Processing of marula (*Sclerocarya birrea subsp. Caffra*) fruits: A case study on health-promoting compounds in marula pulp

Penny Hiwilepo-van Hal

Thesis committee

Thesis supervisor:

Prof. dr. Ir. M. A. J. S. van Boekel
Professor, Product Design and Quality Management,
Wageningen University, Wageningen, NL

Thesis co-supervisors:

Dr. Ir. M. Dekker
Associate Professor, Food Quality and Design,
Wageningen University, Wageningen, NL

Dr. ing. R. Verkerk
Assistant Professor, Food Quality and Design,
Wageningen University, Wageningen, NL

Dr. P.G. Bille
Senior lecturer, Food Science and Technology,
University of Namibia, Windhoek, Namibia

Other members:

Prof. dr. H.J. Wichers, Wageningen University, NL

Prof. dr. E.J. Woltering, Wageningen University, NL

Prof. dr.ir. J. van Camp, University of Ghent, Belgium

Dr. ir I. D. Brouwer, Wageningen University, The Netherlands

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Thesis

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Penny Hiwilepo-van Hal

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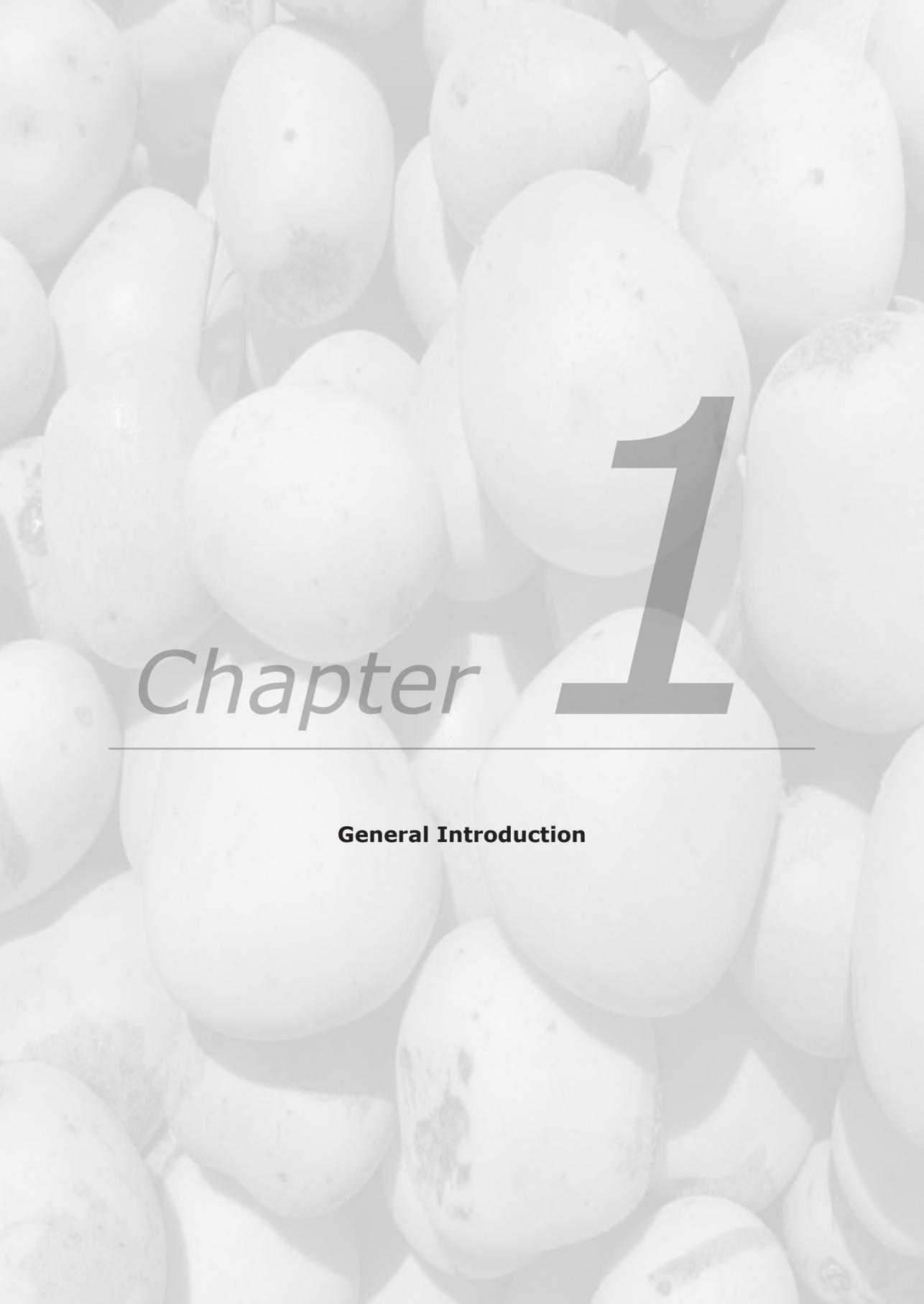
Abstract

Marula is a multipurpose tree from Southern Africa, used by local people for its fruit, and cosmetic oil from the seed and for medicinal products from the bark and leaves. Fruits are eaten raw, or used to prepare juices, jams, conserves, dry fruit rolls, or fermented to make alcoholic beverages like beer, wine and Amarula. The fruit is a vital source of vitamin C for rural people most of whom cannot afford other more expensive sources of vitamin C. The specific processing methods and conditions of making marula juice vary among different regions. This thesis investigated the fate of antioxidants, i.e. vitamin C, and their activities due to heat processing and fermentation of the marula pulps and its juices.

The results showed that marula fruit pulp has a vitamin C content higher than that of most fruits, ranging from 62 mg/100 g fresh weight– to over 400 mg/100 g. Juice production was optimized by an experimental design combined with response surface modelling: adding pectinase (in the range of 0.1 to 0.14%) increased the yield of marula juice by 23%. The optimal extraction temperature for the content of vitamin C and polyphenols as well as for the antioxidant activity ranged between 40 and 60°C. At heating temperatures below 125ºC, ascorbic acid in marula pulp was about 15-fold more stable than in mango and guava pulp. The results further revealed that marula peel contained more volatile compounds (75) including all the identified volatiles (41) of the flesh.

Marula fruit is a rich source of vitamin C and other antioxidants. The use of unfermented juice should be encouraged since it can contribute to the energy intake of the marula juice drinkers. Marula juice is a rich source of natural antioxidants. In addition, marula processors are advised to incorporate (part of) the skin in products such as juices, jams, jellies and alcoholic beverages during processing to enhance the unique characteristic marula flavor in the products which are currently claimed not to have a strong marula like flavour.

Table of Contents



Chapter 1

General Introduction

1. Background information

Evidence from epidemiological studies indicates that diets rich in fruit and vegetables are associated with a lower risk of several degenerative diseases (Nicoli, Anese & Parpinel, 1999). These results have created a new perspective on the potential of diet in preventing life-threatening diseases. The health-promoting capacity of fruit and vegetables depends on their antioxidant activity, which can be attributed to the presence of compounds like vitamin C (Steinberg, 1991). *Sclerocarya birrea subsp. Caffra* (*marula*) fruit juice is known for its very high vitamin C content, providing about 70 to 400 mg of vitamin C per 100 g of fresh juice. Therefore, the fruit serves as an important source of vitamin C for its users for instance, rural community in southern Africa (Nerd, Aronson & Mizrahi, 1990; Nerd, Aronson & Mizrahi, 1994). It is this high vitamin C content of the fresh fruit that makes it so important nutritionally and accounts for the early observations of the ability of the marula fruit to combat scurvy (Mokgolodi, Ding, Setshogo, Ma & Liu, 2011). Vitamin C is an essential nutrient for humans with a recommended daily allowance of 60 mg according to Whitney & Rolfes (1993). This can be obtained from half a cup (125 ml) of orange juice per day or from 30 ml of *marula* juice. Therefore, tropical fruits including *marula* play a prominent role in the diet due to their nutritional value and to the high content of water-soluble vitamins, especially vitamin C.

2. The marula tree and its fruit

Sclerocarya birrea is commonly known as *marula* in southern Africa but other names are used in other countries as well (Mutshinyalo & Tshisevhe, 2003). The genus *Sclerocarya* comprises only 2 species; *birrea* and *gillettii*, but Shackleton *et al.* (2001) noted that there are actually four species with *birrea* having 3 subsp., namely Birrea, Caffra and Multifoliolata. *Sclerocarya birrea* occurs naturally or cultivated in the Sahel, East and Southern Africa outside the humid forest zone (Orwa, Mutua, Kindt, Jamnadass & Simons, 2009). The tree prefers a warm and frost-free climate and is highly salt tolerant (worldagroforestry.org, du Plessis, 2002). The *Sclerocarya birrea* tree can reach heights of up to 18 m and a

trunk diameter of 120 cm (Orwa *et al.*, 2009 & von Teichman, 1982). The tree (figure 1) has a grey bark, short taproot of 2.4 m and lateral roots that can reach up to 30 m (Orwa *et al.*, 2009). The tree prefers clay soils or sandy loam soils and is common in areas receiving 200-1370 mm of rainfall annually. It is a protected species and often planted in crop fields by some farmers in Namibia and Botswana (Shackleton *et al.*, 2001). The fruits of *marula* abscise before ripening when they are still green and the time of fruit abscission varies among trees (Nerd *et al.*, 1994 and Bille & Steippich, 2003). After abscission, the colour changes to yellow (figure 1-3), aroma develops and the flesh softens.

Figure 1. Marula tree (A) and marula tree bark (B).

This happens 7-10 days after abscission (Nerd *et al.*, 1990). The fruits (figure 2) are round and oval drupe and 3-5 cm in diameter when mature and develop in clusters of three to five at the ends of twigs on a new growth (Mojeremane & Tshwenyane, 2004 and Nerd *et al.*, 1994). They have a skin that covers the flesh or pulp and a stone inside, which is about 2-3 cm long with one to four cavities containing the seed (figure 3) (Mojeremane & Tshwenyane, 2004). The edible part of the fruit is very small compared to the fruit size; the average weight of the fruit is 18 g and the peel or skin, stone and flesh make up 41%, 53% and 6% of total weight respectively (von Teichman, 1983).

Figure 2. Marula fruits (A) and squeesing marula juice (B)

Figure 3. Marula stone cracked (A) and extracting its kernel (B)

3. The use of marula

Marula (*Sclerocarya birrea subsp. Caffra*) is one of the most important fruits and potential sources of income for primary producers in the North and Central Regions of Okavango and Caprivi in Namibia (du Plessis, 2002). It is also one of the most commonly utilized indigenous wild fruit in Africa (Shackleton *et al.*, 2001). The tree is highly appreciated by rural communities for its fruits. Female trees bear plum-sized fruits with a thick yellow peel and a translucent, white and highly aromatic sweet-sour fruit which is eaten raw like a small mango, or used to prepare juices (figure 2), jams, conserves, dry fruit rolls, and alcoholic beverages (Nerd & Mizrahi, 1993 and Mizrahi & Nerd, 1996). The taste of the fruit is said to be acidic and bitter but of pleasant flavour when fully ripe (Ogbobe, 1992). Since its fruit kernels (figure 3) are eaten or used for oil extraction, the *marula* is considered a multipurpose

tree (Mutshinyalo & Tshisevhe, 2003). The oil can be used for cooking or for cosmetic purposes (du Plessis, 2002; Mojeremane & Tshwenyane, 2004).

Marula kernels are regarded as delicacy in regions of the tree's natural habitat and are commonly used to supplement the diet during winter season (Shone, 1979). They also make good snacks and can be consumed raw or roasted and for the purpose of adding a unique flavour to the food. The nuts can be mixed with vegetables or meat or may be ground by pounding and formed into a cake before consumption. In some households, the ground nuts are used in baking traditional breads (Shone, 1979).

The wood from marula trees is used for making utensils, fencing poles as well as fuel for cooking in Namibia. For medicinal purposes, leaves, bark and roots are used. The leaves mainly used for coughs while the bark and roots are for stomach-related ailments and other ailments, notably fever, boils, diarrhoea and blood circulation problems. Mixed with other medicinal plants, the bark is used by traditional healers to treat various illnesses such as syphilis, leprosy, dysentery, hepatitis, rheumatism, gonorrhoea, diabetes, dysentery and malaria, particularly bark that is gathered before the first flush of the leaves (Mutshinyalo & Tshisevhe, 2003).

Other uses derived from *marula* tree include caterpillars that are edible, fodder for livestock, nuts for rattles, beads and necklaces, hair relaxers as well as diviners die. At a small scale, the skin of marula fruits can be dried in order to be used as a substitute for coffee. Also at this scale the leaves are cooked as relish (Shackleton, Shackleton, Cunningham, Lombard, Sullivan & Netshiluvhi, 2002).

Like many traditional food plants, this tree species provides food at all times, including times of food scarcity. In periods of the year characterized by shortages of subsistence products such as a season of hunger preceding the first harvest, or in times of food shortage and drought, *Sclerocarya birrea* can become a crucial source of nutrition (Mojeremane & Tshwenyane, 2004). Even for livestock during drought, branches of *Sclerocarya birrea* are cut by livestock owners to get the leaves as fodder for their animals (Mojeremane & Tshwenyane, 2004).

Figure 4.
Mini *marula* festival

4. Implication of commercialization of marula juice and its products in Namibia and South Africa

Marula fruits were found to be part and parcel of community's livelihoods or for daily lives of many people from Namibia and South Africa, who are very poor and depended on natural resources to meet their basic needs (Shackleton *et al.*, 2002). Many social networks and relations are formed during neighbourhood *marula* parties (figure 4) where beer or wine is consumed. The demise of these 'get-together' or gathering was one of the main reasons linked to marula commercialization. There is a clear distinction on ownership of *marula* tree in South Africa and Namibia. In Namibia, almost all *marula* trees are privately owned as they are in people's fields while in South Africa they are on communal land and are accessible to everybody. In Namibia, however, informal institutions have evolved to ensure equity and sharing of *marula* resources. People brew beer and wine in the owner's field and women from the community provide labour so that they can share the benefits accruing from *marula*. In South Africa, *marula* beer is mostly made at home with help of family members and anyone can access it. According to Shackleton *et al.*, (2002) this has potential implications for increased commercialisation such as:

- Decreased willingness to share marula fruit and its products amongst the wider community and thus turning it from something that was shared and seen as a gift, into something that is retained by individual households to sell.
- Breakdown in social cohesion as *marula* pressing and removal of the kernel will be done by individuals to gain more money rather than sharing as was done previously.

5. Rationale of the thesis

The *marula* (*Sclerocarya birrea subsp. Caffra*) fruit is a vital source of vitamin C for rural community, most of whom cannot afford other sources of vitamin C that are sold on the market since they are expensive. Although some rural people might not be aware of the health benefits of *marula*, especially its vitamin C content. In addition, many people outside the production areas are now aware of this. Demand for tropical fruits is steadily increasing due to the natural antioxidants that they contain and *marula* products are no exception. This offers an opportunity for prompting commercialization of *marula* product out of the zone where it is currently commercialized to other zones, thus creating income for rural poor and improving their well-being. Since marula fruits generally grow naturally, they are available at no cost to the wider rural population or at a lower price than other fruits sold in supermarkets.

Several users of *marula* fruits fail to optimise juice yield, due to the fact that squeezing and pressing is not efficient enough because part of *marula* pulp is attached to the central pit and skin. In some trials with a hydraulic press, juice yields varied from less than 20% to more than 40% of fruit weight (du Plessis, Lombard and den Adel, 2002). In order to increase the yield of the juice and the press capacity, the use of an enzyme pectinase can be considered which at the same time clarifies the juice (Sreenath, Sudarshana, & Santhanam, 1995).

The processing methods of making marula juice vary from village to village, region to region and country to country. In many cases the fruits are subjected to a heat treatment before pressing. The heating process can be up to over 3 hours. Heat treatment is always applied in the commercial food industry as an important processing step for inhibiting spoilage caused by microorganisms and enzymes, thereby prolonging shelf life. Even though it is a necessary step for food processing, heating affects nutritional compounds like vitamin C and other antioxidants and often in a negative way. In addition, heating could affect the overall aroma profile of the marula juice and its pulp as compounds causing off-flavours may be formed which is undesirable to consumers. After or before heat treatment, the pressed juice and pulps are stored in freezer for a considerable time, even up to six months by commercial juice processors. Volatile compounds of marula could further be altered by different storage temperature and time conditions even though freezing is a well-known preservation method.

Currently, juices of marula available on the market are classified as inferior in terms of marula-like flavour and this could be due to the effect of heat processing of the pulp or due to the storage conditions of the pulp or juices before they are used by consumers or maybe it is due to the fact that marula-like flavor compounds are left behind in the skin, which in many cases is discarded. Furthermore, very little is known on flavour characteristics of marula product (Schäfer and McGill, 1986) and nothing has been published on the influence of storage and heat treatment on the marula juice, pulp and its derived product flavor compounds.

Therefore, current processing methods of marula products in rural areas where it is mostly processed need to be investigated in order to attain the full potential of marula products in terms of health and nutritional benefits. It is important to use the processing methods that enhance the retention of vitamin C and other nutrients in *marula* for the products to be beneficial to consumers and increase the demand for the products among health conscious users.

Marula is known to be high in vitamin C and it is also known that vitamin C (ascorbic acid) is a compound that can be degraded quite easily as it is influenced by several factors like temperature, water activity, presence of oxygen, acidity, presence of sucrose, metal catalysts, amino acids and enzymes (Solomon, Svanberg & Sahlstrom, 1995; Huelin, 1953; Saguy, Kopelman & Mizrahi, 1978; Greenway & Ongomo, 1990; Uddin, Hawlader, Ding & Mujumdar, 2002). For instance, degradation of vitamin C has been reported in many fruit products as a result of processing or storage, and has been considered one of the major causes of quality and colour changes (Yuan & Chen, 1998). Vitamin C degradation was observed in an aseptically packaged orange drink with 10% orange juice that lost 40% of vitamin C after 6 months of storage and lost up to 75% at storage temperatures of 22 to 30°C (Luque-Perez, Rios, & Valcarcel, 2000). However, in *marula* jam, it was found that the vitamin C content after pasteurisation was as high as 84% of the original content (Hillman, Mizrahi & Beit-Yannai, 2008), indicating its relative stability in jam. However, there is no literature on its stability in juice or pulp. It is believed that when vitamin C is retained during processing and storage, this implies that conditions have been relatively mild, so other nutrients would also be retained (Freedman & Francis, 1984; Starr & Francis, 1968). Therefore vitamin C is often used as an indicator compound to study the effect of processing and storage on food quality in a broader sense.

Although it is known that *marula* fruit contains polyphenolic compounds (Borochov-neori *et al.*, 2008), little is known about their antioxidant activity and their content in processed marula products like fermented juice. In addition to that, stability of these antioxidants is also unknown and the nutritional content of fermented *marula* juice is important, since the most popular uses of marula fruit is its fermented juice.

6. Objective and thesis outline

The overall objective of this thesis was to investigate the effect of processing on quality attributes of marula pulps and its juices, especially the fate of antioxidants and their activities. In order to achieve the overall objective, the

following specific objectives were formulated:

1. To critically evaluate literature on proximate composition and nutritional value of marula in comparison with other tropical and indigenous fruits, in order to identify areas for future research.

2. To determine the optimum processing conditions for maximum juice yield and retention of vitamin C, polyphenols and antioxidant activity of the marula juice.

3. To determine the effects of fermentation temperature and time on the vitamin C, polyphenols and antioxidant activity of naturally fermented marula juice.

4. To investigate the thermal degradation of vitamin C in marula pulp.

5. To identify and characterize the volatile flavor compounds of marula fruit and further investigate their changes under different heating and storage conditions.

This chapter (chapter 1) gives an overview and general information about the marula tree and its fruits. In chapter 2, a critical evaluation of literature on proximate composition and nutritional value of *marula* in comparison with other tropical and indigenous fruits is presented. Moreover, this chapter identifies areas for future research, some of which serve as the base for the specific objectives for chapter 3 to 6 in this thesis. Chapter 3 describes the optimum processing conditions for maximum juice yield, vitamin C, polyphenols, antioxidant activity, and clarification (colour) of the *marula* juice. Subsequently, chapter 4 presents the effects of fermentation temperature and time on the vitamin C, polyphenols and antioxidant activity of the naturally fermented marula juice. In chapter 5, the thermal degradation of vitamin C in marula fruit as compared to other selected tropical fruits is discussed and this is followed by chapter 6 that identifies and characterizes the volatile flavor compounds of *marula* fruit and further investigates their changes under different heating and storage conditions. The final chapter (chapter 7) presents a general discussion of the main findings, together with concluding remarks on how far this thesis has realized its objectives and what could be the next steps.

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Chapter 2

A review of the proximate composition and nutritional value of marula (*Sclerocarya birrea subsp. Caffra*)

Hiwilepo-van Hal, P., Bille, P. G., Verkerk, R., van Boekel, M. A. J. S. & Dekker, M.

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Abstract

This review critically evaluates literature on proximate composition and nutritional value of *marula* in comparison with other tropical and indigenous fruits, to identify areas for future research. The mineral and nutrient content of *marula* fruit varied greatly from study to study according to place of origin, soil, climate and time that lapsed after harvesting before analysis was carried out. Processing methods also vary from author to author; some boiled the fruits before samples were taken and the time and temperature treatment also differed. *Marula* pulp is reported to have a vitamin C content higher than that of most fruits, ranging from 62 mg/100 g – to over 400 mg/100 g. Additionally, *marula* fruit is reported to have an antioxidant capacity of between 8-25 mM ascorbic acid equivalents and a total phenolic content ranging from 7.5 – 24 GAE (gallic acid equivalent) (mg/g dry weight). *Marula* kernels are also a good source of protein, oil, magnesium, phosphorus and potassium and the oil is used in food preparation. The variation found in reported data is due to variation in collection, handling and storage as well as analysis methods used. *Marula* fruits could play a vital role in terms of nutrition to rural populations who rely on the usage of the fruits and do not have easy access to other sources of nutrients. Recommendations given for future research include: 1. improving *marula* fruits juice yields 2. Investigate the effect of processing and storage on the retention of nutrients such as vitamin C and its antioxidant capacity in marula pulp and its products, 3. Identify individual antioxidants and their activity in unprocessed and processed marula products 4. Identify the most important flavour compounds and 5. Further investigate the effect of processing or storage on *marula* flavor compounds.

Key words: *Sclerocarya birrea subsp. Caffra*, marula, vitamin C, and antioxidant.

1. Introduction

Marula (*Sclerocarya birrea subsp. Caffra*) is one of the most commonly utilized indigenous wild fruits in Africa (Shackleton, *et al.*, 2001). The marula tree is a multipurpose tree highly appreciated by rural communities, mainly for its fruits but also for its cosmetic oil from the seed and medicinal properties from the bark and leaves (von Teichman, 1983; Mutshinyalo and Tshisevhe, 2003). Female trees bear plum-sized fruits with a thick yellow peel and a translucent, white, highly aromatic sweet-sour fruit (Nerd and Mizrahi, 1993; Nerd, *et al.*, 1990; Nerd, *et al.*, 1994; Mizrahi and Nerd, 1996). *Marula* fruit has a thick, soft leathery exocarp with tiny, round or oval spots, enclosing a juicy, mucilaginous pulp that adheres tightly to the seed and can be removed only by sucking (von Teichman, 1982).

Fruits are eaten raw, like a small mango or used to prepare juices, jams, conserves, dry fruit rolls and also fermented to make alcoholic beverages such as beer, wine and a liquor called Amarula (Nerd and Mizrahi, 1993; Nerd, et al, 1990; Nerd, et al, 1994, Mizrahi and Nerd, 1996 and Mojeremane and Tshwenyane, 2004). The fruit pulp serves as a base for fruit drinks, nectars and teas, alcoholic beverages such as marula beer and amarula cream, wines, liqueurs and punches. The fresh fruit tastes tart, sweet and refreshing, although the fruit has a slight turpentine-like aroma and can give off a very unpleasant smell when decaying. According to Ogbobe, (1992) the taste of the fruit is described as being acidic and bitter but of pleasant flavor when fully ripe.

The edible flesh part of the fruit is very small compared to the total fruit size. Generally, the average weight per fruit is 20 g in South Africa and 26.7 g in Namibia and the mean skin mass is 8 g in South Africa and 10 g in Namibia, mean flesh mass is 7 g in South Africa and 13 g in Namibia and mean pulp mass (flesh and skin) is 16 g in South Africa and 22 g in Namibia (Leakey *et al.*, 2005).

The fruits are much sought after by humans and animals for their nutritious pulp with high vitamin C content and edible nuts. It has

become a commercial fruit crop in recent years, the fruit pulp being used to produce a jelly/jam and to flavor liqueur (Van Week *et al.*, 2002). The stem, bark, roots and leaves of the plant have been reported to possess medicinal and other properties (Ojewole, Mawoza, Chiwororo and Owira, 2010). In southern and some other parts of Africa, the stem-bark, roots and leaves of *Sclerocarya birrea* are used as traditional medicines that are believed to treat an array of human disorders including: malaria and fevers, diarrhoea and dysentery, stomach ailments, headaches, toothache, backache and body pains, infertility, schistosomiasis, epilepsy and diabetes mellitus (Watt and Breyer-Brandwijk, 1962; Pujol, 1993; Hutchings *et al.*, 1996; and Van Wyk *et al.*, 2002). In Namibia, the use of *marula* as a medicinal plant is known and promoted by the Ministry of Agriculture and natural resources. Today the tree is classified as a medicinal plant like *hoodia* and *devil's claw*, which are the two indigenous natural plants with popular usage. Marula is described as a rich source of various nutrients (Eromosele *et al.*, 1991).

The present review critically evaluated literature on proximate composition and nutritional value of *marula* in comparison with other tropical and indigenous fruits in order to identify areas for future research.

2. Food uses of marula

Ripe *marula* fruit can be consumed by biting or cutting through the thick, leathery skin and sucking the juice or chewing the mucilaginous flesh after removal of the skin (von Teichman, 1982). A popular fermented alcoholic beverage is prepared from the ripe fruit. In some cases the skin is removed and the juice is fermented together with the pulp still on the seed. Other methods include the cutting of the skin and allowing the whole fruit to ferment (Carr, 1957). Yeasts, naturally occurring on the fruit, are traditionally utilized for spontaneous fermentation. This beverage is commonly known as 'marula-beer' or "marula-wine" (Shone, 1979) with an alcohol content of 2-5% (Dlamini and Dube, 2008) and is used for the famous South African "Amarula Cream Liqueur".

Marula kernels found inside the nut of the fruit are regarded as a delicacy in regions of the tree's natural inhabitant; they are commonly used to supplement the diet during winter (Shone, 1979), they make good snacks and can be consumed raw or roasted and for the purpose of adding a unique flavour to the food. The nuts are mixed with vegetables or meat or may be grounded by pounding and formed into a cake before consumption. In some households, the grounded nuts are used in baking of traditional breads (Shone, 1979). Oil for human consumption and for cosmetic purposes can also be extracted from the nuts (Pierre, 2002).

More recently, the fruit has been used to prepare jelly or jam, which is sold on a small-scale (Bille and Steppich, 2003). The taste of marula jam and jelly is reported to be good, and the colour is attractive (waxy yellow) without the need for addition of artificial food colors. The skin of *marula* fruits can be dried in order to use it as substitute for coffee. The leaves are cooked as relish. During droughts, branches of *Sclerocarya birrea* are cut by livestock owners to use leaves as fodder for livestock (Mojeremane and Tshwenyane, 2004).

Like many traditional food plants, the tree species provide food at all times and times of food scarcity. In times of subsistence shortages, such as a season of hunger preceding the first harvest, or in times of famine and drought, *Sclerocarya birrea* can become a crucial source of nutrition (Mojeremane and Tshwenyane, 2004).

3. Problems encountered during processing

There are, however, several disadvantages with the *marula* when it comes to processing. It is said by several users that the skin of the fruit is rather too thick and is of little value at present. It is also argued by Gous *et al.* (1988) that the flesh (pulp) of the fruit is very difficult to remove from the central pit. In addition, the percentage of flesh to skin and pit is rather small (about 20%) (Gous *et al.*, 1988). The traditional way of making *marula* juice is by using a cow horn to puncture the leathery skin

of the *marula* fruit after which the juice is squeezed out of the *marula* (den Adel, 2002). The squeezing is not efficient to gain a high juice yield, because part of the flesh is attached to the central pit and skin. Recently, the hydraulic press has been in use, it is still hard to press all the juice out from *marula* fruits because the pulp is bound by pectin into a gel form. Therefore, finding a way to obtain higher juice yield and one that is clarified will be an area for further research.

Another major problem in assessing *marula* fruit for processing was the difficulty in obtaining consistently ripe and undamaged samples. This usually results in the use of fruits with different degrees of ripeness resulting in the final product being too sour to be palatable. Very little is known in scientific literature and practically nothing has been published on the acceptability and preference for the texture and flavour characteristics of the product (Schäfer and McGill, 1986). Schäfer and McGill, (1986) published on flavour profiling of juice of the *marula* as an index for cultivar selection. They further suggested to undertak an investment in the processing and that the responses from the potential consumers of all cultural groups in the market area should be evaluated.

