# Enzymatic debittering of grapefruit peel juice

**by**

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**Presented in partial fulfillment of the requirements for the degree**

**MSc (Agric) Food Science and Technology**

**Department of Food Science**

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**University of Pretoria**

**South Africa**

**Pretoria**

**January 2012**

I declare that the dissertation, which I hereby submit for the MSc (Agric) degree in Food Science and Technology at the University of Pretoria is my own work and has not been previously submitted by me for a degree at any other University or institution of higher education.

…….

Signature

…….

Date

# ABSTRACT

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by

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Department: Food Science
Course: MSc (Agric) Food Science and Technology

Vast amounts of waste consisting of peels, segment membranes and seeds are generated during grapefruit juice processing. The peels can be used for juice extraction to obtain grapefruit peel juice. Grapefruit peel juice can be a relatively cheap product and can be used as juice fillers. Extreme bitterness due to the compounds naringin and limonin limits the use of grapefruit peel juice in such applications. The aim of this study was to determine the effects of the enzymes aromase and laccase on the bitter compounds naringin and limonin and other physico-chemical properties of grapefruit peel juice.

Grapefruit peel juice was prepared by freezing milled peel residues, defrosting and pressing the juice through a screen. The peel juice was treated with aromase (0, 0.4 and 0.8% w/v) and laccase (0, 1.5 and 3.0% w/v) in a 3 x 3 factorial experiment. Reverse-phase HPLC was used to determine naringin, naringenin and limonin contents. Sugars (glucose, fructose, sucrose and rhamnose) were determined using liquid chromatography, anion-exchange chromatography with pulsed amperometric detection and gas chromatography-mass spectrometry. The colour and clarity were also determined. A 25-member consumer sensory panel was used to rate the juice samples for bitterness.

Treating grapefruit peel juice with increasing concentrations of aromase decreased naringin content by 80% and increased naringenin by 85 times. Increasing concentrations

of laccase only decreased naringin by up to 40% and increased naringenin by 4 times. Aromase-laccase combination treatment at their highest concentrations produced the greatest decrease in naringin. Glucose content increased by 1.2 times on treating with aromase and by 0.95 times on treatment with laccase. The combination enzyme treatment produced the greatest increase in glucose by 2.0 times. There was no evidence of release of rhamnose upon aromase treatment. The rhamnose moiety (from the disaccharide moiety of naringin) may be broken down into other compounds due to other activities of aromase.

Limonin was decreased by 8 times on treatment with aromase and by 1.2 times on treatment with laccase. The combination enzyme treatment decreased limonin by up to 6 times. The untreated grapefruit peel juice showed an increase in limonin content by almost 30% after storage for 7 months while the aromase-treated sample showed a decrease in limonin by 35%, an indication that aromase can be used to prevent delayed bitterness in grapefruit peel juice.

The grapefruit peel juice became darker on treatment with laccase and lighter on treatment with aromase. The combination treatment made the grapefruit peel juice darker compared to treatment with laccase on its own. Treatment with aromase increased clarity by 25% by making it less hazy.

Although the decrease in naringin due to treatment with aromase on its own was less than the combination enzyme treatment, the aromase-treated sample was ranked by the sensory panel as least bitter followed by the combination enzyme-treated, laccase-treated and the untreated samples. This may be due to the greater decrease in limonin in the aromase-treated sample compared to the other samples.

In summary, this research shows that aromase can be used either on its own or in combination with laccase to debitter grapefruit peel juice, although it can also be used in combination with laccase. The use of these enzymes provides the citrus processing

industry with alternative and possibly more cost-effective methods of debittering citrus products.

# ACKNOWLEDGEMENTS

Our Heavenly Father, for all the strength and wisdom during the research period. I was constantly reminded of how almighty He is.

I would like to thank the following people, without whom this project would not have been a success:

Dr KG Duodu and Dr MN Emmambux, my supervisors, for all their valuable advice and guidance and support throughout this study, I do not know what I would have done without it.

Cape Fruit Processors (Pty) Ltd for the financial support of the project.

Amano Enzymes for providing the enzymes and Dr Shotaro Yamaquchi and Mr Masamichi Okada for technical support and assisting with the limonin HPLC analysis.

Marie Smith for assistance and advice on statistical analysis of data.

Lida Barnard for her friendliness and help with typing and layout of the dissertation.

Liza du Bruin and co workers at the laboratory at Cape Fruit Processors (Pty) Ltd.

Elsie Verheem at the Merensky Library, University of Pretoria for her friendliness and willingness to lend a helping hand by introducing me to the current technology used.

CJ Schoeman for asssting in the naringin and naringenin HPLC analysis and all the students in the Department of Food Science, University of Pretoria for their friendship and eagerness to lend a helping hand.

Nico Stolze and Edward Sibya at Transvaal Suiker Beperk for their assistance with the sugar HPLC analysis.

My sister Retha Carelsen and brother in law and their children for their hospitality and providing accommodation during my visits to the University.

My parents for making it all possible through their constant encouragement, advice, love and support, I dedicate this dissertation to them.

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# CHAPTER 1: INTRODUCTION

## 1.1 Statement of the problem

The projected world grapefruit production for 2010 was more than 5.5 million metric tons (Landaniya, 2008). South African grapefruit production was estimated at 370,000 metric tons for marketing year 2011 (USDA Foreign Agricultural Service Global Agricultural Information Network Report). About 60% of the grapefruit commercial crop today is processed into juice and segments (Jaffee, 1999). The fresh weight of mature grapefruit consists of 35 to 50% juice, with the remainder made up of peel, segment membranes and seeds (Sinclair, 1972). After extraction of the juice, the remainder of the fruit peel, membranes, juice vesicles and seeds are discarded as waste (van Heerden et al., 2002). In South Africa waste generated by citrus processing plants in 2007 varied between 165000 to 260000 tonnes. The peel residue contains about 80 – 85% of moisture which is liberated as peel juice on treating the peel residue with calcium salts (Kimball, 1999). The peels are dried and used as ruminant feed (Bampidis et al., 2006). Citrus peel waste can also be composted after peel juice removal (van Heerden et al., 2002).

The peel juice, with chemical oxygen demand (COD) of 152000 ppm and biological oxygen demand (BOD) of 59000 ppm (Crandall, 1980) can contribute to water pollution if not utilized properly. The waste streams containing peel juice are harmful to microorganisms and livestock due to the high concentration of organic compounds including terpene-containing oils and flavonoids. The peel juice also contains significant amounts of complex, insoluble carbohydrates and adds to the organic load of the waste water (Burton et al., 2007). There are many applications for citrus peel juice. It can be used as juice filler or sweetener in juice formulations i.e. drinks and it can also be clarified and used as a substitute for apple or pear juice in 100% juice formulations. It can also be used as a sweetener in canned fruit. However the use of grapefruit peel juice is limited to grapefruit juices and products due to its bitterness. There is a need for research in order to remove bitterness to make the peel juice useful for other applications.

Naringin is the principal bitter substance in grapefruit. Its intense bitterness is said to exceed that of quinine and is detectable in water containing as little as 0.05 mg/kg (Prakash et al., 2002). Naringin occurs in high concentrations in the albedo layer or inner layer of the peel, in seeds, in the core and in membranes of citrus fruit (Carl et al., 1960). Its taste threshold in water is approximately 20 ppm. Naringin is abundant in immature grapefruit but its concentration decreases as fruit ripens (Munish et al., 2000). Naringin ranges from about 218 to 340 ppm with a general trend to diminish towards midseason and increase again towards the end of the season (Tatum et al., 1972). Limonin is another bitter principle in grapefruit and causes delayed bitterness. The sensory threshold is 4 to 6 ppm (Fayoux et al., 2007). Limonin in processed juice acts synergistically with naringin to cause bitterness (Munish et al., 2000).

The enzyme naringinase is currently commercially used to remove naringin, however, this process is rather expensive (Prakash et al., 2002). The use of the enzyme limonoate dehydrogenase to prevent the formation of limonin was found not to be economically viable (Hasegawa et al., 1975). Resins are normally used in industry to remove limonin (Fayoux et al., 2007). There is a growing interest in using other enzymes to remove bitter compounds in citrus products which may be more efficient and possibly more cost-effective. Two of such enzymes that could possibly be used are aromase and laccase. Aromase is a β-primeverosidase as well as a β-glucosidase and will convert di-glycosides to aglycone form efficiently (Merrett, Amano Enzymes, personal communication). Laccase is a polyphenol oxidase oxidizing various aromatic and non-aromatic compounds by a radical reaction mechanism (Claus, 2004). Successful debittering of grapefruit peel juice with aromase and laccase could provide the citrus processing industry with alternative and possibly more cost-effective methods of debittering citrus products.

## 1.2 Literature review

Citrus juices are the most common among the fruit juices around the world and constitute a major portion of the food industry. Citrus fruit date back as far as 2200 BC, when tributes of mandarins and pummelos were presented in the imperial court of Ta Yu in China (Webber, 1967).

Grapefruit and pummelos (shaddocks) are closely related. In fact, many believe that the grapefruit is a hybrid or variety of the pummelo. The grapefruit (so named, it is believed, because the fruit grows in clusters like grapes) is recognized as the separate species *Citrus paradisi*. There are three pummelo species: *Citrus grandis*, the most common, *Citrus maxima*, and *Citrs decumana* (Kimball, 1999).

The United States is the top producer of grapefruit in the world followed by China and South Africa (Landaniya, 2008). South Africa exported approximately 1.5 million metric tons of citrus to growing markets in the Middle and Far East. South Africa ranks as the world's second largest exporter of fresh citrus fruit by volume behind Spain, and is ranked 14$^{th}$ in world citrus production (Jaffee, 1999). South African grapefruit production was estimated to be 370,000 metric tons for marketing year 2011 (USDA Foreign Agricultural Service Global Agricultural Information Network Report). South African grapefruit is harvested from mid-April to late June (Landaniya, 2008). Grapefruit is mainly grown in the Limpopo and Mpumalanga provinces of South Africa. The two provinces are characterized by warm subtropical climatic temperatures which are best suited for grapefruit production. The most popular grapefruit varieties in South Africa are Star Ruby and Marsh (USDA Foreign Agricultural Service Global Agricultural Information Network Report).

The anatomical parts of the grapefruit can be seen in Figure 1.2.1 showing a cross section of grapefruit. The grapefruit juice is localized in the juice sacs, and the flavedo, albedo, segment membranes and seeds form peel waste after the grapefruit juice is extracted.

### 1.2.1 Production of grapefruit peel juice

The extraction of juice from grapefruit creates a large amount of waste by-product in the form of grapefruit peel, seeds, rag (the membranes between the citrus segments) and pulp (Jones, 2006). Peel juice is obtained by shredding the peel waste from the extraction process to provide peel liquid slurry and pressing to produce a spent peel cake and a raw peel juice which consists of water, sugars, flavour components and oils (Chu et al., 2006). Calcium oxide (CaO), commonly known as lime is normally added to the shredded peel to enhance the peel juice extraction. Unlimed peel is very slimy and retains moisture. The slimy nature is probably due to hydrogen bonding of the ester groups of the pectin with water. Lime demethoxylation increases the ionic strength of these bonds giving a less slimy texture (Kimball, 1999).

About 0.15 – 0.25% lime is added to shredded peel and allowed to react for 3 to 15 minutes. The exact mechanism by which the lime enhances peel juice extraction is not known. The peel consists of 80 to 85% water (Kimball, 1999). The lime contacts free water producing calcium hydroxide ($Ca(OH)_2$), which is toxic. The addition of the lime renders any subsequent products unsuitable for human consumption (Jones, 2006). Other methods need to be investigated to remove or reduce the water content of grapefruit peels to make the grapefruit peel juice more fit for human consumption.

### 1.2.2 Bitterness in grapefruit peel juice

In citrus juices, two compounds produce essentially all of the bitter taste, limonin and naringin (Kimball, 1999). The non-bitter precursor (limonoate A-ring lactone) is flavorless and exists in the juice cell cytoplasm and membranes in the fruit. When the fruit is extracted, this precursor comes in contact with the acid environment of the juice and undergoes a slow conversion to the bitter limonin (Zukas et al., 2004). Naringin occurs in high concentrations in the albedo layer, in seeds, in the core and in membranes of vegetables and fruit (Carl et al., 1960).

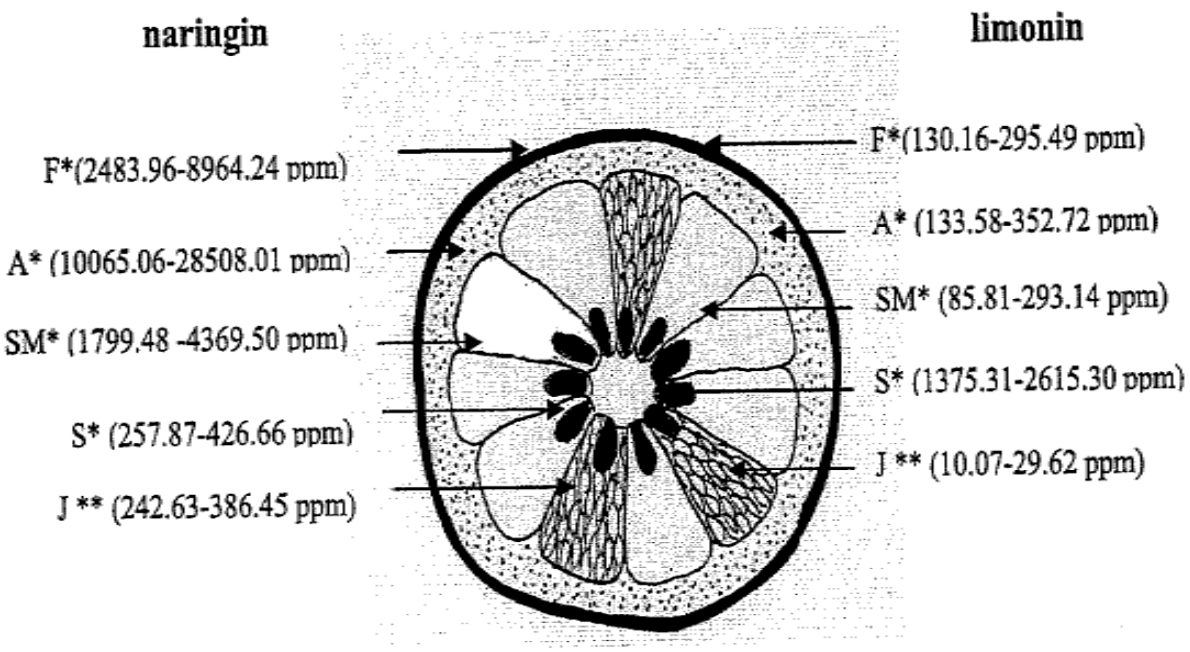



**Figure 1.2.1** Cross section of grapefruit showing distribution of limonin (mg/kg) and naringin (mg/kg) in each fruit part. F=Flavedo, A=Albedo, SM=Segment membranes, S=seeds, J=juice.

*Unit of limonin concentration in flavedo, albedo, segment membranes and seeds from dry weight.

**Unit of limonin concentration in juice from fresh weight. (Pichaiyongvongdee and Haruenkit, 2009)

The distribution of limonin and naringin in each fruit part of grapefruit can be seen in Figure 1.2.1 (Pichaiyongvongdee and Haruenkit, 2009). The juice has a naringin content of 386.45 ppm compared to a total naringin content of 42268.41 ppm in the flavedo, albedo, segment membranes and seeds which are part of the peel waste used to produce peel juice. Limonin content on the other hand is 29.62 ppm in juice compared to a total limonin content of 3556.65 ppm in the flavedo, albedo, segment membranes and seeds which are part of the peel waste used to produce peel juice. One can therefore expect that grapefruit peel juice would be more bitter than the grapefruit juice itself. Naringin was found to be the major flavonoid in grapefruit followed by narirutin and hesperidin (Ross et al., 2000). The intense bitterness due to naringin is said to exceed that of quinine and is detectable in water containing as little as 0.05 mg/kg (Prakash et al., 2002). The development of excessive

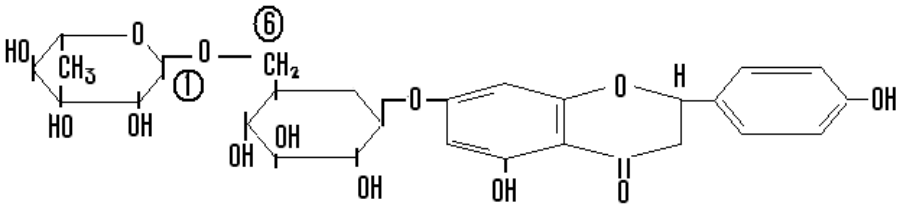
bitterness is a major problem for the citrus industry, especially in processed products (Lindsay, 1996).

### 1.2.3 Structure and chemistry of the major bitter compounds in grapefruit

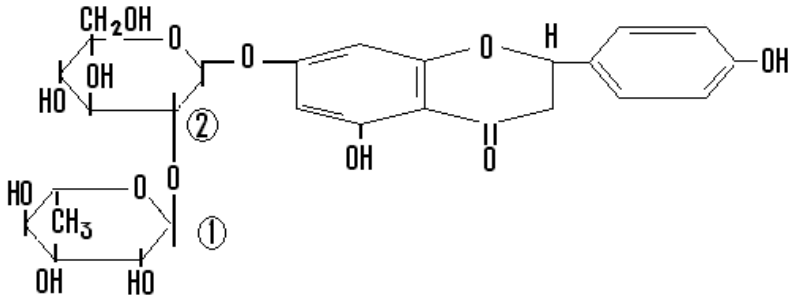
#### 1.2.3.1 Naringin

The flavanone glycoside naringin consists of an aglycone naringenin and a disaccharide consisting of rhamnose and glucose. Naringin is one of the main glucosides of naringenin found in grapefruit with the other being narirutin. The flavanone glycosides exist as structural isomers of which one will be intensely bitter while the other is tasteless. The flavanone portion of the bitter molecule is tasteless, while the glycoside portion is tasteless or slightly sweet (Rouseff et al., 1980). With naringin the sugar attached is neohesperidose and with narirutin, the sugar is rutinose (Figure 1.2.2). Both sugars are disaccharides of rhamnose and glucose. Bitterness is observed only when the sugars and the flavanone aglycone are linked as explained in the next paragraph.

While no single structural feature has been associated with bitterness, the linkage between the sugars rhamnose and glucose and the flavanone is very important. Linked between C-1 in the rhamnose to the C-2 in glucose, the resulting disaccharide is called neohesperidose. If the essentially tasteless neohesperidose is linked to a flavanone through the 7-hydroxy position (as shown in Figure 1.2.2 for naringin), the resulting flavanone glycoside will be intensely bitter. However, if the same sugars are linked C-1 to C-6 to form rutinose, the resulting molecule (as shown in Figure 1.2.2 for narirutin) is tasteless. If the rhamnose portion of the neohesperidoside is removed from naringin, the resulting glucoside (prunin) is still bitter, but at a much reduced intensity.



