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SOLUBILITY RELATIONSHIPS OF LIMONIN AND THE PHENOMENON OF DELAYED BITTERNESS IN CITRUS JUICES

A thesis presented in partial fulfilment
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ABSTRACT

Unsuccessful attempts were made to produce cloud-stable, non-bitter citrus juices by enzymic degradation of juice-soluble pectin. The failure of these attempts demonstrated the necessity for a greater understanding of the solubility relationships of limonin and the phenomenon of delayed bitterness.

The solubility of limonin in model solutions was investigated. It proved impossible to prepare aqueous solutions containing high concentrations of limonin without the use of heat. Even though the addition to such solutions of sugar, pectin and polygalacturonic acid at concentrations commonly found in citrus juices had a statistically significant (95% level) effect on the equilibrium limonin concentrations, the magnitude of the effect was quite small. Moreover, the solubility of limonin shaken at 30 C for five days in model solutions containing citrus proteins and lipids was no greater than its solubility in water, leading to the conclusion that neither lipids nor proteins are responsible for high concentrations of limonin in citrus juices. Direct solubilization of limonin cannot therefore account for high limonin concentrations in citrus juices to which no heat has been applied.

High concentrations of limonin in model solutions could be achieved by the application of heat. In refluxed aqueous solutions, the presence of pectin, sucrose and sucrose, glucose and fructose in combination increased the solubility of limonin, the greatest increase occurring with sucrose alone. Even more important was the effect of solutes on slowing down the rate at which limonin came out of solution on cooling. Again the effect of solutes was different, sucrose being more effective than pectin in holding limonin in solution, but high concentrations were maintained longer in solutions containing the sugar mixture or sucrose and pectin. Saturated solutions of limonin in acidic model solutions prepared under reflux deposited limonin with approximately equal rapidity, irrespective of other solutes.

On the other hand, solutions of similar limonin concentration, prepared by adding components of the model solutions (citric acid, pectin and sucrose at concentrations found in citrus juices) to a hot saturated solution of limonin, retained moderately high concentrations of limonin provided pectin was present.

Limonin analysis by extraction at two pH levels demonstrated that the equilibrium operating in the establishment of high concentrations of limonin in model solutions involved neutral hydrolysis of limonin to one or other of its hydroxyacid forms. On cooling such solutions, limonin crystallized out, affecting the hydroxyacid-lactone equilibrium. The effect of solutes is related to their influence on this equilibrium.

When similar extraction procedures were applied to studying the phenomenon of delayed bitterness in citrus juices, evidence was obtained for the presence in freshly extracted citrus juices of two limonin precursors which each showed differing stabilities, both to the presence of natural citrus enzymes and to the application of heat.

All previous work relating the effects of various treatments to limonoid bitterness in citrus juices and model solutions must be reconsidered in the light of the possible involvement of the two limonin precursors, the associated enzymes, and the hydroxyacid forms of limonin.

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CHAPTER ONE

INTRODUCTION

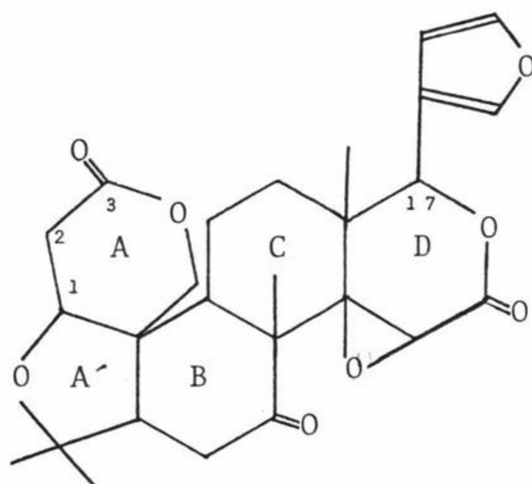
1.1. BITTERNESS IN CITRUS JUICES

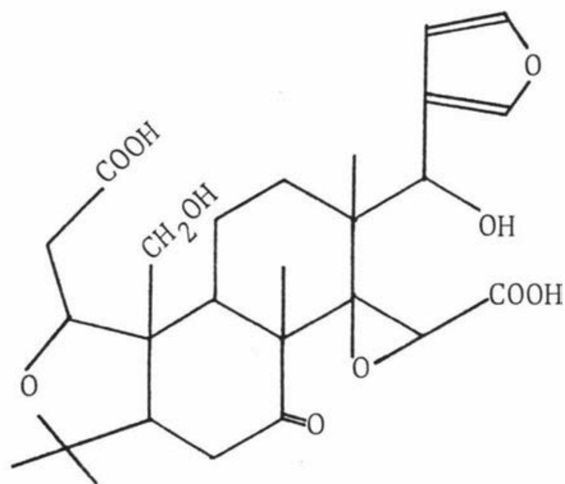
The intense and objectionable bitterness in citrus juices is due to the presence of limonin (I), a $C_{26}H_{30}O_8$ tetra-nortriterpenoid dilactone (Kefford and Chandler 1970; Maier *et al.* 1977). Bitterness appears slowly on standing at room temperature or more rapidly after a heat treatment, and this gradual development is sometimes referred to as delayed bitterness. The absence of bitterness in the intact fruit and the delay in the onset of bitterness after juicing differentiates limonin bitterness from that due to the comparatively water-soluble flavanone neohesper-idosides, such as the naringin of grapefruit, which are distributed throughout the fruit including the intact juice sacs. While most people find the bitterness due to flavanone neohesperidosides to be pleasantly attractive within limits, that due to limonin is regarded as objection-able and detrimental to juice quality (Chandler and Nicol 1975).

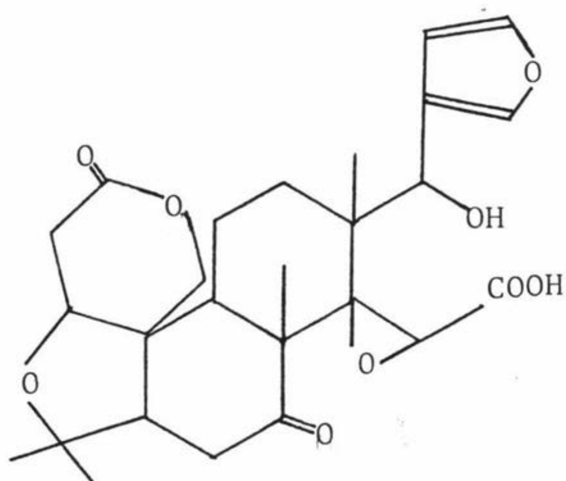
Although limonin appears to be ubiquitous in all citrus species (Dreyer 1966), it may not be present in sufficient quantity at maturity to cause delayed bitterness of the juice. Delayed bitterness is most noticeable in the juice of the Navel orange (of commercial significance in Australia, the U.S.A. and elsewhere) and the Shamouti orange (of commercial significance in Israel). Although not as obvious because of the presence of bitter flavanone neohesperidosides, delayed bitterness also occurs in the juice of the true grapefruit, the Natsudaidai orange (of commercial significance in Japan) and the New Zealand grapefruit (the most important variety of citrus processed in New Zealand).

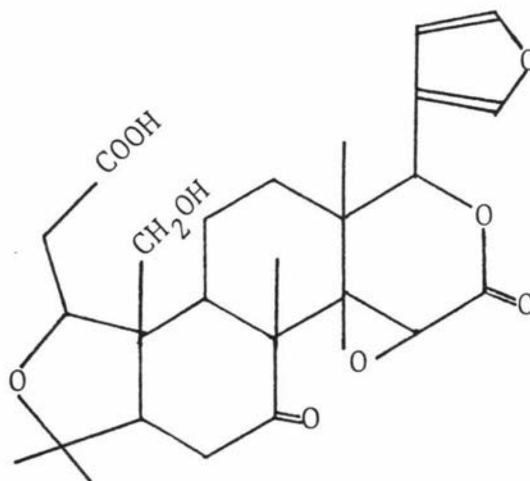
Studies on the problem of bitterness in citrus juices started from the earliest days of the development of the orange juice industry in California (Higby 1938). Although nowadays juice from the above citrus varieties can find consumer acceptance when it is blended with non-bitter juices, there is a limit to the amount of blending that can

Fig. 1 Structural formulae of limonin (I), limonin dihydroxy diacid (II), limonoic acid A-ring lactone (III) and limonoic acid D-ring lactone (IV)









be done. Moreover, with the worldwide expansion of the citrus processing industry, bitterness due to limonin still remains a serious economic problem, particularly in areas where non-bitter cultivars can not be grown. Consequently, bitterness in citrus juices continues to stimulate considerable research from a number of different approaches including the horticultural and technological. The chemistry and biochemistry of limonoids in general have been reviewed by Dreyer (1968) and Connolly *et al.* (1970), while the limonoid constituents of citrus have been reviewed by Kefford and Chandler (1970), Goodwin and Goad (1970) and Maier *et al.* (1977).

1.2. DELAYED BITTERNESS AND THE LIMONIN PRECURSOR

The phenomenon of delayed bitterness was explained by Higby (1941) in terms of a water-soluble, non-bitter precursor which on contact with the acid juice formed or released the bitter principle. He suggested it might be the salt of the dihydroxy diacid (II) (derived from the two lactone groups in limonin) or a glycosidic derivative.

The formation of the bitter substance in Navel orange juice was followed by Emerson (1948) who examined extractions with benzene 20 min after the juice was expressed, then after 4 h and again after standing overnight. The juice after the removal of benzene was not perceptibly bitter and the residues from the first and last extractions were only slightly bitter, the bulk of the bitter material being found in the second extract. Limonin was the only bitter substance isolated from the benzene extracts. Emerson concluded that the precursor was insoluble in benzene and that time was required for limonin formation.

In a later paper Emerson (1949) reported that 90% of the total limonin present in immature Valencia peel was preformed, only 10% being present as precursor limonin. In this paper he also studied the salt of the dihydroxy diacid (II) (hereafter referred to as the disalt) formed on

alkaline hydrolysis of the two lactone rings of limonin, which he found to be non-bitter, and showed that it became bitter in dilute acid solutions or in orange juice. He concluded, however, that this compound lactonized too slowly to be the precursor. He suggested that one of the monolactone acids (III and IV) or perhaps a derived glycoside might be the precursor and that enzymes might be involved in conversion of the precursor to limonin.

Samisch and Ganz (1950) observed delayed bitterness in both reamed juices and albedo suspensions dispersed in water. When the solid particles were removed immediately after extraction, bitterness failed to develop.

When reviewing the evidence available in 1959, Kefford explained the phenomenon of delayed bitterness on physical grounds in what came to be known as the diffusion theory. He argued that initially limonin was present in the juice as a constituent of the tissue fragments. Because of its low solubility, it took an appreciable time (unless heat was applied) for the limonin to diffuse from these particles and reach a concentration necessary for the juice to taste distinctly bitter. He cited the evidence of Samisch and Ganz (*ibid.*) as supporting the physical mechanism, together with the results of Chandler (1958) who had reported that when finely-divided limonin or limonin adsorbed on alumina was suspended in water acidified to pH 3.5, the liquor was not bitter after 8 h at room temperature, but became bitter on further standing or on heating.

In a later review (Kefford and Chandler 1970) it was suggested that Emerson's results with benzene extracts of Navel orange juice could be interpreted to mean that no limonin was present initially because it (or its precursor) had not had time to diffuse into the juice from the tissues. The difficulty in interpreting Emerson's experiments stemmed from the fact that the presence of the precursor was being inferred from indirect evidence.

No further publications on delayed bitterness emerged until 1968, some 19 years after Emerson had somewhat prophetically written that "in view of the sensitivity of the precursor to acid, and the possible presence of a lactone group sensitive to alkali, the isolation of the precursor might be a matter of considerable difficulty".

The necessary ingredients for direct confirmation of the presence of a precursor and proof of its identity emerged in the early 1960's when the structure of limonin was established following the collaborative efforts of four leading research teams (Arnott *et al.* 1960; Arigoni *et al.* 1960; Barton *et al.* 1961).

Evidence in support of the precursor theory was obtained by Maier and Beverly (1968) who reported the presence in albedo extracts of early season Navel oranges and Marsh grapefruit of a non-bitter limonin monolactone which was stable at the pH of the tissues (5.1) but was converted to limonin at the pH of the juice (3.0). They suggested that all the limonin was in the monolactone form in Navel orange tissues, contrary to the finding of Emerson (1949) that at least 90% of the limonin in Valencia peel was preformed limonin.

Maier and Margileth (1969) showed that only one of two possible limonin monolactones occurred naturally and identified it as limonoic acid A-ring lactone (LARL)(III) by comparison with the authentic compound using high-voltage paper electrophoresis and thin-layer chromatography (TLC) on microcrystalline cellulose.

During the work on LARL, Maier and Margileth (*ibid.*) observed that certain membrane extracts from Navel orange and Marsh and Ruby Red grapefruit carpellaries converted LARL to limonin more rapidly than other extracts. Evidence suggesting the presence of a lactonizing enzyme in the tissues was obtained by showing the formation of limonin from synthetic LARL when this substrate was mixed with an

unheated tissue extract. No limonin was formed when the extract was boiled before adding the substrate. Subsequently the lactonizing enzyme was isolated from orange seeds, purified and named limonin D-ring lactone hydrolase (Maier *et al.* 1969).

Levi *et al.* (1974a,b) determined the limonin content of Shamouti orange juice and aqueous extracts of endocarp and peel tissues, before and after heat treatment at 90 C for 5 min, and assumed that the difference would represent the LARL content of the unheated tissues or juice. They reported substantial amounts of LARL and small amounts of limonin in the tissues and about equal amounts in the juice.

Tariq *et al.* (1974) reported the limonin content of freshly extracted mature Valencia orange juice to be 2 - 3 ppm, rising to 11 - 13 ppm after pasteurization, while Di Giacomo *et al.* (1976) reported limonin contents of up to 7 ppm in freshly extracted juice from different varieties of orange grown in Italy, rising to 15 ppm after 13 days storage at 0 C.

The presence of an enzyme in the peel of Shamouti orange capable of degrading limonin monolactone so as to prevent its conversion to limonin was suggested by Flavian and Levi (1970). Chandler (1971a) demonstrated the presence in the albedo of Navel and Valencia oranges of a system, with the properties of an enzyme, which acted on orange extracts so as to reduce the amount of limonin formed on heating and suggested that it was, in effect, identical to the enzyme described by Flavian and Levi. In a subsequent paper (Nicol and Chandler 1978) detailing the optimum conditions for the extraction of this enzyme, it was referred to simply as the limonin precursor degrading (LPD) enzyme without specifically identifying its substrate, the substrate used for the assays being orange albedo.

That LARL was the primary limonoid of fruit tissues was suggested through the use of the enzyme limonoate dehydro-

genase (LD). LD enzymes isolated from bacteria (grown on the disalt as carbon source) by Hasegawa *et al.* (1972; 1974) specifically catalyse the dehydrogenation of the 17-hydroxyl group of limonoids to a keto group, i.e. only limonoids having an open D-ring (free 17-hydroxyl group) can be attacked. It was found that the direct addition of purified LD from *Pseudomonas* sp 321-18 to freshly expressed Navel orange juice resulted in the conversion of essentially all the limonoid present to 17-dehydrolimonoic acid A-ring lactone (Hasegawa *et al.* 1973; Brewster *et al.* 1976).

Following these studies, the suggestion that LARL was the only source of limonin in orange juice became so established that Hasegawa and Maier (1979) stated unequivocally that "limonin is present in citrus seeds but not in the fruit tissue". They had earlier (Maier *et al.* 1977) explained the findings by Levi *et al.* (*ibid.*) of equal amounts of limonin and LARL in freshly extracted juice by suggesting that since no precautions were taken to slow LARL conversion to limonin during preparation and solvent extraction of the juice, the limonin reported most likely came from LARL conversion to limonin after juice extraction.

1.3. SOLUBILITY RELATIONSHIPS OF LIMONIN

Although concentrations of limonin in freshly processed Navel orange juice can reach 35 ppm, preparation of intensely bitter solutions by refluxing limonin with water at a pH below 4.5 (to prevent hydrolysis of limonin to the non-bitter ion of its hydroxy acid form) proved unsuccessful (Chandler 1971b). In a series of experiments, Chandler found that sucrose and pectin alone and in combination substantially increased the solubilization of limonin in hot water. These substances also helped to hold limonin in solution after cooling, with pectin having a greater effect than sucrose. Unfortunately the solutions were filtered prior to analysis using celite (3 g/100 mL) which would have absorbed some of the limonin, resulting in lower levels

being reported in all solutions than would actually have been present prior to filtration. The results also indicated the inherent variability in the rate of crystallization of limonin from identically prepared solutions. For example, in one run the limonin concentrations in model solutions containing 0.1% pectin and 12% sucrose spin-cooked in cans and then stored for 1, 2 and 9 weeks were 60, 43 and 31 ppm respectively, while in another run they were 42, 39 and 31 ppm. Chandler concluded that the combined effect of pectin and sucrose was to create and maintain in the system conditions that may lead to high limonin concentrations.

A number of independent observations were cited to support the above conclusions. Cans of immature Navel orange juice which had been stored for about 15 years at 0 C had deposited hard, sandy crystals of practically pure limonin. It was suggested that the loss of solubility was due to the considerable degradation of the pectin by acid hydrolysis, since the juice failed to hold its pulp in suspension for more than a few seconds.

