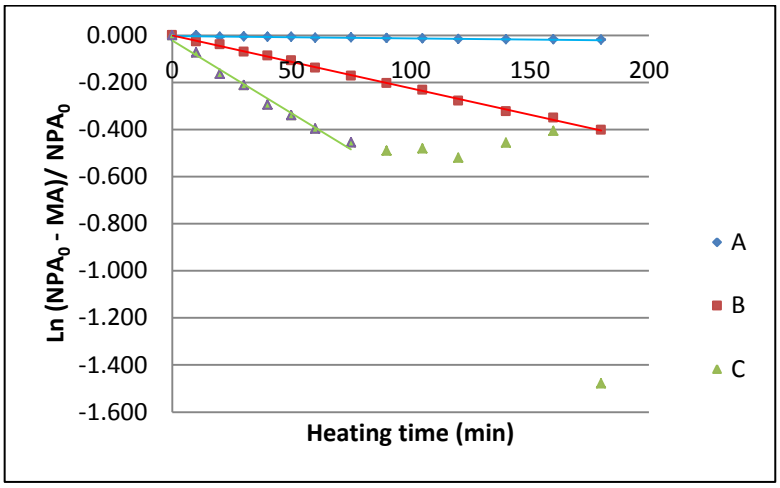
$$k_1'/k_6' = \frac{[H_2SO_4]}{[H^+]}\text{from TFA} = \frac{0.002191\ \text{min}^{-1}}{0.001008\ \text{min}^{-1}} = \frac{1\ \text{M}}{[H^+]}\text{from TFA}$$

Thus, the predicted [H^+] of the TFA solution is 0.46M. If the [H^+] of TFA is calculated using its K_a, the two values would be expected to agree. The $K_{a(TFA)}$ = 0.23, which yields [H^+] = 0.39 M. The values of 0.39 and 0.46 M are within 18% of each other. Further replication of the experiment might be expected to return values in even better agreement. Overall, the NPA degradation reaction is first order in NPA and in H^+ and there is no accumulation of an intermediate between NPA and MA thus, all NPA should degrade to yield MA.

4.2.6 NPA activation energy

The rate constants for NPA degradation at 60, 100 and 120 ºC were found to be 0.00009, 0.00224 and 0.00615 min^{-1} respectively (**Figure 4.13**; raw data can be found in **Appendix C2**). The rate constant for 100 ºC was consistent with the k_1 of 0.00214 min^{-1} (average) previously calculated (**Figure 4.7)**.

The activation energy of NPA degrading to MA was determined from an Arrhenius plot to be76.9 kJ mol^{-1} (**Figure 4.14**).



Trial	Temperature (°C)	Slope	R^2
A	60	-0.0000948x	0.917
B	100	-0.0022462x	0.998
C	120	-0.0061505x	0.985

Figure 4.13 Plot of Ln ([NPA_0]-[MA]/[NPA_0]) versus heating time (min) for a NPA standard of 0.389 mg mL^{-1} for various temperatures.

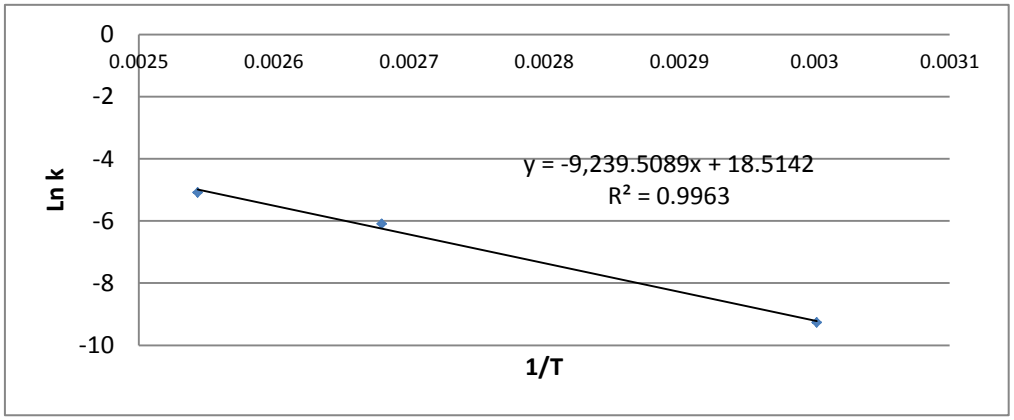
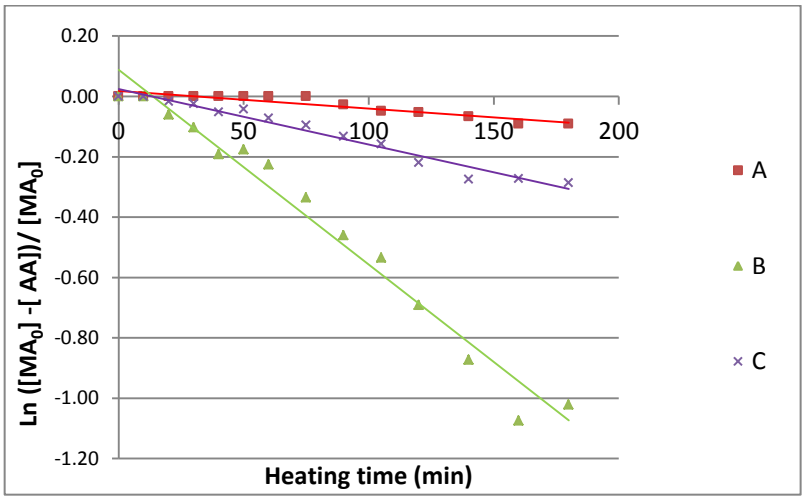


Figure 4.14 Arrhenius plot for the degradation of NPA to MA (mmols).

4.2.7 MA activation energy

The rate constants for MA degrading to yield AA were determined to be 0.00184, 0.00059 and 0.00645 min^{-1}, for 100, 110 and 120 ºC respectively (**Figure 4.15**) (**Appendix C2**). The rate of 0.00059 min^{-1} is comparable with the value of ~ 0.000681 min^{-1} obtained in a previous trial of various MA concentrations (**Figure 4.11)**. The difference between the two is most likely due to the fact that the AA is usually only detectable at low levels, resulting in a greater degree of error in integration.

The activation energy for MA degrading to AA was determined from the Arrhenius plot as being 146.3 kJ mol^{-1} (**Figure 4.16**). This is consistent with the fact that MA degrading to AA has a smaller rate constant than that of NPA degrading to MA. The values obtained for the activation energies of NPA degrading to MA and MA degrading to AA are in the same order of magnitude as values obtained in other research for degradation of other small organic acids (~ 100 – 110 kJ mol^{-1}).[63,64]



Trial	Temperature (°C)	Slope	R^2
A	100	-0.000587x	0.882
B	120	-0.006454x	0.973
C	110	-0.001837x	0.968

Figure 4.15 Plot of Ln ($[MA_0]$-[AA])/$[MA_0]$ versus heating time (min) for a MA standard of 0.2391 mg mL^{-1} for various temperatures.

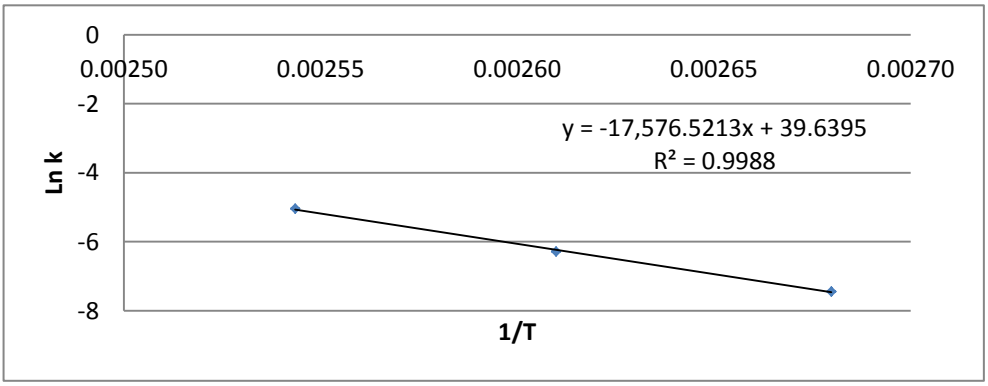


Figure 4.16 Arrhenius plot for the degradation of MA to AA (mmols).

4.2.8 Time at which MA (intermediate) reaches maximum concentration

Assuming the reaction is a sequential first order reaction with rate constants for k_1 and k_2 (0.00214 and 0.00068 min^{-1} respectively) of the reaction seen in **Equation 4.1**. The time at which the intermediate MA will be at maximum concentration can be determined when the derivative of [MA] with respect to time is zero:

$$(d[MA]/dt)_{t=tmax} = 0 \tag{4.19}$$

With the expression for MA in **Equation 4.6** substituted into the prior equation, the time at which maximum [MA] will be observed (t_{max}) can be determined:

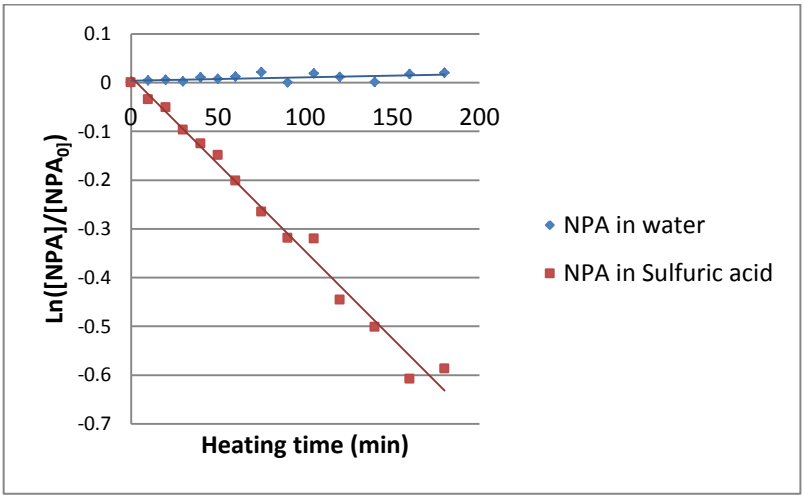
$$t_{max} = \frac{1}{k_1 - k_2} Ln\left(\frac{k_1}{k_2}\right) \tag{4.20}$$

Thus, t_{max} for MA was determined to be 777 minutes. This is an extremely long time, one that well exceeds the proposed method heating of 60 minutes. Therefore, it is reasonable to assume that high concentrations of MA will not be seen. This may result in MA being extremely hard to quantify and AA often unable to be observed. This is consistent with what has been observed in the kinetic degradations of NPA and MA standards.

4.2.9 NPA degradation in water

As seen in **Section 3.2.1.1**, an acid strength of 1 M was most efficient at hydrolysing the NPGs to release NPA. However, to understand what might happen during a cooking process, kinetic trials on degradation of NPA in water were also performed. An NPA standard of 0.3886 mg mL^{-1} was made using deionised water and prepared in the same way as samples for the NPA degradation in acid (**Section 2.4.1**). These were then analysed by HPLC (for raw data see **Appendix C1**).

A plot of $\text{Ln}\frac{[\text{NPA}]}{[\text{NPA}_0]}$ versus time comparing similar concentrations of NPA in water (0.3886 mg mL^{-1}) and 1 M sulphuric acid (0.3890 mg mL^{-1}) can be seen in **Figure 4.17**. The plot was $\text{Ln}\frac{[\text{NPA}]}{[\text{NPA}_0]}$ versus time because with water as the solvent there is no detectable degradation to form MA. Even with an extended heating time of 180 minutes, MA is not seen in the chromatogram. This indicates that even though 180 minutes of boiling nuts, hydrolyses the glycosides it may not further hydrolyse the NPA.



Solvent	Slope	R^2
Water	0.0000695x	0.267
Sulfuric acid (1 M)	-0.0035694x	0.986

Figure 4.17 Plot of Ln([NPA]/[NPA$_0$]) versus heating time comparing two solvents, water and sulfuric acid (1 M).

4.3 Correction factors to calculate the concentration of NPA_0

The toxicity of the karaka nuts arises principally from the presence of NPGs and there are a variety of NPGs present in karaka nuts. The toxicity of these NPGs are currently undetermined, however it has been assumed in this thesis that all NPGs contribute to the toxicity of the nuts. The method that has been developed is based on hydrolysing all of the NPGs to release NPA and measuring the total NPA concentration. It is assumed that all nitropropanoyl ester groups of the various NPGs have been hydrolysed to release NPA, thus the measurement is an NPA equivalents concentration. However, heating in acid which is essential for hydrolysis of glycosides, results in an attendant loss of NPA, as NPA degrades to yield MA and upon further degradation AA and often the MA and AA contents are too low to detect. As a result, a factor that allows for the NPA degradation needs to be incorporated to give an accurate prediction of NPA at time zero, $NPA_{0,}$ which is equivalent to the potential NPA released from the initial NPG content. There are two ways in which this factor has been determined as described in **Section 4.3.1**.

4.3.1 Determination of a factor to account for the loss of NPA during hydrolysis of NPGs.

Option 1 results in a correction factor (F_1) of 1.229 (4 s.f.).
The method is based on the greatest observed loss of NPA under the hydrolysis conditions. During tests to date, when different concentrations of NPA standards were made with sulfuric acid (1 M) as the solvent and heated (1 h, 100 ºC), the average percentage loss of NPA was determined as 16.6% (maximum loss of 18.6%) based on the peak area measurement (**Table 4.1)**. Since underestimation of NPA is undesirable, the greatest loss of 18.6% was used to determine a factor, which gives a rough approximation of original NPA content at a time of zero minutes, accounting for the NPA content of the NPGs found in the karaka nuts.

The greatest loss of NPA with heating time of 1 hour as stated is 18.6% so F_1 can be determined as:

1-([Maximum % NPA lost]/100) for chosen heating time = Y

Thus, 1/Y = Factor (F_1)

F_1 is the factor that the NPA content is multiplied by at the chosen time (must be the same heating time used for % NPA loss). For example, with a method that uses a heating time of 60 minutes, the greatest NPA % loss was found to be 18.6%, thus, Y would be 0.814 and the factor (F_1) for $NPA_{0\ (60\ min)}$, will be 1.229 (4s.f).

There remains potential for concentrations of NPA_0 to be under-estimated; thus, if F_1 is to be used as a correction factor to determine NPA_0 from a measured NPA value, an additional safety component should be included when calculating possible consumption levels.

Table 4.1 Percentage loss of NPA as a result of heating various concentrations of NPA standards at 100 ºC for 60 min

Concentration of NPA (mg mL^{-1})	Peak area of NPA at t =0 min	Peak area of NPA at t=60 min	NPA % loss
0.2700	21472081	18191659	15.3
0.3890	30211866	24701133	18.2
0.5024	38541129	31385603	**18.6**
0.5026	37555604	31486543	16.2
0.8140	61160945	52034472	14.9
		Average	**16.6**

Option 2:

$$\frac{[AA]}{\left(\frac{k_1 e^{-k_2 t} - k_2 e^{-k_1 t}}{k_2 - k_1} + 1\right)} = \frac{[AA]}{X} = [NPA]_0 \qquad (4.9)$$

Using the expected equation (4.9), a correction factor (X) is able to be determined, using the experimentally obtained values of 0.00214 and 0.00068 min^{-1} for k_1 and k_2 respectively. X has been determined as 0.00248. Thus, X has a value of 0.00248 which is much smaller than the projected value for F_1. However, there are some problems associated with derivation of X.