Narirutin (naringenin 7 – ß - rutinoside)

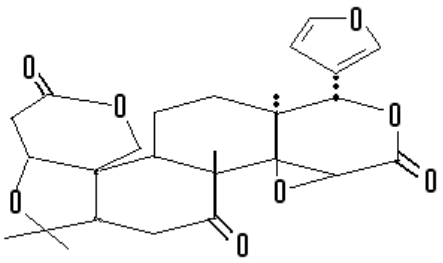


Naringin (naringenin 7 – β – neohesperidoside)

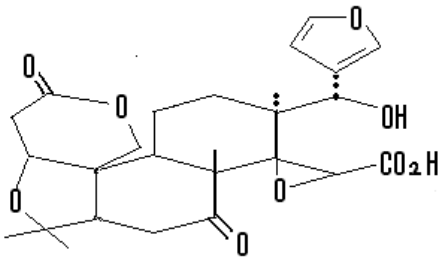
**Figure 1.2.2** Structural isomers of naringenin illustrating the two possible configurations of the sugars attached at the 7 position. Naringin is bitter whereas narirutin is tasteless (Horowitz et al., 1964).

### 1.2.3.2 Limonin

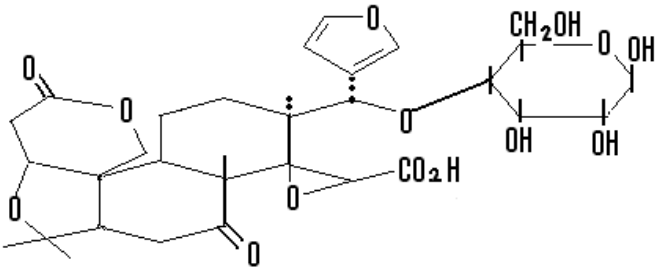
Limonin can be described as a limonoid compound. Citrus limonoids are highly oxygenated triterpenoid compounds present in the Rutaceae and Meliaceae family (Berhow, et al., 2000). Limonoids occur in a variety of citrus tissue in significant quantities as aglycones, glucosides or A-ring lactones (Figure 1.2.3).



Limonin (bitter)



Limonoate A-ring lactone (non-bitter precursor)



Limonin glucoside

**Figure 1.2.3** Structures of limonin, limonin A-ring lactone and limonin glucoside (limonin 17-ß-D-glucopyranoside) (Zukas et al., 2004).

These complex triterpenoids display significant biological activity and have been speculated to function in plants as protective agents against plant predators (Roy et al., 2006). The chemical structure of limonin includes a furan ring, two lactone rings, a five-membered ether ring, and a three-membered ether ring or epoxide. All other citrus limonoids also contain the furan ring and at least one of the lactone rings (Maier et al., 1980). The two lactone rings in limonin can open reversibly (Agrigoni et al., 2005).

### 1.2.4 Biosynthesis and location of limonoids

Nomilin is most likely the initial precursor of all the limonoids. Nomilin biosynthesis begins in the region of the stem tissue and migrates to other tissue such as leaves, fruit

tissue and seeds (Hasegawa et al., 1980. Limonin is synthesized from nomilin via obacunone and ichangin (Figure 1.2.4) (Hasegawa et al., 1985).

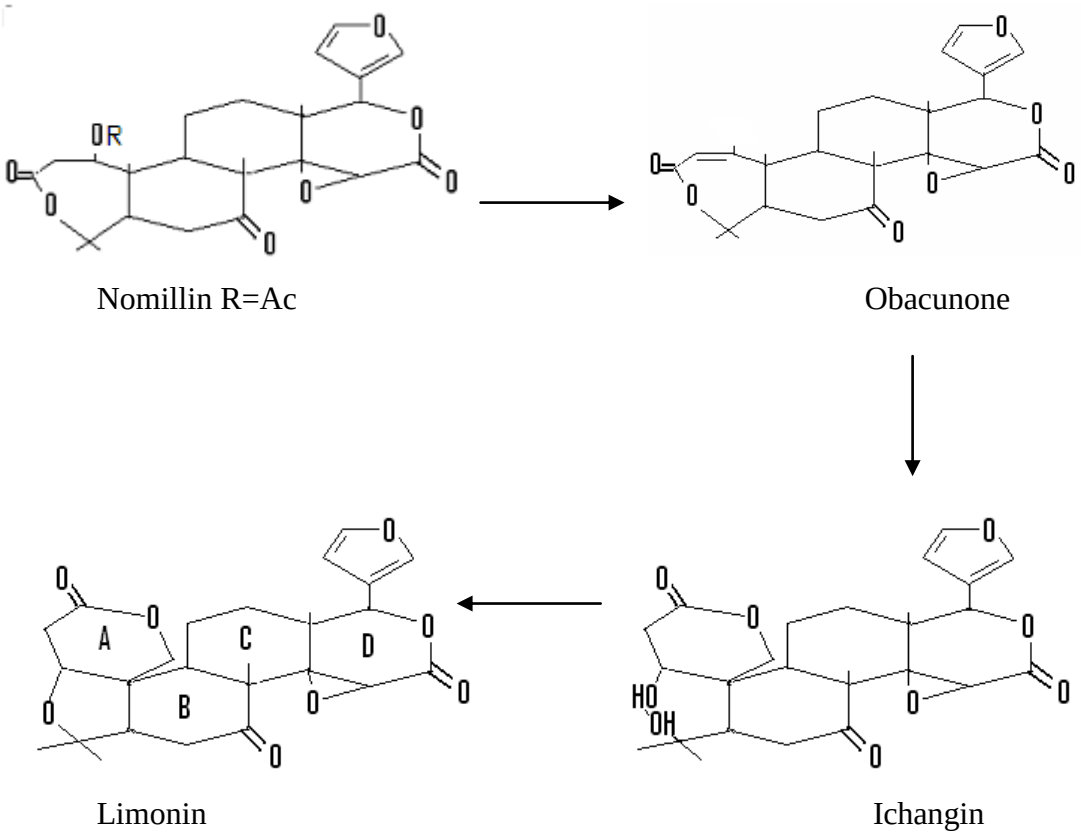



**Figure 1.2.4** Biosynthetic pathway for production of limonin (Hasegawa et al., 1985).

The monolactones such as limonoate A-ring lactone are the predominant limonoids present in citrus leaf and fruit tissue whereas the dilactones such as limonin are the predominant limonoids in seeds. Limonin is the predominant limonoid aglycone (Maier et al., 1969) and limonin glucoside is the predominant limonoid glucoside in fruit tissue (Ozaki et al., 1991). The accumulation of high concentrations of both limonoid aglycones and glucosides in citrus seeds involves the conversion of newly synthesized monolactones (the D-ring open limonoid aglycones) to dilactones (the D-ring closed limonoid aglycones) by the action of the limonoid D-ring lactone hydrolase during fruit growth (Fong et al., 1990). Two enzymes, limonin D-ring lactone hydrolase and limonoid glucosyltransferase (Figure 1.2.5) are competitive with each other for newly biosynthesized monolactones (Herman et al., 1991).

### 1.2.5 The phenomenon of delayed bitterness in citrus fruit

Most citrus fruits generally do not taste bitter if eaten fresh or if the freshly squeezed juice is consumed. However within a few hours at room temperature or overnight in a refrigerator, the juice from some citrus varieties becomes bitter (Berhow et al., 2000). The gradual development of bitterness or delayed bitterness is caused by the formation of the intensely bitter limonin from a non-bitter precursor (Figure 1.2.5) (Fayoux et al., 2007).

The mechanism for delayed bitterness was not fully understood until 1968 when Maier and Beverley (1968) identified a monolactone as the precursor of limonin. There were two possible monolactones occurring naturally, an A-ring lactone and a D-ring lactone (Arigoni, 2005; Maier, 1969). The monolactone, limonoate A-ring lactone was finally identified as the precursor of limonin (Maier and Margileth, 1969).

Citrus fruits do not normally contain limonin but rather the non-bitter precursor limonoate A-ring lactone (Maier et al., 1980). The precursor undergoes a slow conversion to the bitter limonin when in contact with the acid environment of the juice. Thermal processing accelerates this reaction (Barrett et al., 2005) Limonin D-ring lactone hydrolase isolated from citrus will also accelerate the conversion to limonin (Maier et al, 1980).

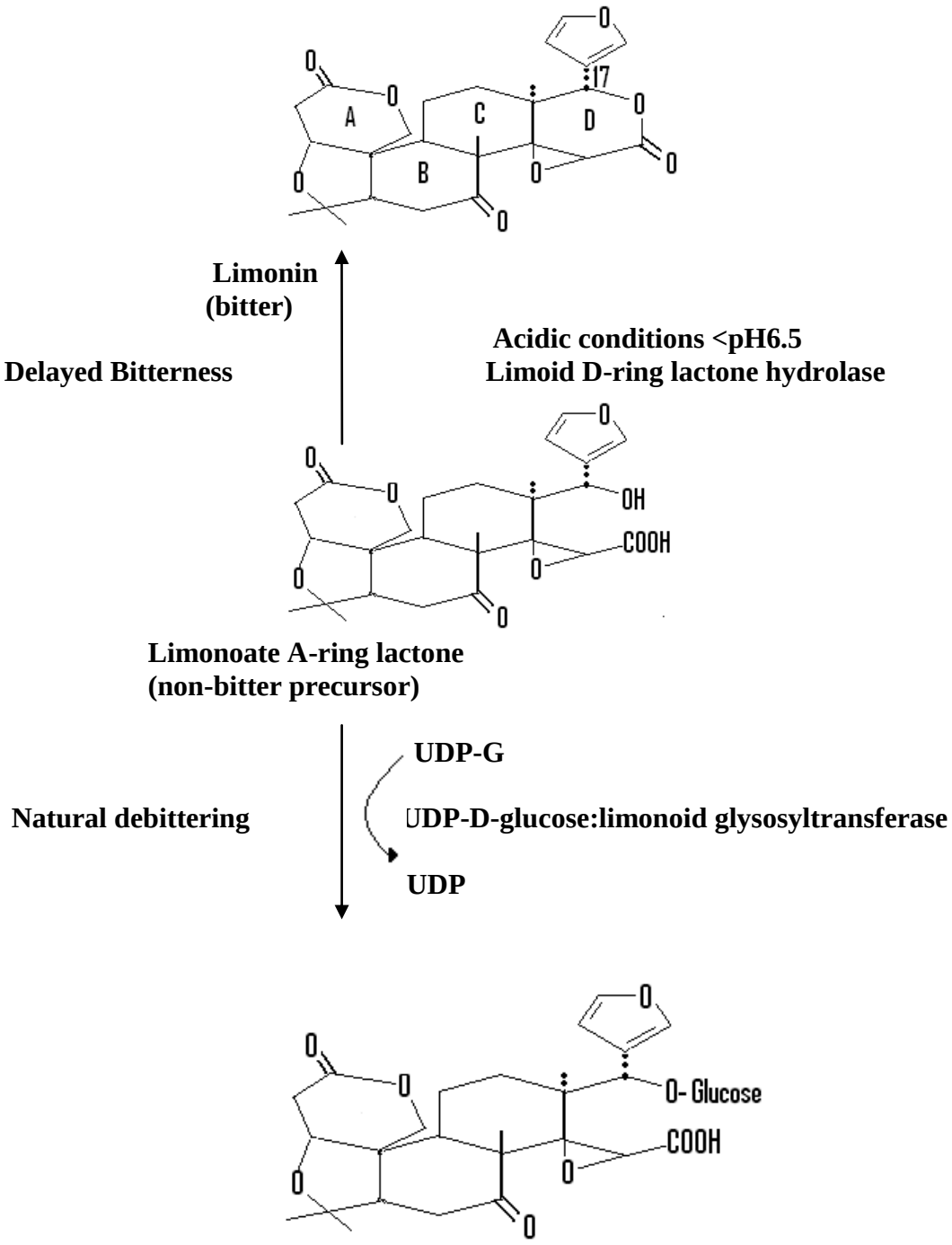



**Figure 1.2.5** Mechanism of delayed bitterness in citrus juice and of the natural debittering process in citrus (Ozaki et al., 1991; Zukas et al., 2004; Hasegawa et al., 1991. Herman et al., 1991; Maier et al., 1969; Fong et al., 1990; Fayoux et al., 2007).

The fruit has a natural debittering process that comes into effect during late stages of fruit growth and maturation (Hasegawa et al, 1991). During this process, there is conversion of limonoate A-ring lactone to tasteless limonin glucosides, such as limonin 17-L-D glucopyranoside (Figure 1.2.5) (Hasegawa et al, 1991). The conversion is catalyzed by the enzyme UDP-D-glucose-limonoid glucosyltransferase (limonoid glucosyltransferase). This enzyme catalyzes the transfer of a glucose unit from uridine diphospho glucose (UDPG) to limonoids to yield non-bitter glucosides, which reduces limonoid bitterness in citrus (Karim & Hashinaga, 2010). Its activity appears to occur only in mature fruit tissue and seeds (Hasegawa et al, 1991). All limonoid glucosides are non-bitter and each has one D-glucose molecule attached to the C17 position of the limonoid molecule via a ß-glucosidic linkage such as limonin 17 ß-D glucopyranoside (Herman et al., 1991). The limonoid content in citrus fruits decreases during the process of ripening and is considered to be due to this natural debittering process involving the conversion of limonoids to corresponding glucosides.

### 1.2.6 Enzymatic debittering of citrus juices and products

#### 1.2.6.1 Use of naringinase

Naringinase is produced by the fungus *Aspergillus niger* using easily available, inexpensive industrial waste such as sugarcane bagasse and citrus peel (Puri and Baneriee, 2000). Naringinase can be used in debittering by its ability to break down naringin (Thomas et al., 1958) due to the fact that it possesses σ-rhamnosidase and ß- glucosidase activities. The enzyme firstly hydrolyses naringin to prunin and rhamnose by its σ-rhamnosidase activity. Prunin is then broken down into naringenin and glucose by the ß-glucosidase activity (Prakash et al., 2002). The enzymatic reaction is shown in Figure 1.2.6.

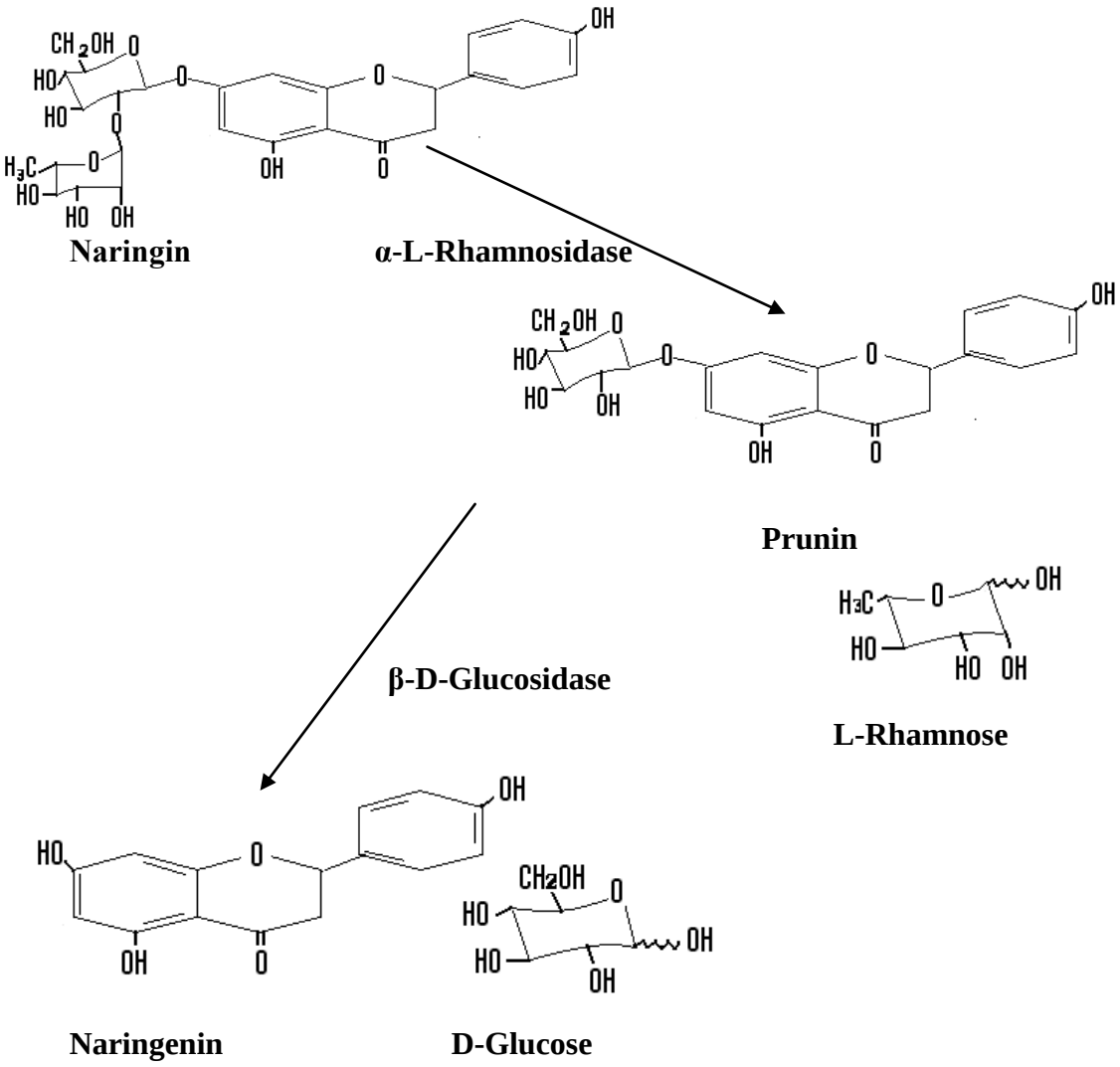



**Figure 1.2.6** Enzymatic hydrolysis of naringin by naringinase (Puri et al., 2011).

Apart from using naringinase, some methods proposed for removing naringin from grapefruit products include acid hydrolysis and the use of activated carbon. Acid hydrolysis of the naringin glycoside yields rhamnose, glucose and the non-bitter aglycone, naringenin, but such a procedure would be too drastic for practical application (Thomas et al., 1958). Under the proper conditions of pH and temperature, activated carbon will almost completely remove naringin (Burdick and Maurer, 1950) but desirable flavor constituents are also removed and this method is not too desirable if flavour compounds are to be retained. Against this background, enzymatic hydrolysis of naringin is regarded as a more

suitable method. The naringinase enzyme is applied in various forms. For example, naringinase from *Aspergillus niger* can be immobilized in a hollow fiber (Olsen et al., 1964), naringinase from *Penicillium* spp can be immobilized in packaging films (Soares and Hotchkiss, 1998) and amberlite and alginate can also be used to entrap the naringinase enzyme (Mishra and Kar, 2003). Table 1.2.1 shows some examples of the use of naringinase to remove naringin from citrus products. As shown in Table 1.2.1, naringinase can be used at various concentrations and under different conditions of pH, temperature and time to reduce naringin in citrus fruit and juices by 21 to 84%.