Schäfer and McGill, (1986) showed that there was not much difference in respect of odour, flavour and aftertaste of juices prepared from different cultivars. One prominent attribute that has been noted concerning flavour of *marula* juice is the extreme sourness in combination with a lack of sweetness (Schäfer and McGill, 1986). The relatively low sugar to acid ratios may have to be adjusted to make juices acceptable for consumption (Gous *et al*, 1988). The flavour of the *marula* fruit is mainly concentrated in the peel (von Teichman, 1983). That could be the reason why most of the *marula* products available in the marked do not contain the typical *marula* flavour since the skin is not incorporated in juice processing. Therefore, investigations still need to be done to identify the important flavour compounds and add them back to the juice to obtain a product of full and natural *marula* flavour.

4. Vitamin C

Marula fruit juice is known for its very high content of vitamin C, ranging from 62 mg/100 g (Carr, 1957) to more than 400 mg/100 g in the fresh fruit (Eromosele et al, 1991 and Hillman, *et al.*, 2008) and thus, the fruit serves as an important source of vitamin C for many rural people (Nerd et al, 1990; Nerd et al, 1994). Even the lowest reported values of vitamin C in *marula* are comparable to the content of vitamin C in other fruits such as orange juice and still higher than that of other citrus juices (Pretorius *et al.*, 1985). Hillman et al, (2008) found very high content of ascorbic acid in *marula* fruit juice, as high as between 700 and 2100 mg/100 g, more than 10 times higher than in orange juice and pomegranate juice, while Mdluli and Owusu-Apenten, (2003); Glew *et al.*, (2004); and Nagy *et al.*, (1990) recorded values that were 3 to 4 times the amounts found in oranges juice. Leakey, (1999) stated that the vitamin C content of *marula* fruits in Nigeria was 403 mg/100 g and to be twice that found in Botswana, although Eromosele *et al.*, (1991) stated that the variation can be considerable depending on the stage of ripening, the content being highest in ripe fruits with 403 mg/100 g and 201 mg/100 g in unripen fruits. According to Leakey (1999), the proximate analyses for different fruits from southern Africa reveal some variation, which may be either genetic or environmental or both and or due to different analytical methods. The causes of this variation are still not known and need to be investigated, as genetic variation of this magnitude would be of importance to domestication programmes. Hillman *et al.*, (2008) found the variation to be due to differences among clones of *marula* and fruits ripening stages.

Most fruits such as grapes, oranges, apple, lemon and papaya amongst many others, have a lower vitamin C content compared to *marula* fruit as shown in Table 1. Only guava has a high vitamin C content of about 300 mg/100 g (Vinci *et al.*, 1995). As shown in Table 1, vitamin C content vary greatly with different studies. This could be due to different analytical methods used, but also due to variation in the place of origin, soil, climate, ripening stage of the fruits and time that lapsed after harvesting before analysis took place.

Table 1: Vitamin C content of marula fruit in comparison to some fruits

Type of fruit	Vitamin C (mg/100 g)		Source
	Pulp	Flesh/Juice	
Marula	62-179.1	403	Eromosele *et al*, (1991)
		200	Nerd, *et al,* (1990)
		275	Carr (1957)
		267	Borochov-neori *et al, (2008)*
		133	Dlamini and Dube, 2008
Orange		50	Eromosele *et al*, (1991) Takeda, 2009
		60	Dlamini and Dube, 2008
		33	Hillman *et al*, 2008
Strawberries	60		Eromosele *et al*, (1991) Takeda, 2009
Grapes		38	Eromosele *et al*, (1991)
Guava		300	Takeda (unknown year)
Baobab		283	Chadare *et al*, 2009
Parinari mobola (hissing tree)	64.1		Carr (1957)
Kiwi		52	Hillman *et al*, 2008
		67	Vinci et al, 1995
Wild grape (*Lannea edulis*)	14		Carr(1957)
Sour plum (*Ximenia caffra)*	49.2		Carr (1957)
Wild mango(*cordyla Africana)*	75.6		Carr (1957)
Avocado pear		10	Vinci *et al*, 1995
Kumquat		55	Vinci et al, 1995
Litchi		22	Vinci et al 1995
Mango		25	Vinci et al, 1995
Papaya		88	Vinci et al, 1995
Passion fruit		65	Vinci et al, 1995
Pineapple	25		Takeda, 2009
		31	Vinci et al, 1995
Apple	6		Takeda, 2009
Lemon	50		Takeda, 2009
		51	Vinci et al, 1995
Apricot	25		Takeda, 2009
Lime	25		Takeda, 2009
Cantaloupe	40		Takeda, 2009
Cherry	6.5		Takeda, 2009
Grapefruit	45		Takeda, 2009
		65	Vinci et al 1995
Peach	7		Takeda, 2009
Pear	4		Takeda, 2009
Tomato	25		Takeda, 2009

5. Antioxidant activity

Borochov-neori *et al.*, (2008) found that *marula* juice had 56 mg/100 ml of pyrogallol equivalence of phenols and an antioxidant capacity of 382 mg/100 ml of vitamin C equivalence. The antioxidant activity remained after pasteurization and only 14% was lost after storage during refrigeration at -18 °C after 4 weeks. Hillman *et al.*, (2008) reported antioxidant capacity of marula juice to be 141 – 440 mg/100 ml ascorbic acid equivalent compared to 44 – 76 mg/100 ml ascorbic acid equivalent for orange and 44–132 mg/100 ml ascorbic acid equivalent for pomegranate.

Mdluli and Owusu-Apenten, (2002) found total antioxidant capacity (TAC) of *marula* fruit in terms of equivalent concentration of L-ascorbic acid (L-ASC-eq.) to be 2960 mg/100 g L ASC-eq (pH 4.5) and 1872 mg /100 g L-ASC-eq. (pH 7). Vitamin C accounted for about 70% of TAC of the *marula* fruit, which is 20 – 40 times higher than reported for most common fruits (Mdluli and Owusu-Apenten, 2002).

It is clear that *marula* fruit and its juice have higher antioxidant activity than other fruits like pomegranate and orange juice, but further investigation is necessary in this aspect since different analysis methods have been used and that makes it difficult to draw up a concrete conclusion towards the antioxidant obtained from different fruits. Antioxidant stability towards heat treatment is important but seems not to have been studied, even though thermal treatment is always applied in the food industry as important processing steps for inhibiting spoilage caused by microorganisms and enzymes in order to increasing shelf life. The other area that is not described in literature is the effect of storage conditions; storage might bring up many changes in the unprocessed or processed *marula* juice and its products.

6. Phenolics and flavonoids

Gous *et al.*, (1988) found that all seven *marula* juice products (from different trees) contained large amounts of polyphenols, ranging between 226 – 414 mg/100 ml tannic acid equivalence for three consecutive years (1985 – 1987). Hillman *et al.*, (2008) evaluated polyphenol contents using gallic acid as a standard and found from 17 clones that the content ranged from 700 to 2500 mg GAE/100 g dry weight. In pineapple, banana and guava, the phenolic and flavonoid content were measured and given in terms of Gallic acid equivalent and Catechin equivalents (CEQ), respectively (Alothman *et al.*, 2009). These ranged from 35 to 55 mg/100 g for different concentrations of methanol, ethanol, acetone and water for pineapple. The phenolic content of banana ranged from 24 to 72 mg/100 g while phenolic content for guava ranged from 109 to 191 mg/100 g (Alothman *et al.*, 2009). The content of soluble phenolics in marula fruit juice was 56 g/100 g (Borochov-neori *et al.*, 2008). The flavonoid CEQ content of pineapple ranged from 1 to 4 mg/100 g, in banana it ranged from 5 to 24 mg/100 g and in guava it ranged from 14 to 45 mg/100 g (Alothman *et al.*, 2009).

The variation among the phenolic contents obtained could be due to different extraction procedures used, to different clones and fruit quality of the selected clones. According to Alothman *et al.*, (2009), the recovery of phenols was dependent on the fruit type and the extraction solution used, indicating that some fruit can be efficiently extracted using 100% methanol or acetone while others were extracted with 50% of the same extraction solution, therefore optimising the method of extraction should be the priority. From three authors who analysed the phenolic content of *marula* the results varied within and between authors, indicating that different results can be obtained from tree to tree, year to year and from one author to next. According to Borochov-neori *et al.*, (2008), and in comparison to other fruits, *marula* contains higher phenolic content and this could contribute negatively toward health status of the *marula* consumers.

7. Minerals

The mineral composition of *marula* fruit varies with its geographical origin where trees are found as shown in table 2. Table 3 shows the mineral content of *marula* fruit in comparison to other fruits. The most abundant minerals found in *marula* are calcium, magnesium, potassium and phosphorus, whereas sodium, iron, copper, zinc, cobalt, lead and manganese were present in smaller amounts (Gous et al, 1988; Holtzhausen *et al.*, 1990 and Eromosele *et al.*, 1991). Studies by Bille and Steppich (2003) concur with those of other authors (Gous et al, 1988; Holtzhausen *et al.*, 1990; and Eromosele *et al.*, 1991). Pulp of the *marula* fruit is high in potassium, calcium and magnesium. It was also concluded that, climate played a bigger role in influencing the mineral concentration of all *marula* products, slightly more than the origin of the tree and this is mostly due to draught or being a wet year. Additionally, the processing method such as heat treatment before puree is prepared or before pressing the juice out could also contribute. It is important to know the concentration of minerals in *marula* fruit but next to that it is also necessary to know the bioavailability that is, if the minerals are not absorbed in the gut, the nutritional value is nil. Compounds such as oxalic acid, or phytic acid may bind minerals and form insoluble complexes that are not absorbed and this area is not well covered in *marula*. According to Gous *et al.*, (1988) the relatively low sugar and high potassium content of *marula* juice can further add to the health benefits since potassium is an essential nutrient to maintain fluid and electrolyte balance in the body. On the other hand, it is debatable whether low sugar is healthy. If the diet is low in energy it may be very healthy to have some sugar. Apart from the sugar content fluctuation arising from geographical origin where trees are found, the variations can be due to other factors like the method used for extraction by different researchers, handling of sample prior to analysis and the analytical method used. Geographical origin is also broader in the sense that other factors can cause variations like soil type, soil fertility and climatic conditions due rainfall partern and sun intensity including genetic variation from region to region and country to country.

Table 2: Mineral composition of Marula fruit in mg/100 g fresh fruit, from different regions in Africa.

Origin	Burkina Faso		Niger	South Africa		Sibasa	SWA- Namibia[1]		SWA- Namibia[2]	
Part of the fruit	Fruit	Seed	Seed	Fruit	Seed	Fruit	Flesh	Skin	Flesh	Skin
	mg/100 g									
Magnesium	310	193	421	10.5	467	14.8	25.3	33.5	74.7	41.5
Calcium	481	156	154	6.2	106	10.4	20.1	44.7	117.9	126.7
Iron	2.5	2.8	2.8	0.10	0.42	0.24	0.5	0.55	0.30	0.27
Copper	-	-	2.5	0.04	2	0.11	0.07	0.08	0.45	0.07
Zinc	-	2.7	6.2		-	0.17	0.10	0.17	0.34	0.12
Sodium	1.5	1.2	4.3	Trace	338	0.64	2.2	1.7	2.5	2.0
Potassium	212	264	364	548	677	163	317	345	490	417

Sources: Fox & Hallowes (1982); Venter (2002); Glew *et al.* (1997); Wehmeyer (1967); Arnold *et al.* (1985); Glew *et al.* (2004) and Bille & Steppich (2003).

[1] Samples from North central Namibia

[2] Samples from North west Namibia

Table 3: Mineral content in mg/100 g fresh weight of marula in comparison to other fruits.

Type of Fruit	Cu	Fe	Mg	Mn	Zn	P	Ca	K	Na	References
	mg/100 g									
Marula fruit	0.04	0.1	10.5			18.7	6.2	54.8	trace	Wehmeyer (1966), Carr (1957)
Marula juice		0.71	44	0.05	0.19		40	328	10	Borochov-neori *et al* (2008)
Marula Fruit		2.5	310			262	480			Mojeremane (2004)
Marula nut		2.8	193.		2.7	212.0	156.0			Mojeremane (2004)
Marula nut	2.81	4.87	462		5.19		808	601	3.81	Arnold *et al* (1985)
African plum	0.29	2.9	35.5	0.47	1.23					Smith *et al* (1996)
African grapes	0.32	1.3	84.5	0.06	0.34					Smith *et al* (1996)
Flour tree pulp	0.25	0.3	56.8	0.12	0.74					Smith *et al* (1996)
Baobab pulp		4.3	195	0.7	1.7	106	302	1794		Chadare *et al* (2009)
Christ thorn	0.64	2.86	91	0.61	1.18		225			Eromosele *et al* (1991)
Blood plum	0.18	2	53.3	0.76	0.72		50.5			Eromosele *et al* (1991)
Wild olive	0.17	1.97	25.3	0.51	0.63		3.3			Eromosele *et al* (1991)
Wild Annon		1.33	42.4	0.43	0.64		28.9			Eromosele *et al* (1991)
Shea butter	0.11	1.93	26.3	0.24	0.47		36.4			Eromosele *et al* (1991)
Chinese date tree	0.6	6.3	227	3.5	1.55		712.5			Eromosele et al (1991)
Date palm	0.12	1.07	16.7	0.36	0.37		13			Eromosele *et al* (1991)
Grewia retinervis	0.4	4.7	172		1.6	60	157	655	31	Taylor (1985)

8. Lipids and fatty acids

Marula nut has a higher lipid/oil content than Baobab, *Adansonia digitata Carissa edulis* and *Hibiscus esculentus* nuts as shown in table 4. Shone (1979) and Von Teichman (1983) reported 5700 mg/100 g of lipids found in the nut of *marula* and Glew *et al.*, (1997) found 19.5 g/100 g dry weight of lipids in the nut. The lipid content of *marula* nut varied from 50 – 85% of dry weight according to Eromosele *et al.*, (1991); Leakey, (1999) and Arnold *et al.*, (1985). According to Glew *et al.*, (2004), the fatty acid composition and content of the *marula* seed (daniya seed) was high (47.0% of dry weight) with the major fatty acid monoenoic oleic acid (18:1 n-9) accounting for 63% of the total fatty acid content (47 g/100 g dry weight). Ogbobe, (1992) reported that *marula* seed contained stearic, palmitic and archidonic acids as predominant, representing 50.7%, 23%, and 8% of total fat, respectively. In addition to that Wehmeyer, (1967) stated that the *marula* oil itself is high in unsaturated fatty acids containing 70% oleic acid and 8% linoleic acid of total fat. According to Mariod and Abdelwahab, (2012), fatty acid and oil composition can be affected by harvesting time and an increase in the oil content up to 63% of dry weight was obtained at the end of the last harvesting date, whereas only 17% of dw was obtained at the first harvesting date (first harvest date March and last harvest date June).

Table 4: Lipid content in marula fruit in comparison to other fruits.

	Lipid content (g/100 g dry weight)		
Part of fruit	**Pulp**	**Nut**	**References**
Marula	13.5	19.5	Glew *et al.* (1997)
	-	5.7	Shone (1979), Von Teichman (1983)
		50 – 85%	Eromosele *et al.* (1991), Leakey (1999)
Baobab	3.6	28	Chadare *et al.* (2009)
Adansonia digitata	15.5	9	Glew *et al.* (1997)
Carissa edulis	3	-	Glew *et al.* (1997)
Hibiscus esculentus	19	-	Glew *et al.* (1997)

9. Macronutrients

Protein content and amino acids content. *Marula* fruits and seeds contain 3600 and 5600 mg/100 g dry weight of total protein, respectively (Glew *et al.*, 1997), indicating that the seeds of marula contain more protein than the fruits. The protein from the seeds varies from country to country, for instance the kernel from Nigerian *marula* was found to contain 36.7% crude protein, which is much more than 5.6% obtained by Glew *et al.*, (1997), whereas the Sudanese one contained only 28.0% with lysine as the limiting amino acid. Protein from *marula* kernel contains sulfur-containing amino acids like methionine and cysteine and its *in vitro* protein digestibility was almost similar to that of soy bean protein (Mariod and Abdelwahab, 2012). 79% of *marula* seed protein and soybean protein could be digested by pancreatic enzymes. Additionally, Wehmeyer, (1967) indicated that the kernel contains considerable amounts of protein ranging between 23-31%. Furthermore, Quin, (1959) indicated that *marula* kernels had a higher protein and oil content than most other popular nuts, including walnuts, hazelnuts, chestnuts and almonds. The amino acid content of *marula* fruit and seed is lower than in baobab as shown in table 5. The content of amino acids in *marula* was comparable to amounts found in other fruits (Glew *et al.*, 1997). WHO/FAO (1973) in Glew *et al.*, (1997) reported that several essential amino acids like leucine, lysine, the phenylalanine/tyrosine pair and threonine in *marula* seeds were rated lower than the World Health Organization protein standard . It should be noted that others amino acids like isoleucine, methionine, cysteine, tryptophan and valine were rated higher than the World Health Organization standard.

Table 5: Amino acid content in g/100 g of dry weight of marula fruit in comparison to baobab content.

Amino acid	Marula nut	Baobab seed	Marula pulp	Baobab pulp
		g/100 g dry weight		
Alanine	2.53	8.0	2.66	5.6
Arginine	6.76	11.5	2.12	6.8
Aspartic acid	5.17	16.9	3.77	7.5
Cysteic acid	1.95	2.8	.97	1.3
Glutamic acid	13.1	35.9	4.52	8.4
Glycine	2.68	8.8	1.98	6.2
Histidine	1.22	3.4	.8	2
Isoleucine	-	5.8	-	3.6
Leucine	3.78	10.6	2.74	5.4
Lysine	1.29	6.9	1.57	4
Methonine	.68	1.9	.51	1.9
Phenylalanine	2.37	7.2	1.6	3.5
Proalanine	-	6.9	-	3.7
Proline	2.52	9.1	3.28	7
Serine	2.64	8.3	1.91	-
Threonine	1.79	5.8	1.45	-
Tryptophan	.83	2.6	.52	3.5
Tyrosine	1.47	3.9	1.32	8.5
Valine	3.03	8.5	2.17	4.9

Source: Glew *et al.*, 1997

The reported moisture content of *marula* fruit juices/flesh varies between 82 and 93% (Gous *et al.*, 1988 and Shone, 1979) as shown in table 6. These variations were attributed to differences in growing conditions of the trees (Gous *et al.*, 1988). In different regions of Southern Africa, reported moisture content of *marula* juice/flesh varied from 79 – 92 g/100 g. It could be also due to the difficulty to obtain a representative sample for moisture of juice/flesh determination since the flesh adhering tightly to the skin and stone, and information on how the sample was prepared was not documented. Oranges, banana, papaya, mango and pineapple when ripe, have moisture contents of 83%, 74%, 90%, 80% and 85%, respectively (Hernandez *et al.*, 2006).

In Table 6 the carbohydrate fraction of the *marula* juice is reported to range between 7 to 14 % of the fresh weight; consisting mainly of sucrose, glucose and fructose and the edible portion (pulp) of *marula* had 2.3% invert sugar (glucose and fructose) and 5.9% sucrose (Gous *et al.* (1988); von Teichman (1983)). In South Africa and Botswana, the Brix values for *marula* fruit can vary between 10.4 and 16.0 degrees according to Leakey (1999). It implies that in some trees the fruit pulp is sweet and in others very sour. It was also found that the variation in total soluble solids of puree and juices varied over

three seasons with the lower value corresponding to drought and the higher value to a wet year. In addition to that, Gous *et al.*, (1988) found the total soluble solid fraction of *marula* puree and juices to vary between 7 and 16 degree brix and appeared to be influenced by severe drought that occurred from 1983 to 1986. According to Taylor and Kwerepe, (1995), as quoted by Leakey, (1999), *marula* contained 3.7% carbohydrate at 96% dry matter of the kernel, but information on the analysis method was not documented.

The energy value of the *marula* fruit is approximately 130 kJ/100 g of fruit flesh (von Teichman, 1983). Wehmeyer, (1967) reported that *marula* nuts represent 3138 kJ/100 g and Wynberg *et al.*, (2002) indicated that the energy value of the kernel is approximately 2699 kJ-2703 kJ/100 g. *Marula* fruit flesh energy value is lower than the compared fruits but the kernel is one with the highest value. As shown in table 6, for instance, *baobab* pulp contains between 848.9-1494.9 kJ/100 g energy, *grewia retinervis* has about 293-1010 kJ/100 g of energy in the flesh while *citrullus lanatus* has 4 kJ/100 g in the flesh and 415 kJ/100 g in the seed (Taylor, 1985).

Marula fruit contained more than 2.9 % of the fresh weight of crude dietary fiber (Taylor and Kwerepe, 1995). Marula fruit juice contained 0.7 g/100 g dietary fiber (Borochov-neori *et al.*, 2008). Table 6 shows the dietary fiber content of *marula* in comparison to other fruits.

The amount of ash that is found in *marula* juice is 1 g/100 g (Borochov-neori *et al.*, 2008). However, Taylor, (1985) found the ash content of the *marula* pulp to be 0.2 g /100 g and for the juice to be 0.09 g /100 g indicating that the skin in marula fruit has the most ash content of about 1 – 4.2 g/100 g. Aganga and Mosase, (2001) found the ash content from the seed of *marula* fruit to be 1.7%. The amount found varied a lot and could be due to the different method of analysis used by the authors in term of time and temperature. The methods for ash determination was not documented by some authors, but Gous *et al.*, (1988) used 2-5 g of *marula* samples and these were ashed for approximately 16 hours at 520°C until light in colour and he found the ash to vary between 0.5 to 0.9 g/100 g. The way the ash was determined by Gous *et al.*, (1988) indicated that they were not precise with time taken and they also used colour as an indicator for sample readiness.

Table 6: Micronutrient composition in comparison to other fruits

Type of fruit	Pulp	Reference	Juice	Reference
Moisture content (g/100 g fresh weight)				
Marula	85 – 87	Carr (1957), Borochov-neori *et al.* (2008)	82 - 93%	Gous *et al.* 1988, Shone 1979
Orange			83%	Hernandez et al, 2006
Baobab	2-28%	Chadare et al, 2009		
Parinari mobola (hissing tree)	69.1	Carr (1957)		
Wild grape (*Lannea edulis*)	70.5	Carr (1957)		
Sour plum (*Ximenia caffra)*	66.4	Carr (1957)		
Wild mango (*cordyla Africana)*	80.9	Carr (1957)		
Papaya			90%,	Hernandez, et al, 2006
Pineapple			85%	Hernandez et al, 2006
Banana			74%	Hernandez et al, 2006
Grewia retinervis	10.6 - 11.7	(Taylor, 1985)		
Citrullus lanatus	86.9 - 97.9	(Taylor 1985)		
Carbohydrate content (mg/100 g fresh weight)				
Marula	700 - 1200	Shone (1979), Gous et al (1988), von Teichman (1983)	1200 - 1440	Gous et al (1988)
Baobab	4660 - 8800	Chadare et al (2009)		
Grewia retinervis	6750	Taylor (1985)		
Citrullus lanatus	90	Taylor (1985)		
Dietary fibre (g/100 g dry weight)				
Marula	2.9	Taylor (1985)	7.95% DM 0.7	Aganga (2001) Borochov-neori *et al.* (2008)
Grewia retinervis	12.6 - 24.7	Taylor (1985)		
Citrullus lanatus	2.3	Taylor (1985)		
Baobab	13.7	(Chadare et al, 2009)		
Ash content (mg/100 g dry weight)				
Marula	10 - 20	Taylor (1985), Carr, (1957) and Gous *et al,* (1988)	10-90	Taylor (1985) Carr, (1957) and Gous *et al.* (1988)
Grewia retinervis	370	Taylor (1985)		
Citrullus lanatus	110	Taylor (1985)		
Baobab	190 - 640	Chadare et al (2009)		

10. Conclusions and Recommendations

Marula fruit mineral and nutrient content varied greatly from study to study. This could be due to variation in place of origin, soil, climate, and time that lapsed after harvesting before analysis took place. Due to the variation found in reported data, we recommend that collection, handling, storage conditions under which the samples were handled and methods used to analyze the samples must be described in detail. *Marula* juice sample preparation could also be the cause of variation, since from country to country the method of extracting the juice out of the fruit is different; it also depends on the strength of the presser/processor. In some cases, authors did not make clear which part of the fruit was used for the analysis. It is very confusing when author use terms like flesh, pulp, and juice or edible portion. For the variation arising due to environmental factors, cannot easily be controlled since most of *marula* trees grow naturally and under no irrigation and fertilizer added and it can grow in open woodlands, bushes, in clay or sandy soil, survives in hot dry climatic conditions with a mean annual rainfall of 200 to 1500 mm. For instance, in some countries like Namibia and South Africa there are extreme variations in rainfall partern from year to year and place to place.

Nonetheless, *marula* fruit is a rich source of antioxidants and vitamin C, which can be eight times higher than that in an orange fruit. The nuts of these trees are also rich in oleic acid, protein, energy and minerals like iron, magnesium, zinc, phosphorus and copper which contribute to the importance of these nuts in the diets of rural communities. *Marula* fruits could play a vital rolein the nutrition of the rural populations who rely on the usage of the fruits and do not have access to other sources of nutrients.

11. Future research recommended

Even though some studies reported on the contents of nutrients found in *marula* fruit, the results reported show great variation in the measured *marula* composition values. What is clear is that *marula* is a rich source of various nutrients, especially vitamin C. However, the way it is processed is of absolute importance as this may have influence on the retention of nutrients such as vitamin C and its antioxidant capacity. This area is not well documented and very limited information is available in the literature. In addition, different methods of analysis have been used and that makes it difficult to draw up a concrete conclusion toward the antioxidant obtained from different fruits and their stability toward heat treatment and storage conditions. Therefore, further investigation on the thermal degradation of vitamin C in *marula* products is needed. In addition, the most made product from *marula* fruit is fermented juice. Little is known about the antioxidant activity and about their content in processed *marula* products like fermented juice and this need further research. Furthermore, it is clear that pressing all the juice out from marula fruits is not easy neither efficient because the pulp is bound by pectin into a gel form. Further work on that is required to improve yields and to get a better-clarified juice. The non-clarified juice is very cloudy, contains a lot of pulp and that is not appealing to a lot of *marula* juice drinkers. The skin of the fruit is rather too thick and is of little value at present. It is also apparent that most of the characteristic flavour of the fruit is contained within the skin, which is lost during processing. That could be the reason why most of the *marula* containing products available in the market do not contain *marula* flavour compounds since the skin is not incorporated in the juice processing. Therefore, investigations need to be done to identify the important flavor compounds and further investigate the effect of processing or storage effect towards *marula* flavour.

The proximate analysis for different fruits from Southern Africa reveal some variation, which may be either genetic or environmental or both or due to different analytical methods. The causes of this variation are still not known and need to be investigated, as genetic variation of this magnitude would be of importance to domestication programmes.

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Chapter 3

Optimising the juice yield and quality of marula fruit (*Sclerocarya birrea subsp. Caffra*) with pectolytic enzymes by a response surface method

Hiwilepo-van Hal, P., Robben, J., Verkerk, R., van Boekel, M. A. J. S. & Dekker, M.

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Abstract

Marula juice is known for its high content of vitamin C. The use of marula juice-based products has been increasing recently. To increase the production of the juice requires development of an efficient extraction process. This study used Response Surface Methodology (RSM) using a Central Composite Design (CCD) to determine the optimum production conditions in order to optimize the marula juice yield, while taking into account the quality attributes of the juice like the content of vitamin C, polyphenols, the antioxidant activity and the colour. The parameters that were studied were temperature (25-60°C), pectinase concentration (0.04-0.2%) and time (5-65 min). The optimal statistical description of the data of juice yield, vitamin C content, polyphenol content, antioxidant activity and the colour of the juice was by a quadratic model. The optimal amount of pectinase (in the range of 0.1 to 0.14%) increased the yield of marula juice by 23% compared to not using it. The optimal temperature for the content of vitamin C and polyphenols as well as for the antioxidant activity ranged between 40 and 60°C. Antioxidant activity showed to be correlated to the content of vitamin C in the juice. Heating time had an effect on the lightness of the marula juice, changing into darker yellow colour at prolonged heating times. The predicted optimal juice yield and quality was validated by additional production runs at the predicted conditions. The quadratic model can be used to perform multi-criteria optimization of the production of *marula* juice, by addressing both the yield and several quality attributes.

Keywords: Marula juice, optimization, pectinase, vitamin C, polyphenols, antioxidant activity.

1. Introduction

Marula (*Sclerocarya birrea*) is one of the most commonly utilized indigenous fruits of Africa (Shackleton *et al.*, 2001). The fruits are round/oval shaped and green when young, becoming butter-yellow at the stage of maturity (Nerd, Aronson & Mizrahi, 1990). It has a thick, soft and leathery skin enclosing a juicy white pulp that adheres tightly to the central pit (von Teichman, 1982). Fruit yield per tree varies greatly from country to country, from area to area and from tree to tree and primarily it is determined by rainfall (Botelle, Du Plessis, Pate & Laamanen, 2002). Pretorius, Rohwer, Rapp, Holtzhausen and Mandery (1985), recorded a yield of 22 000 - 91 000 fruits for four trees in one season. One particular harvest, collected from one tree in 64 days, was reported as high as 91 272 fruits with a mass of 1 647kg (von Teichman, 1983). Marula average fruit yield is about 596 kg per tree with a high standard deviation of 482 kg (Botelle *et al.*, 2002). Leakey, Shackleton and du Plessis (2005), reported that the average weight of marula fruit in South Africa and Namibia was 20 g to 27 g while it was 30 grams in Namibia respectively (Botelle *et al.*, 2002). According to Von Teichman (1983) the edible flesh (pulpy) component of the fruit is very small compared to its size and it is utilized for several purposes by local communities in Southern Africa, for example to prepare juice, jelly or jams, fermented to make alcoholic beverages like local marula beer and the fruit can be eaten raw by children (Nerd & Mizrahi, 1993; Mokgolodi, Ding, Setshogo, Ma, & Liu, 2011). In addition to that, local communities have used marula fruit for generations to cure and prevent scurvy; the anti-scorbutic value of the fresh fruit makes it important to their base diet. There is a wealth of legends around the *marula* fruit and its many uses add to its cultural value (Mokgolodi *et al.*, 2011).