Principally AA is not seen in chromatograms at all after a heating time of 60 minutes because degradation does not occur fast enough and/or once AA is formed in the acidic medium of sulfuric acid, it may decompose completely so is not measurable. Additionally, if and when MA and AA are observed, it is often in

small quantities. This means that quantification of MA or AA is often impossible because peak areas of MA and/or AA are below the MDL or LOQ (when in a matrix of sulfuric acid not in the nut matrix, the latter of which is expected to give higher MDL and LOQs). For these reasons, it would be better to make the assumption that [MA] and [AA] are both negligible and only [NPA] needs to be considered, Thus:

$$[NPA] = [NPA_0]e^{-k_1 t} \quad (4.5)$$

or $[NPA]/X_2 = [NPA]_0$. The value for k_1 comes from the experimental plot of $-\text{Ln}([NPA]/[NPA_0])$ (**Figure 4.6**), and is found to be 0.0034 min^{-1}. Therefore, $X_2 = e^{-(0.0034 \times 60)} = 0.8155$ and $F_2 = 1/X_2 = 1.226$ (4 s.f.).

4.3.2 Comparison of correction factors

Option 1 results in a correction factor (F_1) of 1.229 while option 2 results in a correction factor (F_2) of 1.226, this means there is only a 0.24 % difference between the factors. Option 2 is expected to be more accurate than option 1 because it is based on the actual kinetics of NPA degradation. Both options are convenient and rapid as they only rely on being able to measure the NPA content and not MA and AA also.

4.3.2.1 Final NPA correction factor (CF)

The final correction value that will be used to quote NPA as NPA original at time of zero (NPA_0) is 1.23 (3 s.f.).

4.3.3 Additional safety factor

It is recommended that if in fact this method is to be used at a later date in the food industry, it is refined and an additional safety factor is considered. The addition of a safety factor would ensure that any small or incremental losses in measurement of NPA or incomplete hydrolysis of NPGs to NPA, is accounted for. This is highly important given the inherent toxicity of NPA and the potential toxicity of NPGs. However, for the purposes of this thesis the correction factor (1.23) will be implemented unaccompanied by an additional safety factor.

5 Moisture and NPA quantification in karaka nuts

5.1 Moisture content of karaka nuts

The weight of 10 individual nuts from each of the 16 samples were recorded prior to (wet weight) and after freeze drying (dry weight), and the averages of each sample were calculated. The moisture content of each nut was calculated using **Equation 2.5**. All results are included in **Appendix D1** while a summary can be seen in **Table 5.1.**

The average wet weight of a karaka nut was found to be 2.58 g, with a range of 1.04 g (1.97010 to 3.01097 g), while the average dry weight was found to be 1.45 g, with a range of 1.28 g (0.63383 to 1.91287 g).

The average moisture content between samples varied considerably from 24.1% to 67.8% (**Table 5.1**). The wide range in values is due to a number of factors including the differing stages of ripeness of fruit collected; the location of collection as well as being somewhat dependent on whether or not nuts were collected and then intentionally left outside to weather for periods of time. A summary table of the moisture content in the samples that were collected directly off the trees as drupes (both ripe and unripe, on various dates) was compiled for better comparisons to be made (**Table 5.2)**.

In **Table 5.2**, it can be seen that the nuts from the unripe drupes of both trees A and C have higher average moisture contents of 59.3% and 67.8 % compared with the nuts collected as ripe berries (38.7% and 48.8%, for trees A and C, respectively). Additionally, it was found that moisture content did not only vary with ripeness of berries, but also the location of the tree. This is illustrated as the average moisture content of nuts collected as ripe drupes were found to be 38.7%, 42.6%, 48.8% and 44.3% for trees A to D respectively. The average of these equates to 43.6%. This value is comparable with the moisture contents obtained by McCurdy[17] of 45.8% (range of 41.8 - 48.0%) and Klinac *et al.*, [5] of 43.7% (range of 36.4-48.3%).

When drupes were collected both directly off the tree and the ground for individual trees on a given date, the general trend was that the moisture content of the nuts collected off the ground was slightly lower than those collected directly off the trees. Furthermore, it was shown that if nuts were weathered prior to storage, the moisture content was also found to drop (as expected from natural air drying), a good illustration of this is sample 7. These nuts were weathered (~ 3 weeks) and found to contain an average of 28.4% water. This value is considerably lower than the moisture content of fresh nuts (53.7%) collected from the same tree on the same date that were not weathered (**Table 4.2**). Both of these aforementioned trends coincide with observations of moisture content found in karaka nuts reported by Klinac *et al*.[5]

Table 5.1 Average wet and dry weights (g) and moisture content (%) of the 10 individual samples tested for each of the 16 samples.

			Average weight (g) and moisture content (%) of karaka nuts		
Sample #	**Tree**	**Collection date**	**Wet weight (g)**	**Dry weight (g)**	**% moisture***
1	**C**	**10/1/12**	1.97010	0.63383	67.8
2	**A**	**10/1/12**	1.80457	0.73444	59.3
3	**A**	**24/1/12**	2.81335	1.72503	38.6
4	**B**	**24/1/12**	2.54949	1.50659	42.0
5	**C**	**24/1/12**	2.61069	1.29712	53.7
6	**C**	**24/1/12‡**	2.61616	1.30231	47.7
7	**C**	**24/1/12†**	2.16766	1.51673	28.4
8	**A**	**24/1/12†**	2.54599	1.91287	24.1
9	**C**	**6/2/12**	2.73214	1.52512	44.2
10	**C**	**6/2/12‡**	2.98281	1.63997	45.3
11	**A**	**6/2/12**	2.99344	1.83425	38.7
12	**B**	**8/2/12**	3.00108	1.70356	43.2
13	**B**	**8/2/12‡**	2.19287	1.40153	36.9
14	**C**	**8/2/12**	2.56678	1.32655	48.4
15	**C**	**8/2/12‡**	3.01097	1.66535	44.8
16	**D**	**13/2/12**	2.64587	1.47212	44.3
Average			**2.57525**	**1.44984**	

Notes: *means average of the 10 individual nuts tested for that sample # with outliers determined by Grubbs at 95% CI. All outliers excluded from averages obtained.†= nuts collected this date but exposed to weather and stored in freezer on 12/3/12. ‡= Nuts collected off the ground.

Table 5.2 Summary table of moisture content (%) found in nuts that were collected directly of the trees as drupes (ripe and unripe).

Tree	Sample ID #	Date of collection	Ripeness of drupes	Average moisture content* in nuts (%)	Average of the average moisture content in nuts (%)
A	2	10/1/12	Unripe (green)	59.3	59.3
	3	24/1/12	Ripe	38.6	38.7
	11	6/2/12	Ripe	38.7	
B	4	24/1/12	Ripe	42.0	42.6
	12	8/2/12	Ripe	43.2	
C	1	10/1/12	Unripe (green)	67.8	67.8
	5	24/1/12	Ripe	53.7	
	9	6/2/12	Ripe	44.2	48.8
	14	8/2/12	Ripe	48.4	
D	16	13/2/12	Ripe	44.3	44.3

Notes: *means average of the 10 individual nuts tested for that sample # with outliers determined by Grubbs at 95% CI. All outliers excluded from averages obtained. Only nuts that were collected directly off the tree have been included, all weathered and nuts collected off the ground have been excluded.

5.2 Quantification of nitropropanoic acid in nuts

5.2.1 Concentration of nitropropanoic acid found in individual nuts

Ten individual nuts from each of the 16 samples were randomly selected, prepared separately as in **Section 2.5.1** and analysed by HPLC. The data was processed using the formulae found in **Section 2.5.2**. Any outliers were determined using Grubbs test at 95% CI and were not used in calculating averages. The concentration of NPA found in each nut in g kg^{-1} can be found in **Table 5.3** and all raw data can be found in **Appendix D1**.

Table 5.3 Nitropropanoic acid content found in individual nuts (g kg^{-1}) (dry weight).

			Concentration of NPA in g per kg of nuts (dry weight) (g kg^{-1})											
Sample #	**Tree**	**Collection date**	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Replicate 4**	**Replicate 5**	**Replicate 6**	**Replicate 7**	**Replicate 8**	**Replicate 9**	**Replicate 10**	**Average**	**RSD (%)**
1	**C**	**10/1/12**	132.16	113.72	153.00	170.62	162.76	110.07	112.54	171.27	118.53	141.55	**138.62**	**17.75**
2	**A**	**10/1/12**	153.06	103.35	126.10	147.18	156.85	139.37	117.16	140.90	128.60	157.86	**137.04**	**9.64**
3	**A**	**24/1/12**	89.59	92.84	85.24	102.26	80.55	91.03	95.29	83.48	85.21	92.46	**89.79**	**7.15**
4	**B**	**24/1/12**	83.38	66.42	74.59	72.61	79.59	86.69	89.02	67.36	81.99	69.83	**77.15**	**10.51**
5	**C**	**24/1/12**	87.34	108.67*	72.96	79.53	77.48	87.14	82.09	84.54	95.02	79.06	**82.80**	**12.00**
6	**C**	**24/1/12‡**	85.52	74.25	85.75	160.29*	78.49	84.38	80.27	88.53	78.56	88.86	**82.74**	**27.61**
7	**C**	**24/1/12†**	62.40	47.43	54.68	64.03	46.43	67.21	73.75	79.16	46.40	51.31	**59.28**	**19.93**
8	**A**	**24/1/12†**	83.71	66.50	71.53	63.74	68.06	73.35	75.93	78.03	71.34	84.16	**73.63**	**9.35**
9	**C**	**6/2/12**	77.95	65.72	64.57	72.24	70.85	73.75	77.32	78.08	64.52	75.02	**72.00**	**7.54**
10	**C**	**6/2/12‡**	72.04	77.27	86.09	95.90	80.71	85.59	69.29	79.74	74.80	70.79	**79.22**	**10.42**
11	**A**	**6/2/12**	84.39	97.97	88.62	83.49	88.41	78.87	75.47	88.31	80.70	89.08	**85.53**	**7.47**
12	**B**	**8/2/12**	74.04	76.05	76.74	76.41	79.61	89.38	90.77	70.03	69.58	56.72	**75.93**	**12.90**
13	**B**	**8/2/12‡**	80.13	79.46	104.52	98.18	95.22	71.88	92.19	91.79	74.07	71.09	**85.85**	**13.94**
14	**C**	**8/2/12**	73.29	83.27	68.60	76.96	75.32	84.69	79.94	76.44	66.85	65.01	**75.04**	**8.92**
15	**C**	**8/2/12‡**	82.78	72.66	70.01	94.70	73.77	77.63	88.98	78.25	80.42	74.96	**79.42**	**9.64**
16	**D**	**13/2/12**	40.43	52.66	38.47	60.81	48.87	65.32	49.22	49.03	54.66	43.01	**50.25**	**16.99**

Notes: * = outliers as detected by Grubbs at 95% CI. All data with RSD > 10% was tested for outlier using Grubb's test. †= nuts collected this date but exposed to weather and stored in freezer on 12/3/12. ‡= Nuts collected off the ground.

5.2.2 Average concentration of nitropropanoic acid in karaka nuts

The average concentration of NPA found in karaka nuts ranges from 50.25 - 138.62 g kg^{-1} (**Tables 5.3**), which is equivalent to 5.0 to 13.9%. The levels of NPA appear to vary within a tree, between trees and with collection dates (ripeness of the fruit). It must be noted that these levels are much higher than any previously reported values for karaka. In fact they are orders of magnitude higher than previous reports. However, previous reports often only quoted the concentration as a percentage, for a single NPG (as NPG not NPA). This did not account for the fact that NPGs can yield a varying number of moles of NPA (dependent on the structure of the individual NPG), nor the fact that there is more than one NPG present that will release NPA upon hydrolysis. An example is that McChesney[58] found karakin (an individual NPG) to be present in nuts from ripe berries at a level of 0.08% (w/w). As previously stated, quantification in past research was performed by direct crystallisation or by measuring the release of NO_2, both of which would be expected to give lower levels than a direct assay as used in this research.

5.2.2.1 Variations in the concentration of nitropropanoic acid within nuts

5.2.2.1.1 Ripeness of the nut

The average level of NPA found in nuts from individual trees appears to vary depending upon the stage of ripeness of the drupes. The dates of collection are directly related to the ripeness of the drupes collected. From **Tables 5.3**, **5.4** and **Figure 5.1**, it can be seen that the later the date of collection within the fruiting season, the lower the concentration of NPA detected. This is illustrated by the fact that on the 10/1/12, samples from both trees A and C were collected as very green, unripe drupes. The average concentrations of NPA in the nuts from these drupes were found to be 137.04 and 138.52 g kg^{-1} for tree A and C respectively. In comparison, nuts from riper drupes collected from the same trees (A and C on the 24/1/12) were found to have average NPA concentrations of 89.79 and 82.80 g kg^{-1} respectively. Nuts from very ripe drupes collected on the 8/2/12 were found to have average NPA concentrations of 85.53 and 75.04 g kg^{-1} for trees A and C respectively. The average percentage loss of NPA due to ripening for both trees (A and C) can be calculated using the following formula (**Equation 5.1)**:

$$\%\ \text{NPA loss (w/w)}_{\text{ripening}} = \left\{\frac{[\text{NPA}]_{\text{unripe nuts}} - [\text{NPA}]_{\text{ripe nuts}}}{[\text{NPA}]_{\text{unripe nuts}}}\right\} \times 100 \quad (5.1)$$

The percentage loss of NPA for trees A and C was found to be 37.6 and 45.8% across the course of ripening. These values indicate that natural ripening of drupes, even for just four weeks, appears to result in a significant reduction of NPA content in nuts. This is an important consideration for removal of the toxins in karaka nuts before consumption, as collection of ripe or overripe drupes will yield nuts with naturally lower NPA concentrations. This result is partially contradictory to the findings of McChesney,[58] who found the concentration of karakin to be higher in nuts from ripe drupes compared with unripe drupes (as previously stated). However, the limitations of McChesney's research were that he only isolated one of the NPGs and NPA can also arise from the presence of other NPGs; if NPA is initially found as the free form in unripe drupes and only combines to form glycosides as ripening proceeds. It may be that the major contribution in unripe drupes is from free NPA or NPGs with less than three nitroester groups. The method used in this study detects NPA regardless of whether it is free or bound as NPGs, so McChesney's observations cannot be directly compared.

5.2.2.1.2 Variations due to exposure to weather conditions

When samples were collected on the 24/1/12, additional samples of nuts from trees A and C were collected from the ground (samples 7 and 8). These were then deliberately left outside, exposed to rain, sun and wind (weathering) for ~ 6 weeks prior to being frozen. The results show that the levels of NPA in the nuts exposed to weather conditions are considerably lower than the levels in the fresh nuts that were stored inside. The percentage loss of NPA due to exposure to weather conditions can be calculated using the following formula (**Equation 5.2**):

$$\%\ \text{NPA loss (w/w)}_{\text{weathering}} = \left\{\frac{\text{Average [NPA]}_{\text{fresh nuts}} - \text{Average [NPA]}_{\text{weathered nuts}}}{\text{Average [NPA]}_{\text{fresh nuts}}}\right\} \times 100 \quad (5.2)$$

The result of weathering nuts prior to storage is 18.0 and 28.4% decreases in NPA concentrations for nuts from trees A and C respectively. Although, there were considerable percentage losses of NPA by weathering the nuts, it must be noted that there was a visual change in the appearance of the nuts. The nuts were no longer soft and moist, with a pale white/creamy appearance; but were hard and dry and appeared yellow/slightly brown. The consequent decomposition may affect the nutritional value of the nuts which is undesirable. This needs to be the subject of further investigation.