Russian workers, unaware that they were concerned with limonoid and not flavonoid bitter principles such as naringin, had reported the removal of bitterness from tangerine and other citrus products by the simultaneous addition of peroxide and peroxidase preparations from apples (Markh and Fel'dman 1949, 1950; Markh 1953). Chandler (*ibid.*) explained their results on the basis of pectic enzyme impurities in the crude peroxidase preparations.

Debittering of Navel orange products through the use of fungal and tomato pectic enzymes was observed by McColloch (1950) who noted that this treatment was associated with off-flavour development and clarification. He reasoned that debittering may have resulted from a disruption of the colloidal conditions that held limonin in solution, rather than to disruption of the limonin molecule itself.

A British patent (Hanson 1968) for the preparation of a

non-bitter Navel concentrate treated the extracted juice with an enzyme preparation high in pectinesterase and low in polygalacturonase activity. It was claimed that this procedure counteracted the formation of bitter substances as well as giving a juice of low viscosity which was easier to concentrate. Chandler (*ibid.*) suggested that the function of the pectic enzymes was not to counteract the formation of bitter substances but to decrease the solubility of limonin in the juice.

1.4. A POSSIBLE SOLUTION TO LIMONIN BITTERNESS WITH SIMULTANEOUS CLOUD CONTROL

It appears from the above that, but for the effect of pectin and sucrose in increasing the solubility of limonin in aqueous systems, there would be no such problem as limonin bitterness in citrus juices, and that it might be possible by modifying the pectin content of citrus juices to control their limonin content and consequently their bitterness. Also, since Florida workers (Baker and Bruemmer 1969) had suggested that the cloud in citrus juices could be stabilized by enzymic degradation of the juice-soluble pectin, rather than by the use of heat, it appeared possible that these two problems in citrus processing could be overcome by modification of the pectin content.

The problem of cloud instability in citrus juices arises as a result of the separation of the finely divided particulates normally held in suspension within a colloidal matrix in the fresh juices. Analyses have shown that this fine particulate material is composed almost exclusively of pectin, protein and lipid (Scott *et al.* 1965; Baker and Bruemmer 1969). When this unstable colloidal system collapses, the juice clarifies. Once converted to an unattractive two-phase system of a flocculant sediment in a clear serum, the juice is no longer marketable. In addition, the cloud, which will not remain suspended,

contains most of the characteristic citrus flavour and colour.

Heat is used commercially to stabilize citrus juices against cloud loss by inactivating pectinesterase (PE), an enzyme that initiates a series of reactions leading to clarification. PE demethylates juice-soluble pectin, converting it to low-methoxyl pectin, which reacts with polyvalent cations to form insoluble pectates. Presumably, the precipitation of these pectates occludes the cloud particles and removes them from suspension (Dietz and Rouse 1953).

The use of PE inhibitors to preserve cloud in citrus juices was studied by Kew and Veldhuis (1961) who were granted a patent which claimed that extracts from grape leaves could be used as such. Krop (1974) observed that orange PE was inhibited by pectic acid; similar observations have been made for the PE from alfalfa (Lineweaver and Ballou 1945), banana (Hultin *et al.* 1966) and tomato (Lee and MacMillan 1968). However, pectic acid cannot be used to stabilize cloud, since by itself it causes clarification in concentrations as low as 200 ppm (Krop and Pilnik 1974a).

For many years juice-soluble pectin was presumed to form a colloidal matrix that supported the particulates and was therefore necessary for cloud stability in citrus juices (Rouse and Atkins 1955). However, Baker and Bruemmer (1969) showed that soluble pectin was not itself necessary for cloud support, since a stable suspension of orange particulates could be made in water. Rather, in juices which had lost cloud stability, the pectin had been converted into the destabilizing low-methoxyl pectin. They therefore proposed, as an alternative to heat denaturation of PE for stabilizing orange juice cloud, the addition of a depolymerizing enzyme to degrade this pectin so as to eliminate its destabilizing effect.

Such a process would present advantages because the

temperature of around 90 C, required to inactivate the PE enzyme involved in loss of cloud stability, sometimes results in off-flavours in processed citrus juices (Bissett *et al.* 1953; Kew and Veldhuis 1960). The proposal was supported by the stability of orange juice particulates in centrifugally prepared orange juice serum treated with a commercial depolymerizing enzyme (Baker and Bruemmer *ibid.*). Subsequently they demonstrated that orange juice cloud could be stabilized by the addition of ppm levels of commercial depolymerizing enzymes to the juice (Baker and Bruemmer 1972).

Krop and Pilnik (1974b) showed that the cloud-stabilizing effect of a pure yeast polygalacturonase added to PE active juice was caused by a rapid depolymerization of the low-methoxyl pectin (a product of PE action) so that calcium pectate coagulation could not occur. They also confirmed (Krop and Pilnik 1974a) the presumed role of calcium ions in juice clarification: cloud stability was preserved, in spite of normal PE activity, when calcium ions were previously precipitated as calcium oxalate.

The proposal of Baker and Bruemmer (1972) to stabilize citrus juice cloud by the addition of a pectin-depolymerizing enzyme offered the prospect of simultaneously producing non-bitter, cloud-stable juices in products in which flavonoid bitterness was not a problem. The absence of pectin in the juice would be expected to prevent the solubilization of high concentrations of limonin and, as heat would not be necessary to stabilize the cloud, the flavour of the juice, if marketed fresh or chilled, would be expected to be superior to that of pasteurized juices.

To investigate this possibility, it was initially decided to establish the extent of degradation of the juice-soluble pectin required to produce a stable cloud since the earlier workers (Baker and Bruemmer 1972; Krop and Pilnik 1974b) had merely added depolymerases to degrade the pectin without determining what minimum chain length of pectic acid would

result in stability. Several months after this work had commenced, a paper by Termote *et al.* (1977) was published which demonstrated that the presence of hydrolysates of pectic acid with an average degree of polymerization of 8 to 15 would result in cloud stability since they were too small to cause juice clarification by forming insoluble pectates. As this paper effectively answered the question which the author's own investigations were attempting to solve, this aspect of the problem was not pursued further.

Instead, experiments were undertaken to follow changes in juice turbidity and in limonin and pectin contents of juices which had been subjected to various treatments including pasteurization and the addition of pectic enzymes. Such experiments would, it was hoped, provide valuable information as to the feasibility of producing cloud-stable, non-bitter citrus juices by enzymic degradation of pectin. As this work progressed, it became clear that the studies had shed striking new light on the solubility relationships of limonin, on the phenomenon of delayed bitterness development, and on the nature of the "limonin precursor". The results of this work and their implications are presented in this thesis.

CHAPTER TWO

EXPERIMENTAL METHODS

AND MATERIALS

2.1. MATERIALS

2.1.1. Fruit

In this study three varieties of citrus were used: Washington Navel oranges (*Citrus sinensis* (L) Osbeck) grown at the N.S.W. Department of Agriculture Station at Gosford, Australia, and New Zealand grapefruit (*C. grandis* x *C. reticulata*) and Carter Navel oranges (*Citrus sinensis* (L) Osbeck) both grown at the Massey University orchard, Palmerston North, New Zealand. All varieties were propagated on trifoliata orange (*Poncirus trifoliata*) rootstock. Fruit that was not processed immediately after harvest was stored at 5 C in a storeroom free of any other fruits or vegetables.

2.1.2. Juice

Juice was extracted from the citrus fruits using either a four cup commercial FMC juice extractor, or a domestic Kenwood mixer fitted with a reamer. After extraction the juices were passed through a pulper/finisher fitted with a 100 mesh screen to give a pulp content of approximately 10%.

2.1.3. Sera

For experiments in which only the serum was utilised, the juice was centrifuged at 800 x *g* for 10 min and the supernatant used. The remaining sediment was classified as pulp and, when required, resuspended in a model solution of sucrose (10%) and citric acid (1%) to simulate citrus juices.

2.1.4. Pasteurized Samples

When juices and sera were pasteurized, the criteria used was complete destruction of pectinesterase (PE). However, there are problems in checking that all the PE has been inactivated since the analytical methods available are not sufficiently sensitive to detect low concentrations of PE which can still cause cloud instability (Rothschild *et al.* 1975; Holland *et al.* 1976). Although Eagerman and Rouse (1976) reported that a 2D process (i.e. one achieving a

99% reduction in PE concentration) was sufficient to prevent cloud instability, their results were subsequently shown (Robertson 1978) to be incorrect. To ensure complete destruction of PE a 3.3D process is required (i.e. one achieving a 99.93% reduction in PE concentration) and, to obtain this result, all pasteurized juices in this study were heated to and maintained at 95 C for 5 min before being rapidly cooled. Juice which was not required immediately was, after being held at 95 C for 5 min, filled hot into lacquered cans, cooled and then stored at 2 C.

2.2. DETERMINATION OF COMMON JUICE PARAMETERS

2.2.1. Total Soluble Solids (TSS) Content

This was determined using an Abbe refractometer and expressed as degrees Brix (^{o}B) read directly from the instrument. All reported values were the mean of duplicate determinations.

2.2.2. Titratable Acidity

This was determined by titrating juice (20 mL) and distilled water (100 mL) with sodium hydroxide (0.02N) using phenol-phthalein (0.5% w/v) as an indicator. Results were expressed as grams of citric acid per 100 mL of juice and were the means of duplicate determinations.

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 using a combination electrode connected to either a Radiometer pH meter with analogue output or a Triac pH meter with digital output. The instruments were calibrated with buffers at pH 3.2 and 7.0 prior to measurements being made.

2.2.5. Pulp or Insoluble Solids Content

This was determined by centrifuging 50 mL of juice in a conical graduated centrifuge tube at 800 x *g* for 10 min. The volume of the precipitate was read and the pulp content expressed as a percentage relationship of this volume to the total juice volume.

2.3. DETERMINATION OF JUICE TURBIDITY

2.3.1. Background

The cloud stability of citrus juices is commonly determined by measuring the change in the turbidity of the supernatant phase of the sample after a period of time. Measurements of turbidity are based on the attenuation of a light beam passed through a sample in a cuvette as a result of scattering by the cloud particles present. In the 1950's colourimeters were frequently used and to enable comparisons of results obtained with various instruments, turbidity results were related to the amount of light scattered by standard aqueous suspensions of bentonite and expressed in g/L of bentonite. More recently, spectrophotometers have been used and it is now common (Lankveld 1973; Krop and Pilnik 1974) to express juice turbidity directly as the absorbance of the sample at 660 nm (A_{660}) with distilled water as the standard.

2.3.2. Procedure

Juice samples were mixed thoroughly by inverting the stoppered containers ten times. Aliquots (10 mL) were then withdrawn and placed in conical 15 mL centrifuge tubes. After centrifuging at 800 x *g* for 10 min, about 3 mL of supernatant was transferred into a 10 nm glass cuvette and the absorbance measured at 660 nm in a Varian UV-VIS spectrophotometer.

2.4. DETERMINATION OF CHANGES IN CLOUD PARTICLE SIZE

2.4.1. Background

The measurement of the size of small particles is based on the attenuation, due to scattering, of a light beam incident on a diluted dispersion of the particles.

A detailed outline of basic scattering theory was given by van de Hulst (1957). The most important parameters are the refractive index of the particle relative to that of the medium, the particle's radius or diameter, and the applied wavelength. Another useful parameter is p, a dimensionless parameter of particle size which, in physical terms, represents the phase lag suffered by a wave passing through the centre of the particle. In the region of anomalous diffraction encountered in fruit juice suspensions, Walstra (1964) showed that p was related to Q, the light-scattering quotient, by Equation 1:

$$Q = 2 - \frac{4}{p}\sin p - \frac{4}{p^2}(1 - \cos p) \qquad (1)$$

Later Lankveld (1970) showed that p could itself be calculated from the above mentioned four basic parameters of the suspension according to Equation 2:

$$p = 2\,\Pi\, d\,(n_s - n_c)\,/\,\lambda \qquad (2)$$

where d is the diameter of the particle; n_s is the refractive index of the suspended phase; n_d is the refractive index of the continuous phase; and λ is the applied wavelength.

Lankveld (1973) then used Equation 1 to calculate values of Q using values of p obtained from Equation 2 when there was only a 5% difference between the refractive indices of the suspended and continuous phases. The first maximum in the light scattering quotient occurred at about p = 4 which corresponded to a particle diameter of about 7 micron at λ = 660 nm and n_s/n_c = 1.05.

Lankveld (*ibid.*) also showed that, for a homodisperse suspension of rod-shaped particles, the following expression

held:

$$A = \log (I_o/I)$$

$$= \log_e (\Pi.d^2.Q.N.l) \qquad (3)$$

where A is the measured absorbance; I_o is the intensity of incident light; I is the intensity of transmitted light; d is the diameter of the particle; Q is the light-scattering quotient; N is the number of particles per unit volume; and l is the optical path-length. The absorbance was thus related to the natural logarithms of the optical path-length, the light-scattering quotient, the concentration of the particles and the projected area of the particles.

Although suspensions of fruit particles are very heterogeneous in size, shape and refractive index, Lankveld (1973) was later able to show for diluted (1:9) lemon juice concentrate (65°Brix) a linear relationship between the absorbance (A) and the optical path-length (l) as predicted by Equation 3. He was also able to correlate the relative mean particle size of samples to the ratio of absorbances measured at 600 nm for centrifuged and uncentrifuged samples (A_{600c}/A_{600u}). Similar correlations could be obtained if an uncentrifuged sample was measured at two wavelengths (A_{600u}/A_{430u}). Both a high value for A_{600c}/A_{600u} and a low value for A_{600u}/A_{430u} meant a low mean particle size. The correlation between the two sets of values for samples with a wide range of stabilities was quite good. The dependency of the absorbance on the ratio between the particle size and the wavelength of the light was used as a relative measure of cloud stability. As can be deduced from the above equations, the absorbance A varies not only with changes in the total scattering area ($\Pi d^2 N$) but also with changes in Q as a consequence of changes in particle size p resulting from flocculation.

Krop (1974) made measurements at 550 nm and 720 nm on centrifuged and uncentrifuged samples of orange juice prepared from reconstituted concentrate subjected to various

treatments. He used higher wavelengths than Lankveld (1973) since attenuation of the light beam due to absorption by the yellow pigments of the juice becomes significant at the lower wavelengths. He found that a plot of the ratio of A_{720u}/A_{550u} against storage time gave a horizontal straight line at a constant ratio of about 0.74. This he explained on the basis of the mean particle size in the uncentrifuged samples being so high that the light scattering quotient was not altered by changes in the particle size p due to coagulation. Such a finding does not invalidate Lankveld's work but can be explained by the different systems examined by the two workers. For example, Buslig and Carter (1974) subsequently showed that concentration of citrus juices caused a decrease in the number of particles in the range 1.4 - 3.5 micron and a large increase in the number of particles outside these limits.

When Krop plotted A_{720c}/A_{720u} against storage time, he obtained curves similar in shape to those obtained when A_{660c} was plotted against storage time. As a result all his subsequent studies on cloud stability were made using A_{660c} values. Because Lankveld (1973) was able to study coagulation and flocculation of cloud particles quantitatively using the ratios of absorbance values at different wavelengths for centrifuged and uncentrifuged juices, and because the present study was concerned with single-strength juice rather than juice which had been concentrated and then diluted, it was decided to adopt his approach.

2.4.2. Procedure

Juice samples were mixed thoroughly by inverting the containers ten times. An aliquot (1 mL) was transferred to a 10 mm glass cuvette, distilled water (2 mL) added, the sample mixed and the absorbance measured at both 720 nm and 550 nm in a Varian UV-VIS spectrophotometer. The measured values were multiplied by three. Another 10 mL sample was centrifuged at 800 x *g* for 10 min and the absorbance of the supernatant measured without dilution at 720 nm. Plots were made of A_{720c}/A_{720u} and A_{720u}/A_{550u}

against incubation time of the juice samples.

2.5. QUANTITATIVE DETERMINATION OF PECTIC SUBSTANCES

2.5.1. Background

The pectic substances are polyuronides, present in fruits as complex polymers of galacturonic acid or its methyl ester, with side chains of covalently-linked neutral sugars, typically arabinose, rhamnose and galactose (Pilnik and Voragen 1970). Three distinct classes of pectic substances have been recognised: protopectin, the water-insoluble parent pectic substance; pectinic acids or high-methoxyl pectins and pectinates; and pectic acids or low-methoxyl pectins and pectates. Analysis of pectic substances in plant material generally involves their fractionation into the above three classes by progressive extraction of the alcohol-insoluble solids by a series of solvents: water, a solution of a calcium-complexing agent such as ethylenediaminetetraacetic acid (EDTA) or ammonium oxalate; and cold alkali (Rouse and Atkins 1955). Single extraction procedures have also been reported; one employs a solution of EDTA, followed by alkaline de-esterification and enzymic hydrolysis of the pectin in this extract (McCready and McComb 1952), while another treats the sample with acidified ethanol to remove the cations and alcohol-soluble substances, followed by an acetone wash (Gee *et al.* 1958) to give an extract ready for analysis.

The uronide content of the extracts may be determined by a number of different methods, including estimation of carbon dioxide following decarboxylation, and colourimetric assays following treatment with chromogenic agents. The literature on the analysis of uronic acids by decarboxylation was reviewed by Anderson (1959) who described a simplified apparatus having significant advantages over earlier designs. Further improvements to this apparatus have recently been reported (Whyte and Englar 1974; Castagne and Siddiqui (1975). Colourimetric assays have been described for the quantitative determination of uronic acids using reactions

with carbazole (Dische 1947a) modified by McComb and McCready (1952), Bitter and Muir (1962), Galambos (1967), Berlepsch (1969) and Sarris *et al.* (1975); anthrone (Helbert and Brown 1956, 1957); napthoresorcinol (Almandinger *et al.* 1954); thioglycollic acid (Dische 1947b); cysteine (Dische 1948); 3-hydroxybiphenyl (Blumenkrantz and Asboe-Hansen 1973); and harmine (Wardi *et al.* 1974). Methods have also been reported based on titration (Doesburg 1957; Warren and Woodham 1973), calcium pectate formation (Holt 1954), automated anion-exchange chromatography (Spiro 1977) and ultraviolet estimation of the degradation products resulting from heating with concentrated sulphuric acid (Bath 1958; Katz and Tacker 1972).

