STUDIES ON THE EXTRACTION AND CHARACTERIZATION OF PECTIN AND BITTER PRINCIPLES FROM NEW ZEALAND GRAPEFRUIT AND PHILIPPINE CALAMANSI

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ABSTRACT

A study was conducted to determine the presence of bitter components in NZ grapefruit and Philippine calamansi; describe the effect of maturity on the bitter components and other chemical constituents of grapefruit; reduce the bitterness of grapefruit juice by adsorption on polyvinylpyrrolidone; and to extract and characterize pectin from grapefruit peel.

Naringin (995 ppm), narirutin (187 ppm), and limonoids (7.9 ppm) were detected in NZ grapefruit juice concentrate (27^{o} Brix). Naringin was not detected in the calamansi juice, and limonin was detected at the level of 10.5 ppm in juice containing 5% crushed seeds.

Maturation of the grapefruit caused an increase in pH from 3.00 to 3.50, an increase in total soluble solids from 10.8 to 14.4 with a decline to 13.5^{o} Brix later in the season, a steady fall in acidity from 2.50 to 1.31 g citric acid/100 mL, and a continuous rise in the Brix/acid ratio from 4.2 to 10.3. Juice yield fluctuated throughout the season. Ascorbic acid remained fairly steady in the early- and mid-season fruit but decreased in the late-season fruit. Naringin content was highest at the beginning of the season and fluctuated throughout the season. Naringin content in the grapefruit peel remained constant as the fruit matured. Narirutin was detected in the early-season fruit but disappeared later in the season. Limonoid content in both unpasteurized and pasteurized juices decreased with ripening.

The use of polyvinylpyrrolidone significantly reduced naringin in grapefruit juice by up to 78.1% and limonin by up to 17.5% depending on the amount and reaction time of the adsorbent. A loss of 23.1% in ascorbic acid occurred with 5% PVP with a reaction time of 1 h.

Pectin extraction at 85°C and the use of acidified isopropyl alcohol yielded a product with the following characteristics: 8.9% yield; 1.3% moisture content; 1.9% ash; 759 equivalent weight; 9.2% methoxyl content; 82.2% anhydrogalacturonic acid; 63.2% degree of esterification; 4.2 intrinsic viscosity; 89,362 molecular weight and setting time of 0.55 minute.

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CHAPTER 1

INTRODUCTION

Citrus fruits may fairly be regarded as one of the most important crops directly consumed as food. In New Zealand, grapefruit (C. grandis x C. reticulata) is the most important citrus fruit grown and accounts for almost all of the grapefruit acreage (Anon. 1980). The calamansi (C. mitis) is extensively grown in the Philippines and is now the leading citrus fruit being utilized primarily for its juice.

Several studies had been conducted on the composition of citrus fruits to serve the needs of the food processing and by-products industries for chemical information. However, in New Zealand and Philippines, limited information is available on the seasonal changes of bitter components, pectin extraction and characterization, and the use of adsorbents to reduce the bitter components below the organoleptically detectable level.

This study aims to present information on the bitter components of both grapefruit and calamansi, to describe the physiological and biochemical changes in grapefruit during maturation and ripening, to reduce the bitterness of the grapefruit juice by adsorption on polyvinylpyrrolidone (PVP), and to extract and characterize pectin from grapefruit peel.

1.1 BITTERNESS IN CITRUS FRUITS

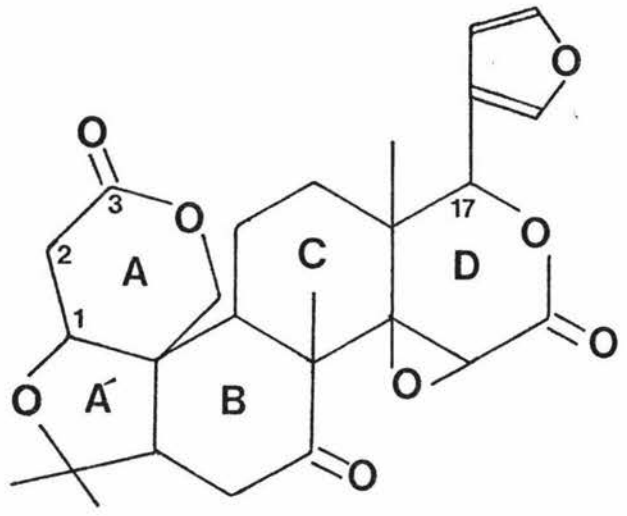
Bitterness in citrus fruits and products is primarily caused by two groups of compounds - limonoids and flavonoids (Kefford and Chandler, 1970; Chandler and Nicol, 1975; Rombouts and Pilnik, 1978).

1.1.1 Limonoid Bitterness

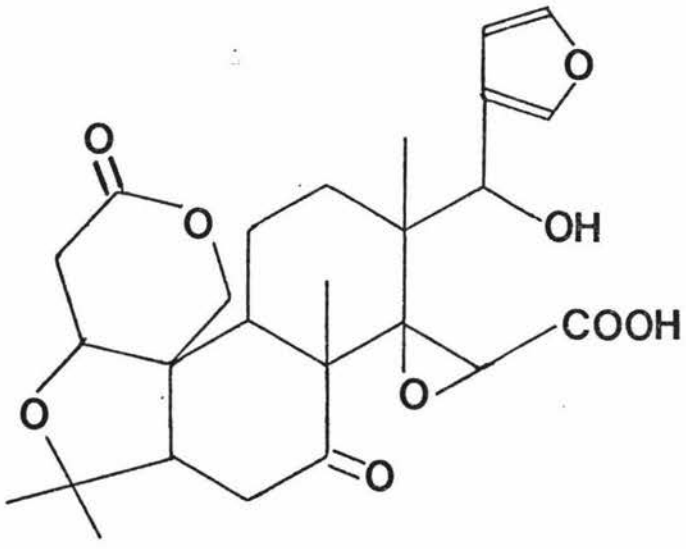
Limonoid bitterness is associated with Navel oranges and grapefruit (Emerson, 1949; Maier and Dreyer, 1965). About 120 years after its first isolation 1841, limonin, the major limonoid was identified as a triterpenoid derivative (I) (Arigoni et al., 1960).

Citrus seeds contain relatively large amounts of limonoids (Hasegawa et al., 1980). They have also been reported to occur in the peel and juice of oranges and grapefruit (Emerson, 1949; Maier and Dreyer, 1965). In citrus juice production, the gradual development of bitterness in the juice after extraction presents a serious problem. This phenomenon is referred to as delayed bitterness since it is not observed either in fresh fruit or in the juice immediately following extraction (Joslyn and Pilnik, 1961). Juice held at room temperature develops a definite bitterness after several hours or, if heated, within a few minutes. This delayed bitterness is characteristic of juice from early-season to mid-season Washington Navel, Australian Valencia and Israeli Shamouti oranges, as well as from Marsh and New Zealand grapefruit (Levi et al., 1974; Chandler and Kefford, 1966; Emerson, 1948; Robertson, 1980). The juice obtained from late-harvested fruit from these same varieties, however, does not develop this bitterness to the same extent.

Two theories have been proposed to explain the phenomenon of delayed bitterness: the precursor theory and the diffusion theory. Higby (1941) proposed that the fruit tissues contain a non-bitter, water-soluble precursor which, after disruption of the fruit tissue in juice manufacture, is extracted into the juice where it is slowly converted to limonin. Emerson (1948, 1949) studied the dihydroxy acid formed on base hydrolysis of limonin and concluded that this compound lactonized too slowly to be the precursor. He



I



II

Fig. 1. Structure of limonin (I) and limonoic acid A-ring lactone (II)

suggested that the monolactone acids or a glycoside could be the precursor, and that enzymes could be involved in its conversion to limonin.

Failure to isolate and identify the precursor led Kefford (1959) to put forth the diffusion theory. He reported that limonin itself is present in the fruit tissues but because of its low solubility, it takes an appreciable time to diffuse from the tissue fragments of the juice into solution and reach a concentration sufficient to impart a bitter taste. Earlier work by Samisch and Ganz (1950) had reported that the bitter principle diffused very slowly from the tissue particles.

Maier and Beverly (1968) studied the capillary membranes, albedo, juice vesicles and seeds of early season Washington Navel oranges and Marsh grapefruit and found that limonin was present only in the juice that developed bitterness. No limonin was detected in the fresh fruit to any significant degree, and the major limonoid present in the albedo and endocarp tissue was limonin monolactone. Their evidence suggested that all the limonin was in the monolactone form, contrary to the findings of the diffusion theory workers. The monolactone itself was not bitter but when it came in contact with the acidic juice, lactonization occurred, resulting in the conversion of the monolactone to the bitter compound limonin. Maier and Margileth (1969) established that A-ring monolactone (II) is the naturally occurring limonin monolactone.

Robertson (1980), on his study of solubility relationships of limonin and the phenomenon of delayed bitterness in citrus juices reported that these processes are very complicated. He presented evidence for the existence of two precursors and argued that the work on which the currently-accepted precursor theory is based lacks quantitative measurements. A need for a reassessment of previous work in

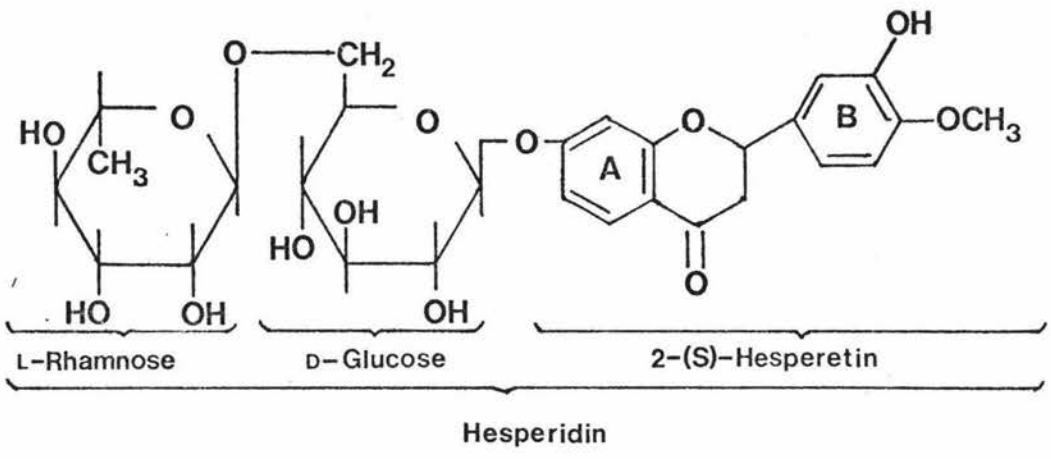
this area was therefore recommended in the light of the possible involvement of two limonin precursors and the associated enzymes and hydroxyacid forms of limonin.

Limonin (which is almost insoluble in water) can give bitter solutions of sickening intensity, especially in the presence of sugar and pectin which increases its solubility (Chandler and Nicol, 1975). Its bitterness is generally unpleasant being particularly persistent on the palate, lingering for a considerable time after swallowing the juice.

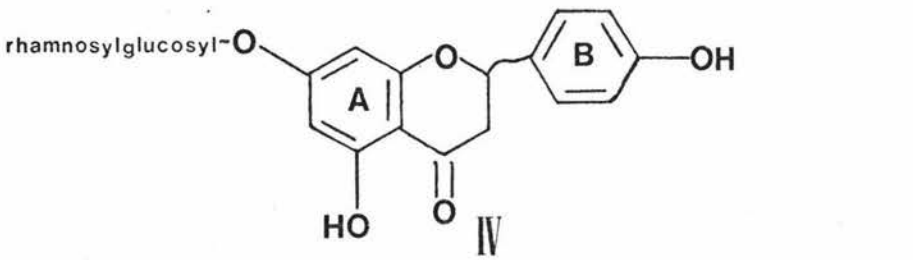
1.1.2 Flavonoid Bitterness

The peels of oranges, lemons and grapefruit contain a wide range of flavanone glycosides. Two of the best known of these compounds are hesperidin (5,7,3'-trihydroxy-4'-methoxyflavanone 7-β-rutinoside), the main flavonoid constituent of oranges and lemons, and naringin (5,7,4'-trihydroxyflavanone 7-β-neohesperidoside), the main flavonoid constituent of grapefruit (Horowitz and Gentili, 1969). Both flavanones are present as the 7-glycosides, and whereas naringin is intensely bitter, hesperidin is tasteless. The structure of hesperidin (III) consists of three parts: L-rhamnose linked α-1,6 to D-glucose, which in turn is linked to the C-7 hydroxy group of the flavanone 2(S)-hesperetin (Horowitz, 1964). The disaccharide portion 6-O-α-rhamnopyranosyl-D-glucopyranose, is referred to by its trivial name rutinose.

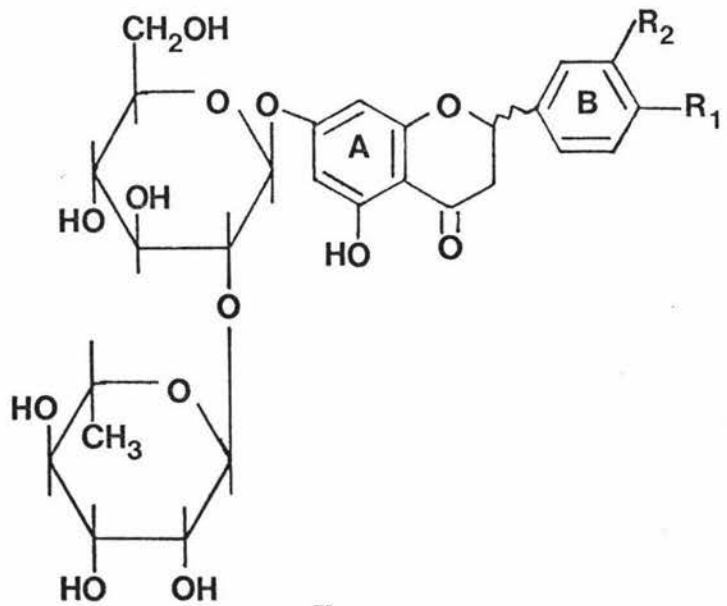
The structure of naringin is thought to be similar to that of hesperidin, differing only in the aglycone moiety, naringenin, in which the β-ring substitution pattern is different (IV).



III



IV



V

Fig. 2. Structural formulae of hesperidin (III), naringin (IV), and neohesperidoses (V).

Horowitz and Gentili (1969) determined the configuration of rhamnose in naringin as well as in two other flavonoids, poncirin (5,7-dihydroxy-4'-methoxyflavanone 7-β-neohesperidoside) and neohesperidin (5,7,3-trihydroxy-4'-methoxyflavanone 7-β-neohesperidoside) in order to explain the sensory differences between naringin and hesperidin. They found that the linkage between rhamnose and glucose was 1⟶2 in all 3 flavonoids, suggesting that they could be considered neohesperidoses (V). However, the linkage between the rhamnose and glucose in hesperidin was 1⟶6. It was therefore apparent that it was the point of attachment between the rhamnose and glucose that determined the bitterness of the flavanone-7-β-neohesperidosides and the tastelessness of the flavanone-7-β-rutinosides. Chandler and Nicol (1975) reported that the bitterness cannot be determined simply by the glycoside bonds. Not all neohesperidosides are bitter, although this disaccharide is a component of the two most bitter citrus flavonoids, naringin and poncirin. According to Horowitz and Gentili (1969), narirutin, didymin, and hesperidin (all rutinosides) are not bitter, but Kamiya *et al.* (1975) who synthesized narirutin described this component as bitter.

Naringin has long been known as the principal flavonoid of the grapefruit, *Citrus paradisi* (Chandler and Nicol, 1975). According to Hagen *et al.* (1966), total flavanone glycosides in grapefruit are made up of naringin (63% w/w), narirutin (28% w/w), and a small percentage of hesperidin, neohesperidin, and poncirin.

The substance now called naringin was first discovered in 1857 by de Vry who isolated it from all of the tissues (but mainly the flowers) of *Citrus decumana* in Java; he apparently did not publish his findings at that time. The structure of naringin was first established by Asahina and Inusube (1929) and then by Horowitz and Gentili (1963a). It was first synthesized by Kamiya (1967).

Naringin occurs in low concentrations in the juice of mature grapefruit, and in higher concentrations in segment membranes, core and peel (Sinclair, 1972). In citrus juice production, bitterness will rapidly increase unless the pulp and rag are removed from the juice. However, the immediate removal of all the pulp particles from the juice will not always give a non-bitter serum because the bitter principles exist in the intact juice sacs, and their concentration there may exceed the bitterness threshold (Chandler and Nicol, 1975). Only at high concentrations is the bitterness due to naringin objectionable, and at moderate levels it may be a desirable constituent. Buffa and Bellenot (1962b) suggested that a level of 0.03-0.07% naringin is required to give grapefruit juice its characteristic bitterness, juices with naringin contents below 0.03% being of poor quality.

Naringin is soluble in aqueous systems, producing a bitter taste. The bitterness of this glycoside is so pronounced that it can be detected when one part is dissolved in 50,000 parts of water (Braverman, 1949). Its bitterness is generally mild and can be readily removed from the palate by normal salivary processes.