5.2.2.1.3 Variations due to the positioning of sample collected (off the tree versus off the ground)

Some of the dates of sampling included drupes or nuts being collected both directly off the tree and some nuts that had already fallen to the ground. The average NPA content in these nuts from various dates can be found in **Table 5.4**. The percentage differences between the nuts collected from the trees and those collected from the ground varies from 0.1% to 13%. The greatest difference of 13% occurred with nuts from tree B (collected on the 8/2/12, 75.93 g kg^{-1} off the tree versus 85.85g kg^{-1} off the ground). Although the percentage differences in NPA concentration varies considerably between nuts off the tree and nuts off the ground, no definitive conclusions can be drawn without further testing as there are numerous possible reasons for this observation. The nuts collected from the ground appear to have higher levels of NPA than the equivalent nuts collected off the tree on the same dates. This may be due to the fact that the nuts off the ground had been blown off the tree at an earlier stage of ripeness than the equivalent nuts on the tree, since nuts from unripe drupes have been found to have higher concentrations of NPA. Alternatively, birds may have fed off the berry flesh, causing the nuts to fall, however it might be expected the birds would feed off the riper berry flesh. Overall, nothing definitive can be concluded about whether it is best to collect the nuts directly off the tree as drupes or wait until they fall to the ground. However, if nuts are collected from the ground, degradation and/or decomposition can occur.

Table 5.4 Summary table of the average NPA concentration of nuts collected off the various trees on the different dates.

	Average concentration of NPA in nuts (g kg^{-1})				
Collection date	**10/1/12**	**24/1/12**	**6/2/12**	**8/2/12**	**13/2/12**
Tree					
A	137.04	89.79, 73.63†	-	85.53	-
B	-	77.15	-	75.93, 85.85‡	-
C	138.62	82.80, 82.74‡, 59.28†	72.00, 79.22‡	75.04, 79.42‡	-
D	-	-	-	-	50.25

†= collected at this date but exposed to weather and stored in freezer on 12/3/12.

‡= nuts collected off the ground.

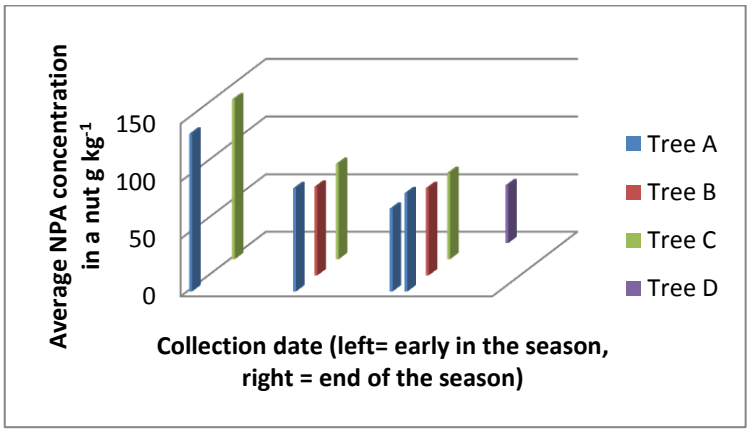


Figure 5.1 Plot of average NPA concentration of each tree versus the date of collection (left is 24/1/12, right 13/2/12), where collection date is proportional to ripeness. Data is only for samples that were not weathered.

5.2.2.2 The difference in nitropropanoic acid concentration within a single nut

Three of the 16 samples were randomly selected (4, 7 and 14), and an individual nut from each of these was randomly selected and prepared as in **Section 2.5.1**. The sample was homogenised and five sub-samples of ~ 0.1 g were prepared and analysed by HPLC. The data was processed using the formulae found in **Section 2.5.2**.The quantity of NPA found in g kg^{-1} can be found in **Table 5.5** and all raw data can be found in **Appendix D2**.

The variance in concentration found within each of the three individual nuts is best represented by the relative standard deviation (RSD), which is a measure of how much variance there is in relation to the mean; RSD= s/m x100, where

s = standard deviation and m = mean. It can be seen that there is only a very small variance in the replicates from samples 4 and 14, with RSD values of 0.91 and 1.15% respectively. In comparison the variance from sample 7 is considerably larger at 8.09% (**Table 5.5**). The most likely reason for the greater variation in sample 7 is that it was from a sample of nuts that had been weathered prior to being frozen. As already discussed, weathering results in visible changes in the nuts. These changes made it extremely difficult to grind the nuts into a homogeneous powder following freeze drying in the preparation stage. Nuts from the two samples that were weathered had to be cut into very small pieces (< 3 mm^3) instead. This means that the nut sample from sample 7 is much less homogeneous than that of samples 4 and 14 and more variance was expected. The value for variance in non-weathered nuts is quite small and indicative that if a nut is homogenised, then the average results of NPA content in a given nut would be expected to be quite accurate.

Table 5.5 The variation in concentration of NPA g kg^{-1} found in individual nuts.

Concentration of NPA in nuts (g kg^{-1})			
Sample #	**4**	**7**	**14**
Replicate			
1	71.59	64.35	77.71
2	70.73	68.50	76.16
3	69.98	76.71	75.69
4	70.63	78.56	76.33
5	71.37	72.43	75.45
Average	70.86	72.11	76.27
RSD	0.91%	8.09%	1.15%

5.2.2.3 The difference in quantity of nitropropanoic acid found in left and right halves of nuts

The NPA concentrations of the left (L) and right (R) halves of individual karaka nuts were compared (**Table 5.6**; raw data in **Appendix D2**). It can be seen that the percentage difference in NPA concentration between halves of a nut ($\%D_{L/R}$) varies from 2.8% to 10.6%, giving a range of 7.8%. The average $\%D_{L/R}$ was 5.8% (intra-nut variation). The value of 7.8% for the range may be considered small given that a value of 20 or 30% difference is readily accepted in biological statistics.[65] The average difference of 5.8% is also acceptable, assuming that there

is some degree of biological variation. The only thing that can be concluded by these results is that there definitely appears to be variance within halves of karaka nuts, which most likely arises from biological differences due to different stages of ripening between individual nuts, or even within a nut, for example if one half of the drupe is more exposed to sunlight.

Table 5.6 Quantity of nitropropanoic acid in vertical halves of karaka nuts.

Sample #	Tree	Concentration g kg^{-1}		% difference between halves ($\%D_{L/R}$)
		Left (L)	Right (R)	
2	A	112.19	115.29	2.8
6	C	91.40	82.61	10.6
11	A	81.29	84.28	3.7
12	B	73.06	69.70	4.8
14	C	90.19	96.40	6.9
			Average	5.8

5.2.2.4 The difference in nitropropanoic acid concentration between top and bottom halves of a nut

The quantity of NPA found in the tops and bottoms in g kg^{-1} can be found in **Table 5.7**, and all raw data can be found in **Appendix D2**. The NPA concentrations of the top (T) and bottom (B) halves of individual karaka nuts were compared (**Table 5.7**). It can be seen that the $\%D_{T/B}$ varies considerably from 0.6% to 23.4%, with a range of 22.8%, while the average $\%D_{T/B}$ was found to be 7.3%. The only thing that can be concluded from these results is that there definitely appears to be a large biological variance that needs to be considered when running tests. Suggestions to overcome this are, if and where possible to homogenise the whole nut as this should give a value that is closer to the true content of NPA in a nut.

Table 5.7 Quantity of nitropropanoic acid in the horizontal halves of karaka nuts.

Sample ID # the nut comes from	Tree that the nut comes from	Concentration g kg^{-1} Top (T)	Bottom (B)	% difference between halves ($\%D_{T/B}$)
1	C	95.81	87.42	9.6
2	A	161.93	131.22	23.4
3	A	99.71	89.19	11.8
4	B	89.21	81.42	9.6
5	C	87.80	85.87	2.3
6	C	79.54	82.80	4.1
7	C	44.55	40.05	11.2
8	A	58.26	77.24	32.6*
9	C	75.25	79.85	6.1
10	C	77.35	74.38	4.0
11	A	82.43	85.11	3.3
12	B	67.29	72.80	8.2
13	B	71.02	75.07	5.7
14	C	73.35	75.23	2.5
15	C	81.35	81.82	0.6
16	D	47.74	44.44	7.4
			Average	7.3

*= outlier at 95% CI using Grubbs test.

5.2.2.4.1 Comparison of percentage differences in nitropropanoic acid concentration found between vertical and horizontal halves of karaka nuts

The average $\%D_{T/B}$ was found to be 7.3% (**Table 5.7)** which is greater than the average $\%D_{L/R}$ of 5.8% (**Table 5.6**). Both of these values indicate a degree of variation between halves of a nut, however these values are considered acceptable given that the most likely cause of variation is biological differences. The top half of a nut may be expected to have higher NPA concentrations due to the fact that it is closer to the stalk, assuming NPA/NPGs are biosynthesised elsewhere in the tree (some literature indicates NPA is biosynthesised in higher levels near the roots of a plant, however nothing conclusive has been found at this point in time). If the glycosides present in karaka nuts are increased with growth of the drupe, this may be expected to be observed. However, this was not always the observation, as 7 out of the 16 bottom halves of the nuts were found to have higher NPA content than the bottoms. Thus, it appears that NPA content is not strictly concentrated around the point of growth of the drupe but random. It is not

possible to account for variation due to a segment of a drupe being exposed to sun, as this also may have an effect, especially if NPG content is affected by ripeness of the drupe. This problem is expected to affect the vertical halves (L and R) of the nuts more, as one half may have been more exposed to sunlight while the other was shaded by the tree. In addition, there is the problem that the drupes grow and ripen as clusters, so are therefore exposed to sunlight by differing amounts and ripen at varying rates, even within a cluster on an individual tree. However, what can be concluded is that there is a considerable amount of variation within an individual nut, therefore if and where possible this should be mitigated by homogenising the whole nut, so that a NPA value closer to the total value of the whole nut can be obtained. In addition, due to the large degree of variation present from nut to nut, in order to make comparisons, when nuts are to be exposed to treatment processes, they will have to be cut in half. This will ensure a more accurate comparison of NPA levels prior to and after treatments. Since, the percentage differences between vertical halves of nuts was found to be smaller than horizontal halves, it is recommended that comparisons be made using vertical halves as this should give more accurate results.

5.2.2.5 Comparison of intra-nut and inter-nut variation

The intra-nut percentage differences between both vertical halves and horizontal halves of nuts tested (**Tables 5.6** and **5.7**), proved to be lower than the values (RSD) obtained for inter-nut tests (**Table 5.3**). This corresponds with the expectation that lower variation is expected from within an individual sample versus a group of samples. There are numerous literature sources confirming this, with an example being that the biological variation in analytes of a healthy human population was found to vary between 1.0 – 36.7% within individuals (intra), while the group variation was greater at 0.54 – 72.4% (inter).[65]

5.2.3 Quantification of NPA in parts of a karaka drupe; berry flesh, shell, pellicle and nut

The concentrations of NPA in g kg^{-1} for each of the individual parts were calculated (**Table 5.8**; for raw data see **Appendix D3**). It can be seen from **Table 5.8** that for both unripe and ripe drupes, the parts that contain the highest concentrations of NPA are the nuts, followed by the berry flesh. The shell and pellicle contain varying amounts with no apparent trend, however it would require further testing and a greater number of replicates to confirm this is the case. In addition, it can be seen that there is a trend whereby nuts from unripe drupes contain higher levels of NPA than nuts from ripe drupes. This coincides with results previously reported (**Section 5.2.2.1.1)** when the concentrations of individual nuts at differing stages of ripeness were compared.

Table 5.8 Concentrations of NPA in g kg^{-1} for individual parts of karaka drupes.

	NPA concentration in g kg^{-1}					
	Nut # 1 (unripe)			Nut # 9 (ripe)		
Part tested	**Nut 1**	**Nut 2**	**Average**	**Nut 1**	**Nut 2**	**Average**
Nut	158.96	89.02	**123.99**	76.96	63.42	**70.19**
Shell	5.58	3.19	**4.38**	2.80	7.20	**5.00**
Pellicle	22.92	12.96	**17.94**	2.60	2.87	**2.74**
Berry flesh	34.95	2.19	**18.57**	28.06	9.34	**18.70**

The results indicate that the NPA measured arising from NPGs in karaka, are present in higher concentrations in the nuts and berry flesh, which are the parts of most nutritional interest. Thus, it will be vitally important that processing techniques be developed in order to eliminate as many toxins as possible. Primarily the focus should be on the nuts as these are of the most nutritional interest. However, the lower concentrations found in the berry flesh may be worth future investigation, especially if nutritional benefits of karaka berries over other fruit can be found. This would aid in their marketability as a food product. Additionally, in future if the only interest is in the nut, when toxin removal techniques are implemented, NPA as a by-product may be able to be obtained from the other 'waste' parts of the drupe, i.e. the berry flesh, shell and pellicle and not only from the solution of the treated nuts. However, this is an idea that will need further investigation.

Furthermore, it must be noted that due to time restrictions and lack of interest in investigating parts of the karaka other than the nuts, the parts trial was only carried out on a limited number of samples (2 ripe and 2 unripe). As a result, caution with the interpretation of NPA content in parts should be applied. Especially considering the large degree of variation seen between nuts, this is extremely evident with sample 1, where the concentration of NPA for nut 1 was found to be 158.96 and 34.95 g kg^{-1} for the nuts and berry flesh, respectively (**Table 5.8**). Although, the trend of ripe berries having lower concentrations of NPA is apparent, future investigation and more replicates are required in order to confirm the concentration of NPA in other parts of the karaka.

6 Effectiveness of different nut treatment procedures

6.1 Introduction

The removal and/or degradation of toxins from karaka nuts is important in order to be able to render them safe to eat. Consequently, some basic industrial techniques that incorporate water and/or heat including autoclaving, microwave cooking, boiling, oven roasting and soaking were investigated. These potential treatments were assessed in order to determine whether heat and/or water is the most important process in eliminating the toxins found in karaka nuts.

When the tests were performed the efficiency of the processing technique was determined by the percentage loss of NPA (**Equation 6.1).** The percentage of NPA remaining in the nut was also calculated (**Equation 6.2**).

$$\%\ \text{NPA loss (w/w)} = \frac{[(\text{NPA})\text{untreated half of the nut} - [\text{NPA}]\text{treated half of the nut})}{[\text{NPA}]\text{untreated half of the nut}} \times 100 \quad (6.1)$$

$$\%\ \text{NPA remaining in the nut (w/w)} = \frac{[\text{NPA}]\text{treated half of the nut}}{[\text{NPA}]\ \text{untreated half of the nut}} \times\ 100 \quad (6.2)$$

In addition, since NPA itself may be a useful by-product (for example due to its potential use in insecticides) when processing treatments involved water, it was worth determining the concentration of NPA that is found in solution as a result. This was achieved by analysing the solution as is (unhydrolysed) to give free NPA and hydrolysed (**Section 2.7**) to give total NPA bound and free (**Equations 6.3** and **6.4**, respectively). Essentially the latter is the most important value as it is the highest concentration of NPA that can be obtained as a by-product of treating the nuts. The amount of NPA degraded by the treatment process is then equivalent to the difference between the total % of NPA lost due to treatment and the % of NPA found in the solution (**Equation 6.5**).

$$\%\ 'free' NPA\ liberated\ into\ the\ solution\ (w/w) = \frac{[NPA] unhydrolysed\ extraction\ solution}{[NPA]\ untreated\ nut} \times\ 100 \quad (6.3)$$

$$Total\ \%\ NPA\ liberated\ into\ the\ solution\ \left(\frac{w}{w}\right) = \frac{[NPA]\ hydrolysed\ extraction\ solution}{[NPA] untreated\ nut} \times\ 100 \quad (6.4)$$

$$Total\ \%\ NPA\ destroyed\ (\mathrm{w/w}) = \frac{[NPA]\ lost\ due\ to\ treatment - [\ NPA] found\ in\ the\ hydrolysed\ solution}{[NPA] untreated\ nut} \times 100 \quad (6.5)$$

The various treatments used different volumes of water and slightly different wet weight of nut for each test. Therefore, in order for the various treatments to be compared, the results were standardised to give the concentration of NPA (mg mL^{-1}) in solution arising from using 1 kg of nuts and 1 L of water. This was done by using the using **Equation 6.6**.

$$\mathrm{NPA\ mg\ mL^{-1}} = \frac{\text{NPA peak area}}{\text{Slope of NPA calibration curve}}\ (\mathrm{mg\ mL^{-1}}) \times \frac{\text{Volume of solution used (mL)}}{1000\ (\mathrm{mL})} \times \frac{1000\ (\mathrm{g})}{\text{weight of nut used (g)}} \quad (6.6)$$

An example of this calculation is as follows, for 1.08 g of nut being treated in a volume of 160 mL:

$$\text{NPA (mg mL}^{-1}) = \frac{\text{NPA peak area}}{\text{Slope of NPA calibration curve}}(\mathrm{mg\ mL^{-1}}) \times \frac{160\ (\mathrm{mL})}{1000\ (\mathrm{mL})} \times \frac{1000\ (\mathrm{g})}{1.08\ (\mathrm{g})}$$

6.2 Treatments to remove NPA from karaka nuts

6.2.1 Autoclaving

The percentage loss of NPA from nuts as a result of autoclaving for various time periods (**Table 6.1**) was calculated using **Equation 6.1** (raw data in **Appendix E1**).