Since colourimetric methods are generally simpler and more rapid than others, first consideration was given to such methods, and particularly to the most widely used method, that involving carbazole. However, it has long been known (Dische 1947) that use of the carbazole reaction for the quantitative determination of uronic acids has the disadvantage that neutral sugars interfere with its specificity because the reagent does not differentiate between uronic acids and hexoses (Dische *ibid.*) or dextrins (Joslyn and Chen 1967). Furthermore, in a long series of analyses, the time dependence of the colour development using carbazole necessitates the application of a factor to correct the absorbance to a constant time (Dekker and Richards 1972).

On the other hand, a recently introduced chromogenic agent 3-hydroxybiphenyl is specific for uronic acids and shows no reaction with even relatively high concentrations of neutral sugars (Blumenkrantz and Asboe-Hansen 1973). Therefore, a procedure which used 3-hydroxybiphenyl for the colourimetric assay was adopted for the determination of pectic substances in citrus juices. For comparative purposes, some juices were assayed using carbazole as well as 3-hydroxybiphenyl.

2.5.2. Procedures

2.5.2.1. Reagents

All reagents were Analar grade unless stated otherwise.
3-Hydroxybiphenyl solution: a solution (0.15%) of 3-hydroxybiphenyl (*m*-phenylphenol, Eastman Organic Chemicals) in sodium hydroxide (0.5%) was prepared and stored in a brown glass container wrapped in aluminium foil at 2 C. This solution was replaced every 2 months.
Sulphuric acid/sodium tetraborate solution: a solution (0.0125M) of sodium tetraborate in concentrated sulphuric acid was prepared using overnight stirring to ensure complete solution.
Carbazole solution: a solution (0.1%) was prepared by dissolving reagent grade carbazole in absolute ethanol.

2.5.2.2. Extraction

Two 16 g quantities of juice were weighed into 50 mL graduated conical-bottom centrifuge tubes. Hot (75 C) 95% ethanol was added to a volume of 40 mL and the mixture heated for 10 min in a water bath at 85 C, with occasional stirring using a glass rod. The volume of the mixture was made up to 45 mL and the tubes were centrifuged at 1000 x *g* for 15 min. After discarding the supernatant solution, the leaching was repeated with hot 63% alcohol for 10 min at 85 C.

The pellet remaining after decanting off the supernatant from the final centrifugation was dispersed in approximately 5 mL of distilled water and the volume made up to 35 mL with distilled water. The tube contents were then agitated for 10 min by bubbling air through it from a capillary. The tube was centrifuged at 1000 x *g* for 15 min and the liquid decanted into a 100 mL volumetric flask. The extraction procedure was then repeated with the residue and 1 N sodium hydroxide (5 mL) was added to the combined extracts. After making up to volume with distilled water and mixing, the solution was allowed to stand for 15 min before beginning the colourimetric procedure.

The residue in the centrifuge tube was dispersed in 0.75%

ammonium oxalate solution (5 mL). A similar procedure to that outlined above was then followed, with ammonium oxalate solution taking the place of distilled water.

The residue remaining in the centrifuge tube after the double oxalate extraction was washed into a 100 mL volumetric flask, 1 N sodium hydroxide (5 mL) was added, and the contents were made up to volume with distilled water. After mixing, the solution was allowed to stand 15 min with occasional shaking before filtering through Whatman No. 1 paper.

Aliquots were taken from each of the above three extracts for analysis by the colourimetric method.

Standard solutions (1.0 mL) containing 0 to 80 µg galacturonic acid (Sigma) or polygalacturonic acid (Sigma Grade III), which had been dried 5 h in a vacuum oven at 30 C, were analysed by the same colourimetric method.

2.5.2.3. Colourimetric method

3-Hydroxybiphenyl: an aliquot of each extract was mixed with distilled water in three test tubes (20 x 150 mm) to a total volume of 1.0 mL, the actual proportions depending on the anhydrogalacturonic acid content of the extract. To each tube was added sulphuric acid/tetraborate solution (5 mL) and immediately after mixing the tubes were placed in a water-ice bath. They were then heated in a boiling water bath for 6 min and immediately cooled again in a water-ice bath. To two of the three tubes 3-hydroxybiphenyl reagent (100 µL) was added, while to the third tube was added 0.5% sodium hydroxide (100 µL). This tube acted as a blank to correct for the slight pink colour produced when neutral sugar-containing materials are heated in sulphuric acid/tetraborate solution. After mixing the tubes were left to stand for 15 min before reading the absorbance at 520 nm in a Spectronic 20 spectrophotometer.

Carbazole: aliquots of each extract and distilled water were added to each of three test tubes (20 x 150 mm) to a total volume of 1.0 mL, the actual proportion depending on

the anhydrogalacturonic acid content of the extract. To two of the tubes was added carbazole solution (0.5 mL), while absolute ethanol (0.5 mL) was added to the third tube. A white flocculent precipitate formed in the sample tubes. To each tube sulphuric acid/tetraborate solution (5 mL) was added and immediately after mixing the tubes were placed in a water-ice bath. The tubes were subsequently heated in a boiling water bath for 6 min and immediately cooled again in a water-ice bath. After 30 min the absorbance of the solutions was read at 525 nm in a Spectronic 20 spectrophotometer.

It was found that three samples of juice could be analysed in duplicate for each class of pectic substance in 8 h.

2.5.3. Reliability of method

A New Zealand grapefruit juice was analysed according to the above procedure. Six samples of the juice were fractionally extracted and duplicate analyses made of each extract. Chromogens were formed using both 3-hydroxybiphenyl and carbazole.

The results of the analysis of the pectic substances in the grapefruit juice are shown in Table I. They are of a similar magnitude to those reported by Rouse *et al.* (1958).

There is no significant difference between the results obtained using 3-hydroxybiphenyl or carbazole as the chromogenic reagent. However, because 3-hydroxybiphenyl resulted in a smaller standard error and a developed colour stable over 8 h, it was used to determine the pectic substances in citrus juices in this work.

Table I Pectin analyses for a New Zealand grapefruit juice using two chromogenic reagents

Chromogenic reagent	Pectin as anhydrogalacturonic acid (mg/100 g juice)			
	Soluble in: Water	Oxalate	Hydroxide	TOTAL
3-Hydroxybiphenyl				
Mean of 6 determinations	54	31	5	90
Standard error	2	2	1	
Carbazole				
Mean of 6 determinations	53	30	7	90
Standard error	3	3	1	

2.6. DETERMINATION OF PECTIC ENZYMES

2.6.1. Background

Pectic enzymes may be divided into two main groups: the de-esterifying enzymes or pectinesterases (PE) and the depolymerizing enzymes (Pilnik and Voragen 1970). The depolymerizing enzymes can be further classified into eight sub-groups according to the following criteria: hydrolytic or transeliminative cleavage of glycosidic bonds; endo or exo mechanism of the splitting reaction; and preference for pectin or pectic acid as the substrate (Kulp 1975). Those depolymerases which cleave the glycosidic bonds hydrolytically are known as polygalacturonases (PG) while those that cleave them transeliminatively are known as lyases. While evidence for the existence of a PE in citrus juices is unequivocal, it is now considered that depolymerizing enzymes do not occur in citrus juices (Pilnik and Voragen *ibid.*; Robertson 1976). However, both types of

enzymes were involved in this study since the PE naturally present in the juices was inactivated by heat in some treatments while commercial depolymerizing enzymes from microbial sources, containing principally PG, were added to the juices in others. Therefore, assay procedures for both PE and PG were required.

2.6.2. Pectinesterase

The method of Robertson (1976) was used. Briefly, it measured the amount of alkali required to keep a synthetic pectin substrate at pH 7.5 when a sample of juice was added to it. This determined the carboxyl groups freed by enzyme action per unit of time. PE activity was expressed in milliequivalents of ester hydrolysed per minute per gram of soluble solids in the sample. Each determination was carried out in duplicate.

2.6.3. Polygalacturonase

The method of Vas *et al.* (1967) was used. Briefly, it measured the flow-time of a reaction mixture in a Cannon-Fenske viscometer at frequent intervals (typically every 2 min) for about 12 min. The reaction mixture consisted of 1% polygalacturonic acid (Sigma) (6 mL) and a 0.1% aqueous solution of enzyme (0.1 mL). The viscometers were placed in a water bath (Tamson TXV40) maintained at 40 ± 0.01 C. From the flow times (t), the specific viscosities $(t-t_o)/t_o$ were calculated, where t_o was the flow time of distilled water. When these were plotted as a percentage of the initial specific viscosity against the logarithm of the corresponding flow time, a straight line was obtained from which, by interpolation, the time required for a definite (25%) change in specific viscosity was obtained. One unit of PG was defined as the amount of enzyme required for this reduction in viscosity to occur in 100 min. Each determination was carried out in triplicate and the mean values used to calculate the specific viscosities and the corresponding flow times.

2.7. QUANTITATIVE ANALYSIS OF LIMONIN

2.7.1. Background

Since the concentration of limonin in most samples to be analysed was low and many other substances could have been present, an assay method which was both sensitive and specific was required. A usual first step is extraction with a solvent to separate limonin from more hydrophilic material. Further purification can then be achieved by some type of chromatography.

Although assay methods have been published utilizing spectrophotometry (Wilson and Crutchfield 1968; Nomura and Santo 1965), gas chromatography (Kruger and Colter 1972; Basker *et al.* 1973), fluorometry (Fisher 1973) and high-performance liquid chromatography (Fisher 1975), they have not been widely adopted by researchers for the routine determination of limonin. Instead, methods involving thin-layer chromatography (TLC) have been used.

The first TLC method for the quantitative analysis of limonin, developed by Chandler and Kefford (1966), involved extraction with chloroform-ethanol containing BHA to prevent oxidation of the limonin, conversion to the dinitrophenylhydrazone, separation by TLC, elution, measurement of the UV absorption of the eluate, and comparison with that of eluates from standard spots. Specificity was achieved by the extraction, TLC and detection steps.

Maier and Grant (1970) developed a method involving extraction with chloroform, separation by TLC and formation of orange-brown spots with Ehrlich's reagent which Dreyer (1965) reported constituted good evidence for the presence of a furan ring in the substrate. The limonin concentration was estimated by visual comparison with co-chromatographed limonin standards on the TLC plate. On chromatograms of orange juice extracts, a yellow spot slightly behind but overlapping the limonin spot was present. This spot faded within 2 h when the plate was placed in the draft of a fume hood out of direct light. Although other types of compounds

also react with Ehrlich's reagent, the limonoid colour was characteristic enough that it could readily be differentiated (Maier *et al.* 1977).

Another method using quantitation on TLC plates was reported by Chandler (1971c). After extraction with chloroform-ethanol, the extract was treated with saturated sodium tetraborate to remove sugars and glycosides. After TLC of the extract along with limonin standards, the spots were revealed by exposure to bromine vapour and spraying with Tollen's reagent (specific for the furan ring) and were measured by densitometry. When this method was applied to orange juice, five compounds reacting to the bromine-silver nitrate treatment were encountered. While one of these was positively identified as limonin, the other four spots were similar to limonin in colour and in the time they took to appear. It was suggested that these four spots were furanoids and, by implication limonoids, since the only similarly reacting compounds known to be present in citrus fruits were the comparatively soluble furano-coumarins which had much higher R_f values than the limonoids. The identification of the four spots was not made.

Tatum and Berry (1973) reported a TLC method for estimating the limonin content of citrus juices which involved direct spotting of the juice onto the TLC plate. After development, the limonin spots were visualised by spraying with either 10% sulphuric acid in ethanol or 2% sulphuric acid and 1% *p*-dimethylaminobenzaldehyde in ethanol. The concentration of the limonin was estimated by visual comparison with standard co-chromatographed limonin.

After critically considering the above TLC methods, a modification of the 1971 Chandler method was developed which was more rapid, required a smaller sample of juice, and was less dependent on operator skill. This last factor was an important one since attempts to visualise TLC plates with bromine vapour and Tollen's reagent frequently ended in failure.

2.7.2. Preparation of extract

The juice sample (20 mL) was shaken for 20 min with chloroform (10 mL), ethanol (10 mL), ammonium sulphate (5 g) and butylated hydroxyanisole (0.5 mg) in a centrifuge bottle containing a stopper which was impervious to chloroform. The mixture was centrifuged for 10 min 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 rapid addition of approximately 25 mL chloroform to the extract immediately prior to filtration was found to improve considerably the effectiveness of the separation of the organic and aqueous phases. The residue after evaporation to dryness was dissolved in chloroform and the solution transferred to a 5 mL volumetric flask and made up to volume for quantitative TLC with washings of the residue using the same solvent; for juices having less than 10 ppm limonin a 2 mL volumetric flask was used.

For the experiments involving model solutions of limonin, the following sampling procedure was used. A clean, dry pipette was used to sample each flask. A rubber stopper was fitted to the lower portion of the pipette, and a length of glass tubing fitted over the stopper. A sheet of filter paper (Whatman No. 541) was then fastened around the end and lower sides of the glass tube and held in place with a rubber band. This procedure was adopted to prevent any limonin crystals from passing into the pipette during sampling. A sample of solution was then withdrawn and transferred into a glass centrifuge tube. After drying the outside of the pipette, the index finger was placed over the end and the pipette filled with chloroform:ethanol:BHA (1:1:0.005%). This procedure was adopted to recover any limonin that may have crystallized on the walls of the pipette during the withdrawal process. The pipette was then drained into the centrifuge tube. After the addition of ammonium sulphate, the extraction procedure was as described above except that the step involving the addition of saturated sodium tetraborate was omitted.

2.7.3. Chromatography

Commercial pre-coated glass plates with a 0.25 mm layer of Silica Gel G (Merck) were used as received from the supplier without any activation. Aliquots (10 µL) of eight limonin-containing extracts were spotted down, together with four reference samples (each of 10 µL) from chloroform solutions containing 0.01 and 0.02% (w/v) limonin and 0.005% BHA. A 25 µL syringe with a needle cut at right angles was used for spotting down to avoid damaging the surface of the plates. The plates were developed twice for juice samples and once for those from model solutions with benzene:ethyl acetate:acetic anhydride (50:50:2) in equilibrated tanks. The solvent front was allowed to move to a height of 16.5 cm each time, with a single developing time of about 50 min. Duplicate spots were made from the same extract on separate plates.

2.7.4. Visualization of spots

After chromatography, the plates were air-dried at room temperature, sprayed evenly with concentrated sulphuric acid in ethanol (25 mL; 1:10), and then placed in an oven at 110 C for 8 min. The limonin spots appeared as dark brown areas on an almost white background.

2.7.5. Densitometric measurements

For the analyses carried out at CSIRO Food Research Laboratories, Sydney, the densities of the spots were measured from the reflectance of white light as recorded by a Schoeffel SD2000 Scanning Spectrodensitometer operating in a single beam mode. The plate was held on a motor-driven stage and moved at a speed of 3 cm/min under a light beam which scanned a lane carrying a limonin spot. The signal at any instant was recorded on a Type 3046 Yew Laboratory Recorder, operating at 6 cm/min. From the curve so obtained for each of the limonin spots on the plate, the height of each peak was measured, initial trials having indicated that peak height rather than peak area gave a more consistent estimate of spot density.

For the analyses carried out at Massey University, Palmerston

North, the densities of the spots were measured from the transmittance of yellow light as recorded by a Canalco Scanner Model G. Other operating parameters were as for the Schoeffel Spectrodensitometer. To determine the spot density, the curve obtained on the recorder paper for each of the limonin spots on the plate was cut out and weighed, initial trials having indicated that this method rather than one based on measurement of peak height or area gave a more consistent estimate of spot density.

2.7.6. Determination of limonin content

The absolute amount (in μg) of limonin (L_x) in a spot which on application of the above procedure gave an average peak height or peak weight of P_x was calculated from the expression:

$$L_x = \frac{(P_x - P_2 - 2P_1)}{P_2 - P_1}$$

where P_1 is the average height or weight of the peaks from the 0.01% limonin standard; and P_2 is the average height or weight of the peaks from 0.02% limonin standard. The absolute amount of limonin in a spot was converted to the concentration of limonin (in ppm) in the juice or model solution by multiplying by the relevant factor as follows:

Sample volume of juice or solution	Capacity of volumetric flask	Multiplying factor
20 mL	5 mL	25
20 mL	2 mL	10
10 mL	5 mL	50
10 mL	2 mL	20

2.7.7. Reliability of method

The results obtained when the above procedure was applied to two Navel orange juices (J1 and J2) are shown in Table II. Three samples of each juice (A, B and C) were extracted and each extract was spotted once onto two different TLC plates.