**Table 1.2.1** Some examples of the use of naringinase to reduce naringin in citrus juices

| Fruit | Usage Conditions | | | | Concentrations | Level of reduction | Reference |
|---|---|---|---|---|---|---|---|
| | °Brix | pH | Time | Temp | | | |
| Grapefruit juice | Natural* | 4.0 | 4 hr | 40°C | 1 g/l | 75% | Prakash et al., 2002 |
| Grapefruit peel juice | 11.8 | 2.88 | 96 min | 60°C | 404.51 mg/l | 21.55% | Yalim et al., 2004 |
| Orange peel juice | 11.8 | 4.03 | 85 min | 60°C | 108.61 mg/l | 35.25% | Yalim et al., 2004 |
| Grapefruit juice | 10.5 | Natural | 10 hr | 80°C | 0.018 g/100 g | 79% | Olsen and Hill, 1964 |
| Grapefruit juice | 10.5 | | 180 min | 55°C | Alginate-entrapped naringinase (1.98 enzyme units/ml juice) | 83.84% | Mishra and Kar, 2003 |
| Grapefruit juice | 10.0 | | 15 days | 7°C | Naringinase immobilized in cellulose acetate films (7.2 $cm^2$/ml); 23% efficiency at 7°C | 60% | Soares and Hotchkiss, 1998 |

*Natural – °Brix as is

### 1.2.6.2 Use of limonoate dehydrogenase

Limonoate dehydrogenase (limonoate-NAD oxidoreductase) is isolated from *Arthrobacter globiformis* and as mentioned earlier, catalyzes the conversion of limonoate or limonoate A-ring lactone to non-bitter 17-dehydrolimonoate or 17-dehydrolimonoate A-ring lactone (Hasegawa et al., 1974). Limonoate dehydrogenase requires for its action the presence of nicotinamide-adenine nucleotide (NAD). The reaction is shown in Figure 1.2.7 (Hasegawa et al., 1975). Some examples of the application of limonoate dehydrogenase to reduce limonin in citrus juices are given in Table 1.2.2.

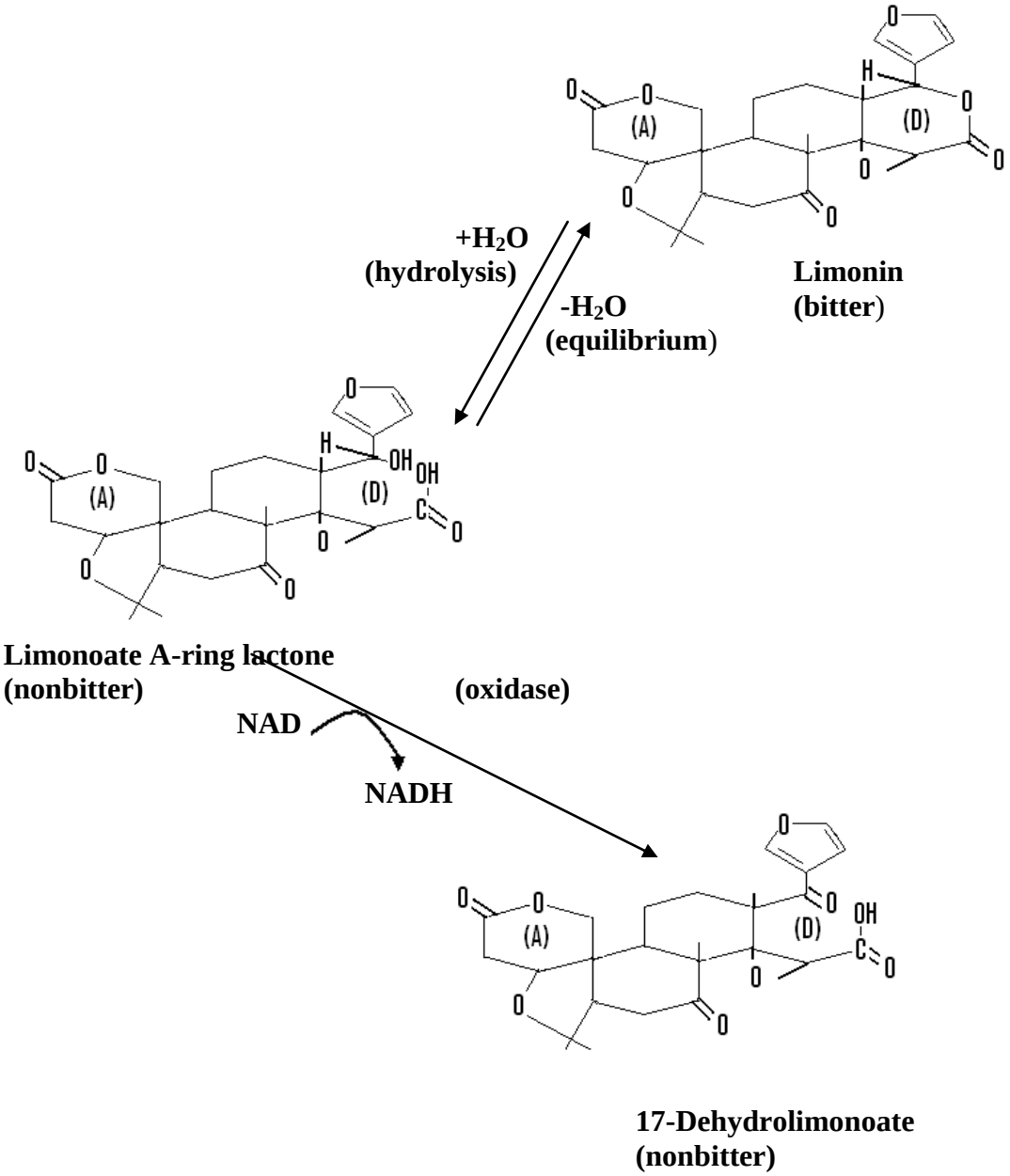



**Figure 1.2.7** The formation of the non-bitter 17-dehydro-limonoate A-ring lactone by action of limonoate dehydrogenase on limonoate A-ring lactone (Lindsay, 1996).

A new limonoid-metabolizing bacterium has been isolated from soil *Pseudomonas sp* that metabolizes limonoate mainly through deoxylimonin (Hasegawa et al., 1974). This enzyme contains considerable amounts of limonoate dehydrogenase activity with properties quite different from the dehydrogenase (limonoate-NAD oxidoreductase) of *Arthrobacter globiformis*. This enzyme isolated from *Pseudomonas* is characterized as a limonoate-NAD(P) oxidoreductase (Hasegawa et al., 1974). It requires Zn ions and sulphydryl groups for its catalytic action, uses both NAD and NADP as cofactors, and has a wide pH activity range with the optimum at pH 8.0. Some examples of its use are given in Table 1.2.2.

Both limonate dehydrogenase and limonoate-NAD(P) oxidoreductase exhibit the ability to convert limonoate A-ring lactone into 17-dehydrolimonoate A-ring lactone as well as converting limonoic acid, or its salts into 17-dehydrolimonoate. However, limonoate-NAD(P) oxidoreductase is a different enzyme from limonate dehydrogenase because it has many properties distinct from those of limonate dehydrogenase. For example, limonoate-NAD(P) oxidoreductase has maximum activity at pH 8.0, and is active over a wide pH range, including acidic pH's. On the other hand, limonate dehydrogenase has maximum activity at pH 9.5 and exhibits low activity at acidic pH's.

**Table 1.2.2** Some examples of the use of limonoate dehydrogenase and limonoate:NAD(P) oxidoreductase to reduce limonin in citrus juices

| Fruit | Enzyme used | Usage Conditions | | | | Level of reduction | Reference |
|---|---|---|---|---|---|---|---|
| | | pH | Time (hours) | Enzyme units per 100 g juice, incubated at ambient temperature | NAD and /or NADP added, micromoles per 100 g juice | | |
| Navel orange juice | Limonoate dehydrogenase | 9.5 | 17 | 0.85 | NAD = 2.5 | 97% | Hasegawa et al., 1975 |
| Navel orange juice | Limonoate dehydrogenase | 8.0 | 1 | 12.0 | NAD = 100 | 58% | Hasegawa et al., 1975 |
| Navel orange peel | Limonoate dehydrogenase | 5.5 | 2 | 19.0 | NAD = 6.0 | 73% | Hasegawa et al., 1975 |
| Lemon seeds | Limonoate dehydrogenase | 7.5 | 1 | 31 | NAD = 250 | 64% | Hasegawa et al., 1975 |
| Navel orange juice | Limonoate:NAD(P) oxidoreductase | 3.5 | 1.5 | 2.5 | NADP = 100<br>NAD = 100 | 48% | Hasegawa et al., 1975 |
| Navel orange juice | Limonoate:NAD(P) oxidoreductase | 7.5 | 20 | 1.18 | NADP = 200 | 80% | Hasegawa et al., 1975 |

NAD = Nicotinamide adenine dinucleotide NADP = Nicotinamide adenine dinucleotide phosphate

### 1.2.6.3 Use of aromase

Aromase is a ß-primeverosidase manufactured by a unique fermentation process with a selected strain belonging to *Penicillium multicolor* (Yamaguchi, Amano Enzymes, personal communication). Aromase converts diglycosides (ß-primeveroside) to aglycone form. In plants, aroma precursors and some functional compounds are present as di-glycosides. Therefore, aromase has been shown to enhance the aroma of wine, fruit juice and tea (Yamaguchi, Amano Enzymes, personal communication). Aromase has never been used before in applications to debitter citrus products. It has ß-glucosidase activity and might also be used to inactivate naringin. It can remove the disaccharide consisting of glucose and rhamnose from the naringin, releasing the aglycone (Merrett, Amano Enzymes, personal communication). The hydrolytic activity of the enzyme was found to be most effective at 50°C and pH 3-4 (Merrett, Amano Enzymes, personal communication).

ß-primeverosidase enzymes hydrolyze naturally occurring diglycosides such as a ß-primeveroside and are key enzymes involved in aroma formation during the tea manufacturing process (Murata et al., 1999). ß-primeverosides are aroma precursors of monoterpenes and aromatic alcohols from tea leaves (Murata et al., 1999). Crude enzyme extracts obtained from the manufacturing process of oolong tea and black tea mainly showed ß-primeverosidase activity although monoglycosidase activity was present to some extent (Mitzutani et al., 2002). These enzymes belong to the family of hydrolases, specifically those glycosidases that hydrolyse O- and S- glycosyl compounds. ß-primeverosidase has been found to be a real diglycoside-specific glycosidase (Sakata et al., 2003).

### 1.2.6.4 Use of laccase

Laccase can be produced by the fungus *Trametes versicolor* (Minussi, 2007). Generally, laccase can be obtained from bacteria and occurs widely in fungi. Laccase was first obtained from the juice of the Japanese tree *Rhus vernicufera* and occur widely in fungi (Yaropolov et al., 1994). Until recently laccases were found only in eukaryotes, e.g.

fungi, plants, insects (Mayer et al., 2002). There is now increasing evidence for the existence in prokaryotes of proteins with typical features of the multi-copper oxidase enzyme family (Alexander et al., 2000).

Laccase is a polyphenol oxidase (para-diphenol oxidase) which belongs to the family of blue multicopper oxidases that oxidize polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds (Minussi, 2002). It appears there is no documented example in the literature of the use of laccase to debitter citrus products. However, judging from its potential action, it may be used to debitter grapefruit peel juice. Laccase can oxidize various aromatic and non-aromatic compounds by a radical reaction mechanism (Claus, 2004). Laccase will oxidize the hydroxyl groups on naringin and limonin. Fungal laccases exhibit an enlarged substrate range and are then able to oxidize compounds with a redox potential exceeding their own. The oxidation of substrates creates reactive radicals that can undergo non-enzymatic reactions (Alexander et al., 2000). Figure 1.2.8 shows typical laccase reactions where a phenolic compound undergoes a one-electron oxidation to form an oxygen-centered free radical. This species can be converted to the quinone in a second enzyme-catalyzed step or by spontaneous disproportionation (Claus, 2004) and the quinone can in turn undergo polymerization (Claus, 2004). The reduction of oxygen to water is accompanied by the oxidation, typically, of a phenolic substrate (Thurston, 1994). These enzymes catalyze the one-electron oxidation of four reducing substrate molecules concominant with the four-electron reduction of molecular oxygen to water.

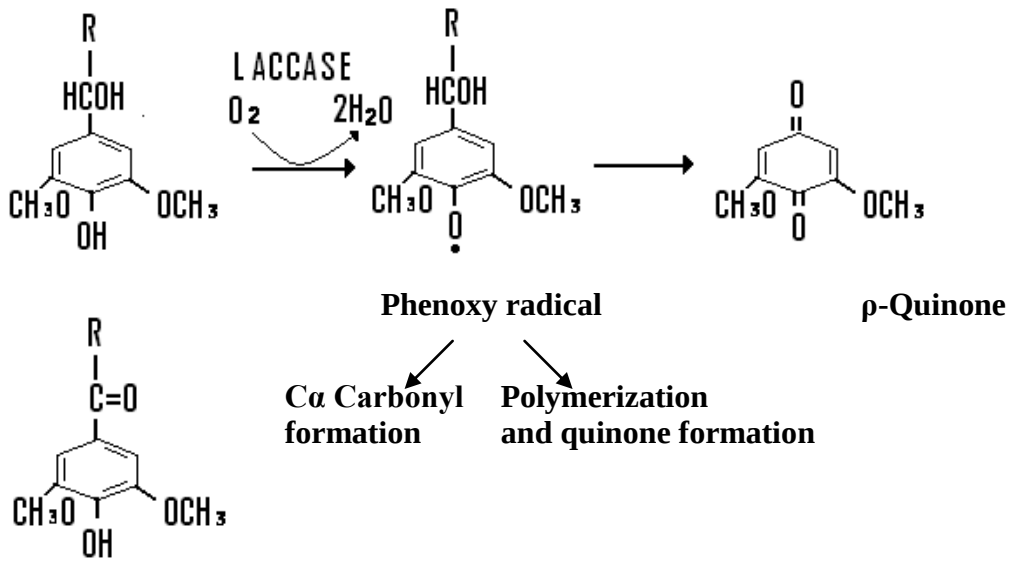



**Figure 1.2.8** Typical reaction of laccase (Minussi et al., 2002).

Laccases are involved in the degradation of complex natural polymers, such as lignin or humic acids (Claus and Filip, 1998) The reactive radicals generated lead to the cleavage of covalent bonds and to the release of monomers (Claus, 2004). In several cases a laccase-catalyzed ring-cleavage of aromatic compounds has been reported (Duran and Esposito 2000, Claus, 2004). The phenoxyl free radical specie can undergo polymerization reactions to form quinones (Minussi, 2002). Laccase is used as cross linking agent in food applications of cereal, dairy and meat (Selinheimo, 2008). Owing to their high non-specific oxidation capacity, laccases are useful biocatalysts for diverse biotechnological applications (Claus, 2004). In several cases a laccase catalyzed ring-cleavage of aromatic compounds has been reported (Duran and Esposite, 2000; Claus, 2004).

### 1.2.7 Bitterness perception

There are a variety of chemical compounds which are bitter, such as polyphenols, organic acids, peptides, salts, sulfimides, and acyl sugars (Drewnowski, 2001). Studies of sweet and bitter taste mechanisms and sweet and bitter compounds indicate that there may exist

some relationship between sweet taste receptors and bitter taste receptors (Walters and Roy, 1996). Some bitter substances possess an AH/B entity identical to that found in sweet molecules as well as the hydrophobic group. In this concept, the orientation of AH/B units within specific receptor sites, which are located on the flat bottom of receptor cavities of the tongue, provides the discrimination between sweetness and bitterness for molecules possessing the required molecular features. Molecules that fit into sites that were oriented for bitter compounds give a bitter response and those fitting the orientation for sweetness elicit a sweet response. If the geometry of a molecule were such that it could orient in either direction, it would give bitter-sweet responses (Lindsay, 1996).

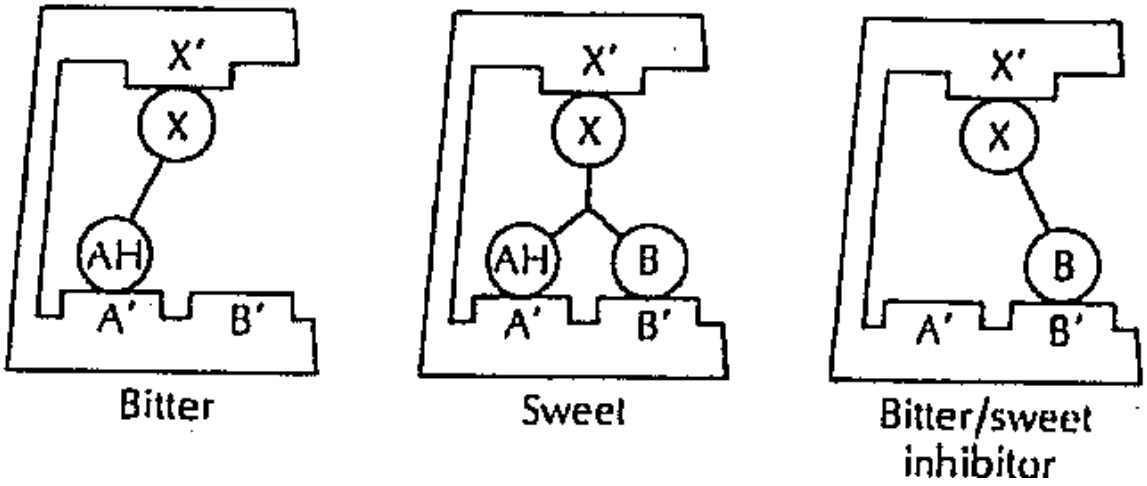



**Figure 1.2.9** The Okai unified bitter/sweet taste receptor model (Roy, 1992).

The electrophilic group (AH) binds to the receptor site A' via an amino or hydrophobic group. In order for a compound to be perceived as bitter, the AH group must bind to A'; a second hydrophobic group X must bind to a second site X', and a third site, B', must be left open (Roy, 1992). Concomitant binding of a hydrophobic region (X) in the compound potentiates a bitter taste and is the minimum requirement for bitterness. Only these two groups (AH and X) are required in Okai's simple model for the bitter receptor, which defines an optimum AH-B distance of 4.1 Å. In Okai's unified model of sweet/bitter taste Figure 1.2.9, a third receptor site (B') must be free in order to produce bitterness; if a sulphonic acid group such as taurine blocks it, no bitterness is perceived.

Okai further claims that high overall hydrophobicity of the molecule is the determinant of the potency of bitterness, and that there is an optimum molecular diameter (15 Å) to fit receptor complex to produce bitterness. Bitterness by naringin is observed only when the sugars and the flavanone aglycone are linked as explained earlier. The limonoid compound limonoate A-ring lactone is only bitter when both lactone rings are closed forming the bitter compound limonin with the bond between the carboxyl and hydroxyl groups as seen in Figure 1.2.3. The taste threshold of naringin in water is approximately 20 mg/kg (Munish et al., 2000) and for limonin 4 to 6 mg/kg (Fayoux et al., 2007). Quinine is an alkaloid that is generally accepted as the standard for the bitter taste sensation. The detection threshold for quinine hydrochloride (IV) is about 10 ppm (Lindsay, 1996). Many bitter compounds are detectable at very low thresholds and because of this it is apparent that bitterness is a receptor-based sensation, meaning that there are specific structures on the tongue which respond to specific molecules. Another factor that affects perception of bitterness is age. Bitter and salty perception declines with age, although it has been shown that sweet and sour perception is relatively unaffected (Lawless, 1985). Some citrus juice constituents like sugar and acids have an effect on taste thresholds for limonin and naringin bitterness (Guadagni et al., 1973).

### 1.3 Concluding remarks

Bitterness in citrus juices and products is mainly caused by two compounds, naringin and limonin and is a major problem in the citrus industry worldwide. In grapefruit, flavedo, albedo, segment membranes and citrus seeds are known to accumulate relatively large amounts of limonin and naringin. Limonin is also responsible for the phenomenon of delayed bitterness in citrus juices. The debittering of citrus products such as grapefruit peel juice by removal of the bitter compounds can expand its application in other food products.

The enzymes naringinase and limonoate dehydrogenase can be used to remove naringin and limonin respectively in citrus products. Alternative enzymes that could be used are aromase and laccase. Aromase is a ß-primeverosidase cable of hydrolyzing di-glycosides

(ß-primeveroside) to aglycones. Laccase contains phenol oxidase that oxidizes various aromatic compounds by a radical-catalyzed reaction.