The average percentage loss of NPA from karaka nuts was found to be 59.3% when autoclaved for 2 minutes and 65.2% when autoclaved for 5 minutes, while longer autoclaving times of 20, 60 and 120 minutes resulted in average NPA losses of 79.7%, 88.4% and 94.4% respectively. These results indicate that autoclaving does significantly reduce the NPA content in karaka nuts; however it is unknown whether it is the temperature or pressure that has the most significant effect. It is also unknown whether the NPA is liberated into solution and/or degraded.

It must also be noted that although NPA concentrations reduce as a result of autoclaving, the nuts are visibly degraded. For short autoclaving times of 2 and 5 minutes, the nuts are dark brown/orange instead of the usual pale white creamy colour. Upon being autoclaved for longer periods of time (20, 60 and 120 min) the nuts appear sticky and dark brown/black (**Figure 6.1**). Ultimately, autoclaving for any amount of time does not result in a product that would appeal to consumers. This degradation is undesirable as there is no way autoclaving, even for short periods of time leaves karaka nuts in a visually appealing state.

Figure 6.1 Appearance of nuts after autoclaving: left to right; nut autoclaved for 120 minutes, nut prior to autoclaving and far right is a nut that was autoclaved as a drupe for 5 minutes.

Table 6.1 Percentage of NPA loss from karaka nuts as a result of autoclaving for various time periods

		NPA content in g kg^{-1} (dry weight)		
	Sample	**Prior to treatment**	**After treatment**	**% loss of NPA (g kg^{-1})**
Autoclaving time 120 min	**1**	66.10	3.71	94.4 (62.39)
	2	71.74	3.46	95.2 (68.28)
	3	63.06	4.12	93.5 (58.94)
	Average			***94.4 (63.20)***
Autoclaving time 60 min	**1**	84.69	9.23	89.1 (75.46)
	2	82.51	10.23	87.6 (72.28)
	3	71.53	8.14	88.6 (63.39)
	Average			***88.4 (70.38)***
Autoclaving time 20 min	**1**	78.20	18.13	76.8 (60.07)
	2	89.73	16.27	81.9 (73.46)
	3	89.59	17.57	80.4 (72.02)
	Average			***79.7 (68.52)***
Autoclaving time 5 min	**1**	76.66	26.23	65.8 (50.43)
	2	84.26	27.24	67.7 (57.02)
	3	62.59	23.63	62.2 (38.96)
	Average			***65.2 (48.80)***
Autoclaving time 2min	**1**	70.75	31.07	56.1 (39.68)
	2	69.28	28.76	58.5 (40.52)
	3	81.75	30.09	63.2 (51.66)
	Average			***59.3 (43.95)***

Whole drupes were autoclaved for the four shorter time periods (2, 5, 20 and 60 min) to assess if karaka nuts could be left in visually appealingly states while still reducing the NPA content of the nuts (**Table 6.2**). Since the whole drupe was autoclaved, individual halves of the nuts could not be compared in terms of NPA content prior to and after autoclaving. Consequently, **Equation 6.7** was used, whereby half a nut from an autoclaved drupe was compared to the average NPA content found in nuts from sample 9.

$$\%\ of\ NPA\ loss(w/w) = \frac{(Average\ [NPA]\ in\ nuts\ from\ ID\ \#\ 9\ -\ [NPA]\ treated\ half\ of\ the\ nut)}{Average\ [NPA]\ found\ in\ nuts\ from\ ID\ \#\ 9} \times 100 \qquad (6.7)$$

Table 6.2 NPA content and percentage loss of NPA from karaka nuts that were autoclaved as whole drupes.

		NPA concentration in g kg^{-1} (dry weight)		
	Sample	**Prior to treatment**	**After treatment**	**% NPA loss (g kg^{-1})**
	1	72.00*	8.55	88.1 (63.45)
Autoclaving	**2**	72.00*	7.75	89.2 (64.25)
time	**3**	72.00*	6.90	90.4 (65.10)
60 min	***Average***			***89.2 (64.27)***
Autoclaving	**1**	72.00*	14.25	80.2 (57.75)
time	**2**	72.00*	10.47	85.5 (61.55)
20 min	**3**	72.00*	13.51	81.2 (58.49)
	Average			***82.3 (59.26)***
Autoclaving	**1**	72.00*	22.60	68.6 (49.40)
time	**2**	72.00*	21.09	70.7 (50.91)
5 min	**3**	72.00*	17.81	75.3 (54.19)
	Average			***71.5 (51.50)***
Autoclaving	**1**	72.00*	27.28	62.1 (44.72)
time	**2**	72.00*	26.53	63.2 (45.47)
2 min	**3**	72.00*	23.88	66.8 (48.12)
	Average			***64.0 (46.10)***

*= average NPA content found in nuts from sample # 9 when 10 individual nuts were tested (**Table 5.3**).

It can be seen from the results in **Table 6.2** that the average percentage losses of NPA in nuts when autoclaved as drupes were between 0.81- 6.3% higher than nuts alone. This indicates that NPA may be liberated more easily from the NPGs if nuts are autoclaved as whole drupes. The moisture content of the berry flesh may be aiding in hydrolysis of the ester bonds in NPGs to release water soluble NPA. However, nothing definitive can be concluded at this point without further testing. Also, there may be some inaccuracies, particularly for longer treatment times (where the higher percentage NPA losses are actually associated with lower g kg^{-1} values) because the percentage loss of NPA in the nuts treated as whole drupes were compared to a previously found average NPA content for nuts (sample 9). Conversely, the nuts tested as halves were compared against the concentration of NPA of the individual nut being tested, thus more accurate comparisons were achieved. In addition, it was found that although NPA content

was diminished significantly even if the nut was autoclaved as a drupe, the nut after autoclaving was still visibly degraded, brown/black in colour similar to nuts treated without berry flesh; with the extent of discolouration directly relating to autoclaving time.

Since no solutions were obtained from autoclaving, no solution tests were conducted. However, it is expected that an autoclaving time of one hour, would significantly degrade or destroy NPA content in solution almost completely. This is based on the findings of Murray *et al.*,[55] who demonstrated that autoclaving of NPA isolated from *Indigofera* and synthesised NPA for one hour was found to completely decompose NPA.

In summary, autoclaving of karaka nuts, with or without berry flesh results in a significant reduction in NPA content. However, as a consequence the nuts are left in visually unappealing states which defeats the purpose of this project. Thus, autoclaving is not an effective treatment process for eliminating the NPGs found in karaka nuts.

6.2.2 Soxhlet extraction

Karaka nuts with wet weights between 1.46 to 1.84 g were treated overnight with soxhlet extraction in water (160 mL). The average % loss of NPA in karaka nuts as a result of overnight soxhlet extraction was found to be 98.7% (**Table 6.3**) (Raw data in **Appendix E2**). This indicates that soxhlet extraction does result in a considerable amount of NPA loss. However, it was unknown whether the NPGs are being degraded and/or liberated and whether it is heat or water that has the most significant effect. Additionally, there were some changes observed in the nuts that were treated; the nuts became very moist (this could be reduced if nuts were subsequently dried) however, there were not significant changes in the colour of the nuts meaning the nuts were left in visually appealing states.

Table 6.3 Concentration and percentage loss of NPA from karaka nuts as a result of overnight (17 h) soxhlet extraction

Sample	[NPA] prior to treatment (g kg^{-1})	[NPA] after treatment % (g kg^{-1})	Total NPA loss due to treatment % (g kg^{-1})
1	71.80	1.8 (1.31)	98.2 (70.49)
2	91.01	2.1 (1.87)	97.9 (89.14)
3	67.04	1.1 (0.73)	98.9 (66.31)
4	72.58	0.1 (0.07)	99.9 (72.51)
Average		**1.3 (1.00)**	**98.7 (74.61)**

Table 6.4 Standardised NPA concentrations in mg mL^{-1} in solution as a result of soxhlet extraction

Treatment	Sample	Standardised‡ [NPA] (mg mL^{-1})	% NPA in solution that was free* NPA
Soxhlet extraction overnight(~ 17h)	**Solution 1**	17.26	-
	Solution 2	18.35	-
	Solution 3	14.97	-
	Solution 4	15.81	-
	Average	**16.60**	-
	Hydrolysed solution 1	29.86	57.8
	Hydrolysed solution 2	34.23	53.6
	Hydrolysed solution 3	23.80	62.9
	Hydrolysed solution 4	27.71	57.1
	Average	**28.90**	**57.8**

Notes: * = free NPA is NPA that is released into water either as free NPA from the nuts, or is NPA that is released from hydrolysed NPGs as result of the cooking process.‡ = Standardised concentration of NPA that would arise from treating 1 kg of nuts in 1 L of water.

The standardised concentrations of NPA in mg mL^{-1} were calculated and can be seen in **Table 6.4** (for individual nut weights treated see **Appendix E2**). Soxhlet extraction results in 53.6 to 62.9% of the NPA being released as the free acid. The additional material observed after hydrolysis arises from a mixture of glycosides in the solution. The total standardised NPA concentrations released into hydrolysed solutions are between 23.80 and 34.22 mg mL^{-1} of NPA.

In summary, it appears that soxhlet extraction may be a viable means of removing toxins from nuts as well as liberating considerable concentrations of NPA to be obtained as a by-product in solution. Typically, soxhlet extraction would not be a commercial treatment process; however, the results are indicative that the right combination of heat and water could prove to be beneficial in toxin removal.

6.2.3 Boiling

Karaka nuts with wet weights between 1.23 to 1.87 g were boiled for various time periods (0.5 to 6 h) in water (150 mL). The effectiveness of boiling as a detoxification treatment of karaka nuts varied, with shorter boiling periods of half an hour and one hour resulting in average NPA losses of 65.7% and 82.1%, respectively. Longer boiling periods of 3 and 6 hours resulted in NPA losses greater than 90% (92.3% and 96.0%, respectively; **Table 6.5**, raw data in **Appendix E3**). These results indicate that boiling can result in significant reduction of NPA content in nuts. Although the NPA content was found to reduce with boiling time, it is not yet established whether water is the greatest contributor or whether it is a combination of water and heat that result in the considerable NPA losses from nuts. This will need to be investigated further.

In addition, it must be noted that although longer boiling periods resulted in greater NPA loss, the additional boiling results in structural degradation of the nuts. The pale yellow/cream nuts become light brown, at boiling times in excess of 3 hours (still a visually appealing colour), but more importantly the nuts became “water-logged” or soggy. This resulted in them breaking or mashing into pieces when touched (**Figure 6.2**). If karaka flour is required this may not be an issue.

Table 6.5 Concentration and changes of NPA content of karaka nuts, with boiling (100 °C) for various time periods.

	Sample	[NPA] prior to treatment (g kg^{-1})	[NPA] after treatment (g kg^{-1})	Total NPA loss in the nut due to treatment % (g kg^{-1})
Boiling time 0.5 h	**1**	76.84	16.74	78.2 (60.10)
	2	71.98	30.57	57.5 (41.41)
	3	71.05	27.46	61.4 (43.59)
Average				***65.7 (48.37)***
Boiling time 1 h	**1**	83.05	19.08	77.0 (63.97)
	2	69.32	12.07	82.6 (57.25)
	3	63.06	8.33	86.8 (54.73)
Average				***82.1 (58.65)***
Boiling time 3 h	**1**	76.68	5.96	92.2 (70.72)
	2	80.19	6.87	91.4 (73.32)
	3	70.99	4.76	93.3 (66.23)
Average				***92.3 (70.09)***
Boiling time 6 h	**1**	67.75	2.50	96.3 (65.25)
	2	78.47	3.98	94.9 (74.49)
	3	67.23	2.10	96.9 (65.13)
Average				***96.0 (68.29)***

Figure 6.2 Appearance of a karaka nut that has been boiled for 1 hour at 100 °C, the water-logged and mushy state, as well as colouration changes can be seen.

The standardised concentrations of NPA in mg mL^{-1} for the solutions of the various boiling times were calculated and can be seen in **Table 6.6** (for individual nut weights treated see **Appendix E3**).

It can be seen from **Table 6.6** that boiling for various periods of time releases a variable range of NPA , between 22.80 and 30.71 mg mL^{-1} of NPA (0.5 and 3 h, respectively). As expected, the longer the nuts are boiled, the greater the percentage of free NPA (average percentage values of NPA of 10.9, 16.8, 22.6 and 31.1% for 0.5,1, 3 and 6 h, respectively). The values seen in the table of both free and total NPA found in the solutions indicate that there is a large degree of liberation of NPA from karaka nuts. This means that boiling may well be a viable technique for obtaining solutions of NPA naturally. However, at a certain time of boiling, the simultaneous liberation and degradation of NPA needs to be considered, in order to maximise the yields of NPA in solution. For example, if maximal NPA content is the desired outcome, perhaps for use in insecticides and repellents, the additional 3 hours of boiling between 3 to 6 hours would be counter-productive. This is because a boiling time of 3 hours can obtain more NPA in solution than 6 hours boiling time, due to the consequential degradation of NPA with the prolonged exposure to heat. These results are consistent with previous reports. Murray *et al*.,[55] state that boiling for 1 hour at 100 °C can completely degrade NPA, while McChesney[58] and Carter[30] state that boiling the NPG karakin for 6 hours or more results in complete hydrolysis to NPA. Meaning that with long boiling times in water, one may still expect some NPA to be present, as the NPGs are gradually hydrolysed to release NPA over time in order for it to be degraded.

In summary, boiling appears to cause a significant reduction in NPA content in nuts. It is unknown whether heat or water has the most significant effect on the observed NPA reduction, thus this will need further investigation in the future. Moreover, at present there are changes observed in the nuts boiled for longer periods of time which leave them soggy. The process of boiling or at least use of heat and/or water in toxin removal should be further investigated, as boiling appears to be beneficial. Boiling may be most usefully implemented as part of a processing technique, rather than as the sole processing technique. Furthermore, it appears that boiling karaka nuts may be a viable processing method if looking to

obtain NPA in solution, however conditions to avoid unnecessary degradation and loss would need to be further investigated in order to get maximum efficiency.

Table 6.6 Standardised NPA concentrations in mg mL^{-1} as a result of boiling for various time periods.