Table II TLC-Densitometric determination of limonin in Navel orange juice samples

Sample code	Limonin content (ppm)		Mean	Average of means ± SD
J1A	32.1	29.9	31.0	
J1B	30.8	34.7	32.8	31.4 ± 1.9
J1C	31.5	29.4	30.5	
J2A	15.8	14.5	15.2	
J2B	15.2	14.0	14.6	14.9 ± 0.7
J2C	14.5	15.4	15.0	

From the results in Table II it was concluded that for juices having a limonin content greater than 30 ppm, a difference greater than 3.8 ppm in the mean limonin contents from duplicate analyses was a real difference and was not due to experimental error. Likewise, for juices having a limonin content of 15 ppm, a difference in mean limonin contents greater than 1.4 ppm was considered to be a real difference. By extrapolation from these results, in analyses of juices containing 10, 20 and 25 ppm limonin, respective differences of 0.6, 2.2 and 2.9 ppm were considered to be significantly (95% confidence limits) different. The above ranges apply to juices; greater accuracy would be expected in model solutions containing limonin.

Examination of the plates obtained when extracts of Navel orange juice were spotted and treated as outlined above revealed the presence of three yellow spots adjacent to the limonin, with one spot very close to the baseline side of the limonin spot. These spots were not visible until the plates were sprayed. In the case of extracts of New Zealand grapefruit juice only two yellow spots were visible adjacent to the limonin spot.

Using the above TLC procedure, samples of the limonoids

nomilin, obacunone, deacetylnomilin, limonilic acid, limonexic acid and rutaevin could be easily detected as dark brown spots and all had R_f's sufficiently different from that of limonin to avoid interference. In all samples of citrus juices examined, a purple spot appeared with an R_f of 0.32 (limonin R_f 0.53). There was no evidence to suggest that either the purple or the yellow spots were limonoids and they were not considered further. Furthermore, on many TLC plates of Navel orange and New Zealand grapefruit juice extracts, there were up to 3 dark brown spots similar in appearance to limonin. They did not interfere with the limonin assay and no attempt was made to identify them.

CHAPTER THREE

EFFECT OF VARIOUS TREATMENTS ON CITRUS JUICES

3.1. CHANGES IN JUICE TURBIDITY AND LIMONIN CONTENT WITH TIME

3.1.1. Experimental

Fresh Navel orange juice from the FMC extractor was divided into two equal portions. One portion was pasteurized to inactivate the cloud-destabilizing enzyme pectinesterase (PE). Each portion was then halved; to one half, sodium metabisulphite was added to give an SO_2 level of 1000 ppm, while to the other 1000 ppm of benzoic acid was added. The usual parameters used to characterize citrus juices were measured. The four solutions were then placed in flasks and kept in a stationary water bath at 30 C. The limonin content of the upper layer of the juices was measured over a period of months, as was the turbidity of the serum and the juice.

3.1.2. Results

The pasteurized samples of Navel orange juice used in this trial had a pH of 3.0; a soluble solids content of $12.2^{o}B$; a titratable acidity of 1.91 g citric acid/100 mL; a Brix:acid ratio of 6.4:1; and a pulp content of 17%. Other results are summarized in the Tables III and IV.

3.1.3. Discussion

Reference to Table III shows that the unpasteurized samples lost their cloud rapidly. Pasteurized juices retained considerable cloud even at the end of the holding period, while the unpasteurized juices preservatized with sulphite or benzoate were virtually clear after 9 and 48 days respectively. The difference in the rate of clarification between the two unpasteurized juices can be explained on the basis of enzyme activation since Edwards and Joslyn (1952) reported that sodium bisulphite increased PE activity in citrus juices.

In both the pasteurized and unpasteurized juices there was a significant decline in limonin concentration with time (Table IV). After 70 days the pasteurized and unpasteurized juices held half and one third their initial limonin content.

Table III Changes in turbidity (A_{660c}) of Navel orange juices held at 30 C in the presence of sulphite or benzoate

Holding period (days)	Pasteurized				Unpasteurized			
	Sulphite		Benzoate		Sulphite		Benzoate	
	Serum	Juice	Serum	Juice	Serum	Juice	Serum	Juice
0	1.195	1.232	1.196	1.232	0.570	0.517	0.775	0.669
5	0.930	1.439	0.986	1.509	0.089	0.068	0.382	0.601
9	0.993	1.481	0.961	1.489	0.013	0.051	0.306	0.540
13	0.946	1.492	0.876	1.545	0.007	0.045	0.242	0.483
20	0.869	1.437	0.874	1.534	0.013	0.035	0.242	0.387
27	0.770	1.501	0.856	1.632	0.007	0.028	0.179	0.214
48	0.592	1.424	0.651	1.645	0.000	0.026	0.027	0.067

Table IV Changes in limonin content (ppm) of Navel orange juices held at 30 C in the presence of sulphite or benzoate

Holding period (days)	Pasteurized		Unpasteurized	
	Sulphite	Benzoate	Sulphite	Benzoate
0	32	32	n.d.	n.d.
5	31	30	30	32
13	22	25	21	25
20	25	21	19	24
27	23	16	24	19
37	28	17	17	18
48	24	23	16	16
70	19	17	10	10
157	17	17	10	9

n.d. = not determined

There was no significant difference between juices preservatized with benzoic acid and those preservatized with sulphite. The fact that the greater decline in limonin content was observed in the unpasteurized juices suggests that it could have been due to the presence of a limonin precursor degrading (LPD) enzyme in the unpasteurized juices (Nicol and Chandler 1978). On the other hand, the cloud-destabilizing effect of PE could also have contributed to the limonin decrease.

However, despite the very rapid clarification of the sulphited unpasteurized sample, there was a considerable lag before the limonin content decreased. Furthermore, in pasteurized juices which retained relatively stable cloud, the limonin content still declined by 50%. It is not possible to draw any firm conclusions from this experiment regarding the influence of cloud particulates on limonin levels in citrus juices, except that rapid loss of cloud is not associated with a rapid fall in limonin content.

Several other points arise from Table III; in particular the marked changes in turbidity of the serum from the pasteurized juices compared to the relatively constant turbidity for the juice samples. The lower turbidities in the sera from pasteurized juices indicate that even in the absence of PE changes do take place in the cloud. This could be as a result of acid hydrolysis of the pectin at 30 C, leading to a lowering of juice viscosity and a decrease in the size of the particles which could be supported in suspension as cloud. If the pectin was being hydrolysed in the pasteurized juices, then this would support the suggestion of Chandler (1971b) that limonin had crystallized out of 15 year old juice because the pectin had been hydrolysed and the cloud destabilized.

Therefore it was decided to conduct a further trial in which the changes in the pectic substances would be monitored as well as changes in limonin content and juice turbidity. In addition, enzymes would be used to degrade the pectin in

an attempt to stabilize the cloud without inactivating PE by heat.

3.2. CHANGES IN LIMONIN CONTENT, JUICE TURBIDITY, CLOUD PARTICLE SIZE AND PECTIC SUBSTANCES WITH TIME

3.2.1. Experimental

Fresh Navel orange juice from the FMC extractor was divided into two portions. One portion was then pasteurized to inactivate the PE. Each portion was further subdivided into six flasks and sodium metabisulphite was added to give an SO_2 level of 350 ppm. Two flasks were treated as controls, two flasks were treated with Irgazyme M10 (100 ppm) and the remaining two with Ultrazym 100 (200 ppm).

The enzymes Irgazyme M10 and Ultrazym 100, supplied by Ciba-Geigy, Basel, Switzerland, were previously assayed for their pectin-hydrolysing activity. The levels of these enzymes added to the juices were chosen to give pectin-hydrolysing activities similar to those recommended by Baker and Bruemmer (1972) as sufficient to stabilize the cloud in unpasteurized citrus juices.

One flask from each of the above pairs was placed in a stationary water bath maintained at 30 C, while the remaining flasks were placed in a chill room at 7 C. The juices were analysed periodically for limonin content, juice turbidity, cloud particle size and the concentration of pectic substances. The usual parameters used to characterize citrus juices were measured.

3.2.2. Results

The unpasteurized juice had a pH of 3.6; a soluble solids content of $13.3^{o}B$; a titratable acidity of 1.33 g citric acid/100 mL; a Brix:acid ratio of 10.7:1; a pulp content of 19%; and a PE content of 73.8×10^{-4} units/g soluble solids. The corresponding values for the pasteurized juices were 3.6; 12.9; 1.24; 9.66:1; 11%; and zero.

Changes in the limonin content of the juices are presented in Tables V and VI. Up to and including day 42, the limonin content of the upper layer of the unshaken juice was determined, while on day 62 the limonin content of the whole juice after shaking was determined. Changes in juice turbidity and cloud particle size are summarized in Tables VII and VIII, while changes in the pectic substances of the juices during storage are presented in Tables IX and X.

3.2.3. Discussion

3.2.3.1. Limonin contents

Examination of the results in Table V in the light of the reliability of the method for limonin determination (see Table II) reveals that there were significant differences between certain successive limonin determinations. Less apparent from Table V is any definite trend in the limonin content of the juices. However, if it is assumed for the purposes of discussion that the holding temperature and the addition of pectic enzymes did not affect the limonin content of the juices (i.e. that the only factor influencing limonin content during storage was whether or not the juice had been pasteurized), the mean values presented in Table VI can be calculated.

Two points emerge from Table VI. The first is that the limonin content of both the pasteurized and unpasteurized juices increased during storage. The second is that, when the range of each mean is taken into consideration, there is no significant difference between the mean values for the pasteurized and unpasteurized juices after any given holding period. Thus the only positive conclusion from the results in Table V is that there was a slight but significant increase in limonin content of the juices during storage.

Such a result is in sharp contrast to the results from the first trial (Section 3.1.2.) which demonstrated a dramatic reduction in limonin content of both pasteurized and unpasteurized juices on storage. For example, after 48 days storage the limonin content of the pasteurized juices had declined by 25% and that of the unpasteurized juices by 50%.

Table V Changes in limonin content (ppm) of Navel orange juices held at 7 C and 30 C with and without the addition of pectic enzymes

Holding period (days)	Pasteurized juices[a]						Unpasteurized juices[b]					
	Control		Irgazyme		Ultrazym		Control		Irgazyme		Ultrazym	
	7 C	30 C	7 C	30 C	7 C	30 C	7 C	30 C	7 C	30 C	7 C	30 C
1	13.0	15.0	n.d.	n.d.	n.d.	n.d.	12.0	12.0	n.d.	n.d.	n.d.	n.d.
4	n.d.	n.d.	16.5	16.0	14.5	14.0	n.d.	n.d.	13.0	12.5	13.5	14.5
7	13.5	15.0	15.0	14.5	15.0	16.0	13.5	13.0	12.5	13.0	14.5	13.5
15	14.5	14.0	16.0	n.d.	15.0	14.0	13.5	13.0	13.5	14.5	12.5	14.0
21	14.0	15.0	13.5	13.0	14.5	15.5	12.0	14.5	11.5	12.5	13.0	13.5
28	14.5	15.5	18.0	15.5	16.5	16.5	12.5	14.0	12.5	12.0	15.5	14.5
42	14.5	16.0	16.5	17.5	15.0	16.5	14.5	15.0	15.5	15.0	14.0	14.5
62	17.0	17.0	17.0	18.0	15.0	18.0	16.0	16.5	16.5	16.5	16.0	15.5

a = limonin content at zero time 13.5 ppm

b = limonin content at zero time 12.0 ppm

Table VI Changes in the mean values of the limonin content (ppm) of Navel orange juices

Holding period (days)	Pasteurized juice	Unpasteurized juice
0	13.5	12.0
1	14.0	12.0
4	15.2 ± 1.2	13.4 ± 1.1
7	14.8 ± 1.2	13.3 ± 1.2
15	14.7 ± 1.3	13.5 ± 1.0
21	14.3 ± 1.3	12.8 ± 1.7
28	16.1 ± 1.9	13.5 ± 2.0
42	16.0 ± 1.5	14.8 ± 0.8
62	17.0 ± 2.0	16.2 ± 0.7

The much higher initial concentrations of limonin in the juices used in the first trial cannot account for this difference in behaviour since, even when the limonin content had fallen to comparable levels (16 ppm in the unpasteurized juices after 48 days storage), it still declined significantly (down to 10 ppm) within another 22 days.

The difference in the changes in the limonin content of the juices in the two trials might have been related to the maturity of the fruit used (Brix:acid ratio 6.4:1 for fruit in the earlier trial and 10.2:1 in the present trial) and thus differences in the various limonin components in the fruit at the time of juicing. It is well established (Maier *et al.* 1977) that, during the maturation of citrus fruits, a complex series of reactions involving the formation and degradation of limonin and associated compounds occurs. Therefore, differences in the changes in the limonin contents of various orange juices during storage may be related to the presence or absence of compounds closely related to limonin, and to variations in the activity of the associated enzyme systems. For example, if it is assumed that three compounds are related to each other as indicated by the following reaction sequence:

then if compound B were limonin and A and C were present in the juice in high concentrations, some A may be converted to limonin; however, little limonin would be converted to C due to product inhibition. As a result there would be a small increase in limonin concentration until equilibrium was established. On the other hand, if the concentration of A or B were high and C low, then A might be converted to limonin and limonin converted to C. In this case there would be a decrease in the limonin concentration until equilibrium was established. There is no direct evidence from either trial to indicate that the above conditions exist, but such a sequence of events would provide a possible explanation for the differences observed in the results from these trials.

The limonin content of the shaken juices after a holding

period of 62 days was significantly greater than the limonin content of the supernatant serum from juices stored 42 days in all but two cases (the exceptions being the pasteurized juices containing Irgazyme). However, since this difference was merely the continuation of a trend for increasing limonin content with holding period, it would appear that shaken juice contains no more limonin than the upper layer of unshaken juice. Therefore there is no evidence that limonin was being leached from the cloud to account for the increase in limonin content with holding period.

3.2.3.2. Juice turbidities

Apart from significantly lower levels of juice turbidity in certain samples stored at 7 C (to be discussed in detail later), there was little difference in the pattern of the results obtained at the two holding temperatures (Tables VII and VIII), and the general effects will be discussed by reference to the treatments at 30 C.

In pasteurized control juices, there was a slight increase in juice turbidity within 3 days but thereafter it remained relatively constant. In unpasteurized control juices, juice turbidity (already considerably lower initially than in the pasteurized juices) fell very rapidly to give almost clear solutions above the sedimented cloud. On the other hand, the control sera showed rapid loss of turbidity in both pasteurized and unpasteurized samples, though much more rapid in the latter, and this fall continued throughout the period of the experiment. There was an apparent temperature effect in pasteurized samples, although it was less marked in unpasteurized samples, in that juice turbidity was higher in samples stored at 30 C than at 7 C, presumably due to temperature effects on natural enzyme activity.

In Ultrazym-treated juices, there was a rapid decline in turbidity with time, especially in the unpasteurized samples, and a similar though even more rapid loss of turbidity in the sera. With unpasteurized juices, but not with pasteurized juices, turbidity was retained better in the Ultrazym-treated samples than in the controls, possibly due to the

Table VII Changes in juice turbidity (A_{660c}) of Navel orange juices held at 7 C with and without the addition of pectic enzymes

Holding period (days)	Pasteurized juice[a]						Unpasteurized juice[b]					
	Control		Irgazyme		Ultrazym		Control		Irgazyme		Ultrazym	
	Serum	Juice	Serum	Juice	Serum	Juice	Serum	Juice	Serum	Juice	Serum	Juice
3	0.361	0.874	0.224	0.385	0.109	0.264	0.146	0.094	0.040	0.067	0.017	0.051
6	0.318	1.020	0.111	0.452	0.078	0.296	0.060	0.065	0.019	0.110	0.018	0.062
10	0.279	0.947	0.060	0.389	0.045	0.211	0.009	0.026	0.008	0.102	0.056	0.016
13	0.329	0.969	0.074	0.418	0.050	0.226	0.011	0.035	0.027	0.130	0.034	0.062
20	0.271	n.d.	0.035	0.458	0.026	0.200	0.002	0.010	0.010	0.192	0.009	0.064
27	0.259	0.906	0.033	0.382	0.020	0.151	0.004	0.012	0.014	0.141	0.007	0.057
42	0.319	0.965	0.034	0.471	0.022	0.164	0.008	0.020	0.015	0.178	0.010	0.077
61	0.200	0.810	0.022	0.403	0.018	0.158	0.002	0.025	0.008	0.158	0.004	0.073

a = juice turbidity at zero time 0.851

b = juice turbidity at zero time 0.131

Table VIII Changes in juice turbidity (A_{660c}) of Navel orange juices held at 30 C with and without the addition of pectic enzymes

Holding period (days)	Pasteurized juice[a]						Unpasteurized juice[b]					
	Control		Irgazyme		Ultrazym		Control		Irgazyme		Ultrazym	
	Serum	Juice	Serum	Juice	Serum	Juice	Serum	Juice	Serum	Juice	Serum	Juice
3	0.438	1.150	0.154	0.666	0.120	0.298	0.094	0.093	0.046	0.281	0.034	0.104
6	0.429	1.189	0.097	0.703	0.045	0.207	0.037	0.081	0.026	0.270	0.026	0.084
10	0.409	1.139	0.077	0.749	0.034	0.223	0.018	0.056	0.023	0.311	0.015	0.088
13	0.458	1.138	0.082	0.667	0.031	0.204	0.013	0.033	0.020	0.337	0.014	0.068
20	0.407	1.158	0.058	0.686	0.025	0.161	0.003	0.022	0.024	0.346	0.009	0.073
27	0.342	1.200	0.046	0.683	0.023	0.129	0.004	0.019	0.024	0.554	0.008	0.057
42	0.262	1.246	0.052	0.640	0.031	0.110	0.010	0.035	0.042	0.500	0.014	0.083
61	0.261	1.102	0.045	0.749	0.028	0.111	0.008	0.021	0.031	0.402	0.010	0.055

a = juice turbidity at zero time 0.851

b = juice turbidity at zero time 0.131

macerating effect of the enzyme on cloud particles. This result agrees with the reported stabilization of cloud by treatment with pectic enzymes (Baker and Bruemmer 1972) but the magnitude of the effect in the present experiments was commercially insignificant. There was no significant temperature effect in the Ultrazym treatments.