From the available literature, it appears aromase and laccase have not been used in debittering citrus products and specifically not used on grapefruit peel juice. Even though there is general knowledge of how these enzymes could work, it is not known precisely what their mechanisms of action would be in removing bitter compounds from grapefruit peel juice. The possibility of synergistic effects between the two enzymes could exist. Apart from debittering, it is not known what their effect will be on other properties of grapefruit peel juice, e.g. colour and clarity. Insights provided by this research regarding these issues will be of benefit to the citrus processing industry. Efficient production of a debittered grapefruit peel juice would open avenues for beneficiation of a waste product from citrus juice processing such as grepfruit peel waste.

## 1.4 Hypotheses and Objectives

### 1.4.1 Hypotheses

Treating grapefruit peel juice with aromase on its own will reduce the levels of naringin and limonin and increase sugar levels. Aromase has β-primeverosidase as well as ß-glucosidase activity. It will hydrolyze naringin to release the naringenin aglycone and a disaccharide of glucose and rhamnose, and further hydrolyze the disaccharide into glucose and rhamnose (Merrett, Amano Enzymes, personal communication). Aromase will reduce limonin levels by opening the A and D rings and furan ring of limonin by hydrolysis forming hydroxyl and carbonyl groups (Merrett, Amano Enzymes, personal communication).

Treating grapefruit peel juice with laccase on its own will reduce the levels of naringin and limonin. Laccase is a phenol oxidase and can act on phenolic and various aromatic compounds by a radical-catalyzed mechanism (Selinheimo, 2008). It will oxidize the

hydroxyl groups on naringin and limonin to form oxygen-centered free radicals, and to quinones which in turn undergo polymerization to form other products (Minussi et al., 2002).

Treating grapefruit peel juice with aromase and laccase will reduce the levels of naringin and limonin and reduce its bitterness. Quinine is an alkaloid that is generally accepted as the standard for the bitter taste sensation. The detection threshold for quinine hydrochloride (IV) is about 10 ppm (Lindsay, 1996). The taste threshold for naringin in water is approximately 20 ppm (Munish et al., 2000) and that for limonin is 4 to 6 ppm (Fayoux et al., 2007). The enzyme treatment will reduce levels of naringin and limonin to levels close to or below these thresholds. As a result, sensory panelists will perceive enzyme-treated grapefruit peel juice samples as less bitter or not bitter at all.

Treating grapefruit peel juice with laccase will make the juice darker. Laccase is a polyphenol oxidase which can cause enzymatic browning (Rocha and Morais, 2001) leading to a darker coloured grapefruit peel juice. It will act on phenolic and various aromatic compounds (Selinheimo, 2008) forming quinones. Rapid polymerization of quinones to produce black, brown or red insoluble polymers known as melanin (Ruangchakpet and Sajjaanantakul, 2007) which will result in darker coloured grapefruit peel juice.

Treating grapefruit peel juice with aromase will increase the clarity of the juice. Haze is formed by the interaction between phenolic compounds and proteins (Siebert et al., 1996) and formation of haze reduces clarity of juices. Hydroxyl groups in phenolic compounds can bind with more than one polypeptide chain to cross link that leads to haze (Emmambux, 2004). Hydrolysis of naringin (a glycoside with many hydroxyl groups) to naringenin (an aglycone with relatively less hydroxyl groups) by the aromase enzyme will lead to decreased interactions between phenolic compounds and proteins and thus decrease in haze formation or increase in clarity of the grapefruit peel juice.

### 1.4.2 Objectives

To determine the effect of aromase and laccase on their own and in combination on the bitter compounds naringin and limonin and sugars in grapefruit peel juice.

To determine the effect of aromase and laccase on their own and in combination on the colour and clarity of grapefruit peel juice.

To determine the sensory perception by consumer sensory evaluation of bitterness in grapefruit peel juice samples treated with aromase and laccase on their own and in combination.

# CHAPTER 2: RESEARCH

## 2. Naringin and limonin contents, physico-chemical properties and consumer acceptability of grapefruit peel juice debittered with aromase and laccase

### 2.1 Abstract

The juice from the waste products of grapefruit juice extraction (consisting mainly of outer parts of the fruit namely flavedo, albedo, segment membranes and seeds) was extracted to obtain grapefruit peel juice. The peel juice was treated with the enzymes, aromase and laccase in an attempt to remove the bitter compounds naringin and limonin and render it non-bitter. Treatment with aromase (0.8% w/v) decreased naringin by almost 80% and decreased limonin by almost 8 times. In the aromase-treated peel juice, there was no sign of formation of limonin due to delayed bitterness after 7 months of storage. Treatment with laccase (3.0% w/v) only decreased naringin by 40% and decreased limonin by only 1.2 times. Treatment with a combination of aromase (0.8% w/v) and laccase (3.0% w/v) showed the greatest decrease in naringin of 95% and decreased limonin by up to 6 times. The grapefruit peel juice became lighter on treatment with aromase and the clarity increased. The grapefruit peel juice became darker on treatment with laccase. Respondents in a sensory panel identified the aromase-treated sample as the least bitter. These results indicate that aromase can be used on its own to reduce bitterness in grapefruit peel juice.

## 2.2 Introduction

Production of grapefruit juice and other citrus juices generates vast quantities of processing by-products, mainly peels, cores, and segment membranes (Sreenath et al., 1995). Citrus waste and citrus peel juice can contribute to environmental problems and contamination of water resources. The peel juice has high chemical oxygen demand (152000 ppm) and biological oxygen demand (59000 ppm) when dumped into the water system (Crandall, 1980) and therefore can contribute to water pollution if not utilized properly. Grapefruit peel juice consists of water, sugars, flavour compounds and oils (Grohmann et al., 1999). However, it is extremely bitter due to the presence of naringin and limonin (Singh et al., 2008). If the grapefruit peel juice is debittered, it can be made useful for other applications, for example as an ingredient in fruit juice formulations.

Enzymes are used extensively to debitter citrus juices. The enzyme naringinase can be used in debittering due to its ability to break down naringin. Naringinase possesses α-rhamnosidase and β-glucosidase activities (Prakash et al., 2002). The enzyme firstly hydrolyses naringin to prunin and rhamnose by its α-rhamnosidase activity. Prunin is then broken down into naringenin and glucose by the β-glucosidase activity of naringinase (Prakash et al., 2002). The limonin precursor limonoate A-ring lactone can be converted to limonin in acidic conditions and this causes delayed bitterness. The enzyme limonoate A-ring dehydrogenase can inactivate the limonoate A-ring lactone precursor (Hasegawa et al., 1975).

The enzymes laccase and aromase may also be used for debittering. Laccase is a type of copper-containing polyphenol oxidase that oxidizes polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds (Minussi, 2002). Laccase is produced by the fungus *Trametes versicolor* (Minussi, 2007) and may be used to oxidize naringin and reduce bitterness. Based on its action, laccase could also oxidize limonoate A-Ring lactone and prevent the formation of limonin. Aromase is a β-primeverosidase manufactured by unique fermentation process with a selected strain belonging to *Penicillium multicolor* (Yamaguchi, Amano Enzymes, personal

communication). Aromase converts di-glycosides (β-primeveroside) to aglycone form efficiently. It has β-glucosidase activity and can be used to inactivate naringin. It removes the disaccharide glucose and rhamnose from the naringin and releases the aglycone. Aromase might therefore be more effective than naringinase which removes glucose and rhamnose in two separate successive steps.

The objective of this study was to determine the effect of aromase and laccase on the bitter compounds naringin and limonin and the physico-chemical and sensory properties of grapefruit peel juice.

## 2.3 Materials and Methods

### 2.3.1 Materials

Peel waste products consisting of flavedo, albedo, segment membranes and seeds were collected after the juice extraction of grapefruit (Star Ruby variety) at Capefruit Processors (Pty) Ltd, Malelane, South Africa. These were the raw materials from which peel juice was extracted. The enzymes aromase and laccase were procured from Amano Enzymes, Technoplaza, Kakamigahara, Gifu, Japan.

#### 2.3.1.1 Preparation of grapefruit peel juice

The grapefruit peel waste was milled using a colloid mill and frozen in 20 kg boxes at a temperature of –18°C in order to break the cells to release moisture. After defrosting, the milled peels were pressed by hand through a cheese cloth to extract the peel juice. The extracted juice was screened to remove solids by pressing through a 100 μm bag filter.

#### 2.3.1.2 Enzymatic treatment of grapefruit peel juice

The grapefruit peel juice was treated with aromase and laccase enzymes in a 3 x 3 factorial experiment. Grapefruit peel juice quantities of volume 170 ml were placed in 250 ml Schott bottles. These were then treated with the enzymes as follows:

Aromase

- $C_0$ as control, grapefruit peel juice with no enzyme treatment.
- $C_{0.4,}$ aromase at 0.4% (w/w) of the grapefruit peel juice
- $C_{0.8,}$ aromase at 0.8% (w/w) of the grapefruit peel juice

Laccase

- $C_0$ as control, grapefruit peel juice with no enzyme treatment.
- $C_{1.5}$, laccase at 1.5% (w/w) of the grapefruit peel juice
- $C_{3.0}$, laccase at 3.0% (w/w) of the grapefruit peel juice

All the bottles were incubated in a water bath at 55°C for 480 min. The samples were then incubated at 80°C for 15 min to inactivate the enzyme. The treated grapefruit peel juice samples were then equally distributed in 10 ml sample bottles and frozen until required for analysis. All the treatments were done in duplicate. The optimum conditions for aromase are pH 3.0 – 4.0 at 50°C and laccase pH 4.0 – 4.5 at 60°C.

### 2.3.2 Methods

#### 2.3.2.1 Physico-chemical characterization of grapefruit peel juice

The following physico-chemical tests were conducted to characterize the grapefruit peel juice: Total soluble solids were determined with the Atago refractometer (Model RX-5000α, Atago Co. Ltd, Japan) and the results expressed in degrees Brix. Titratable acidity was assessed by titrating dilute samples with 0.1 N NaOH to the phenolphthalein end point and ash was determined according to the Association of Official Analytical Chemists methods 932.12 and 940.26 respectively (AOAC, 1990). The pH was measured with a pH meter (Model 702 SM Titrino, Metrohm Herisau, Switzerland). Percent pulp was determined using the International Federation of Fruit Juice Producers Analysis No 60 (IFFJP, 1991). Percent oil was determined using the International Federation of Fruit Juice Producers Analysis No 45 (IFFJP, 1972). Proximate analyses for protein (micro Kjeldahl method), moisture and fat (Soxhlet extraction apparatus) were carried out according to AOAC methods 14.086, 14.084 and 14.089 respectively (AOAC, 1980).

Total carbohydrate was determined by difference between 100 and the sum of the percentages of water, protein, total lipid (fat), ash, and when present, alcohol. Starch was qualitatively measured according to AOAC method 37.1.54 (AOAC, 1980). An Inductively Coupled Plasma – atomic emission spectrophotometer (Spectoflame, Spectro Analytical Instruments, Germany) was used to determine the sodium content. Energy values were calculated (Food and Agriculture Organization of the United Nations, 1998).

#### 2.3.2.2 Determination of naringin and naringenin

Naringin and naringenin contents of the untreated and enzyme-treated grapefruit peel juice were determined using reverse-phase HPLC (Rouseff, 1988). All the solvents and chemicals used during this assay were of HPLC grade.

The grapefruit peel juice samples were filtered through a 0.2 µm PTFE syringe filter prior to HPLC injection. The HPLC system consisted of a Waters 1525 binary HPLC pump and a Waters 2487 dual wavelength absorbance detector (Waters, Milford, MA, USA). The separation was accomplished by means of a YMC-Pack ODS-AM-303 (250 mm x 4.6 mm i.d., 5 µm particle size) column (Waters, MD, USA). Breeze ™ software (Waters, Milford, MA, USA) was used to monitor the separation process and after analysis a chromatogram was obtained.

The injection volume for all samples was 20 µl with the analysis conducted at a flow rate of 0.8 ml/min and monitored at 280 nm. The mobile phase consisted of 0.1% acetic acid in distilled water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). The linear gradient of the solvents was as follows: solvent B was increased from 8 to 10% in 2 min, then increased to 30% in 25 min, followed by an increase to 90% in 23 min, then increased to 100% in 2 min, kept at 100% of B for 4 min, and finally returned to the initial condition. Running time was 60 min and the column temperature was held at 25 °C during the run.

Pure standards of naringin and naringenin were purchased from Sigma Aldrich (USA). The standards were prepared in dimethyl sulfoxide at concentrations of 200, 150, 100, 50,

25, 20, 10 and 5 ppm (mg/l). Standards of 20 µl aliquots were chromatographed singly and as mixtures by injection into the HPLC system. Calibration curves were obtained for each standard by plotting peak areas versus concentrations. Regression equations that showed high degree of linearity ($R^2$> 0.995) were obtained for each standard from the calibration curves. Naringin and naringenin in the samples were identified by comparing the retention time of the unknown with those of the standards. The concentrations of the identified naringin and naringenin were calculated using the regression equations obtained and expressed as mg/ 100 g of sample on dry basis.

#### 2.3.2.3 Determination of limonin

Limonin content of the untreated and enzyme-treated grapefruit peel juice samples was determined using reverse-phase HPLC (Shaw and Wilson, 1984). All the solvents and chemicals used during this assay were of HPLC grade. The grapefruit peel juice samples were filtered through a 0.2 µm PTFE syringe filter prior to HPLC injection. The HPLC system consisted of a Shimadzu LC-20A HPLC and a Shimadzu SPD-M20A UV detector (Shimadzu, Japan). The separation was accomplished by means of a TSKgel ODS-100V (Tosoh, 4.6 mm x 7.5 cm i.d., 3 µm particle size) column (Shimadzu, Japan). Shimadzu software was used to monitor the separation process and after analysis a chromatogram was obtained. The injection volume for all samples was 10 µl with the analysis conducted at a flow rate of 1.0 ml/min and monitored at 207 nm. The mobile phase consisted of $H_2O$ : Acetonitrile : Tetrahydrofuran = 65 : 17.5 :17.5

Pure limonin standard was purchased from Wako Pure Chemical Industries Ltd (Japan). Limonin standard solutions with concentrations of 1, 5, 10, 15, and 25 ppm (mg/l) were prepared using the mobile phase. The standards were run on the HPLC system and standard calibration curves were obtained by plotting peak areas versus concentrations. Regression equations that showed high degree of linearity ($R^2$= 0.9982) were obtained from the calibration curves. Limonin in the grapefruit peel juice samples was identified by comparing the retention time of the unknown with those of the standard. The concentration of the identified limonin was calculated using the regression equations obtained and expressed as mg/100 g of sample on dry basis.

### 2.3.2.4 Sugars (glucose, sucrose, fructose and rhamnose)

Glucose, fructose and sucrose concentrations in the untreated and enzyme-treated grapefruit peel juice samples were determined using HPLC. All the solvents and chemicals used during this assay were of HPLC grade.

The grapefruit peel juice samples were filtered through a 0.2 µm PTFE syringe filter prior to HPLC injection. The HPLC system consisted of a Waters 1525 binary HPLC pump (Waters, Milford, MA, USA) and an ERC 7515A refractive index detector (Saitama, Japan). The separation was accomplished by means of a carbohydrate (30 cm x 0.78 mm i.d.) column. Breeze $^{TM}$ software was used to monitor the separation process and after analysis a chromatogram was obtained. The injection volume for all samples was 20 µl with the analysis conducted at a flow rate of 0.5 ml/min. The mobile phase consisted of distilled water.

Pure standards of glucose, sucrose, fructose and rhamnose were purchased from Sigma Aldrich (USA). The sugar standards were prepared in distilled water at concentrations of 20, 30, 50, 80, 100 and 300 (g/l). Standards of 20 µl aliquots were chromatographed singly and as mixtures by injection into the HPLC system. Standard calibration curves were obtained for each sugar by plotting peak areas versus concentrations. Regression equations that showed high degree of linearity ($R^2 > 0.995$) were obtained for each sugar from the calibration curves. Sugars in the grapefruit peel juice samples were identified by comparing the retention time of the unknown with those of the standard sugars.

Disaccharides were analysed using High performance anion-exchange chromatography – pulsed amperometric detection (HPAEC-PAD) method. The equipment used consisted of a Perkin Elmer PE 200 pump (Perkin Elmer, Japan) and ALS 2016 Purge and Trap accessories (Perkin Elmer, Japan). Separation of 20 µl sample aliquots was carried out using CarboPac PA-100 anion-exchange column (4 x 250 mm) with a Dionex CarboPac PA 100 guard column (4 x 50 mm) attached and detection was by a Dionex PAD-11 detector (Dionex Corporation, Sunnyvale, California). The eluent was 150 mM NaOH at a flow rate of 1 ml/min.

Further confirmation of the sugars present in the grapefruit peel juice samples was done by Gas Chromatography – Mass Spectrometry (GC-MS). The GC-MS was carried out with a Varian 3800 GC coupled to a Varian 4000 ITD MS (Varian, Inc., California, USA). Chromatography was accomplished on a Varian factor four VF-5ms, 0.25 mm x 30 m x 0.25 µm column (Varian, Inc., California, USA). The injection volume was 1 µl with an injector split of 1:20 at 280°C. The temperature was 100°C for 1 min and 100°C to 130°C at a program rate of 30 °C/min with a 2 min hold and 130 to 320°C at a program rate of 5°C/min with a 7 min hold. Helium was used as a GC carrier gas at a flow rate of 1 ml/min.

#### 2.3.2.5 Colour and clarity

Colour was measured using a HunterLab LICO 200 spectrophotometer (Hunter Asssociates Laboratory, Reston, Virginia). The measurements were based on the L, a, b Hunter tristimulus scale, (L= lightness; +a = red, -a = green, +b = yellow, -b = blue).
Clarity was measured at 625 nm using a Metertek SP-839 spectrophotometer (Metertek Inc, Nangang, Taipei, Taiwan).

### 2.3.3 Consumer acceptability

Quantitive Descriptive sensory method using a Williams design (Lawless and Heymann, 2010) was used. Twenty-five panelists were pre-screened for ability to taste bitterness. Panelists were introduced to the sensory evaluation software (Compusense ® five 4.8 (1986-2007) Ontario, Canada). Four grapefruit peel juice samples (treated at the highest concentrations) control, 0.8% aromase, 3.0% laccase and both 0.8% aromase and 3.0% laccase were presented to panelists in polystyrene cups with a randomized 3-digit code. An untreated grapefruit peel juice sample was included as a control. Panelists were requested to rate the juices for bitterness.

### 2.3.4 Statistical analysis

The experiment (limonin and naringin concentrations as dependent variables and aromase and laccase enzyme treatments as independent variables) was established as a completely random design (CRD) with three replicates. Factorial analysis of variance (ANOVA) was used to test for differences between 3 laccase and 3 aromase enzymes (treatments), as well as the laccase by aromase interaction. The data was acceptably normal with homogeneous treatments variances. Treatment means were separated using Fisher's protected least significant difference test (LSD) at the 5% level of significance (Snedecor & Cochran, 1980).

The analysis of the factorial experiment was performed by a computerized statistical analysis software system (GenStat ®, 2009).