Marula fruits are rich in nutrients such as vitamin C, phenolic compounds and other minerals like potassium, calcium and magnesium (Hiwilepo-van Hal, Bille, Dekker, & van Boekel, 2013). The vitamin C content of fresh marula juice is about four times higher compared to orange juice and pomegranate juice (Hillman, Mizrahi & Beit-Yanni, 2008). According

to Eromosele, Eromosele and Kuzhkuzha (1991), marula juice had an ascorbic acid content of (403mg/100g), as compared to other fruits like grapes (38 mg/100g), oranges (50 mg/100g) and strawberries (59 mg/100g). Ndhlala, Kasiyamhuru, Mupure, Chitindingu, Benhura, and Muchuweti (2007), measured the phenolic acid composition of the peel and pulp of *marula* (*Sclerocarya birrea*), F. indica and O.megacantha. It was found that the *marula* pulp had the highest total phenolic, flavanoids and condensed tannins, 2262 µg GAE/g, 202 µg catechin/g and 6.0% condensed tannins, respectively. The total phenolic content of marula pulp was eight times higher than that of the other fruits.

The traditional way of extracting *marula* juice is by using a cow horn to puncture the leathery skin of the *marula* fruit after which the juice is squeezed out of the *marula* (den Adel, 2002). This squeezing is not efficient to gain a high juice yield, because part of the flesh is attached to the central pit and skin. According to Gous, Weinert and van Wyk, (1988) the fleshy part of the *marula* fruit contains more than 2.0% pectin. In Namibia the use of a small pedal-operated hydraulic press designed and disseminated by CRIAA SA-DC/ Katutura Artisans Project, has been used and *marula* juice can be extracted at a much faster rate (du Plessis, Lombard & den Adel, 2002). One press capacity per day yields 200 L/day of *marula* juice (4 kg of fruits yields about 1 L of juice), while without the press only about 20 L/day per person. In some trials with a hydraulic press, juice yields varied from less than 20% to more than 40% of the fruit weight (du Plessis *et al.*, 2002). Even though the hydraulic press has been in use, it is still hard to press all the juice out from marula fruits because the pulp is bound by pectin into a gel form. By adding pectinases, the gel is broken down so that the juice can be extracted easly. Although pectinases have been the principal enzymes used (Sreenath, Sudarshana, & Santhanam, 1995), a mixture of cellulolytic and pectinolytic enzymes is frequently used for complete liquefaction of fruits pulps resulting into not only higher juice yield but also juice with high dry matter content (Sreenath, Nanjundaswamy, & Sreekantiah, 1987). Response Surface Methodology (RSM) is a tool of collection of statistical and mathematical technique which is effective for developing, optimising and improving the process and the same time reduced the use of samples. This present study

used Response Surface Methodology (RSM) to determine the optimum processing conditions (enzyme concentration, extraction temperature and time) for maximum juice yield, vitamin C, polyphenols, antioxidant activity and clarification (colour) of the marula juice. These conditions will serve as a preliminary basis for further studies on the Brix, viscosity of the juice and to validate the optimum condition for vitamin C, polyphenols, antioxidant activity and clarification (colour) of the marula juice.

2. Materials and methods

2.1. Marula fruits collection

Marula fruits were obtained from the northern part of Namibia with the help of Eudafano Women's Cooperative (EWC) in Ondangwa, Namibia. Fruits were selected by hand sorting for their stage of maturity and visual appearance. Damaged fruits were discarded and green, unripe fruits were held back to ripen in a heap. Fruits that were classified as ripe were selected on colour, going from green to yellow and another important factor was their firmness as they become softer when ripe. These ripe fruits were transported to Wageningen University in frozen conditions and stored in the freezer at -20°C experimentation.

2.2. Enzyme

Pectinase enzyme from *Aspergillus niger* spp. was obtained from Sigma, and stored at +4°C prior to the experiments.

2.3. Sample preparation

The total amount of frozen *marula* fruits was first weighed, and then thawed in lukewarm water of about 40 ºC till the fruits were semi-frozen. After that, the peel of the marula fruits was removed, sliced in small pieces and subsequently poured in liquid nitrogen. The flesh was separated from the kernel, then the kernel was weighed and the percentage of pulp to nut was calculated. The frozen flesh and peel were blended with Waring commercial blender (model HGB 2WTS3) and stored in the freezer at -20°C.

2.4. Heating and juice pressing

The experimental design is according to the Response Surface Methodology and shown in Table 1. About 120 g of marula flesh and skin were used for each sample in this experiment. Samples were treated according to the established time, temperature and enzyme concentration combinations as can be seen in the experimental design in Table 1. For heating, first the samples were heated in a water bath at 100°C till the required temperature was reached as shown in Table 1. During heating, the samples were continuously stirred by using an automatic stirrer and kept in a temperature controlled water bath and subjected to treatment given in Table 1. After heating, the samples were cooled on ice and the juice was pressed out using ISO hydraulic press 100 bars. The amount of juice was weighed and the juice yield was calculated as percentage from the amount of juice obtained based on the amount of initial pulp (flesh + skin) in g.

2.5. Experimental design and Response Surface Methodology

Design-Expert 8.0.5 software was used to make an experimental design to obtain the combinations of the settings for the three process variables that were used for the experimental productions. A Central Composite Design (CCD) was used and this design is mostly used to study linear interactions and the quadratic effects between the independent variables similar to Rastogi and Rashmi (1999). In the present study these independent variables were i) enzyme concentration X_1 ranging from 0.04 - 0.2%, ii) temperature X_2 ranging between 25 – 60 °C and iii) incubation time X_3 of 5 – 65 min. Each independent variable could be set at five different values. A total of seventeen combinations were taken in random order according to CCD configuration for the three factors as shown in Table 1. The response variable/dependent variables Y were 1) juice yield, 2) vitamin C, 3) polyphenol content, 4) antioxidant activity and 5) clarification of the juice (colour). After obtaining the experimental data on juice yield and the four qualities attributes, Response Surface Methodology (RSM) was used to analyse the results.

This general equation relating to each response, the coded variables (X_1, X_2, X_3) by a second-degree polynomial is using **Equation 1**. The coefficients of the polynomial were represented by b_0 (constant term),

$b_{1,}$ b_2 and b_3 (linear effects), $b_{11,}$ b_{22} and b_{33} (quadratic effects), and b_{13} (interaction effects):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad \textbf{Equation 1}$$

Analysis of variance (ANOVA) tables were generated and the effect of independent variable and regression coefficients of individual linear, quadratic and interaction terms were determined. Different model complexities were fitted to the data sets: linear, two-factor-interaction and quadratic. Based on sequential and lack-of-fit p-values the best fitting significant model ($p<0.05$) was selected.

Table 1: Settings for the three independent variables: enzyme concentration (X_1), Time (X_2), Temperature (X_3) for the central composite experimental design.

Run	Enzyme conc. X_1 (%)	Time X_2 (min)	Temperature. X_3 (°C)
1	0.1	35	42.5
2	0.1	35	42.5
3	0	35	42.5
4	0.1	35	42.5
5	0.04	17.5	53
6	0.1	35	25
7	0.2	35	42.5
8	0.16	53	32
9	0.1	35	60
10	0.04	17.5	32
11	0.04	53	32
12	0.16	53	53
13	0.04	53	53
14	0.1	65	42.5
15	0.1	5	42.5
16	0.16	17.5	53
17	0.16	17.5	32

The significance of all terms in the polynomial was judged statistically by computing the F-value and P-value at a probability (p) of 0.05. Non-significant interaction and quadratic terms in the models were removed by backward elimination, resulting in simplified models with less parameter. The models were then used to generate contour maps to visualise the effects of the process variables on the five responses.

2.6. Ascorbic acid extraction

The procedure used for extracting L-ascorbic acid (AA) for the pressed juice was a modification of the method described by Hernandez, Lobo and Gonzealez, (2006). 2.5 ml of the juice was transferred into 10 ml tubes. The juice was mixed with 2.5 ml of the extracting solution containing 3% MPA (Metaphosphoric Acid) and 0.001Mol/L TBHQ (tert - butylhydroquinone) then the mixture was homogenized. After homogenization, the mixture was centrifuged (ALC PK131R) for 5 minutes at 2255xg at 4°C. The extracts were diluted up to six times with distilled water. All extractions were carried out under reduced light and on ice. For the standard, a commercial L-ascorbic acid with the range 1.56 µg/ml – 200 µg/ml was made. Subsequently, about 2 ml of the standard and extract was filtered through 0.45µm filter and used for high performance liquid chromatography (HPLC) analysis.

2.7. HPLC analysis

The method that was used for the determination of L-ascorbic acid (AA) for pressed *marula* juice was as described by Hernandez *et al.*, (2006) with modifications. The specifications of the HPLC system were a thermo separation products model with P-2000 Binary Gradient Pump and UV 2000 detector. Separations were carried out on a Varian Polaris C18-A column, 150 x 4.6 mm with 5.5 minutes running time and 20 µl injection volume. The mobile phase employed was a mixture of (Orthophosphoric Acid 0.2% in distilled water). The flow rate of the mobile phase was 1 ml/min with a UV- detector at a wavelength of 245 nm. AA peaks were identified by comparing their UV-visible spectral characteristics and retention time with a commercial standard of AA. The amount of vitamin C was expressed as ascorbic acid equivalents in mg/100g dry weight (dw) contents.

2.8. Total phenolic content

Total phenolic content of the pressed *marula* juice was determined using The Folin-Ciocalteu method as described by Georgé, Brat, Alter and Amoit, (2005) and by Swain and Hillis, (1959) with some modifications. 0.25 g of fruit pulp was homogenized in 5 ml of methanol/distilled water (1:1 v/ v). The homogenate was centrifuged (ALC PK131R) for 5 minutes at 2255xg

at 4°C and the supernatant collected were diluted to 1:10 with distilled water. A 70% Na_2CO_3 solution was prepared; this solution was stirred at room temperature for 1 hour and then used during the extraction. For the standard a calibration curve for tannic acid from sigma 1 mg/ml and diluted to 1: 32 was done. Briefly, in a volumetric flask of 25 ml, 5 ml distilled-water, 1 ml of Folin-Ciocalteu reagent, 1 ml of 70% Na_2CO_3 solution and 1 ml of the juice extracts or tannic acid was added. Then the flask was filled with distilled-water up to 25 ml and mixed thoroughly. After full development of the blue colour the absorption was measured with a spectrophotometer (Cary 50, Probe UV visible, Varian) at 725 nm. The total phenolic content was expressed as tannic acid equivalents in mg/100g dw of *marula* fruit juice.

2.9. Antioxidant activity determination

The antioxidant activity of the *marula* extract was studied by evaluating the free- radical scavenging effect on the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. The method used was as described by Brand-Williams, Cuveliere and Berset (1995) with some modification. The extract was mixed with 50% aqueous methanol, then the mixture was homogenised and centrifuged (ALC PK131R) for 5 minutes at 2255xg at 4°C. The highest concentration used for the juice was 25 mg/ml, which was diluted with aqueous methanol solution to achieve the lowest concentration of 6.25 mg/ml. Fruit extract (0.1 ml) was mixed with 3.9 ml of 0.02 Mol/L DPPH (Sigma) methanolic solution. The mixture was thoroughly mixed and kept in the dark for 30 min. The absorbance was measured at 515 nm using the spectrophotometer (Cary 50 Probe). For the determination of the scavenging effect on DPPH radicals, the amount of DPPH which did not react for all the dilutions of sample was determined using a DPPH calibration curve. The EC_{50} value was determined graphically by plotting the disappearance of DPPH as a function of the sample concentration. Trolox (Aldrich) 1.5 to 0.094 mMol was used as a standard. The EC_{50} values from the curve for Trolox and from the juice extract was used to calculate the EC_{50} expressed in Trolox Equivalent of Antioxidant Activity (TEAC).

2.10. Colour

The colour of the pressed *marula* juice was measured with the Hunter LAB apparatus, which measures L, a and b values. The L value stands for lightness, positive-a refers to red, negative-a to green, positive-b to yellow and negative-b to blue (purple). The apparatus was first standardized with a black and white plate and a green control plate was used to check if the calibration was correct. The assessment of colour differences (delta) was done in order to have only one colour value and expressed as $\Delta E^* = [(\Delta L^2) + (\Delta a^2) + (\Delta b^2)]^{1/2}$. The sample with the shortest processing time resulting in the lightest colour (run 15) was used as reference sample for the calculation.

2.11. Validation of predicted optimal conditions

The optimum conditions for the extraction maximum yield from *marula* fruit using the RSM were validated experimentally and predicted values were compared with the experimental ones in order to determine the validity of the model prediction.

3. Results and Discussion

3.1. Juice yield

Table 3 summarizes the results of each dependent variable with their coefficients of determination (R^2). While the full quadratic model contains a total of 10 parameters (equation 1) only 5 to 7 parameters were significant in the obtained five RSM models of the measured responses. The obtained model p-values indicate that the model representations of the effects were significant. The lack-of-fit p-values were all non-significant ($p>0.05$), which is another criteria for a good fit of the data by the model (data not shown).

The contour plot (Figure 1) indicates that increasing enzyme concentration between increases the marula juice yield. This correlation was also found by Sreenath *et al.*, (1995) and many other authors. An enzyme concentration higher than 0.15% was found not to increase juice yield. The observed experimental maximum juice yield of 56.4% was produced

with 0.1% enzyme at a temperature of 42.5 °C and an incubation time of 5 min. The experimental values were very close to the model values, which confirm the validity and adequacy of the models. Increase in yield by the use of enzyme was found to be 23% in comparison to the (reference) average control yield of 33.9 ± 4.6%. In the studied range the effect of time was found to be very small, indicating that 5 minutes is enough for the enzymes to act. The interactions between enzyme concentration, incubation time and temperature were all not significant.

Table 2: Experimental results for the 17 runs for the responses for juice yield (Y_1), vitamin C (Y_2), polyphenols (Y_3), antioxidant activity (AOAA) (Y_4) and colour (Y_5) of the marula juice. ND = not detected.

Run	Enzyme conc. X_1 (%)	Time X_2 (min)	Temp. X_3 (°C)	Yield Y_1 (%)	Vitamin C Y_2 (mg/100g dw)	Polyphenols Y_3 (mg/100g dw)	AOAA Y_4 (µmol/mg)	Colour Y_5 Delta-E
1	0.1	35	42.5	52.4	430	$2.26*10^3$	$7.1*10^{-3}$	7.02
2	0.1	35	42.5	50.5	270	$2.49*10^3$	$7.4*10^{-3}$	7.40
3	0	35	42.5	16.2	ND	$3.04*10^3$	$7.7*10^{-3}$	ND
4	0.1	35	42.5	53.9	400	$2.07*10^3$	$8.5*10^{-3}$	6.40
5	0.04	17.5	53	43.8	1040	$2.62*10^3$	$1.7*10^{-2}$	2.42
6	0.1	35	25	53.4	470	$2.51*10^3$	$9.7*10^{-3}$	4.05
7	0.2	35	42.5	55.2	580	$2.02*10^3$	$7.1*10^{-3}$	6.49
8	0.16	53	32	52.7	330	$1.54*10^3$	$1.1*10^{-2}$	8.31
9	0.1	35	60	45.2	490	$2.75*10^3$	$1.9*10^{-2}$	6.18
10	0.04	17.5	32	44.4	520	$1.73*10^3$	$4.3*10^{-3}$	3.37
11	0.04	53	32	48.9	ND	$1.48*10^3$	$7.5*10^{-3}$	6.18
12	0.16	53	53	53.1	760	$1.96*10^3$	$1.7*10^{-2}$	8.84
13	0.04	53	53	43.4	680	$2.73*10^3$	$1.4*10^{-2}$	6.30
14	0.1	65	42.5	50.8	ND	$2.26*10^3$	$4.8*10^{-3}$	7.60
15	0.1	5	42.5	56.4	930	$2.30*10^3$	$6.2*10^{-3}$	ND
16	0.16	17.5	53	51.6	1240	$2.01*10^3$	$1.6*10^{-2}$	7.01
17	0.16	17.5	32	48.3	740	$1.65*10^3$	$1.2*10^{-3}$	3.82

Table 3: Significant ($p<0.05$) model coefficients, R^2 and p-values for the obtained RSM models for the 5 responses for the marula juice production.

Model coefficient	Response				
	Yield	Vitamin C	Polyphenols	AOAA	Colour
b_0	30.9	649	514	$3.86*10^{-2}$	-6.22
b_1	417	1084	7539	$5.96*10^{-2}$	-2.38*10
b_2	$3.32*10^{-3}$	-39.7	-1.67	$-1.04*10^{-5}$	0.29
b_3	-0.113	13.3	50.1	$-1.95*10^{-3}$	0.25
b_{11}	-1533			0.17	
b_{22}		0.45			$-2.67*10^{-3}$
b_{33}				$2.88*10^{-5}$	$-3.49*10^3$
b_{13}			-267	$-1.88*10^{-3}$	0.903
R^2	0.74	0.67	0.54	0.86	0.89
p-value	$1.6*10^{-3}$	$2.66*10^{-2}$	0.04	0.001	$8.0*10^{-4}$

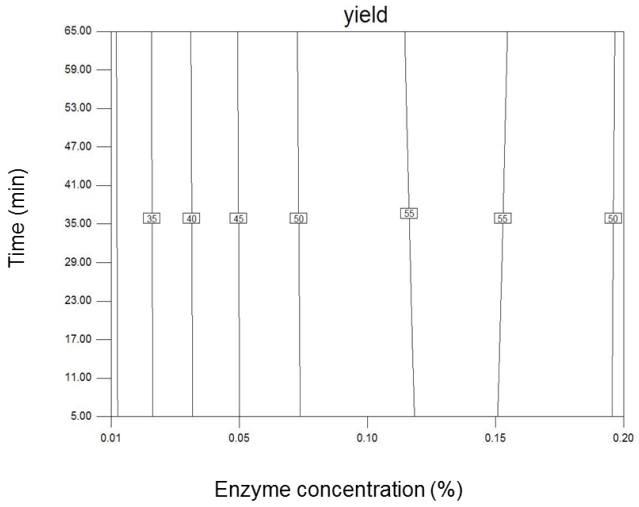

Figure 1. Contour plot representing the effect of enzyme concentration and time on juice yield (%), temperature was fixed at 35 °C.

3.2. L-ascorbic acid content

The L-ascorbic acid content of the untreated **marula** juice was found to be 1150 ± 140 mg/100g dw. In figure 2 the result shows an increase in vitamin C with temperature above 40°C and with increasing enzyme concentration. The optimum value by RSM was higher than 1000 mg/100g dw at an enzyme concentration higher than 0.1%, for temperature higher than 40°C and incubation time of about 5 min. The optimum observed value was 1240 ± 30 mg/100g dw at an enzyme concentration of 0.16 %, temperature of 53°C and incubation time of 17.5 min. There was no correlation between enzyme concentration, time and temperature on the vitamin C concentration.

Vitamin C in *marula* juice seemed to be quite stable during heating. According to Hiwilepo-van Hal, Bosschaart, van Twisk, Verkerk and Dekker (2012) one explanation for this stability might be that the vitamin C molecules are protected by the *marula* fruit matrix, where they are released during heating. Or maybe part of the ascorbic acid is more stable due to a different location in the fruit matrix, or due to a limiting amount of a reactant in the degradation reaction (e.g. oxidizing agent). That might end up at the rate at which vitamin C breakdown occurring is lower than the rate of its release. Similar result was found in *marula* jam pasteurised

at 93 °C for 14 min, in which the vitamin C content was as high as 84% of the original content (Hillman *et al.*, 2008). This shows that vitamin C in marula fruit juice is stable upon heating even at high temperatures.

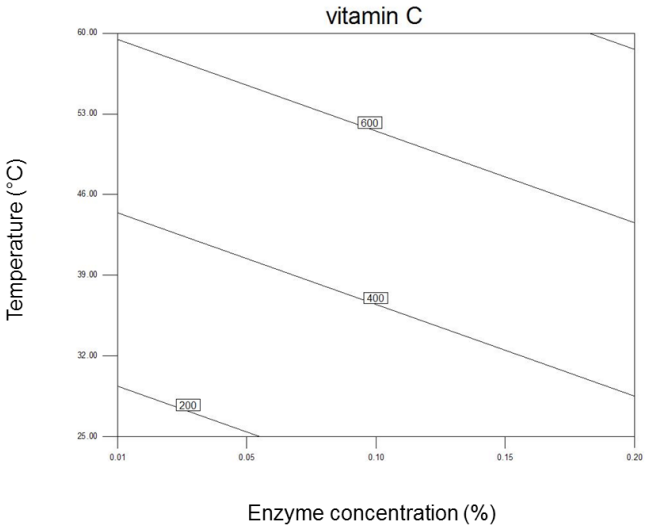

Figure 2. Contour plot representing the effect of enzyme concentration and temperature on vitamin C (mg/100g dw) of *marula* juice, heating time was fixed at 36 min.

3.3. Polyphenol content

The polyphenol content of the untreated *marula* juice was found to be 3000 ± 360 mg/100g dw. The effect of enzyme concentration, heating time and temperature on total polyphenol content of *marula* fruit juice was measured and modelled. The shaped curve fits the measured polyphenol content as can be seen in Figure 3. The polyphenol content increases with increasing temperature and decreases with increasing enzyme concentration. Increasing processing time leads to a decrease in the content of polyphenols (negative b_2 value, see Table 3). According to Xu, Ye, Chen and Liu, (2007) a drop in polyphenol content was expected with increasing heating temperature. This increase can be explained by the breakdown of polyphenols into phenolic acids like Gallic acid. Another explanation could be the breakdown of the *marula* matrix by which the rate of polyphenol released was higher than the breakdown of the molecules. From literature, it is known that the enzyme polyphenol oxidase is activated during juice processing, since plant cells are destroyed. This enzyme is able to oxidize polyphenols causing brown discoloration and

results in loss of antioxidant activity. Therefore, it will be interesting to further analyze the stability and activity of polyphenol oxidase during marula juice processing. In figure 3 the polyphenol content is plotted against enzyme concentration and temperature.

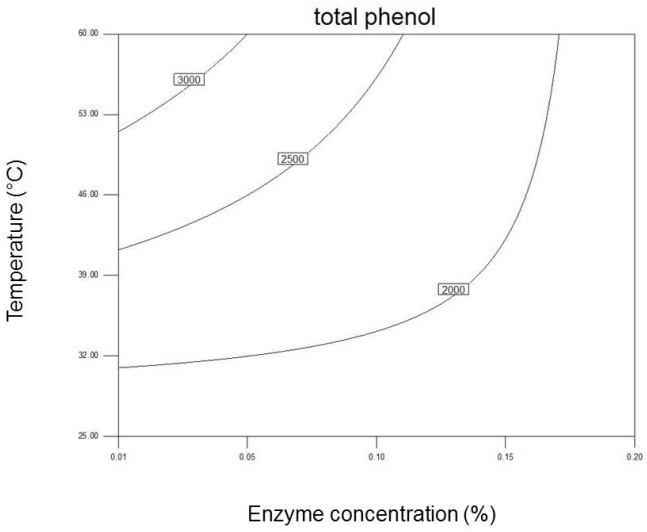

Figure 3. Contour plot representing the effect of enzyme concentration and temperature on polyphenol content (mg/100g DW) of *marula* juice, heating time was fixed at 36 min.

3.4. Antioxidant activity

In Table 3 the results indicates that the proposed model was able to fit measured antioxidant activity. The antioxidant activity of the untreated *marula* juice was found to be 0.011 ± 0.003 µmol/g TEAC. There was a strong correlation between temperature and antioxidant activity (p=0.0026). The temperature range of 25–32ºC showed a small decrease in antioxidant activity, while the temperature range of 39–60ºC showed a remarkable increase in antioxidant activity (Figure 4). Since the hydrophilic antioxidant activity was measured, the radical scavenging capacity strongly depended on vitamin C and polyphenol content. Mdluli and Owusu-Apenten, (2003) reported that vitamin C content of *marula* fruit accounts for about 70% of the total antioxidant capacity. According to Kennedy, Rivera, Lioyd, Warner and Jumel, (1992) vitamin C is unstable during heating; however, an increase in vitamin C concentration during heating was found in our results. The increase in antioxidant activity might be correlated to the increase in vitamin C. Hiwilepo-van Hal, Bille, Verkerk

and Dekker, (2012) also found a positive correlation between antioxidant activity and polyphenols (R^2=0.64) and between antioxidant and vitamin C (R^2=0.59) of marula juice. For a better understanding, a further study on evaluation of *marula* antioxidant capacity and its correlation with the content of specific compounds in the juice is needed.

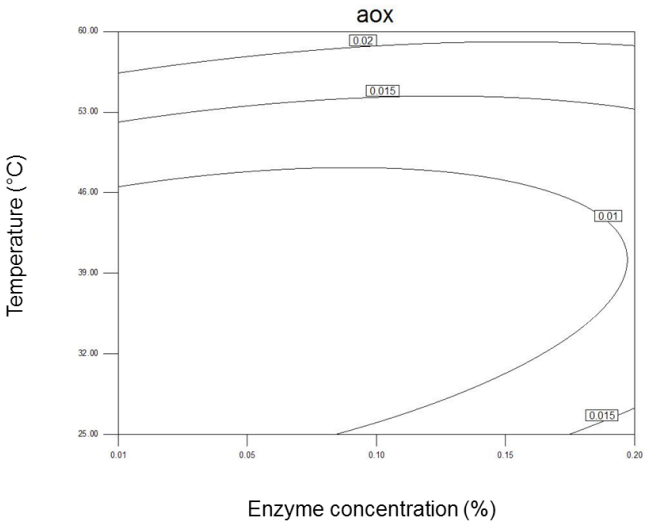

Figure 4. Contour plot representing the effect of enzyme concentration (%) and temperature (°C) on the antioxidant activity (µmol/g TEAC) of *marula* juice. Heating time was set at 36 min.

3.5. Juice colour

The L*, a* and b* values of the untreated samples (reference) were found to be 47.3 ± 2.4, -2.3 ± 0.2 and 13.0 ± 0.7 respectively. In Table 3 the results show that the proposed model was able to fit total color difference (delta-E) of juice (R^2=0.89). For the (L*) the parameter, time had a p-value of 0.0004 which implies that this factor significantly influenced the lightness of the juice. However, the factors of temperature and enzyme concentration did not significantly influence lightness, with p=0.18 and p = 0.12, respectively. It is notable that the factor of temperature was not significantly related to lightness, since temperature is an important parameter in the activity of polyphenol oxidase. Polyphenol oxidase causes brown discoloration when oxidizing polyphenols. This only occurs when plant tissue is damaged, like during juice processing (Yemenicioglu, Ozkan & Cemeroglu, 1997). According to Mdluli, (2005) at 60 °C *marula* fruit polyphenol oxidase was relatively heat-stable and retained up to

60% activity after 16 min of heating, while 70% activity was retained for peroxidase at the same temperature. For the colour (a*), the p-values for heating time, temperature and enzyme concentration were 0.0003, 0.48 and 0.06, respectively. The color of the juice was becoming more reddish with prolonging heating time. For colour b* the p-values for heating time, temperature and enzyme concentration were 0.006, 0.68 and 0.06 respectively. The colour of the juice became more yellow with prolonged heating time. The combination of yellow and reddish colour is referring to orange/brown discoloration, possibly by enzymatic browning. Non-enzymatic browning is another possibility, since *marula* fruits are rich in sugars. The Maillard reaction occurs when sugars react with free amino acid groups and this leads to brown colour pigments (Turkmen, Sari, Poyrazoglu & Velioglu, 2006). Partly, it could be due to non-enzymatic browning because of carbonyl break down products of L-ascorbic acid like furan-type components, lactones, acids and 3-hydroxy-2-pyrone. According to Roig, Bello, Rivera and Kennedy, (1999), browning is followed by vitamin C loss and breakdown products like furan-type components, lactones, acids and 3-hydroxy-2-pyrone were identified as non-enzymatic browning products. Therefore, further work on identifying degradation product of vitamin C in *marula* might give a better understanding.

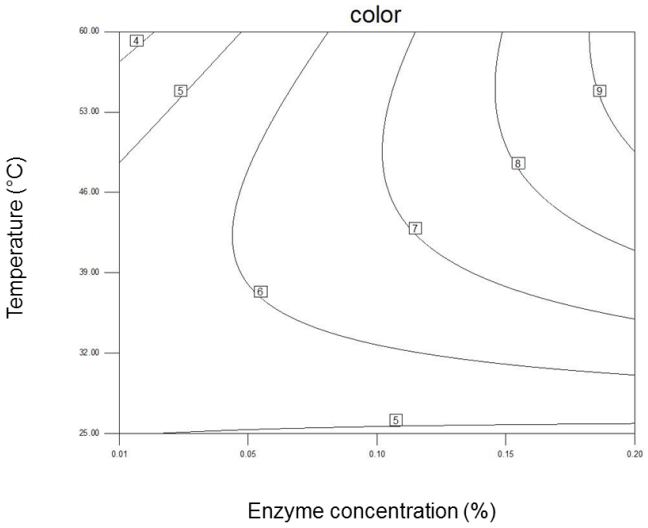

Figure 5.
Contour plot representing the effect of enzyme concentration and temperature on the total color difference (ΔE) of *marula* juice; heating time was fixed at 36 min.