Treatment	Sample	Standardised‡ [NPA] (mg mL^{-1})	% NPA in solution that was free* NPA
Boiling (0.5 h)	Solution 1	2.75	
	Solution 2	2.22	
	Solution 3	2.50	
	Average	**2.49**	
	Hydrolysed solution 1	24.46	11.3
	Hydrolysed solution 2	22.69	9.8
	Hydrolysed solution 3	21.46	11.7
	Average	**22.87**	**10.9**
Boiling (1 h)	Solution 1	4.25	
	Solution 2	4.24	
	Solution 3	4.09	
	Average	**4.19**	
	Hydrolysed solution 1	31.51	13.5
	Hydrolysed solution 2	22.92	18.5
	Hydrolysed solution 3	22.02	18.6
	Average	**25.48**	**16.8**
Boiling (3 h)	Solution 1	6.97	
	Solution 2	6.75	
	Solution 3	7.03	
	Average	**6.92**	
	Hydrolysed solution 1	30.05	23.2
	Hydrolysed solution 2	32.48	20.8
	Hydrolysed solution 3	29.60	23.8
	Average	**30.71**	**22.6**
Boiling (6 h)	Solution 1	8.58	
	Solution 2	8.69	
	Solution 3	8.63	
	Average	**8.64**	
	Hydrolysed solution 1	26.81	32.0
	Hydrolysed solution 2	32.52	26.7
	Hydrolysed solution 3	25.06	34.4
	Average	**28.13**	**31.1**

Notes: * = free NPA is NPA that is released into water either as free NPA from the nuts, or is NPA that is released from hydrolysed NPGs as result of the cooking process.‡ = Standardised concentration of NPA that would arise from treating 1 kg of nuts in 1 L of water.

6.2.4 Oven roasting

The average percentages of NPA lost from karaka nuts as a result of oven roasting nuts at a temperature of 80 °C were found to be 4.6, 9.6 and 9.3% for roasting times of 10, 20 and 30 minutes respectively. A hotter roasting temperature of 120 °C resulted in higher percentage losses of NPA, 12.8, 24.8 and 25.6%, while as expected the highest temperature of 175 °C, resulted in even greater percentage losses, 12.7, 36.9 and 65.4% for 10, 20 and 30 minutes, respectively (**Table 6.7**, **Figure 6.3**; raw data in **Appendix E4**). These results indicate that oven roasting can reduce the NPA content in nuts; however the loss is not as large as could be hoped for. These results also indicate that heat alone does degrade NPA and as expected higher temperatures are more efficient at doing so.

It must also be noted that at an individual heating temperature, the range between the three individual results for each heating time varied significantly. This is most likely due to the fact that the moisture content and weight of each nut (half) that was roasted varied. When undergoing the roasting process moisture loss is likely to happen first, followed by the degradation of NPA, however these processes may have happened simultaneously. For future tests, weights should be recorded prior to and after treatment thus the percentage losses in terms of % per gram of nut treated could be compared. This would be expected to give less variable results as it accounts for water content. However, nuts will always vary in weight and water content. Therefore, when a treatment process is developed in the future, one should be aware of and expect the variable range that can occur, irrespective of weight treated and water content, as it will be impractical to know this for every nut processed.

Table 6.7 Percentage loss of NPA from karaka nuts as a result of oven roasting at 80, 120 and 175 °C, for time periods of 10, 20 and 30 minutes.

			NPA content in g kg^{-1}			
			Prior to treatment	**After treatment**	**% loss of NPA**	**Average % loss of NPA**
Oven roasting temperature 80 °C	**Oven roasting time 10 min**	**Nut 1**	75.36	69.92	7.2	**4.3**
		Nut 2	76.82	74.74	2.7	
		Nut 3	80.15	77.83	2.9	
	Oven roasting time 20 min	**Nut 1**	69.02	67.26	2.6	**9.6**
		Nut 2	80.70	64.01	20.7	
		Nut 3	74.33	70.29	5.4	
	Oven roasting time 30 min	**Nut 1**	70.05	64.27	8.3	**9.3**
		Nut 2	65.18	63.75	2.2	
		Nut 3	75.32	62.15	17.5	
Oven roasting temperature 120 °C	**Oven roasting time 10 min**	**Nut 1**	86.60	71.81	17.1	**12.8**
		Nut 2	78.05	66.27	15.1	
		Nut 3	69.37	64.96	6.4	
	Oven roasting time 20 min	**Nut 1**	82.08	56.98	30.6	**24.8**
		Nut 2	84.79	69.10	18.5	
		Nut 3	77.15	57.66	25.3	
	Oven roasting time 30 min	**Nut 1**	74.82	61.88	17.3	**25.6**
		Nut 2	79.52	59.77	24.8	
		Nut 3	77.40	50.50	34.8	
Oven roasting temperature 175 °C	**Oven roasting time 10 min**	**Nut 1**	85.12	81.71	4.0	**12.7**
		Nut 2	75.98	67.62	11.0	
		Nut 3	70.79	54.45	23.1	
	Oven roasting time 20 min	**Nut 1**	75.13	43.41	42.2	**36.9**
		Nut 2	68.08	43.66	35.9	
		Nut 3	63.88	43.14	32.5	
	Oven roasting time 30 min	**Nut 1**	78.59	31.81	59.5	**65.4**
		Nut 2	74.30	29.92	59.7	
		Nut 3	75.78	17.58	76.8	

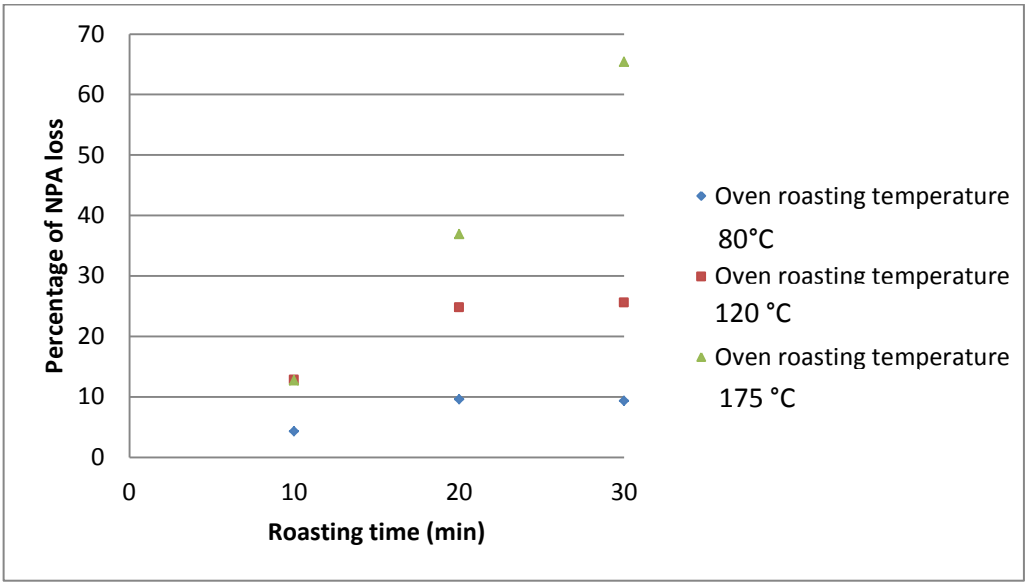


Figure 6.3 Percentage of NPA loss from nuts that were oven roasted at various temperatures for time periods of 10, 20 and 30 minutes.

The highest temperature tested (175 °C) was equivalent to the temperature at which other raw nuts are roasted. This is inclusive of other poisonous nuts, for example cashew nuts.[66,67] However, it is important to note that when cashews are purchased 'raw', they have already been pre-treated to remove the shell oil which contains the poisonous qualities, and the toxins are not within the nut as such. The technique of roasting cashews involves 5 minutes roasting followed by a cooling period, this is repeated four times giving a total roasting period of 20 minutes.[67] The cooling period prevents the nuts burning or becoming too coloured. A cooling period was not used in the roasting technique of karaka nuts and consequently it was observed that the nuts coloured significantly after the longer roasting times (20 and 30 minutes) especially at the temperature of 175 °C (**Figure 6.4**). However, the resulting colour is a visually appealing dark orange/brown with a roasting temperature of 120 °C. In contrast, the brown/black colouration observed when nuts were oven roasted at 175 °C for 30 minutes, leaves nuts in a visually unappealing state. If the implementation of cooling periods were incorporated, it may be expected that NPA losses will still be observed, with nuts potentially being left in acceptable states. Accordingly, oven roasting as a potential processing technique for toxin removal should not be excluded as a possibility. Skey[14] reported that baking karaka nuts for four hours at 212 °C was believed to remove toxins (based on the bitter taste). The temperatures of roasting used in the

present study are considerably lower than what karaka were typically exposed to in a 'hangi' (300-350 °C).[68] However, the pressure in the hangi pit and moisture content from berry flesh as well as the vegetation used to cover and keep the 'hangi' from burning may also have been contributing factors to the reduction in NPA content. There are no written reports of what karaka looked like after traditional preparation to compare results with.

Figure 6.4 Visual appearance of oven roasted karaka nuts: left to right; oven temperature 80 °C, unroasted, 10, 20 and 30 minutes; 120 °C, unroasted, 10, 20 and 30 minutes; 175 °C, unroasted, 10, 20 and 30 minutes.

In summary, oven roasting of karaka nuts does result in reduction of NPA content, indicating heat alone can degrade NPA. The reduction in NPA is greatest when roasted at higher temperatures and for longer time periods. However, the highest reductions observed (~ 65%), are not as high as desired. In addition, depending on the exact conditions, oven roasting can leave nuts in an attractive condition which is desirable. Thus, oven roasting or the use of heat in toxin removal should be further investigated as it appears to be beneficial. However, it may be best implemented as part of a processing technique, as it does not appear to be sufficient as a sole processing technique. Additionally, the implementation of a step to pre-soak the nuts in order to prevent scorching may be worth investigating, as this would allow for the possibility of longer roasting times and higher temperatures to be tested.

6.2.5 Microwave cooking

The effectiveness of microwave cooking karaka nuts while immersed in water (150 mL) was found to vary (raw data in **Appendix E5**). Cooking time periods of 1, 3 and 5 minutes resulted in average NPA losses of 7.3, 31.5 and 41.5% (**Table 6.8**). These results indicate that microwave cooking can reduce NPA content; however, the losses observed are not as high as required for adequate detoxification. In addition, it must be noted that treated nuts were observed to

change in colour from a pale cream to light orange/brown (**Figure 6.5**) however, the changes observed still left nuts in a visually appealing state.

Table 6.8 Concentration and changes of NPA content of karaka nuts, as a result of microwave cooking (850 Watts) for time periods of 1, 3 and 5 minutes.

		Sample	[NPA] prior to treatment (g kg^{-1})	[NPA] after treatment (g kg^{-1})	Total NPA loss due to treatment % (g kg^{-1})
Microwave cooking time 1 min		**1**	72.05	64.93	9.9 (7.12)
		2	69.40	65.37	5.8 (4.03)
		3	65.93	61.79	6.3 (4.14)
	Average				***7.3 (5.10)***
Microwave cooking time 3 min		**1**	75.56	44.41	41.2 (31.15)
		2	68.90	51.76	24.9 (17.14)
		3	78.19	56.11	28.2 (22.08)
	Average				***31.5 (23.46)***
Microwave cooking time 5 min		**1**	72.24	43.36	40.0 (28.88)
		2	73.22	44.51	39.2 (28.71)
		3	62.97	34.37	45.4 (28.6)
	Average				***41.5 (28.73)***

Figure 6.5 Visual comparison of an un-treated half of a nut with the half of the nut that was microwave cooked (850 Watts) for a time period of 5 minutes (left to right; untreated half, treated half).

The standardised concentrations of NPA in mg mL^{-1} for the solutions were calculated and can be seen in **Table 6.9** (for individual nut weights treated see **Appendix E5**). It can be seen that of the NPA found in the solutions only 6.5 to 11.0% was free NPA (1 and 5 min, respectively). The total average standardised concentrations of NPA found were between 2.57 and 9.95 mg mL^{-1}. These values are low and indicate that microwave cooking may not be the best means of trying to obtain NPA from nuts. However, it may be worth further investigating higher power microwave cooking and longer time periods. It must also be noted that the difference in concentrations obtained for cooking times of 3 and 5 minutes, do not indicate a significant increases in NPA content, indicating longer cooking times may actually prove inefficient.

In summary, microwave cooking does not appear to be an effective processing technique as it gives relatively low reductions in NPA content in karaka nuts regardless of the fact that nuts are still visually appealing. However, it is recommended that further investigation into longer microwave cooking time periods or higher powered cooking be investigated. This will clarify if additional or higher NPA losses can be obtained.

Table 6.9 Standardised NPA concentrations in mg mL^{-1} in solution as a result of microwave cooking for various time periods.

Treatment	Sample	Standardised‡ [NPA] (mg mL^{-1})	% NPA in solution that was free* NPA
Microwave cooking (1 min)	Solution 1	0.17	
	Solution 2	0.14	
	Solution 3	0.18	
	Average	**0.16**	
	Hydrolysed solution 1	2.21	7.7
	Hydrolysed solution 2	3.01	4.7
	Hydrolysed solution 3	2.50	7.2
	Average	**2.57**	**6.5**
Microwave cooking (3 min)	Solution 1	1.00	
	Solution 2	0.70	
	Solution 3	0.67	
	Average	**0.79**	
	Hydrolysed solution 1	10.62	9.4
	Hydrolysed solution 2	9.69	7.3
	Hydrolysed solution 3	9.54	7.0
	Average	**9.95**	**7.9**
Microwave cooking (5 min)	Solution 1	0.92	
	Solution 2	1.11	
	Solution 3	1.22	
	Average	**1.08**	
	Hydrolysed solution 1	9.98	9.3
	Hydrolysed solution 2	9.99	11.1
	Hydrolysed solution 3	9.60	12.7
	Average	**9.86**	**11.0**

Notes: * = free NPA is NPA that is released into water either as free NPA from the nuts, or is NPA that is released from hydrolysed NPGs as result of the cooking process.‡ = Standardised concentration of NPA that would arise from treating 1 kg of nuts in 1 L of water.

6.2.6 Cold-water treatments

The soaking of karaka nuts proved to be beneficial with average NPA losses of 75.9 and 87.0% found for soaking periods of 4 and 14 days respectively. The cold-water flow treatment also proved to be effective with higher average NPA losses found of 81.3 and 96.0% (**Table 6.10**; raw data in **Appendix E6**). These results indicate that cold-water soaking or the use of cold-water alone can significantly reduce the NPA content in nuts. Although, the cold-water flow was found to reduce NPA by greater amounts than soaking did, further investigation into optimum flow rates and treatment times is required.

A considerable degree of toxin loss was expected to be observed from cold-water treatments, even after the shorter time period of 4 days, as Skey[14] reported that when he soaked nuts with successive removal and replacement of water for 2 days, the bitterness of karaka nuts (based on taste) was no longer present. The losses of NPA observed in this trial did not leave nuts free of NPA but the losses of NPA were between 75 – 96% (**Table 6.10**) which are high losses in comparison to other treatments.

With the cold-water treatments there were a few visual observations and changes that are important and must be noted. Firstly, the density of nuts treated with cold-water flow appeared to change (sticky/static when ground, not as hard as untreated nuts). Secondly, there were some visual changes after treatments. The nuts that were soaked appeared grey, while the nuts that were subjected to constant water flow appeared to have yellow/orange tinged patches (**Figure 6.6**). The yellow/orange tinge appears to be leaching or withdrawal of compounds (inclusive of the toxins) to the surface of nuts, while the grey colouring appears to be the beginning of mould formation. This is an important observation as the solutions in which nuts were soaked, also appeared to have the beginning of mould formation; the 14 day solutions were noticeably mouldier than the 4 day solutions. This is indicative that a fresh water flow may be more beneficial than a set-volume as it prevents stagnation occurring. Lastly, the glass-wool that was used in the water flow columns was found to change to orange/brown colour after a few days (2 - 4 days) of water flow indicating most removal of compounds/toxins occurred in this period of time (**Figure 2.5**, in methods).