1.2 CHANGES IN THE BITTER COMPONENTS AND OTHER CHEMICAL CONSTITUENTS OF N.Z. GRAPEFRUIT DURING MATURATION

1.2.1 Limonin

The concentration of bitter principles in all parts of citrus fruits decreases with advancing maturity (Higby, 1938). In oranges, the effect of maturity on bitterness has been studied by Kefford and Chandler (1961) and Bowden (1968); their results showed that there is a decrease in bitterness with maturity. The crude bitter principle content of the dried peel of Navel oranges on rough lemon rootstock decreased from 0.10 to 0.06% while that of Valencias on rough lemon stock decreased from 0.07 to 0.00%

during three months (Chandler, 1958).

Limonoid bitter principles generally decrease with maturity of the fruit (Emerson, 1949). Maier and Dreyer (1965) reported the presence of limonin in grapefruit juice at levels above its taste threshold. They found that limonin was present six months after the fruit had reached commercial maturity. The endocarp of the grapefruit retained limonin (in amounts up to 140 ppm on a wet weight basis) far longer after commercial maturity than did the Navel oranges where limonin decreased and disappeared as the fruit ripened beyond commercial maturity (Higby, 1938). Maier and Beverly (1968) reported that in Navel oranges, the concentration of A-ring monolactone gradually decreased in the tissue as the fruits developed beyond commercial maturity, and that the limonin content of the extracted juice showed a corresponding decrease.

1.2.2 Flavonoids

Camp *et al.* (1932) reported that the intensity of flavonoid bitterness is a function of fruit maturity, method of preparation and subsequent treatment of the product. Albach *et al.* (1969) investigated the production of naringin in grapefruit and found that naringenin rhamnoglucosides accumulated throughout the entire growing period from development of the ovary to fruit maturity. The rate of naringin formation, however, appeared to be several thousand times greater during the initial period of fruit development than in the subsequent periods. These workers also observed periodic increases in the production of naringenin glucosides which accompanied growth flushes, suggesting that it might be possible to anticipate increases in bitterness so that the processing industry could adjust its harvesting times accordingly. This would ensure the production of grapefruit products of uniform quality. The accumulation of naringin during the initial stages of fruit development appeared to coincide with the formation of new cells.

Maier (1969) also reported a rapid increase in naringin during the initial development of Marsh seedless grapefruit. The naringin accounted for 9% of the weight of the fruit (41% of the dry weight) at the end of the first month, levelling off when the fruit had reached one-quarter of its maturity. The peel thickness also reached a maximum when the amount of naringin started to level off. Kesterton and Hendrickson (1952) found that the naringin content became rather constant after the equatorial diameter had reached 50.8 mm. As the fruit grew larger, the naringin content decreased as a percentage of its overall weight.

A decrease in naringin content was observed as the season progressed (Maurer et al., 1950) and as the fruit ripened (Hagen et al., 1966). The juice at all times contained less naringin than did the peel, membrane or core.

1.2.3 Ascorbic Acid

Cattoni and Gonzales (1932) stated that grapefruit juice had a scurvy-preventing potency equal to that of lemon juice, and that its curative value was superior. The grapefruit peel was subsequently found to be especially rich in ascorbic acid (Atkins et al., 1945). They found that the concentration of ascorbic acid in the juice was only one-seventh and one-fifth of the vitamin C concentration in the flavedo and albedo respectively. On a whole fruit basis, the juice contained about 17% of the total ascorbic acid of the fruit.

New Zealand grapefruit juice contains from 21 to 49 mg/100 mL ascorbic acid (Hyatt, 1936; Strachen, 1967; Anon., 1966; Dawes, 1970; Robertson, 1975). As fruit ripens and increases in size, the concentration generally decreases (Harding and Fisher, 1945). Metcalfe et al. (1940), in the Rio Grande Valley of Texas, concluded that there is a very definite decrease in the ascorbic acid content of the fruit (6 varieties) by the end of the season.

Ugon and Bertullo (1944) and Krezdorn and Cain (1952) stated that the amount of ascorbic acid in grapefruit juice decreased as the season advanced. Previous work, however, had reported that the more mature fruits from a given tree sometimes contained more ascorbic acid than the less mature ones (Melas-Joannides, 1939). A later paper by Ugon and Bertullo (1945) reported that the ascorbic acid concentration in the grapefruit juice increased as the season advanced.

The seasonal decrease in concentration of ascorbic acid in grapefruit should be considered in relation to the increase in volume of the juice and in the size and weight per fruit during growth and maturation. Harding and Fisher (1945) showed that when expressed as mg per mL of juice, the ascorbic acid decreased significantly during the season. Since growth of the fruit is accompanied by a large increase in juice volume and fruit weight, the concentration of ascorbic acid would decrease, resulting in less ascorbic acid per mL of the juice. On the other hand, if the ascorbic acid values are reported on the basis of mg per fruit, an increase occurs during the growth and development.

1.2.4 Acidity

pH and titratable acidity are the two measures of acidity used in physiological studies on grapefruit. Grapefruit contains a relatively high concentration of organic acids. The titratable acidity may constitute 8 to 15 percent of the total soluble solids of the juice from the edible portion (Sinclair, 1972). Wolf (1958) determined the organic acids in grapefruit juice and reported that the total acidity was composed of 87.2% citric acid, 2.17% malic acid, 1.7% quinic and phosphoric acid, and 7.0% unidentified acid. Traces of oxalic and tartaric acids in grapefruit were reported in earlier work (Braverman, 1949).

Many investigators have shown that the concentration of titratable acids in grapefruit gradually decreases as the fruit develops and matures (e.g. Sinclair and Bartholomew 1944; Harding and Fisher 1945). Ting and Vines (1966) on their study of Marsh grapefruit found that the citric acid content increased throughout the growth cycle, declined up to about 5 months from fruit set and then remained roughly constant. Karaoulanis and Margaris (1975) in their study of the seasonal changes in grapefruit reported that as the fruit ripened, there was a decrease in acidity. Sinclair and Ramsey (1944) reported that the decrease in titratable acidity was considered to be due to dilution as the fruit increased in size and juice content.

Decrease in the concentration of acid with the gradual increase in total sugars during development results in an increase in the ratio of total soluble solids to acidity, which is the basis for determining the legal maturity of the fruit as well as its palatability. In view of this, titratable acidity of the juice is highly associated with quality of the fruit (Sinclair, 1972).

Harding and Fisher (1945) observed a gradual increase in the pH of grapefruit as the fruit matured. The change in pH was slight, indicating the strong buffer capacity of grapefruit juice. Karaoulanis and Margaris (1975) observed the same trend, with a marked increase in the pH of the juice during ripening.

A linear relationship between the pH of grapefruit and the log of the free acid concentration expressed as milliequivalents per gram of soluble solids was demonstrated by Kilburn (1958) and Kilburn and Davis (1959) who were able to draw a common regression line through the points. This means that at a uniform soluble solids content (^{o}Brix), the pH of citrus juices is proportional to the log of the titratable acidity.

1.2.5 Total Soluble Solids

There is a fairly close correlation between total soluble solids and sugar content because a high proportion of the soluble solids are sugars. Grapefruit juice contains 6-13% soluble solids, of which more than half are sugars (Kefford, 1959; Sinclair, 1972). The major sugars are reported to be sucrose, glucose and fructose with trace quantities of acid-hydrolyzable glycosides (flavanones) containing galactose and rhamnose (McReady et al., 1950). Ting and Deszyck (1961) reported that in the peel, the total sugar content of grapefruit was about 80% of the total soluble solids and the sugars present were glucose, fructose and sucrose with traces of xylose and rhamnose.

Many investigators have reported that sugars increase as the season advances. According to Harding and Fisher (1945) the reducing, non-reducing and total sugars increased as grapefruit continued to ripen on the tree. Usually before or at maturity, the sucrose decreases and the reducing sugars increase due to the breaking down of sucrose into glucose and fructose. On the other hand, Grebinskii (1940) reported that the sucrose content of Rumanian grapefruit juice reached a maximum and then began to decrease long before the fruit was fully mature. Hilgeman and Smith (1940), in studies of Marsh grapefruit from September to May reported that total sugars increased until January and then decreased. Results of a 2-year study in the Naples area showed that the fruits reached maximum soluble solids during the month of February and then decreased (Gioffe, 1976). A marked increase in the soluble solids and total sugars was observed in grapefruit harvested from three different regions of Greece (Karaoulanis and Margaris, 1975). Such a pattern of increasing to a maximum and then levelling off or declining with advancing maturity has also been reported in other areas, e.g. in California

and Arizona (Rygg and Getty, 1955); Texas (Krezdorn and Cain, 1952; Burdick, 1961; Lime et al. 1954, 1956); Florida (Stenstron and Westbrook, 1956) and New Zealand (Robertson, 1975).

1.3 METHODS OF DEBITTERING CITRUS JUICES

Because bitterness continues to be an important economic problem in the citrus industry, several methods have been developed and research is still being conducted to reduce (or preferably totally eliminate) bitterness. Maier et al. (1977) reported that there are several approaches to controlling juice bitterness among which are pre-harvest factors, post-harvest fruit treatment, processing conditions and treatment of the juice. Processors try to minimize limonoid and flavonoid bitterness in grapefruit products by carefully selecting the fruit, by controlling the pressure used in juice extraction and by blending bitter and non-bitter juices where possible.

1.3.1 Removal of Limonoid Bitterness

1.3.1.1 Enzymic Methods

As early as 1950, McColloch reported that citrus juices could be debittered by enzymic degradation of the pectic substances which caused a change in the physical state of limonin. Pectin-destroying enzymes from fruits or fungi were found to debitter orange juice after several hours of treatment at 4-10°C, but the process was associated with the development of off-flavour and loss of cloud. Recently Robertson (1980) attempted to reduce limonoid bitterness by degrading the pectin in Navel orange juice. However, he was unable to demonstrate any reduction in limonin concentration, even after all the pectin has been degraded.

Several studies have been directed towards the use of enzymes of bacterial origin to degrade limonin. An enzyme, limonoate dehydrogenase was isolated from Arthrobacter globiformis (Hasegawa et al., 1972) and Pseudomonas sp. (Hasegawa et al., 1974b) which catalyzed the dehydrogenation of the free 17-hydroxyl group of limonoate A-ring lactone to produce 17-dehydrolimonoate A-ring lactone which is not bitter.

In addition to limonoate dehydrogenase, Hasegawa et al. (1974c) isolated another limonoid metabolic system from Pseudomonas sp. 321-18. The addition of limonin to this system produced exocellular traces of deoxylimonin together with considerable quantities of deoxylimonoate as metabolites.

Vaks and Lifshitz (1976) identified an enzyme from Acinetobacter sp. capable of attacking limonin close to the natural pH of the juice. This enzyme was distinct from those discussed previously and was inactive at pH 8.5, where limonin is in the open-ring limonoate form. However, the mechanism of the reaction catalyzed and the nature of the products formed were not discussed.

Limonin-degrading enzymes are also present in orange albedo (Chandler, 1971). Hasegawa et al., (1974a) found limonoate dehydrogenase activity in the albedo tissues of Navel oranges. This confirmed the ability of citrus fruits to form 17-dehydrolimonoate A-ring lactone from limonin.

The interruption of limonoid metabolism in postharves fruit in the absence of oxygen supported the limonoate dehydrogenase pathway as the one operating in citrus fruits (Eskin, 1979). Nicol and Chandler (1978) in a paper detailing the optimum conditions for the extraction of this enzyme, referred to it simply as the limonin precursor degrading (LPD) enzyme without specifically identifying its substrate. The substrate used for their assays was orange albedo.

Enzymic methods for reducing limonoid bitterness pose several problems. The enzymes of bacterial origin are expensive and have very limited activity at the natural pH of citrus juices. The high limonin-degrading activity of citrus albedo is encountered only over a narrow period during maturation of the fruit, and the isolation of extracts of high activity is difficult (Chandler and Nicol, 1975). Although enzymic methods pose a number of problems, the search for more suitable enzymes is still in progress (Brewster et al., 1976).

1.3.1.2 Adsorption Methods

The problem of bitterness appears to have been solved through the recent use of chemical sorbents. An early attempt to reduce limonin was reported by McColloch (1950) using activated carbon. However, this material was too selective to be of any practical value.

More recent interest in chemical sorbents led to an examination of polyamide powders by Chandler et al. (1968). They found that much of the limonin from pasteurized Navel orange juice could be adsorbed by polyamides. The most efficient adsorption involved a two-step treatment bringing the limonin concentration below the organoleptically detectable level. The powder was, however, found to be more effective in removing flavonoids as well as some ascorbic acid and this lack of specificity was a major drawback in the industrial utilization of these powders.

A series of sorbents were examined by Chandler and Johnson (1977), who showed that cellulose acetate was far more specific than polyamide powders. Treatment of orange juice serum with the powder removed 44-70% of the limonin content in less than an hour, at the same time removing relatively negligible amounts of hesperidin and ascorbic acid. Other materials, including related carbohydrate derivatives and polymers were tested but only cellulose acetate butyrate shared the unusual sorptive properties of cellulose acetate.

Chandler (1977), in reviewing this bitterness problem discussed the production of cellulose acetate gel beads that were successfully used in a "gel debittering process". This process provided a simple and inexpensive method for the removal of limonin, thus permitting the commercial use of citrus juices affected by the delayed bitterness phenomenon. The cellulose ester adsorbents may be used in gel bead form packed in columns, or as a powder. A drawback is the regeneration of the adsorbent, which requires rather large volumes of water (Rombouts and Pilnik, 1978).

The use of other chemical sorbents has been investigated by Maeda et al. (1979) who used PVP, Nylon-66 and High Porous Polymer in the adsorption of naringin but not limonin from Natsudaidai juice.

1.3.2 Removal of Flavonoid Bitterness

1.3.2.1 Enzymic Methods

The bitter flavour of naringin is associated with the 1⟶2 linkage between rhamnose and glucose as discussed in Section 1.1.2. The susceptibility of this glycosidic bond to hydrolytic enzymes suggested the possibility of hydrolyzing naringin to rhamnose and prunin (5,7,4'-trihydroxyflavanone 7-ß-D-glucoside). Prunin can be further hydrolyzed to glucose and naringenin, neither of which is bitter. Naringenin is very poorly water soluble and has no bitter taste. Prunin seems to retain at least part of the bitterness (Horowitz and Gentili, 1969) but is certainly less soluble than naringin. Ting (1958) found in certain commercial pectic enzyme preparations (Pectinol 10-M and Pectinol 100-D), an enzyme that would hydrolyze naringin to rhamnose, glucose and naringenin. An enzyme was isolated from the preparation, naringinase, which was active over the pH range 3-5. This enzyme was partially purified by Thomas et al. (1958) who found that it was composed of two glycosidases. Dunlap et al. (1962) separated these two enzymes and identified

them as rhamnosidase and glucosidase.

Versteeg et al. (1977) studied the mode of action of naringinase using a thin-layer chromatographic-fluorodensitometric method. Two enzyme preparations were used which both hydrolyzed naringin and narirutin to prunin (rhamnosidase activity) and prunin to naringenin (glucosidase activity). In grapefruit juice, hydrolysis of both naringin and narirutin to prunin went at approximately the same rate. The prunin accumulated in the intermediary stage of the reaction, but was finally converted to naringenin. The glucosidase was inhibited by glucose but other inhibitors which may affect the activities of both enzymes may also be present in the juice.

The enzymatic approach has since been the subject of many patents and the three most recent patents are by Ito and Takiguchi (1970), and Fukumoto and Okada (1972, 1973). The enzyme naringinase has been isolated from a wide range of moulds grown on media containing naringin or rhamnose and in tests of 132 strains from 39 genera, Aspergillus niger and Coniella diplodiella have been reported to yield the most active preparations, hydrolyzing naringin within a few hours at room temperature and at the natural pH of most citrus juices (Chandler and Nicol, 1975). The first commercial preparations of naringinase were so heavily contaminated with pectinase that undesirable loss of cloud resulted from their use. This problem has led to attempts to compensate for the added costs of naringinase purification by immobilizing the enzyme onto solid supports, thereby allowing it to be re-used. Goldstein and co-workers (1971) claimed that an immobilized naringinase system could be used in a continuously generated bath or column process for removing the bitterness from clarified juices. Further work has been done in this area (Dinelli and Morisi, 1972; Krasnobaev, 1973, 1974) but attempts to obtain stable immobilized naringinase preparations have not been successful so far (Chandler and Nicol, 1975).

1.3.2.2 Adsorption Methods

The use of chemical sorbents to remove flavonoid bitterness was first reported by Horhammer et al. (1957) who found that polyamides have a pronounced affinity towards flavonoids due to phenolic groups on the flavonoids. Chandler et al. (1968) in their attempt to remove limonin from bitter orange juice, found that in a double batch treatment, most of the polyphenolics were removed in the first treatment while limonin was removed only during the second treatment. Polyvinylpyrrolidone (PVP) was recently used to remove naringin from Natsudaidai juice (Maeda et al., 1979). Other chemical sorbents were also used: Nylon-66 which adsorbed more than 74% of the naringin present in the juice and High Porous Polymer (HPP) which adsorbed about 59%.