3.6. Validation of yield results

The result from the validation experiment of juice yield at an enzyme concentration of 0.14%), heating time of 65 min and temperature of 60°C was 55.6 ± 1.1 % and it shows that the experimental values were found to be close to the predicted one 54.6 %. This confirms the validity and adequacy of the predicted model for yield.

4. Conclusion

The use of the pectinase enzyme concentration can increase the yield of *marula* juice by 23%, an enzyme concentration around 0.14% seems to be optimal for marula juice processing. The use of RSM revealed that there is a strong positive correlation between increasing enzyme concentration and juice yield, indicating that the *marula* yield is mostly affected by the enzyme concentration and less by the processing time (5-65 min) and the heating temperature used (25-60 °C). Furthermore increasing enzyme concentration to a level greater than 0.14% with temperature ranging between 40 and 60ºC can significantly increase the vitamin C content and antioxidant activity. The antioxidant activity seems to be correlated to the increase in vitamin C. The polyphenol content increased with increasing temperature and decreased with increasing enzyme concentration. The processing time had a significant effect on the lightness of the juice. However, the factors of temperature and enzyme concentration did not significantly influence the lightness. The predicted optimal conditions for maximizing yield were experimentally validated. The obtained model can also be used to perform multi-criteria optimisation by setting desirability scores not only for yield, but also for the four measured quality responses. In this way the yield can be also optimised under the constraints of the desired quality of the juice.

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Chapter 4

The effect of temperature and time on the quality of naturally fermented marula (*Sclerocarya birrea subsp. Caffra*) juice

Hiwilepo-van Hal, P. Bille, P. G., Verkerk, R., & Dekker, M. (2013).

LWT-Food Science and Technology, *53*, 70 – 75.

Abstract

This paper presents the effects of fermentation on the retention of vitamin C, total polyphenols and antioxidant activity in the naturally fermented *marula* juice. The fermentation conditions have been varied; temperature ranged between 20 and 40 ºC and fermentation time from 1 to 8 days. Marula juice fermented at higher temperatures ranged between 30 to 40 ºC for 6 to 4 days retained high antioxidant activities, and they were positively correlated to its ascorbic acid and phenolic content. The values obtained ranged between 0.0239 ± 0.0051 to 0.029 ± 0.0038 µmol/mg for Trolox Equivalence Antioxidant Capacity, 870 ± 80 to 960 ± 130 mg/100 ml for total phenolic content and 90 ± 6 to 159 ± 15 mg/100 ml for ascorbic acid. In general, fermented marula juice can be used as a good source for natural antioxidants.

Key words: Marula, *Sclerocarya birrea subsp. Caffra*, vitamin C, polyphenols and antioxidant activity

1. Introduction

Marula (*Sclerocarya birrea subsp. Caffra*) is an important tropical fruit in Southern Africa. In certain areas like in the North Central Regions of Kavango and Caprivi in Namibia it is one of the most important fruits and potential sources of income for primary producers (Botelle, Du Plessis, Pate, and Laamanen, 2002). It is also one of the most commonly utilized indigenous wild fruit in Africa (Shackleton *et al.*, 2001). In Namibia domestication and integration of *marula* into the Ovawambo system of farming is common practice for several centuries (Botelle *et al.*, 2002). Lately, *marula* has acquired significant commercial value since its fruits and other products have entered local, regional and international market (Mokgolodi, Ding, Setshogo, Ma, and Liu, 2011). The commercial use of the plant has increased in recent years such that the fruit is used for preparation of juices, jams, conserves, jellies and alcoholic beverages (Bille & Steppich, 2003).

The *marula* juice can be fermented to give a refreshing drink and in many parts of southern Africa, it is used for brewing beer and distilled spirits (Ojewole, Mawoza, Chiwororo, & Owira, 2010). Traditional *marula* beer and wine are produced and traded in Botswana, South Africa, Swaziland, Namibia and Zimbabwe. The alcohol content in *marula* wine is about 5% and it depends on the fermentation time (Mokgolodi *et al.*, 2011). Traditionally in Namibia, *marula* juice can be used to produce either lower-alcoholic or higher alcoholic drinks. The difference in the two types is the fermentation period; the lower-alcoholic drink is fermented for less than two days while the higher-alcoholic drink is fermented for 4 to 5 days. In traditional fermentation, no starter cultures are added, only the natural microflora causes fermentation and these are mainly yeasts that are introduced by drosophila or fruit flies that feed on ripe fruits. These microorganisms use the sugar for energy and in the process, they break down sucrose into glucose and fructose that are fermented into ethanol (Cancalon & Parish, 1995). According to Dlamini and Dube, (2008) in traditional fermentation of *marula* juice, the lactic acid bacterial population was found to drop with an increase in yeast level and decreasing pH. Dlamini and Dube,

(2008) found out that the content alcohol of the fermented *marula* juice to be dependent on the sugar content and yeast presence in the juice. Vitamin C was also found to decrease during fermentation due to low sugar concentration, pH, oxygen and enzymes (Dlamini & Dube, 2008).

Several authors reported that unfermented *marula* fruit juice contains vitamin C, sucrose, glucose, fructose, phenolic compounds, dietary fibre, minerals such as K, Na, Ca, Mg, Fe, Zn, Mn as well as many other compounds (Borochov-Neori *et al.*, 2008; Eromosele, Eromosele, & Kuzhkuzha, 1991). The vitamin C content in *marula* fruits was found to be more than 4 times that of oranges and is reported to have a range of 67 – 403 mg/100 g fresh weight (Borochov-Neori *et al.*, 2008; Carr, 1957; Eromosele *et al.*, 1991; Hillman, Mizrahi, & Beit-Yannai, 2008 and Hiwilepo-van Hal, Bosschaart, van Twisk, Verkerk and Dekker, 2012). In addition, the *marula* juice was high in polyphenols (2262 µg GAE/g) and flavanoids (202 µg catechin/g) (Ndhlala, Kasiyamhuru, Mupure, Chitindingu, Benhura, & Muchuweti, 2007). *Marula* wine was also reported to contain small amounts of protein and amino acids, which contributed to the protein demand of the consumer (Steinkraus, 1983). Fermenting yeast is known to increase the content of B vitamins. Baxter, (2000) showed that limited consumption of alcohol helps to reduce the risk of cardiovascular disease and the majority of clinical studies show that most of the protective effect is due to the ethanol itself.

Fermented foods are important to rural populations because of the preservation effects, mostly due to the lower pH and the alcohol content. In fermentation, there could be an added advantage of vitamin C, which is retained in substantial amounts during the four days of fermentation (Dlamini and Dube, 2008). The stability of vitamin C is known to be higher at lower pH of foods.

Since the nutritional content of fermented *marula* juice is important, this study seeks to determine the effects of fermentation temperature and time on the quality aspects of the naturally fermented *marula* juice. This study specifically investigated the retention of vitamin C, total polyphenols and antioxidant activity in fermented *marula* juice. Other quality parameters that were evaluated were the pH, alcohol level, individual sugars and degrees brix in the fermented juices.

2. Materials and methods

2.1. Sample collection

Marula fruits were obtained from the northern part of Namibia with the help of Eudafano Women's Cooperative (EWC) in Ondangwa, Namibia. Ripe *marula* fruits were pressed by a Hydraulic jack 10 ton/ 10.7 bar (designed and made by the project EWC). About 30 litres of marula juice was divided into twelve parts; ten for fermentation (in duplicate) at different temperatures and two for analysing the fresh juice as a reference point. The fresh and the fermented juices were kept frozen at -20 ºC for analysis.

2.2. Sample fermentation

600 ml *marula* juice was poured in sterile 1 litre plastic containers and naturally fermented at temperatures ranging between of 20°C and 40°C for 1 to 8 days. The fermentation scheme can be seen in table 1.

Table1. Fermentation days and temperature of the *marula* samples.

Temperature (°C)	Fermentation time (days)			
20	2	4	6	8
25	2	4	6	8
30	1	2	4	6
35	1	2	3	5
40	1	2	3	4

2.3. Ascorbic acid extraction

The procedure used for extracting L-ascorbic acid (AA) and L-dehydroascorbic acid (DHA) for fermented and unfermented marula fruit juice was a modification of the method described by Hernández, Gloria Lobo and González, (2006). Fermented and unfermented juice (2.5 ml) was transferred into 10 ml tubes. The juice was mixed with 2.5 ml of the extracting solution containing 3% MPA (Metaphosphoric Acid) and 1 mMol/L TBHQ (tert-butylhydroquinone) then the mixture was homogenized with UltraTurrax T20B for 1 min. After homogenization, the mixture was centrifuged (ALC PK131R) for 5 minutes at 2255 x g at 4°C.

The extract was diluted up to six times with distilled water. All extractions were carried out under reduced light and on ice. For the standard, a commercial L-ascorbic acid with the concentration range of 1.56 – 200 µg/ml was made. Subsequently, 2 ml of the standard and extract were filtered through 0.45µm filter and were used for high performance liquid chromatography (HPLC) analysis.

To determine DHA, 2.0µl Dithiothreitol (DTT) was added to the extract and then incubated at 25ºC for 15 minutes in the dark to convert any DHA to AA before HPLC analysis. The DHA content of the sample was calculated by subtracting the initial AA content from the total AA content, after conversion.

2.4. HPLC analysis of ascorbic acid

The method that was used for the determination of L-ascorbic acid (AA) and L- dehydroascorbic acid (DHA) for fermented and unfermented *marula* fruit juice was as described by Hernandez *et al.*, (2006) with modifications. The HPLC system used was a Thermo Separation Products Model with P-2000 Binary Gradient Pump and UV 2000 detector. Separations were carried out on a Varian Polaris C18-A column, 150 x 4.6 mm with 5.5 minutes running time and 20 µl injection volumes using an auto-sampler. The mobile phase employed was a solution of 0.2 % orthophosphoric acid in distilled water. The flow rate of the mobile phase was 1 ml/min. Detection was done by UV/VIS-DAD at a wavelength of 245 nm. The AA peak was identified by comparing its UV-visible spectral characteristics and retention time with the commercial standard of AA.

2.5. Total phenolic content

Total phenolic content of the fruit extract was determined using the Folin-Ciocalteu method as described by Georgé, Brat, Alter and Amoit, (2005) and Swain and Hillis (1959) with few modifications. About 0.25 ml of fermented and unfermented *marula* juice was homogenized with Ultra Turrax T20B for 1 min in 5 ml of methanol/distilled water (1:1 v/v). The homogenate was centrifuged (ALC PK131R) for 5 minutes at 2255 x g

at 4°C and the supernatant collected were diluted to 1:10 with distilled water. A 70% Na_2CO_3 solution was prepared; this solution was stirred at room temperature for 1 hour and then used during the extraction. For the standard, a calibration curve for Gallic acid 1mg/ml and diluted to 1: 32 was used. In a volumetric flask of 25 ml, 5 ml distilled-water, 1 ml of Folin-Ciocalteu reagent, 1 ml of 70% Na_2CO_3 solution and 1 ml of the juice extracts or a Gallic acid standard were added. Then the flask was filled with distilled-water up to 25 ml and mixed thoroughly. After 15 minutes a full development of the blue colour appeared and the absorption was measured with a spectrophotometer (Cary 50, Probe UV visible, Varian) at 725 nm. The total phenolic content was expressed as Gallic acid equivalents (GAE) in mg/ml of *marula* fruit juice.

2.6. Antioxidant activity evaluation: Off line DPPH free Radical-Scavenging assay

The method used was as described by Brand-Williams, Cuveliere and Berset, (1995) with some modification. Fermented and unfermented *marula* juice extracts were each mixed with 50 % aqueous methanol, then the mixture was homogenised with Ultra Turrax T20B for 1 min and centrifuged (ALC PK131R) for 5 minutes at 2255 x g at 4°C. The highest concentration used for the *marula* fermented and unfermented juice was 25 mg/ml of *marula* juice, which was diluted with aqueous methanol solution to achieve the lowest concentration of 6.25 mg/ml of *marula* juice. Each juice extract was tested in triplicate at three concentrations, such that the juice concentration given a 50% fall in absorbance of the DPPH can be calculated. Juice extracts (0.1 ml) was mixed with 3.9 ml of 0.02 mol/L DPPH methanolic solution. The mixture was thoroughly mixed and kept in the dark for 30 min. The absorbance was measured at 515 nm using a spectrophotometer (Cary 50 Probe). For the determination of the scavenging effect on DPPH radicals, the amount of DPPH that did not react with all the dilutions of sample was determined using a DPPH calibration curve. The EC_{50} value was determined graphically by plotting the disappearance of DPPH as a function of the sample concentration. Trolox 1.5 to 0.094 mmol was used as a standard. The EC_{50} values from the curve for Trolox and from the juice extract was used to calculate the EC_{50} expressed in Trolox Equivalent of Antioxidant Activity (TEAC).

2.7. Individual Sugar analysis

The individual sugars in fermented and unfermented *marula* juice were separated by HPLC. The HPLC system used was a Thermo Separation Products model with P-2000 Pump and ELSD-2100 polymer labs detector. Separations were carried out on Alltech prevail carbohydrates column, 250 x 4.6 mm with an evaporator temperature of 80 °C and nebuliser temperature of 60 °C. The running time was 14 minutes with a flow rate 1 ml/min on isocratic 75/25 % acetonitrile/water. In brief, 1ml of samples were mixed with distilled water of about 80 ºC, after that the solution was incubated in 80 °C water bath for 5 min, then homogenised with Ultra Turrax T20B for 1 min and centrifuged (ALC PK131R) for 5 minutes at 2255 x g at 20 °C. Some extracts were diluted up to eighty times with distilled water. For the external standard, sucrose, glucose and fructose with the range 45 – 680 µg/ml were used. Subsequently, about 2 ml of the standard and extract were filtered through a 0.45 µm filter and used for high performance liquid chromatography (HPLC) analysis. The sugar peaks were identified by comparing retention times with those of the external standards of sucrose, glucose, and fructose. All the sugars were quantified by the external standard method.

2.8. Statistical analysis

Results were statistically analyzed using SPSS software analysis of variance (ANOVA) with Tukey test to compare any significant differences between the means. Values were expressed as means ± standard deviations. Differences were considered significant at $P < 0.05$. All the analyses were carried out in triplicates.

3. Results and discussion

3.1. Statistical results

The analysis of variance (ANOVA) showed a significant difference ($p < 0.001$) for glucose, sucrose and fructose in fresh sample and fermented samples of the *marula* juices. However, for ascorbic acid, no significant difference ($p > 0.05$) was found between fresh and 40 ºC, 25 and 20 ºC, and between 30

and 20 ºC. For dehydroascorbic acid, fresh *marula* juice differed significantly ($p<0.05$) from other juices fermented at temperature 20, 25, 30, 35 and 40 ºC. For the total phenolic content, no significant difference ($p > 0.05$) was found between fresh and 30 ºC, fresh and 40 ºC, 20 and 25 ºC, 20 and 30 ºC, 20 and 35 ºC, 25 and 35 ºC, 30 and 35 ºC, 30 and 40 ºC and 35 and 40 ºC. For Trolox Equivalent Antioxidant Capacity (TEAC), there was no significance difference ($p > 0.05$).

Statistical analysis showed that the ascorbic acid in fermented *marula* juice is not affected by the fermentation temperature and time taken to ferment, even though high amount of ascorbic acid can be retained in juices fermented under 30, 35 and 40 ºC. There was no significant difference for the phenolic content and Trolox Equivalent Antioxidant Capacity (TEAC), and this shows the role of the ascorbic acid and other antioxidants being stable during fermentation of *marula* juice. The sugar content was lower in the fermented juice than in the fresh juice, due to conversion of sugars into alcohol.

3.2. Individual sugars and brix

The identified individual sugars that were present in unfermented *marula* juice were sucrose (47 ± 6.7 mg/ml), glucose (4.9 ± 0.8 mg/ml) and fructose (22 ± 3.5 mg/ml), with similar results found by Weinert, van Wyk and Holtzhausen, (1990). During fermentation the disaccharide sucrose was enzymatically broken down by the action of the microbial sucrase into its monomeric sugars fructose and glucose, which were then readily fermented by the yeast naturally present on the fruit into ethanol (Dlamini & Dube, 2008). At all fermentation conditions, the fermented *marula* juice contained very little fructose and almost no sucrose and glucose at the end of fermentation as shown in figure 1 (B-D). Looking at this result, sucrose was already converted into simple sugars before the samples were completely fermented. During fermentation, glucose and fructose was converted into alcohol by the yeast and lactic acid bacteria. The fermented juice was relatively low in sugar. The average brix values of fermented and unfermented marula juice are presented in figure 1A. The brix dropped from 11.8 to 2.6 % during fermentation and that is due to the disappearance of the sugars.

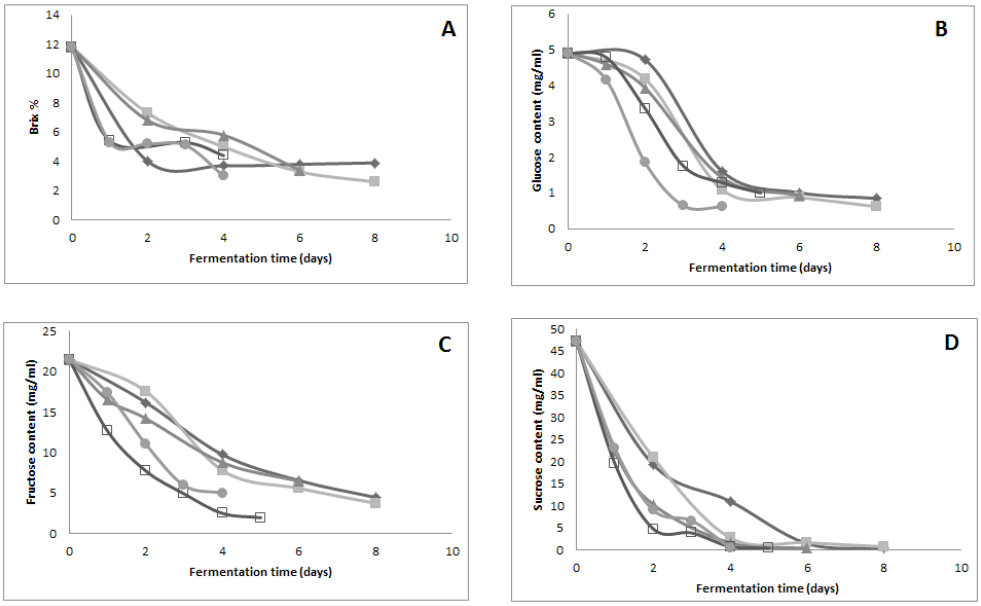


Figure 1. Changes in Brix (A), Glucose (B), Fructose (C) and Sucrose (D) content during *marula* juice fermentation at different temperature: 20ºC (♦), 25 ºC (■), 30 ºC (▲), 35 ºC (□) and 40 ºC (●).

4

3.3. Other parameters measured: pH, alcohol and dry matter content

During fermentation, lactic acid bacteria metabolized the sugars to mainly lactic acid, that caused the initial drop in pH from 4.38 before fermenting to 3.44 at the end of fermentation. The alcohol content of fermented *marula* juice ranged between 0.9% (v/v) for the first days of fermentation and 5.5% (v/v) at the end of fermentation. According to Dlamini and Dube, (2008) for any fruit that is fermented, the alcohol level reached depends on the levels of fermentable sugars in the juice and on the characteristics of the yeast present. The dry matter content dropped considerably during fermentation from 11.8% in fresh juice to 1.8% in fermented juice, mainly caused by the conversion of sugars to alcohol and CO_2.

3.4. Ascorbic and dehydroascorbic acid

The ascorbic acid of the *marula* juice used for fermentation was 172 ± 8 mg/100 ml with DHA of 59 ± 3 mg/100 ml. Figure 2 shows the ascorbic acid and DHA before and at the end of fermentation. Fermentation at the two lowest temperatures at the longest times (20 and 25 °C for 8 days) resulted in the lowest retention of vitamin C (69 ± 5 and 52 ± 6 mg/100 ml) when compared to the retention of (130 ± 12 and 159 ± 15 mg/100ml) for the two highest temperatures with the shortest times (35 and 40 °C for 5 and 4 days, respectively). The intermediate temperature and time of 30 °C for 6 days resulted in an intermediate retention of ascorbic acid (90 ± 6 mg/100 ml). In contrast, DHA was only high in the juice fermented at 30°C (30 ± 5mg/100 ml), followed by 25 and 35 °C with (24 ± 6 and 22 ± 1 mg/100 ml, respectively) while the highest and lowest temperatures 40 and 20 °C, resulted in lower DHA (21 ± 2 and 21 ± 5mg/100 ml). Dlamini and Dube, (2008) stated that the decrease in pH and depletion of oxygen caused by the fermenting organisms during *marula* juice fermentation can contribute to the stability of vitamin C. Furthermore, they stated that the apparent differences in vitamin C stabilities between fermented *marula* juice and orange juice could be due to differences in the intrinsic composition of the two types of juices, which is not accounted for by pH alone.

Looking at these results, it can be concluded that traditional fermented *marula* juice is a good source of ascorbic acid, noting that traditionally *marula* juice is fermented for four days at ambient tropical temperatures of around 30 to 40 °C. The study carried out by Dlamini and Dube, (2008) also indicated that *marula* wine is a good source of ascorbic acid; after four days of fermentation it retained 96 mg/100 g (72 %) of the ascorbic acid content of the starting juice 133 mg/100 g, and this is in agreement with our findings of 159 mg/100 ml (70%) after 4 days of fermentation at 40°C of the starting juice which contained 172 mg/100 ml.

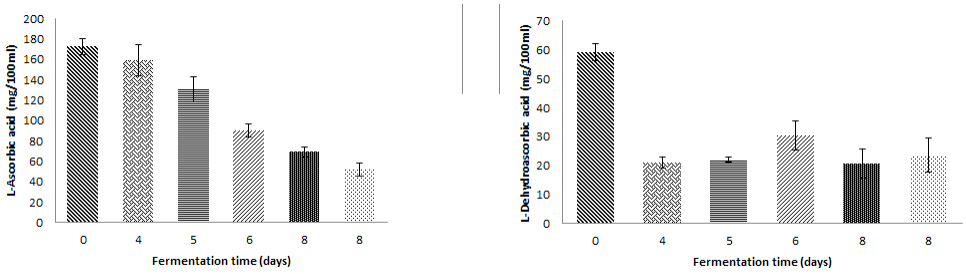
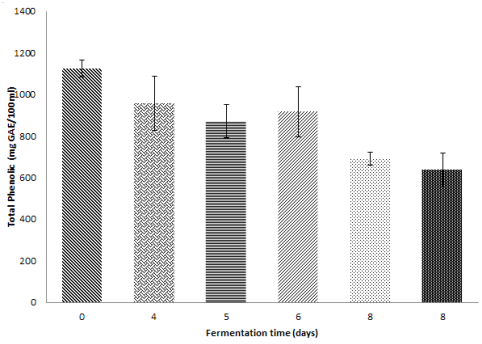


Figure 2. L-Ascorbic acid and L-Dehydroa c acid at the end of fermentation at different temperature: Fresh 40°C 35°C 30°C 20°C

3.5. Total phenolic content

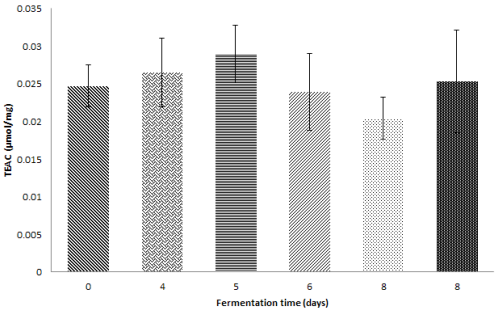
The total phenolic content of unfermented *marula* juice was 1130 ± 40 mg GAE/100 ml as shown in figure 3.This value was higher than the range (506 – 872 mg GAE/100 g) reported by Lamien-Meda *et al.*, (2008) for the whole fruit and almost five times higher than the value of 226 mg/100 g reported by Ndhlala *et al.*, (2007) in the pulp. According to Lamien-Meda *et al.*, (2008), these variations could be explained by the climate and by the extraction solvent used. Pissard *et al.*, (2012) did a study on apple and they also stated that polyphenol content varied greatly depending on variety, which is a possible explanation why the polyphenols in this study differed considerably from those reported in literature.

After fermentation between 20 and 40 ºC for 4 to 8 days, the polyphenol content dropped from 1130 ± 40 mg/100 ml to 630 ± 80, 690 ± 30, 870 ± 80, 920 ± 120 and 960 ± 130 mg/100 ml for 20, 25, 30, 35 and 40 ºC, respectively. The results clearly show that a high polyphenol content can be retained if the juice is fermented under high temperature of 30, 35, and 40 ºC for a maximum of 6, 5 and 4 days. Overall, the phenolic content was high in all the juices, even after fermenting for 8 days. This result shows that phenolic compounds in *marula* juice can be considered as fairly stable during fermentation, irrespective of the fermenting temperature and duration.



3.6. Off line DPPH free Radical-Scavenging activities

The Trolox Equivalent Antioxidant Capacity (TEAC) assay was used for measuring total free radical scavenging capacities of the fresh and fermented *marula* juices. Fresh *marula* juice had a TEAC value of 0.0247 µmol/mg, while after fermentation the TEAC values of the juices ranged from 0.0204 ± 0.0028 to 0.029 ± 0.0038 µmol/mg. The highest activity was measured in the juice fermented for 5 days at 35 ºC. However, there was no clear trend on the effect of temperature on the antioxidant activities in the fermented juice. Unexpectedly, almost all the juice had an increase in the activity after fermentation with an exception for the juice fermented at 25 and 30 ºC, which showed a lower activity than the initial activity, as shown in figure 4. Overall, fermented *marula* juice retained over 80% of its initial antioxidant activities. The decrease in activity could be due to the fact that during fermentation some antioxidant compounds were degraded or transformed while the increase could be polyphenols were attached to the fiber contained in the juice and can only be released during fermentation, or might be due to the increase of gallic acid observed during fermentation.



4. Correlations

The correlation between the contents of total polyphenols, ascorbic acid and the identified individual phenolic compounds and the antioxidant activity was determined. The results show a positive correlation between the antioxidant activity and the total polyphenol content (r^2=0.64) and between the antioxidant activity and the ascorbic acid content (r^2=0.59). Previous studies conducted with wines by Alonso, Dominguez, Guillen and Barroso, (2002) also found a positive correlation between the total phenolic content and the antioxidant activity measured using an electrochemical method and other methods by Brenna and Pagliarini, (2001) and by Sanchez-Moreno, Satue-Gracia and Frankel, (2000).

According to Gil, Tomas-Barberan, Hess-Pierce and Kader, (2002) the fruits showing high antioxidant capacity also contained high amount of phenolics. Furthermore, they stated that antioxidant capacity of fruits such as strawberry, raspberry and other berries showed that vitamin C is not the main antioxidant in these fruits but polyphenols are mainly responsible for the observed activity. Another study carried out by Velioglu, Mazza, Gao and Oomah, (1998) showed a positive correlation between total phenolic content and antioxidative activities in some selected fruits. Tsao, Yang, Xiel, Sockovie and Khanizadeh, (2005) also found out in their study that antioxidant activity of apple was positively correlated with the total polyphenolic concentrations measured by the Folin-Ciocalteu method and the total polyphenolic index obtained by HPLC. In contrast, Pissard

et al., (2012) found no relationship between the three quality parameters of sugar, polyphenol and vitamin C contents. According to Ainsworth and Gillespie, (2007) the Folin-Ciocalteu assay eliminates approximately 85% of ascorbic acid and other potentially interfering compounds although the electron transfer reaction is not specific for phenolic compounds. Based on that the high polyphenol obtained in this paper could not be due to the interference of ascorbic acid but rather due to the nutritional status of the plant or the environmental conditions and the maturity of the fruits.

For a better understanding, further study on evaluation of *marula* antioxidant capacity and investigation on their correlations will be required since many methods can be used to determine this activity.

5. Conclusion

With this finding fermenting, *marula* juice is a good source of antioxidants and their activities were positively correlated to vitamin C and phenolics. Fermenting at lower temperatures of 20 and 25 °C resulted in a lower retention of antioxidant activities, ascorbic acid and phenolic content and vice versa for high temperature 30 to 40 °C. Processors of *marula* fermented juice should note that fermenting *marula* juice under temperature ranging between 30 and 40 °C for 4 to 6 days will end up retain high antioxidant activity which is positively correlated to its vitamin C and phenolic content. For that reason they should ferment at temperature ranging between 30 and 40 °C for 4 to 6 days to produce an alcoholic product high in antioxidant. Therefore, *marula* juice can be used as a good source of natural antioxidants. Further investigations will be important to identify unknown phenolic compounds in *marula* juice and to study their health effects, bioavailability and metabolism in vivo.

6. Acknowledgment

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Chapter 5

Kinetics of thermal degradation of vitamin C in marula fruit (*Sclerocarya birrea subsp. Caffra*) as compared to other selected tropical fruits.

Hiwilepo-van Hal, P., Bosschaart, C., van Twisk, C., Verkerk, R. & Dekker, M. (2012).