In Irgazyme-treated pasteurized juice, there was some evidence of a slight increase in turbidity after an initial fall, but values soon stabilized at a lower than initial level; serum samples showed rapid and complete loss of turbidity. In the unpasteurized juices, more obvious at 30 C than 7 C, there was a notable increase in turbidity, again possibly due to a macerating effect of the enzyme on the larger particles. Since this effect of Irgazyme was to increase rather than maintain cloud stability and since the effect was not produced in pasteurized juices, it seems that the natural fruit enzymes are involved as well as Irgazyme. At both temperatures the absolute differences between the control and the Irgazyme-treated juices were very similar, suggesting that the effect of Irgazyme is independent of temperature.

Furthermore, in view of the results recorded in Tables VII and VIII, the convention (Baker and Bruemmer 1972; Krop 1974) of shaking juices prior to withdrawing a sample to determine the turbidity should not be followed since it leads to erroneous conclusions. For example, although the unpasteurized juice held at 30 C containing Irgazyme showed an increase in turbidity when measured conventionally, the turbidity of the serum decreased rapidly. Since consumer acceptability is based largely on the state of the cloud in unshaken juices, turbidity measurements of the serum give a more meaningful result.

3.2.3.3. Cloud particle size

When the ratio of the absorbances at one wavelength of centrifuged and uncentrifuged samples (A_{720c}/A_{720u}) were plotted against the ratio of the absorbances at two wavelengths for the corresponding uncentrifuged samples

(A_{720u}/A_{550u}) (Fig. 2 and 3), information regarding the relative stability of the juices was obtained. Fig. 2 presents a plot of these ratios for juice sera after three days; since similar regressions were obtained at both 7 C and 30 C, these results have been plotted together. From the observations of Lankveld (1973), samples having a high A_{720c}/A_{720u} value and a low A_{720u}/A_{550u} value (i.e. samples located in the upper left-hand quadrant) would be expected to have a lower mean particle size than those in the lower right-hand quadrant. In other words, they would be less inclined to flocculate and would therefore be more stable. On the other hand, particles with a high mean particle size would be less stable and more likely to flocculate.

In confirmation of Lankveld's observations, flocculation did occur where expected, as reflected in Fig. 3 which is the equivalent plot to Fig. 2 but three days later. On comparing the two figures it can be seen that the points for sera from six juices (A7, A30, C7, D30, E30 and F30 - see legend to Fig. 2 for the meaning of these designations) moved up the line between days three and six. This movement would be the result of the larger particles in these juices flocculating and settling out as expected from Lankveld's theory. As a consequence the turbidity of these sera (Tables VII and VIII) decreased, although the magnitude of the decrease was very samll in two cases (A30 and F30). However, for the same six samples, the turbidity of the juice increased in two cases (A7 and C7), remained virtually constant in another two (A30 and E30) and decreased in two cases (D30 and F30). This confirms the recommendation (Section 3.2.3.2.) that juices should not be shaken prior to withdrawing a sample for turbidity measurement.

The points for two juices (B7 and F7) barely moved at all in relation to the lines of best fit, and while the turbidity of the juice increased in both cases, the turbidity of the serum decreased in B7 and remained constant in F7.

The points for the remaining four juices (B30, C30, D7 and E7) all moved down the line of best fit between days three

Fig. 2 Plot of A_{720c}/A_{720u} values versus A_{720u}/A_{550u} values for samples of the serum of Navel orange juices after three days storage at 7 C and 30 C

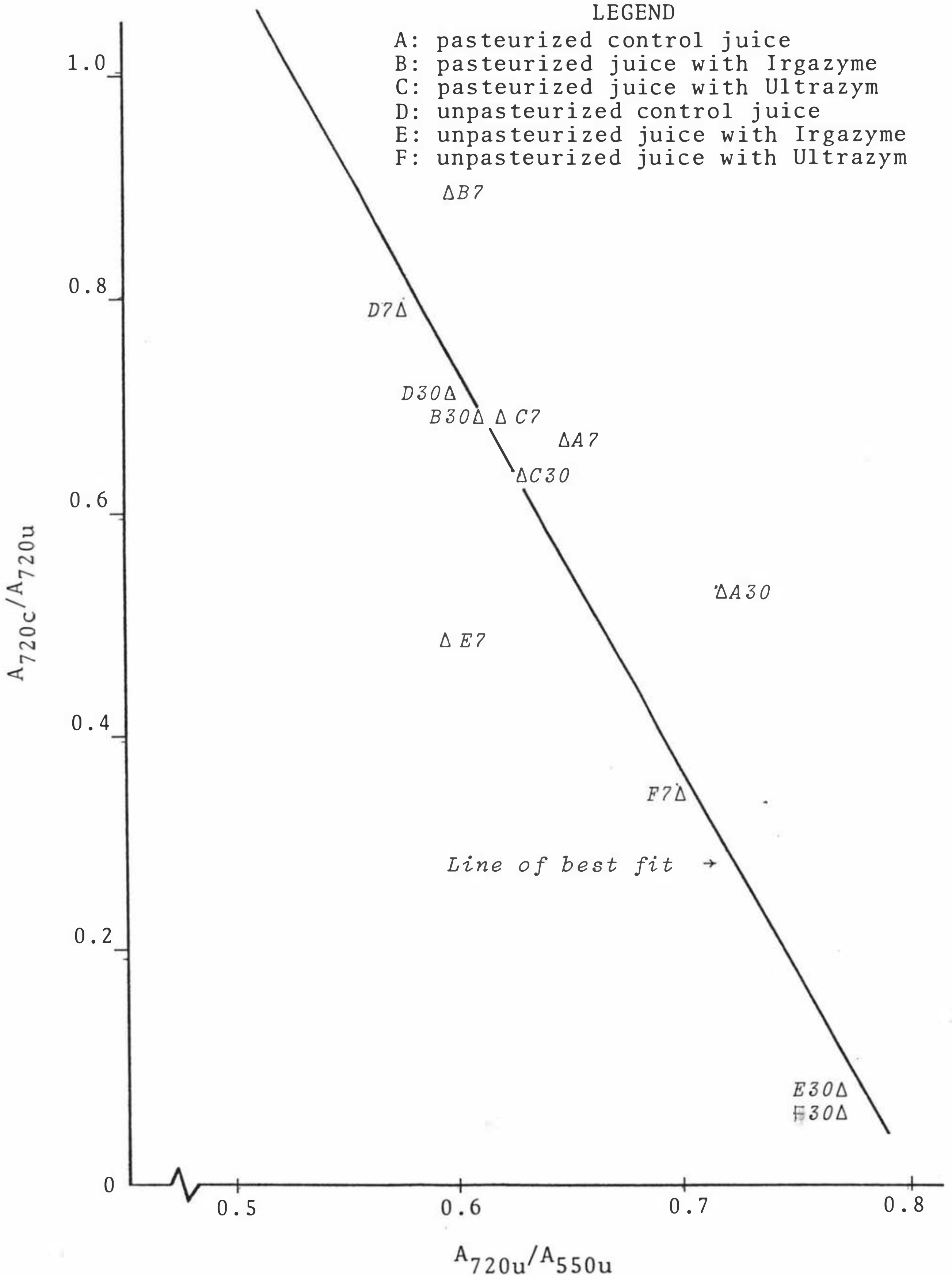
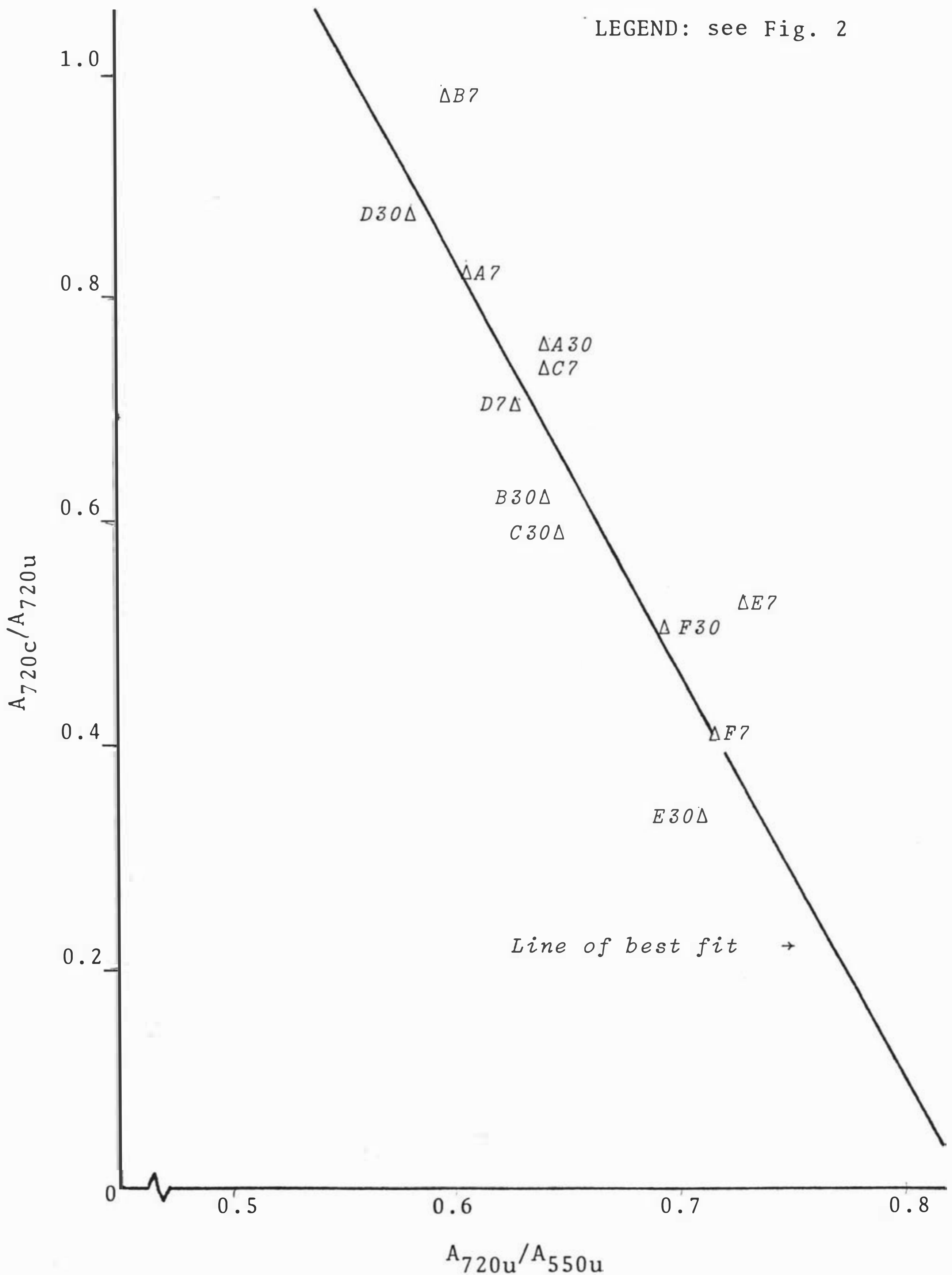


Fig. 3 Plot of A_{720c}/A_{720u} values versus A_{720u}/A_{550u} values for samples of the serum of Navel orange juices after six days storage at 7 C and 30 C



and six, and in all cases there was decline in the turbidity values of the sera. For the juices the turbidity values increased in two cases (B30 and E7) and decreased in the other two cases (C30 and D7).

It was not possible to relate the changes in juice turbidity to the action of the pectic enzymes in the juices, since a decrease in juice turbidity was observed in juices where these enzymes (both natural and added) were both present and absent. Obviously there are many factors which determine the stability of cloud in citrus juices.

3.2.3.4. Pectic substances
Several interesting points arise from a consideration of the pectin analyses of the juices (Tables IX and X). While some hydrolysis occurred in the pasteurized control juice held at 7 C, the pectin in the corresponding juice held at 30 C had undergone considerable hydrolysis. The total pectin content after 63 days at 7 C was double that at 30 C for the pasteurized control juice. The unpasteurized control juices held at 7 C and 30 C had lower water-soluble (i.e. high-methoxyl) pectin but higher oxalate-soluble (i.e. low-methoxyl) pectin contents than the corresponding pasteurized juices. These results are to be expected since PE was present in the unpasteurized juices and would have been expected to de-esterify high-methoxyl pectin and convert it to low-methoxyl pectin.

Pectin in juices containing Irgazyme underwent rapid degradation at both 7 C and 30 C, there being no significant differences between pasteurized and unpasteurized samples. Changes in pectin in juices containing Ultrazym and Irgazyme were almost identical, the only difference being that the former enzyme acted more rapidly than the latter. In juices containing these enzymes, most of the pectin was degraded within four days, the juices held at 30 C being degraded more rapidly than those held at 7 C. This is to be expected since the temperature optimum for pectic enzymes is close to 50 C (Neubeck 1975).

Table IX Changes in pectic substances of juices stored at 7 C

Juice sample	Holding period (days)	Pectin[a] soluble in:			
		Water	Oxalate	Hydroxide	TOTAL
Pasteurized control juice	0	38	27	20	85
	4	22	24	29	75
	28	25	8	15	49
	63	20	14	13	47
Pasteurized juice with Irgazyme	4	14	4	5	24
	28	8	3	5	16
	63	2	2	3	7
Pasteurized juice with Ultrazym	4	4	3	5	12
	28	2	2	5	9
	63	2	2	4	8
Unpasteurized control juice	0	23	23	26	72
	4	18	37	14	69
	28	11	31	15	57
	63	9	27	4	40
Unpasteurized juice with Irgazyme	4	7	12	7	25
	28	3	7	6	16
	63	2	3	2	7
Unpasteurized juice with Ultrazym	4	5	5	7	17
	28	3	3	5	11
	63	3	3	3	9

a = as mg anhydrogalacturonic acid/100 mL juice.

Table X Changes in pectic substances of juices stored at 30 C

Juice sample	Holding period (days)	Pectin[a] soluble in: Water	Oxalate	Hydroxide	TOTAL
Pasteurized control juice	0	38	27	20	85
	4	27	24	16	67
	28	34	19	14	67
	63	11	6	6	23
Pasteurized juice with Irgazyme	4	7	4	5	16
	28	9	2	2	13
	63	2	3	2	7
Pasteurized juice with Ultrazym	4	3	3	5	11
	28	3	2	4	9
	63	2	1	2	5
Unpasteurized control juice	0	23	23	26	72
	4	21	38	13	72
	28	14	32	9	55
	63	8	17	3	28
Unpasteurized juice with Irgazyme	4	5	5	6	16
	28	5	4	5	14
	63	2	2	2	6
Unpasteurized juice with Ultrazym	4	4	5	6	15
	28	4	2	5	11
	63	2	2	1	5

a = as mg anhydrogalacturonic acid/100 mL juice.

Baker and Bruemmer (1972) reported that, to achieve a stable cloud, 61% of the total pectin should be hydrolysed to oligogalacturonides (OG) which would form soluble pectates with divalent cations and thus maintain a stable cloud. However, their method of calculating OG was crude, since they simply took the difference between the total pectin in the control and treated juices. No account was taken of the fact that OG could be degraded right down to galacturonic acid. In their enzyme-treated juice with a stable cloud, 61% of the total pectin had been hydrolysed in eight days. In the present trial, pectin in Irgazyme-treated juices (whether pasteurized or not) underwent similar hydrolysis to this in four days at 7 C, while those held at 30 C were hydrolysed even more rapidly. Despite the similarity in these degradations with the degradation which Baker and Bruemmer (*ibid.*) reported to lead to stability, a stable cloud was not achieved in the present work.

The explanation for the lack of a stable cloud in the enzyme-treated juices was provided by the paper of Termote *et al.* (1977) which became available as these experiments were concluding. They demonstrated that the function of OG was to inhibit PE, rather than just to form soluble pectates with the divalent cations, and that 1000 ppm of OG with a degree of polymerization (DP) in the range 8 - 15 was required for this inhibition. The presence of OG with DP exceeding 16 resulted in cloud destabilization within 30 min. They favoured addition of chemically or enzymically prepared OG rather than the formation of them *in situ* as proposed by Baker and Bruemmer (*ibid.*). Furthermore, they reported the addition of enzymically prepared OG (DP 8 - 15) only extended the shelf life of juices held at 30 C by 50 to 90 h, a juice without added OG showing unacceptable cloud loss after 40 h. Juices held at 3 C maintained stability six times longer than juices held at 30 C.

In the light of the above paper and the results of this trial, the following picture emerges. Enzymic modification of juice pectin *in situ* is not a satisfactory method to achieve cloud stability in citrus juices. Moreover, limonin

cannot be rapidly removed from citrus juices by hydrolysing the juice pectin, since high limonin contents were maintained for up to 60 days even in those juices in which pectin was almost completely degraded.

3.3. EFFECT OF VARIOUS TREATMENTS ON THE LIMONIN CONTENT OF NAVEL ORANGE JUICE

3.3.1. Experimental

The juices from the first trial (Section 3.1) were subjected to the following sequential treatments 158 days after extraction:

(a) shaking for 2½ h;
(b) centrifuging at 800 x *g* for 10 min;
(c) centrifuging at 40,000 x *g* for 20 min;
(d) aerating for 4h.