1.4 PRODUCTION OF DIHYDROCHALCONES AND PECTIN FROM CITRUS PEEL

1.4.1 Types of Dihydrochalcones

Flavonoids found in grapefruit are classified as 7-β neohesperidosides and 7-β rutinosides. These bitter and non-bitter flavonoids are available in large quantities and can be readily reduced to extremely sweet dihydrochalcones. These findings have considerable commercial potential. Because of the comparatively high quantities which can be isolated, the flavonoids have often attracted attention as a means of increasing the profitability of citrus processing (Vincent, 1962; Hendrickson and Kesterton, 1965). In Florida, about 7,000 tonnes of hesperidin can be recovered annually from the residues of the processors and although their processed grapefruit contains several thousand tonnes of naringin, little is recovered.

Presently accepted artificial sweeteners such as saccharin and cyclamate have enjoyed steadily increasing demand during the last decade. With waste materials from the

citrus processing industry as starting material, dihydrochalcones which are low-calorie sweeteners could be produced to replace sucrose in the diet.

Currently, the three most promising dihydrochalcone sweeteners are neohesperidin dihydrochalcone, naringin dihydrochalcone and hesperidin dihydrochalcone.

Neohesperidin dihydrochalcone. Neohesperidin is the predominant bitter principle of the Seville orange peel and can be readily converted to the intensely sweet dihydrochalcone derivative upon alkaline hydrogenation. Neohesperidin dihydrochalcone is 1,000-1,900 times sweeter than sucrose or 20 times sweeter than saccharin (Horowitz and Gentili, 1963; 1969). However, because the starting material neohesperidin occurs in the sparsely cultivated Seville or bitter orange (Citrus aurantium), it is not a commercial product. Much work has therefore been done in the production of neohesperidin using naringin as starting material.

Naringin dihydrochalcone. The large scale preparation of naringin dihydrochalcone from commercially available naringin can be accomplished easily and in high yield by catalytic hydrogenation in alkaline solution. Naringin dihydrochalcone is reported to have the same sweetness as saccharin or 20 times sweeter than sucrose (Horowitz and Gentili, 1969).

Hesperetin dihydrochalcone. Hesperetin dihydrochalcone is derived from hesperidin which is the most ubiquitous of the citrus flavonoids (Horowitz and Gentili, 1969b; 1971b). The hesperidin is reduced in alkali to hesperidin dihydrochalcone followed by a partial acid or enzymic hydrolysis to remove L-rhamnose (Horowitz and Gentili, 1969b; 1971b). This compound can also be made by partial hydrolysis of neohesperidin dihydrochalcone.

This type of dihydrochalcone exhibits a sweetness about equal to that of saccharin, on a molar basis. Moreover, hesperetin dihydrochalcone glucoside imparts a more agreeable sweetness, i.e., less clinging and absence of bitter or other taste effects, than some of the dihydrochalcone sweeteners such as naringin dihydrochalcone or prunin dihydrochalcone (Horowitz and Gentili, 1971a).

Studies at Dynapol, Palo Alto, California have now conclusively established that the structural elements of the dihydrochalcones responsible for inducing the sweet-taste response reside entirely on the aromatic nucleus (Crosby, 1976).

1.4.2 Extraction and Characterization of Pectin

Pectic substances are found in fruits and vegetables, being most abundant in limes, lemons, grapefruit and oranges. They are the major constituents of the middle lamella as well as structural elements in the primary cell wall (McClendon, 1964; Talmadge et al., 1973). Three distinct classes of pectic substances have been recognized: protopectin, the water-insoluble parent pectic material; pectinic acids or high methoxyl pectins and pectinates; and pectic acids or low methoxyl pectins and pectates (Pilnik and Voragen, 1970). Pectin is composed of anhydrogalacturonic acid units that exist in a chain-like combination with each unit connected through the 1,4 glycosidic linkages forming a polygalacturonic acid (Rouse, 1977; Doesburg, 1965). Some of the carboxyl groups are esterified with methanol, some are neutralized with cations and some are free acids (Fig. 3). Secondary acetyl groups occur in many pectins. Schultz (1965a) reported values from 0.2% in apple, citrus and cherry pectins to 3-4% for peach, pear and sugar beet pectins. Pectin acetyls are important because they affect the gelation of pectin.

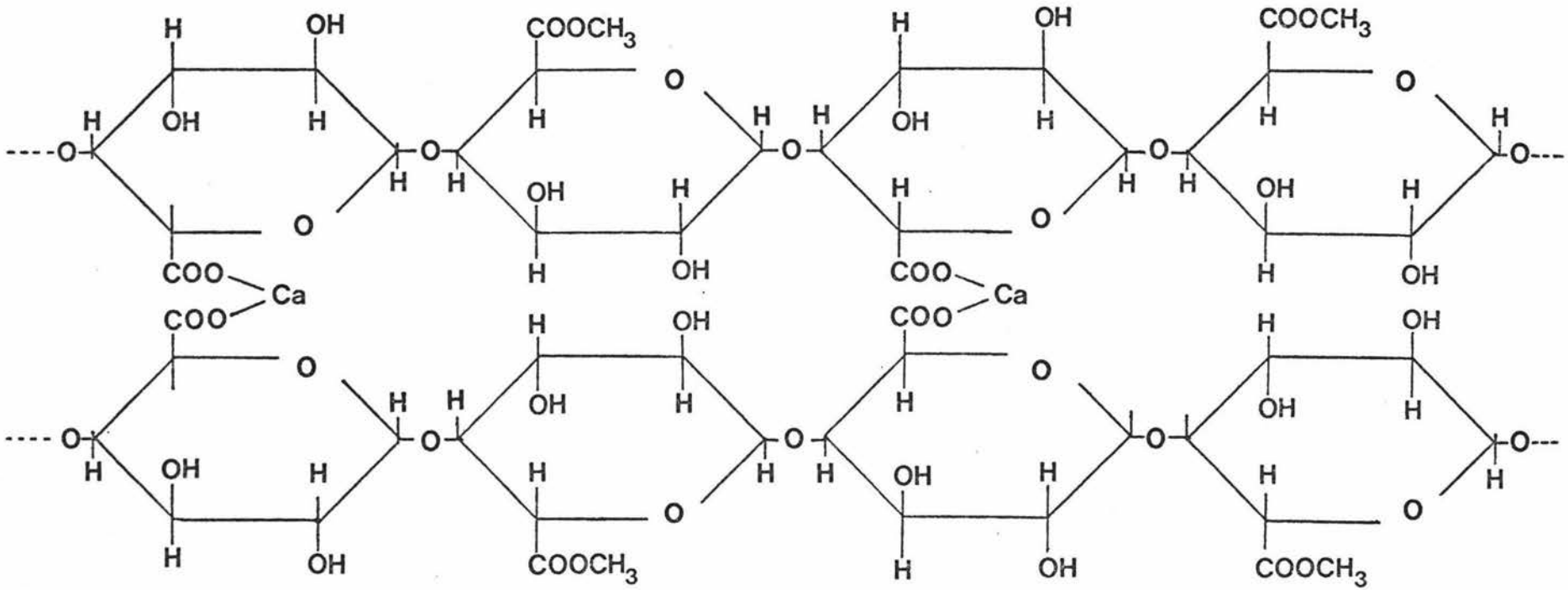


Fig. 3. Part of polygalacturonic acid molecule esterified with methanol, also showing cross linkage through a polyvalent ion. (From Baker and Goodwin, 1941).

Generally, extraction temperatures range from 80 to 100°C, time of extraction from 20 to 60 minutes and pH from 1.4 to 2.6. Crandall and Kesterton (1976) used a hot water leach (88-90°C) for the recovery of pectin, with naringin being recovered as a specialty product from the hot water leach.

Precipitation of the filtered, clarified and concentrated pectin is accomplished using acetone (Hinton, 1940), alcohol (ethyl or isopropyl) or aluminium hydroxide (Rouse, 1977). Sinclair and Joliffe (1958) reported that recovery of acetone-insoluble solids is much more difficult than recovery of alcohol-insoluble solids, owing to the poor filtration and sticky adhesion of the former to the filter paper and sides of the container, possibly because of the precipitation of more colloidal materials. Thus alcohol is preferable to acetone for the recovery of pectic substances from citrus because of the greater ease of recovery of the insoluble solids. Aluminium hydroxide prepcipitation proved to be superior, but the method was more lengthy due to pH adjustments and a series of washings and rinsings to remove excess aluminum and acid.

The alcohol-insoluble solids resulting from the citrus peel extraction are composed chiefly of the structural constituents of the cell, i.e., cellulose, hemicellulose, pectin, protein, etc. (Sinclair and Crandall, 1949a). Pectin is composed of long chains of galacturonic acid residues with varying degrees of esterification of the carboxyl groups. The chemical nature of the pectin molecule can be ascertained by taking advantage of certain chemical reactions of polygalacturonic acid which can be determined quantitatively. Chemical and physical properties of pectin are affected by the extent of acetylation of the hydroxyl groups, of methyl esterification of carboxyl groups, and by the degree of polymerization.

In citrus fruits, pectin is the naturally occurring colloidal stabilizer that gives juice its viscosity or "body" (Rouse, 1977). Pectin is produced from the white spongy albedo or from the whole peel of citrus fruits where primary walls are said to contain a large proportion of protopectin (Kertesz, 1951). Royo et al. (1980) in a study of the preparation of dried peel for pectin production from different citrus fruits reported that dried lemon peel yields the highest proportion of crude pectin, followed by orange and then by grapefruit. Rouse and Crandall (1976) reported a similar finding.

Pectin content generally increases with maturity (Rygg and Harvey, 1938; Ting and Deszyck, 1961). Sinclair and Joliffe (1958, 1961) observed a steep initial rise in the pectin substances in the peel and pulp followed by a gradual decrease throughout the season. Gaddum (1934) reported that as Valencia oranges ripened, the water-soluble pectin in both the albedo and the pulp increased to a peak and then declined. Pectin in the peel, membrane, juice and juice sacs was found to be generally higher in the fruits from young trees.

The commercial extraction of pectin is usually accomplished with mineral acids such as sulphurous (Wilson, 1925; Joseph and Havighorst, 1952; Snyder, 1970; Wiles and Smit, 1971), sulphuric (Myers and Baker, 1929, 1931, 1932), a combination of hydrochloric with ion-exchange resins (Doesburg, 1973; Huang, 1973a, 1973b), or nitric (Rouse and Crandall, 1976). Many organic acids and their salts such as oxalic acid and ammonium oxalate (Manabe and Tarutani, 1966), tartaric acid (Myers and Baker, 1929), polyphosphates (McReady et al., 1947) and many others (Joslyn and Deuel, 1963) have also been used. Meyers and Rouse (1943) reported the use of a H-Zeolite ion exchange resin for the extraction of pectin. A very low yield of pectin obtained from dried orange peel was reported (Bailly, 1956) using Zeocarb as extractant at 85-90°C.

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In foods, regular pectin is used in the manufacture of fruit jellies, jams, preserves, confectionery jelly pieces, thickeners for low-calorie fruit syrup and beverages, flavour emulsions and salad dressings (Glicksman, 1969). Low methoxyl pectin is used in the manufacture of salad and dressing gels with imitation flavour and colour, salad and dressing gels of fruit and vegetables juices, milk gels and puddings, low-calorie jam-like fruit gels for dietetic use and, fruit and berry gels for ice cream use. Non-food uses include pharmaceutical and medicinal. Ahmadsiddiqui et al. (1971) demonstrated the beneficial medicinal effect of pectin in disorders of the alimentary canal. Works on the effect of pectin on blood had been conducted by Keys et al. (1961) and Fisher et al. (1964). Rouse (1977) suggested further research on the application of metallic pectinates to relieve physical suffering.

New Zealand imports pectin mainly from Denmark, United Kingdom and United States. Other sources are Italy, Switzerland, Fed. Rep. of Germany, Australia, Israel and Sweden. For the year 1979-80, New Zealand imported 20,805 kg pectin substances from Denmark, 15,375 kg from U.K. and 1,376 from U.S.A. with the cost (including insurance and freight) being NZ $150,964, $69,990 and $14,882 respectively (New Zealand Department of Statistics, 1981). Ten years earlier the comparable figures were 17,954 kg from Denmark, 17,219 kg from U.K. and 7,864 kg from U.S.A. with the cost (including insurance and freight) being NZ $4,962, $11,377 and $20,337, respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Fruit

The New Zealand grapefruit (*C. grandis* x *C. reticulata*) used in this study were picked from the Massey University Orchard, Palmerston North. Fruit that was not processed immediately after harvest was stored at 5°C.

Calamansi fruit (*C. mitis* Blanco) was purchased from the local market in College, Laguna, Philippines and processed as described below.

2.1.2 Juice

2.1.2.1 Fresh Juice

Juice was extracted from fruit using a domestic Kenwood mixer fitted with a reamer. The extracted juice was screened through four layers of cheesecloth to separate the seeds and pulp from the juice.

2.1.2.2 Pasteurized Juice

2.1.2.2.1 Grapefruit Juice

The juice was heated and maintained at 95°C for five minutes and cooled rapidly.

2.1.2.2.2 Calamansi Juice

The pasteurized calamansi juice was prepared as follows (Nisperos, 1977). The stem-end was cut and the juice was extracted by squeezing manually since the fruits were small (about 2 to 2.5 cm in diameter). It was then strained through a stainless steel sieve. A water-soluble cloud stabilizer (gum tragacanth) was added at 4% (w/v) of the total volume of the juice. The juice was then homogenized in a Waring blendor for one minute. The homogenate was heated in a water bath to a temperature of 70°C and held there for one minute to inactivate pectinesterase and facilitate deaeration.

The hot juice was filled into 215-mL capacity glass bottles, capped, pasteurized for 15 minutes in boiling water and cooled.

Another batch of juice was processed as above except that crushed seeds were included at a level of 5% of the total volume of the juice. The above juice samples were then posted to Massey University, Palmerston North for analysis.

2.2 DETERMINATION OF COMMON JUICE PARAMETERS

2.2.1 Total Soluble Solids (TSS)

This was determined using an Abbe refractometer at room temperature and expressed as degrees Brix (^{o}Brix).

2.2.2 Titratable Acidity

Juice (10 mL) was diluted to 100 mL with distilled water and titrated with sodium hydroxide (0.1 N) using phenolphthalein as an indicator. Titratable acidity was calculated using the relationship one mL of 0.1 N NaOH = 0.0064 g citric acid, results being expressed as g citric acid/100 mL juice.

2.2.3 Brix:Acid Ratio

This was calculated by dividing the total soluble solids reading by the titratable acidity.

2.2.4 pH

This was determined by using a pH meter calibrated with buffers at pH 3.2 and 7.0.

2.2.5 Pulp or Insoluble Solids Content

Juice (50 mL) was placed in a conical graduated centrifuge tube and centrifuged at 800 x g for 10 minutes. The volume of the precipitate was read and the pulp content expressed as a percentage of the total juice volume.

All reported values for the above analyses are the mean results of duplicate determinations.

2.3 DETERMINATION OF ASCORBIC ACID

2.3.1 Background

The ascorbic acid content of fruit juices can be determined by titration and colorimetric methods. The visual titration method is based on the reduction of the dye 2,6-dichlorophenolindophenol by the acid solution of ascorbic acid (AOAC, 1980). At the end point, excess unreduced dye is rose pink in acid solution. This method is applicable to the determination of reduced ascorbic acid, but in samples where ferrous Fe, stannous Sn, cuprous Cu, SO_2, sulfite and thiosulfate are present, the method is not applicable. Also, in coloured samples like grapefruit juice, detection of the end point is difficult thereby producing inaccurate results.

Another method used in ascorbic acid determination is based on colorimetry using 2,4-dinitrophenylhydrazine (AOAC, 1960; Roe and Kuether, 1943). It gives an estimation of the total ascorbic acid after oxidation. However, it is very lengthy, requiring more than three hours to complete the determination.

The method used in the study is a spectrophotometric method as modified by the Canada Department of Agriculture (Pearson, 1976). Like the visual titration method, it is based on the oxidation-reduction of ascorbic acid and

the dye 2,6-dichlorophenolindophenol but in this case the concentration of the dye is measured spectrophotometrically. Due to simplicity of the method, a duplicate analysis can be done in about ten minutes and the results were found to be accurate and reliable.

2.3.2 Procedure

2.3.2.1 Special Reagents

Stock ascorbic acid solution. A solution of ascorbic acid (0.1%) was prepared in oxalic acid (0.4%) solution.

Working standards. 1, 2, 3, 4 and 5 mL of the stock ascorbic acid solution were transferred to 100 mL volumetric flasks and made to volume using the 0.4% oxalic acid solution. These working standard solutions (numbered WS1 to WS5) contained 1, 2, 3, 4 and 5 mg ascorbic acid per 100 mL, respectively.

Standard dye solution. 12 mg of 2,6-dichlorophenolindophenol dye (BDH) was dissolved in distilled water (1 L).

2.3.2.2 Standard Curve

The standard curve was prepared by using four 0.5 inch colorimeter tubes containing the following:

DW - 10 mL distilled water
No. 1 - 1 mL 0.4% oxalic acid
S - 1 mL WS No. 1 + 9 mL distilled water
No. 2 - 1 mL WS No. 1

The spectrophotometer (Spectronic 70) was adjusted to zero using distilled water with the wavelength set at 520 nm. To tube No. 1, 9 mL standard dye solution was added, mixed and read exactly 15 seconds after adding the dye. This reading was recorded as L_1. The instrument was then adjusted to zero using tube S. To tube No. 2, 9 mL of the standard dye was added, mixed and read after 15 seconds (L_2). Readings of L_1 and L_2 were recorded for each working

standard and the standard curve was constructed with the concentration of ascorbic acid (mg/100 mL) as abcissa and L_1-L_2 for each working standard as ordinates.