## 2.4 Results and discussion

### 2.4.1 Characterization of grapefruit peel juice

The physico-chemical properties of the grapefruit peel juice are shown in Table 2.1.1

**Table 2.1.1** Physico-chemical properties of untreated grapefruit peel juice as is basis

| Test Performed | Untreated Grapefruit peel juice |
|---|---|
| °Brix Refractometer, °Brix | 10.80 (0.02)[1] |
| Citric Acid, %Acid (w/w)[2] | 0.51 (0.01) |
| Brix/acid Ratio | 21.94 (0.51) |
| pH | 3.83 (0.03) |
| Pulp, % (v/v) | 5.0 (0.0) |
| Oil, % (v/v) | 0.005 (0.000) |
| Protein content (N x 6.25), g/100 ml | 0.78 (0.01) |
| Fat content, g/100 ml | 0.14 (0.03) |
| Moisture content, g/100 ml | 91.23 (0.17) |
| Ash, g/100 ml | 0.67 (0.02) |
| Carbohydrates, g/100 ml | 7.18 (0.12) |
| Energy value, kJ/100 ml | 133 (3) |
| Sodium content, mg/100 ml | 96.4 (2.7) |
| Starch (qualitative) | nd |

nd = not detected

[1] Standard deviation in brackets

[2] Units in brackets

The °Brix of 10.80 in the untreated grapefruit peel juice was higher than 8.00 – 9.45 reported in commercial grapefruit juices (Pichaiyongvongdee and Haruenkit, 2009). The citric acid content of 0.51 g/100 g and pH of 3.83 in the untreated grapefruit peel juice related well to 0.49 – 0.58 g/100 ml and 3.80 – 3.96 reported in commercial grapefruit

juices (Pichaiyongvongdee and Haruenkit, 2009). Citric acid content of 0.32 – 0.38 g/100 ml and pH of 5.65 – 5.70 has been reported in grapefruit peel juice (Sinclair, 1972). The citric acid content and pH can vary according to season and fruit maturity (Sinclair, 1972). The Brix/acid ratio is an important parameter related to the quality of citrus (Xu et al., 2008). The acid taste of citrus fruits is measured by the organic acid content of the juice while the sweet taste is measured by the content of soluble sugars. This balance between sweetness and tartness is defined by the legal standards of quality in terms of a parameter known as the °Brix:acid ratio (or Ratio), a value commonly used in the citrus industry to express the relationship between sweetness and tartness (Sinclair, 1972). The higher Ratio of 28.24 in the treated grapefruit peel juice compared well with Ratio 15 – 30 in apple juice (Board and Woods, 2007) which makes debittered grapefruit peel juice ideal to be used as a substitute for apple or pear or grape in formulated 100% grapefruit juices. Apple or pear or grape juice is used in 100% grapefruit juice formulations to make the balance between sweetness and tartness more acceptable in South Africa. Debittered grapefruit peel juice can also potentially be used as a substitute for apple or pear or grape in other formulated 100% juices if the grapefruit flavour and aroma are removed. Debittered grapefruit peel juice would also be ideal to be used in canned grapefruit segments in juice. The debittered grapefruit peel juice will also enhance the grapefruit flavour and aroma of the final product.

### 2.4.2 The effect of aromase and laccase on naringin and naringenin content of grapefruit peel juice

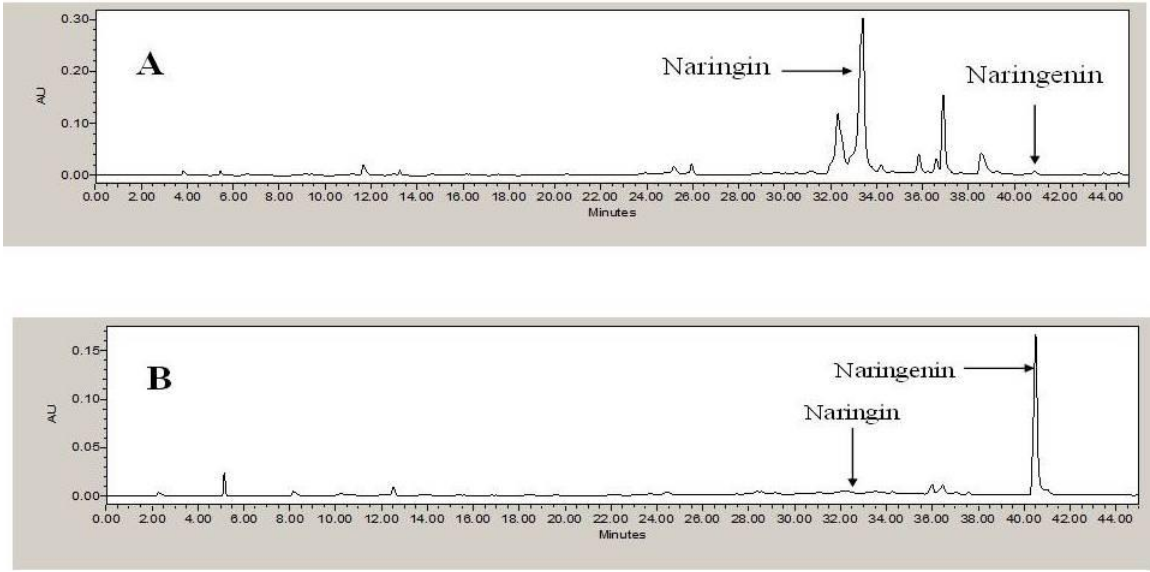



**Figure 2.1.1** HPLC (High-Performance Liquid Chromatography) chromatograms showing the effect of enzyme treatment on naringin and naringenin content of grapefruit peel juice. A: untreated grapefruit peel juice B: grapefruit peel juice treated with a combination of laccase (3.0%) and aromase (0.8%).

The chromatograms in Figure 2.1.1 are shown as an example of the effect of aromase and laccase on naringin and naringenin in grapefruit peel juice. For the untreated grapefruit peel juice (Figure 2.1.1A), a prominent naringin peak was observed at a retention time of 33.5 min. A very small naringenin peak appeared at 40.9 min. The effect of the enzyme treatment was clearly visible in the chromatogram (Figure 2.1.1B) of the treated grapefruit juice with the almost total disappearance of the naringin peak and appearance of a prominent naringenin peak. The peaks were integrated to the concentrations of the bitter compounds from the standard curves and the results presented in the Tables.

**Table 2.1.2** Effect of aromase and laccase on naringin content (mg/kg) of grapefruit peel juice[5]

| | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| Laccase conc. (%)[1] | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 447 g[2] (16)[3] | 152 e (7) | 77 c (1) | 225 a[4] |
| 1.5 | 430 g (41) | 119 d (10) | 48 b (4) | 199 a |
| 3.0 | 260 f (20) | 75 c (4) | 27 a (2) | 121 a |
| Aromase main effect | 379 b | 115 a | 51 a | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.
[5] as is

Treating grapefruit peel juice with increasing concentrations of aromase alone decreased naringin content by up to about 80%. On the other hand, increasing concentrations of laccase only decreased naringin by up to 40%. Overall, the main effects showed decrease in naringin content by up to 85% due to aromase treatment and a lower decrease by 45% due to laccase treatment. The combination treatment of aromase (0.8%) and laccase (3.0%) produced the greatest decrease in naringin content of grapefruit peel juice by 95%.

**Table 2.1.3** Effect of aromase and laccase on naringenin content (mg/kg) of grapefruit peel juice[5]

| | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| Laccase conc. (%)[1] | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 3 a[2] (0)[3] | 223 c (5) | 252 d (15) | 135 a[4] |
| 1.5 | 4 a (0) | 284 e (6) | 282 e (14) | 161 a |
| 3.0 | 12 b (0) | 294 e (3) | 314 f (12) | 169 a |
| Aromase main effect | 6 a | 267 b | 283 b | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.
[5] as is

Treatment of grapefruit peel juice with aromase alone increased naringenin content by about 99%. On the other hand, treatment with laccase alone appeared to increase naringenin content by 75% though this did not seem to be significant. Overall, the main effects showed a significant increase in naringenin content by 98% due to treatment with aromase while laccase treatment did not produce a significant overall increase in naringenin content. The combination treatment of aromase (0.8%) and laccase (3.0%) produced the greatest increase in naringenin content of grapefruit peel juice by 99%.

As mentioned earlier in the Materials and Methods, the grapefruit peel waste collected in this research consisted of the flavedo and albedo parts of the peel, some segment membranes and seeds. The naringin content of 447 mg/kg reported in Table 2.1.2 in the grapefruit peel juice is within the range of total naringin content of grapefruit peel waste (consisting of flavedo, albedo, segment membranes and seeds) reported by Pichaiyongvongdee and Haruenkit (2009) as 435.1 – 850.9 mg/kg. These results indicate that in grapefruit, naringin appears to be more concentrated in the peels than in the fluid-filled sacs (vesicles).

As expected, the naringenin content of the grapefruit peel juice (3 mg/kg) was low (Table 2.1.3). Naringenin content of 4.2 mg/kg has been reported in commercial grapefruit juice (Gattuso et al., 2007). Due to the bitter taste of the fruit, it would be expected that naringin content would be significantly higher in grapefruit than its non-bitter product naringenin.

The decrease by almost 80% in naringin content of the aromase-treated grapefruit peel juice (Table 2.1.2) accompanied with the significant increase in naringenin (Table 2.1.3) could be due to ß-primeverosidase activity and to a lesser extent the ß-glucosidase activity of the aromase enzyme. The ß-primeverosidase activity hydrolyzes the di-glycoside naringin (Sakata et al., 2003) and releases the aglycone naringenin and the disaccharide neohesperidoside or rutinoside. The β-glucosidase activity may bring about hydrolysis of the glycosidic bond between glucose and the aglycone (Prakash et al., 2002). The 95% decrease in naringin due to the combination treatment of aromase (0.8%)

and laccase (3.0%) (Table 2.1.2) and increase in naringenin by 99% (Table 2.1.3) suggests that the two enzymes may be exerting synergistic effects. Naringin may first be hydrolysed by aromase to produce products such as naringenin and sugars which would have higher numbers of free hydroxyl groups. The hydroxyl groups may then further be oxidized by the laccase enzyme.

### 2.4.3 The effect of aromase and laccase on limonin content of grapefruit peel juice

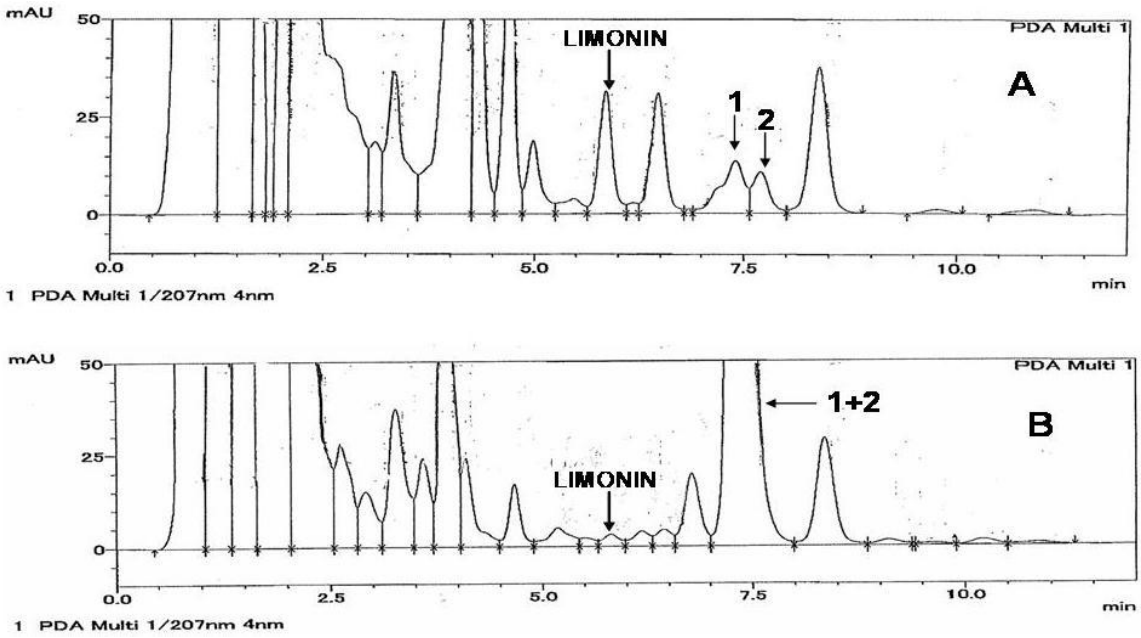



**Figure 2.1.2** HPLC (High-Performance Liquid Chromatography) chromatograms showing the effect of enzyme treatment on limonin content of grapefruit peel juice. A: untreated grapefruit peel juice B: grapefruit peel juice treated with aromase (0.8%).

The chromatograms in Figure 2.1.2 are shown as an example of the effect of aromase and laccase on limonin in grapefruit peel juice. For the untreated grapefruit peel juice (Figure 2.1.2A), the limonin peak appeared at a retention time of about 5.9 min. Peaks 1 and 2 in the chromatogram of the grapefruit peel juice sample were also present in the chromatogram of all the limonin standards used for calibration (results not shown). On treatment with aromase (at 0.8%) alone (Figure 2.1.2B), there was a significant reduction in the area of the limonin peak as well as a significant increase in the areas of peaks 1 and 2.

**Table 2.1.4** Effect of aromase and laccase on limonin content (mg/kg) of grapefruit peel juice[5]

| | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| Laccase conc. (%)[1] | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 45 f[2] (0)[3] | 6 a (1) | 6 a (1) | 19 a[4] |
| 1.5 | 40 e (0) | 8 b (1) | 10 c (1) | 19 a |
| 3.0 | 38 d (2) | 6 a (0) | 8 b (0) | 17 a |
| Aromase main effect | 41 b | 7 a | 8 a | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.
[5] as is

Treatment of grapefruit peel juice with aromase alone decreased limonin content by about 87% (Table 2.1.4). Treatment with laccase alone appeared to decrease limonin content by only about 16%. Overall, the main effects showed a significant decrease in limonin content by 5 times due to aromase treatment while laccase treatment appeared to have no significant effect on limonin content. The combination treatments decreased limonin content by up to 6 times.

The limonin content of untreated grapefruit peel juice reported in Table 2.1.4 (45 mg/kg) was higher than the level reported in commercial grapefruit juice (average of 18 mg/kg) (Ohta and Hasegawa, 2006). This observation is similar to what was observed with the naringin content and provides further indication that in the grapefruit, the bitter compounds appear to be more concentrated in the peels than in the juice sacs. Citrus fruit do not normally contain limonin but rather a non-bitter precursor, limonoic acid A-ring lactone, which is converted gradually to limonin in the juice after extraction from the fruit (Hasegawa et al., 1980).

The reduction in limonin content of the grapefruit peel juice on treating with aromase could be due to the ß-glucosidase activity of the enzyme. By this activity, aromase could open the A and D ring of limonin by hydrolysis (as seen in Figure 3.2.3, page 74)

forming hydroxyl groups. Aromase might also hydrolyze the furan ring in the limonin structure forming two hydroxyl groups. These would lead to reduction in the levels of limonin.

### 2.4.3.1 Effect of storage on limonin content of aromase-treated grapefruit peel juice

**Table 2.1.5** Effect of storage for 7 months on limonin content of aromase-treated grapefruit peel juice

| Enzyme conc. (%)[2] treated samples | Limonin content (mg/kg) | Limonin content (mg/kg) after 7 months storage |
|---|---|---|
| Untreated | 44.53 (1.30)[1] | 54.97 (1.86) |
| 0.8% Aromase | 4.63 (1.44) | 3.20 (1.54) |

[1] Standard deviation in brackets

[2] Enzyme concentrations expressed as percentage of grapefruit peel juice

Table 2.1.5 shows the limonin content of untreated and aromase- treated grapefruit peel juice after frozen storage for 7 months. The untreated control showed an increase in limonin concentration by almost 30%. On the other hand, there was a decrease in limonin content of the aromase- treated sample by 35% after storage for 7 months.

The 30% increase in limonin in the untreated grapefruit peel juice is due to the phenomenon of delayed bitterness. This phenomenon is attributed to the formation of limonin from the limonoate A-ring lactone present (Fayoux et al., 2007). The result in Table 2.1.5 suggests that in the untreated grapefruit peel juice sample, there may have been limonoate A-ring lactone (the limonin precursor) present that could have been converted to limonin over the 7 month storage period. Treatment with aromase may have inactivated limonin as well as its precursor. As a result, there was no available limonate A-ring lactone to be converted to limonin over the 7 month storage period.

### 2.4.4 The effect of aromase and laccase on the sugar content of grapefruit peel juice

#### 2.4.4.1 Glucose

**Table 2.1.6** Effect of aromase and laccase on glucose content (g/l) of grapefruit peel juice[5]

| Laccase conc. (%)[1] | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 47.6 ab[2] (7.4)[3] | 64.2 c (6.5) | 58.4 bc (9.7) | 56.7 a[4] |
| 1.5 | 46.5 ab (4.0) | 65.9 c (10.3) | 81.6 d (1.5) | 64.7 a |
| 3.0 | 45.4 a (3.4) | 85.7 de (5.3) | 98.4 e (5.4) | 76.5 a |
| Aromase main effect | 46.5 a | 71.9 ab | 79.5 b | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.
[5] as is

Treatment of grapefruit peel juice with aromase alone increased glucose content by about 1.2 times. Treatment with laccase alone also increased glucose content by about 0.95 times. Overall, the main effects showed an increase in glucose content by 1.7 times due to aromase treatment and by 1.3 times due to laccase treatment. The combination treatment of aromase (0.8%) and laccase (3.0%) produced the greatest increase in glucose content of grapefruit peel juice by 2.1 times.

### 2.4.4.2 Sucrose

**Table 2.1.7** Effect of aromase and laccase on sucrose content (g/l) of grapefruit peel juice[5]

| Laccase conc. (%)[1] | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 57.2 abc[2] (6.8)[3] | 59.0 bc (9.3) | 48.1 a (5.5) | 54.8 a[4] |
| 1.5 | 62.8 c (2.3) | 53.0 ab (9.9) | 56.4 abc (1.4) | 57.4 a |
| 3.0 | 59.2 bc (5.1) | 64.0 c (1.8) | 58.9 bc (9.1) | 60.7 a |
| Aromase main effect | 59.7 a | 58.7 a | 54.5 a | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.
[5] as is

Treatment of grapefruit peel juice with aromase alone non-significantly decreased sucrose content by about 0.84 times. Treatment with laccase alone non-significantly increased sucrose by about 1.03 times. Overall, the main effects had no significant effect on sucrose content. The combination treatment of aromase (0.8%) and laccase (3.0%) increased sucrose non-significantly by 1.03 times.

### 2.4.4.3 Fructose

**Table 2.1.8** Effect of aromase and laccase on fructose content (g/l) of grapefruit peel juice[5]

| Laccase conc. (%)[1] | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 53.5 bc[2] (6.8)[3] | 57.0 c (6.2) | 46.7 a (5.5) | 52.4 a[4] |
| 1.5 | 51.4 abc (2.2) | 46.0 a (4.9) | 49.5 ab (1.8) | 49.0 a |
| 3.0 | 49.5 ab (1.4) | 49.8 ab (1.9) | 49.2 ab (3.3) | 49.5 a |
| Aromase main effect | 51.5 a | 50.9 a | 48.4 a | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.
[5] as is

Treatment of grapefruit peel juice with aromase alone non-significantly decreased fructose content by about 0.87 times. Treatment with laccase alone non-significantly decreased sucrose by about 0.93 times. Overall, the main effects had no significant effect on sucrose content. The combination treatment of aromase (0.8%) and laccase (3.0%) increased sucrose by 0.92 times.