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Abstract

The kinetics of the thermal degradation of vitamin C of *marula*, mango and guava pulp at different heat treatments ranging from 80 to 150°C was investigated. For temperatures lower than 125ºC, the ascorbic acid in *marula* pulp was about 15 fold more stable to heat than the ascorbic acid in mango and guava pulp. The results showed that a simple first order degradation model could not describe the vitamin C degradation as biphasic behaviour was observed. Therefore, the model was transformed in a two-fraction model in which the vitamin C content was divided into relatively stable and instable fractions. *Marula* had a low $k_{d1,100°C}$ of $7.2*10^{-3}$ min^{-1} compared to $k_{d1,100°C}$ of $1.2*10^{-1}$ min^{-1} for guava and $1.3*10^{-1}$ min^{-1} for mango. Guava had the highest activation energy, E_a of 58 kJ/mol, followed by mango with 39 kJ/mol and last *marula* with 29 kJ/mol.

Keywords: *Marula*, Vitamin C, Degradation, Thermal treatment, Guava and Mango.

1. Introduction

Marula (*Sclerocarya birrea ssp. caffra*) tree grows in the savannah regions of sub-Saharan Africa. The *marula* fruit is the size of a small plum with pale-yellow colour. The fruits are highly aromatic and can be eaten fresh or used in making juices, jams and alcoholic beverages, like Amarula (Hillman, Mizrahi, & Beit-Yannai, 2008). Guava (*Psidium guajava)* is common in tropical and subtropical countries where it is important food and medicinal plant (Gutiérrez, Mitchell, & Solis, 2008). Mango fruit (*Mangifera indica* L.) is a common commercial fruits, growing in the tropics and used to make products such as jam, chutney, juices and concentrates (Iagher, Reicher, & Ganter, 2002).

Marula fruits were found to have a vitamin C content of more than 4 times that of oranges (Hillman *et al.*, 2008; Eromosele, Eromosele, & Kuzhkuzha, 1991; Borochov-neori *et al.*, 2008). *Marula* juice was reported to have a vitamin C content range of 67–403mg/100g fresh weight (Carr, 1957; Eromosele *et al.*, 1991; Hillman *et al.*, 2008), guava juice contains 72–300mg/100g (Luximon-Ramma, Bahorun, & Crozier, 2003; Uddin, Hawlader & Ding, 2002) and mango juice contains 37–74mg/100g (Luximon-Ramma *et al.*, 2003; Hernández, Lobo & González, 2006).

Degradation of vitamin C has been reported in many fruit products as a result of processing or storage, and has been considered one of the major causes of quality deterioration during processing and storage of food products (Yuan & Chen, 1998). Vitamin C degradation was observed in an aseptically packaged orange drink with 10% orange juice which lost 40% of vitamin C after 6 months at storage and lost up to 75% at storage temperatures of 22 to 30°C (Luque-Perez, Rios & Valcarcel, 2000). Heat treatment or pasteurisation has a significant effect on loss of vitamin C. However, in *marula* jam, it was found that vitamin C content after pasteurisation was as high as 84% of the original content (Hillman *et al.*, 2008) indicating its relative stability.

When vitamin C is retained during processing and storage, this implies that

conditions have been relatively mild, so other nutrients would also be retained. Therefore, vitamin C is often used as an indicator compound to study the effect of processing and storage on food quality in a broader sense. The aim of this study is therefore to compare the kinetics of the thermal degradation of vitamin C of *marula*, mango and guava pulp after different heat treatments at temperatures ranging from 80 to 150 °C.

2. Materials and Methods

2.1. Sample collection

Marula fruits were obtained from the northern part of Namibia with the help of Eudafano Women's Cooperative (EWC) in Ondangwa, Namibia. Guava was obtained from Fruit and Veg City in Windhoek, Namibia. Mango was obtained from Dutch retailer Albert Heijn, Wageningen, The Netherlands.

2.2. Sample preparation

The fruits were peeled and the edible part cut into small pieces, frozen in liquid nitrogen and blended with Waring commercial blender (model HGB 2WTS3). Part of the pulp was used for unheated analysis while the other part was stored at -20 ºC before heating for experimentation.

2.3. Heat treatments

Preliminary results by Donkelaar, (2009) showed that *marula* pulp needed more heating time in comparison to mango and guava pulp. The heating scheme for *marula* was therefore different from that of guava and mango as shown in Table 1. Frozen fruit pulp (7 grams) was placed in stainless steel heating tubes with screw caps. A thermocouple was placed in 3 tubes through the cap to monitor the temperature inside the tubes during heating. Then the tubes were heated in a heating block (Liebisch 33649, Bielefeld, Germany). Time taken to reach the required heating temperature (2-3 min) was excluded from the kinetic parameter analysis. After heating, the samples were cooled on ice and analysed.

Table 1: Heating times and temperatures of the marula, guava and mango samples.

Fruit	Marula				Mango and Guava			
Heating Temperature (°C)	80	100	125	150	80	100	125	150
Heating time (min)	120	60	10	3	30	15	5	1.5
	300	120	30	6	60	30	10	3
	480	240	60	10	120	60	20	6
	1440	480	120	15	240	120	40	12
			240	30				

2.4. Ascorbic acid extraction

This procedure is a modification of the method described by Hernandez *et al.*, (2006). Heated and unheated frozen fruit pulp (0.25g) was transferred into 10ml tubes. In case of mango the sample size was 2g, due to the relatively low vitamin C content. Fruit pulp was mixed with 3.5ml of the extracting solution containing 3% MPA (Metaphosphoric Acid) and 0.001Mol/L TBHQ (tert - butylhydroquinone) then the mixture was homogenized. After homogenization, the mixture was centrifuged (ALC PK131R) for 5 minutes at 2255xg at 4ºC. All extractions were carried out under reduced light. For the standard, a commercial L-Ascorbic acid with the range 1.56µg/ml - 200µg/ml was made. Subsequently about 2 ml of the standard and extract were filtered through 0.45µm filter and used for high performance liquid chromatography (HPLC) analysis.

2.5. HPLC analysis

The method that was used for the determination of L-ascorbic acid (AA) for heated and unheated fruit pulp was as described by Hernandez *et al.*, (2006) with modifications. The specifications for the HPLC system were a thermo separation products model with P-2000 Binary Gradient Pump and UV 2000 detector. Separations were carried out on a Varian Polaris C18-A column, 150 x 4.6 mm with 5.5 minutes running time and 20 µl injection volume. The mobile phase employed was a mixture of (Orthophosphoric Acid 0.2% in distilled water). The flow rate of the mobile phase was 1ml/min with a UV-detector at a wavelength of 245 nm.

2.6. Kinetic modelling and statistics

To analyse the kinetics of the breakdown of vitamin C, two different reactions models (simple first order and two-fraction first order), combined with the Arrhenius equation, were compared. The models were compared by the PPAIC (Posterior Probability and the Akaike Information Criterium) method (Akaike, 1974). The results showed that a simple first order degradation model could not describe the vitamin C degradation, as biphasic behaviour was observed. Therefore, the model was adapted to a two-fraction first order model in which the vitamin C content was divided in a relatively stable and unstable fraction which together made the total concentration (Equations 1-3).

$$c_s = c_t \cdot SF \qquad \textbf{Equation 1}$$

$$c_u = c_t \cdot (1 - SF) \qquad \textbf{Equation 2}$$

$$c_t = c_s + c_u \qquad \textbf{Equation 3}$$

With:

C_s = 'stable' concentration (mg/g)
C_u = 'unstable' concentration (mg/g)
C_t = total concentration (mg/g)
SF = 'stable' fraction no unit (-)

Equation 4 and 5 show the first order kinetic degradation with two different rate constants for the stable and unstable concentrations.

$$\frac{dC_S}{d_t} = -k_d \cdot C_s \qquad \textbf{Equation 4}$$

$$\frac{dC_u}{d_t} = -k_d \cdot C_u \qquad \textbf{Equation 5}$$

The temperature dependency of the first order rate constant, k_d, is described by the Arrhenius equation (Oerlemans, Barrett, Suades, Verkerk, & Dekker, 2004). Equations 6 and 7 show the Arrhenius equations with two different activation energies for the two reactions (stable and unstable fractions).

$$k_{ds} = k_{ds,ref} \exp\left\{\left(\frac{E_{as}}{R}\right)\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right\} \quad \textbf{Equation 6}$$

$$k_{du} = k_{du,ref} \exp\left\{\left(\frac{E_{au}}{R}\right)\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right\} \quad \textbf{Equation 7}$$

wIn these equations the parameters are:

k_d = Degradation rate constant of stable ($_s$) or unstable ($_u$) fraction (min^{-1})

R = Gas constant (J/mol) = 8.314 $J.mol^{-1}.k^{-1}$

E_a = Activation energy (J/mol)

T = Temperature in Kelvin

$_{ref}$ = refers to reference temperature (set to 100 °C = 373 K)

Reaction kinetics modelling and parameter estimations were done by global fitting of the data sets using the determinant criterion (Stewart, Caracotsios, & Sorensen, 1992] as quoted by Oerlemans *et al.*, (2006). Global fitting implies that the data sets from different incubation temperatures and times for each compound were fitted simultaneously to the degradation model to obtain the degradation parameters. The software package Athena Visual Workbench (www.athenavisual.com) was used for the numerical integration of differential equations and for estimation of the rate constants in the differential equations following minimization of the determinant in order to obtain the reaction kinetic parameters with their confidence interval (rate constant $k_{d,100°C}$ and activation energy E_a).

3. Results and discussion

3.1. Thermal degradation of Ascorbic acid and modeling

The degradation of ascorbic acid is shown below in Figure1. The results showed that ascorbic acid content decreased with increasing temperature treatments; this confirms what previous studies found (Van den Broeck, Ludikhuyze, Weemaes, Loey & Hendricks, 1998). The thermal degradation of ascorbic acid is usually described in literature by a first order model. However, the figures of the degradation of ascorbic acid in this study clearly showed that a first order model could not fit this degradation process. For each temperature setting it was clear that a certain fraction of the ascorbic acid was degrading with a high rate and the remaining fraction was degrading with a lower rate. According to this observation, a model was made, combining the two fractions of the vitamin C degradation.

An explanation for this behaviour could be due to effect of the limited amount of oxygen present in the fruit samples or in the headspace of the heating tubes during heat treatment. By a fast aerobic degradation of ascorbic acid, oxygen will be consumed and the remaining ascorbic acid will subsequently be degraded by an anaerobic degradation pathway with a lower reaction rate. Based on the different initial concentration of ascorbic acid in *marula* compared to the other fruits, it could be expected that a relative smaller part of the total ascorbic acid pool would react with oxygen in *marula*. On the other hand the relative stable fraction (SF) of ascorbic acid was similar for all fruits. Further research is needed to investigate this effect in more details. Another reason for this behaviour could be that part of the ascorbic acid is more stable due to complex formation with other compounds in the fruit matrix. The pH for *marula*, guava and mango were 3.6, 3.9 and 3.4 respectively. The pH did not change as a result of heat treatment.

The degradation kinetic parameters were calculated and displayed in table 2. The reference temperature used was 100ºC (373 K) to calculate the degradation reaction rate constant (k_d) and the activation energy (E_a).

Table 2: Kinetic degradation parameters of ascorbic acid in marula, guava and mango.

		Marula	Guava	Mango
$k_{d1,100}$	(min^{-1})	$7.2*10^{-3} \pm 2.1*10^{-3}$	$1.2*10^{-1} \pm 0.3*10^{-1}$	$1.3*10^{-1} \pm 0.5*10^{-1}$
$k_{d2,100}$	(min^{-1})	[a] $7.9*10^{-4} \pm 5.6*10^{-4}$	[b] $5.3*10^{-5} \pm 1.8*10^{-4}$	NRE
$E_{a,d1}$	(kJ/mol)	29 ± 16	58 ± 10	39 ± 14
$E_{a,d2}$	(kJ/mol)	[a] 119 ± 26	[b] 190 ± 89	NRE
SF	(-)	0.54 ± 0.12	0.51 ± 0.04	0.53 ± 0.04

[a] =correlation coefficient of 0.885 between $E_{a,d2}$ and $k_{d2,100}$; [b]= correlation coefficient of 0.996 between $E_{a,d2}$ and $k_{d2,100}$; NRE: no reliable estimate obtained.

This analysis shows that the degradation rates of ascorbic acid in *marula* are much lower compared to guava and mango. The stable fraction (SF) of ascorbic acid is almost identical for the three fruits. Guava had the highest activation energy, so the ascorbic acid degradation rate showed the strongest dependence on temperature.

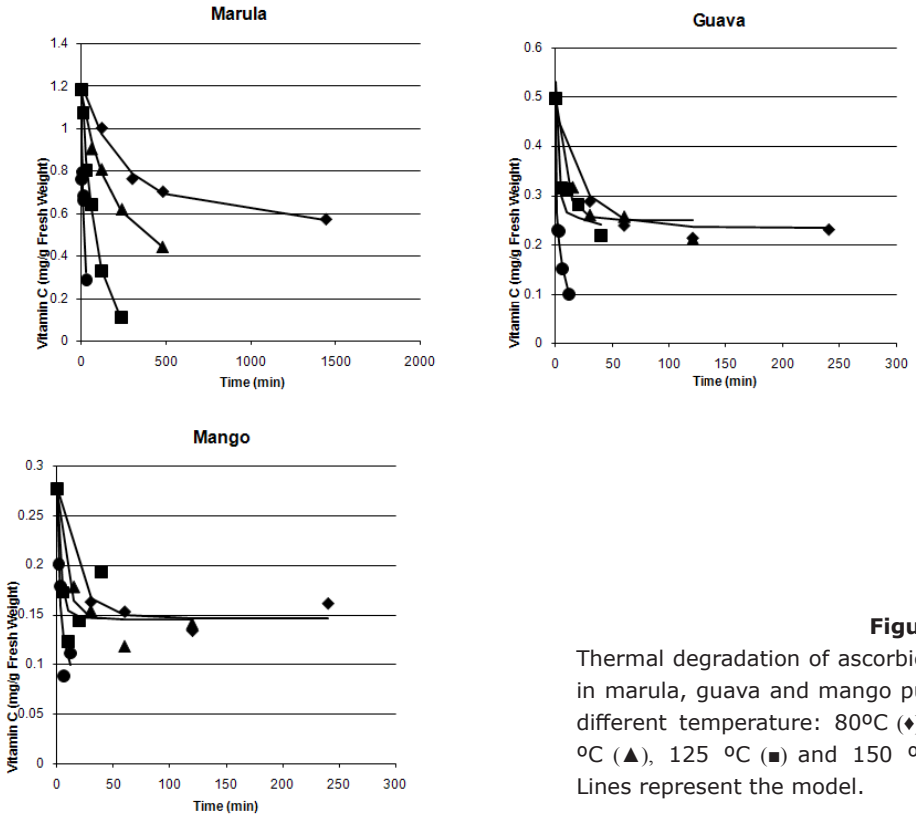

Figure 1: Thermal degradation of ascorbic acid in marula, guava and mango pulp at different temperature: 80ºC (♦), 100 ºC (▲), 125 ºC (■) and 150 ºC (●). Lines represent the model.

4. Conclusion

Marula fruit pulp is a rich source of ascorbic acid and it is 15 times more stable to heat as compared to mango and guava pulp. Simple first order degradation model could not describe the vitamin C degradation as biphasic behaviour was observed. Therefore, the model was adapted to a two-fraction model in which the ascorbic acid content was divided in a relatively stable and unstable fraction.

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Chapter 6

Extraction and characterization of volatile compounds of the peel and flesh of marula fruit (*Sclerocarya birrea subsp. Caffra*)

Hiwilepo-van Hal, P., Li, G.,
Verkerk, R., & Dekker, M.

To be submitted for publication

Abstract

Volatile compounds of the flesh and peel of *marula* fruit collected in Namibia were investigated. In addition, the changes in volatile compounds for different heating and storage conditions were characterized. Headspace-solid phase micro extraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) were used for the analysis. In total, 75 volatile compounds were identified in *Marula* peel and 41 in flesh. Sesquiterpene compounds dominated the volatile fraction in flesh and peel with β-caryophyllene, α-humulene, (E)-germacrene D and β-selinene being the most abundant constituents. *Marula* peel contained more volatile compounds including all the identified volatiles of the flesh. Heating at 110°C for longer than 10 min had an effect on *marula* volatile compounds. New compounds, such as oxygenated terpene, were found while esters and ketones disappeared during heating. Storage at 4°C over 30 days had no major influence on volatiles of *marula* flesh and peel, except that sesquiterpene and alcohols compounds gradually increased during 30 days storage.

Keywords: *marula* volatiles, *Sclerocarya birrea subsp. Caffra*, GC-MS, SPME, sesquiterpenes

1. Introduction

Marula (*Sclerocarya birrea subsp. Caffra*) is a tropical tree that mainly grows in Southern Africa south of the Zambezi river (Nerd and Mizrahi, 1993) and it contributes a lot to local communities because of its high nutritional and commercial values (Mokgolodi, Ding, Setshogo, Ma and Liu, 2011). The trees bear round shape fruits with thick plain skin and juicy sour-sweet flesh (Nerd and Mizrahi, 1993). Although different parts of the tree, such as stem-barks, leaves and roots have different ethnomedical and commercial uses because of their own characteristics, the fruit is considered as the major product (Ojewole, Mawoza, Chiwororo and Owira, 2010). With the development of local society and economics, rather than being eaten directly, *marula* fruit is more used nowadays in food industries for juice production, beer brewing and jelly making (Bille & Steppich, 2003). *Marula* fruits are claimed to contain high concentrations of nutrients (Hiwilepo-van Hal, Bille, Dekker and van Boekel, 2013). Vitamin C content, for example, was reported to be much higher in *marula* pulp than in lemon, orange, guava and mango (Ojewole *et al.*, 2010; Hiwilepo-van Hal, Bosschaart, van Twisk, Verkerk and Dekker, 2012).

Numerous evaluation criteria could be considered important for the quality of the fruit and products derived thereof. Of these, flavour and odour are no doubt important ones because of the significant influence they have on consumers' choices as well as other marketing facts such as price and availability.

In a recent research, a comparison of *marula* fruit volatile compounds of pulp and intact fruit was done by Viljoen, Kamatou and Baser, (2008). A novel extraction for *marula* using HS-SPME (headspace solid phase micro-extraction) was employed in this research instead of the traditional liquid-liquid extraction method used by Pretorius, Rohwer, Rapp, Holtzhausen and Mandery, (1985). The results showed that, compared to pulp samples, volatile profiles of intact fruits were more complex. This may indicate that more volatile compounds occur in the *marula* skin than in the flesh. In other words, *marula* skin could play an important role in determining/enhancing organoleptic properties of *marula* products rather than being just wasted after processing steps.

Aroma as main characteristic of fresh fruits is a complex combination of several volatile compounds. It frequently contains terpenes but also other compounds such as esters, aldehydes and alcohols that contribute to the aroma of *marula* fruits. Since the odour quality of *marula* perceived by people is described as similar to that of grapefruit, odour active compounds in common, might be found in both kinds of fruits. The odour active compounds detected by gas chromatography in grapefruits included esters, aldehydes, alcohols, ketones as well as monoterpene d-limonene (Verlet, 1993). However, it is worth noticing that compounds considered as major contributors for grapefruits aroma, are p-1-menthene-8-thiol, ethyl butanoates and nootkatone (Buettner & Schieberle, 1999; Moshonas & Shaw, 1971) were not found in *marula* fruit, pulp and juice according to Viljoen *et al.*, (2008). Besides grapefruit, *marula* juice odour is also linked to that of pineapples or mangos. According to Dube, Dlamini and Sibanda, (2011) this could be due to the presence of compounds like ethyl acetate, benzaldehyde and linalool that are considered as pleasant aroma compounds in both type of fruits.

From a chemical standpoint, it is known that the aroma compound mixture of a certain kind of food usually has a very complex composition. Different results can be obtained by using different extraction methods. Two related studies on *marula* volatiles, for example, showed some differences. Monoterpene compounds were found by Pretourius *et al.*, (1985) when using a solvent extraction method, whereas they were absent in both the intact fruit and the pulp after SPME extraction according to Viljoen *et al.*, (2008).

Thermal treatment is frequently applied in the food industry as an important processing step for inhibiting spoilage caused by microorganisms and enzymes in order to increase shelf life. Although it is considered a necessary step for food processing, heating could affect the overall aroma profile and sometimes not in a positive way. Certain aroma compounds may be lost while other compounds causing off-flavours may be formed by derivation or by interaction of amino acid and reducing sugar during heat processing, which is undesirable from a consumers' point of view. For

example, aroma changes of thermally processed cupuacu pulp, the aroma compounds of which contribute to the unique odour of the fruit, were lost while compounds causing off-flavour were generated (Prosen & Zupančič-Kralj, 1999). Because of that, research on heating effects on food aroma quality is necessary for identifying and minimizing significant undesired aroma alterations.

After heat treatment, the volatile compounds profile of food products could be further altered by different storage temperature and time conditions. Under certain storage conditions, compounds causing off-flavour might increase or decrease and reduce the sensory quality of stored food. For example, in pasteurized guava puree stored at frozen temperature, development of off-flavour could still be observed, which indicated that even low temperature storage is not a guarantee for guava fruit quality (Augusto, Valente, Dos Santos Tada and Rivellino, 2000). On the other hand, results obtained from similar research on volatile profile changes of raspberry during long time frozen storage, did not show significant differences when compared to fresh fruits (Silva , Sims, balaban, Silva and O'Keefe, 2000). Considering the limited information about the volatile profile of *marula* fruits, including peel and the possible changes under varied heating and storage conditions. This study was aimed at characterization of *marula* volatile compounds and also investigating the changes under different heating and storage conditions.

2. Material and methods

2.1. Sample collection

Marula fruits were collected from trees grown in the northern part of Namibia. With help of Eudafano Women's Cooperative (EWC) in Ondangwa, ripe fruits were gathered in rural areas and brought to the factory Eudafano women cooperative centre (EWC). After that, they were selected and sorted by hand whereby damaged fruits were discarded while green fruits were held back for ripening. Selected ripe fruits that were considered to

be at similar ripening stage were transported to Wageningen University under frozen condition and stored at -20°C until experimentation, which took an average of about 4 weeks.

2.2. Sample preparation for aroma characterization

Marula fruits collected and stored at -20°C were used. The fruits were separated into skin and flesh parts by peeling with a sharp knife. The inside kernel was discarded. Liquid nitrogen was used immediately to freeze the separated skin/flesh parts. The frozen skin/flesh parts were made into homogenized powders by using a stainless-steel Waring blender. Freshly prepared samples of homogenized powder were passed into sealed glass vials and stored in labelled jars at -20 °C waiting for later GC-MS analysis (waiting time 0 to 14 days). Because the samples were kept frozen and no additional chemicals were added into the analysed samples, the released volatile compounds of prepared samples were assumed to be as those from the usual consumed fresh fruit, although enzymatic changes could have occurred.

2.3. Sample prepared for processing effects

2.3.1. Thermal treatment

Stored samples (flesh/skin powder) were taken out from the freezer and 2 g of each was passed into heating vials for further heating by using heating blocks. The chosen temperatures and times for thermal process are shown in Table 1. Preliminary results showed that high temperatures inside a glass tube were difficult to achieve. Therefore, glass tubes were only used for samples heated at lower temperature, from 40 to 85 °C. For temperature of 110 °C, metal tubes were used instead (Hiwilepo-van Hal *et al.*, 2012).

Table 1: Temperature and time combinations used in this study.

Temperature/°C	Heating time/min			
40	5	15	30	60
85	3	5	10	20
110	1	3	5	10

2.3.2. Storage effects

Samples were heated first at 110°C for 15 min to inactivate bacteria nd enzymes and to prevent spoilage during storage. After that, processed samples were stored at 4°C for three different time periods, 10, 20 and 30 days. Labelled samples after fixed storage time were taken out and stored at -20 °C until later analysis.

2.4. Preliminary experiment for compounds extraction optimization

To optimize the SPME extraction conditions, PDMS and CAR-PDMS fibres were first compared for their performance. Various conditions of influencing factors such as desorption time (3, 5 and 10 min), pre-incubation time (1, 5 and 10 min), extraction time (4, 10 and 20 min) and temperature (40, 60 and 80 °C) were used for extraction condition optimization.

2.5. Volatile compounds analysis

Samples of 1 g of *marula* flesh or skin were placed into a sealed gas chromatography (GC) glass vial (1.15 X 3.15 cm). Headspace solid phase micro-extraction (HS-SPME) was employed in this experiment for *marula* volatile compounds characterization. A TRACE GC ultra-gas chromatography coupled with a DSQ II (Thermo, USA) Mass spectrometer (MS) was used to analyse and determine fruit volatile compound compositions. The GC was equipped with a stabilwax column (30 m length x 0.32mm I.D, 0.25 µm film thickness). Helium was used as carrier gas at a constant rate of 1.2 ml/min. MS were obtained on 70ev. Mass range m/z was from 35-225. Oven temperature for experiments on *marula* volatile characterization was gradually increased from initial temperature of 40 to 240 °C at a rate of 10°C/min, then when it reached 240 °C, it was held for another 1 min. To optimise the extraction method, a restriction coil (2m length x 0.10mm I.D) was attached to the column. A blank vial was analysed in each run for background subtraction to eliminate possible effects caused by air. Each sample was measured in duplicate.

2.6. Identification and quantification of volatile profiles

The data was analysed by using Xcalibur and AMDIS Software Thermo Fisher Scientific, Takkebijsters, Breda, The Netherlands. Compounds were identified mainly according to the NIST library with a match over 70%. Mass spectra data obtained from literature were used for further confirmation. Considering the large number and the complexity of volatile compounds as well as the lack of available GC standards, compounds were roughly quantified by peak area calculation. The quantitative GC correction factors for each compound were assumed to be the same.

3. Results and discussion

3.1. Preliminary experiment for compounds extraction optimization

The preliminary results for the optimization of the extraction methods showed that, more volatile compounds could be extracted and identified while using CAR-PDMS compared to the PDMS fibre. Optimal conditions were: 10 min desorption time, 1 min pre-incubation time, 60°C extraction temperature and 4 minutes extraction time.

3.2. Volatile compounds profiling

Comparing the separate analysis results of peel and flesh, revealed that, under the same analysing condition, more compounds and higher peak intensity could be observed in the peel, which indicated that higher concentrations of the same volatile compounds were present in peel compared to flesh. Clear differences between the volatile composition of *marula* fruit flesh and peel were found. More different flavour compounds were detected in the headspace of the peel (75 in total), which was 34 more than what was found in the flesh sample (41 in total). All 41 compounds present in the flesh were also present in the peel. Detailed information can be found in Table 2.

Table 2: Flavor compounds identified in fresh peel and flesh, RT= retention time, * = Compound present in peel but not in flesh.

Peak No.	RT (min)	Compounds in peel and flesh	Odour quality
1.	2.24	acetaldehyde	fruity, pungent [a]
2.	4.24	methyl acetate	sweet, fruity [k]
3.	5.84	ethyl acetate	green, fruity [e]
4.	6.53	*2-methyl butyraldehyde	musty [p]
5.	7.22	*ethanol	alcoholic [k]
6.	9.53	*1-penten-3-one	peppery, garlic
7.	10.48	*ethyl isovalerate	fruity, pineapple-like [h]
8.	10.92	*hexanal	green, fruity, grass [k]
9.	11.96	*dihydro-3-methyl- 2(3H)-furanone	-
10.	12.28	*propanoic acid, anhydride	-
11.	12.81	*2,3,5-trimethyl- heptane	-
12.	12.93	*isobutyl isovalerate	sweet, fruity [l]
13.	12.98	*heptanal	green, herbal [d]
14.	13.16	isoamyl alcohol	alcoholic, fruity, banana-like [l]
15.	13.26	*d-limonene	citrus, orange-like [g]
16.	13.91	amyl alcohol	sweet, balsam [h]
17.	14.07	*3-carene	sweet, citrus [b]
18.	14.27	*1-butanol, 3-methyl-, carbonate (2:1)	-
19.	14.47	I*soamyl 2-methyl butyrate ester	sweet, fruity [l]
20.	14.57	*1-pentanone,1-(4-methylphenyl)	-
21.	14.72	isoamyl isovalerate	sweet, fruity [e]
22.	14.87	acetoin	sweet ,fatty [k]
23.	15.15	*2-Pentyn-4-one	-
24.	15.56	*1-hexanol	green, fruity [g]

25.	15.94	*3-isopentenyl isovalerate	-
26.	16.11	*3-hexen-1-ol,formate,(z)	sweet, green [o]
27.	16.45	nonanal	waxy, citrus [c]
28.	16.57	*butyl hexanoate	fruity, pineapple [k]
29.	16.60	*hexyl butyrate	green, fruity [k]
30.	16.76	*hexyl 2-methyl butyrate	green, fruity, spicy [k]
31.	17.40	α-cubebene	Herbal, waxy [g]
32.	17.55	δ-elemene	woody [g]
33.	17.63	*n-valeric acid cis-3-hexenyl ester	-
34.	17.87	ylangene	herbal [i]
35.	17.92	*decanal	sweet, soapy [c]
36.	18.01	copaene	woody, spice [j]
37.	18.41	β-bourbonene	herbal woody [g]
38.	18.44	octyl formate	fruity, orange-like, waxy [a]
39.	18.58	β-cubebene	citrus, fruity [g]
40.	18.65	benzaldehyde	sweet, sharp [g]
41.	18.84	(Z)-3-octen-1-ol	fruity, melon-like [a]
42.	19.09	*1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)cyclohexane	-
43.	19.18	*asesquiterpene hydrocarbon	
44.	19.25	β-elemene	fresh herbal [g]
45.	19.43	(+)-epi-bicyclosesquiphellandrene	-
46.	19.52	β -caryophyllene	sweet, woody [g]
47.	19.77	α-gurjunene	woody [a]
48.	19.83	ç -elemene	-
49.	19.98	*n-cubebene	citrus [g]
50.	20.16	aromadendrene	-
51.	20.21	δ-cadinene	herbal, woody [j]

52.	20.46	α-humulene	woody [g]
53.	20.57	α-muurolene	woody [g]
54.	20.65	*γ-selinene	-
55.	20.74	*δ-selinene	-
56.	20.81	epizonarene	-
57.	20.92	(E)-germacrene D	woody, spicy [g]
58.	21.11	β-selinene	herbal [a]
59.	21.22	γ-elemene	-
60.	21.34	β-cadinene	green, woody [a]
61.	21.47	(R)-γ –cadinene	herbal, woody [a]
62.	21.62	(-)-α-Panasinsen	-
63.	21.74	valencene	sweet, citrus [k]
64.	21.83	α-cadinene	woody [a]
65.	22.32	calamenene	herbal, spice [a]
66.	22.41	*elemene	-
67.	22.59	benzyl Alcohol	floral [h]
68.	23.31	n-calacorene	woody [a]
69.	23.57	*Z-4-dodecenol	oily [k]
70.	23.77	*calacorene	woody [a]
71.	24.12	*10-undecyn-1-ol	-
72.	24.21	caryophyllene oxide	sweet, woody [g]
73.	25.76	bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene	-
74.	25.94	*T-muurolol	woody, spice [k]
75.	26.44	n-cadinol	herbal [k]

a. (Pino *et al.*, 2001); b. (Boatright & Lei, 1999); c. (Aparicio *et al.*, 2000); d. (Carrapiso *et al.*, 2002); e. (Ong *et al.*, 2008); f. (Vermeulen & Collin, 2006); g. (Choi, 2003); h. (Peinado *et al.*, 2004); i. (Eyres *et al.*, 2005); j. (Minh Tu *et al.*, 2002); k. (Bauer *et al.*, 2001); l. (Aznar *et al.*, 2001); m. (Weenen *et al.*, 1996); n. (Milo & Grosch, 1995); o.(Vermeulen & Collin, 2006); p. (Farah *et al.*, 2006).