2.3.2.3 Method

Juice (25 mL) was blended with 0.4% oxalic acid (175 mL) for three minutes. L_1 was obtained as above. The instrument was adjusted to zero with tube S containing 1 mL filtrate plus 9 mL distilled water. To tube No. 2 containing 1 mL filtrate, 9 mL dye was added, mixed and read after 15 seconds (L_2). L_1-L_2 was calculated and the concentration of ascorbic acid was obtained from the standard curve. Dilution of the juice was accounted for during calculations. For accurate results, it was necessary that duplicate readings for L_1 be done before doing triplicate readings for L_2.

2.3.3 Reliability of the Method

The method was used to determine the ascorbic acid content of fresh grapefruit juice. Results are given in Table I.

Table I. Ascorbic acid content of fresh N.Z. grapefruit juice.

Sample No.	Ascorbic acid (mg/100 mL)
1	36.8
2	41.6
3	41.6
4	41.2
5	41.6
6	41.6
7	41.6
Mean ± SD	40.86 ± 1.80

2.4 DETERMINATION OF LIMONIN

2.4.1 Background

The method developed by Robertson (1980) was used in the study.

2.4.2 Procedure

2.4.2.1 Preparation of Extract

The juice sample (20 mL) was shaken for 20 minutes with chloroform (10 mL), ethanol (10 mL), ammonium sulphate (5 g) and butylated hydroxyanilose (0.5 mg) in a centrifuge bottle provided with a rubber stopper. The mixture was centrifuged for 10 minutes at 800 x g, the upper layer removed by suction and the lower layer shaken with saturated sodium tetraborate (20 mL) for 15 sec. The mixture was again centrifuged, the upper layer withdrawn and the extract filtered through Whatman 1PS phase separating paper. The addition of about 25 mL chloroform to the extract prior to filtration was found to improve the separation of the aqueous and the organic phases. The organic layer was evaporated to dryness using a rotary evaporator at 45°C. The residue was diluted with small quantities of chloroform, transferred to 5 mL volumetric flask and made to volume with the washings of the residue using the same solvent.

2.4.2.2 Chromatography

Commercial pre-coated Silica Gel G (Merck) plates were used. On an area about 2.0 cm from the bottom of the plate, 30 uL of the aliquots of the extracted juice samples were applied using a 25-uL Hamilton syringe with a needle cut at right angles to avoid damaging the surface of the plates. Four reference spots (each 15 uL) of the limonin standards (0.01 and 0.02 percent, w/v limonin and 0.005 percent BHA) were also applied. Total spots amounted to 10, 6 for samples and 4 for standards. The spots were dried with a steam of cold air before placing in a well-equilibrated tank containing

50 mL of benzene:ethyl acetate:acetic anhydride (50:50:2). The plates were developed twice, each development requiring 50 minutes for the solvent to travel to a height of 17.0 cm. Duplicate spots were made from the same extract on separate plates.

2.4.2.3 Visualization of Spots

After chromatography, the plates were air-dried at room temperature, sprayed evenly with 25 mL of 10% concentrated sulphuric acid in ethanol, and then dried in an oven at 110°C for 8 minutes. The limonin spots appeared as dark brown areas on an almost white background.

2.4.2.4 Densitometric Measurements

The densities of the spots were measured by a Shimadzu Dual-wavelength TLC Scanner (CS-910) at 556 nm equipped with a Beckman recorder operating at 20 mV with a speed of 10 cm/minute. Curves obtained from each sample were cut out and weighed.

2.4.2.5 Determination of Limonin Content

The absolute amount (in ug) of limonin per spot was calculated using the formula:

$$L_x = \frac{P_x + P_2 - P_1}{P_2 - P_1}$$

where L_x = amount of limonin in ug
P_x = weight of the sample curve
P_2 = average weight of the 0.02% limonin standard curve
P_1 = average weight of the 0.01% limonin standard curve

The absolute amount of limonin in a spot was converted to the concentration of limonin (in ppm) in the juice by multiplying by the factor 8.33

2.4.3 Reliability of the Method

The method was used to determine the limonin content of New Zealand grapefruit juice concentration (27^{o} Brix) and calamansi juice with 5 percent crushed seeds. Results of the analyses gave the following:

Table II. Limonin content of N.Z. grapefruit juice concentrate and Philippine calamansi juice with 5% crushed seeds.

Sample	Limonin content (ppm)		Mean	Ave of means ± SD
NZ grapefruit juice conc.	8.0	8.2	8.1	7.9 ± 0.22
	8.0	7.8	7.9	
	7.7	7.6	7.6	
Calamansi juice with 5% crushed seeds	10.0	9.7	9.8	10.5 ± 1.22
	12.3	12.0	12.2	
	9.7	9.6	9.6	

2.5 DETERMINATION OF NARINGIN

2.5.1 Background

A general test developed by Davis (1947) is the most widely used assay procedure and is based on the yellow colour given by flavanone glycosides in alkali. While it is simple, it is not specific for naringin, since all flavanones give a yellow colour under these conditions, as does ascorbic acid. The method usually gives high values compared with other methods and is clearly unsuitable for measuring naringin in grapefruit products (Kefford and Chandler, 1970). Gerngross and Renda (1966) proposed a method for naringin estimation in citrus fruits whereby albedo extracts were treated with 1-nitroso-2-napthol in the presence of nitric

acid to give a blood red colour. Since neither the extraction nor the reaction are selective, the results should be regarded as applying to total flavanones rather than specificially for naringin.

A direct spectrophotometric method (Hendrickson et al., 1958b) and a quantitative paper chromatographic method (Dunlap et al., 1962a) have been developed but a selective method has to be used to separate the multiplicity of similar compounds encountered in the analysis of citrus flavonoids.

A detailed procedure was given by Hagen et al. (1965) which could determine individual components (naringin, narirutin, poncirin, didymin, neohesperidin, and hesperidin) but the method is complex and lengthy. It involves preliminary polyvinylpyrollidone column chromatography, thin-layer chromatography and finally fluorometry.

Fisher et al. (1966) simplified the process into a relatively rapid and reasonably accurate procedure for determining naringin and its isomer, but the extraction procedure was still quite lengthy. Tatum and Berry (1973) developed a simplified procedure based on the method of Fisher et al. (ibid.) which is reliable for naringin determination, can be performed easily on a routine basis, and could provide a basis for quality control tests on grapefruit juice. This method (with a slight modification as presented by Versteeg et al., 1977) was adopted in this study. It requires no sample preparation, the juice being spotted directly onto the plates. This method determines the naringin and narirutin in a relatively short period of time.

2.5.2 Procedure

2.5.2.1 Chromatography

Thin-layer chromatography was done using plates coated in the laboratory with Silica Gel G (Merck, Darmstadt), of thickness 0.25 mm. Samples were applied as bands (100 uL) using a 25 mL Hamilton syringe on an area about 2.0 cm from the bottom of the plates. When 100 uL was applied as a single band, the plates were dried in a gentle stream of cold air after application of the first half of the sample. The dried plates were developed twice at room temperature in tanks equilibrated for two hours with 50 mL solvent (nitromethane: methanol, 10:3 v/v) and a filter paper lining around the walls. The solvent was allowed to travel to a height of 17.0 cm which required about 40 minutes. Plates were dried in a fume hood for 10 minutes between development.

For precision and accuracy of the results, it was found that thorough drying of the plates was needed before placing them into the well-equilibrated tanks. Also, the use of fresh solvent is advisable for a better separation of the naringin from its isomer, narirutin.

2.5.2.2 Collection of Naringin and Narirutin Bands

After development and drying, the plates were sprayed with 25 mL of 1% solution of $AlCl_3$ in ethanol. Naringin and narirutin bands appeared as yellow fluorescent bands when viewed under UV light at 367 nm. The bands were marked and the areas to be collected were sprayed with water to prevent development of an electrostatic charge which could cause loss in scraping when transferring the material to 10 mL centrifuge tubes. Scraping was done using a spatula and the powder was transferred to centrifuge tubes with the aid of a soft brush.

2.5.2.3 Standard Curve

A standard curve was prepared by spotting known amounts (0 to 40 uL) of naringin (1 ug/uL) on a plate and

carrying this material through the complete procedure.

2.5.2.4 Colorimetric Method

The scraped bands were transferred to the centrifuge tubes, 4 mL of the Davis reagent (methanol: diethylene glycol:4N aqueous sodium hydroxide, 125:112:20 by volume) was added. The mixture was stirred with a glass rod and the tubes were capped and centrifuged at 800 x g for 10 minutes. The colour began to develop as soon as the test reagent was added and reached a maximum after 15 minutes with no further change in absorbance after one hour. The supernatant was transferred in 1 cm absorption cells and read in a spectrophotometer (Hitachi, Model 101) at 420 nm against a blank of modified Davis reagent. The concentration of naringin was obtained from the standard curve and converted to ppm by multiplying by 1000. Since no narirutin was available, narirutin content was estimated using the same standard curve for naringin.

2.5.3 Reliability of the Method

A preliminary study was done to determine the naringin content of grapefruit juice. Because the presence of other flavanones in addition to naringin and narirutin might complicate the study, 10 uL of standard solutions (1 ug/uL) of naringin (pure, Koch Light), naringenin (Sigma), hesperidin (pure, Koch Light) and hesperetin (crystalline, Sigma) were spotted along with the juice sample. Table III presents the Rf values of the different flavanones.

Narirutin was not available but it was reported to have the same R_f value as that of naringin (Versteeg, 1977). His study showed that naringin and narirutin had the same R_f values when developed in water, ethanol, methyl ethyl ketone and acetyl acetone (65:15:15:5) but they can be separated by the procedure of Tatum and Berry (1973). The band or spot from grapefruit juice with an R_f of 0.58 was therefore assumed to be narirutin.

Table III. Average R_f values of flavanones chromatographed on Silica Gel G plates developed with nitromethane:methanol (10:3).

Flavanone	R_f values *
Naringin	0.32
Hesperidin	0.38
Hesperetin	0.91
Naringenin	0.89
Grapefruit juice	0.32
	0.58

* mean of duplicate determinations

Table IV. Naringin content of fresh NZ grapefruit juice.

Plate No.	Naringin (ppm)
1	615
2	615
3	610
4	570
5	628
Mean ± SD	607.6 ± 22.05

The naringin content of fresh NZ grapefruit juice was determined using the above method. Results gave an average of 607.6 ± 22.05 (Table IV).

A comparison between the above method was done with that of the Davis method (Table V). It was found that the Davis method gave a value for the naringin content which was about twice that obtained using the modified Tatum and Berry method. The same finding was reported by Tatum and Berry (1973) who found that the glycoside concentration by the Davis method was approximately 2.2 times that of the above method.

Table V. Comparison of the Davis and the Tatum and Berry method for naringin determination of NZ grapefruit juice concentrate.

Method	Naringin (A) (ppm)	Narirutin (B) (ppm)	A/B
Tatum and Berry			
Plate 1	1,029	n.d.*	
2	993	192	5.2
3	993	186	5.3
Davis	2,000		
Davis/naringin	2.0		

* not determined

2.6 EXTRACTION AND CHARACTERIZATION OF PECTIN

2.6.1 Recovery of Pectin from Grapefruit Peel

The method used in the recovery of pectin from grapefruit peel was based on nitric acid extraction and alcohol precipitation (Crandall and Kesterton, 1976) with some minor modifications.

Fresh peel (1 part) was added to boiling water (3 parts) and stirred for 5 minutes. The mixture was blended for 3 minutes using a Jeffco wet disintegrator and then drained and pressed by hand using cheesecloth. The pressed peel was immediately cooled by dropping into two parts of cold water, stirred, drained and pressed again. The peel was oven-dried overnight at 80°C. To 1 part dried peel, 70 parts distilled water was added and the pH was adjusted to 1.6 ± 0.05 using 1 N nitric acid. The mixture was heated to 90°C for 45 minutes with occasional stirring to break the bigger lumps of dried peel. After extraction, the mixture was quickly cooled, centrifuged and the liquor decanted into a beaker. The solids from the centrifugation were re-extracted with distilled water, stirred, centrifuged and the liquor decanted into the beaker containing the original liquor. The combined liquors were filtered and the filtrate was precipitated in 2 volumes of acidified isopropyl alcohol (2 mL of 1 N nitric acid/L of alcohol) to remove metallic contaminants. The resulting pectin was separated by filtration through a nylon mesh. After filtration, the pectin was washed with 70% isopropyl alcohol to remove the sugars and any residual acid. The pectin was dried under vacuum at 60°C for 14 hours to a moisture content of about 10-20% and ground to pass a 60-mesh screen for further analysis.

2.6.2 Characterization of Pectin

2.6.2.1 Yield of Pectin

Yield of pectin was calculated as the weight of dried alcohol precipitate extracted from a given weight of peel.

2.6.2.2 Moisture Content

Moisture content was determined by drying 1 g of pectin at 60°C in vacuum to constant weight.

2.6.2.3 Ash content

Ash was estimated by weighing 1 g of pectin into a tared crucible, igniting slowly and then heating at 600°C for 4 hours.

2.6.2.4 Anhydrogalacturonic Acid Content

The uronide content of pectin preparations has been determined by titration, decarboxylation and colorimetric methods. Titration can only be applied if the pectin preparation does not retain mineral acids from its preparation, and the results are reproducible only if the pectin is completely dispersed or if the marc preparations are finely ground (Joslyn, 1962).

Decarboxylation has been widely used since its introduction by Lefevre and Tollens (1907), and a number of papers have been published in this area. Uronic acids may be decarboxylated by heating with hydrochloric acids, by heating alone at elevated temperatures, or by metal catalysis (Anderson, 1958; Barker et al., 1958; McReady et al., 1946; Dore, 1926).

Because of the ease of determining pectic substances by colour reactions, colorimetric methods are often preferred for analysis. Colorimetric methods based on the carbazole method of Dische (1950) are widely used. These procedures have been criticized on the grounds that they are not

sufficiently specific (Joslyn, 1962). A recent method by Blumenkrantz and Asboe-Hansen (1973) used m-hydroxydiphenyl to form a chromogen which is specific for uronic acids. A procedure based on m-hydroxydiphenyl was developed and reported to be sensitive, specific and reproducible (Robertson, 1979).

A method for the determination of anhydrouronic acid content was based on that of McComb and McReady (1952) for the de-esterification of pectin solution, and the colorimetric method of Robertson (1979).

Procedure:

Reagents - 0.0125 M Sodium tetraborate. 10 H_2O (A.R.) in H_2SO_4, sp. gr. 1.84 (A.R.)

0.15% Meta-hydroxydiphenyl solution (m-phenylphenol, Eastman Organic Chemicals) in 0.5% sodium hydroxide, stored in a brown glass container wrapped in aluminium foil and kept at 2°C.

Polygalacturonic acid (Sigma, Grade III) standards of 0 to 100 ug/mL. The polygalacturonic acid had been dried for 5 hours in a vacuum oven at 30°C before diluting in deionized water.

A solution of 0.1% sugar-free (washed with 70% isopropyl alcohol) pectin was de-esterified by holding in 0.05 N sodium hydroxide for 30 minutes at 25-30°C and diluted to 0.002%. Into each of three test tubes (20 x 150 mm) was added 1 mL of the de-esterified pectin solution. To each tube was added 5 mL of sulphuric acid/tetraborate and immediately after mixing the tubes were placed in a

water-ice bath. They were then heated for 6 minutes and immediately cooled again in the water-ice bath. To two of the three tubes, 100 uL of m-hydroxydiphenyl reagent was added, while to the third tube was added 100 uL of 0.5% sodium hydroxide. This tube acted as a blank to correct for the slight pink colour produced when neutral sugar-containing materials are heated in sulphuric/tetraborate. After mixing, the tubes were left to stand for 15 minutes before reading the absorbance at 520 nm in a Spectronic 70 spectrophotometer. Standard solutions were analyzed by the same colorimetric method.

2.6.2.5 Equivalent Weight

Equivalent weight was estimated by the titration method of Joslyn (1970).

A 0.5 g of pectin sample was weighed into a 250 mL Erlenmeyer flask and was moistened with 5 mL of 95% ethyl alcohol. One gram of sodium chloride was added to sharpen the end point. One hundred mL of carbon dioxide-free distilled water and 6 drops of phenol red indicator were added. The mixture was slowly titrated until the indicator changed (pH 7.5). Equivalent weight was calculated according to the formula:

$$\text{Equivalent weight} = \frac{\text{Weight of sample (mg)}}{\text{meq. of sodium hydroxide}}$$

2.6.2.6 Methoxyl Content

Methoxyl Content was based on the titration method of Joslyn (1970).