After each treatment, the limonin content of the juices was determined, in the case of the first treatment without allowing sedimentation of the cloud.

3.3.2. Results

These are presented in Table XI.

3.3.3. Discussion

Unpasteurized juices shaken for 2½ h and sampled before cloud separation showed no significant differences in limonin contents from the original juices. Thus the limonin which disappeared from solution during storage (Table III) must have crystallized out onto the walls of the flasks or become occluded in the precipitated cloud particles and been resuspended during the shaking process. The fact that the initial (day 1) limonin levels were not achieved in pasteurized juices after agitation could possibly be due to the fact that, being pasteurized, these juices were more viscous, so that the process of resuspension was slower. The viscosity difference might also explain why the limonin contents of the unpasteurized sera were lower after 158 days compared with the pasteurized sera.

Table XI Effect of various treatments on the limonin content of Navel orange juice samples

Treatment	Pasteurized juices		Unpasteurized juices	
	Sulphite	Benzoate	Sulphite	Benzoate
Control juice at day 1	32	32	n.d.	n.d.
Natural serum at day 158	17	17	10	9
Agitation (2½ h)	21	21	35	28
Centrifugation (10 min at 800 x *g*)	19	21	11	11
Centrifugation (20 min at 40,000 x *g*)	16	17	10	9
Aeration (4 h)	18	19	11	10

On the other hand, the centrifugation results indicate that the limonin contents of the juices after centrifugation, as well as the natural sera at day 158, represent soluble limonin, there being more soluble limonin in pasteurized (mean value 16.5 ppm after 20 min at 40,000 x *g*) than unpasteurized (mean value 9.5 ppm) samples.

The effect of the pasteurization treatment on the limonin precursor may have been responsible, at least in part, for the differences in behaviour of the pasteurized and unpasteurized juices during storage. However, at this stage it is not possible to attribute the differences in behaviour to any one particular cause.

3.4. DEBITTERING OF JUICES WITH MODIFIED PECTIN CONTENTS

3.4.1. Background

Although the use of cellulose acetate (CA) gel beads to debitter citrus juices is a commercially viable process (Kefford and Chandler 1977; Chandler and Johnson 1979), no theory has yet been presented which satisfactorily explains why these beads selectively absorb limonin from citrus juices. One plausible theory which may be called the 'exclusion-precipitation' theory (Johnson 1977, personal communication), suggests that the pectin is excluded from the pores in the gel bead by its molecular size. Since it has been suggested that pectin is necessary to hold the limonin in solution (Chandler 1971b), limonin would be expected to precipitate onto the pore walls from the pectin-free juice entering the pores. To provide further evidence to support this theory, several experiments were performed using juices in which the pectin had been enzymically degraded.

3.4.2. Experimental

Three cans of Washington Navel orange juice which had been extracted using an FMC juice extractor, processed as described in Section 2.1.4. and then stored at 1 C for five

months, were opened and the contents mixed. All the following procedures were performed in a laboratory maintained at 20 C. The juice was divided into two portions; to one portion of juice the enzyme Ultrazym 100 (0.1% w/v) was added with thorough mixing, and the other portion was untreated. After 24 h, half of each portion of juice was centrifuged at 800 x *g* for 10 min. Samples of all four portions of juice were analysed for limonin and the juices were then debittered as follows.

A 100 mL sample of each juice was placed in a 150 mL conical flask, and CA gel beads were added to give 2% CA on a dry weight basis. The flasks were then stoppered with a rubber bung containing a suba-seal in a glass tube through which nitrogen gas was passed to sparge the flasks. The flasks were then shaken mechanically for 60 min, after which time the juice was separated from the beads and analysed for limonin, corrections being made for dilution of the juice (measured by change in the total soluble solids content) caused by water present in the gel beads. Controls were similarly run but without addition of the beads. The temperature of the juices rose by 1 C during the shaking treatment. The experiment was duplicated on another batch of the same juice. The partition coefficients for the distribution of limonin between the juice and the beads were calculated using the formula:

$$\text{partition coefficient} = \frac{(C_i - C_d) \times \text{vol. juice (L)}}{C_d \times \text{dry wt. beads (kg)}}$$

where C_i is the initial concentration of limonin in juice; and C_d is the limonin concentration of juice after debittering.

3.4.3. Results

These are summarized in Table XII.

3.4.4. Discussion

After 24 h of enzyme action, the limonin contents of the control and the enzyme-treated juices were not significantly different, except for the centrifuged samples of the enzyme-treated juices where they were significantly lower (about

Table XII Results of cellulose acetate (CA) treatment[a] of orange juices with and without prior treatment with pectic enzymes[b]

	Limonin concentration (ppm)	
	Trial 1	Trial 2
Juices without enzyme treatment:		
Uncentrifuged		
Without CA treatment	22.4	20.4
With CA treatment[c]	13.3	13.8
Centrifuged		
Without CA treatment	20.4	22.5
With CA treatment[c]	11.4	11.7
Juices with enzyme treatment:		
Uncentrifuged		
Without CA treatment	21.0	20.2
With CA treatment[c]	13.8	12.7
Centrifuged		
Without CA treatment	18.3	16.9
With CA treatment[c]	11.7	8.9

a = agitation for 60 min with CA gel beads; 2% CA (dry wt.).
b = 0.1% w/v Ultrazym 100 for 24 h at 20 C.
c = after allowance for water present in CA gel beads.

3 - 4 ppm or 13 - 20%) than the mean value (21.2 ± 1.8) for the other six juices. This would suggest that the enzyme treatment resulted in some change in the amount of limonin present in solution which showed up when the serum was analysed after centrifugation. Possibly, the limonin precipitated onto the insoluble cloud particles which were brought down during centrifugation, or the limonin crystallized out of solution, the crystal s being centrifuged down with the cloud. This decrease in solubility of limonin in enzyme-treated juices provides partial support for the 'exclusion-precipitation' theory since it demonstrates a fall in limonin solubility accompanying a reduction in the pectin content of the juices.

Several other points arise from these results. Firstly, CA beads removed about 2 ppm more limonin from juice if it was centrifuged beforehand. This result can be explained on the basis of centrifugation removing interfering cloud particles which could clog up the pores or block the absorption sites in the beads. Secondly, the effect of prior enzyme treatment on the efficiency of the debittering treatment remains uncertain since in one trial no significant difference was observed and in the second trial the difference was just significant, although showing up more clearly in centrifuged juices. Whatever the effect of prior enzyme treatment, it was too small to be conclusively demonstrated using the present analytical techniques.

Similarly, because of experimental errors inherent in the analysis of limonin, no significant differences would be detected in the partition coefficients of limonin between CA and the various orange juices: calculations show that when a juice of 17.0 ± 1.5 ppm limonin content is debittered by CA beads to a juice with 11.0 ± 1.0 ppm limonin content, reductions in limonin contents anywhere between 18.5 to 10 ppm and 15.5 to 12 ppm could be recorded. Thus partition coefficients ranging from 14.5 to 42.5 are to be expected. Under these circumstances, the lower affinity of enzyme-treated juices for limonin to be expected from the lower soluble limonin contents of such juices could not be

demonstrated in terms of partition coefficients.

Therefore, these experiments failed to provide any confirmation of the 'exclusion-precipitation' theory for CA absorption of limonin by demonstrating an increase in the partition coefficient of limonin between CA and juice after enzyme treatment. More clear-cut trends might have been obvious if a longer period of time had elapsed between enzyme addition and centrifugation, or if greater centrifugal forces were employed.

3.5. EFFECT OF CENTRIFUGATION AND EXTENDED PECTIC ENZYME TREATMENT ON LIMONIN SOLUBILIZATION

3.5.1. Experimental

Two further trials were undertaken in which similar raw materials and experimental conditions as described in Section 3.4.2. were used apart from certain modifications: no debittering trials took place; in the first trial four additional centrifugation conditions were used; and in the second trial, enzyme action was allowed to continue for nearly four weeks.

3.5.2. Results

These are summarized in Tables XIII and XIV.

3.5.3. Discussion

The results in Tables XIII and XIV show that no significant differences could be detected in these samples between the limonin content of control juices and those that had been subjected to enzyme treatment and centrifugation under a variety of conditions. If there were any differences in limonin solubility as a result of enzyme treatment and/or centrifugation in these two trials, they were too small to be detected by the analytical methods used.

Differences can be seen in the results in Tables XII, XIII and XIV when they are considered together. In Table XII, there are two sets of results which show a significant

Table XIII Effect of centrifugation on limonin content of orange juice with and without enzyme treatment

Sample	Limonin content (ppm)	
	Control juice	Enzyme-treated juice[a]
Juice	26.7	25.0
Natural serum	24.1	24.8
Centrifuged juices:		
360 x *g* x 10"	28.0	26.6
800 x *g* x 10"	22.5	23.0
1150 x *g* x 10"	24.0	26.2
2000 x *g* x 10"	25.4	26.0
2640 x *g* x 10"	23.8	23.5
Mean	24.9 ± 1.4	25.0 ± 1.0

a = 0.1% w/v Ultrazym 100 for 66 h at 20 C.

Table XIV Effect of prolonged enzyme treatment[a] on limonin content of orange juice

Sample	Limonin content (ppm)	
	Control juice	Enzyme-treated juice[a]
Natural serum	29.9	28.6
Centrifuged juices:		
2640 x *g* x 45"	29.8	30.7
40000 x *g* x 20"	31.6	29.0
Mean	30.4 ± 1.2	29.4 ± 1.3

a = 0.5% w/v Ultrazym 100 for 8 days at 30 C and then 17 days at 20 C.

reduction (13 - 20%) in limonin solubility as a result of enzyme treatment followed by centrifugation, and in one of these, enzyme treatment appeared to increase limonin adsorption by CA, further indicating an effect of enzyme treatment on limonin solubility. However, in two sets of results (in Tables XIII and XIV) no significant differences could be detected before and after enzyme treatment.

While further work would be desirable to resolve this apparent discrepancy, differences in raw material could account for the results from these trials. These differences are evident in the initial limonin contents of uncentrifuged juices in Tables XII and XIII (about 21 and 27 ppm respectively) and of natural sera in Tables XIII and XIV (about 24 and 29 ppm respectively). Such differences in limonin content suggest differences in limonoid composition and in the activities of the associated enzymes in these juices which could account for the results reported.

3.6. EFFECT OF ENZYME TREATMENTS ON LIMONIN CONTENTS AND BITTERNESS RATINGS OF PASTEURIZED AND UNPASTEURIZED JUICES

3.6.1. Experimental

The juice samples remaining at the conclusion (day 62) of an earlier experiment (Section 3.2.), in which juices were stored with and without enzyme treatment, were allowed to equilibrate at 20 C before being tasted by five trained tasters. These tasters had previously shown themselves capable of discriminating between juices containing varying concentrations of limonin. All samples were presented to the tasters in identical containers which were differentiated only by a random code number written on the outside. Each trial was repeated the following day, the 30 C samples being tasted on days one and two, and the 7 C samples on days three and four. As well, samples of the juices were debittered prior to tasting according to the procedure described in Section 3.4.2. Both bitter and debittered juices were tasted at the one session. Tasters scored each

juice for bitterness on a 0 - 10 scale, 0 being given when no bitterness was detectable and 10 when juices were intensely bitter.

Prior to debittering and tasting, the free SO_2 content of all the juices was determined using a specific ion electrode according to the procedure of Barnett (1975).

3.6.2. Results

The taste panel scores and limonin contents of the juices are summarized in Table XV. Table XVI gives the free SO_2 contents of the juice prior to tasting and/or debittering with CA gel beads.

3.6.3. Discussion

The taste panel results (Table XV) showed quite reasonable consistency both between the repeat tastings of the same juices, and between the tastings of juices of different physico-chemical composition but similar limonin contents. Even although the pectin had been completely degraded in the juices containing added enzymes, this did not interfere with the perception of bitterness by the tasters. This suggests that the limonin is still in solution in all the juices, a suggestion confirmed by the limonin analyses. Thus, the enzymic degradation of the pectin in these samples had not caused the limonin to precipitate or crystallize out to the extent that consistent significant differences were recorded in the limonin contents.

Such a conclusion disagrees with the results reported in Section 3.4.3. but agrees with the results reported in Section 3.5.2. However, there was a difference between the juices used in Sections 3.4. and 3.5. and those used here: the juices used here had the pectic enzymes added to them immediately after extraction and (where necessary) pasteurization, whereas the juices used in Sections 3.4. and 3.5. were canned and stored for five months at 1 C before the enzymes were added to them. With the exception of the results in Table XII, the addition of pectic enzymes does not seem to have significantly affected either the solubilization or the insolubilization of limonin in the

Table XV[a] Average bitterness scores[b] and limonin contents (ppm)[b] for Navel orange juices[c] with and without prior treatment with enzymes[d] and CA[e]

Juices	Bitterness[f]		Limonin content	Partition coefficient[g]
	Day 1	Day 2		
Juices without CA treatment:				
Pasteurized; no enzyme added	3.2(4.5)	3.0(3.0)	17.1(16.8)	
Pasteurized; Irgazyme added	3.6(3.6)	2.5(3.4)	16.8(18.2)	
Pasteurized; Ultrazym added	4.4(3.4)	3.0(2.6)	15.6(17.7)	
Unpasteurized; no enzyme added	2.4(3.2)	4.2(3.8)	16.1(16.5)	
Unpasteurized; Irgazyme added	4.2(2.0)	4.8(3.2)	16.5(16.7)	
Unpasteurized; Ultrazym added	3.6(2.8)	4.8(3.8)	15.9(15.5)	
Juices with CA treament[h]:				
Pasteurized; no enzyme added	1.8(2.0)	1.0(1.2)	10.5(9.2)	81.5(91.5)
Pasteurized; Irgazyme added	1.2(1.8)	1.0(1.2)	10.2(8.4)	82.5(108.5)
Pasteurized; Ultrazym added	2.2(1.4)	2.0(1.0)	10.2(10.2)	76.5(87.0)
Unpasteurized; no enzyme added	0.6(1.6)	0.8(1.0)	9.9(8.6)	81.5(96.0)
Unpasteurized; Irgazyme added	1.2(2.2)	1.8(1.2)	9.6(9.6)	86.0(87.0)
Unpasteurized; Ultrazym added	1.2(1.8)	1.8(1.4)	9.1(9.4)	87.5(82.5)

a = see next page for footnotes.

Footnotes for Table XV:

b = after storage of juice for 62 days at 7 C and 30 C (values for 30 C storage are given in brackets after those for 7 C storage).

c = see Section 3.2.2. for details of initial juice composition.

d = 100 mg/L Irgazyme M10 or 200 mg/L Ultrazym 100.

e = agitation for 60 min with cellulose acetate (CA) gel beads; 2% CA (dry weight).

f = mean score from five tasters who scored each juice for bitterness on a 0 - 10 scale, 0 being given when no bitterness was detectable and 10 when juices were intensely bitter.

g = partition coefficient = $\frac{(C_i - C_d) \times \text{vol. juice (L)}}{C_d \times \text{dry wt. CA beads (kg)}}$

where C_i is the initial concentration of limonin in the juice; and C_d is the limonin concentration of juice after debittering.

h = limonin contents have been corrected for dilution from water in the CA beads.

Table XVI Free SO_2 contents (ppm) of orange juices[a] after storage at 7 C and 30 C

Treatment	Storage at 7 C	Storage at 30 C
Pasteurized[b]; no enzyme added	290	2
Pasteurized; Irgazyme added[c]	230	1.5
Pasteurized; Ultrazym added[d]	210	1.0
Unpasteurized; no enzyme added	260	59
Unpasteurized; Irgazyme added[c]	250	19
Unpasteurized; Ultrazym added[d]	230	28

a = initial SO_2 content in all flasks was 350 ppm (by calculation).

b = held at 95 C for 5 min and then rapidly cooled.

c = 100 mg/L Irgazyme M10.

d = 200 mg/L Ultrazym 100.

3.6.3. Discussion (continued)
orange juices.

The variation in partition coefficients is more a reflection of the accuracy of the limonin determinations rather than any real difference in behaviour of the various juices with CA gel beads. Again, the similar behaviour of limonin during the debittering process in both the presence and absence of juice-soluble pectin provides further evidence that the 'exclusion-precipitation' theory does not adequately explain the mechanisms of limonin adsorption by CA gel beads.

As would be expected, the juices held at 30 C had lost considerably more SO_2 than those held at 7 C (Table XVI), presumably due to the greater volatility of the SO_2 at the higher temperature. Less expected was the greater SO_2 contents in the unpasteurized juices held at 30 C compared to the pasteurized juices. Presumably the SO_2 reacted with products of the Maillard non-enzymic browning reaction which would have been occurring at a greater rate at 30 C than at 7 C. Also the heat received during pasteurization would have been sufficient to initiate the Maillard reaction and create a heavier demand on the SO_2 than in the unpasteurized samples.

3.7. GENERAL DISCUSSION

At this stage, several conclusions can be drawn from the preceding experiments. Firstly, the role of pectin in maintaining limonin in solution in citrus juices does not appear to be as dominant as suggested earlier by Chandler (1971b). Certainly the addition of enzymes to accelerate degradation of pectin did not result in the precipitation or crystallization of limonin from juices in one trial (Table V) even after 62 days storage, and in fact limonin contents increased slightly over the period. This was contrary to earlier results (Table III) which showed a marked decrease in limonin contents (contemporaneous with but apparently not directly associated with loss of cloud)

in both pasteurized and unpasteurized juices, but more particularly in the latter.