The analysis revealed that sesquiterpene hydrocarbons were the major volatile compounds in peel and flesh. Among the 75 detected flavour components in the peel sample, 39 were identified as sesquiterpene hydrocarbons. They constituted over 98% of the *marula* peel volatiles and over 89% of the flesh volatiles according to a rough calculation based on total peak area.

Several esters as well as aldehyde compounds were detected in flesh and peel: isoamyl isovalerate, isobutyl isovalerate, isoamyl 2-methyl butyrate, 2-propenoic acid, 2-methyl-, 2-propenyl ester, (Z)-3-hexen-1-yl valerate and ethyl lactate are ester compounds listed in Table 2. Most of them were described as having sweet fruity aroma qualities except description of green (unripe) fruity for (Z)-3-hexen-1-yl valerate (Ong *et al.*, 2008). From those identified ester compounds, only isoamyl isovalerate and ethyl lactate were present in both peel and flesh samples but more were found in peel. Among all peel and flesh samples, the compounds β-caryophyllene, α-humulene, E-germacrene D and β-selinene were the most abundant compounds in the volatile fraction. It is the first time that β-selinene is identified as a volatile in *marula.* It is a compound reported as an important constituent in aroma profile of essential plant oils (Fazzalari, 1978). B-caryophyllene as well as α-humulene were found to be the most abundant volatile compounds in the *marula* flavour profile. They both have sweet fruity odour characteristics but odour strengths of these two compounds were not strong (Tam, Yang, Zhang, Guan and Li, 2007). Their published threshold values were relatively high, even higher than limonene, which was described before (64 ppb in water) (Tam *et al.*, 2007). However, considering their large contents, the contribution of these two compounds to *marula* flavour can probably not be neglected. In research on volatile components of guava, another tropical fruit, these two compounds were, besides β-selinene, mentioned as having clear guava flavour in odour assessment (Macleod & Gonzalez de Troconis, 1982). This might explain the "pleasant, sour sweet, guava-like" flavour description often stated in relation to *marula* flavor.

Dodecane, a long chain alkane with undesired petrolic odour, was present

in the *marula* volatile profile. This odour compound is not usually reported as a component found in fruit products. But in the study of volatile constituents of fresh lulo (*Solanum uestissimum* D.) fruits, which grow in north-western South America, its occurrence was reported (Suarez & Duque, 1991).

The typical Namibia *marula* derived product is *omaongo*, it is a fermented *marula* drink, which is widely welcomed by local people since ancient times. The traditional way of making *omaongo* is to first use a sharp cow horn to pierce the peel and to squeeze juice out for later fermentation. The nut is squeezed out in another container for later use, as is dried flesh. Only peel would be discarded (den Adel, 2002). However, since the peel is now found to be rich in aroma volatiles, it is suggested to involve skin into the fermentation procedure rather than throwing it away. This opens up possibilities to use *marula* peel in the processing of *marula*-based products because of its pleasant aroma characteristics. A related study was done on wine making via fermentation of pineapple peel and the developed wine was reported to be acceptable wine and richer in flavour compared to the one without the addition of the peel (Graham, Majeed, Wilson, Wickham and Lynda, 2004). Considering their flavour similarity described by panellists (du Plessis, 2002), it might also be possible for the development of *marula* peel-based wine.

3.3. Heating and storage effects on marula volatile compounds

3.3.1. Influence of heating on marula flesh volatile compounds

In Table 3, the volatile compounds in the *marula* flesh that changed during heating are listed. Although observed peak intensities of many volatile compounds were significantly reduced after heating, no big differences were found in the presence of the major compounds. Sesquiterpenes such as β-caryophyllene, α-humulene, (E)-germacrene D and β-selinene still dominated as major compounds in volatile profile of *marula* flesh. But for some minor volatile compounds, thermally induced reactions, such as decomposition or new generation occurred. For instance, by comparing the aroma profile of flesh samples heated at 40 °C and unheated flesh

samples, short chain hydrocarbons that could not be detected in unheated samples such as pentanal, 1-hexanol and 2-ethyl-1-hexanol could be found as flesh aroma compounds after thermal treatment of longer than 5 min. In contrast, for this same short heating period, no decomposition of originally existed compounds were observed. Ethyl acetate disappeared after 15 min, whereas 1-pentanone, 1-(4-methylphenyl) was found to be generated as new odour compound. After heating for 30 min, monoterpene 4-(+)-carene began to accumulate.

Heating at 85 °C did not change flesh volatile composition when compared with heating at 40 °C. However, at 40 °C veridiflorol was detected after 3 min of heating time. It is a sesquiterpen alcohol and was reported as a major compound of African mango (Sakho, crouzet and Seck, 1985) and its generation, might be caused by existing tricyclic sesquiterpenes in flesh samples such as aromadendrene or α-gurjunene through epoxidation (Tressl, Engel, Kossa and Koeppler, 1983).

Besides that, acetone was also detected in samples after 3 min of heating at 85°C and D-limonene, a monoterpene with orange-like aroma, also accumulated after 5 min of heating at the same temperature. Possible explanation for D-limonene formation could be due to the hydrolysis of existing terpenyl glucosides in *marula* flesh as was found for grapes (Williams, Strauss and Wilson, 1980).

Compared to 40 and 85 °C, new oxygenated terpenes such as linalool and humulane-1, 6-dien-3-ol were found upon heating at 110 °C. According to previous model studies, their formation could be caused by two possible pathways: either the acid-catalysed rearrangement of polyols (Sakho *et al.*, 1985; Yen *et al.*, 1992) or the oxidation of hydrocarbon terpenes, for example, formation of linool in flesh samples after heating could result from a pathway involving limonene oxidation (Williams *et al.*, 1980). Besides the generation of oxygenated terpene, 3-furaldehyde was also found in heated samples. The presence of furan compounds in food is usually considered to be the result of thermal decomposition of carbohydrates or the Maillard reaction between reducing sugars and amino groups, or from acid or base-catalyzed dehydration of sugars. According to Pretorius *et al.*, (1985), 2-furfural and 5-hydroxymethyl-2-furfural occuring in fresh *marula* juice probably arose from acid-catalyzed dehydration reactions.

Table 3: Changes in volatile compounds in marula flesh samples after various heating treatments. RT = retention time.

Peak No.	RT (min)	Compounds	Odour quality	Heating Time and Temperature														
				40 °C, time: min					85 °C, time: min					110 °C, time: min				
				0	5	15	30	60	0	3	5	10	20	0	1	3	5	10
1	3.95	Acetone	light fruity, apple-like [a]	ND	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	+
2	5.00	Tetrahydrofuran	-	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+
3	5.84	ethyl acetate	banana-like, fruity [a]	+	+	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND
4	8.47	Pentanal	oxidized, nutty [b]	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+
5	8.50	1-propen-2-ol,formate	-	ND	ND	+	+	+	ND	ND	+	+	+	ND	ND	ND	+	+
6	9.95	dimethyl amine	-	ND	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	+
7	13.27	d-limonene	citrus, orange-like [c]	ND	ND	ND	ND	ND	ND	ND	+	+	+	ND	+	+	+	+
8	14.57	1-Pentanone, 1-(4-methylphenyl)	-	ND	ND	+	+	+	ND	ND	+	+	+	ND	+	+	+	+
9	14.72	isoamyl isovalerate	sweet, fruity [a]	+	+	+	+	+	+	+	+	+	+	+	ND	ND	ND	ND
10	14.76	(+)-4-Carene	-	ND	ND	ND	+	+	ND	ND	+	+	+	ND	ND	ND	+	+
11	15.57	1-hexanol	fruity, fatty [d]	ND	+	+	+	+	ND	+	+	+	+	ND	ND	+	+	+
12	16.11	3-hexen-1-ol,formate,(z)	sweet, green	ND	+	+	+	+	ND	ND	+	+	+	ND	ND	+	+	+
13	17.49	2-ethyl-1-hexanol	floral, citrus [d]	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+
14	17.62	3-furaldehyde	almond [f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	+
15	18.29	Linalool	sweet, floral, citrus [d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	+
16	24.94	humulane-1,6-dien-3-ol	spicy [f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	+
17	25.34	ethyl lactate	buttery, fruity, pineapple-lik[e] e [b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	+
18	25.01	Veridiflorol	-	ND	ND	ND	ND	ND	ND	+	+	+	+	ND	ND	ND	ND	ND

a. (Ong *et al.*, 2008); b. (Boatright & Lei, 1999); c. (Choi, 2003); d. (Pino *et al.*, 2001); e. (Peinado *et al.*, 2004); f. (Bauer *et al.*, 2001)
ND: not detected; +: identified compounds.

3.3.2. Influence of heating on marula peel volatile compounds

In Table 4 the volatile compounds in the marula peel that changed during heating are listed. Changes on the marula peel mainly occurred in simple short chain hydrocarbons rather than in the dominant sesquiterpene constituents. Disappearance of the ester compound ethyl acetate, the aldehyde compounds decanal 2-methyl butyraldehyde and heptanal, the alkene compound 1-penten-3-one was observed after heating at 40 °C. Only 2-methyl butyraldehyde was described as the cause for off-flavour with a musty odour. For example, by comparing all the heated samples, the ester ethylisovalerate that was previously detected in unheated peel could not be found in samples heated longer than 60 min at 40 °C (table 4). The furan derivate dihydro-3-methyl-2(3H)-furanone could not be detected in peel samples after a heat treatment longer than 5 min.

More marked changes can be observed at higher temperatures and longer time. For instance, in the samples heated at 110 °C for 20 min, the 76 volatiles that were detected in unheated fresh peel were reduced to 60. Sixteen compounds, including 7 esters (methyl acetate, ethyl acetate, isobutyl isovalerate, 3-isopentenyl isovalerate, 3-hexen-1-ol,formate(z), hexyl butyrate, octyl formate), 3 kinds of aldehyde (2-methyl butyraldehyde, heptanal and decanal), 2 kinds of ketone (1-penten-3-one and 2-Pentyn-4-one), furan derivate dihydro-3-methyl-2(3H)-furanone, propanoic anhydride, alkyl 2,3,5-trimethyl-heptane and 3-methyl-1-butanol carbonate disappeared. Most of them are associated with sweet, fruity odour and might contribute to the whole marula peel aroma profile.

Table 4: Changes on volatile compounds in marula peel samples after various heating treatment. RT = retention time.

Peak No.	RT (min)	Compounds	Odour quality	Heating Time and Temperature														
				40 °C, time :min					85 °C, time :min					110 °C, time :min				
				0	5	15	30	60	0	3	5	10	20	0	1	3	5	10
1	3.97	Acetone	apple-like [a]	ND	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	+
2	4.24	methyl acetate	sweet, fruity [b]	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	ND
3	5.00	Tetrahydrofuran	-	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+
4	5.84	ethyl acetate	banana-like, fruity [a]	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND
5	6.53	2-methyl butyraldehyde	musty [g]	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND
6	8.49	Pentanal	fermented, fruity [c]	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+
7	9.53	1-penten-3-one	rotten, fruity [h]	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND
8	10.48	ethyl isovalerate	fruity, pineapple-like [d]	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
9	10.60	4-hexen-3-one	spicy, green [i]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
10	11.96	dihydro-3-methyl- 2(3H)-furanone	-	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	+	+	+	ND
11	12.28	propanoic acid, anhydride	-	+	+	+	+	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND
12	12.81	2,3,5-trimethyl- heptane	-	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	ND
13	12.93	isobutyl isovalerate	sweet, fruity [j]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND
14	12.98	heptanal	fatty, fruity [h]	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND
15	14.07	3-carene	sweet, citrus [b]	+	+	+	ND	ND	+	+	+	+	ND	+	+	+	+	+
16	14.27	1-butanol, 3-methyl-, carbonate (2:1)	-	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND
17	14.76	(+)-4-carene	-	ND	ND	+	+	+	ND	+	+	+	+	ND	+	+	+	+
18	15.10	4-Penten-1-ol, propanoate	-	ND	+	+	+	+	ND	ND	+	+	+	ND	ND	ND	ND	ND

19	15.15	2-Pentyn-4-one	-	+	ND	ND	ND	ND	+	+	ND	ND	ND	+	ND	ND	ND	ND
20	15.94	3-isopentenyl isovalerate	-	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	ND
21	16.11	3-hexen-1-ol,formate,(z)	sweet, green [i]	+	+	ND	ND	ND	+	+	ND	ND	ND	+	+	+	+	ND
22	16.59	dimethyl trisulfide	cooked onion, cabbage-like [k]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
23	16.60	hexyl butyrate	green, fruity [l]	+	+	+	+	+	+	+	+	+	+	+	ND	ND	ND	ND
24	17.49	2-ethyl-1-hexanol	floral, citrus [h]	ND	ND	+	+	+	ND	ND	+	+	+	ND	ND	ND	+	+
25	17.92	decanal	sweet, soapy, orange-peel [e]	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND
26	18.28	Linalool	sweet, citrus [f]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	+
27	18.44	octyl formate	fruity, orange-like, waxy [k]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND
28	22.81	Pentanoic acid, phenylmethyl ester	-	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+
29	24.48	naphthalene, 1,5-dimethyl-	-	ND	+	+	+	+	ND	+	+	+	+	ND	+	+	+	+

a. (Ong *et al.*, 2008); b. (Pino *et al.*, 2001); c. (Boatright & Lei, 1999); d. (Peinado *et al.*, 2004); e. (Aparicio *et al.*, 2000); f. (Choi, 2003); g. (Farah *et al.*, 2006); h. (Carrapiso *et al.*, 2002); i. (Vermeulen & Collin, 2006); j. (Aznar *et al.*, 2001); k. (Milo & Grosch, 1995); l. (Bauer *et al.*, 2001)

ND: not detected; +: identified compounds.

3.3.3. Storage effects on the flavour compounds of marula flesh

Marula flesh samples heated at 110 °C for 15 min and then sealed and stored at 4 °C for a maximum of 30 days were compared to heated samples without storage. The compounds are listed in Table 5. No marked difference was observed for sesquiterpenes, which were considered as major aroma compounds of the aroma of *marula* flesh. However, for minor compounds of storage effects could be found. For instance, after 30 days of storage, the number of esters was reduced from 4 to 3 and a new compound isoamyl isovalerate was detected. 1-propen-2-ol, formate as well as ethyl lactate disappeared and the same happened with monoterpenes.

A new compound, dimethyl sulfone, was detected. It is an organosulfur compound reported before as aroma constituent in young port wine (Boulanger & Crouzet, 2001). Its formation was described as strictly depended on the presence of oxygen (Silva Ferreira *et al.*, 2002).

Except for the changes in the profile of peel volatiles, the content of each compound also changed during storage time. Absolute peak area of the major sesquiterpene compounds, namely copaene, caryophyllene, α-humulene, (E)-germacrene D, β-selinene and β-cadinene after different storage time were compared in Figure 1 and 4. The response factors of all these six compounds were considered equal because of their molecular structure as isomers. In Figure 1, the major sesquiterpene contents in *marula* flesh were found to increase during the first 10 days of storage. Caryophyllene, for example, had an absolute peak area almost 10 fold as large as its area in non-stored samples. The amounts decreased relatively more between 10 and 20 days of storage and after 20 days, they tended to stabilize. Another noticeable change was the increase of the peak area of alcohol compounds, such as ethanol or isoamyl alcohol in figure 2 and figure 3.

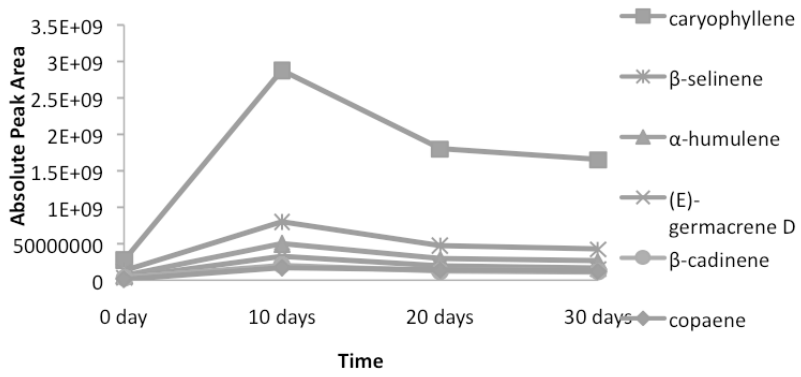

Figure 1. Changes in sesquiterpene compounds in pulp during storage at 4°C.

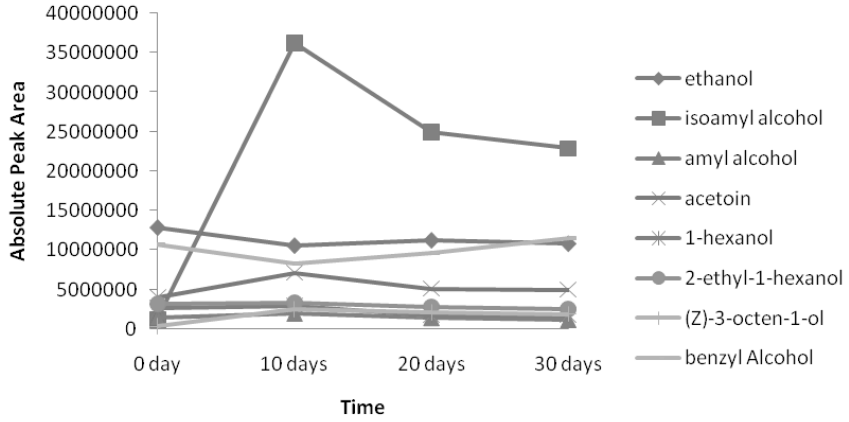

Figure 2. Alcohol compounds detected in flesh during storage at4°C.

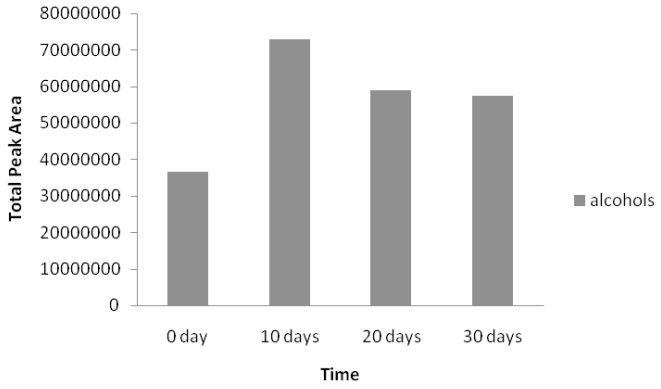

Figure 3. Amount of alcohol content in flesh during storage at 4°C.

3.3.4. Storage effects on *marula* peel

The presence of volatile compounds in samples stored at 4 °C for different times are listed in Tables 5 and 6. Similar to flesh samples, the dominating volatile compounds sesquiterpene did not change much. But at high temperature, compounds such as 4-hexen-3-one, tetrahydrofuran or dimethyl trisulfide disappeared after 10 days of storage. Besides that, the monoterpene compound 3-carene as well as its isomer (+)-4-carene could also not be detected after storage. Except for dimethyl sulfone, mentioned earlier in the discussion of newly generated compounds in marula flesh after storage, the compound 2-pentyl furan also appeared as new volatile constituent in the profile of peel samples stored longer than10 days. Like dimethyl sulfone and 2- pentyl furan were also regarded as a compound causing off-flavour in food products (Silva Ferreira *et al.*, 2002). Their formation were reported resulting from oxidation of linoleic acid in soybean oil product (Yen & Lin, 1999).

Compared to *marula* flesh, sesquiterpene compounds in peel samples showed a different change during storage. The effect of storage on 6 major sesquiterpene compounds, caryophyllene, β-selinene, (E)-germacrene D, α-humulene, copaene and β-cadinene, is shown in figure 4. The measured peak areas of those compounds remained relatively stable in the first 20 days.

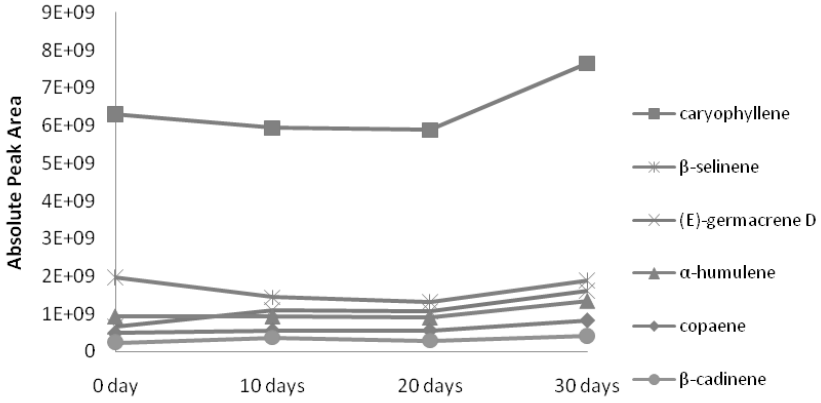

Figure 4. Comparison of major sesquiterpene compounds in peel samples at different storage time.

Table 5: Comparison of the presence of flavour compounds in *marula* flesh samples for different storage time at 4 °C. RT = retention time

Peak No.	RT (min)	Compounds	Odour quality	Storage time (days)			
				0 day	10 days	20 days	30 days
		Aldehydes					
1	2.24	acetaldehyde	fruity, pungent [a]	+	+	+	+
5	8.47	pentanal	fermented, fruity [b]	+	+	+	+
18	16.45	nonanal	waxy, citrus [c]	+	+	+	+
29	18.66	benzaldehyde	almond [b]	+	+	+	+
		Alcohols					
4	7.22	ethanol	alcoholic [k]	+	+	+	+
9	13.13	isoamyl alcohol	alcoholic, fruity, bitter, harsh [l] isoamyl alcohol	+	+	+	+
11	13.92	amyl alcohol	sweet, balsam [h]	+	+	+	+
15	14.85	acetoin	sweet, fatty [h]	+	+	+	+
16	15.57	1-hexanol	fruity, green [g]	+	+	+	+
20	17.49	2-ethyl-1-hexanol	floral, citrus [d]	+	+	+	+
30	18.84	(Z)-3-octen-1-ol	fruity, melon-like [a]	+	+	+	+
50	22.59	benzyl Alcohol	floral [h]	+	+	+	+
		Ketones					
2	3.95	acetone	apple-like [e]	+	+	+	+
12	14.57	1-Pentanone, 1-(4-methylphenyl)	-	+	ND	ND	ND
		Fruans					
3	5.00	tetrahydrofuran	-	+	ND	ND	ND
22	17.62	3-furaldehyde	almond [h]	+	+	+	+
		Esters					
6	8.50	1-propen-2-ol,formate	-	+	ND	ND	ND
13	14.72	isoamyl isovalerate	sweet, fruity [e]	ND	+	+	+
17	16.11	3-hexen-1-ol,formate,(z)	sweet, green [f]	+	+	+	+
27	18.44	octyl formate	fruity, orange-like, waxy [a]	+	+	+	+
55	25.34	ethyl lactate	fruity, pineapple-like [h]	+	ND	ND	ND
		Monoterpenes/ Monoterpenoids					
10	13.27	d-limonene	citrus, orange-like [g]	+	+	ND	ND
14	14.76	(+)-4-Carene	-	+	ND	ND	ND
25	18.29	linalool	sweet, citrus [g]	+	+	+	+
		Sesquiterpenes/ Sesquiterpenoids					
19	17.40	α-cubebene	herbal, waxy [g]	+	+	+	+
21	17.55	δ-elemene	woody [g]	+	+	+	+

23	17.87	ylangene	herbal [i]	+	+	+	+
24	18.01	copaene	woody, spice [j]	+	+	+	+
26	18.41	β-bourbonene	herbal woody [g]	+	+	+	+
28	18.58	β-cubebene	citrus, fruity [g]	+	+	+	+
31	19.25	β-elemene	herbal [g]	+	+	+	+
32	19.43	(+)-epi-bicyclosesquiphellandrene	-	+	+	+	+
33	19.52	β -caryophyllene	sweet, woody [g]	+	+	+	+
34	19.77	α-gurjunene	woody [a]	+	+	+	+
35	19.83	ç -elemene	-	+	+	+	+
36	20.16	aromadendrene	-	+	+	+	+
37	20.21	δ-cadinene	herbal, woody [j]	+	+	+	+
38	20.46	α-humulene	woody [g]	+	+	+	+
39	20.57	α-muurolene	woody [g]	+	+	+	+
40	20.81	epizonarene	-	+	+	+	+
41	20.92	(E)-germacrene D	woody, spicy [g]	+	+	+	+
42	21.11	β-selinene	herbal [a]	+	+	+	+
43	21.22	γ-elemene	-	+	+	+	+
44	21.34	β-cadinene	green, woody [a]	+	+	+	+
45	21.47	(R)-γ -cadinene	herbal, woody [a]	+	+	+	+
46	21.62	(-)-π-Panasinsen	-	+	+	+	+
47	21.79	valencene	sweet, citrus	+	+	+	+
48	21.83	α-cadinene	woody [a]	+	+	+	+
49	22.32	calamenene	herbal, spice [a]	+	+	+	+
51	23.31	π-calacorene	woody [a]	+	+	+	+
53	24.82	(-)-cubenol	herbal [k]	+	+	+	+
56	25.76	bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	-	+	+	+	+
57	26.44	π-cadinol	herbal [k]	+	+	+	+
54	24.94	humulane-1,6-dien-3-ol	spicy [k]	+	+	+	+
52	24.21	caryophyllene oxide	sweet, woody [g]	+	+	+	+
		Others					
7	9.95	dimethyl amine	-	+	+	+	+
8	10.80	dimethyl sulfone	sulphurous, burnt [m]	ND	+	+	+

a. (Pino *et al.*, 2001); b. (Boatright & Lei, 1999); c. (Aparicio *et al.*, 2000); d. (Carrapiso *et al.*, 2002); e. (Ong *et al.*, 2008); f. (Vermeulen & Collin, 2006); g. (Choi, 2003); h. (Peinado *et al.*, 2004); i. (Eyres *et al.*, 2005); j. (Minh Tu *et al.*, 2002); k. (Bauer *et al.*, 2001); l. (Aznar *et al.*, 2001); m. (Weenen *et al.*, 1996).

ND: not detected; +: identified compounds.

Table 6: Comparison of the presence of flavor compounds in marula peel samples for different storage time at 4°C. RT = retention time.