Procedure:

To the solution with pH 7.5 titrated for equivalent weight containing 0.5 g of pectin, 25 mL of 0.25 N sodium hydroxide was added. The mixture was thoroughly

shaken and allowed to stand 30 minutes at room temperature in a stoppered flask. Twenty-five mL of 0.25 N hydrochloric acid (or an amount equivalent to the base) was added and the mixture was titrated with 0.1 N sodium hydroxide to the same end point as described in Sect. 2.6.2.5 for equivalent weight. Percent methoxyl content was calculated as:

$$\% \text{ Methoxyl} = \frac{\text{meq. of sodium hydroxide} \times 31 \times 100}{\text{weight of sample (mg)}}$$

2.6.2.7 Degree of Esterification (DE)

This was calculated on the basis of methoxyl and anhydrouronic acid (AUA) contents according to the formula of Schultz (1965):

$$\% \text{ DE} = \frac{176}{31} \times \frac{\% \text{ methoxyl}}{\% \text{ AUA}} \times 100$$

2.6.2.8 Setting Time

The setting time of pectins is of importance to jam, marmalade and preserve processors. Several methods have been proposed to measure this factor (Olsen et al. 1939; Joseph and Baier, 1949; Olliver, 1950; Doesburg and Grevers, 1960). Owens et al. (1952) described a simple method which was similar to the international method for preparing standard jellies (Institute of Food Technologists, 1959).

Procedure:

The weight of pectin to be used for the standard jelly was calculated by dividing 650.0 by the value of an assumed firmness grade (say, 150). Into a dry container, sugar was weighed which was equivalent to 650.0 minus the weight of pectin used. About 20-30 grams of the weighed

sugar was transferred and mixed by stirring with a spatula or glass rod. Distilled water (410 mL) was measured and poured into a tared 3 L stainless steel saucepan provided with a stirrer, into which was poured the pectin-sugar mixture and gently stirred for about 2 minutes. The saucepan was then placed on a stove and heated with stirring until the contents came to a full rolling boil. The remaining sugar was then added. Heating and stirring continued until all the sugar was dissolved. Heating was stopped when the net weight of the jelly batch reached 1015.0 g. The rate of heating was controlled to maintain the heating time from 5 to 8 minutes. When the jelly had reached 95°C, it was poured into a beaker placed in a water bath maintained at 30°C. Setting time was obtained from the time of filling to the time when the jelly at the top just congealed.

2.6.2.9 Viscosity determinations

Relative viscosities of 0.15, 0.10 and 0.05 g/100 mL pectin were determined by the following procedure (Owens et al., 1946):

The pectin sample (ash- and moisture-free basis) was dissolved in 50 mL distilled water with pH adjusted to 4.8. The solution was stirred for 2 hours. Sodium chloride (0.80 g) and sodium hexametaphosphate (0.20 g) dissolved in 15 mL distilled water were added and the solution was stirred for another hour. The pH was adjusted to 6.0 ± 0.2. The solution was transferred to a 100 mL volumetric flask and made to volume using distilled water. The solution was filtered through a dry ash-free filter paper (Whatman No. 41) to remove dust, fibre or any possible undissolved substances. Viscosity of the solution was determined within an hour after the pH adjustment by means of a Cannon-Fenske No. 50 viscometer with 10 mL solution at 25 ± 0.03°C. The efflux time for the solvent which consisted of 0.8% sodium chloride and 0.2% sodium hexametaphosphate was determined using the

same instrument. The relative viscosity (η_r) was calculated by dividing the time of efflux of the solution with that of solvent. Intrinsic viscosity [η] was then determined by plotting $(\eta_r - 1)/C$ against C, where C is the pectin concentration, on graph paper and extrapolating to zero concentration.

2.6.2.10 Molecular weight determination

The molecular weight was calculated by using Staudinger's equation (1932) as described by Christensen (1954):

$$\text{Intrinsic viscosity, } [\eta] = KM$$

where K is a constant and M is the molecular weight. The value for K had been shown by Vollmert (1950) to be nearly constant for high methoxyl pectins and was assumed to be 4.7×10^{-5}.

CHAPTER 3

BITTER COMPONENTS OF NEW ZEALAND GRAPEFRUIT AND PHILIPPINE CALAMANSI

3.1 EXPERIMENTAL

Pasteurized grapefruit juice concentrate and calamansi juice were analyzed for their flavonoid and limonoid content according to the procedures described previously (2.4 and 2.5). For the flavonoid determination, standard solutions (1 ug/uL) of naringin (pure, Koch Light), hesperetin (crystalline, Sigma), naringenin (Sigma) and hesperidin (pure, Koch Light) were spotted together with the juice samples on separate plates for identification purposes. Naringin and narirutin were determined by applying bands of juice samples across thin-layer plates.

3.2 RESULTS

R_f values of New Zealand grapefruit juice concentrate (3.34 pH, 27°Brix and 0.91 g citric acid/100 mL) and calamansi juice (2.44 pH, 8.5°Brix and 3.57 g citric acid/100 mL) are presented in Table VI.

Grapefruit contained fluorescent spots with R_f values of 0.32 (equal to that of the naringin standard) and 0.58. Preliminary studies (as described previously in Section 2.5.3) showed that narirutin could be separated from naringin by applying bands of the juice across thin-layer plates and developing in nitromethane:methanol as described by Tatum and Berry (1973). The spot or layer with an R_f of 0.58 was then assumed to be narirutin. Naringin is absent in calamansi juice but a fluorescent spot with an R_f of 0.29 was detected. This compound was not identified due to the unavailability of other standard flavanones which are useful for identification of such compounds.

Table VI. Average R_f value of standard flavanones and juice samples on Silica Gel G plates, developed with nitromethane:methanol (10:3 v/v).

Flavanone/Sample	R_f
Naringin	.32
Hesperidin	.38
Hesperetin	.91
Naringenin	.89
Grapefruit juice conc.	.32, .58
Calamansi juice, plain	.29
Calamansi juice, with 5% crushed seeds	.29

Table VII. Replicate determinations of naringin and narirutin with 70 uL aliquots of New Zealand grapefruit juice concentrate.

Plate No.	Naringin		Narirutin	
	ug	ppm	ug	ppm
1	72	1,029	13.7	196
2	69.5	993	13	186
3	69.5	993	12	171
4	68.5	979	13	186
5	68.5	979	13.7	196
Mean	69.5	995	13.08	187
S.D.	1.43	20.46	0.70	10.25

Table VIII. Limonin content of New Zealand grapefruit and Philippine calamansi juice.

Sample	R_f (ave.)	Limonin (ppm)		Mean	Ave. of Means ± SD
Grapefruit juice	0.55	8.0	8.2	8.1	7.9 ± 0.22
		8.0	7.8	7.9	
		7.7	7.6	7.6	
Calamansi juice (with 5% crushed seeds	0.56	10.0	9.7	9.8	
		12.3	12.0	12.2	10.5 ± 1.22
		9.7	9.6	9.6	
	0.59	(light brown spot also present, 1.1 ppm)			

Table IX. Summary of grapefruit and calamansi juice determinations.

Property	Grapefruit juice	Calamansi juice (plain)	Calamansi juice (with 5% crushed seeds)
pH	3.34	2.44	2.44
Soluble solids (°Brix)	27.0	8.50	9.50
Titratable acidity (g citric acid/100 mL)	0.91	3.57	3.57
Naringin (ppm)	995.0	none	none
Narirutin (ppm)	187.0	none	none
Limonin (ppm)	7.9	none	10.5

Table VII gives the result of the naringin determinations using 70 uL juice across thin-layer plates. Limonin determination was carried out for the juice samples and the results are given in Table VIII. Table IX summarizes the chemical properties of both the grapefruit and calamansi juice.

3.3 DISCUSSION

The Silica Gel thin-layer chromatography using nitromethane:methanol gave good separation of naringin from its isomer narirutin. This can be explained by the thorough drying of plates before placing in well-equilibrated tanks, and also by the use of fresh solvents for each determination as suggested by Tatum and Berry (1973).

Analyses revealed that grapefruit juice concentrate contain 995 ppm naringin, 187 ppm narirutin, and 7.9 ppm limonin. Naringin accounts for most of the bitterness in the grapefruit juice as can be seen from the values obtained for naringin and limonin. This finding supports the earlier reports that naringin is the compound which is primarily responsible for the bitterness of grapefruit (Horowitz, 1964).

Naringin is present in grapefruit juice at levels ranging from 130 to 950 ppm (Maurer *et al* 1950). The obtained value for naringin is higher than the given range and this could probably be due to storage and processing condition, i.e., the juice had been concentrated to 27°Brix. Camp *et al*. (1932) noted that the bitter taste in the stored products on aging was of glycosidal origin. They also found that the intensity of the bitterness was a function of fruit maturity, method of preparation and subsequent treatment of the product and that the glycosides had to be controlled or removed before a satisfactory frozen or cold-stored product

could be obtained. Maurer et al. (1950) stated that when naringin in the juice samples exceeded 0.070% (700 ppm), the juice was inferior and had a bitter taste, while juice containing less than 0.050% (500 ppm) naringin appeared to have superior flavour and quality.

Narirutin is present at 187 ppm level. This compound had been reported to be bitter (Horowitz and Gentili, 1969) and non-bitter (Kamiya et al., 1975). Such a level of narirutin could contribute to the bitterness of the juice if proven to be bitter, so that a study on the nature and extent of narirutin bitterness is helpful before any conclusion can be made on its effect to the over-all bitterness of grapefruit juice. Narirutin makes up about 28% (w/w) of the total flavonoids in grapefruit while naringin makes up 63% (w/w) (Hagen et al., 1966).

The limonin (7.9 ppm) present would be a minor contributor to the bitterness of the grapefruit juice. Maier and Dreyer (1965) isolated limonin from grapefruit juice in concentrations up to 9.5 ppm and believed that it contributed to the bitterness of the juice. Delayed bitterness caused by limonin would not, however, be noticeable due to the presence of naringin. Limonoid bitterness is most noticeable in the juice of the Navel orange and Shamouti orange where it is present at levels of up to 42 and 25 ppm, respectively (Wilson and Crutchfield, 1968; Levi et al., 1974). The amount of limonin that must be present in a juice before bitterness becomes detectable varies with the sweetness and acidity of the juice as well as the sensitivity of the taster, but as a general rule, a juice containing less than 6 ppm is unlikely to taste bitter while a juice with more than 9 ppm will seem bitter to most tasters (Chandler and Kefford, 1966). The juice in this study with 7.9 ppm limonin is thus expected to possess a slight bitterness.

Calamansi fruit is extensively grown in the Philippines and it is now the leading citrus fruit being utilized primarily for its juice. It bears a small, seedy fruit with a highly sour (as indicated by the low pH and high titratable acidity) but flavourful juice. The fruits are largely consumed fresh but some progress has already been made on processing the juice (Mendoza et al., 1976). The presence of the common grapefruit flavonoids was not detected in the calamansi juice but thin-layer chromatography revealed the presence of a fluorescent spot with an R_f of 0.29. This compound was not identified due to the lack of other flavonoids.

Calamansi juice with 5% crushed seeds contain 10.5 ppm limonin. Plain juice does not contain detectable levels of limonin but when seeds are incorporated, limonin was detected at a fairly high level. During processing operations, the seeds are easily damaged due to the small size of the fruit (about 2-2.5 cm in diameter). This damage to the seeds results in bitterness development in the juice after several hours at room temperature or if heated, within a few minutes. The presence of limonin in the seeds would therefore impart bitterness to the juice above the organoleptically detectable level. Radioactive tracer experiments showed that citrus seeds accumulate limonoids as they are translocated from fruit tissue during growth of the fruit (Hasegawa et al. 1980). Other limonoids aside from limonin have been reported. Nomilin, nomilinic acid and ichangin are also bitter but they are insignificant because of their low levels in the seeds. Neutral limonoids in citrus seeds consist of limonin, nomilin, obacunone and deacetylnomilin while the acidic limonoids are nomilinic acid, deacetylnomilinic acid and isonomilinic acid. Table VIII shows the presence of a light brown spot just above the limonin layer. This spot was suspected to be a limonoid and was found to be present at 1.1 ppm level.

However, no attempt was done to identify such compound. Maier and Beverly (1968) reported the R_f values of the different limonoids. The solvent system was, however, different from that used in this study and therefore the identification of the above spot was not possible.

CHAPTER 4

CHANGES IN THE BITTER COMPONENTS AND OTHER CHEMICAL CONSTITUENTS OF NEW ZEALAND GRAPEFRUIT DURING MATURATION

4.1 EXPERIMENTAL

Determination of the chemical constituents of grapefruit was done immediately after picking. Analyses were conducted every three weeks from June 2 to October 26, 1981. Methods of analysis were as described in Section 2.1 to 2.3.

Naringin content in the juice and peel was determined the day after picking. One part of the peel was blended with three parts hot water (88-90°C) in a Jeffco wet disintegrator for 3 minutes. Separation of the extract from the solids was done using 6 layers of cheesecloth. The extract (50 uL) was spotted directly onto Silica Gel G plates while the ground peel was further treated for pectin extraction. Fresh juice (100 uL) was spotted directly onto Silica Gel G plates and treated as described in Section 2.5.

Limonin content was determined for unpasteurized and pasteurized juices. Pasteurized juice at normal pH and with pH adjusted to 5.6 using 1 N NaOH was analysed for limonin immediately after pasteurization and again after standing for 48 h at room temperature.

Data for the different properties were separately analysed using a completely randomized design with 2 replications and 8 treatments (picking dates). Specific differences between treatments were determined by Tukey's test (Honestly Significant Difference or HSD). All comparisons were made at a 5% level of significance. Results of the statistical analyses are presented in Appendix 1.

4.2 RESULTS

Seasonal changes in the chemical constituents of grapefruit juice are summarized in Table X and Fig. 4. There was a fluctuation in the juice yield as the fruit ripened. A general increase in pH was observed as the season progressed. Statistical analyses revealed that ripening of the fruit significantly affected the soluble solids, titratable acidity, ^{o}Brix/Acid ratio and ascorbic acid of the juice. Soluble solids markedly increased from 10.8 in the early-season fruit to 13.5^{o}Brix in the late-season fruit. A downward trend in titratable acidity was observed as the season progressed. The maturity index (the ratio of ^{o}Brix to acid) showed a continuous increase as the fruits ripened. Insoluble solids or pulp content was high initially but remained rather constant throughout the season. Statistical analysis, however, revealed that ripening of the fruit did not significantly affect this property. The observed decrease is attributed more to the juicing procedure rather than to the maturity of the fruit. Ascorbic acid was affected by ripening but a significant decrease occurred only in the fruits harvested late in the season.

Table XI and Fig. 5 present the seasonal change in the naringin and narirutin content of juice and peel in NZ grapefruit. Naringin content in the juice was highest at the beginning of the season but fluctuated widely throughout the season, reaching the second highest concentration at the end of the season. Narirutin was present in the early-season fruit but disappeared as the season advanced. No significant effect was observed in the naringin content of the peel with ripening of the fruit. Narirutin was also present in the peel of the early-season fruit but disappeared later in the season.

A general decrease in limonoids was observed in the unpasteurized and pasteurized juices, as shown in Table

XII and Fig. 6. Limonoid content was lowest in the juice from the fruit harvested on September 14. Juice stored at room temperature for 48 h showed a slight increase in limonoid content in the case of pasteurized juice with normal pH, while the pasteurized juice with pH adjusted to 5.6 showed a slight decrease.

Fluctuations in the total extractable limonin (TEL) caused by heating occurred as the season progressed as shown in Table XIII. Protolimonin decreased with ripening. Actual limonin values fluctuated throughout the season.