Table 6.10 Concentration and percentage changes in NPA due to cold-water treatments of nuts, for 4 and 14 day time periods.

	Treatment time period	Sample	[NPA] prior to treatment (g kg^{-1})	[NPA] after treatment (g kg^{-1})	Total NPA loss due to treatment % (g kg^{-1})
Cold-water soaking	4 days	1	60.24	9.80	83.7 (50.44)
		2	86.78	30.39	65.0 (56.39)
		3	74.11	15.53	79.0 (58.58)
		Average			***75.9 (55.14)***
	14 days	1	70.38	2.98	95.8 (67.40)
		2	74.38	19.70	73.5 (54.68)
		3	67.89	5.69	91.6 (62.20)
		Average			***87.0 (61.43)***
Cold-water flow	4 days	1	75.77	10.98	85.5 (64.79)
		2	66.37	17.01	74.4 (49.36)
		3	81.38	13.08	83.9 (68.30)
		Average			***81.3 (60.82)***
	14 days	1	77.21	3.49	95.5 (73.72)
		2	65.40	3.07	95.3 (62.33)
		3	70.05	1.86	97.3 (68.19)
		Average			***96.0 (68.08)***

Figure 6.6 Visual appearance of nuts that have been cold-water treated for 14 days; left to right, 2 images of nuts treated for 14 days with soaking, 2 images of nuts that have been treated with cold-water flow.

In summary, cold-water treatments appear to cause a significant reduction in NPA content of karaka nuts. The results are also indicative that water alone without heat is a significant contributor to NPA loss, thus should be implemented in any future testing or development of a processing technique. In particular, fresh water-flow appears to be better as it has higher NPA losses and there is less chance of mould formation. However, future investigation into optimum treatment time and flow-rate (water volume) is required. In future development of a processing technique it would be recommended that water-flow or soaking are implemented as part of a processing technique, rather than as sole treatment techniques.

When the karaka nuts were soaked in water for 4 and 14 days the standardised concentrations of NPA in mg mL^{-1} for the solutions were calculated and can be seen in **Table 6.11** (for individual nut weights treated see **Appendix E6**). The average standardised concentrations for NPA in mg mL^{-1} were 31.34 and 28.49 for time periods of 4 and 14 days respectively. Of which 52 and 71.9% (4, 14 days respectively) was found as 'free' NPA. These results indicate that cold-water alone affects significant liberation and natural hydrolysis of NPA from NPGs in the nuts. However, the size of the effect may have been exacerbated, as a part of the observation may have arisen due to microbial action (as a small amount of mould began forming-NPA is known to be produced by some common moulds[15]), even though precautions such as a sealed system were taken to avoid this. For the sake of consistency, NPA measured is assumed to have arisen from the nuts or compounds in the nuts. If microbial action only had little influence on the results, it indicates that heat is not necessarily an essential component of any process designed to extract NPA into solution for applications such as pesticide. In addition, the lack of heat results in less degradation of NPA, which is desirable if one is seeking to obtain the NPA as a by-product (heat is counterproductive as it degrades NPA).

Table 6.11 Standardised NPA concentrations in mg mL^{-1} in solution as a result of soaking for time periods of 4 and 14 days.

Treatment	Sample	Standardised‡ [NPA] (mg mL^{-1})	% NPA in solution that was 'free'* NPA
Cold water soaking (4 days)	Solution 1	15.16	
	Solution 2	14.42	
	Solution 3	19.07	
	Average	**16.21**	
	Hydrolysed solution 1	26.83	56.5
	Hydrolysed solution 2	33.40	43.2
	Hydrolysed solution 3	33.78	56.5
	Average	**31.34**	**52.0**
Cold water soaking (14 days)	Solution 1	24.56	
	Solution 2	20.28	
	Solution 3	14.93	
	Average	**19.92**	
	Hydrolysed solution 1	35.42	69.3
	Hydrolysed solution 2	32.09	63.2
	Hydrolysed solution 3	17.95	83.1
	Average	**28.49**	**71.9**

Notes: * = free NPA is NPA that is released into water either as free NPA from the nuts, or is NPA that is released from hydrolysed NPGs as result of the cooking process.‡ = Standardised concentration of NPA that would arise from treating 1 kg of nuts in 1 L of water.

6.3 Comparison of NPA loss in nuts as a result of the various treatments

6.3.1 Most effective detoxification processing treatment

The average loss of NPA from karaka nuts due to various treatment types, times and conditions, expressed as both % and g kg^{-1}, can be found in **Table 6.12** (raw data in **Appendix E7**), the ranges of NPA loss for each treatment are also indicated. The average percentage loss for each treatment can also be seen in **Figure 6.7**. The average percentage loss of NPA from nuts varied considerably between treatments, from 4.3% (oven roasting at a temperature of 80 °C for 10 min) to 98.7% (overnight soxhlet extraction).

Table 6.12 Comparison of the average NPA losses from karaka nuts (both % and g kg^{-1}) for the various treatment types, times and conditions.

Comparison of nut treatments

		%		g kg^{-1}		
Treatment	**Time period**	**Average NPA loss**	**Range (x_L-x_H)$^{\#}$**	**Average [NPA] loss**	**Range (x_L-x_H)$^{\#}$**	**Visually appealing state (Y or N)$^{\#}$**
Autoclave (nut)	**2 (min)**	59.3	7.1 (56.1-63.2)	43.95	11.98 (39.68-51.66)	N
	5 (min)	65.2	5.5 (62.2-67.7)	48.80	18.07(38.95-57.02)	N
	20 (min)	79.7	5.1 (76.8-81.9)	68.51	13.38 (60.07-73.45)	N
	60 (min)	88.4	1.5 (87.6-89.1)	70.37	12.07(63.39-75.46)	N
	120 (min)	94.4	1.7 (93.5-95.2)	63.21	9.33 (58.95-68.28)	N
Autoclave (drupe)	**2 (min)**	64.0	4.7 (62.1-66.8)	46.10	3.39 (44.73-48.12)	N
	5 (min)	71.5	6.7 (68.6-75.3)	51.50	4.79 (49.40-54.19)	N
	20 (min)	82.3	5.3 (80.2-85.5)	59.26	3.78 (57.75-61.53)	N
	60 (min)	89.2	2.3 (88.1-90.4)	64.27	1.65 (63.45-65.10)	N
Soxhlet extraction	**~ 17 h**	98.7	1.9 (98.0-99.9)	74.62	22.84 (66.31-89.15)	Y
Boiling	**0.5 (h)**	65.7	20.7 (57.5-78.2)	48.37	18.69 (41.41-60.10)	Y
	1 (h)	82.1	9.8 (77.0-86.8)	58.65	9.23 (54.74-63.97)	Y
	3 (h)	92.3	1.9 (91.4-93.3)	70.09	7.09 (66.23-73.32)	Y*
	6 (h)	96.0	2.0 (94.9-96.9)	68.29	9.29 (65.13-74.42)	Y*
Oven roasting (80 °C)	**10 (min)**	4.3	4.5 (2.7-7.2)	3.28	3.36 (2.08-5.44)	Y
	20 (min)	9.6	18.1 (2.6-20.7)	7.50	14.91 (1.77-16.68)	Y
	30 (min)	9.3	15.3 (2.2-17.5)	6.79	11.75 (1.42-13.17)	Y
Oven roasting (120 °C)	**10 (min)**	12.8	10.7 (6.4-17.1)	10.32	10.38 (4.41-14.79)	Y
	20 (min)	24.8	14.3 (18.5-30.1)	20.09	9.41 (15.69-25.10)	Y*
	30 (min)	25.6	17.5 (17.3-34.8)	19.86	13.96 (12.94-26.90)	Y
Oven roasting (175 °C)	**10 (min)**	12.7	19.1 (4.0-23.1)	9.37	12.92 (3.42-16.34)	Y
	20 (min)	36.9	9.7 (32.5-42.2)	25.60	10.97 (20.74-31.71)	Y*
	30 (min)	65.4	17.3 (59.5-76.8)	49.79	13. 82 (44.39-58.21)	N
Microwave cooking	**1 (min)**	7.3	4.1 (5.8-9.9)	5.10	3.09 (4.03-7.12)	Y
	3 (min)	31.5	16.3 (24.9-41.2)	23.46	14.01 (17.14-31.15)	Y
	5 (min)	41.5	6.2 (39.2-45.4)	28.73	0.28 (28.60-28.88)	Y
Cold water soaking	**4 (days)**	75.9	18.7 (65.0-83.7)	55.14	8.14 (50.44-58.58)	Y
	14 (days)	87.0	22.3 (73.5-95.8)	61.43	12.72 (54.68-67.40)	Y*
Cold water flow	**4 (days)**	81.3	11.1 (74.4-85.5)	60.82	18.94 (49.36-68.30)	Y
	14 (days)	96.0	2.0 (95.3-97.3)	68.08	10.99 (62.33-73.32)	Y*

Note: #= $\mathbf{x_L}$ means lowest value, $\mathbf{x_H}$ highest value

Y= yes, N = no, and Y* means borderline, ie: there are definite observed changes, but believed these may be able to be overcome to leave the nut in a consumable state (colour changes, too soggy etc)

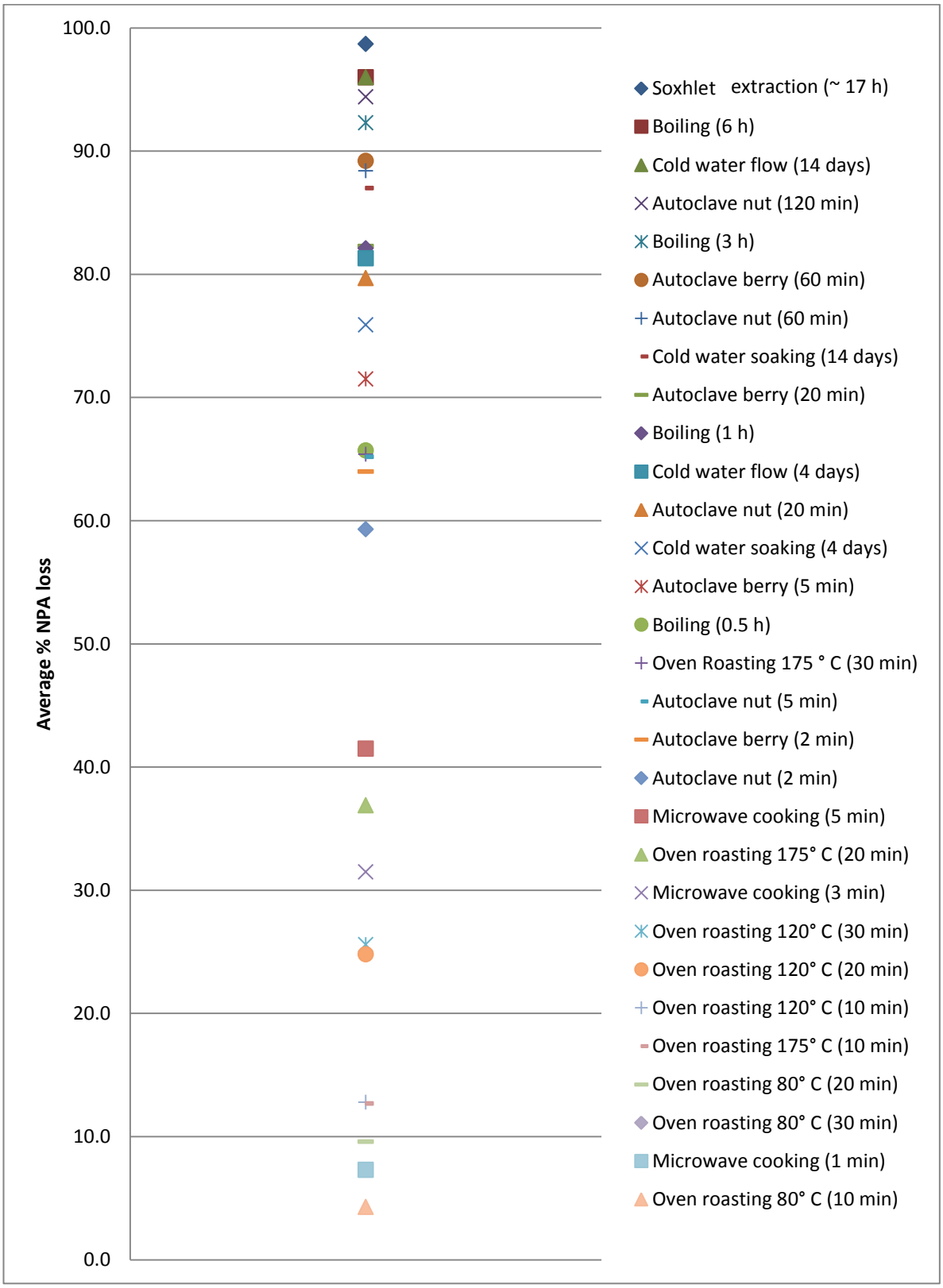


Figure 6.7 Comparison plot of average percentage NPA losses observed from nuts due to the various treatments

Using 75% NPA loss as an arbitrary measure for evaluating if a processing technique is effective, it was found there are 13 different treatment conditions that meet this criterion and these can be seen in **Figure 6.8**. Of these 13, it can be seen that all of the treatments involve water and a small number also utilise heat. None of these 13 most effective techniques use heat alone (oven roasting), which indicates that water is essential for inducing significant NPA loss from karaka nuts.