Peak No.	RT (min)	Compounds	Odour quality	Storage time (days) 0 day	10 days	20 days	30 days
		Aldehydes					
1	2.24	Acetaldehyde	Fruity, pungent [a]	+	+	+	+
5	8.49	Pentanal	Fermented, fruity [b]	+	+	+	+
22	16.45	Nonanal	Waxy, citrus [c]	+	+	+	+
35	18.65	Benzaldehyde	Sweet, sharp [b]	+	+	+	+
		Alcohols					
4	7.22	Ethanol	Alcoholic [k]	+	+	+	+
9	10.92	Hexanal	Green, fruity, grass	+	+	+	+
11	13.16	Isoamyl alcohol	Alcoholic fruity, banana-like [l]	+	+	+	+
14	13.91	Amyl alcohol	Sweet, balsam [h]	+	+	+	+
20	14.87	Acetoin	Sweet, fatty [h]	+	+	+	+
21	15.56	1-hexanol	Green, fruity [g]	+	+	+	+
27	17.49	2-ethyl-1-hexanol	Floral, citrus [d]	+	+	+	+
36	18.84	(Z)-3-octen-1-ol	Fruity, melon-like [a]	+	+	+	+
62	22.59	benzyl Alcohol	Floral [h]	+	+	+	+
65	23.57	Z-4-dodecenol	Oily [n]	+	+	+	+
67	24.12	10-undecyn-1-ol	-	+	+	+	+
		Ketones					
2	3.97	Acetone	Apple-like [e]	+	+	+	+
7	10.60	4-hexen-3-one	Pungent, green [a]	+	ND	ND	ND
17	14.57	1-Pentanone, 1-(4-methylphenyl)	-	+	+	+	+
		Furans					
3	5.00	Tetrahydrofuran	-	+	ND	ND	ND
13	13.77	2-pentyl furan	fruity , green [b]	ND	+	+	+
		Esters					
6	10.48	Ethyl isovalerate	pineapple-like [l]	+	+	+	+
10	12.93	isobutyl isovalerate	sweet, fruity [l]	ND	+	+	+
16	14.47	isoamyl 2-methyl butyrate ester	sweet, fruity	+	+	+	+
18	14.72	isoamyl isovalerate	sweet, fruity [e]	+	+	+	+
23	16.57	butyl hexanoate	fruity, pineapple [k]	+	ND	ND	ND
25	16.76	hexyl 2-methyl butyrate	green, fruity, spicy [k]	+	+	+	+

29	17.63	n-valeric acid cis-3-hexenyl Ester	-	+	+	+	+
63	22.81	benzyl valerate	fruity, floral [a]	+	+	+	+
		Monoterpene					
12	13.26	d-limonene	citrus, orange-like [g]	+	+	+	+
15	14.07	3-carene	sweet, citrus [a]	+	ND	ND	ND
19	14.76	(+)-4-carene	-	+	ND	ND	ND
32	18.28	Linalool	sweet, citrus [g]	+	+	+	+
		Sesquiterpenes					
26	17.40	α-cubebene	herbal, waxy [g]	+	+	+	+
28	17.55	δ-elemene	woody [g]	+	+	+	+
30	17.87	ylangene	herbal [i]	+	+	+	+
31	18.01	copaene	woody, spice [j]	+	+	+	+
33	18.41	β-bourbonene	herbal woody [g]	+	+	+	+
34	18.58	β-cubebene	citrus, fruity [g]	+	+	+	+
37	19.09	cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	-	+	+	+	+
38	19.18	a sesquiterpene hydrocarbon	-	+	+	+	+
39	19.25	β-elemene	herbal [g]	+	+	+	+
40	19.43	(+)-epi-bicyclosesquiphellandrene	-	+	+	+	+
41	19.52	β -caryophyllene	sweet, woody [g]	+	+	+	+
42	19.77	α-gurjunene	woody [a]	+	+	+	+
43	19.83	ç -elemene	-	+	+	+	+
44	19.98	n-cubebene	citrus [a]	+	+	+	+
45	20.16	aromadendrene	-	+	+	+	+
46	20.21	δ-cadinene	herbal, woody [j]	+	+	+	+
47	20.46	α-humulene	woody [g]	+	+	+	+
48	20.57	α-muurolene	woody [g]	+	+	+	+
49	20.65	γ-selinene	woody [g]	+	+	+	+
50	20.74	δ-selinene	-	+	+	+	+
51	20.81	epizonarene	-	+	+	+	+
52	20.92	(E)-germacrene D	woody, spicy [g]	+	+	+	+
53	21.11	β-selinene	herbal [a]	+	+	+	+
54	21.22	γ-elemene	-	+	+	+	+
55	21.34	β-cadinene	green, woody [a]	+	+	+	+
56	21.47	(R)-γ –cadinene	herbal, woody [a]	+	+	+	+
57	21.62	(-)-α-Panasinsen	-	+	+	+	+
58	21.74	valencene	sweet, citrus [k]	+	+	+	+

59	21.83	α-cadinene	woody [a]	+	+	+	+
60	22.32	calamenene	herbal, spice [a]	+	+	+	+
61	22.41	elemene	-	+	+	+	+
64	23.31	n-calacorene	woody [a]	+	+	+	+
66	23.77	calacorene	-	+	+	+	+
68	24.21	caryophyllene oxide	sweet, woody [g]	+	+	+	+
70	25.76	isopropyl-5-methyl-9-methylene-bicyclo[4.4.0] dec-1-ene, 2-	-	+	+	+	+
71	25.94	T-muurolol	woody, spice [a]	+	+	+	+
72	26.44	n-cadinol	Herbal [k]	+	+	+	+
		Others					
8	10.82	dimethyl sulfone	sulphurous, burnt [m]	ND	+	+	+
24	16.59	dimethyl trisulfide	cooked onion [f]	+	ND	ND	ND
69	24.48	1,5-dimethylnaphthalene	-	+	+	+	+

a. (Pino *et al.*, 2001); b. (Boatright & Lei, 1999); c. (Aparicio *et al.*, 2000); d. (Carrapiso *et al.*, 2002); e. (Ong *et al.*, 2008); f. (Milo & Grosch, 1995) ; g. (Choi, 2003); h. (Peinado *et al.*, 2004); i. (Eyres *et al.*, 2005); j. (Minh Tu *et al.*, 2002); k. (Bauer *et al.*, 2001); l. (Aznar *et al.*, 2001); m. (Weenen *et al.*, 1996); n. (Hatanaka, 1996)
ND: not detected; +: identified compounds.

4. Conclusion

For the first time, the volatile compounds in *marula* peel and flesh were characterized and compared. 75 compounds were identified in *marula* peel and 41 were found in *marula* flesh. The identified compounds can be classified into several classes: alkyl, aldehyde, ester, furan compound, alcohol, monoterpene and sesquiterpene. Among them, sesquiterpene hydrocarbons (β-caryophyllene, α-humulene, E-germacrene D and β-selinene) were found to be most abundant compounds in flesh and peel, with β-caryophyllene as dominating compound. For the first time β-selinene was identified in *marula*. This is a compound usually reported as important constituent in aroma profile of essential plant oils.

The results obtained on volatile compounds of *marula* peel and flesh provides information on how storage and heating can affect the volatile compounds. No dramatic changes occurred in the concentration of

sesquiterpene compounds at relatively low temperatures, 40 °C and 85 °C, respectively. More marked changes could be observed at the higher temperature of 110 °C for 10 minutes or longer. New compounds such as oxgenated terpene were found while flavour compounds like esters and ketones disappeared during heating. When considering storage effects, no big differences could be observed in the distribution of sesquiterpene as major volatile compounds after storage at 4 °C for a maximum of 30 days exept for minor changes on ketones, furans, esters and monoterpene.

Overall conclusion is that heating at 110°C for 10 min or longer has an effect on the volatile compounds of *marula* flesh and peel as it reduces the amount of flavour compounds. Most *marula* products are processed through heat treatment that might result in volatiles being lost. That could be the reason why most of the *marula* products available in the market are claimed not to contain *marula* like flavour. It might be interesting for *marula* processors to know that heating the flesh, for instance during sterilization, can have an effect on *marul*a flavour. Heating the flesh or juice no longer than 10 min if the temperature is 110°C or higher might be better. This is so since all the major changes were observed after heating for longer than 10 min at temperature of 110°C in this study. Storage at 4 °C for about 30 days had minor effect on flavour compounds of the *marula* flesh and its peel. This means that *marula* flesh can be stored at 4 °C for 30 days without its flavor compounds being lost. It could be interesting to incooperate the skin part in the juice processing since the peel had more flavour compounds in higher concentration than the flesh.

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Chapter 7

General Discussion

1. Introduction

There have been several attempts since the 1970s to commercialise *marula* products, because *marula* is ubiquitously distributed and has multiple uses in Southern Africa. The International Centre has identified the *marula* tree as a key species for Research in Agroforestry (Leakey & Simons, 1998). Increasingly, a variety of *marula*-based products is entering markets either through the efforts of rural communities themselves, or by private companies (Leakey & Simons, 1998). Rather than being eaten directly, *marula* fruits are applied more today in food industries to process products such as *Amarula*' cream liqueur production, juice production, beer and wines brewing and jelly/jam making (Bille & Steppich, 2003; Leakey, Shackleton & Du Plessis, 2005). All of these add value to this species in the eyes of the rural community and provides reasons for the concern expressed regarding the potential 'erosive' effects of *marula* commercialisation on community cohesion and culture (Leakey & Simons, 1998).

Although different parts of the tree such as stem-barks, leaves and roots have different ethnomedical and commercial uses, due to their own characteristics, the fruit in particular is considered as the major product (Ojewole, Mawoza, Chiwororo and Owira, 2010). This thesis's overall objective was to investigate the fate of antioxidants and their activities due to the processing and storage conditions of the *marula* pulp and its juice. This chapter discusses the progress made toward accomplishment of the overall objective for this thesis. The specific objectives were outlined in chapter 1 and were as follows:

1. To critically evaluate literature on proximate composition and nutritional value of *marula* in comparison with other tropical and indigenous fruits, in order to identify areas for future research,

2. To determine the optimum processing conditions for maximum juice yield, vitamin C, polyphenols, antioxidant activity and clarification of the *marula* juice,

3. To determine the effects of fermentation temperature and time on the quality aspects of the naturally fermented *marula* juice,

4. To investigate the thermal degradation of vitamin C in *marula* pulp,

5. To indentify and characterize the volatile flavor compounds of *marula* fruit and to further investigate their changes under different heating and storage conditions.

2. Discussion and interpretation of results

2.1. To critically evaluate literature on proximate composition and nutritional value of *marula*

The critical review of literature on proximate composition and nutritional value of *marula* (chapter 2) revealed that the mineral and nutrient content varied greatly from study to study. This could be due to variation in the place of origin, soil, climate and time that lapsed after harvesting before analyses were carried out. Due to the variability of the results found in reported data, we recommend that collection and storage conditions under which the samples were handled and methods used to analyze the samples must be marmonized and described in detail. In some cases, it was found that authors were not clear or specific in indicating which part of the fruit was used during their analysis. Terms used like flesh, pulp and juice or edible portion, were found to be very confusing and this makes it difficult to group and compare similar results. It was thus recommended that authors should be very specific and clear in outlining which part of the sample was used in their materials and methods. The variation arising due to environmental factors cannot easily be controlled since most of the *marula* trees grow naturally and under no irrigation and fertilizer added. The *marula* tree can grow in open woodlands, bushes, in clay or sandy soil and survives in hot dry climatic conditions with a mean annual rainfall of between 200 to 1500 mm. The above mentioned factors arise also naturally, hence the causes for variation remain unknown as

for now, whether it is due to real biological variation or to experimental uncertainty.
Despite this high variability, several authors have reported that *marula* fruit is known for its very high vitamin C content, and is a rich source of antioxidants. Its vitamin C content can be eight times higher than that found in an orange fruit. The nuts of these trees are also rich in oleic acid, protein, energy content and minerals like iron, magnesium, zinc, phosphorus and copper, which contribute to the importance of these nuts in the diets of some rural communities. *Marula* fruits could play a vital role to the rural populations who rely on the usage of the fruits and have limited access to other sources of nutrients.

The critical review on proximate composition and nutritional value of *marula* was the starting point for the research reported in this thesis. In the review, several recommendations were given for future research and they served as the base for the specific objectives that were addressed in chapter 3 to 6 of this thesis. The main recommendations were:

1. To optimize *marula* fruit juice yield,

2. To investigate the effect of processing and storage on the retention of nutrients such as vitamin C and its antioxidant capacity in *marula* pulp and its products,

3. To identify individual antioxidants and their activity in processed and unprocessed *marula* products and,

4 To identify the most important flavour compounds and further investigate the effect of processing and storage toward the *marula* flavour.

2.2. Optimum processing conditions

The commercial use of *marula* fruit and juice-based products have increased in recent years such that the fruit is used for preparation of juices, jams, conserves, jellies and alcoholic beverages (Bille & Steppich, 2003). In order to increase the production of the juice, developing an efficient extraction process to increase juice yield is an attractive option.

Based on the recommendation given in chapter 2, the next step was to determine the optimum processing conditions for maximum juice yield. To achieve this objective, Response Surface Methodology (RSM) was used and we could not only optimise the yield but also the content of vitamin C, polyphenols, antioxidant activity and clarification of the *marula* juice since those are the important quality aspects. In order to do that, determination of the optimum processing conditions (pectinase enzyme concentration, extraction temperature and time) for maximum juice yield, vitamin C, polyphenols, antioxidant activity and clarification of the *marula* juice is important. By adding an enzyme like pectinase, the gel can be broken down so that the juice can be extracted more efficiently resulting into higher juice yield and also juice with high dry matter content (Sreenath, Sudarshana & Santhanam, 1995).

Different concentrations of pectolytic enzymes were used with different combinations of heating time and temperature. This work is presented in chapter 3 of this thesis. The results indicate that the use of pectinase enzymes can increase the yield of *marula* juice by 23%. The experimental results showed an optimum yield of marula (56.4%) and that was used to build a model that predicted 54.6% optimum *marula* juice yield at an enzyme concentration in the range of 0.1 to 0.14%. Then the results were validated via a new experiment and 55.6% yield of *marula* juice was obtained at enzyme concentration (0.14%), heating time (65 min) and temperature of 60°C.

Furthermore, applying an enzyme concentration of 0.1 to 0.14% and a temperature between 40 and 60 ºC, can significantly increase the vitamin C content, polyphenols and antioxidant activity next to an increased yield. Therefore, *marula* processors can optimise their yield and at the same time have juice high in antioxidant content. The processors are able to achieve this, currently most of the traditional *marula* processing centres are able to sell all their juice and the demand is always higher than the supply in Southern Africa. One of the current difficulties faced by *marula* processors is not being able to obtain high yields. This is due to difficulties that arise during pressing the juice out, and ends up causing high postharvest

losses of fruits not being pressed. Partly, that could be attributed by the slow rate of extraction and cleaning the mess after a single load is pressed. By using the enzyme, it might resolve that problem completely, and at the same time more money can be collected via selling the juice and that money can compensate for the cost of the enzyme purchased. For controlling the temperature, currently most *marula* processors boil the fruits for about 3 hours in order to soften the skin and to facilitate the pressing process. This research has shown that it is best to heat between 40 and 60 minutes, therefore, this finding could be disseminated via Indigenous Plant Task Teams (IPTT) to advice the processors to control the temperature in order to retain high vitamin C and other antioxidants present in *marula* juice. IPTT is a government-chaired multi stakeholder strategic task team with a common purpose of exploring, developing and promoting the potential of Nambia's indigenous plants and their possible products. IPTT also aims that communities living on the margins of the normal economy should be the significant beneficiaries of these efforts in order to expand the sustainable exploitation of value from indigenous natural plants.

The parameter of 'time' had a significant effect on the lightness of the juice. However, the factors of temperature and enzyme concentration did not significantly influence the lightness. Therefore, for processors who want to improve lightness of *marula* juice should consider the heating time to be able to produce a light yellowish beige colour the colour that is almost similar to the pineapple juice colour.

2.3. Quality aspects of naturally fermented *marula* juice

After optimization of the *marula* yield and knowing that traditionally, the main use of the juice is to be fermented into an alcoholic drink, it was considered very important to investigate the effect of temperature and time of fermentation on the quality of naturally fermented *marula* juice (Chapter 4). The results showed that fermented *marula* juice is a source of antioxidants and their activities were positively correlated to vitamin C and phenolic contents. Fermentation at elevated temperatures (30 to 40 °C) for 4 to 5 days resulted in a high retention of ascorbic acid and vice versa,

for lower temperature (20 to 25 °C) for 6 to 8 days. Phenolic compounds in *marula* juice seem to be stable during fermentation irrespective of the fermenting temperature and duration. Processors should ferment *marula* juice at temperatures between 30 and 40 °C for 4 to 6 days to produce an alcoholic product high in antioxidants. The conclusion is that both fermented and unfermented *marula* juice is a good source of natural antioxidants.

Even though the fermented *marula* juice contained higher concentrations of antioxidants, it is worthwhile to advice and promote the use of unfermented *marula* juice since it has considerably higher dry matter content than the fermented one. The fermented *marula* juice had low dry matter content and a very low sugar level after fermentation. In terms of nutrition it probably only delivers alcohol and antioxidants.

2.4.Thermal degradation of vitamin C of *marula*

The findings in chapter 3 and 4 were in line with other authors that *marula* is rich in vitamin C. However, very limited information about the stability of vitamin C in *marula* is available in literature. Therefore, chapter 5 of this thesis investigated the thermal degradation of vitamin C in *marula* fruit in comparison to two other tropical fruits (mango and guava). The results showed that the degradation rates of ascorbic acid in *marula* were much lower; vitamin C was 15 times more stable to heat at 100 °C in *marula* juice as compared to mango and guava pulp (chapter 5). Even though the thermal degradation of ascorbic acid is usually described in literature by a first order model, this could not fit the degradation process in *marula*. A two-stage first-order model fitted better; the first stage describing the faster degradation (called unstable fraction) and the second the slower degradation part (called stable fraction). The stable fraction (SF) for the three fruits was almost identical (table 1), whereas degradation of vitamin C in guava had the highest activation energy (but still rather low) showing that its ascorbic acid degradation rate was more dependent on temperature than for *marula* and mango pulps.

An explanation for this two-stage behaviour could be an effect of the limited amount of oxygen present in the fruit samples or in the headspace of the heating tubes during heat treatment. In other words, a fast aerobic degradation of ascorbic acid is via reaction with oxygen while the remaining ascorbic acid is subsequently degraded by an anaerobic degradation pathway with a lower reaction rate. Based on the different initial concentrations of ascorbic acid in *marula* compared to the other fruits, it can be expected that a relatively smaller part of the total ascorbic acid pool would react with oxygen in *marula* and that might be the reason why the degradation rate of most ascorbic acid in *marula* had a slower rate than that for guava and mango pulps. Further research is needed to investigate this effect in more details, such as investigating the effect of oxygen on the degradation of ascorbic acid in *marula* since in this chapter we did not eliminate oxygen. Another reason for this behaviour could be that the ascorbic acid is more stable due to complex formation with other compounds in the fruit matrix. It has been reported in literature that the rate of degradation increases remarkably when pH > 5.7, but this cannot be the cause for the three fruits since all fruits had a pH value much lower than 5.7 (*marula* 3.6, guava 3.9 and mango 3.4).

Besides the lower rate constant for the degradation rate of the unstable fraction of vitamin C at 100 °C in *marula* compared to mango and guava, also the activation energy (29 kJ/mol) was much lower (39 and 58 kJ/mol) than that of mango and guava respectively. The activation energy indicates the sensitivity towards temperature of the degradation reaction of a compound like vitamin C. Low activation energy indicates that the reaction does not depend very strongly on temperature. So, the degradation rate of vitamin C in *marula* is less influenced by the temperature at which it is processed when compared with mango and guava. This effect is shown in Figure 1. For a heating time of 10 minutes, the vitamin C loss is predicted as a function of heating temperature. For this prediction the two phases, the First Order Kinetic Model was used (Chapter 5).

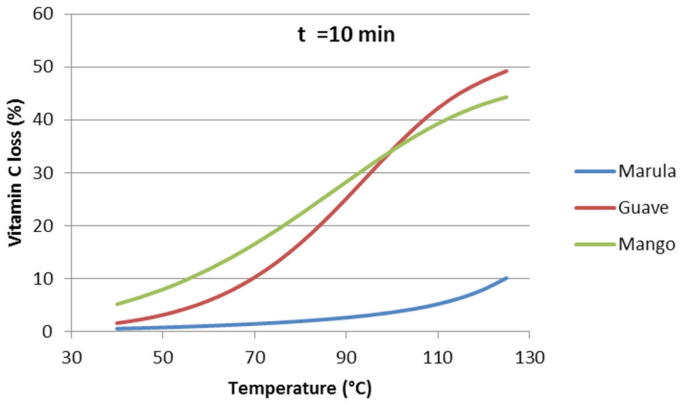

Figure 1. Prediction of vitamin C loss as function of heating temperature for heating for 10 minutes, using the kinetic model as described in Chapter 5.

These results indicate that *marula* processors will be able to subject *marula* juice by basic heat processing treatments like sterilization without degrading much of its vitamin C. Sterilization is a very important processing step as pathogens and spore formers in the juice that might be present are inactivated. Hence, the juice is given a much high self-life, which can be kept at any home of the *marula* juice prpcessors even those without proper cold storage facilities. Sterilized juice can be stored at room temperature or in a refrigerator, such storage condition will be ideal for *marula* processors to use as have limited cold room facilities for storing the pulps prior processing while the processed juice can even be stored at room temperature.

2.5. Identification and characterization of the volatile flavor compounds of *marula* fruit

Nowadays, the pulp of *marula* fruit is also used for the preparation of various food products, such as juices, jams, jellies and alcoholic beverages. These products are claimed not to contain any *marula* flavour and that could be due to processing methods or storage condition that the pulp went through during preparation of those products. This area is not well documented and very limited information is available in the literature. Therefore, chapter 6 of this thesis identified the important flavour compounds and further investigated the effect of processing and storage conditions toward the *marula* flavour.

In *marula* skin, 75 flavour compounds were detected while 41 flavour compounds were found in the flesh. All the flavour compounds that were identified in the flesh were also identified in the skin. The skin had more variety of flavour compounds than in the flesh, therefore it will be advisable for *marula* processors to incorporate the skin in the products such as juices, jams, jellies and alcoholic beverages during processing. This will enhance the unique characteristic of *marula* flavour in the products. Sesquiterpene compounds dominated the volatile fraction in both samples, with β-caryophyllene, α-humulene, (E)-germacrene D and β-selinene being most abundant aroma constituents.

With heating, no major changes occurred in the concentration of sesquiterpene compounds at relatively low temperatures of 40 °C and 85 °C, respectively. More marked changes could be observed at the higher temperature 110 °C for 10 minutes or longer. New compounds such as oxygenated terpene were found while flavour constituents like esters and ketones disappeared during heating. *Marula* processors need to know that heating the pulp, for instance during sterilization, can have an effect on *marula* flavour. Therefore, heating the pulp or juice for not longer than 10 min at the temperature of 110°C or higher might be better. This is so since all the major changes were observed after heating for longer than 10 min at temperature of 110°C in this study. Based on the findings in Chapter 5, it was recommended that the processor of *marula* juice to consider sterilization of the juice as it gives the juice a longer shelf life. But by doing so there might be a possibility of heat induced flavour changes and some might not be desirable. Although this study did not carry out sensory evaluation after heating and since there is a possibility of flavour changes, it is importance to consider sterilisation in order to prolong the shelf life of the processed *marula* juice. For this reason, future studies should consider looking at sensory changes after sterilization of *marula* juice.

When considering storage effects, no big differences could be observed in the distribution of sesquiterpene as major volatile compounds after storage at 4 °C for 30 days except for minor changes on ketones, furans,

esters and monoterpene. This means that *marula* pulp or juice can be stored at 4 °C for 30 days without its flavour compounds being lost. Lower temperature than 4 °C can maintain the flavour compounds for longer than 30 days; therefore *marula* processors can store *marula* pulp or its juice at temperature lower than 4 °C after extraction prior final processing. That can easily happen at the processing centres' like Eudafano Women's Cooperative (EWC) in Namibia where small storage room and other facilities are already in place. After processing the final juice can be sterilized and be stored at room temperature before dispatch.

3. Implications and recommendations of this thesis

3.1. Sample preparation

During this reseach, sample preparations were quite challenging since the fruit was very sticky due to its high sugar content and separation of the skin and pulp was not easy due to juice leaking out. In order to this problem, the procedure was to prepare semi frozen fruits to minimise the leaking of juice and at the same time to reduced the stickiness. In chapter 3, an increased enzyme concentration to 0.2% showed a decrease in juice yield rather than remaining constant after reaching its optimum. An explanation could be that samples were not always homogeneous, some samples contained more flesh or peel than the others and samples with lower yield had high dry matter content and vice versa. Therefore, a better mixing method will be required such as the use of an automated mixer. This experiment showed that working with real foods is not an easy task and is an obstacle to being able to work efficiently. It was already remarked when discussing literature results that this was a real problem that was also experienced in this work; it could well be the reason for the highly variable results found in literature. However, it was worthwhile to study real foods otherwise the remarkable stability of vitamin C would not have been found.

3.2. Fixing time and temperature

During this research, *marula* pulp was treated with several concentrations of enzyme and different time/temperature combinations (chapter 3). Although pectinase was the principal enzymes used, a mixture of cellulolytic and pectinolytic enzymes are frequently used for complete liquefaction of fruit pulps resulting into not only higher juice yield, but also juice with high dry matter content. It was therefore, recommended to further test the effect of different enzymes and concentrations with a mixture of cellulolytic and pectinolytic enzymes on juice yield, vitamin C content, polyphenols and antioxidant activity. In this work, pectinase enzyme was used alone because it is one of the first enzymes to be used in homes for processing juices, and the processing of *marula* is still done at a small scale. Pectinase is the upcoming enzyme for juice production as it increases yield and at the same time speeds up the extraction process (meaning it is widely used in juice production sectors).

3.3. DHA measurement from the pulp

For the conversion of dehydroascorbic acid (DHA) to ascorbic acid (AA) in *marula* pulp (chapter 5 and 6) it was recommended to search for a better converter than dithiotrheitol (DTT), for example tris-2-carboxyethyl-phosphin (TCEP) mentioned by Wechtersbach and Cigić, (2007), since TCEP works at lower pH, even at pH 2. Alternatively, it would be better if DHA could be measured directly with HPLC, as was done previously by Yuan and Chen, (1998). DHA is an intermediate product of degradation of AA which can be further degraded to form diketogulonic acid (DKGA). In heating fruit juices, the browning process can be non enzymatic and it starts with degradation of DHA through the Maillard reaction and that is regardless of the oxygen presence. It is also important to know the DHA content because it is still active as vitamin C.

3.4. Individual phenolic compounds in *marula*

An explorative study was done using online HPLC DPPH radical scavenging activity for identifying phenolic compounds and their potential antioxidant activity in fermented and unfermented *marula* juice (as described in chapter 4). Results obtained could not be presented in this thesis, as they

were not complete due to limited time. The preliminary results obtained where as follow: gallic acid, catechin, ascorbic acid and epicatechin, were identified in fermented and unfermented *marula* juice. Chlorogenic and protocatechuic acids were identified only in unfermented *marula* juice. Among the identified individual antioxidants, ascorbic acid and catechin were the main active antioxidants in unfermented juice, while in fermented juice gallic acid and ascorbic acid were the most active ones. No activity was exhibited by chlorogenic acid at its concentration in unfermented juice. According to Balasundram, Sundram and Samman, (2006) the potential health benefit derived from dietary phenolic compounds depends strongly on their absorption in the gut and metabolism. With *marula,* there is no information covering that, therefore further investigation will be needed on *marula* to study their health benefits, bioavailability and metabolic conversion in vivo.

3.5. Carotenoids from *marula* pulp

Based on a preliminary study not reported in this thesis, it is likely that *marula* contains a major carotenoid, which belongs to the Xanthophyll class. The experiment tried to identify it by its adsorption spectrum, %III/II (ratio of peak three and two in the adsorption spectrum) and mass spectrum, by comparing it to literature, but it was an unknown carotenoid belonging to the xanthophyll class. There is a chance that *marula* contains Lutein but also alpha-cryptoxanthin and antheraxanthin; this is based on preliminary identification which was done. There are many other possibilities for identification, as these carotenes are well known. Other colorants present were pheophytin a and b (= chlorophyll a or b without magnesium) and pheophorbide a (= pheophytin a without phytyl residue). Furthermore, the experiment found a trace of a beta-carotene-like structure. A lot of other compounds were also present in the extract, which have no specific colour, but might be flavonoids. To know more about molecular structures, purification is needed and analysis using, for instance, Nuclear Magnetic Resonance (NMR).

3.6. Marula flavour compounds

In Chapter 6 of this thesis it describes the use of Headspace-Solid Phase Micro Extraction (HS-SPME) and Gas Chromatography-Mass Spectrometry (GC-MS) to identify the major flavour compounds found in *marula* and to investigate the effect of processing and storage conditions on *marula* flavour compounds. Based on this finding, the following recommendation were given: it is known in literature that compounds in high concentration with a low perception level might not be responsible for typical food flavour, for example, the most abundant compound d- limonene in mandarin juices contributed less than oxygenated terpene in less concentration (Min, Callison & Lee, 2003). This means, for a better understanding of *marula* flavour, perception threshold values need to be known. However, only limited information especially for large variation of sesquiterpene found in this research can be found in literature. In this case, Aroma Extract Dilution Analysis (AEDA) was recommended for determination of most active flavour compounds in *marula* samples. Aroma extract dilution analysis is a quantitative gas chromatography olfactometry procedure used for determining the strength of odorants in food extracts. When the odorants with the highest flavour dilution factors have been identified their concentrations in foods are quantified and their odour activity values are calculated.

Furthermore, with regard to quantification of identified aroma compounds, described in chapter 6, it was done by calculating obtained peak area, assuming that response factors of compounds were the same. This semi-quantification method was done because of the lack of commercial GC standards for identified compounds. Therefore, to get more accurate data, an internal standard such as deuterated chlorobenzene can be used.