4.3 DISCUSSION

During the maturation of grapefruit, the major changes in composition were an increase in pH, an increase in soluble solids up to a maximum in mid-September followed by a small decline towards the end of the season, a steady fall in acidity and a continuous rise in the Brix/Acid ratio. Juice yield did not increase with maturity but fluctuated throughout the season. The low value obtained from the late-season fruit is attributed to "drying out" of the fruit. The peel and endocarp of the fruit harvested late in the season were relatively dry. Juice yield of grapefruit was reported to increase with maturity until the fruit ripened, after which the value remained constant (Harding and Fisher, 1945). Krezdorn and Cain (1952) noted that juice yield increased as the season advanced but levelled off towards the end of the season, and that variety had no effect on juice yield. Hilgeman (1941) stated that juice yield was not closely correlated with maturity. Croucher (1935) reported that juice yield rapidly increased and reached a maximum considerably before the fruit reached maturity. Results of the present study showed that juice yield was not affected by ripening and maturation, although a

Table X. Seasonal changes in chemical constituents of NZ grapefruit juice

Picking date	Juice Yield (%)	pH	Soluble solids (°Brix) [1]	Titratable acidity (g citric acid/ 100 mL) [1]	Brix/Acid [1]	Insoluble Solids (%)	Ascorbic acid (mg/100 mL) [1]
June 2	31.02	3.00	10.8 a	2.56 a	4.22 a	12.00	n.d. *
June 22	34.09	3.05	11.6 b	2.40 b	4.84 a	11.00	37.8 a
July 13	35.02	3.07	12.1 c	2.12 c	5.72 b	3.00	36.5 a
Aug. 3	30.95	3.18	12.8 d	2.08 c	6.16 b	3.00	37.8 a
Aug. 24	33.56	3.29	13.6 e	1.86 d	7.34 c	3.00	38.3 a
Sept. 24	33.67	3.38	14.4 f	1.84 d	7.83 ce	2.50	37.8 a
Oct. 5	31.00	3.37	13.5 e	1.63 e	8.28 de	3.00	37.3 a
Oct. 26	26.14	3.50	13.5 e	1.31 f	10.31 f	2.50	33.2 b
HSD			0.5	0.12	0.71		1.84

* not determined

[1] means followed by a common letter are not significantly different at 5% level of significance

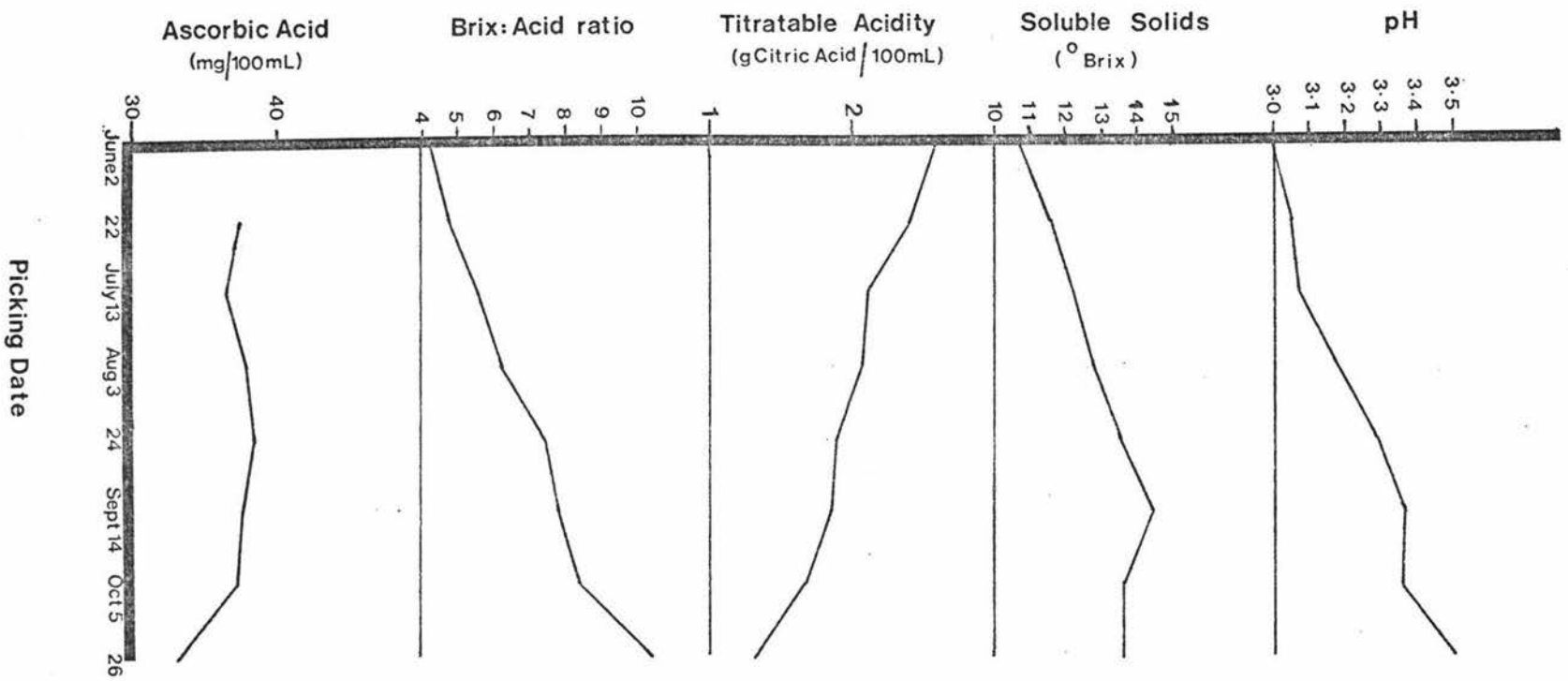

Fig. 4. Effect of maturity on the chemical constituents of New Zealand grapefruit juice.

Table XI. Seasonal changes in naringin and narirutin content of NZ grapefruit juice and peel.

Picking date	Juice		Peel	
	Naringin 1/ (ppm)	Narirutin (ppm)	Naringin 1/ * (%)	Narirutin (ppm)
June 2	743 [a]	42	0.33	255
June 22	615 [c]	-	0.38	-
July 13	575 [d]	-	0.37	-
Aug. 3	655 [bcd]	-	0.39	-
Aug. 24	662 [abc]	-	0.34	-
Sept. 14	528 [e]	-	0.36	-
Oct. 5	670 [abc]	-	0.36	-
Oct. 26	735 [ab]	-	0.34	-
HSD	82			

* expressed on a wet-weight basis

1/ means followed by a common letter are not significantly different at 5% level of significance

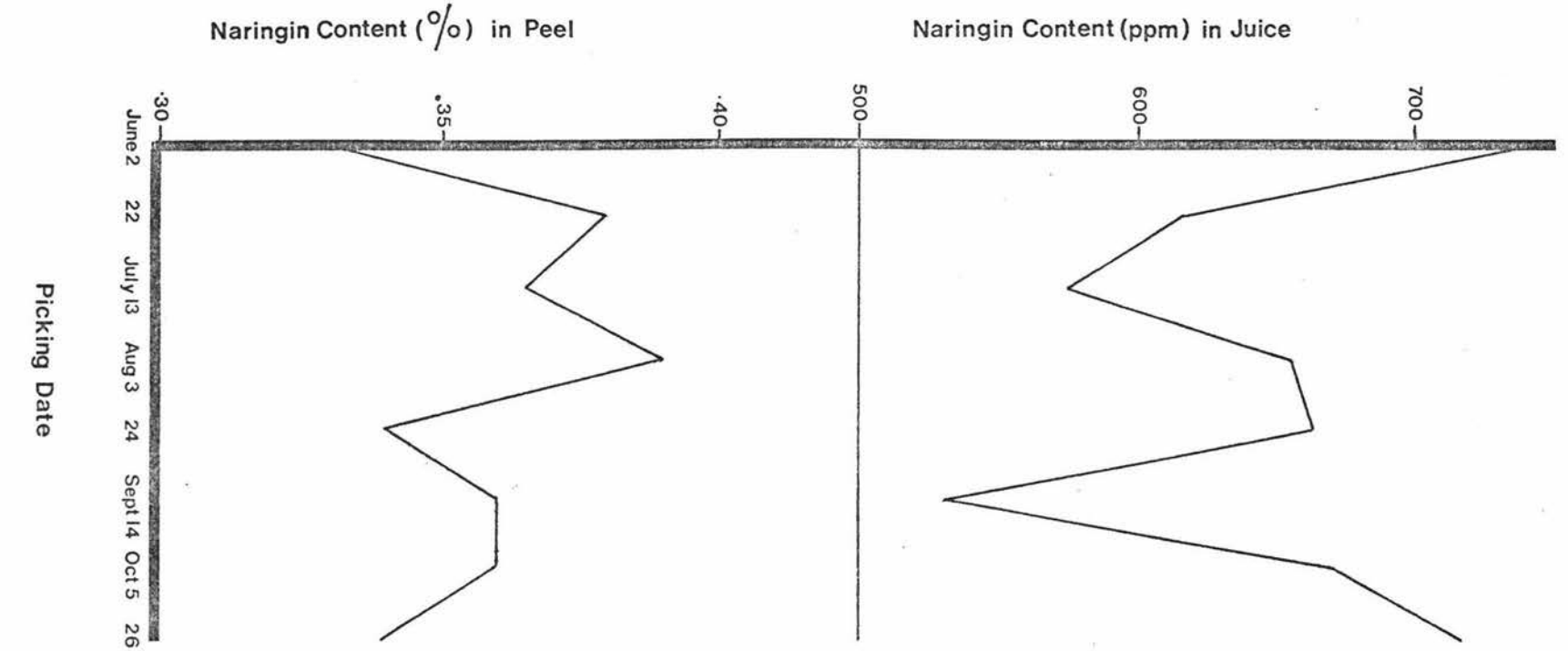

Fig. 5. Effect of maturity on the naringin content of the juice and peel of N.Z. grapefruit

Table XII. Seasonal changes in the limonoid content of NZ grapefruit juice

Picking date	Limonoid content				
	A*[1]	B*[1]	C*[1]	D*[1]	E*[1]
June 2	7.5 b	10.0 a	10.6 a	5.4 abc	5.4 abc
June 22	8.8 a	10.0 a	10.6 a	5.4 abc	5.1 abc
July 13	6.1 c	7.8 ab	7.8 bc	4.8 abc	5.1 abc
Aug. 3	5.7 d	6.8 ab	7.1 bc	4.1 b	3.4 c
Aug. 24	4.8 f	8.0 ab	8.7 b	5.8 a	5.6 ab
Sept. 14	4.8 f	6.2 b	6.5 c	3.9 c	3.8 bc
Oct. 5	5.3 e	7.4 ab	7.4 bc	5.7 ab	5.4 abc
Oct. 26	4.7 f	7.4 ab	7.3 bc	6.1 a	6.0 a
HSD	0.4	3.7	1.7	1.7	2.1

*A - total extractable limonoids (TEL) of unpasteurized juice at normal pH after 2 h

B - TEL of pasteurized juice at normal pH after 2 h

C - TEL of pasteurized juice at normal pH after 48 h

D - TEL of pasteurized juice with pH adjusted to 5.6 after 2 h

E - TEL of pasteurized juice with pH adjusted to 5.6 after 48 h

[1] means followed by a common letter are not significantly different at 5% level of significance

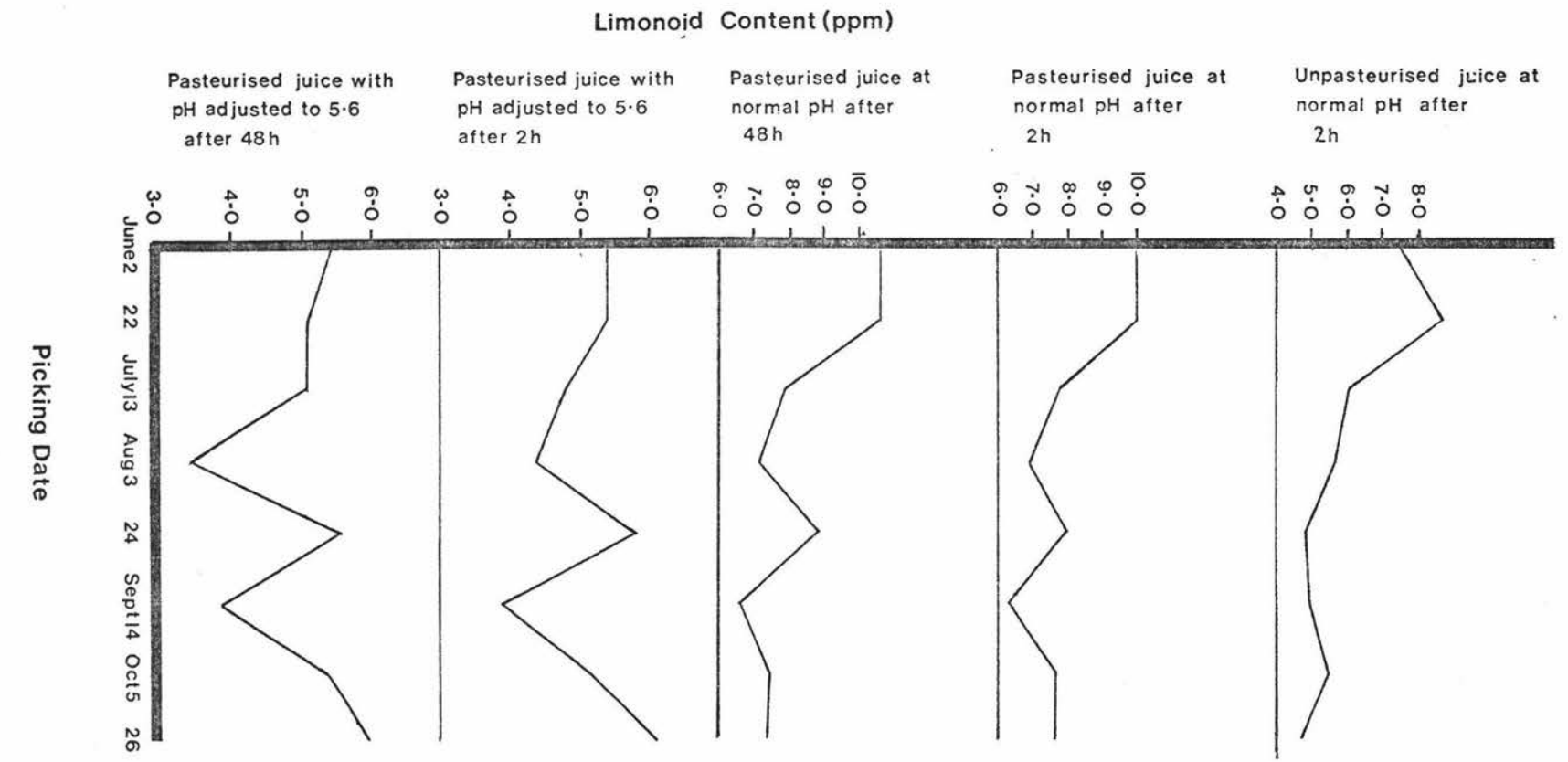

Fig. 6. Effect of maturity on the limonoid content of NZ grapefruit juice.

Table XIII. Changes in the limonoid content of NZ grapefruit juice.

Picking date	Limonoid content (ppm) Increase in TEL after heating [a]	Protolimonin [b]	Limonin [c]
June 2	2.5	4.6	5.4
June 22	1.2	4.6	5.4
July 13	1.7	3.0	4.8
Aug. 3	1.1	2.7	4.1
Aug. 24	3.2	2.2	5.8
Sept. 14	1.4	2.3	3.9
Oct. 5	2.1	1.7	5.7
Oct. 26	2.7	1.3	6.1

[a] represent the difference between TEL of pasteurized and unpasteurized juice at normal pH (B-A)

[b] estimated as the difference between TEL of pasteurized juice at pH 3.2 and 5.6 (B-D)

[c] measured by extraction after pH adjustment to 5.6

lower yield was obtained from the late-season fruit due to "drying out".

The results obtained for titratable acidity and pH are in general agreement with the findings of Sinclair and Bartholomew (1944), Harding and Fisher (1945) and Karaoulanis and Margaris (1975). These workers showed that the concentration of the acids in grapefruit gradually decreased as the fruit developed and matured. This decrease in free acid with maturity results from the increase in the water content of the fruit while the free acid per fruit remains relatively constant (Sinclair, 1972). In the present study, values for titratable acidity ranged from 1.31-2.56 g citric acid/100 mL juice, and for pH from 3.00-3.50. These values indicate that the juice has a high buffering capacity as reported earlier by Sinclair and Bartholomew (1947), who stated that in citrus juices, wide variations in titratable acidity may cause only small changes in pH. Ranges for acidity reported for New Zealand grapefruit are 0.69-1.98 g citric acid/100 mL juice with pH range of 2.95-3.35 (Dawes, 1970) and 1.29-2.11 g citric acid/100 mL with pH range of 2.90-3.40 (Robertson, 1975). Values obtained for titratable acidity and pH were slightly higher than the reported values, and this could be due to differences in variety, maturity levels and season.

It has been reported that during maturation of citrus fruits, the major changes in composition are slow increases in the concentration of soluble solids and a steady fall in acidity (Kefford, 1959), but in some studies of maturity trends, the change in composition have taken a slightly different form. The decrease in acidity is still a definite and consistent trend, but the soluble solids content increases to a maximum and then levels out and declines with advancing maturity. This pattern of change was shown frequently in grapefruit, e.g. in California and Arizona by Rygg and Getty (1955), in Texas by Burdick (1961) and Lime

et al. (1954, 1956), in Florida by Stenstrom and Westbrook (1956), and in New Zealand by Robertson (1975) and this present study. Soluble solids obtained from this study (10.8-14.4 °Brix) are in agreement with the reported value of 8.7-16.8° and 11.0-13.5 °Brix for New Zealand grapefruit juice (Dawes, 1970; Robertson, 1975).

The ratio of soluble solids to titratable acidity is the most widely used criterion of maturity in determining the proper time for harvest (Sinclair, 1972). The palatability of the grapefruit depends largely upon the balance of sweetness and acidity to the human palate (Harding, 1954) and accordingly, the ratio has come to be widely accepted as a useful index of palatability (Baier, 1954). Results of this study reveal a relatively smooth increase in the ratio with maturity of the fruit. Other workers have reported a similar finding, e.g. Krezdorn and Cain on Marsh, Thompson and Webb grapefruit (1952), Harding and Fisher on Florida grapefruit (1945), Karaoulanis and Margaris on grapefruit from three different regions of Greece (1975) and Robertson (1975) on New Zealand grapefruit.

Insoluble solids was high in the early-season fruit but declined and remained rather constant through the remainder of the season. Caldwell (1934) determined both soluble and insoluble solids in Florida grapefruit from July to November and found that insoluble solids was highest at July and lowest at November. Values from this study revealed the same trend, although the results are attributed more to the juicing procedures than to the maturity of the fruit.