In earlier experiments, some evidence (Table XII) was obtained for a decrease in limonin solubility following treatment with pectic enzymes, but not in all juices studied (Tables XIII, XIV, and XV). Some previously unconsidered factors are involved in the establishment and/or maintenance of high limonin concentrations in orange juices. Because of the apparent complexity of these processes, the possibility of degrading juice-soluble pectin in a simple procedure to reduce the concentration of limonin in orange juice does not deserve further consideration at this stage. Much more must be known about the factors involved in the solubilization of limonin in orange juice before commercially viable processes can be proposed to prevent the development of bitterness. More basic research is required before any comprehensive rational explanation can be put forward to account for the conflicting results reported here (and elsewhere) on changes in the limonin content of citrus juices under various experimental conditions.

Secondly, the stabilization of citrus juice cloud by enzymatic degradation of the juice-soluble pectin does not appear to be an acceptable alternative procedure to the use of heat. The results presented earlier indicated that, even when the pectin in freshly extracted, unpasteurized juices was degraded rapidly, there was complete and irreversible cloud loss in the juices. It is now apparent (Termote *et al.* 1977) that the function of degraded pectin is to inhibit PE and prevent it from demethylating juice-soluble pectin which would then form insoluble calcium pectates. Degrading the pectin *in situ* merely speeds up calcium pectate formation and the subsequent cloud destabilization process.

Since modifying the juice-soluble pectin does not reduce limonin solubility or stabilize the cloud, no further investigations were carried out in this area. Instead, it was decided to study the phenomenon of limonin solubilization

in more detail in an attempt to understand more fully the mechanisms involved. It was hoped that results from these studies would then suggest possible experiments aimed at elucidating this phenomenon in model solutions, which are easier to manipulate than actual juices. Results from model systems might, in turn, be used to interpret later work looking at the solubilization phenomenon in citrus juices themselves.

CHAPTER FOUR

THE SOLUBILITY OF LIMONIN IN MODEL SOLUTIONS

4.1. SOLUBILITY OF LIMONIN IN DISTILLED AND ACIDIFIED WATER

4.1.1. Experimental

Three groups of three 300 mL conical flasks were filled with 250 mL of one of the following solutions:

Group A - glass distilled water (pH 6.3);

Group B - 1% citric acid adjusted to pH 3.2 with 0.1 N NaOH;

Group C - 0.123% benzoic acid adjusted to pH 3.2 with 0.1 N NaOH.

To each flask was added finely powdered limonin (50 mg) giving an equivalent concentration of 200 ppm limonin in each solution if all the limonin dissolved. The flasks were then flushed with nitrogen gas to minimise any oxidation of limonin and sealed with rubber stoppers containing a glass tube. The tubes were fitted with suba-seals through which the syringe used to flush the flasks after each withdrawal could pass. The flasks were placed in a reciprocating water bath in which the temperature could be controlled to within ± 1 C over the range of 2 C to 85 C, and shaken continuously. The procedure described in Section 2.7.2. was used to withdraw samples from the flasks for limonin analysis; sampling continued until the concentration of dissolved limonin reached an equilibrium value. Shaking of the flasks was stopped 60 min prior to sampling to allow the solutions to settle, after which they were restoppered, flushed with nitrogen, and shaking recommenced. When the limonin concentrations in solution in the three flasks within each group agreed to within 2 ppm on two successive samplings, the temperature in the water bath was raised to the next desired value. It was found that equilibrium was established within 72 h at all temperatures.

For the solubility determinations at 100 C, fresh solutions were made up in 250 mL round-bottomed flasks. The flasks were placed in isomantles and fitted to reflux condensers, the tops of which were connected to a supply of nitrogen gas to prevent any air entering the system. The flasks were then run under reflux conditions until the concentrations

of limonin in the solutions reached equilibrium values. The sampling procedure was as described above, the solutions being withdrawn seconds after boiling ceased.

The saturated solutions of limonin prepared by equilibration at 85 C were held at 75 C for 4 days. When analyses of the limonin concentration in the solutions showed negligible change, the solutions were held at 25 C and sampled periodically.

4.1.2. Results

The effect of temperature on the solubility of limonin in aqueous solutions is shown in Table XVII, while the multiple regression equations for predicting the change in limonin solubility with temperature are presented in Table XVIII. Changes in the solubility of limonin in water with temperature are shown in Fig. 4, following the usual conventions of plotting solubility against the reciprocal of absolute temperature. The effect of time on the limonin concentration in saturated solutions held at 25 C is presented in Table XIX.

Table XVII Solubility of limonin in aqueous solutions

Temperature	Solubility of limonin (ppm)[a] in:		
(C)	Water	Benzoic acid[b]	Citric acid[c]
2	3.7 ± 0.3	3.7 ± 0.3	3.7 ± 0.2
25	4.6 ± 0.2	5.2 ± 0.3	4.6 ± 0.2
40	8.0 ± 0.2	9.3 ± 0.2	8.4 ± 0.2
55	14.2 ± 0.6	16.0 ± 0.4	15.8 ± 0.9
70	20.9 ± 0.8	24.2 ± 1.4	23.3 ± 1.2
85	42.0 ± 1.3	38.0 ± 1.8	42.2 ± 0.8
100	107 ± 6	113 ± 3	107 ± 3

a = means of duplicate spots from triplicated experiments are given together with the standard error.

b = 0.123% benzoic acid adjusted to pH 3.2 with 0.1 N NaOH.

c = 1% citric acid adjusted to pH 3.2 with 0.1 N NaOH.

Table XVIII Multiple regression equations for predicting the change in limonin solubility with temperature

Solvent	Equation	Multiple correlation coefficient	Variation accounted for
Water	$\log_e S = 47.13 - 26.10T + 3.59T^2$	0.994	98.8%
Benzoic acid solution[a]	$\log_e S = 41.08 - 22.21T + 2.98T^2$	0.988	97.7%
Citric acid solution[b]	$\log_e S = 44.09 - 24.10T + 3.27T^2$	0.993	98.7%

S = solubility of limonin in moles per litre x 10^{-5}.

T = 1000 x K^{-1}.

a = 0.123% benzoic acid adjusted to pH 3.2 with 0.1 N NaOH.

b = 1% citric acid adjusted to pH 3.2 with 0.1 N NaOH.

Fig. 4 Solubility of limonin in water versus reciprocal of absolute temperature

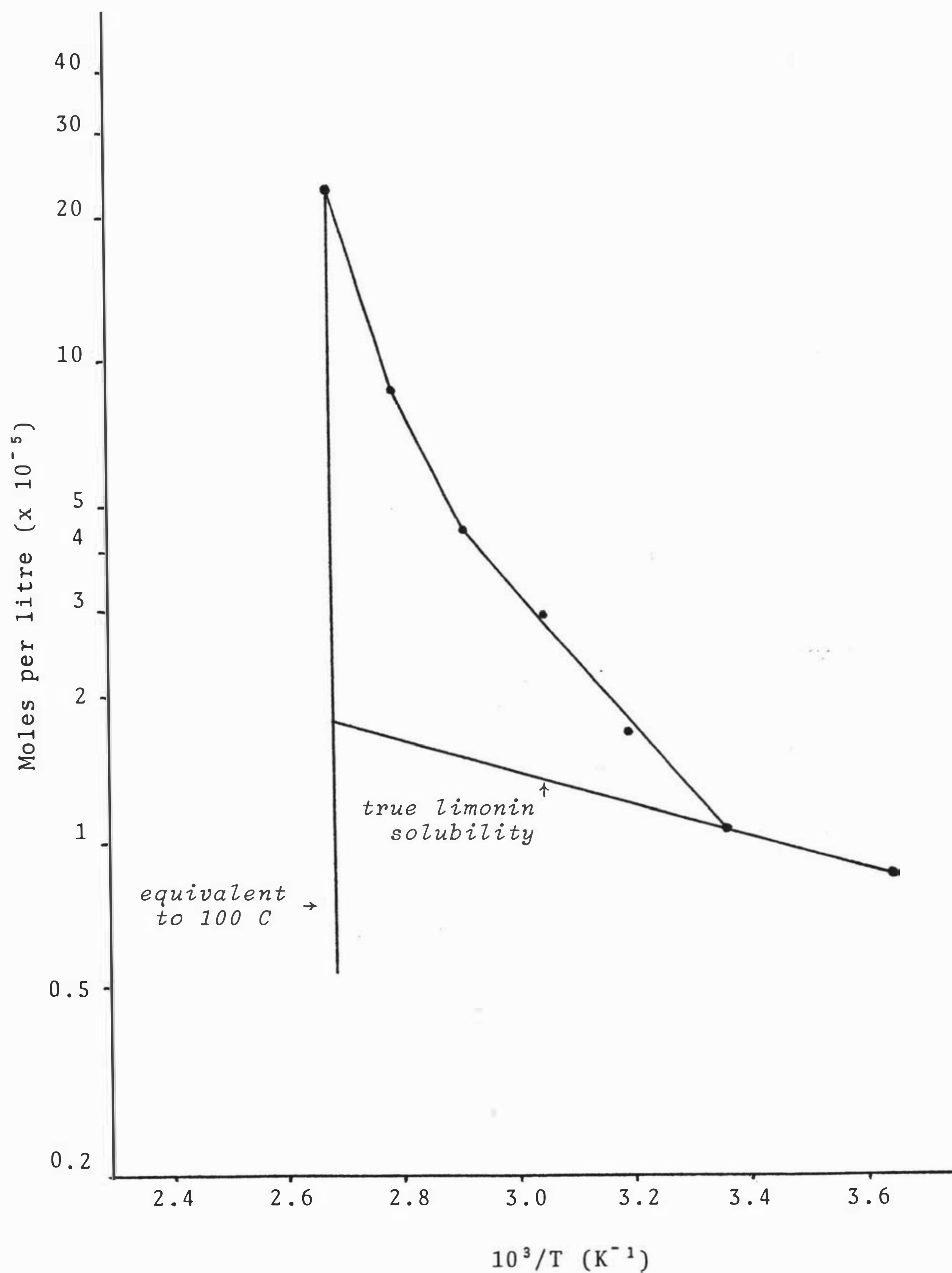


Table XIX Limonin concentration (ppm) in supersaturated solutions stored at 25 C

Solvent	Storage period (days)			
	0	11	39	88
Water	43.5	25.5	21.6	25.6
	40.5	24.8	18.7	24.3
	n.d.	22.9	15.9	24.6
Benzoic acid[a]	35.2	9.0	5.7	6.1
	40.9	8.7	4.0	6.8
Citric acid[b]	41.1	13.2	13.0	10.1
	43.1	13.2	9.7	4.1
	42.3	10.6	6.9	6.7

a = 0.123% benzoic acid adjusted to pH 3.2 with 1 N NaOH.
b = 1% citric acid adjusted to pH 3.2 with 1 N NaOH.

4.1.3. Discussion

The results in Table XVII suggest that it is not possible to obtain aqueous solutions of limonin above about 5 ppm by the simple passage of limonin into solution at room temperature. However, as the curve in Fig. 4 demonstrates, there is a steep rise in the solubility of limonin as the temperature is increased. Inspection of the points on the curve indicates that a number of straight lines can be drawn through the various points. For example, if the points corresponding to the temperatures 2 C and 25 C are joined by a straight line which is extrapolated so that it intersects the perpendicular drawn from the point corresponding to a temperature of 100 C, the corresponding solubility is 8.5 ppm. This is much less than the actual limonin solubility of 107 ppm determined at 100 C.

It is clear that limonin solubility does not obey the standard ideal form in its temperature relationship (Hildebrand and Scott 1970), since, instead of a straight

line plot of log molar concentration against reciprocal absolute temperature, all points appear to be in a smooth curve. Under these circumstances it is reasonable to consider whether limonin actually undergoes any change in chemical form under the conditions of solubilization. Consideration of the structure of limonin as a dilactone suggests that hydrolysis of one or both of the lactone rings could occur under these conditions. A recent review on the hydrolysis of organic compounds in dilute aqueous solutions (Mabey and Mill 1978) indicates that lactone groups (as internal esters of a hydroxyacid) would indeed be readily subject to hydrolysis in aqueous solutions to an extent varying with temperature. Moreover, in weakly acid dilute solutions, the hydrolysis would proceed directly by reaction between the lactone groups and water; in weakly acid solutions of certain esters, hydrolysis by this mechanism can proceed more rapidly than by acid-catalysed hydrolysis. This could account for the similarities in the degrees of hydrolysis and limonin solubilities in all three systems studied.

While there is no direct supporting evidence at this stage, it is suggested that the differences between the recorded limonin solubility at 100 C (107 ppm) and the theoretical value from extrapolation of solubilities at low temperature (8.5 ppm) represents the contribution of hydrolysed limonin to the equilibrium solution at 100 C. This suggestion assumes that any resultant hydroxyacid would be extracted from these solutions and analysed as limonin, having undergone lactonization under the experimental conditions operating. The gradual change in values as the temperature rises is the result of the equilibrium in the hydrolytic process shifting towards the hydroxyacid form. Solubilities recorded represent a continuous smooth transition from a condition where most of the material in solution is limonin to one where most of the material in solution is hydrolysed limonin. The various solubilities recorded would then represent the limonin present plus the amount of hydrolysed limonin in solution in equilibrium with it.

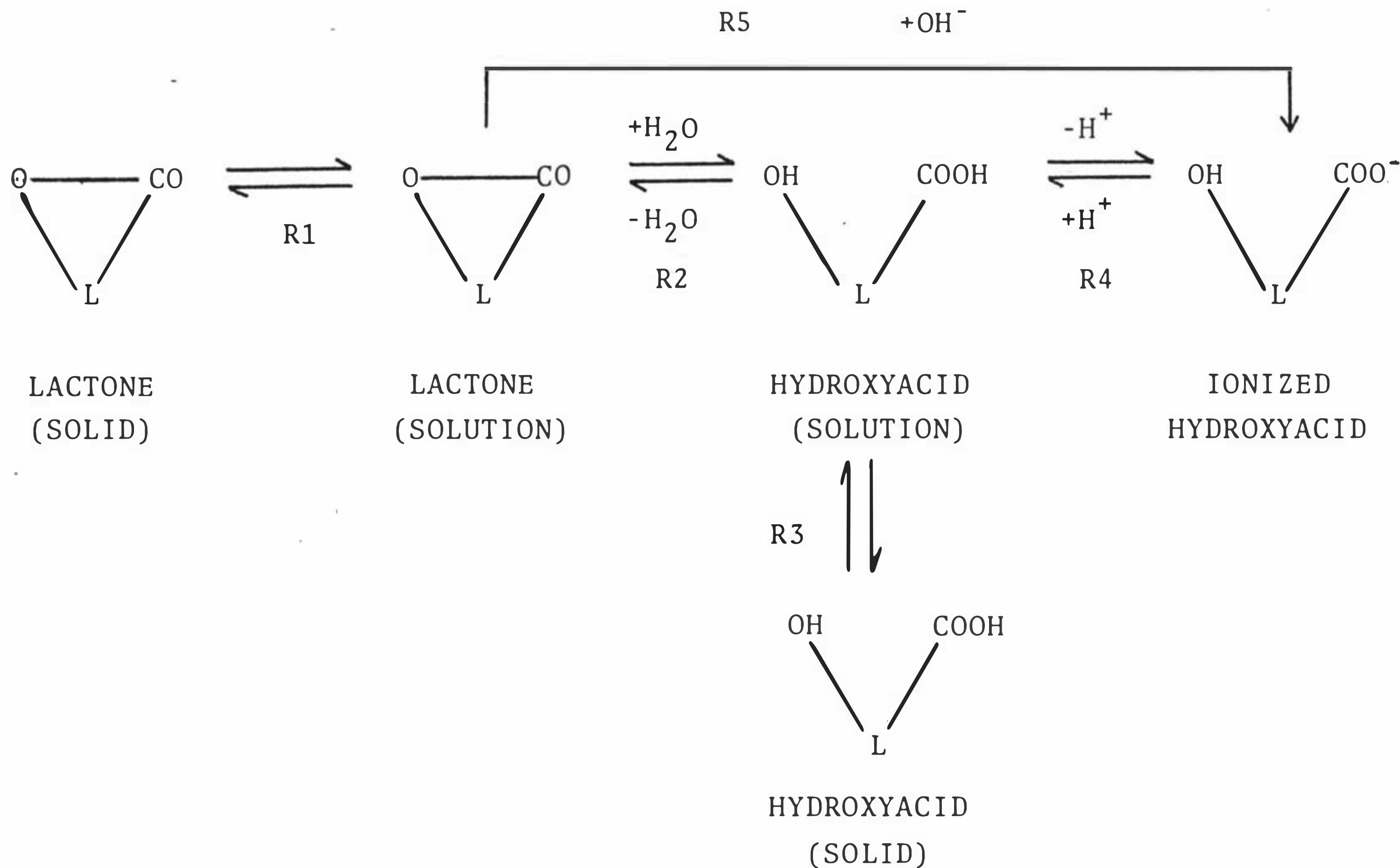
The multiple regression equations (Table XVIII) give useful

empirical relations for predicting the change in limonin solubility with temperature. They show that limonin solubility was very similar in all three systems (water, benzoic acid and citric acid).