The concentration of glucose (Table 2.1.6), fructose (Table 2.1.8) and sucrose (Table 2.1.7) in the untreated grapefruit peel juice reported in this work were all almost twice the levels reported by Kelebek (2010) in grapefruit juice (23.3 g/l for glucose, 34.99 g/l for sucrose and 22.32 g/l for fructose). Citrus peel in general contains a large percentage of sugars, especially as the fruit nears maturity with an average of 13.5% glucose, 13.5% fructose and 11.9% sucrose all calculated on the percentage of dry weight of the peel (Sinclair, 1972).

### 2.4.4.4 Rhamnose

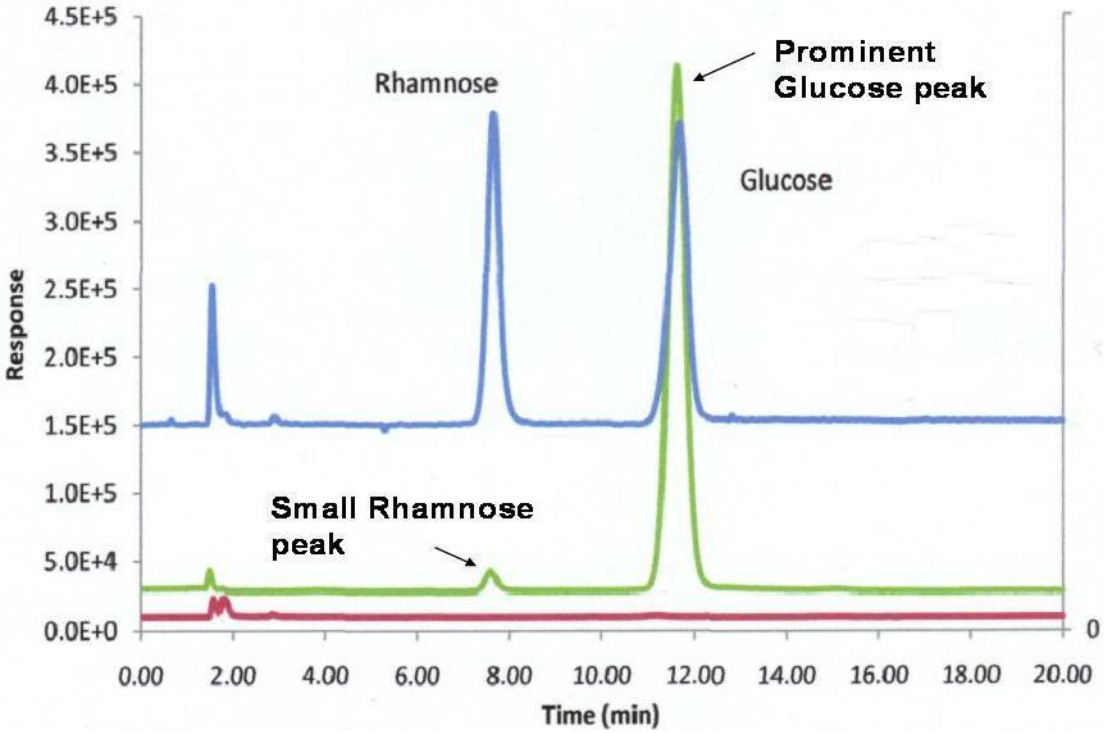



**Figure 2.1.3** HPAEC (High-Performance Anion-Exchange Chromatography) chromatograms of rhamnose and glucose standards (Blue), untreated naringin standard (Red), and aromase treated naringin standard (Green).

The enzyme-treated grapefruit peel juice samples did not provide conclusive evidence for the presence of rhamnose in their chromatograms (results not shown). A confirmatory analysis was conducted on aromase-treated (aromase concentration of 0.8% m/v) naringin standard using HPAEC in order to verify the possible formation of rhamnose after enzyme treatment. Figure 2.1.3 shows that no sugars were present in the untreated naringin standard. The aromase-treated naringin standard showed a prominent glucose peak in its chromatogram and what appeared to be a very small rhamnose peak. No peaks attributable to disaccharides could be identified. The aromase-treated naringin standard and the rhamnose standard were further analysed using GCMS and the result is shown in Figure 2.1.4.

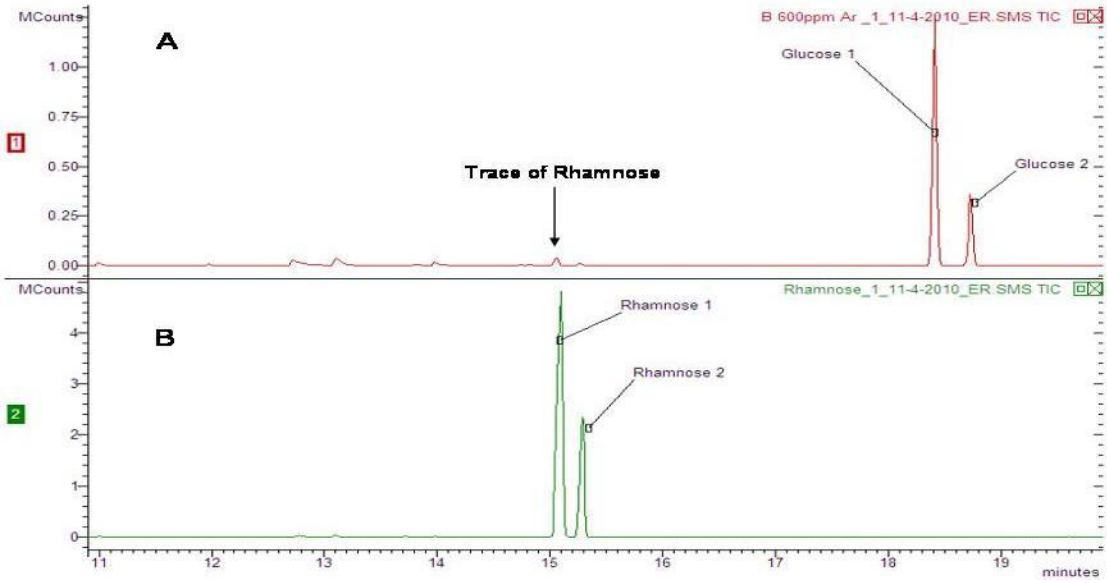



**Figure 2.1.4** GC MS (Gas Chromatography-Mass Spectrometry) chromatograms of the aromase-treated naringin standard (A) and rhamnose standard (B).

The top chromatogram (Figure 2.1.4A) of the aromase-treated naringin standard shows the presence of glucose and a trace of rhamnose. The lower chromatogram (Figure 2.1.4B) of the rhamnose standard is for retention time comparison purpose. The GCMS chromatogram obtained for the aromase-treated naringin standard is in agreement with the HPAEC chromatogram obtained for the same sample in Figure 2.1.3.

These results in Figure 2.1.3 and Figure 2.1.4 may offer some insights into the mode of action of aromase on naringin. Taken in combination with the earlier observations of the naringin and naringenin contents of the aromase-treated grapefruit peel juice, these results indicate that the action of aromase on naringin produces predominantly naringenin and glucose. It appears that contrary to what was expected, neither a disaccharide of glucose and rhamnose nor rhamnose on its own are produced in this reaction. The β-primeverosidase and β-glucosidase activities of aromase would be expected to hydrolyze naringin into naringenin, glucose and rhamnose. However, the fate of the rhamnose moiety is not clear. It may be that aromase has other activities that break down the rhamnose further which may explain the observed absence of rhamnose after treating the grapefruit peel juice with aromase.

### 2.4.5 The effect of aromase and laccase on pH, acidity and °Brix and Ratio of grapefruit peel juice

**Table 2.1.9** Effect of aromase and laccase on pH, acidity, °Brix and Ratio of grapefruit peel juice

| Laccase conc. (%)[1] | Aromase conc. (%) | pH | Acidity g/100 g | °Brix | °Brix:acid Ratio |
|---|---|---|---|---|---|
| 0 | 0 | 3.75 a[2] (0.03)[3] | 0.45 a (0.06) | 9.72 ab (1.37) | 21.94 ab (0.51) |
| 0 | 0.4 | 3.77 a (0.06) | 0.44 a (0.02) | 9.40 a (0.41) | 21.48 a (0.35) |
| 0 | 0.8 | 3.75 a (0.29) | 0.45 a (0.10) | 9.78 ab (2.24) | 22.13 ab (0.91) |
| 1.5 | 0 | 3.75 a (0.13) | 0.45 a (0.05) | 11.03 abc (1.03) | 24.83 abc (0.32) |
| 1.5 | 0.4 | 3.71 a (0.19) | 0.46 a (0.03) | 11.47 bc (0.65) | 24.96 bc (0.31) |
| 1.5 | 0.8 | 3.75 a (0.11) | 0.47 a (0.01) | 11.90 c (0.30) | 25.53 c (0.08) |
| 3 | 0 | 3.87 a (0.01) | 0.38 a (0.04) | 10.47 abc (1.31) | 27.27 abc (0.69) |
| 3 | 0.4 | 3.83 a (0.04) | 0.44 a (0.05) | 12.15 c (1.22) | 28.06 c (1.19) |
| 3 | 0.8 | 3.83 a (0.04) | 0.45 a (0.03) | 12.85 c (0.79) | 28.24 c (0.31) |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice.
[2] Mean values in a column with different letters are significantly different ($p<0.05$)
[3] Standard deviation in brackets

Treatment of grapefruit peel juice with aromase or laccase or their combinations had no significant effect on its pH and acidity. Treatment of grapefruit peel juice with aromase alone produced no difference in °Brix value. On the other hand, treatment with laccase on its own increased °Brix by up to 10% compared to the control. The combination treatments of laccase at 3% with aromase (0.4 and 0.8%) produced the greatest increase in °Brix compared to the control.

Brix (°Brix) is a relative density scale used in the sugar industry (Ball, 2006) and it indicates the percent of sucrose by weight (gram per 100 milliliter of Brix water) in a solution or juice (Moresi and Spinosi, 1980). It is the most commonly used refractrometer scale for measuring solids dissolved in water and it corresponds directly to the refractive index scale (Kappes et al., 2006). If a solution contains dissolved solids other than pure sucrose, such as other sugars (glucose, fructose and rhamnose), minerals etc., then the °Brix only approximates the dissolved solid content (Pancoast, 1980). Of the four sugars

analysed, glucose showed the most significant increase as a result of enzyme treatment. This might be the reason for the increase in °Brix.

As mentioned earlier, the Brix:acid ratio (or soluble solids:acids ratio) is calculated by dividing the percentage of soluble solids (°Brix) by the percentage of titratable acids (Sinclair, 1972). Enzyme treatment of the grapefruit peel juice increased glucose resulting in an increase in °Brix with no significant effect on titratable acids. The increased °Brix accompanied with a constant acidity resulted in increased °Brix/acid Ratio. This may be considered desirable as there is an overall reduction in the effect of the acid and makes the grapefruit peel juice more acceptable to be used as a substitute for apple, pear or grape juice in juice formulations.

### 2.4.6 The effect of aromase and laccase on the colour and clarity of grapefruit peel juice

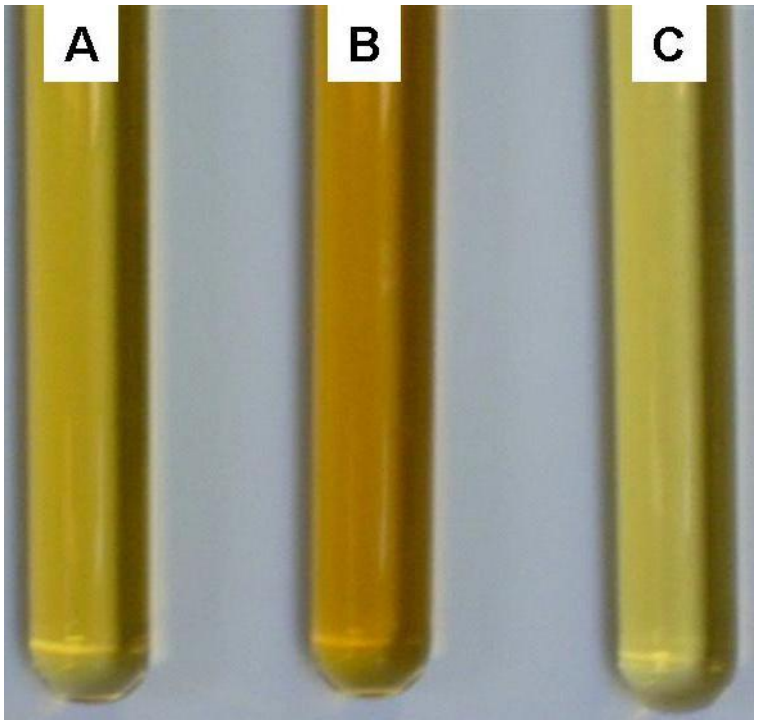



**Figure 2.1.5** Appearance of untreated grapefruit peel juice (A) and grapefruit peel juice after treatment with laccase (3.0%) (B) and aromase (0.8%) (C).

The grapefruit peel juice became darker on treatment with laccase and lighter on treatment with aromase. Laccase is a polyphenol oxidase which can cause enzymatic browning (Rocha and Morais, 2001) leading to a darker coloured grapefruit peel juice.

**Table 2.1.10** Effect of aromase and laccase on the L-value of grapefruit peel juice

| | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| Laccase conc. (%)[1] | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 78 e[2] (2)[3] | 89 f (4) | 93 g (1) | 87 b[4] |
| 1.5 | 70 d (2) | 40 c (4) | 37 b (3) | 49 a |
| 3.0 | 69 d (2) | 33 a (2) | 33 a (1) | 45 a |
| Aromase main effect | 72 b | 54 a | 54 a | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.

Treatment of grapefruit peel juice with aromase alone increased the L-value by about 19%. Treatment with laccase alone decreased the L-value by 14%. Overall, the main effects showed a significant decrease in L-value by 25% due to aromase treatment while laccase treatment had a greater significant effect by decreasing the L-value by 49%. The combination treatment of aromase (0.8%) and laccase (3.0%) produced the greatest decrease in L-value of 58%.

**Table 2.1.11** Effect of aromase and laccase on the a-value of grapefruit peel juice

| | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| Laccase conc. (%)[1] | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | -4.0 b[2] (0.8)[3] | -3.2 b (0.1) | -7.4 a (0.3) | - 4.9 a[4] |
| 1.5 | 7.3 d (0.9) | 4.0 c (0.3) | 4.1 c (0.2) | 5.1 b |
| 3.0 | 9.1 e (0.7) | 4.4 c (0.4) | 4.6 c (0.3) | 6.0 c |
| Aromase main effect | 4.1 c | 1.7 b | 0.4 a | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.

Treatment of grapefruit peel juice with aromase alone decreased the a-value by 84%. Treatment with laccase alone increased the a-value by 2.3 times. Overall, the main effects

showed a significant decrease in a-value by 90% due to aromase treatment while laccase treatment increased the a-value by 3 times. The combination treatment of aromase (0.8%) and laccase (3.0%) also increased the a-value by 3 times.

**Table 2.1.12** Effect of aromase and laccase on the b-value of grapefruit peel juice

| | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| Laccase conc. (%)[1] | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 38 c[2] (2)[3] | 25 b (3) | 27 b (3) | 30 b[4] |
| 1.5 | 40 c (1) | 21 a (1) | 20 a (1) | 27 a |
| 3.0 | 41 c (1) | 18 a (2) | 20 a (1) | 26 a |
| Aromase main effect | 40 b | 22 a | 22 a | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.

Treatment of grapefruit peel juice with aromase alone decrease the b-value by about 30% Treatment with laccase alone appeared to increase the b-value by 10% however this was not significant. Overall, the main effects showed a significant decrease in b-value by 45% due to treatment with aromase while laccase treatment reduced the b-value by 13%. The combination treatment of aromase (0.4%) and laccase (3.0%) produced the greatest decrease in b-value by 54%.

The 20% increase in L value or lightness of the grapefruit peel juice on treatment with aromase may have been caused by the hydrolysis of naringin, making the grapefruit peel juice lighter. The decrease in lightness of the grapefruit peel juice by 15% on treatment with laccase could have been due to the phenol oxidase activity of laccase by making the grapefruit peel juice darker due to enzymatic browning.

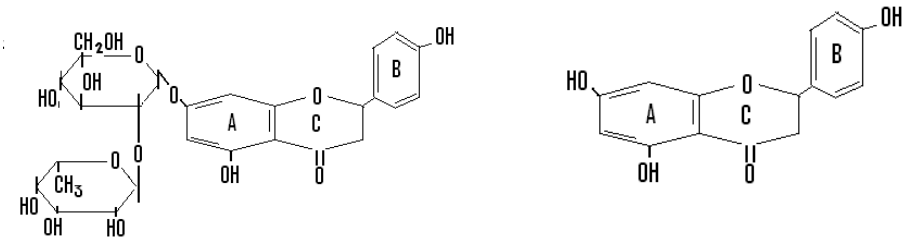
**Table 2.1.13** Effect of aromase and laccase on clarity (% Transmission) of grapefruit peel juice

| | Aromase conc. (%)[1] | | | |
|---|---|---|---|---|
| Laccase conc. (%)[1] | 0 | 0.4 | 0.8 | Laccase main effect |
| 0 | 71 ab[2] (5)[3] | 98 e (4) | 98 e (5) | 89 b[4] |
| 1.5 | 63 a (3) | 93 de (4) | 90 de (6) | 82 a |
| 3.0 | 78 bc (1) | 90 de (4) | 85 cd (8) | 84 ab |
| Aromase main effect | 70 a | 93 b | 91 b | |

[1] Enzyme concentration expressed as percentage (w/v) of grapefruit peel juice
[2] Mean values in a column and row with different letters are significantly different ($p < 0.05$) for the individual and combination effects
[3] Standard deviation in brackets
[4] Main effect values with different letters are significantly different at $p < 0.05$. Laccase main effect compared in the column and aromase main effect compared in the row.

Treatment of grapefruit peel juice with aromase alone increased clarity by 25%. This was also observable considering the aromase main effect. However overall, laccase treatment did not appear to have a pronounced effect on clarity although there was a slight decrease.

Haze is normally formed by the interaction between phenolic compounds and proteins. The amount of haze formed depends on both the concentrations of protein and polyphenol and on their ratio (Siebert et al., 1996). Hydroxyl groups are present in tannins (and other polyphenolic compounds) that can bind with more than one polypeptide chain to cross link that leads to haze (Emmambux, 2004).