Trends in ascorbic acid content with advancing maturity are not so consistent between citrus varieties (Kefford, 1959). Harding and co-workers (Harding, 1944; Harding and Fisher, 1945) noted in their work that ascorbic acid in grapefruit decreased during ripening. Immature fruit contained the highest concentration of ascorbic acid whereas

ripe fruit contained the least. Results of the study do not agree with the mentioned findings due to the observed insignificant differences between ascorbic acid content of the early and mid-season fruit. Significant decreases were observed only from the late-season fruit. Constant values in ascorbic acid have recently been reported in early- and mid-season grapefruit by Albach et al. (1981b). Harding et al. (1940) also observed that fruit harvested late in the season showed a reduction in water content (drying out) and the least amount of ascorbic acid. Values obtained from this study (33.2-37.8 mg/100 mL) are within the 21-49 mg/100 mL range reported by Dawes (1970) but slightly higher than the 23.0-32.7 mg/100 mL reported by Robertson (1975) for NZ grapefruit.

The results for the seasonal changes of flavonoids are not in agreement with the findings reported by Maurer et al. (1950), Kesterton and Hendrickson (1953a), Lime et al. (1954) and Hagen et al. (1966). These workers found that as the fruit matured and ripened, the bitterness decreased in conjunction with a decrease in naringin. Fluctuations in the naringin values occurred with ripening of the fruit. This fluctuation in the naringin content with advancing maturity was reported by Albach et al. (1981a) in their study of the annual and seasonal changes in naringin concentration of Ruby Red grapefruit juice. In their study, naringin concentration of juice from the same grove and tree fluctuated during the season and varied considerably between crop years. In another study, Albach et al. (1981b) noted relatively constant values for the naringin content of grapefruit from November to February, after which an increase of about 20% occurred. Naringin concentration in grapefruit peel did not vary considerably with the ripening of the fruit. Maurer et al. (1950) noted a decrease in the naringin content of the flavedo and albedo of Texas grapefruit as the season progressed, but this study revealed otherwise.

The disappearance of narirutin in both the juice and peel after the first sampling could indicate that the flavanone totally disappeared from the fruit, or that the concentration levelled-off abruptly and was not able to be detected using the present method. A decrease in narirutin content during the season was reported by Hagen et al. (1966) using a sensitive chromatographic-fluorometric method.

The general decrease in limonoids obtained for the unpasteurized and pasteurized juices are in agreement with the findings of Nomura (1963), Maier and Margileth (1968) and Scott (1970). These workers observed that the limonoid content of citrus juices decreased with advancing maturity. Recent study on the seasonal variation of bitter components in grapefruit juice revealed that the limonin content decreased almost linearly as the season progressed (Albach et al., 1981b). Early-season grapefruit contain limonoids which can contribute to the bitterness of the juice in addition to naringin. Chandler (1969) reported that limonin was detectable by the human palate at very low levels (5 mg/kg) and becomes objectionably bitter at levels above 8 mg/kg. In the present study a limonin concentration of 7.4 mg/kg, was detected in the pasteurized juice from the late-season fruit, indicating that limonin still contributes to the total bitterness of the juice from very mature fruit. The presence of limonin in late-season grapefruit has been observed by other workers. For example, Maier and Dreyer (1965) found that limonin persisted longer after commercial maturity in grapefruit than it does in the Navel orange.

The effect of heat on limonoids is clearly demonstrated by the values in Table XIII. Kefford and Chandler (1970) and Maier et al. (1977) reported that on standing or on pasteurization, fruit juices develop bitterness, referred to as delayed bitterness. Robertson (1980) in his study on this area postulated that heat treatment caused the conversion of the non-extractable precursor to protolimonin

or limonin. Protolimonin is believed to be a weakly acidic compound, extractable by organic solvents and readily converted to limonin. This compound has been found to decrease with ripening as reflected by the values in Table XIII. Holding the pasteurized juice for 48 h caused only slight increases in limonoid content. This suggests that very little precursor which could be converted to total extractable limonin remained after pasteurization. The juice at pH 5.6 showed a slight decrease in limonoid content after 48 h, possibly because of limonoid degradation. Robertson (1980) reported the same finding, stating that the conversion of the precursor on standing to extractable limonin appeared to be more rapid at natural juice pH than at pH 5.6.

Analyses of the bitter components of grapefruit revealed that fruit harvested in September showed the most suitable characteristic for consumption in the fresh state and for the extraction of juice, due to the low levels of naringin and limonin.

CHAPTER 5

REMOVAL OF FLAVONOIDS AND LIMONOIDS FROM GRAPEFRUIT JUICE

5.1 EXPERIMENTAL

Polyvinylpyrrolidone (Polyclar, 212 um) was added to the grapefruit juice concentrate at different levels (0, 1, 2, 2.5, 3, 4 and 5 percent w/v) and shaken for 30 and 60 minutes. The juices were analysed for flavonoid and limonin content using the methods described in Sections 2.5 and 2.4. Another batch of freshly extracted grapefruit juice was treated in a similar way and ascorbic acid determinations made (Section 3.3).

The data on the different properties were separately analysed using a completely randomized design with 2 replications and 5 or 6 treatments (levels of PVP). Specific differences between treatments were determined by Tukey's test (Honestly Significant Difference or HSD). All comparisons were made at 5% level of significance. Results of the statistical analyses are presented in Appendix 2.

5.2 RESULTS

Tables XIV and XV present the results of the duplicate determinations for flavonoids and limonin in grapefruit juice concentrate. Statistical analyses revealed that the different levels of PVP brought about a significant decrease in the concentration of naringin, narirutin and limonin.

A significant decrease of 28.1% and 69.5% occurred at 1.0 and 2.5% PVP level, respectively, for the juice shaken for 30 minutes. At 60 minutes, significant decreases of 32.16, 64.62 and 78.1 occurred at 1, 2.5 and 5% PVP level, respectively.

Narirutin content was significantly reduced by 76.0 and 59.4% after treatment with PVP at 30 and 60 minutes, respectively. No further decrease was observed when the concentration of PVP was increased beyond 1%.

Table XIV. Average flavonoid and limonin contents of grapefruit juice treated with different levels of PVP and shaken for 30 minutes. [a]

% PVP (w/v)	Naringin (ppm)	Narirutin (ppm)	Limonin (ppm)
0	995 a	187 a	7.9
1	715 b	45 b	7.0
2	708 b	47.5 b	7.2
2.5	304 c	47.5 b	n.d.*
3	290 c	45 b	7.0
4	270 c	47.5 b	7.0
5	262 c	42.5 b	6.3
HSD	150	24.2	6.3

*n.d. - not determined

Table XV. Average flavonoid and limonin contents of grapefruit juice treated with different levels of PVP and shaken for 60 minutes. [a]

% PVP (ppm)	Naringin (ppm)	Narirutin (ppm)	Limonin (ppm)
0	995 a	187 a	7.9 a
1	675 b	76 b	7.2 b
2	655 b	80 b	6.7 bc
2.5	352 c	76 b	n.d.*
3	362 c	75 b	6.5 cd
4	345 c	75 b	6.6 cd
5	218 d	75 b	7.1 bc
HSD	65	34	0.6

*n.d. - not determined

[a] Means followed by a common letter are not significantly different at 5% level of significance

Table XVI. Average ascorbic acid content of freshly extracted grapefruit juice treated with different levels of PVP and shaken for 30 and 60 minutes. [a]

% PVP (w/v)	Ascorbic acid	
	30 minutes	60 minutes
0	41.6 a	41.6 a
1	35.2 b	35.0 b
2	35.2 b	34.72 b
3	33.2 b	32.8 c
4	33.52 b	32.6 c
5	33.2 b	32.0 c
HSD	2.1	1.3

[a] Means followed by a common letter are not significantly different at 5% level of significance.

No significant decrease in limonin content was observed after treating the juice with the varying PVP levels for 30 minutes. However, at 60 minutes, limonin content significantly decreased by 8.1 and 17.5% after treatment with 1 and 3% PVP, respectively.

Table XVI shows the results of the ascorbic acid determinations made on the juices treated with PVP. Ascorbic acid was significantly reduced by 15.4% after treatment with 1% PVP for 30 minutes. No further decrease was observed with increasing levels of PVP. Shaking for 60 minutes significantly reduced the ascorbic acid by 15.9% and 23.1% at 1 and 3% PVP levels.

5.3 DISCUSSION

Insoluble polyvinylpyrrolidone is a kind of polyamide commercially marketed as Polyclar. The compound was reported to remove phenolic compounds (Loomis and Battaile, 1966), various lactones (Wang and Wang, 1966) and flavanones (Neu, 1960) from plant tissues and fruit juices.

This present study has shown that PVP is effective in removing up to 78.1% of the naringin originally present giving a juice of higher quality and better flavour due to reduced bitterness. In fact, shaking the juice with 2.5% PVP for 30 minutes could lower the naringin to a concentration below the organoleptically detectable level. The significant reduction of narirutin may or may not reduce the bitterness since, as previously mentioned, it has been reported to be both bitter (Kamiya *et al.*, 1975) and non-bitter (Horowitz and Gentili, 1969).

The present findings support the earlier report that polyamides (e.g. PVP) are effective adsorbents for flavanones (Neu, 1960). Most flavanones contain phenolic

groups for which PVP and other polyamides have a pronounced affinity (Horhammer et al.,1957). The oxygen in the peptide linkage of PVP forms unusually strong hydrogen bonds with the proton of phenolic hydroxy groups making PVP an effective adsorbent for flavanones (Wrolstad and Putnam, 1969).

Maeda et al. (1979) reported a 65% adsorption of naringin on PVP using a level of 5% for 1 hour. The difference in the values obtained between this study and the present one could be due to variations in the method of analysis, the nature of the juices used and the treatments employed. Maeda and co-workers used the Davis method for the determination of naringin in Natsudaidai juice, while this study used the more specific Tatum and Berry method.

Although it has been shown that PVP is effective in removing up to 55.7% of the limonin from the bitter orange sera by gentle stirring for 30 minutes (Chandler et al., 1968), results from the present study proved otherwise. No significant reduction in limonin was observed after shaking the juice with PVP for 30 minutes. With 60 minutes shaking, a decrease of 17.5% was observed. This apparent discrepancy in the effectiveness of limonin removal by PVP can be explained on the grounds that the juice used by Chandler contained 19.2 ppm limonin initially compared to 7.9 ppm limonin in the present study.

Wang and Wang (1966) reported that polyamides are used in thin-layer chromatography of various lactones, mainly sesquiterpenoids. This indicates that PVP could be suitable as an adsorbent in removing limonin from citrus juices. However, the use of PVP would require a preliminary separation of cloud from the juice before treating the sera with the sorbent (Chandler et al., 1968). This could explain why no significant reduction was observed in the juice treated with different levels of PVP for 30 minutes and only a low reduction was observed in that treated for 60

minutes. The use of PVP to reduce limonin levels apparently requires a preliminary separation of cloud from the juice, treatment of the serum with the sorbent powder, and recombination of the debittered serum with the cloud.

Determination of the ascorbic acid content of the juice before and after PVP treatment indicated that up to 23.1% loss could occur. Polyamides are used in thin-layer chromatography of ascorbic acid (Wang and Wang, 1966) and therefore it is expected that PVP would adsorb this important grapefruit juice constituent. This loss of ascorbic acid may be considered a drawback, but it can be minimized by using the right combination of the amount and reaction time of PVP just sufficient to bring the concentration of these components below the organoleptically detectable level (700 ppm for naringin and 8 ppm for limonin) and by controlling atmosphere oxidation during reaction times by flushing the container with an inert gas such as nitrogen.

This study had shown the effectiveness of PVP in removing the bitter components in grapefruit juice. A non-bitter grapefruit juice can be prepared from highly bitter, fresh grapefruit juice by simple shaking with PVP. PVP adsorbs up to 78.1% of the naringin originally present and up to 17.5% of the limonin bringing their concentration down to below the organoleptically detectable level. Adsorption of the ascorbic acid can be considered a drawback, but this can be minimized by the above-mentioned factors.

CHAPTER 6

EXTRACTION AND CHARACTERIZATION OF PECTIN FROM NEW ZEALAND GRAPEFRUIT PEEL

6.1 EXPERIMENTAL

Extraction of pectin from the peel of fruit harvested on 22 June, 13 July and 24 August was done according to the method described in Section 2.6.1. Extraction of pectin from fruit harvested on 5 October was done as described below for the pilot plant scale production. The peel utilized for pectin extraction was obtained from the grapefruit picked from the Massey University orchard.

A pilot plant scale production of pectin was done using grapefruit grown commercially in Gisborne. Juice extracted from the fruit was pasteurized, canned in A-10 cans and stored at 5°C. The peel was minced using a Bauknecht mincer, washed with 2.5 parts of tap water to remove low-quality pectic substances and soluble sugars, and dried in a cabinet dryer for 12 hrs. Modifications to the prcoedures of Crandall and Kesterton (1976) was done using concentrated sulphuric acid to adjust the pH to 1.6 $\pm$ 0.05 during extraction at 85°C for 1 hr, and acidified isopropyl alcohol (2 mL conc. sulphuric acid/L isopropyl alcohol) to remove metallic contaminants. The pectin was dried at 60°C in vacuo for 16 hrs in a Cuddons freeze dryer, powdered and stored in glass-tight bottles for further analysis.

6.2 RESULTS

Table XVII presents some general characteristics of New Zealand grapefruit used for pectin extraction.

Average weight and juice yield was highest at the beginning of the sampling period. There was a general increase in the pH, soluble solids and Brix:acid ratio throughout the season for the grapefruit juice. A decrease in the titratable acidity was observed. No general trend was obtained for the peel yield throughout the season.

Effects of seasonal changes on characteristics of pectin extracted from grapefruit peel are reflected by values presented in Table XVIII.

Yield of pectin was greatest from the early-season grapefruit. Ash content was highest from the early-season grapefruit and lowest in the late-season grapefruit. Equivalent weights were low for the early-season fruit but increased towards the end of the season. There was a general increase in the methoxyl content, anhydrogalacturonic acid content, intrinsic viscosity and molecular weight as the season progressed. No definite trend was observed for the degree of esterification. A general decrease in setting time was observed as the season advanced. Fig. 7 summarizes the effects of the different properties on the setting time of pectins.

Values for the extraction and characterization of the pectin on a pilot plant scale production are presented in Table XIX.

6.3 DISCUSSION

The production of a good quality pectin was unsuccessful using fruit harvested in June, July and August. Although yields were high, the pectin quality was inferior compared to the pectin recovered from fruit harvested in October. Such differences in characteristics could be due to the method of extraction and the purification procedures. Extractions for June, July and August were done at 90°C with the pectin precipitated by isopropyl alcohol. The temperature for succeeding extractions was lowered to 85°C due to a reported effect of temperature on pectin yield and grade. Rouse (1977) reported that conditions of extraction giving the highest yield of pectin will not produce the highest jelly grade. Extraction at the higher temperature produced

Table XVII. Comparison of New Zealand grapefruit characteristics in relation to maturity.

Characteristic	Harvest date			
	22 June	13 July	24 August	5 October
Ave. weight (g)	207.7	198.6	180.7	194.5
Juice (%)	34.09	35.02	33.56	31.00
pH	3.05	3.07	3.29	3.37
Soluble solids (°Brix)	11.6	12.1	13.6	13.5
Titratable acidity (g citric acid/ 100 ml)	2.40	2.12	1.86	1.63
Brix:acid ratio	4.84	5.72	7.34	8.28
Peel (%, w/w of whole fruit)	60.33	62.98	60.27	62.75

Table XVIII. Seasonal effects on the characteristics of pectin extracted from grapefruit peel.