4. Concluding remarks

Mineral and nutrients composition of *marula* fruits vary greatly and cannot be compared across different geographical areas. However, it is important to note that *marula* fruit remains high in nutrients, especially vitamin C, despite differences in actual amounts in comparison to other tropical fruits. The use of enzymes such as pectinase in processing *marula* juice should be encouraged as they result in increased yields of juice and antioxidants. Moreover, from the effect of temperature and time on the quality of naturally fermented *marula* juice, it was concluded that fermenting at temperatures between 30 and 40°C for 4 to 5 days will achieve high retention of vitamin C. Furthermore, the use of unfermented *marula* should be encouraged since it retained much higher dry matter content than the fermented one. Vitamin C in *marula* was more stable than in guava and mango and processors can use heat treatment such as sterilization in processing without degrading most of the vitamin C and other antioxidants found in *marula* pulp and its juice. However, in order to maintain flavour compounds during processing of *marula* juice and its products, heating the pulp or juice for less than 10 min at the temperature of 110°C or higher might be better during processing. Lower storage temperature than 4 °C can maintain the *marula* flavour compounds for longer than 30 days without changes. Therefore, *marula* processors can store *marula* pulp or its juice at temperature lower than 4 °C, and hence be able to maintain the flavour compound for a period longer than 30 days. The skin had more variety of flavour compounds than the flesh; therefore it will be advisable for *marula* processors to incorporate the skin in the products such as juices, jams, jellies and alcoholic beverages during processing. This will enhance the unique characteristic of *marula* flavour in the products, which are currently claimed not to contain *marula* like flavour.

Although local processing is not yet fully fledged, its feasibility is enormous. Local processing of *marula* products is feasible. At the moment the only impediment to local processing for *marula* juice and its oil is lack of equipments especially for commercial purposes. M*arula* oil is currently

being processed in Europe and distributed worldwide. The *Amarula* liqueur has been processed in South Africa and sold in over 100 countries (Ref). With the help of donors or government funding especially for equipment procurement, local processing can be a success and marketing thereof will be achieved easily locally and internationally given that *marula* fruits are known and are already approved and certified for consumption in Africa and in Europe, as stipulated in vision 2030 for Namibia and Namibia Development Plan 4 (NDP4). Phytotrade Africa and other stakeholders like CRIAA, Indigenous Plant Task Team (IPTT) and the Ministry of Agriculture in Namibia are already trying to resolve this by allocating funds in uplifting the processing of indigenous plant products via supporting local community groups like Tulongeni Twahangana and Eudafano Women's Cooperative (EWC) in Namibia. This concept is not only happening in Namibia but it is reciprocated in other countries like Southern African where most *marula* trees are found. This jointly works with one purpose, and that is to alleviate poverty and protect biodiversity in the region by developing an industry that is not only economically viable but also ethical and sustainable.

Apart from oil production, *marula* juice can successfully be extracted locally and by using this finding (chapter 2) the yield can be increased with the use of enzymes such as pectinase. This can be achieved locally by training people on the use of the enzymes as well as the use of low cost procedures that can improve the juice yield. In most cases, with necessary financial and training support, local producers can procure all the necessary equipments that are necessary especially for processing under optimal conditions such as temperature controls during processing and storage as suggested in chapter 2 to 6.

Given the abundance of *marula* fruits and a systematic harvesting method that is well coordinated with collection points in all areas, an economically viable supply chain can be established especially when organised groups such as EWC in Namibia are in existence. Higher value products such as *marula* oil can be produced seasonally as well as juice since *marula* fruits are also seasonal. During the season when the fruits ripen, juice can

be produced including other related products for marketing. Afterwards, kernel oil can be produced when operations for juice processing ends and this will ensure continuous supply of marula products. This could be the basis for a continuous sustainability and contribution to livelihoods.

In general, it can be concluded that *marula* fruit is rich in vitamin C and antioxidants. It is a vital source of nutrients for many rural communities as well as a means for a livelihood. This thesis showed that improved processing methods of *marula* fruits can enhance yield as well as aid in retention of essential nutrients. Furthermore, incorporating *marula* peel during processing of *marula* juice can enhance the unique *marula*-like flavour in the final product. The most commonly used method of preservation by the rural communities is fermentation, although it leads to reduced energy content, still leads in significant levels of antioxidants and nutrient content. This thesis gives a basis for further areas of investigation on *marula* compounds, nutrients and improving processing methods. Improving the processing methods can add value to the *marula* products with better nutrition and may fetch higher prices in the market. However, the objectives were not fully achieved. Areas for future research were identified but not all areas were investigated and hence can be investigated further and this includes identification of individual antioxidants and their activity in processed and unprocessed *marula* products.

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Summary

Summary in English

Marula is a multipurpose tree from Southern Africa, highly appreciated by local people, mainly for its fruit, but also for its cosmetic oil from the seed and for medicinal products from the bark and leaves. Fruits are eaten raw, like a small mango, or used to prepare juices, jams, conserves, dry fruit rolls, and also fermented to make alcoholic beverages like beer, wine and creamy liquor called *Amarula*. The fruit is a vital source of Vitamin C for rural people most of whom cannot afford other expensive sources of vitamin C.

The specific processing methods and conditions of making *marula* juice vary among different regions. In many cases, the fruits are subjected to heat treatment before extracting the juice to soften the fruits outer skin in order to make it easier to press the juice out and the juice can also be heated after it is pressed out. The heating process can even be up to over 3 hours. In the food industry, heat treatment is always applied as an important processing step to inhibit spoilage caused by microorganisms and enzymes to prolong food shelf life. Even though it is considered as a necessary step for obtaining a more stable product, heating can affect the nutritional compounds like vitamin C and other compounds possibly in a negative way. Although it is known that *marula* fruit contains polyphenolic compounds, little is known about antioxidant activity and contents in processed *marula* products like fermented juices. In addition, the stability of these antioxidants is also unknown and the nutritional content of fermented *marula* juice is important, since the most popular use of *marula* fruit is its fermented juice.

This thesis investigated the fate of antioxidants and their activities due to heat processing and fermentation of *marula* pulp and juice. The specific objectives of this thesis were (i) to critically evaluate literature on proximate composition and nutritional value of *marula* in comparison with other tropical and indigenous fruits, in order to identify areas for further research, (ii) to determine the optimum processing conditions for maximum juice yield and retention of vitamin C, polyphenols, antioxidant activity and also clarification (colour) of the *marula* juice, (iii) to determine

the effects of fermentation temperature and time on the vitamin C, polyphenols and antioxidant activity of the naturally fermented *marula* juice, (iv) to investigate the thermal degradation of vitamin C of *marula* and (v) to identify volatile compounds in *marula* fruit and to investigate the effect of processing and storage on *marula* volatile compounds.

Chapter 2 is a critical review showing that *marula* fruit pulp has vitamin C content higher than that of most fruits, ranging from 62 mg/100 g – to over 400 mg/100 g. Additionally, *marula* fruit is reported to have an antioxidant capacity of between 8-25 mM ascorbic acid equivalents and a total phenolic content ranging from 7.5 – 24 GAE (gallic acid equivalent) mg/g dry weight. *Marula* kernels are also a good source of protein, oil, magnesium, phosphorus and potassium. *Marula* oil is used for cosmetics and in food applications. Therefore, *marula* fruits could play a vital role to the rural community who rely on the usage of the fruits and have limited access to other sources of nutrients. However, the results reported show a large variation in the measured *marula* compositional values. The cause of variation can be due to several factors like: variation in place of origin, soil and climate, storage time after harvesting before analyses were carried out and methods used to analyze the samples. Due to the variation found in the reported data, it is recommended that collection, handling and storage conditions under which the samples were handled and methods used to analyze the samples must be described in detail and authors should be more specific and clear about which part of the fruit was used during the analysis. Several authors concluded that the climate played a large role in influencing the mineral content of all *marula* products, slightly more than the origin of the tree and this is mostly due to drought or being a wet year (Ref). For the variation arising due to environmental factors one cannot easily control since most of *marula* trees grow naturally in open woodlands, bushes, in clay or sandy soil and survive in hot dry climatic conditions without irrigation or fertilization. Recommendations for future research on processing the *marula* fruit were formulated: 1. improving *marula* juice yield 2. Investigating the effect of processing and storage on the retention of nutrients such as vitamin C and its antioxidant capacity in *marula* pulp and its products, 3. Identifying

individual antioxidants and their activities in processed and unprocessed *marula* products, 4. Identifying volatile compounds in *marula* fruit and 5. investigating the effect of processing and storage on the *marula* volatile compounds.

The effect of variation in processing conditions on juice yield and quality were investigated and is described in **chapter 3.** It was shown that using pectinase (in the range of 0.1 to 0.14%) increased the yield of *marula* juice by 23% compared to not using it. The optimal extraction temperature for the content of *vitamin* C and polyphenols as well as for the antioxidant activity ranged between 40 and 60°C. Antioxidant activity was correlated to the content of vitamin C in the juice. Heating time had an effect on the lightness of the *marula* juice, changing into a darker yellow colour at prolonged heating times. The predicted optimal juice yield (54.6%) was validated by additional production runs at the predicted optimal conditions: enzyme concentration of 0.14%, heating time of 65 min. and temperature of 60°C, gave an average yield of 55.6%.

In **chapter 4**, the effects of fermentation on the retention of vitamin C, the concentration of total polyphenols and several identified individual phenols, and antioxidant activity in the naturally fermented *marula* juice were investigated. The fermentation conditions were varied: temperature ranged between 20 and 40 ºC and fermentation time from 1 to 8 days. *Marula* juice fermented at 30 to 40 ºC for 6 to 4 days, retained high antioxidant activities, which were positively correlated to their ascorbic acid and phenolic content. Overall, it was found that fermented *marula* juice is a good source of antioxidants and vitamin C.

In **Chapter 5**, the kinetics of the thermal degradation of vitamin C in *marula,* mango and guava pulp at temperatures ranging from 80 to 150°C was investigated. The results showed that for temperatures lower than 125ºC, the ascorbic acid in *marula* pulp was about 15 fold more stable than the ascorbic acid in mango and guava pulp. A First Order Degradation Model could not describe the vitamin C degradation because a biphasic behaviour was observed. Therefore, the model was transformed into a two-fraction model in which the vitamin C content was divided

into relatively stable and unstable fractions. The effect of increased in temperature was lower than expected for chemical reactions. This showed that, the degradation rate of vitamin C in *marula* was less influenced by the temperature at which it was processed when compared with mango and guava. For instance heating for 10 min at 100°C, the loss of vitamin C for the three fruits were 4% for marula, 33% for guava and 34% for mango.

In **chapter 6**, volatile flavour compounds of *marula* peel and flesh were identified and characterized and the changes in the profiles at different heating and storage conditions were investigated. The samples were heated for 1 to 60 minutes at temperatures of 40 to 110°C. The juice was stored at 4°C for three different time periods of 10, 20 and 30 days. The results revealed that *marula* peel contained more volatile compounds (75) including all the identified volatiles (41) of the flesh. The identified compounds were classified into: alkyl, aldehyde, ester, furan compound, alcohol, monoterpene and sesquiterpene. Sesquiterpene hydrocarbons (β-caryophyllene, α-humulene, E-germacrene D and β-selinene) were the most abundant compounds in flesh and peel, with β-caryophyllene as dominating compound. β-selinene, a compound usually reported as an important constituent in aroma profile of essential plant oils, was identified in *marula* for the first time. Heating conditions did not cause drastic changes in the concentration of sesquiterpene compounds at low temperatures of 40 °C and 85 °C. More marked changes could be observed at higher temperature of 110 °C for 10 minutes or longer heating time. New compounds such as oxygenated terpene, were found while flavour compounds like esters and ketones disappeared during heating. When considering storage effects, no big differences could be observed after storage at 4 °C for a maximum of 30 days. Distribution of sesquiterpene as major volatile compounds did not change much except for minor changes on ketones, furans, esters and monoterpene. Storing *marula* pulp at 4°C for up to 30 days did not affect the amount of flavour compounds. It could be advisable to incooperate the skin part in the *marula* juice processing since the peel had more flavour compounds in higher concentration than in the flesh or pulp.

In **chapter 7**, the main findings and implications of this thesis were discussed and interpreted. Moreover, recommendations for future work and advice to the *marula* processors based on the findings from this thesis are given. The main findings were that *marula* fruit was a rich source of antioxidants, especially vitamin C. The stability of vitamin C during heating in *marula* was much higher compared to mango and guava pulps. Furthermore, *marula* juice antioxidants could be retained during fermentation at 30 to 40 degrees for 4 to 5 days. The use of fresh or unfermented juice was better nutritionally since unfermented juice contained more sugar and that contributed more to energy intake of *marula* juice consumers. In addition, the nuts of these trees are also rich in oleic acid, protein, energy and minerals like iron, magnesium, zinc, phosphorus and copper. Furthermore, optimum processing conditions found in this study, could increase the juice yield to 56% and at the same time have a juice high in vitamin C and other antioxidants. Therefore, there is an opportunity for *marula* juice to be used as a source for natural antioxidants and hence benefit the rural poor communities.

Marula is one of the most utilized indigenous trees in Southern Africa because of its juice and oil products. The results presented in this thesis contribute to the knowledge on the nutritional value of *marula* juice and its pulp. Future research on *marula* should concentrate on identification of individual antioxidants and their activity in the processed and unprocessed *marula* products and to study their bioavailability and metabolism in vivo.

Samenvatting

Summary in Dutch/ Samenvatting

Marula is een veelzijdige boom uit Zuid-Afrika, die door de lokale bevolking vooral wordt gewaardeerd voor zijn vruchten, maar ook de cosmetische olie uit de zaden en de medische producten van de schors en de blaadjes zijn populair. De marulavruchten kunnen rauw gegeten worden, maar worden ook gebruikt voor vruchtensappen, jams, conserven, gedroogde fruitrollen of voor fermentatie om er alcoholische dranken zoals bier, wijn en likeur (Amarula) van te maken. Marula is een belangrijke bron van vitamine C voor de plaatselijke bevolking, die geen andere, vaak duurdere bronnen met vitamine C kunnen betalen.

Het sap van de marula wordt op verschillende manieren bereid in verschillende regio's in Afrika. In veel gevallen worden de vruchten eerst verhit om de buitenste schil zacht te maken voor het uitpersen. Na het persen kan het sap worden verhit. Het verhittingsproces kan 3 uur of langer duren. De verhittingsstap is belangrijk om microbiologisch en enzymatisch bederf tegen te gaan en daarmee de houdbaarheid te verlengen. Deze verhittingsstap kan echter ook de voedingswaarde verlagen, zoals bijvoorbeeld het vitamine-C gehalte. Hoewel het bekend is dat er polyfenolen in marulavruchten zitten, is er tot dusver weinig bekend over het gehalte aan polyfenolen en de antioxidant werking van verwerkte marulaproducten zoals bijvoorbeeld (gefermenteerde) sappen. De stabiliteit van deze antioxidanten is eveneens onbekend. De voedingswaarde van gefermenteerd marulasap is van belang omdat het gefermenteerde sap het meest geconsumeerde product van marula is.

In dit proefschrift zijn de gehaltes aan verschillende antioxidanten van marulapulp en marulasap en is hun activiteiten bepaald onder invloed van verschillende verhittingsprocessen en fermentaties. De doelen van deze thesis waren (i) het kritisch evalueren van de literatuur om de voedingswaarde van marula te vergelijken met andere tropische en inheemse vruchten, teneinde mogelijkheden voor verder onderzoek te identificeren, (ii) het bepalen van de optimale procescondities om de sapopbrengst te optimaliseren met zoveel mogelijk behoud van vitamine C, polyfenolen, antioxidantactiviteit en kleur van het marulasap, (iii) het bepalen van de effecten van temperatuur en tijd van fermentatie op het gehalte aan vitamine C, polyfenolen, en op de antioxidant

activiteit van het natuurlijk gefermenteerde marulasap, (iv) het onderzoeken van de thermische afbraak van vitamine C van marula en (v) de identificatie van vluchtige bestanddelen in de marulavrucht en het onderzoeken van het effect van de verwerking en opslag van marula op deze vluchtige bestanddelen.

Hoofdstuk 2 geeft een kritisch overzicht van de literatuur waaruit blijkt dat het vitamine C-gehalte in het vruchtvlees van marula hoger is dan dat van de meeste andere vruchten. Dit gehalte varieert van 62 mg/100 g versgewicht tot meer dan 400 mg/100 g versgewicht. Bovendien heeft marula een antioxidantcapaciteit van 8 tot 25 mM ascorbinezuur-equivalenten en een gehalte aan fenolen (totaal) variërend tussen 7.5 en 24 GAE (galluszuur-equivalenten) mg/g drooggewicht. Marulapitten zijn een goede bron van eiwitten, vetten, magnesium, fosfor en kalium. Marula-olie wordt zowel gebruikt voor cosmetica als in voeding. Marulavruchten spelen daarom een belangrijke rol voor de lokale plattelandsbevolking in Zuidelijk Afrika die afhankelijk zijn van de nutriënten uit marula en slechts beperkte toegang heeft tot andere bronnen van deze nutriënten. Er is een echter grote variatie in deze nutritionele waarden van marula gerapporteerd. Deze variatie kan worden veroorzaakt door verschillen in de plaats van oorsprong, bodem, klimaat en de opslagtijd na het oogsten, voordat de analyses werden uitgevoerd en in variatie in de methoden die worden gebruikt voor de analyse.

De variatie in de gerapporteerde samenstelling van marula door de genoemde oorzaken, maakt duidelijk dat het aan te bevelen is om in detail te beschrijven hoe de oogst, behandeling en opslagomstandigheden van de marulavruchten zijn geweest en welke methoden zijn gebruikt voor de analyse van de componenten. Bovendien zouden onderzoekers specifieker en duidelijker moeten zijn over welk deel van de vrucht is gebruikt tijdens de analyse. Verschillende auteurs concludeerden dat het klimaat een grote rol speelt op het gehalte aan mineralen in marula, iets meer dan de locatie van de boom. Variatie als gevolg van omgevingsfactoren is lastig te beïnvloeden omdat de meeste marulabomen ongecultiveerd groeien in open bossen en struiken, op klei- of zandgrond, en overleven in warme en droge klimatologische omstandigheden, zonder irrigatie of bemesting.

Aanbevelingen voor toekomstig onderzoek van de verwerking van de marulavrucht zijn: 1. Verbeteren van de sapopbrengst van de marula 2. Onderzoeken naar het effect van verwerking en opslag op het behoud van voedingsstoffen zoals vitamine C en de antioxidantcapaciteit in marulapulp en producten hiervan, 3. Identificatie van individuele antioxidanten en hun activiteit in marula en verwerkte marulaproducten, 4. Identificatie van vluchtige bestanddelen in marulafruit en onderzoeken van het effect van de verwerking en opslag op deze vluchtige bestanddelen in marula.

Het effect van variatie in de procesomstandigheden op de opbrengst en kwaliteit van marulasap is beschreven in hoofdstuk 3. Het bleek dat gebruik van pectinase (van 0.1 tot 0.14%) de opbrengst van marulasap met 23% verhoogde. De optimale extractietemperatuur voor behoud van vitamine C, polyfenolen alsmede van de antioxidantactiviteit, lag tussen de 40 en 60 °C. De antioxidantactiviteit bleek gecorreleerd te zijn met het vitamine C-gehalte in het sap. De verhittingstijd had een effect op de kleur van het marulasap. Een langdurige verwarmingstijd resulteerde in een meer donkergele kleur. De voorspelde optimale sapopbrengst (54,6%) is bij de volgende omstandigheden: enzymconcentratie (0.14%), verhittingstijd (65 min) en een temperatuur van 60 °C. Dit optimum is gevalideerd door extra producties uit te voeren bij deze condities, dit gaf een gemiddelde opbrengst van 55,6%.

In hoofdstuk 4 zijn de effecten van fermentatie op het behoud van vitamine C, de concentratie van (totaal)fenolen, van verschillende geïdentificeerde individuele fenolen, en van de antioxidantactiviteit in het natuurlijk gefermenteerde marulasap onderzocht. De fermentatie omstandigheden varieerden in temperatuur van 20 en 40 ºC, en in tijd tussen de 1 en 8 dagen. Marulasap dat 4 tot 6 dagen was gefermenteerd bij een temperatuur tussen de 30 en 40 ºC had de hoogste antioxidantactiviteit, die positief gecorreleerd was aan de gehaltes aan ascorbinezuur en de fenolische componenten. Over het geheel genomen, bleek dat gefermenteerd marulasap een goede bron van antioxidanten en vitamine C is.

In hoofdstuk 5 is het onderzoek naar de kinetiek van de thermische degradatie van vitamine C in marula-, mango- en guavepulp bij temperaturen variërend van 80 tot 150 ºC beschreven. De resultaten toonden aan dat bij temperaturen

lager dan 125 ºC, het ascorbinezuur in marulapulp ongeveer 15 keer stabieler was dan ascorbinezuur in mango- en guavepulp. De thermische afbraak van vitamine C kon niet met een eerste orde model worden beschreven. Er werden duidelijk twee fasen waargenomen in de afbraaksnelheid. Het model werd daarom aangepast tot een twee-fase model waarbij aangenomen werd dat het vitamine-C in marula aanwezig is een stabiel en een onstabiel deel. Het effect van de stijging van de temperatuur op de afbraaksnelheid is lager dan verwacht voor chemische reacties. In vergelijking met mango en guava wordt de afbraaksnelheid van vitamine C in marula minder beïnvloed door temperatuurstijging. Bij verhitting gedurende tien minuten bij 100 ºC, bijvoorbeeld, is het verlies aan vitamine C in de drie vruchten: 4% voor marula, 33% voor guava en 34% voor mango.

In hoofdstuk 6 zijn de vluchtige aromaverbindingen van de marulaschil en het vruchtvlees geïdentificeerd en gekarakteriseerd. Tevens zijn de veranderingen in de aromaprofielen bij verschillende verhittings- en opslagomstandigheden onderzocht. De monsters werden tot 60 minuten verhit bij temperaturen van 40 tot 110 ºC. Het sap werd tot 30 dagen bij 4 ºC opgeslagen. Uit de resultaten bleek dat de marulaschil meer geïdentificeerde vluchtige componenten (75) bevatte dan het vruchtvlees, dat 41 componenten bevatte, die allen ook in de schil gevonden werden. De geïdentificeerde componenten werden ingedeeld in: alkyl, aldehyde, ester, furan, alcohol, monoterpeen en sesquiterpeen verbindingen. Sesquiterpeenkoolwaterstoffen (β-caryofylleen, α-humuleen, E-germacreen D en β-selineen) waren de meest voorkomende stoffen in het vruchtvlees en de schil, met β-caryofylleen als hoofdbestanddeel. β-selineen, een component die gerapporteerd wordt als een belangrijk bestanddeel in aromaprofielen van essentiële plantaardige oliën, is voor het eerst in marula geïdentificeerd.

De omstandigheden van de verhitting leidden niet tot dramatische veranderingen in de concentratie aan sesquiterpenen bij temperaturen van 40 °C tot 85 °C. Meer opvallende veranderingen konden worden waargenomen bij een hogere verhittingstemperatuur van 110 °C en een verhittingstijd van 10 minuten of langer. Nieuwe verbindingen zoals geoxideerde terpenen werden gevonden, terwijl esters en ketonen tijdens verwarming verdwenen. Bij het onderzoek

naar de effecten van de opslagcondities konden geen grote verschillen in de verdeling van de sesquiterpenen worden waargenomen na een opslag tot 30 dagen bij 4 °C, met uitzondering van kleine wijzigingen in ketonen, furanen, esters en monoterpenen. De opslag van marulapulp voor 30 dagen bij 4 °C heeft dus weinig invloed op de vluchtige stoffen. Aanbevolen wordt om een deel van de schil mee te verwerken bij de bereiding van marulasap, omdat de schil meer aromatische componenten bevat dan het vruchtvlees.

In hoofdstuk 7 zijn de belangrijkste bevindingen en implicaties van het onderzoek besproken en geïnterpreteerd. Bovendien zijn aanbevelingen gegeven voor toekomstig onderzoek en adviezen gegeven voor verwerkers van marula op basis van de bevindingen van deze thesis. De belangrijkste bevindingen waren dat marulavrucht een rijke bron van antioxidanten en met name van vitamine C is. De stabiliteit van vitamine C tijdens verhitting in marula is veel hoger in vergelijking met mango- en guavepulp. Antioxidanten in marulasap zijn bovendien 4 tot 5 dagen stabiel tijdens de fermentatie op 30 tot 40 ºC. Het gebruik van vers of niet-gefermenteerd sap is beter vanuit een nutritioneel oogpunt, omdat niet-gefermenteerd sap meer suiker bevat dat bijdraagt aan de energie-inname.

Naast de nutritionele waarde van het sap van de marula, zijn de noten van de marula rijk aan oliezuur, eiwitten, energie en mineralen zoals ijzer, magnesium, zink, fosfor en koper. De optimale procescondities die werden gevonden in deze studie kunnen de sapopbrengst verhogen tot 56% met tegelijkertijd een hoog gehalte aan vitamine C en andere antioxidanten. Marulasap kan daarom worden gebruikt als een bron van natuurlijke antioxidanten voor arme plattelands gemeenschappen.

Marula is één van de meest gebruikte inheemse bomen in zuidelijk Afrika vanwege haar sap en olieproducten. De resultaten uit dit proefschrift dragen bij aan de kennis van de voedingswaarde van marulasap en marulapulp. Toekomstig onderzoek naar marula kan zich concentreren op de identificatie van individuele antioxidanten en hun activiteit in onverwerkte en verwerkte marulaproducten. Ook is het nuttig om hun biologische beschikbaarheid en metabolisme *in vivo* te bestuderen.

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About the author

Curriculum Vitae

Publications

Overview of the completed training activities

Curriculum Vitae

Penny Hiwilepo Van Hal was born on the 13th of March 1976 in Ohangwena Region, Namibia. Her career started as a FAO enumerator at the Ministry of Agriculture, Water and Forestry (MAWF) during the period of 1997 and 1999 whilst pursuing her studies towards a Bachelor of Science degree in Food Science and Technology at the University of Namibia, which she completed in 2000. In 2001, Ms. Hiwilepo-van Hal got an employment offer from the University of Namibia as a Staff Development Fellow from which she was able to do her MSc in Food Safety at Wageningen University (2002 – 2004). During 2002, she did a research with CIRAD-France on Marula processing and a great interest developed that prompted her to further research on Marula fruits. Upon completion of her MSc degree, she then returned to her staff development position at University of Namibia since 2004-2007 from which she ultimately became a lecturer at the Department of Food Science and Technology. However, because she still had a dream to know more about marula fruits set aside, the interest continued to grow intensely, hence prompted her to enroll for PhD in the Product Design and Quality Management at Wageningen University in collaboration with the University of Namibia as from 2008. Throughout her academic career, Ms. Hiwilepo-van Hal gained over 10 years of experience gained largely in educational institutions alliances and development. Penny is married to Bart van Hal and they have one daughter Payton-Lao.

List of publications

Accepted papers

Hiwilepo-van Hal, P., Bosschaart, C., van Twisk, C., Verkerk, R. & Dekker, M. (2012). Kinetics of thermal degradation of vitamin C in marula fruit (*Sclerocarya birrea subsp. Caffra*) as compared to other selected tropical fruits. *LWT-Food Science and Technology*, *49*, 188 – 191.

Hiwilepo-van Hal, P. Bille, P. G., Verkerk, R., & Dekker, M. (2013). The effect of temperature and time on the quality of naturally fermented marula (*Sclerocarya birrea subsp. Caffra*) juice. *LWT-Food Science and Technology*, *53*, 70 – 75.

Submitted and in preparation to be submitted papers

Hiwilepo-van Hal, P., Bille, P. G., Verkerk, R., van Boekel, M. A. J. S. & Dekker, M. A review of the proximate composition and nutritional value of Marula (*Sclerocarya birrea subsp. Caffra*).

Hiwilepo-van Hal, P., Robben, J., Verkerk, R., van Boekel, M. A. J. S. & Dekker, M. Optimising the juice yield and quality of marula fruit (*Sclerocarya birrea subsp. Caffra*) with pectolytic enzymes by a response surface method.

Hiwilepo-van Hal, P., Li, G., Verkerk, R., & Dekker, M. Extraction and characterization of volatile compounds of the peel and flesh of marula fruit (*Sclerocarya birrea subsp. Caffra*).

Overview of the completed training activities

Discipline specific activities and courses

Food Fermentation, 2008, VLAG Wageningen, NL

International Food Safety, 2008, Michigan State University, USA

Master Class starting with the Client: New Approaches to effective health promotion, 2009, VLAG Wageningen, NL

University of Namibia FST Trip, 2009, Stellenbosch University, ZA

Reaction kinetics in food science (6th edition), 2009, VLAG Wageningen, NL

Food perception and food preference, 2009, VLAG Wageningen, NL

Scientific methodology for proposal writing & presentation workshop, 2009, IFS-Sweden

Euro-mediterranean symposium Fruit & Veg processing: poster, 2011, Avignon University, FR

General courses

VLAG PhD week, 2009, VLAG Wageningen, NL

PhD Competence Assessment, 2009, WUR Wageningen, NL

Information Literacy, including introduction Endnote, 2009, WUR Wageningen, NL

Techniques for writing and presenting a scientific paper, 2009, WUR Wageningen, NL

Teaching and supervising MSc students, 2009, WUR Wageningen, NL

Project and time management, 2011, WGS Wageningen, NL

Career orientation course, 2012, WUR Wageningen, NL

Optionals courses and activities

Dairy Science and technology, 2008, PDQ Wageningen, NL

PhD tour to Australia, 2010

PhD Proposal writing, 2008 - 2009

PDQ meetings, 2008 – 2013, PDQ Wageningen, NL

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