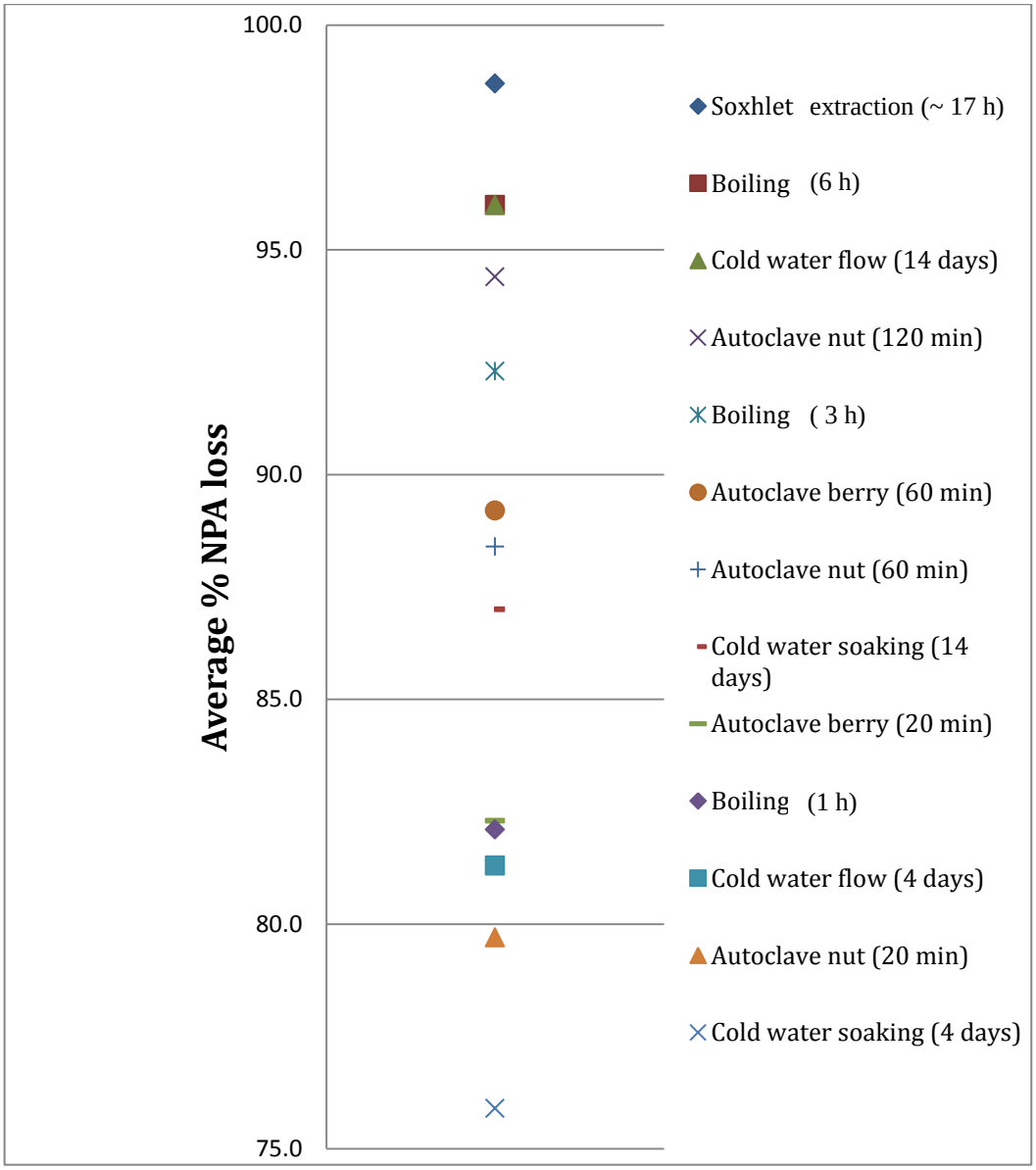


Figure 6.8 Comparison plot of average percentage NPA losses observed from nuts due to treatment, where the loss is greater than 75%.

In addition, it must be noted that some of the treatments degrade the nuts significantly and do not leave nuts in visually appealing states. As a result, the visual appeal of the nuts has been indicated as yes (Y), no (N) or borderline appealing (Y^*) in **Table 6.12**. **Figure 6.9** is a comparison plot of the average NPA percentage losses from nuts, whereby nuts are left in visually appealing or borderline appealing states and NPA loss is greater than 75%. It can be seen that there are eight effective techniques: soxhlet extraction, boiling (1, 3 and 6 h), cold water flow (4 and 14 days), cold-water soaking (4 and 14 days). The fact that all the most effective processing techniques involve water is consistent with what may be expected, as the NPA being measured arises from NPGs found in the nuts. Since NPGs are glucose esters of nitropropanoic acid, it would be expected that water would aid in solubilising the NPGs and allow for easier liberation or degradation. This is also consistent with the traditional process whereby the nuts were soaked in streams or rivers for a period of time (usually a few days), to remove the toxins.[5,11,14-16]

Current tests indicate that treatment processes involving water alone such as cold water soaking and water flow for periods of 4 and 14 days, appear to be beneficial for toxin removal, however there are some undesirable changes observed as already discussed (**Section 6.2.6**). The techniques of boiling and soxhlet extraction which involve water and heat also appear to be effective. Again some undesirable changes are observed with longer treatment times; such as nuts becoming ‘water-logged’ (as already discussed, **Section 6.2.3**). Although, sometimes undesirable visual changes are observed, this does not mean the process should be ruled out as a possibility. This is because future developments or modifications to the processes may prove favourable if the undesirable changes could be avoided, perhaps by combining techniques to get the maximum benefit out of each. For example, using shorter 1 - 3 hour boiling times, combined with drying periods (to avoid water logging) and then repeating the process. Another example is the implementation of a pre-soaking step followed by oven roasting. There are numerous combinations and modifications that could prove to be beneficial in developing an effective toxin removal process and this is an area that needs further investigation.

In summary, further development of a processing technique for NPA removal is still required and it is recommended that the processing technique will involve water either alone, or in combination with heat. It must also be noted that in future, composition tests will need to be conducted to see if the effective toxin removal technique has a significant impact on the nutritional composition of the nuts.

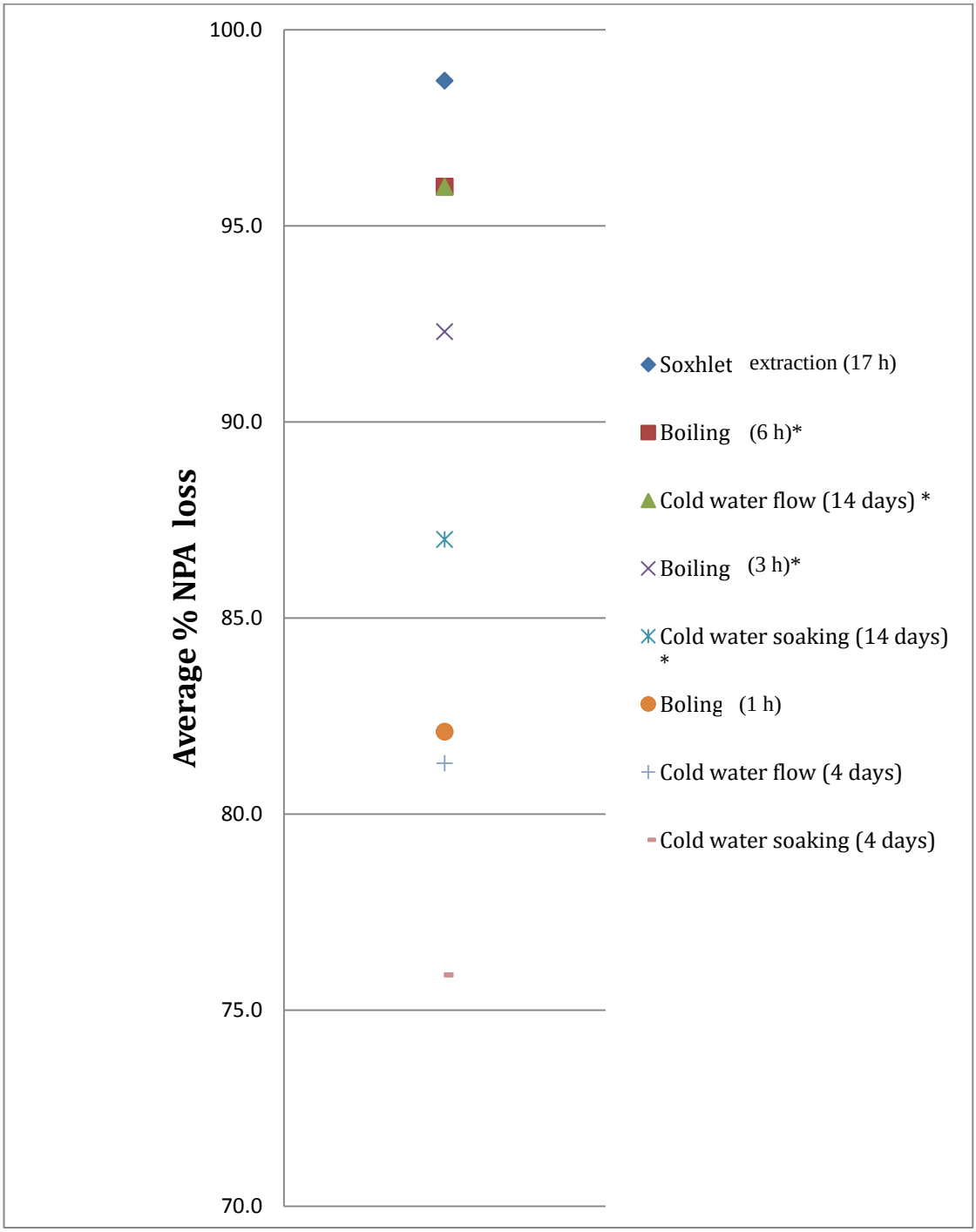


Figure 6.9 Comparison plot of the average NPA percentage loss from nuts, whereby nuts are left in visually appealing states and loss is greater than 75%.

6.3.2 Safe levels of consumption and the numbers of nuts that could be consumed as a result of the various processing techniques

According to literature,[15] an estimated safe level of NPA consumption, based on a no observed adverse effect limit on rodents is 25 μg kg^{-1} day^{-1} or 1.75 mg day^{-1} for a 70 kg adult, with a recommended safety factor of 100. Therefore, the NPA concentration remaining in nuts was compared with the value of 1.75 mg, as well as 175 mg (after the recommended safety factor was removed[15]), in order to determine the efficacy of the various treatments. Treated nuts were also compared to see if they had concentrations of NPA < 5.5 mg, as this is the estimated concentration that Japanese consume on a daily basis from trace amounts in common foods, such as miso, soy sauce, cheese, peanuts and more. The results are given in **Table 6.13**.

It can be seen that out of all the treatments type, times and various conditions only soxhlet extraction left nuts with a concentration of < 1.75 mg g^{-1} NPA. However, the weight of nut that could be consumed is ~ 1.77 g and this equates to less than the average weight of a single nut (2.0 g). Hence, none of the treatments have effectively left nuts in a state that is verifiably safe for consumption.

If the safety factor of 100 is removed, it can be seen that all of the treatments leave nuts with < 175 mg g^{-1} NPA, however it should be noted that the pre-treatment concentrations in individual nuts (< 85 mg g^{-1}) are well below this and presumably untreated nuts would be considered to be toxic. If the weight of nuts (g) that could be safely consumed at this level is converted to number of nuts, it can be seen that the number of nuts that could be considered safe to consume ranges from 1 to 88 nuts, dependent upon the treatment conditions implemented. Of the 30 treatments and conditions tested, 12 resulted in > 5 nuts being able to be consumed, while 6 resulted in > 10 nuts being considered safe to consume. However, removal of the safety factor of 100 probably would not be acceptable to food authorities. In addition, if an arbitrary single consumption value of 25 nuts is consumed, there are only three effective treatments; soxhlet extraction, cold water flow (14 days) and boiling (6 h). However, the undesirable physical changes observed (as stated previously) have implications on whether they are actually the best processes to use.

In summary, all that can be concluded is that processing techniques of karaka nuts require further investigation. It currently appears that water has a more significant effect on toxin removal than heat alone. However, future developments and modifications of processes involving heat and water may also prove to be effective, especially given the considerable advantageous loss of NPA in nuts that were found with soxhlet extraction, a process that involves both heat and water. In addition, studies on storage and natural ageing of nuts should be further investigated, especially considering the observed natural decline in NPA found in nuts, even with ripening and age.

Table 6.13 The weight and number of nuts (in parentheses) that could be safely consumed according to three different guidelines as a result of the different treatments used.

Treatment	Treatment time	[NPA] prior to treatment (g kg^{-1})	[NPA] after treatment (g kg^{-1})	Weight of nuts (g) that may be safely consumed (number of nuts) [a,d]	Weight of nuts (g) that may be safely consumed (number of nuts)[b,d]	Weight of nuts (g) that may be safely consumed (number of nuts)[c,d]
Soxhlet extraction	**~17 hours**	75.61	0.99	1.77 (0)	176.78 (88)	5.56 (2)
Cold water flow	**14 (days)**	70.89	2.81		62.28 (31)	1.96 (0)
Boiling	**6 (h)**	71.15	2.86		61.19 (30)	1.92 (0)
Autoclave (nut)	**120 (min)**	66.97	3.76		46.54 (23)	1.46 (0)
Boiling	**3 (h)**	75.95	5.86		29.86 (14)	
Autoclave (drupe)	**60 (min)**	72.00	7.73		22.64 (11)	
Autoclave (nut)	**60 (min)**	79.57	9.20		19.02 (9)	
Cold water soaking	**14 (days)**	70.89	9.46		18.50 (9)	
Autoclave (drupe)	**20 (min)**	72.00	12.74		13.74 (6)	
Boiling	**1 (h)**	71.81	13.16		13.30 (6)	
Cold water flow	**4 (days)**	74.51	13.69		12.78 (6)	
Autoclave (nut)	**20 (min)**	85.84	17.33		10.10 (5)	
Cold water soaking	**4 (days)**	73.71	18.57		9.42 (4)	
Autoclave (drupe)	**5 (min)**	72.00	20.50		8.54 (4)	
Boiling	**0.5 (h)**	73.29	24.92		7.02 (3)	
Autoclave (nut)	**5 (min)**	74.50	25.70		6.81 (3)	
Autoclave (drupe)	**2 (min)**	72.00	25.90		6.76 (3)	
Oven roasting (175 °C)	**30 (min)**	76.23	26.44		6.62 (3)	
Autoclave (nut)	**2 (min)**	73.93	29.98		5.84 (2)	
Microwave cooking	**5 (min)**	69.50	40.77		4.29 (2)	
Oven roasting (175 °C)	**20 (min)**	69.03	43.43		4.03 (2)	
Microwave cooking	**3 (min)**	74.20	50.74		3.45 (1)	
Oven roasting (120 °C)	**30 (min)**	77.25	57.39		3.05 (1)	
Oven roasting (120 °C)	**20 (min)**	81.34	61.25		2.86 (1)	
Oven roasting (80 °C)	**30 (min)**	70.18	63.39		2.76 (1)	
Microwave cooking	**1 (min)**	69.10	64.00		2.73 (1)	
Oven roasting (80 °C)	**20 (min)**	74.68	67.18		2.60 (1)	
Oven roasting (120 °C)	**10 (min)**	78.01	67.69		2.59 (1)	
Oven roasting (175 °C)	**10 (min)**	77.30	67.93		2.58 (1)	
Oven roasting (80 °C)	**10 (min)**	77.44	74.16		2.36 (1)	

[a] based on the estimated safe daily consumption of NPA for a 70 kg adult, with a safety factor of 100. (< 1.75 mg day^{-1}) [15]

[b] based on the estimated safe daily consumption of NPA for a 70 kg adult, with the safety factor of 100 removed (< 175 mg day^{-1})[15]

[c] based on the average maximum level of NPA that is estimated to be consumed on daily basis by the Japanese (< 5.5 mg day^{-1}) (Kinosita *et al*., 1968, as cited in Burdock *et al*., 2001)[15]

[d] calculated based on the average weight of a nut 2.0 g, obtained from the wet and dry weight averages of nuts (160) used in determining average NPA content of individual nuts (**Table 5.3**). Nut number was always rounded down to the nearest whole number.

6.4 Comparison of NPA liberated into solution as a result of the various treatments

The standardised NPA concentrations of NPA in mg mL^{-1} for treating 1 kg of nuts in 1 L for the various treatments, times and conditions that had solutions were calculated and have been compared (**Table 6.14).**

Table 6.14 Standardised NPA concentrations mg mL^{-1} found in solution as a result of the various treatments, treatment times and conditions, involving water; soxhlet extraction, boiling, microwave cooking and soaking.

	Standardised‡ NPA concentration (mg mL^{-1})		
Treatment	**Unhydrolysed solution**	**Hydrolysed solution**	**% NPA in solution that is free NPA**
Cold water soaking (4 days)	16.21	31.34	52.0
Boiling (3 h)	6.92	30.71	22.6
Soxhlet extraction overnight(~ 17h)	16.60	28.90	57.8
Cold water soaking (14 days)	19.92	28.49	71.9
Boiling (6 h)	8.63	28.13	31.1
Boiling (1 h)	4.19	25.48	16.8
Boiling (0.5 h)	2.48	22.80	10.9
Microwave cooking (3 min)	0.79	9.95	7.9
Microwave cooking (5 min)	1.08	9.86	11.0
Microwave cooking (1 min)	0.16	2.57	6.5

Notes: * = free NPA is NPA that is released into water either as free NPA from the nuts, or is NPA that is released from hydrolysed NPGs as result of the cooking process.‡ = Standardised concentration of NPA that would arise from treating 1 kg of nuts in 1 L of water.