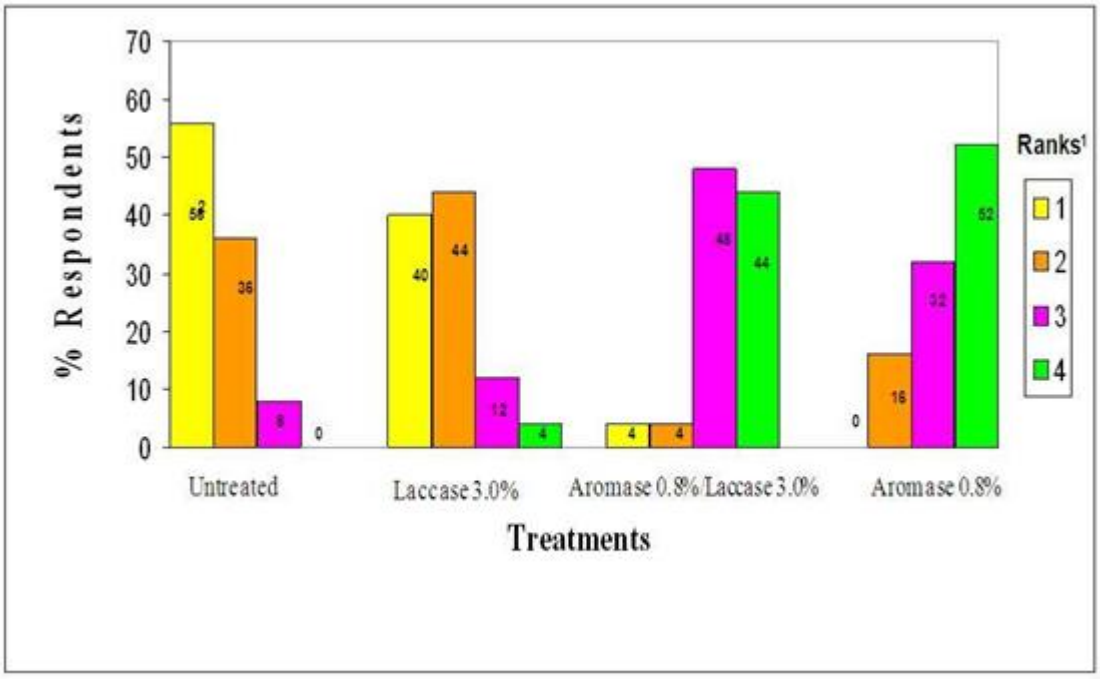



**Naringin** **Naringenin**

**Figure 2.1.6** Structures of naringin and naringenin.

The structures of naringin and naringenin are shown in Figure 2.1.6. It can be seen that naringin has more hydroxyl groups due to the fact that it is a glycoside with sugar molecules attached. Therefore the hydrolysis of naringin to naringenin by the aromase enzyme leads to less available hydroxyl groups for interaction with proteins. The resultant effect would be less haze formation and greater clarity of the grapefruit peel juice.

### 2.4.7 Sensory evaluation




[1]Ranks

1st Most bitter

2nd Second most bitter

3rd Second least bitter

4th Least bitter

[2]Rank total (%) given by the panelists (n=25) for each of the grapefruit peel juice samples

**Figure 2.1.7** % Respondents for each ranking level of the grapefruit peel juice samples.

Figure 2.1.7 shows the percentage of respondents obtained during sensory evaluation of the grapefruit peel juice samples for each ranking level. A total of 92% of the panelists perceived the untreated sample as either most bitter or second most bitter while only 8% perceived it as either least bitter or second least bitter. The lacase-treated sample was perceived by a total of 84% of the panelists as either the most bitter or second most bitter while only 16% perceived it as least bitter or second least bitter. Only 16% of the panelists perceived the aromase-treated sample as either the most bitter or second most bitter while a total of 84% perceived it as least bitter or second least bitter. The combination-treated sample was perceived by only 8% of the panelists as either most bitter or second most bitter while a total of 92% perceived it as either least bitter or second least bitter. Ranking and Rank totals given by the panelists to the four grapefruit peel juice samples can be seen in Table 2.1.14.

**Table 2.1.14** Ranking totals for bitterness by 25 panelists to four enzyme treated grapefruit peel juice samples

| Treatment | Ranks[2] | Rank total |
|---|---|---|
| Aromase 0.8% | 1st | 84.00 a[1] |
| Aromase 0.8%, laccase 3.0% | 2nd | 83.00 a |
| Laccase 3.0% | 3rd | 45.00 b |
| Untreated | 4th | 38.00 b |

[1]Rank totals (given by the panelists to the four grapefruit peel juice samples) in a column with different letter are significant different ($p<0.05$)
[2]Ranks

| | |
|---|---|
| 1st | Least bitter |
| 2nd | Second least bitter |
| 3rd | Second most bitter |
| 4th | Most bitter |

The aromase-treated grapefruit peel juice was ranked least bitter and the untreated as most bitter. Generally, the sensory ratings of the grapefruit peel juice samples could be related to their content of these bitter compounds. The taste threshold of naringin in water is approximately 20 mg/kg (Munish et al., 2000) and for limonin 4 to 6 mg/kg (Fayoux et al., 2007). Quinine is an alkaloid that is generally accepted as the standard for the bitter taste sensation. The detection threshold for quinine hydrochloride (IV) is about 10 ppm

(Lindsay, 1996).The respondents in the sensory analysis rated the grapefruit peel juice treated with aromase alone as the least bitter which can be related to what was found with the HPLC results where treatment with increased concentrations of aromase alone produced the biggest decrease in naringin and limonin. The untreated grapefruit peel juice sample with the highest concentrations of naringin and limonin was rated most bitter by the respondents in the sensory analysis.

## 2.5 Conclusion

Treatment of grapefruit peel juice with aromase and laccase reduces levels of the bitter compounds naringin and limonin significantly. The reduction in bitter compounds seems to be predominantly due to the action of aromase rather than laccase. This is shown in sensory results with respondents rating aromase-treated grapefruit peel juice samples as least bitter. This work shows that aromase can inactivate both bitter compounds naringin and limonin and can be used in debittering applications to reduce bitterness. Apart from the effect on bitterness, treatment of the grapefruit peel juice with the enzymes also affects colour and clarity of the juice and this may influence application of the debittered peel juice. Laccase makes the juice darker while aromase makes it lighter and increases clarity.

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# CHAPTER 3: DISCUSSION

## 3.1 Methodologies

In the citrus juice processing industry, peel juice is obtained by shredding the peel waste from the extraction process to obtain a peel liquid slurry and pressing to produce a spent peel cake and a raw peel juice which consists of water, sugars, flavour components and oils (Chu et al., 2006). This is done by using the following basic steps as outlined in Figure 3.1.1.

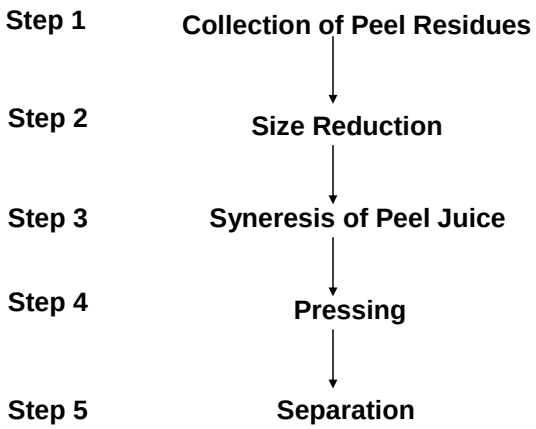



**Figure 3.1.1** The process of citrus peel juice extraction. (Chu et al., 2006; Wilkins et al., 2007)

Currently the effective syneresis of peel juice to give a product that is fit for human consumption is a challenge. Syneresis of peel juice is done in the industry by using either lime or enzymes consisting of a combination of pectinases, hemicellulases and cellulases. Untreated peel is very slimy and retains moisture. The slimy nature is probably due to hydrogen bonding of the ester groups of the pectin with water. The industry primarily makes use of lime and enzymes to enhance the syneresis of the peel juice. The advantages and disadvantages of these methods are summarized in Table 3.1.1.

**Table 3.1.1** The advantages and disadvantages of peel juice syneresis with lime and enzymes

| Treatment | Advantages | Disadvantages |
|---|---|---|
| Lime | Lime demethoxylation of the pectin ester groups will give a less slimy texture (Kimball, 1999) | The Lime contacts free water producing calcium hydroxide $Ca(OH)_2$, which is toxic. |
| Enzymes | Cellulose, hemicellulose and pectin can be hydrolyzed using pectinase, cellulose and beta-glucosidase enzymes (Wilkins et al., 2007). The peel juice is fit for human consumption | A combination of enzymes is needed. Enzymes need ideal pH and temperature conditions to be effective. |

A different method for syneresis of peel juice was used in this study to ensure the same advantages, but reduce the disadvantages. The milled peels were frozen at – 18°C followed by thawing. Bulk freezing is a slow process during which big ice crystals are formed (Miyawaki, 2001). The ice crystals break down the cell membranes around each cell (Arthey, 1993). The osmotic potential changes during freezing and thawing and this can also cause cell wall degradation (Cheftel et al., 2000). The peel liquid then leaks out of the unprotected cells during thawing. The main advantage of the method used in this study is that it produces a peel juice that is fit for human consumption as it does not involve addition of lime which produces toxic $Ca(OH)_2$. The process will also be easy to carry out by using simple, readily available and non-sophisticated equipment in a juice processing facility.

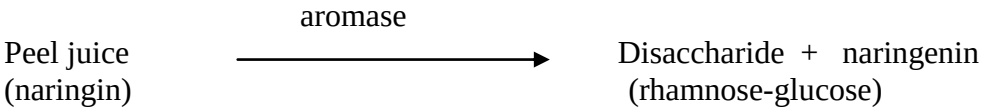
Grapefruit peel juice is extremely bitter mainly due to the compounds limonin and naringin. In this study the aim was to produce a grapefruit peel juice that can be used as a substitute for apple and pear juice in juice formulations. The peel juice was debittered by inactivating the bitter compounds limonin and naringin. This study made use of the commercial enzymes aromase and laccase for debittering. Aromase has ß-primeverosidase activity where it converts di-glycosides (ß-primeverosides) to aglycone form and releases the

glycoside, as well as ß-glucosidase activity where it hydrolyses the o-glycosidic bond between the aglycone and the glycoside. Laccase has polyphenol oxidase activity and can oxidize various aromatic compounds forming free radicals that can undergo polymerization and be removed. Three chromatographic techniques were used for analysis of the treated and untreated grapefruit peel juice as shown in Table 3.1.2.

**Table 3.1.2** Chromatographic techniques used for analysis of enzyme-treated and untreated grapefruit peel juice

| Technique | Sample | Compounds analysed |
|---|---|---|
| HPLC | Untreated and enzyme-treated grapefruit peel juice | Sugars (glucose, fructose, sucrose and rhamnose)<br>naringenin<br>naringin<br>limonin |
| HPAEC-PAD | Enzyme-treated naringin standard | Sugars (glucose, fructose, sucrose and rhamnose) |
| GC-MS | Enzyme-treated naringin standard | naringin<br>naringenin<br>rhamnose<br>glucose |

Sugars (glucose, fructose and sucrose), naringin, naringenin and limonin were analysed using three different HPLC methods. It was expected that the action of aromase on naringin would produce naringenin and a disaccharide (as shown in the reaction below) due to the ß-primeverosidase and ß-glucosidase activity of aromase.




Contrary to this expectation, when the HPLC method for sugars was run on the aromase-treated peel juice sample, no disaccharide consisting of rhamnose and glucose was detected. Only glucose was detected. More research was therefore needed to gain some insight into

the mechanism of action of aromase on naringin. A decision was made to determine the sugars released when a naringin standard was treated with aromase. Another HPLC method using a carbohydrate column with $H_2O$ as mobile phase with refractive index detection was used for sugar analysis of the aromase-treated naringin standard. The result obtained still indicated the absence of rhamnose as well as a disaccharide.

A usual occurrence during HPLC analysis of sugars is that rhamnose and glucose tend to elute at similar retention times and as a result their peaks overlap. Another chromatographic technique, HPAEC-PAD (High Performance Anion Exchange Chromatogram with Pulsed Amperometric Detection) was used to investigate this. HPAEC-PAD separates carbohydrates via specific interactions between the hydroxyl groups of the glycan and the stationary phase at high pH (Edge, 2003). Glycans usually consist solely of O-glycosidic linkages of monosaccharides. HPAEC-PAD is more resolution efficient and can prevent overlaps between different sugars (Guignard et al., 2005). The HPAEC-PAD of the aromase-treated naringin standard showed a prominent glucose peak in its chromatogram and what appeared to be a very small rhamnose peak which was a similar result to what was found with HPLC analysis of the sugars.

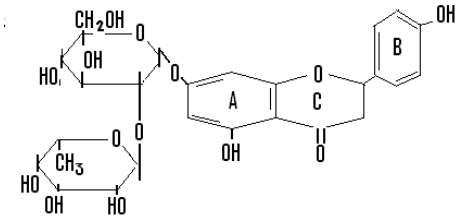
Both glucose and rhamnose can exist as isomers (Forsman and Leino, 2010). GC-MS (Gas Chromatography- Mass Spectrometry) was used in an attempt to identify the isomers, if existent, and to confirm if both isomers were not being detected by HPAEC-PAD analysis. An aromase-treated naringin standard as well as a rhamnose standard for retention time comparison purpose were analysed by GC-MS. The aromase-treated naringin standard showed two peaks representing the glucose isomers and only a trace of rhamnose. This confirmed the HPLC result where only glucose was detected.

The results from the three chromatographic techniques (HPLC, HPAEC-PAD and GC-MS) provided some insight about the possible mechanism of action of aromase on naringin. It is clear that glucose is a major product when aromase acts on naringin as shown in the reaction below. However, what is not clear is the fate of the rhamnose moiety of naringin. This is discussed later in the document.

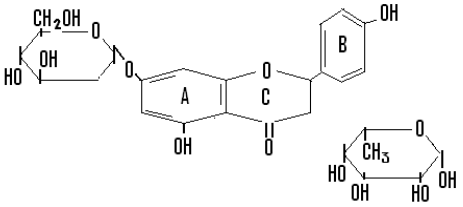
$$\text{Peel juice (naringin)} \xrightarrow{\text{aromase}} \text{glucose + naringenin}$$

Sensory evaluation was used to determine the perception of bitterness in the samples. Evaluation was conducted through a Ranking test and panelists were in separate tasting booths to avoid any influence from each other and to minimize distractions from noise. There was no training for the consumers except introducing them to the session, which saved a lot of time. Consumers used were screened for ability to taste bitterness. Ideally all 9 samples of the different enzyme treatments should have been evaluated but having 9 samples with different degrees of bitterness could be too much to differentiate. Four grapefruit peel juice samples consisting of the untreated and the three highest enzyme-treated samples, laccase-treated (0.3%), aromase-treated (0.8%) and the combination-treated aromase (0.8%), laccase (3.0%) samples were evaluated by the panelists. These four samples were a smaller number to handle and easier to distinguish unlike when a large number is involved.

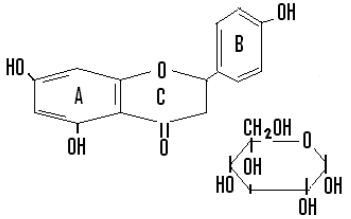
### 3.2 The effect of aromase and laccase on naringin and naringenin and limonin content of grapefruit peel juice



**Naringin**



**Prunin + Rhamnose**



**Naringenin + Glucose**

**Naringinase**

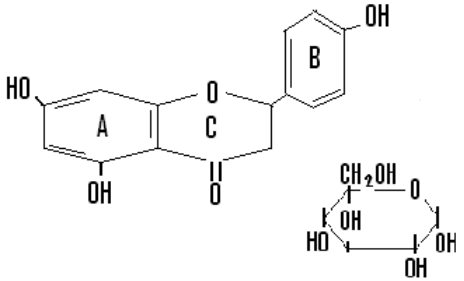
**Rhamnosidase**

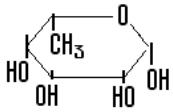
**Naringinase**

**Glucosidase**

**Aromase**

**β-primeverosidase**
**β-glucosidase**





**Naringenin + Glucose + Rhamnose (may be broken down further into other compounds)**

**Figure 3.2.1** Comparison of the mode of action of naringinase on naringin (Puri et al., 2011) and of aromase on naringin.

Naringinase is commercially used to inactivate the bitter compound naringin (Figure 1.2.6). It has σ-rhamnosidase and ß-glucosidase activities which will enable it to hydrolyze naringin in two successive steps. The aromase enzyme used in this study showed some similarity to naringinase (Figure 3.2.1). The decrease in naringin and increase in naringenin content of the aromase-treated grapefruit peel juice could be hypothesized to be mainly the result of the ß-primeverosidase activity and to a lesser extent due to the ß-glucosidase activity of aromase.

The main flavanone glycosides in citrus fruit are the structural isomers, narirutin (naringenin 7-ß-rutinoside) and naringin (naringenin 7-ß-neohesperidoside) (Horowitz and Gentili, 1964). The ß-primeverosidase activity of aromase hydrolyzes the di-glycoside naringin or its isomer narirutin by releasing the aglycone naringenin and the disaccharide neohesperidoside or rutinoside. The ß-glucosidase activity of aromase would be expected to inactivate naringin by hydrolyzing the glycosidic bond between glucose and the aglycone. By doing this it removes the disaccharide of glucose and rhamnose from naringin and releases the aglycone naringenin as shown in Figure 3.2.1. An aromase-treated naringin would be therefore be expected to yield a disaccharide (neohesperidoside consisting of L-rhamnose and D-glucose) or the monosaccharides glucose and rhamnose probably in the ratio 50:50. However, in this study, chromatographic analyses of the aromase-treated samples showed that the enzyme treatment produced an increase in naringenin and glucose with only a trace of rhamnose. It is possible that the rhamnose may be converted into other compounds (Figure 3.2.1) and could suggest that aromase may have other activities above the known β-primeverosidase and β-glucosidase activity. Aromase is a commercial enzyme and can have some side activities.

The fermentative action of micro-organisms which involves action of various enzymes such as isomerases, kinases and aldolases can convert rhamnose into other compounds. As a result of the action of enzymes produced by *Bacillus macerans*, L-rhamnose can split into glyceraldehyde and L-lactaldehyde (Weimer, 1984) and the reduction of the latter can yield propylene glycol (Saxena et al., 2010). The action of enzymes produced by *Escherichia coli* may bring about isomerization of L-rhamnose

to L-rhamnulose which can be converted to L-rhamnulose 1-phosphate as shown in Figure 3.2.2 (Takagi and Sawada, 1964)).

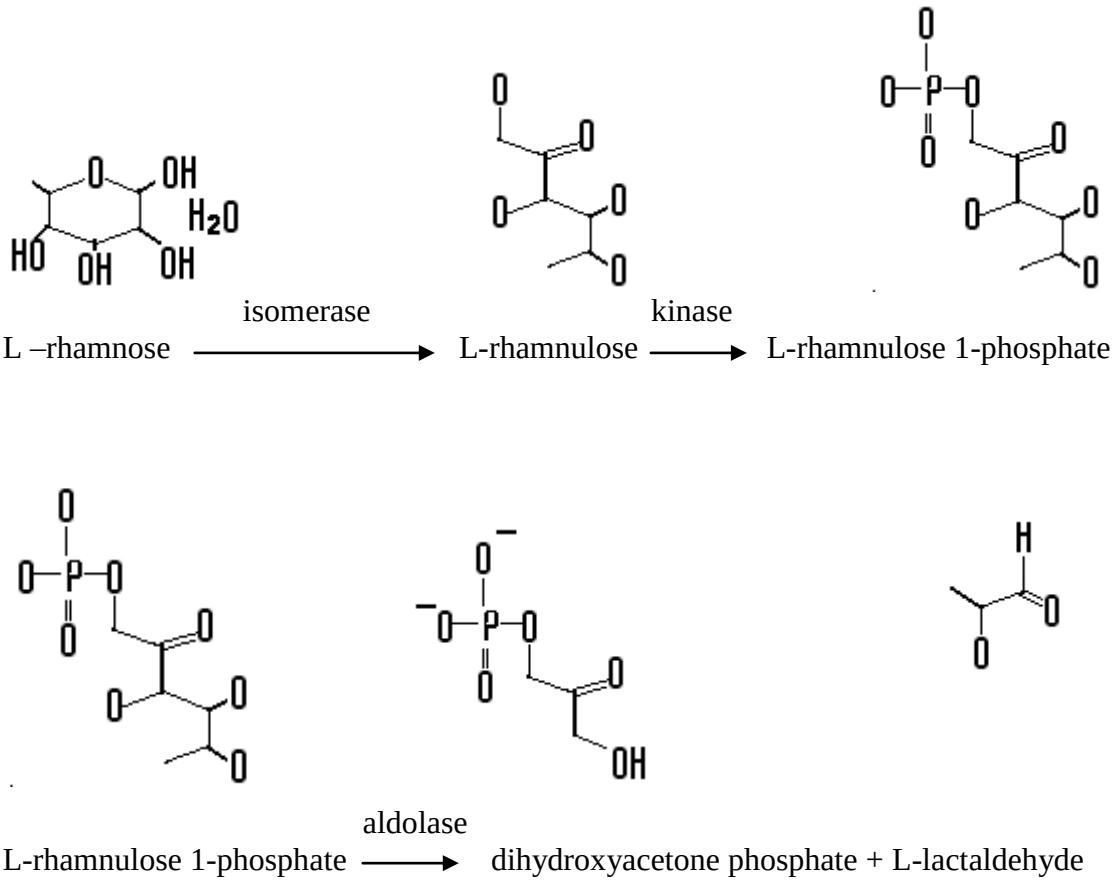



**Figure 3.2.2** The effect of L-rhamnose isomerase, L-rhamnulose kinase and L-rhamnulose-1-phosphate aldolase on L-rhamnose (Weimer, 1984; Saxena et al., 2010; Takagi and Sawada, 1964).