Characteristic	Harvest date			
	22 June[c]	13 July[c]	24 August[c]	5 October[d]
Yield (%)[a]	25.19	14.04	11.28	8.89
Moisture (%)	6.78	8.31	7.89	1.30
Ash (%)	6.06	6.04	2.83	1.87
Equivalent weight[b]	307.48	288.02	704.00	759.00
Methoxyl (%)[b]	6.23	6.38	8.84	9.16
Anhydrogalacturonic acid (%)[b]	67.50	71.88	71.25	82.25
Degree of esterification (%)[b]	52.40	50.39	70.44	63.20
Intrinsic viscosity[b]	n.d.*	1.9	3.0	4.2
Molecular weight ($\times 10^3$)	n.d.*	40.425	63.830	89.362
Setting time (min).	>15.00	>15.00	11.3	0.55

[a] dry-basis

[b] moisture- and ash-free basis

[c] extracted at 90°C for 45 min using 1 N nitric acid and precipitated by isopropyl alcohol

[d] extracted at 85°C for 1 hr using conc. sulphuric acid and precipitated by acidified isopropyl alochol

* not determined

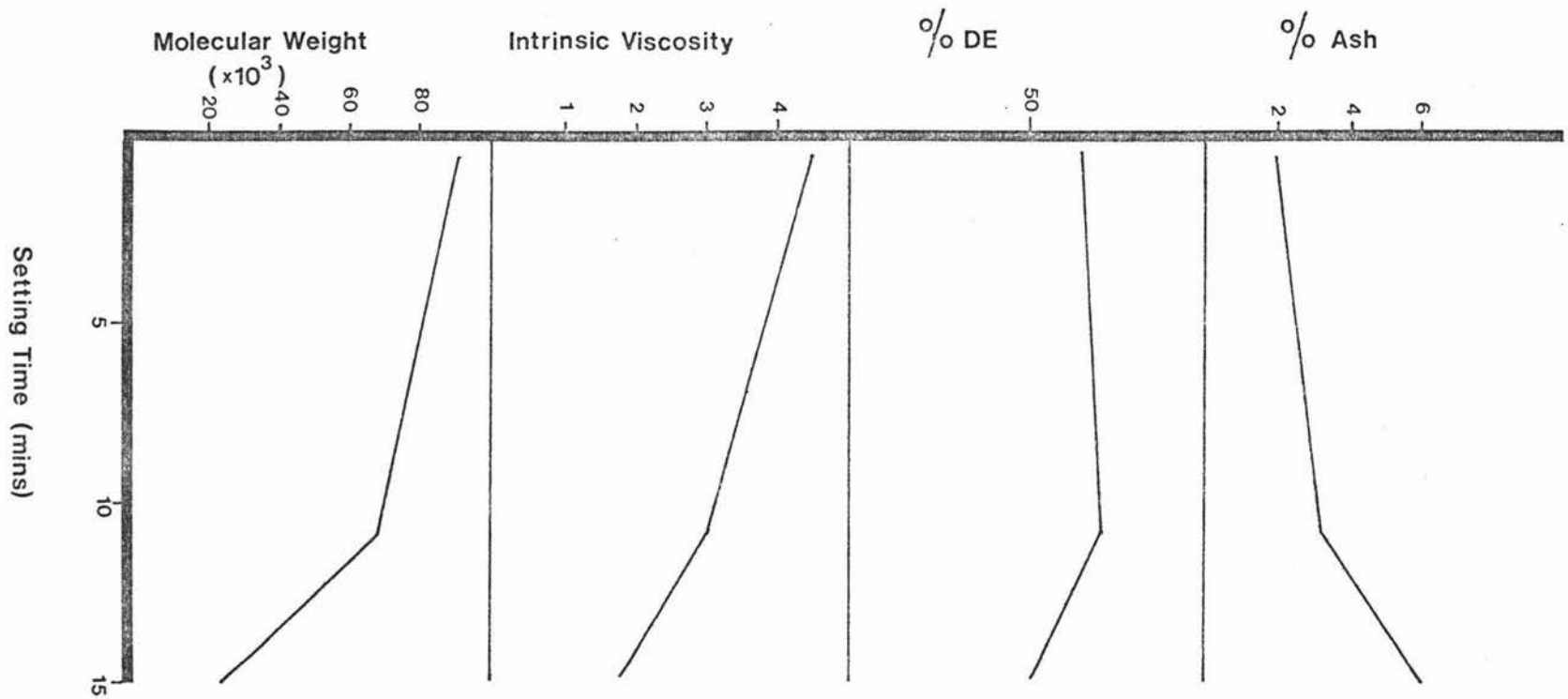

Fig. 7. Effect of % Ash, % DE, intrinsic viscosity and molecular weight on the setting time of pectin.

Table XIX. Comparison of the different characteristics between the locally made pectin and commercial pectin samples.

Characteristic	Pectin from this study	Sigma (Citrus pectin, Grade I)
Yield (%)[a]	15.96	n.d.*
Moisture (%)	3.26	14.06
Ash (%)	1.48	16.82
Equivalent weight[b]	686.00	1038.81
Methoxyl (%)[b]	9.28	8.30
Anhydrogalacturonic acid (%)[b]	89.00	87.50
Degree of esterification (%)	59.20	54.11
Intrinsic viscosity (dl/g)[b]	2.8	4.6
Molecular weight ($\times 10^3$)	59.575	97.872
Setting time (min.)	1.2	1.25

[a] dry-basis

[b] moisture- and ash-free basis

[c] extracted at 85°C for 1 hr using conc. sulphuric acid and precipitated by acidified isopropyl alcohol from fruit harvested on 5 October

* not determined

a high yield of pectin but it was of low quality compared to that extracted at the lower temperature. Another factor which could have influenced the properties of the pectin isolates is the purification procedure (Potter, 1966). The pectin produced using the method of Crandall and Kesterton (1976) had high impurities, as shown by the high ash content and low anhydrogalacturonic acid content. Improvement in the purity of the pectin was done during the extraction from the fruit harvested in October and those used for the pilot plant scale production by using acidified isopropyl alcohol to remove metallic contaminants (Cole and Holton, 1942).

Yield of pectin was highest from the grapefruit harvested in June, the value decreasing towards the end of the season. This observed decrease in pectin yield throughout the season is in agreement with the results obtained by Sinclair and Joliffe (1958). They reported that pectic substances in the peel and pulp of Valencia oranges gradually decreased throughout the season. Ripening and maturing of fruit involves the breakdown of pectin to sugars and acids, and consequently, the quality and quantity of extracted pectin will depend upon the age and maturity of its source (Doesburg, 1965). Other studies reported that pectin yield in Valencia oranges (Gaddum, 1934) increased to a maximum at first and then declined, while in lemons (Rouse and Knorr, 1968, 1970) pectin yield increased with maturity with the exception of some lemon varieties. Observations from this study indicate that pectin recovery is highest during the early part of the season. However, the recovery yield was lower than the reported 30.17% yield from Duncan grapefruit using the same temperature and time of extraction (Crandall and Kesterton, 1976), although caution has to be exercised in comparing values since cultivars and method of extraction affect the values for pectin actually recovered.

The high ash value of the pectin extracted from the early-season grapefruit indicates the presence of

impurities. Ash content indicates the percentage of inorganic impurities, this value being used in the determination of pectin purity (Francis and Bell, 1975). Further washing of the precipitate during the August extraction using 70% isopropyl alcohol markedly reduced the ash content from 6.04 to 2.83%. Ash content was further reduced to 1.87% during the pectin extraction for October due to the use of acidified alcohol which removed the metallic contaminants (Cole and Holton, 1942). Precipitation in acidified alcohol and washing of the precipitate with 70% isopropyl alcohol at least three times was therefore found necessary in the production of pectin of greater purity.

A general increase in the equivalent weight of pectin was observed throughout the season. These values are used to calculate anhydrogalacturonic acid and degree of esterification (McCready, 1970) but in this study, they were not used due to differences in the methods employed.

The data on methoxyl content is of particular interest in view of its effect on the jellying power of pectin (Wilson, 1928; McCready, 1970). Luers and Lockmuller (1927) have presented considerable data showing that the jellying properties of pectin are approximately proportional to its methoxyl content. They found that pectins under 7.3% methoxyl will not gel, while those between 11-12% methoxyl have the best jellying properties. The present study has shown that the increasing methoxyl content throughout the season gave a corresponding decrease in the setting time. Results agree with the finding of Luers and Lockmuller (ibid.) although Myers and Baker (1929, 1934) reported that the methoxyl content is not a measure of jellying power. They stated that a decrease in jellying power is due to the depolymerization of the pectin molecule.

It can be seen that the extraction at lower temperature (85°C) caused an increase in the methoxyl content. Myers and Baker (1934) reported that the higher the temperature, the lower is the methoxyl content of the pectin. Prolonged heating at a high hydrogen-ion concentration causes the breaking down of larger molecules into smaller ones and the splitting-off of the methoxyl groups from the pectin molecule. Early-season grapefruit extractions produced pectins with low methoxyl content and poor gelling properties. This could probably be due to the high temperature combined with high pH used during the extraction. The required pH for extraction was not maintained throughout the heating period and this could have caused reduction in the methoxyl content. A need to control the pH and temperature is therefore recommended for the production of pectin with high methoxyl content.

Anhydrogalacturonic acid content gives a measure of the purity of the product (Francis and Bell, 1975). This value is an indication of the percentage of organic materials present, generally neutral polysaccharides. The increasing value for anhydrogalacturonic acid throughout the season was due mainly to the improved extraction and purification procedures. Francis and Bell (1975) stated the dry citrus pectin is usually around 83% anhydrogalacturonic and 10% methoxyl content. The pectin extracted from the fruits harvested on October is therefore of good quality, as reflected by the 82.25% anhydrogalacturonic acid and 9.16% methoxyl.

Pectin with the highest degree of esterification (DE) was obtained from the grapefruit harvested in August. The DE is an important factor which determines the setting time and solubility of pectins and depends on the uronic acid and methoxyl content of pectin (Schultz, 1965; Smit and Bryant, 1968; Mizote et al., 1975). Pectins with 70% DE or above are reported to form a jelly more rapidly or at a

higher temperature than those with a DE between 50 and 70% (McCready, 1970). Results of the study revealed, however, that the pectin with the highest DE (70.44%) did not have the shortest setting time. This is in agreement with the observations reported by Pilnik (1964) and Doesburg (unpub.) that citrus pectins do not show such a strong relationship between their DE and setting time. This somewhat different effect of DE of citrus pectins may be related to a slightly different distribution of esterified carboxyl groups over the pectin molecule. Heri (1962) reported that, in relation to the DE, apple pectins were found to be more homogeneous than citrus pectins.

Measurement of the viscosity of dilute solutions provides one of the simplest methods of obtaining information about the size and shape of macromolecules (Kragh, 1961). Values from Table XVIII indicate that an increase in the viscosity gives a corresponding increase in the molecular weight of the pectin molecule. Usually during polymerization of an organic compound, the viscosity increases as the size of the molecule increases. Doesburg (1965) reported that the greater the viscosity, the higher the molecular weight.

The pectin extracted at the lower temperature had a higher degree of polymerization, as indicated by the high values in intrinsic viscosity and molecular weight obtained from the fruits in October. Although pectin is a high carbohydrate polymer, its molecular weight is much below that of cellulose and starch (McCready, 1970). The number average molecular weight of pectins is reported to be between 10,000-400,000 (Doesburg, 1965). The molecular weights obtained from this study (40,425; 63,830 and 89,362) are within the range given for molecular weight of pectins. The low viscosity and molecular weight values of the pectins extracted from June, July and August grapefruit are due mainly to depolymerization. Depolymerization is more extensive at high temperatures, producing a pectin with low viscosity and jellying power (Myers and Baker, 1934).

The final test of pectin quality must always be a jelly test (Potter, 1966). Standard jellies with 65% total solids were prepared according to standard procedures for the determination of setting time. Pectin grade for the standard jelly was not determined due to the unavailability of the Exchange Ridgelimeter which is used for determining the firmness of pectin test-jellies. Setting time is the period immediately after the batch of jelly has been mixed to when the jelly at the top just begins to congeal. Rate of cooling, presence of metal ions, pH and solids content influence setting time and temperature (McCready, 1970). Usually high molecular weight pectins with 70% DE and above will be rapid-set while those with 50-60% DE will be slow-set.

The effect of the different properties on setting time is summarized in Fig. 7. An increase in ash content brought about a decrease in the setting time. Degree of esterification did not have a marked effect on the setting time. Citrus pectins do not show such a strong relationship between their setting time and degree of esterification due to a slight difference in esterified carboxyl group distribution over the pectin molecule and heterogeneity of the citrus pectin as compared to apple pectin (Pilnik, 1964; Doesburg, unpub.; Heri, 1962). Degree of polymerization greatly influenced the setting time; an increase in the value brought about a corresponding decrease in setting time. This observation supports the earlier finding by Doesburg and Grevers (1960) that there is a rather strong correlation between jellying power and degree of polymerization.

A high yield of pectin was obtained from the pilot-plant scale production utilizing grapefruit obtained from a nearby orchard. The yield is slightly higher than the 14.5% yield reported by Alexander and Sulebele (1980) for pectin extracted from Indian grapefruit but lower than the 22% reported by Rouse et al. (1964c, 1965b) for Silver Cluster grapefruit. Results of the analyses showed that the pectin

is of good quality as reflected by the 1.5% ash, 9.3% methoxyl, 89.0% anhydrogalacturonic acid, 59.2% DE, 2.8 intrinsic viscosity, 59,575 molecular weight and setting time of 1.2 minutes. The production of a good quality pectin has been largely determined by the method of extraction and purification procedures. Control of pH and temperature during extraction and the use of acidified isopropyl alcohol were found to significantly improve the pectin quality.

CHAPTER 7

CONCLUSIONS

Results of this study have shown that naringin accounts for most of the bitterness in grapefruit, with limonin being a secondary contributor. In calamansi juice, flavonoids do not contribute to the bitterness, only limonoids which were detected in juice with 5% crushed seeds. The incorporation of the fruit seeds during processing would therefore impart a bitter taste which would lower the quality and flavour of the product.

During the ripening of grapefruit, major changes in composition were an increase in pH, an increase in soluble solids to a maximum which declined slightly towards the end of the season, a continuous rise in the Brix/acid ratio, and a steady fall in acidity. Juice yield was not affected by ripening. Ascorbic acid remained relatively constant in the early- and mid-season grapefruit but decreased in the late-season fruit. Naringin values fluctuated throughout the season but were highest in the early-season fruit. Naringin content in the peel was not affected by the ripening of the fruit. Narirutin was detected in early-season fruit but disappeared later in the season. The limonoid content was observed to decrease with the advancing maturity of the fruit.

Grapefruit juice with bitterness below the organoleptically detectable level was prepared from the highly bitter fresh juice by simple treatment with PVP. PVP adsorbed naringin and limonin up to 78.09% and 17.51%, respectively, depending on the amount and reaction time with the adsorbent. The loss of ascorbic acid can be considered a drawback but this can be minimized by using the right amount and reaction time of PVP just sufficient to bring the concentration of these components below the organoleptically detectable level and by controlling atmospheric oxidation of the ascorbic acid.

The production of a good quality pectin was largely influenced by extraction and purification procedures. Extraction at 85°C for 1 h and the use of acidified isopropyl alcohol yielded pectin with a low ash content and high values for methoxyl, anhydrogalacturonic acid, intrinsic viscosity and molecular weight with a relatively good gelling property.

APPENDICES

1. Analysis of Variance (ANOVA) for the different properties presented in Section 4.2.

a. Total Soluble Solids

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	20.3161	2.9023	199.64**	3.50
Error	8	0.1163	0.0145375		
Total	15	20.4324			

b. Titratable Acidity

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	2.2952	0.3278857	327.8857**	3.50
Error	8	0.008	0.001		
Total	15	2.3032			

c. Brix:Acid Ratio

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	55.934	7.9905714	246.86**	3.50
Error	8	0.25895	0.0323687		
Total	15	56.19295			

** - highly significant

d. Ascorbic Acid

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	6	36.755	6.1258333	28.59**	3.87
Error	7	1.50	0.2142857		
Total	13	38.255			

e. Naringin (in juice)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	76,610.50	10,944.357	25.20**	3.50
Error	8	3,475.00	434.375		
Total	15	80,055.50			

f. Naringin (in peel)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	0.005575	0.000796428	$0.78^{n.s.}$	3.50
Error	8	0.0084	0.00105		
Total	15	0.013975			

** - highly significant
n.s. - not significant

g. Limonin (for unpasteurized juice)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	30.95	4.4214285	505.31**	3.50
Error	8	0.07	0.008		
Total	15	31.02			

h. Limonin (for pasteurized juice with normal pH)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	26.3894	3.7699142	4.31**	3.50
Error	8	7.005	0.875625		
Total	15	33.3944			

i. Limonin (for pasteurized juice with normal pH, after 48 h)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	35.8444	5.1206285	28.55**	3.50
Error	8	1.435	0.179375		
Total	15	37.2794			

** - highly significant

j. Limonin (for pasteurized juice with pH adjusted to 5.6)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	9.0075	1.2867857	7.30**	3.50
Error	8	1.41	0.17625		
Total	15	10.4175			

k. Limonin (for pasteurized juice with pH adjusted to 5.6, after 48 h)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	7	11.09938	1.5856257	5.43**	3.50
Error	8	2.335	0.291875		
Total	15	13.43438			

** - highly significant

2. ANOVA for the different properties presented in Section 5.2

a. Naringin (30 minutes)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	6	1,641,067.4	273,511.23	158.07**	3.22
Error	10	17,303.2	1,730.32		
Total	16	1,658,370.6			

b. Naringin (60 minutes)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	6	1,454,808.9	242,468.15	756.60**	3.22
Error	10	3,204.7	320.47		
Total	16	1,458,013.6			

c. Narirutin (30 minutes)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	6	70,375.89	11,729.315	104.73**	3.22
Error	10	1,120.00	112.00		
Total	16	71,495.89			

** - highly significant

d. Narirutin (60 minutes)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	6	43,393.06	7,232.18	80.00*	3.22
Error	10	904.00	90.4		
Total	16	44,297.06			

e. Limonin (30 minutes)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	5	4.72151	0.944302	$2.71^{n.s.}$	3.33
Error	10	3.40809	0.340809		
Total	15	8.1296			

f. Limonin (60 minutes)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	5	5.02269	1.004538	39.61**	3.33
Error	10	0.24934	0.024934		
Total	15	5.27203			

** - highly significant

n.s. - not significant

g. Ascorbic acid (30 minutes)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	5	103.392	20.6784	73.99**	4.39
Error	6	1.677	0.2795		
Total	11	105.069			

h. Ascorbic acid (60 minutes)

SV	d.f.	SS	MS	Fc	F_t (.05)
Treatment	5	125.931	25.1862	243.42**	4.39
Error	6	0.261	0.1035		
Total	11	126.552			

** - highly significant

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