It can be seen that the highest concentrations of NPA in solution obtained from treating nuts are achieved with cold water soaking (4 days), boiling (3 h) and soxhlet extraction. All three of these treatments result in standardised concentrations of NPA > 28.50 mg mL^{-1}.

The various treatments have shown that water is essential in obtaining NPA in solution. The cold water treatments without heat were found to be more effective than treatments involving heat as they result in higher concentrations of NPA in solution. It has been shown that although heat aids in hydrolysing NPGs in the nuts to release NPA, prolonged exposure to heat results in not only hydrolysis of NPGs to NPA, but degradation of NPA which is undesirable.

In summary, depending on the processing techniques utilised to obtain solutions of NPA, the best treatment depends on the desired outcome and resources available. That is if NPA is desired to be obtained as the free acid without the expense of hydrolysis, then cold water soaking for 14 days appears to be the best option. However, if highest concentration of NPA is the desirable outcome then the top three individual treatments appear to be cold water soaking (4 days), boiling (3 h) and soxhlet extraction.

7 Conclusions and recommendations

Conclusions

7.1 Method development

A relatively robust method was developed that enables the release of NPA *via* acid hydrolysis of the nitropropanoyl ester groups of the NPGs found in karaka nuts. The NPA is able to be measured and subsequently quantified as a NPA equivalence value; that is it quotes the total NPA concentration that arises from all NPGs, allowing for the fact that most NPGs release more than one mole of NPA. The conditions of the hydrolysis were optimised with the best combination found to be sulfuric acid (1 M) and heating (100 °C, 1 h).

7.2 Development of an adjustment factor to account for the degradation of NPA as a result of the hydrolysis

Due to the fact that NPA degrades because of the combination of acid and heat used in the hydrolysis of NPGs, an adjustment factor to account for NPA loss was determined. This was vital as it is important not to underestimate NPA or NPG content owing to their inherent toxicities. Kinetic trials were conducted to account for the NPA loss, as it degrades to MA and eventually AA. From the kinetic trials, a correction factor (CF) was determined to account for what the original NPA concentration at a time of zero (NPA_0) would be if there was no consequent degradation. The factor (F_2) was determined to be 1.226 which is comparable with an alternative factor (F_1) of 1.229 that was determined by NPA standards treated under the same hydrolysis method used for nuts. Thus, a final correction factor of 1.23 (3 s.f.) was used to correct all NPA concentrations determined when acid hydrolysis was used.

7.3 Quantification of NPA in karaka nuts

The average concentration of NPA found in karaka nuts ranges from 50.25 - 138.62 g kg^{-1} (dry weight) (**Tables 4.4, 4.5**), which is equivalent to 5.0 to 13.9%. These are much higher percentages than any previously found because the method used in this study measured total NPA whereas earlier methods measured single NPGs unhydrolysed without accounting for other compounds that contribute to total toxicity. Additionally, the levels of NPA appear to vary within a tree, between trees and with collection date (stage of ripeness).

7.3.1 Variations of NPA concentrations within a nut

7.3.1.1 Ripeness of the nut

The effect of ripening nuts was significant in reducing the amount of NPA measured. When unripe green drupes from trees A and C were collected (10/1/12), the average NPA levels found in nuts were found to be 137.04 and 138.52 g kg^{-1}. When nuts off the same two trees were collected ~ 4 weeks later (8/2/12), they were found to have NPA levels of 85.53 and 75.04 g kg^{-1} for trees A and C respectively. This equates to a significant reduction of 37.6 and 45.8% of NPA in nuts as a result of natural ripening. This is an important consideration for removal of the toxins from karaka nuts before consumption as collection of ripe or overripe drupes will yield nuts with naturally lower NPA concentrations.

7.3.1.2 Variations due to weather conditions

Nuts that were weathered outside were found to have reductions of NPA content between 18.0 and 28.4%. Although, this is a considerable percentage loss, as a consequence of weathering the nuts degraded. This apparent decomposition may affect nutritional value and requires further investigation.

7.3.1.3 Variations due to the positioning of nuts collected (directly off the tree versus the ground)

There was found to be no conclusive trend as to whether it was most beneficial to collect the nuts directly off the tree (as ripe drupes), or off the ground in order to obtain nuts with naturally lower NPA levels. However, the NPA levels in nuts that

fall to the ground are expected to vary more, as the nuts may fall as unripe drupes (thus have more NPA naturally). In addition, nuts that fall to the ground are more likely to degrade/decompose which is undesirable.

7.3.1.4 Variation within individual nuts

The average percentage difference between halves of nuts, both vertical ($\%D_{L/R}$) and horizontal ($\%D_{T/B}$) were calculated and found to be 5.8% (**Table 4.7**) and 7.3% (**Table 4.8)** respectively. Both of these values indicate a degree of variation between halves of a nut, however these values are considered acceptable given that the most likely cause of variation is biological differences. As a result of the variation within a single nut, if and where possible the sample was homogenised to give a more accurate NPA concentration. In addition, due to the lower average percentage difference between vertical halves of the nuts, when all treatments were performed, nuts were cut vertically in order to give the most accurate comparison of pre and post treatment NPA concentrations.

7.3.2 Variations and concentrations of NPA in the various parts of karaka drupe; berry flesh, shell, pellicle and nut

It was found that all parts of the karaka drupe have measurable NPA content however, the highest concentrations of NPA arising from NPGs in karaka are found in the nuts followed by the berry flesh. Ripe drupes have an average NPA content of 18.70 g kg^{-1} and 70.19 g kg^{-1} for the berry flesh and nuts respectively. In comparison, unripe drupes contained higher levels with the average NPA concentrations for the berry flesh and nuts of 34.95 g $kg^{-1}$and 158.96 g kg^{-1}, respectively.

7.4 The effectiveness of various treatments at removing NPA from nuts

Nuts were subjected to various potential industrial processing treatments, treatment times and treatment conditions in order to determine their efficacy at toxin removal. The treatments included boiling, microwave cooking, oven roasting, autoclaving, soxhlet extraction and cold-water treatments (soaking and constant cold-water flow). It was found that there were eight techniques which effectively resulted in > 75% NPA loss from karaka nuts and still left the nuts in a

visually appealling state: soxhlet extraction, boiling (1, 3 and 6 h), cold water flow (4 and 14 days) and cold water soaking (4 and 14 days).

All the most effective processing techniques involve water. This is consistent with what may be expected, as the NPA being measured arises from NPGs found in the nuts. Since NPGs are glucose esters of nitropropanoic acid, it would be expected that water would aid in solubilising the NPGs and allow for easier liberation or degradation of NPGs. This finding is consistent with the traditional process whereby the nuts were soaked in streams or rivers for a period of time (usually a few days or weeks), after being cooked in a 'hangi' to remove the toxins. Thus, the traditional preparation may have in fact been the best as it incorporated a combination of heat, water and pressure.

Out of all of the treatment types, times and various conditions, none proved to leave nuts with a concentration lower than the estimated safe level of daily NPA consumption of < 1.75 mg, whereby a safety factor of 100 is implemented (based on average 70 kg adult).[15] If the safety factor is removed, 6 of the 30 treatments result in > 10 nuts being able to be consumed. If an arbitrary value of 25 nuts (in one day) are to be consumed then only three treatments (soxhlet extraction, constant cold water flow (14 days) and boiling (6 h)) are effective at toxin removal. However, the undesirable physical changes observed have implications on whether they are actually the best processes to use. In addition, given the safety factor is not present, this would probably not be acceptable to food authorities.

At present, toxin removal in order to render karaka nuts safe to consume appears to be an impractical activity by a single process. Future modifications to methods and treatments (including combining methods) as well as further testing are required before anything definitive can be concluded.

7.5 Liberation of NPA in solutions as a result of treatments

The NPA liberated into solution as a result of detoxifying karaka nuts, could be obtained as a by-product and has the potential for its own commercial applications, for example as insecticides or repellents.

The NPA was measured in solutions as both free NPA (unhydrolysed solution) and total NPA (hydrolysed solution). The standardised levels for treating 1 kg of nuts in 1 L of water were found to give a concentration range of free NPA between 0.16 mg mL^{-1} (microwave cooking, 1 min) to 19.92 mg mL^{-1} 10 (overnight soxhlet extraction). While the total NPA levels ranged from 2.57 to 31.34 mg mL^{-1} for microwave cooking (1 min) and cold-water soaking (4 days), respectively.

It was apparent that cold-water treatments appeared to be more effective in obtaining higher concentrations of NPA in solution. This is as expected because although heat aids in the hydrolysis of NPGs to release NPA, heat also results in degradation of NPA and consequently results in lower concentrations of NPA in solution. Overall, if NPA is desired to be obtained as the free acid without the expense of acid hydrolysis, then cold water soaking for 14 days appears to be the best option. However, if highest concentration of NPA (assuming the solution will be further hydrolysed) is the desirable outcome, then the top three individual options are cold water soaking (4 days), boiling (3 h) and soxhlet extraction. It is also evident that obtaining NPA from a natural source (karaka) as a by-product of toxin removal appears to be feasible. Especially given that it could possibly happen on a large scale with relatively low costs, possibly lower than those of synthesis

Recommendations and future work

7.6 Method development

7.6.1 HPLC Run-time

The current method is relatively rapid; however the HPLC run-time is one area where better efficiency could be obtained. The run time of 51 minutes is inefficient time-wise. Once it was determined only NPA was to be measured the reduction of run time to 35 minutes, resulted in greater time efficiency with later eluting peaks overlapping before the peak of interest at 23 minutes. It is recommended in future that for better efficiency, a gradient elution scheme be designed for the HPLC method, so that all peaks prior to the NPA peak elute faster, leaving NPA able to be measured as well as speeding up the elution time of the small unidentified peak that elutes currently at ~ 41 minutes. This will result in a more efficient process, with more samples being able to be analysed in a given time period. In addition, if the technique is to be used industrially, a faster run-time would be essential in order to lower costs (time, solvent etc).

7.6.2 Elucidation and quantification of individual NPGs

The method developed to date hydrolyses all nitropropanoyl ester groups of NPGs to release NPA, and a total NPA count is obtained. However, it is unknown how much of the NPA is associated with each of the NPGs, as the proportions of NPGs in karaka nuts are undetermined. A future advancement of this method could allow for the individual NPGs to be isolated from a batch of karaka nuts and for the NPG ratios to be determined. If this was done, one could develop a formula and from the NPA concentration determine the approximate concentration of each individual NPG, accounting for the proportion that it is usually found in nuts and by the amount of moles of NPA each NPG releases. This may be an important advance if in future some of the NPGs are found to be more or less toxic than others. Alternatively, a method could be developed to quantify each individual NPG separately.

7.6.3 Alternative uses for the current method

The three main methods currently used in literature for NPA determination are TLC, GC and HPLC, with HPLC being the most recently used, due to its advantages such as greater sensitivity and no requirement for volatility. The method developed is therefore relatively modern and suitable to quantify NPA. At present it is designed to quantify NPA in karaka nuts, however with small method modifications or suitable extraction procedures, the method could be used to quantify NPA in other plant extracts across various genera.

7.7 Recommendation for toxicology tests

Currently, there is limited research on the toxicological differences between NPA and NPGs. However, due to the fact that NPGs are able to be hydrolysed to release NPA, it is vitally important that future research will look at comparing the toxicities of these two. These values will need to be reported clearly and allow for the fact that toxicities differ according to the ability of animals (ie: ruminants vs non-ruminants) to digest the two compounds The primary aim of future toxicological research should focus on determining a safe level of consumption of both NPGs and NPA, so that treated nuts have a definitive safe consumption value to be compared with.

7.8 Future treatments to be investigated

The results indicated that the best treatments for removing NPGs and lowering the NPA measured in karaka nuts were those that involved water. However, heat also proved to be beneficial as it assists in degradation of NPA. In future, alternative treatments that were not tested here but could be worth investigating include; steaming, higher power microwave cooking, pressure cooking and autoclaving with the drupes submerged in water. In addition, to date the treatments tested were all one stage because this would be the most cost effective way of treating nuts. It is worth investigating if multi-stage treatments will result in additional gains or more beneficial results (higher NPA loss). An example of this could be boiling for one hour followed by dry roasting, followed by another hour boiling before roasting again, or any combination of treatments, especially those that appear to be most beneficial. This advancement in the treatment method would be

consistent with the traditional methods, whereby two techniques, soaking and baking of karaka nuts were implemented before consumption. While treatments are being designed, the mitigation or avoidance of undesirable changes such as nuts becoming excessively 'soggy' (due to boiling for long time periods), should also be investigated. For example, to avoid nuts scorching when being roasted, a pre-treatment process of soaking should be trialled.

In terms of future investigation into treatments to release NPA; any of the treatments that had high NPA loss from the nuts would be the best starting points, as in order for NPA to be liberated into solution, it first needs to be removed from the nuts. In addition, obtaining NPA in solution as a result of processing the unused parts of the karaka drupe; the berry flesh, shell and pellicle may be worth investigation as an efficient use of resources. Moreover, if nuts are to be used as a viable natural source for obtaining NPA (as appears likely), the development of an efficient method for obtaining NPA from the water would be essential. This is because depending on the treatment, a high percentage of NPA in solution may not actually be in the free form but still as partial or unhydrolysed NPGs.

7.9 Investigation of the karaka berry as a food source

In a test that was conducted on the parts of the karaka drupe, it was found that the two parts containing the highest NPA concentrations were the nut and berry flesh. However, the considerably lower levels of NPA in the berry flesh make it a potential area for future investigation. If the toxins could be removed from the berry flesh and it is found to have comparable nutritional value or health benefits over other fruit, it may be a more viable option for investigation of a potential 'traditional Māori' food.

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8 Appendices

8.1 Appendix A: Raw data for calibration curves

8.1.1 Nitropropanoic acid calibration curve data

Concentration of NPA (mg mL^{-1})	Peak Area
0.003	206569
0.115	8248021
0.544	41187846
1.077	77240743
1.466	95796243

8.1.2 Malonic acid calibration curve data

Concentration of MA (mg mL^{-1})	Peak Area
0.08	164933
0.11	218797
0.31	679805
0.53	1149861
0.81	1813395

8.1.3 Acetic acid calibration curve data

Concentration of AA (mg mL^{-1})	Peak Area
0.115	8248021
0.544	41187846
1.077	77240743
1.466	95796243

8.2 Appendix B: Method development - On CD

Appendix B1: Selection of hydrolysis acid concentration, Section 3.2.1.1.

Appendix B2: Optimisation of hydrolysis conditions, Section 3.2.2.

Appendix B3: Differences in NPA and MA concentrations due to experimental factors, Sections 3.4.1 and 3.4.2.

8.3 Appendix C: Kinetic data - On CD

Appendix C1: Raw data for kinetic degradation trials; NPA (trials 1-6), MA and NPA in water.

Appendix C2: Kinetics of NPA and MA degradation at different temperatures.

8.4 Appendix D: NPA quantification in karaka - On CD

Appendix D1: NPA content in individual nuts

Appendix D2: Variations in NPA content in individual nuts

Appendix D3: NPA content in parts of ripe and unripe karaka

8.5 Appendix E: Karaka treatments - On CD

Appendix E1: Autoclaving

Appendix E2: Soxhlet extraction

Appendix E3: Boiling

Appendix E4: Oven roasting

Appendix E5: Microwave cooking

Appendix E6: Cold-water treatments

Appendix E7: Treatment comparisons