Rhamnose isomerase (L-rhamnose ketol isomerase, E.C. 5.3.1.14) converts L-rhamnose to L-rhamnulose (6-deoxy-L-fructose) (Figure 3.2.2), which is followed by phosphorylation of the primary hydroxyl group mediated by a specific rhamnulose kinase, and finally, by an aldol cleavage into dihydroxyacetone phosphate, and L-lactaldehyde is catalyzed by L-rhamnulose 1-phosphate aldolase (Prabhu et al., 2011). All these are possible reactions that rhamnose can undergo after being released from naringin.

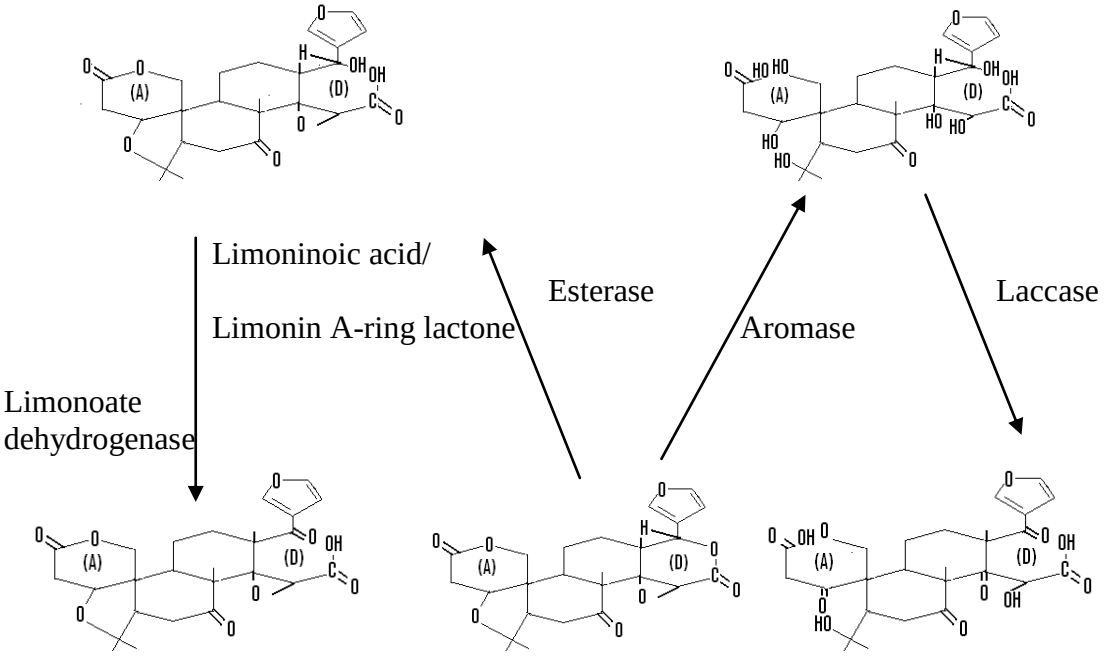



**Figure 3.2.3** Comparison of the mode of action of esterase and limonoate dehydrogenase on limonin (Lindsay, 1996) and of aromase and laccase on limonin.

The aromase-treated grapefruit peel juice showed a reduction of almost 40% in limonin. The ß-glucosidase activity of aromase could open the A and D ring of limonin by hydrolysis as seen in Figure 3.2.3 forming hydroxyl and carbonyl groups. Aromase might also hydrolyze the furan ring in the limonin structure forming two hydroxyl groups.

It appears that limonin was hydrolyzed into more than one product as shown by the appearance of peaks 1 and 2 in Figure 2.1.2 (Research chapter). These two peaks were also present in the calibration curves of the limonin standard which suggests that these compounds could be traces of the precursor material of limonin during its biosynthesis. It may be hypothesized that one of the compounds may be formed as a result of hydrolysis of the furan ring attached to the A-ring of limonin, forming a compound with two hydroxyl groups similar to the precursor compound Ichangin as seen in Figure 1.2.4-(Biosynthetic pathway of limonin in literature review). The other peak could be of limonoate A- Ring lactone formed after the opening of the D ring of

limonin. Both peaks showed visible increases in area and height after aromase treatment.

Limonin is largely responsible for the delayed development of bitterness in citrus juice (Maier et al., 1980) as seen in Figure 1.2.5 of the literature review. The limonin concentration increased by almost 30% over time in the untreated grapefruit peel juice as seen in Table 2.1.5 of the results. As mentioned earlier, this phenomenon of delayed bitterness is attributed to the formation of limonin from the limonoate A-ring lactone present (Fayoux et al., 2007). On the other hand, aromase treatment decreased the limonin content which decreased further over time. This suggests further breakdown of limonin and limonoate A-ring lactone in the aromase-treated sample during storage due to the action of aromase. The apparent lack of delayed bitterness in the aromase-treated sample may be an indication that there was no available limonoate A-ring lactone present that could be converted to limonin over time. This may be regarded as a positive result and offers a way of producing a more stable grapefruit peel juice with a constant level of bitterness over time.

It appears that treatment of the grapefruit peel juice with laccase alone decreased naringin, increased naringenin and also decreased limonin. However these changes were marginal.

Treating grapefruit peel juice with a combination of aromase and laccase produced synergistic effects on the levels of naringin and naringenin. It may be proposed that this may be possibly due to two successive events. Naringin is first hydrolyzed by aromase to naringenin followed by laccase oxidizing hydroxyl groups on the naringenin and converting it into other compounds which were probably not detected by the HPLC method used in this work for naringenin. The combination enzyme treatment also produced a synergistic effect on reduction of limonin possibly via hydrolysis of ester and ether bonds by aromase followed by oxidation of free hydroxyl groups by laccase. This showed some similarity with previous research done using the enzyme esterase on limonin as substrate followed by limonoate dehydrogenase on limonin A-ring lactone as substrate (Lindsay, 1996). One of the actions of aromase and laccase was possibly similar to esterase and limonoate dehydrogenase.

Generally, the sensory ratings of the grapefruit peel juice samples could be related to their content of the bitter compounds naringin and limonin. The respondents in the sensory analysis rated the grapefruit peel juice treated with aromase alone as the least bitter which can be related to what was found with the HPLC results where treatment with increased concentrations of aromase alone produced the biggest decrease in naringin and limonin. The untreated grapefruit peel juice sample with the highest concentrations of naringin and limonin was rated most bitter by the respondents in the sensory analysis. Both treatments with aromase on its own (at its highest concentration), and the combination treatment of aromases and laccase (at its highest concentration) decreased naringin and limonin in grapefruit peel juice more than the other enzyme treatments and were perceived as least bitter by the sensory panel.

### 3.3 The effect of aromase and laccase on the colour and clarity of grapefruit peel juice

The 20% increase in L value or lightness of the grapefruit peel juice on treatment with aromase was probably caused by the hydrolysis of naringin and naringenin, making the grapefruit peel juice lighter due to a decrease in available phenolic compounds (flavonoids). The decrease in lightness of the grapefruit peel juice by 15% on treatment with laccase could have been due to the phenol oxidase activity of laccase (Mayer, 2006) by making the grapefruit peel juice darker due to enzymatic browning. Most phenolic compounds (flavonoids) like naringin and naringenin (Gattuso et al., 2007) in grapefruit peel juice can serve as substrates for enzymatic browning (Ruangchakpet and Sajjaanantakul, 2007). Laccase is an example of a phenol oxidase which catalyzes the transformation of an array of aromatic compounds that have two adjacent phenolic groups on them like naringin and naringenin (Mayer, 2006). This includes phenolic compounds that act as antioxidants. These copper-containing enzymes like laccase oxidize the phenolic groups to reactive molecules known as quinones, which continue reacting with each other and other cellular factors to form brown pigments known as melanin (Mayer, 2006) that are responsible for the dark colour of the grapefruit peel juice. The synergistic effect of the combination of aromase and laccase was shown by the highest decrease by 58% in the lightness. This may be due to the hydrolysis by aromase and the increased availability of hydroxyl groups that could be oxidized by laccase.

Flavonoids (flavanones and flavones) are the most common phenolic compounds found in citrus fruit (Ignat et al., 2011). Polyphenolics provide a number of different functionalities in foods including colour and astringency. Tannin-protein interactions are important in foods like beer where the tannins from the hops react with protein to form "haze" (Siebert and Troukhanova, 1996). Numerous aromatic rings with available hydroxyl groups are present in tannins that can bind with more than one polypeptide chain to cross link that leads to haze (Emmambux, 2004). It may be hypothesized that such phenolic-protein interactions may also contribute to lack of clarity in the grapefruit peel juice. The clarity of the grapefruit peel juice increased by almost 25% on treatment with aromase alone. The combination of aromase and laccase produced a similar effect by increasing the clarity by 20%. Aromase hydrolyzes naringin to the aglycone naringenin which has less hydroxyl groups that can interact with proteins. The resultant effect would be less haze formation and greater clarity of the grapefruit peel juice.

### 3.4 Proposed method for producing debittered grapefruit peel juice

The process of debittering currently used in the industry can either be with enzymes (naringinase) (Puri and Banerjee, 2000) or resins which are able to remove phenolic compounds by adsorption on activated carbon or by anion-exchange (Grohmann et al., 1999). The resin needs to be used in specified equipment containing resin columns. An ultrafiltration unit is also needed to clarify the juice before using resin columns (Kimball, 1999). As a result, the use of resins can be an expensive exercise due to the need for sophisticated pieces of equipment. With regard to the use of naringinase, a possible disadvantage is that it can only reduce naringin levels and not inactivate limonin if present. In this regard, the proposed method for debittering grapefruit peel juice in this research provides a significant advantage in that a single enzyme, aromase (at the most effective concentration of 0.8% w/v as shown in this research) can be used to inactivate both naringin and limonin and its precursor limonoate A-ring lactone.

From the results of this study, a method for producing debittered grapefruit peel juice may be proposed as shown in Figure 3.3.1 below.

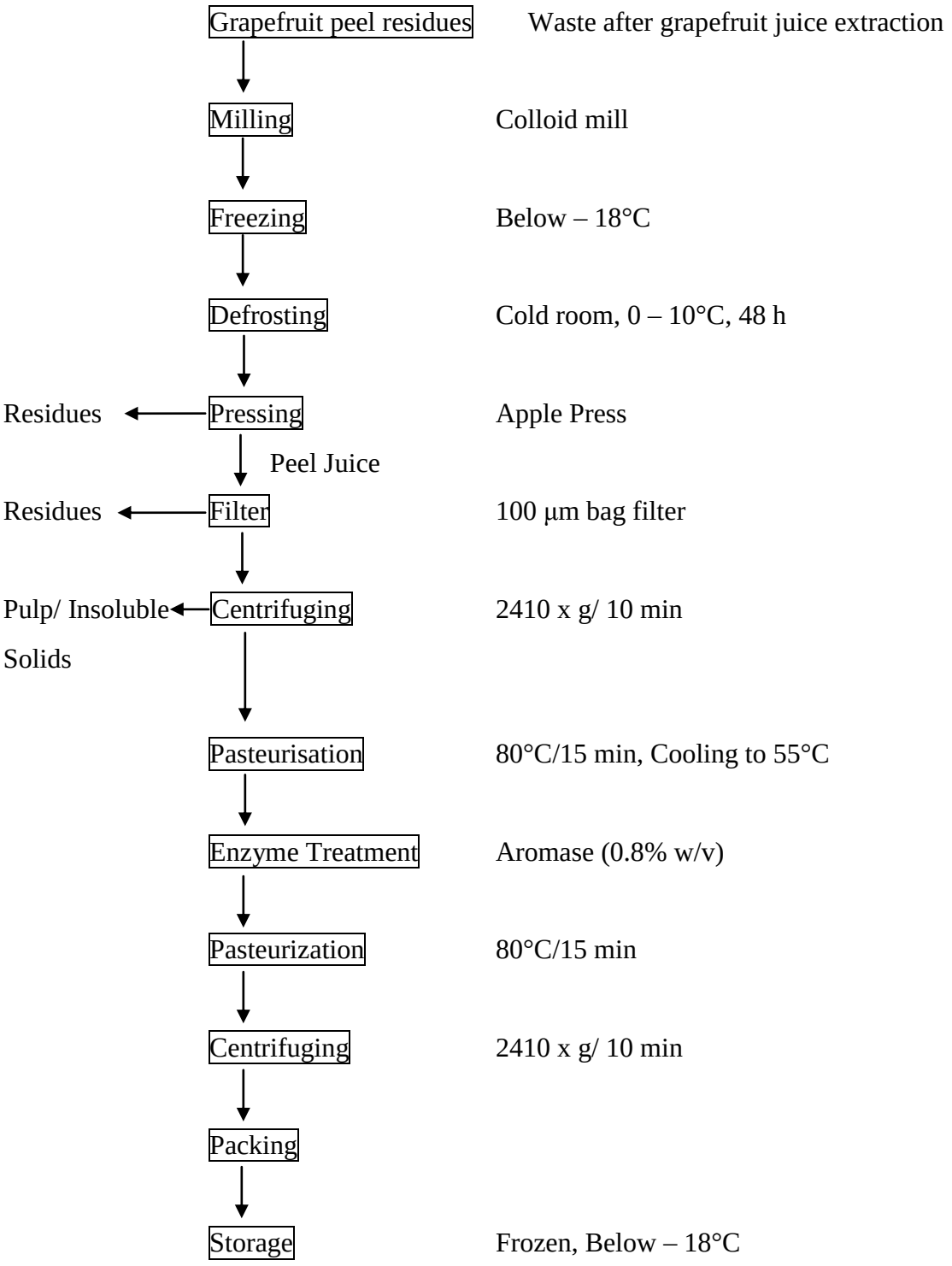



**Figure 3.3.1** Flow chart showing a proposed method for producing debittered grapefruit peel juice.

The proposed method in this research also lends itself to further simplification so it can be used by small scale citrus farmers or processors to further process peel waste.

Another advantage of the proposed method is that it requires only standard equipment for example a freezer, mill, press, tanks, centrifuge and pasteurizer which are equipment commonly found in fruit juice processing factories and therefore could be less capital intensive. This is in contrast to for instance, methods based on the use of resins where more sophisticated technology such as membrane filtration is required.

Peel waste currently removed from citrus factories by contractors at a high cost can be used as raw material to extract peel juice that can be enzymatically debittered to create a value added product that can be used as a substitute for apple or pear concentrate in fruit juice formulations.

### 3.5 Future research

One of the limitations of this research was that although the results did provide some evidence of breakdown of limonin through the action of aromase, the exact mechanism and degradation products could not be verified. More sophisticated chromatographic techniques coupled with mass spectroscopy could be used in further research to determine the products formed by the action of aromase on limonin and provide more insights into the mechanism of action of the enzyme. This will enable more efficient use of the enzyme in the debittering process.

The results of this research also appeared to suggest that the aromase enzyme had other activities apart from the known β-primeverosidase and β-glucosidase activity. The fate of rhamnose produced from hydrolysis of naringin by aromase could not be verified. It was hypothesized that the rhamnose could be converted into other compounds by aromase exerting other possible activities like isomerase, kinase and aldolase which are also shown by *Bacillus macerans* and *Escherichia coli*. More research is needed to identify and verify these other hypothesized activities of aromase. This will provide more insight on the full spectrum of activities of aromase and the mechanism of action of the enzyme aromase on naringin and resulting breakdown products.

# CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

This study shows that the enzymes aromase and laccase impact differently on the bitter compounds, limonin and naringin, and other physico-chemical properties of grapefruit peel juice.

Treatment of grapefruit peel juice with aromase reduces levels of naringin significantly with an increase in naringenin and glucose. It appears that the ß-primeverosidase activity of aromase may hydrolyze naringin into the aglycone naringenin and the disaccharide glucose-rhamnose. It also seems that the ß-glucosidase activity of aromase may hydrolyze the disaccharide glucose-rhamnose. It appears aromase may have other activities that involve further breakdown of the rhamnose moiety.

Treatment of grapefruit juice with aromase reduces levels of limonin significantly without any sign of an increase in limonin after a 7 month storage period. This may be due to hydrolysis of limonin as well as its precursor limonoate A-ring lactone by the aromase enzyme and this may leave no available limonoate A-ring to be converted to limonin. Aromase may therefore be effective at preventing delayed bitterness in grapefruit peel juice.

Treatment of grapefruit peel juice with laccase reduces levels of naringin with no effect on glucose. Laccase, a polyphenol oxidase may break down naringin by oxidizing the hydroxyl groups on the naringin molecule.

As with treatment with aromase on its own (at its highest concentration), the combination treatment of aromase and laccase (at their highest concentrations) also decreases naringin and limonin in grapefruit peel juice much more than the other enzyme treatments. As a result the debittered grapefruit peel juice resulting from these two treatments are perceived as least bitter by a sensory panel.

Treatment of grapefruit peel juice with aromase lightens the colour and increases the clarity. The hydrolysis of naringin leads to formation of other phenolic compounds

(naringenin) with less hydroxyl groups that can interact with proteins. On the other hand, treatment of grapefruit peel juice with laccase darkens the colour. Laccase, a polyphenol oxidase oxidizes phenolic groups to quinones, which continue reacting with each other to form brown pigments known as melanins.

This research has shown that aromase can be used to reduce bitterness in grapefruit peel juice. The debittered grapefruit juice can be used as a substitute for apple, pear and grape juice in any juice formulation. However, the grapefruit aroma and flavour still remain in the debittered peel juice. To make the debittered peel juice more useful in other applications, more research is needed to find appropriate ways of removing the grapefruit aroma and flavour. Citrus fruit flavour and aroma is due to complex combinations of soluble compounds (mostly acids, sugars, and flavonoids) and volatile compounds. The latter consists mostly of mono- and sesquiterpenes (Sharon-Asa et al., 2003). To remove these compounds, techniques such as vacuum evaporation, enzymes in combination with ion exchange resins, fining agents, adsorbents and precipitating agents can be investigated. The colour changes due to pigment formation resulting from the use of enzymes such as laccase can also be removed together with flavour and aroma compounds by using activated carbon columns.

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