The results in Table XIX demonstrate that limonin comes out of simple supersaturated solutions fairly readily at 25 C, reaching what appear to be limiting values within 11 days. However, the fall in limonin concentration was much more marked in acid solutions; although all solutions started with somewhat similar concentrations, the final concentration was about four times higher in distilled water than in acidified water. Indeed, the acid solutions approached the maximum limonin concentration (about 5 ppm) achieved when limonin passes into solution directly without the application of heat (Table XVII), i.e. the "true" solubility of limonin in water. This suggests an involvement of hydrogen ions in the complex equilibrium established in cooling refluxed solutions of limonin. This effect of hydrogen ions would be explained if the molecular species being analysed as limonin in the neutral solutions was the hydroxyacid, since the lactonization of this to limonin would be accelerated by hydrogen ions.

The various equilibria that could operate in aqueous solutions of limonin are shown in Fig. 5. In the initial step, solid limonin passes into solution as the dilactone, with equilibrium R1 being established between the solid and solution states. Although only one lactone group is shown in Fig. 5, both the A-ring and the D-ring lactones could establish the various equilibria shown. With increasing temperatures, the lactone is hydrolysed to the hydroxyacid, with establishment of the equilibrium R2 being accelerated by hydrogen ions. The equilibrium would be shifted to the left by any process which removed the lactone from solution. The equilibrium between the solid and solution states of the hydroxyacid (R3) has only theoretical significance in the present context because the hydroxyacid is, on physico-chemical grounds, likely to be considerably more soluble than the lactone. From entropy considerations, the lactone

Fig. 5 Equilibria operating in aqueous solutions of limonin



would cease to pass into solution before the hydroxyacid could reach sufficient concentration to separate out of solution. The equilibrium would operate if hydroxyacid could be introduced into the solution from the solid state, and the hydroxyacid would continue to pass into solution as the lactone separated out.

The equilibrium between limonin in the solution and solid states would be effected by ionization of the hydroxyacid which would shift it to the right. The dissociation of the hydroxyacid (equilibrium R4) would occur very rapidly, as is the case with most ionic reactions; the degree of dissociation would depend on the concentration of the acid and its dissociation constant, and would be changed by the addition of other ions, i.e. by buffering.

The base-catalysed hydrolysis of the lactone to the ionized hydroxyacid (equilibrium R5) is impossible under the conditions being examined in this study and will not be considered further.

Now the unusual feature of the results is the close similarity of the solubilities recorded for the three solutions under conditions favouring solubilization (i.e. under heating conditions - Table XVII) in contrast to the difference in the solubilities recorded under conditions favouring crystallization (i.e. under cooling conditions - Table XIX). In unbuffered and buffered (pH 3.2) solutions, solubilizing conditions induced the same total limonin content at the end state of equilibrium. While this may have been the result of differing concentrations of all possible components adding up to the same total concentration, an attempt to find a simpler explanation is justified. Indeed, all that is required is for ionization to occur to the same extent in the unbuffered and buffered solutions of the hydroxyacid, i.e. for ionization not to be markedly inhibited by the excess of hydrogen ions in the buffered solutions.

On the other hand, under cooling conditions the end state of the equilibrium showed a higher concentration of total

limonin (mean value 24.8 ppm) in the unbuffered solutions than in the solutions buffered at pH 3.2 (mean value 6.8 ppm), the concentration in the latter approaching the equilibrium solubility value for limonin at 25 C.

The origin of the difference of 18 ppm in the total limonin concentration in buffered and unbuffered cooled supersaturated solutions may be found in the excess hydrogen ions in the buffered solutions. These allowed the reactions R4, R2 and R1 to move freely to the left, until small amounts of hydroxyacid remained in solution in equilibrium with the lactone when it reached its solubility limit. In the unbuffered solutions, on the other hand, the shortage of hydrogen ions allowed a considerable amount of limonin (18 ppm) to remain in solution as the hydroxyacid (both ionized and unionized) for the maintenance of equilibrium R4.

The question now arises as to which of the hydroxyacids was involved in the above equilibria. While the possibility that both are involved cannot be ruled out, calculations can be made which suggest that it is very probable that one rather than both were present. The ionization exponent pKa for the D-ring hydroxyacid is 2.7, while that for the A-ring hydroxyacid is 4.7 (Emerson 1949). Calculations using the standard equation (Glasstone and Lewis 1965):

$$Ka = \frac{\alpha^2 c}{1 - \alpha}$$

where Ka is the ionization concentration quotient; α is the degree of ionization of acid; and c is the total acid concentration, indicate that at pH 3.2, the D-ring hydroxyacid would be 71% dissociated while the A-ring hydroxyacid would be 3% dissociated. Thus a great difference would not be expected between the total solubilities of the D-ring hydroxyacid at pH 3.2 and 6.3. On the other hand, the A-ring hydroxyacid, being only 3% ionized at pH 3.2 would not be held in solution in the ionized state at this pH and would come out of solution according to equilibria R2 and R1.

These calculations suggest that the acid in the cooled,

unbuffered saturated solutions would be the A-ring hydroxyacid, since there is little change in the degree of dissociation of the D-ring hydroxyacid at the pH of the buffered and unbuffered solutions.

The above suggestion is in agreement with the known greater ease with which the D-ring hydroxyacid lactonizes. For example, Maier and Marglith (1969) were able to prepare A-ring hydroxyacid in substantially homogeneous state but not the D-ring hydroxyacid. This difference probably originates in the fact that the D-ring lactone is less strained than the A-ring lactone. Because of the strain in the A-ring lactone, this ring would be more easily hydrolyzed than the D-ring lactone under acid or near neutral conditions.

4.1.4. Conclusion

From the above results and in light of the discussion, it is now proposed to assume the presence of limonin plus hydroxyacid in all heated solutions. Therefore, all limonin analyses (carried out as described in Section 2.7.2.) will be referred to as total extractable limonin (TEL) in the remaining sections of this thesis in cases where heat has been applied to the solutions.

4.2. FACTORS AFFECTING THE SOLUBILITY OF LIMONIN IN SOLUTIONS MODELLING CITRUS JUICES

4.2.1. Introduction

Work by Chandler (1971b) suggested that sugar increased the solubility of limonin in citrus juices, while pectin held the limonin in solution. In view of the results presented in Section 2 which showed that degradation of juice pectin *in situ* did not, in the short term at least, cause limonin to come out of solution, it was decided to examine in more detail the role of sugar and pectin in limonin solubility in model solutions. Unlike the experiments of Chandler (*ibid.*), the direct solution of limonin into these systems was measured at 30 C, instead of preparing saturated solutions of limonin by the application of heat and then allowing these solutions

to cool to 30 C. The approach adopted here is in line with the fact that, even without the application of heat, limonin concentrations in citrus juices reach 35 ppm or more, far higher than the solubility of limonin in water at 30 C recorded in the previous section.

4.2.2. Experimental

The three juice constituents investigated were pectin, polygalacturonic acid (PGA) and sugars.

The concentrations of pectin used were the approximate upper and lower levels found in citrus juices for total pectic substances (0.05% and 0.10% respectively). The pectin used was a high-methoxyl pectin of citrus origin (Sigma Grade I) which normally accounts for approximately one third of the total pectic substances in freshly extracted citrus juices.

The PGA was demethylated citrus pectin (Sigma Grade I) and it was used at the same levels as the pectin with the proviso that the combined levels of pectin and PGA in any model solution did not exceed 0.10%. PGA is found in similar concentrations to pectin in freshly extracted citrus juices.

The sugars used were sucrose:glucose:fructose in the ratio 2:1:1, which is approximately the ratio in which they are found in citrus juices. The concentrations of 9% and 15% were chosen as being representative of the lower and upper concentrations of sugar in citrus juices.

To demonstrate any possible interaction between the various components in the model solutions, a full factorial experimental design was employed consisting of the three components at three levels as indicated in Table XX. Because of the proviso that the levels of pectin and PGA in combination should not exceed 0.10%, nine of the possible permutations were eliminated so that the number of treatments was reduced from 27 to 18. To permit an estimate of the significance of the effects and interactions, the treatments were all done in duplicate. Because only nine treatments could be handled experimentally at any one time, the 36 treatments were

randomized into four blocks of nine to counteract any "between block" effects.

The model solutions were each made up to a total volume of 200 mL as follows. All the solid components, including citric acid (2 g) and sodium metabisulphite (200 mg, which gave an effective level of 50 ppm SO_2 to inhibit any microbial growth in the flasks), were first added to a 300 mL conical flask and mixed thoroughly. Distilled water (150 mL) was added and the solution was stirred until all the solids dissolved. The pH of the solution was then adjusted to pH 3.2 with 0.1 N NaOH and the total volume of the solutions made up to 200 mL. Finely powdered limonin (20 mg) was then added to each flask prior to the flasks being flushed with nitrogen gas and fitted with a rubber stopper.

The flasks were then placed in a water bath maintained at 30 ± 1 C and shaken continuously. Shaking and sampling were continued until the solutions had reached equilibrium. The sampling procedure used was as outlined in Section 2.7.2., with the following alterations: the volume of solution withdrawn each time was 40 mL and the pipette was rinsed with water prior to filling with chloroform:ethanol to prevent any pectin or PGA from precipitating out in the pipette.

The results from the experiment were analyzed on the CSIRO Cyber 76 computer using the GENSTAT Mark 4 statistical package.

4.2.3. Results

Table XX gives the mean values and residuals of the equilibrium limonin solubilities in the various solutions, while Table XXI shows the analysis of variance of the results.

Table XX Mean values and residuals[a] of equilibrium limonin solubility (ppm) in various solutions

	PC1	PC2	PC3	PC4	PC5	PC6	Mean
0% sugar[b]	4.2	5.1	5.3	5.0	3.6	6.0	4.9
	(0.04)	(0.50)	(0.18)	(-0.16)	(-1.21)	(0.65)	
9% sugar	5.2	5.3	6.2	6.8	6.7	6.0	6.0
	(-0.14)	(-0.46)	(-0.07)	(0.42)	(0.74)	(-0.50)	
15% sugar	5.3	5.7	6.0	6.0	6.4	6.3	6.0
	(0.10)	(-0.05)	(-0.12)	(-0.26)	(0.47)	(-0.15)	
Mean	4.9	5.4	5.8	5.9	5.6	6.1	5.6[c]

a = residuals are shown in brackets below corresponding means.
b = sugar composed of sucrose:glucose:fructose in ratio 2:1:1.
c = grand mean.
PC1 = no pectin; no PGA.
PC2 = 0.05% pectin; no PGA.
PC3 = 0.10% pectin; no PGA.
PC4 = no pectin; 0.05% PGA.
PC5 = 0.05% pectin; 0.05% PGA.
PC6 = no pectin; 0.10% PGA.

Table XXI Analysis of variance of experimental design

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance (F)
Block stratum:				
Pectic component	2	6.061	3.030	7.969
Residual	1	0.380	0.380	2.036
TOTAL	3	6.441	2.147	11.497[a]
Blocks/Units stratum:				
Sugar	2	9.854	4.927	26.385[a]
Pectic components	5	2.971	0.594	3.182[b]
Sugar/PC	10	5.716	0.572	3.061[b]
Residual	15	2.801	0.188	
TOTAL	32	21.342	0.667	
GRAND TOTAL	35			

a = significant at 0.1% level.
b = significant at 5% level.

4.2.4. Discussion

The first point to note is the significant (0.1% level) "between-block" effect, i.e. some unexplained variations occurred between the four sets of nine flasks each. Since there was no conscious change in methodology, procedure or equipment during these experiments, these variations can best be ascribed to inadvertent differences. Although shown to be significant in this statistically designed experiment, these variations are comparatively small and would normally be absorbed into the differences encountered in the results of replicated limonin analyses (Section 2.7.7.). The "between-block" effect was taken into account by the computer programme in calculating the means in Table XX.

The analysis of variance indicates further statistically significant interactions. The greatest of these is that due to sugar (significant at the 0.1% level). In the absence of sugar, the mean limonin concentration was 4.9 ppm, while at sugar concentrations of 9% and 15%, the mean concentrations were 6.0 ppm. The effect of pectic components was significant at the 5% level; solutions containing no pectin or PGA gave the lowest mean limonin concentration (4.9 ppm), while solutions containing no pectin but 0.10% PGA gave the highest concentration (6.1 ppm). Also significant at the 5% level were the sugar/pectic component interactions, demonstrable in those residuals in Table XX which deviate by more than ± 0.37 (± 2 standard deviations) from zero, the residual mean square being 0.188. Thus, limonin was least soluble in a solution containing no sugar, 0.05% PGA and 0.05% pectin (3.6 ppm) and most soluble in a solution containing 9% sugar, 0.05% PGA and no pectin (6.8 ppm). Attempted interpretation of these differences does not seem possible because no consistencies are apparent and their statistical significance is comparatively small.

A comparison of the effects of pectin and PGA on limonin solubility can be demonstrated by averaging the relevant pectin component means in Table XX. Thus solutions containing no pectin (PC1, PC4 and PC6) had an average limonin

concentration of 5.6. The various values are shown in Table XXII.

Table XXII Effect of pectin and PGA on limonin solubility

Pectin (%)	PGA (%)	Limonin (ppm)
0		5.6
0.05		5.5
0.10		5.8
	0	5.4
	0.05	5.8
	0.10	6.1

The trend evident from Table XXII is that increasing the concentration of either pectin or PGA increased the solubility of limonin, PGA having a somewhat greater effect than pectin. This result appears to be contradictory when compared with the mean values in Table XX but this is not so since the results in Table XXII ignore the effect of sugar; the mean values obtained when sugar is taken into account indicate that some interaction occurs between the three solutes. It is not possible from these experiments to draw any conclusions about or quantify in any way these interactions.

While these results have demonstrated that sugar, pectin and PGA have a statistically significant effect on the equilibrium limonin concentrations in aqueous systems prepared directly without the use of heat, the magnitude of the effect is quite small. Compared to the values obtained by Chandler (1971b) with heated limonin solutions containing sugar and pectin, the results obtained in this experiment where heat was not used are effectively insignificant, thus supporting the premise (Section 4.1.3.) that it is not possible to produce aqueous solutions

containing high concentrations of limonin from the cold. Further work on limonin solubility in heated model solutions was therefore undertaken.

4.3. SOLUBILITY OF LIMONIN IN SOLUTIONS PREPARED BY REFLUXING LIMONIN IN MODEL SOLUTIONS

4.3.1. Experimental

Five solutions containing limonin (20 mg) were refluxed. Each solution had different sugar/pectin concentrations: no sugar or pectin; 10% sucrose; 10% sucrose, glucose and fructose in the ratio 2:1:1; 0.10% pectin; and 10% sucrose and 0.10% pectin. All concentrations were on a weight/weight basis, and the experiment was performed in duplicate.

The flasks were refluxed for 45 h and after cooling the contents were transferred to 250 mL conical flasks and stoppered. Sodium metabisulphite (200 ppm) was added and the flasks were stored in the dark at room temperature (20 ± 4 C). The total extractable limonin (TEL) concentrations were measured at various times.

4.3.2. Results

These are presented in Tables XXIII and XXIV, results from duplicated experiments being reported separately.

4.3.3. Discussion

When the solutions containing sucrose were analysed for limonin, a large black spot appeared (R_f = 0.48) below the limonin spot (R_f = 0.53) on the TLC plate. To ascertain whether or not this spot was a limonin-derived compound, solutions of sucrose (10%) were refluxed for 45 h and then analysed as for limonin. In the absence of limonin a black spot of similar intensity still appeared, thus demonstrating that the black spot from the limonin solutions was a degradation product of sucrose and was not derived from limonin.

As the solutions containing sucrose had TEL concentrations

Table XXIII Initial changes in pH of refluxed solutions

Composition of solution	pH					
	Before reflux		One h after reflux[a]		After addition metabisulphite[b]	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Water	5.8	6.5	5.2	5.2	5.0	5.1
10% sucrose	6.0	6.6	3.2	3.0	3.1	3.2
10% sugar[c]	6.5	6.5	3.0	3.0	3.2	3.2
0.10% pectin	4.0	4.2	3.8	3.7	3.8	3.8
10% sucrose; 0.10% pectin	4.0	4.2	3.4	3.0	3.4	3.2

a = temperature was 51 C.
b = temperature was 30 C.
c = sucrose:glucose:fructose in ratio 2:1:1.

Table XXIV Total extractable limonin concentration (ppm) in refluxed model solutions

Composition of solution	Days in storage									
	0		7		17		31		65	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Water	113.5	111.0	7.4	4.3	n.d.	3.4	3.7	n.d.	n.d.	n.d.
10% sucrose	186.0	179.0	184.0	83.0	95.5	91.5	31.4	83.5	31.0	41.5
10% sugar[a]	138.0	148.0	126.5	112.5	96.0	100.0	93.0	n.d.	73.5	45.5
0.10% pectin	132.5	134.5	56.0	33.4	33.4	n.d.	17.4	25.0	18.1	16.2
10% sucrose plus 0.10% pectin	137.0	146.5	133.5	120.5	51.0	74.5	32.1	85.0	35.3	82.5

a = sucrose:glucose:fructose in the ratio 2:1:1.

close to that obtained if all the limonin present had dissolved, the true equilibrium concentrations may well have exceeded the values obtained. Therefore, solutions of sucrose (10%) containing powdered limonin at a level equivalent to 400 ppm were refluxed for 45 h and the TEL concentration determined in the cooled solutions. Values of 182 ppm were obtained, indicating that the concentration of TEL in refluxed solutions containing 10% sucrose (179 - 186 ppm) was the equilibrium value.

From Table XXIII it can be seen that in all solutions the pH had decreased after refluxing. The decrease was least in those solutions which contained pectin, because the pH of these solutions was low before reflux due to free acids in the pectin (approximately 70% methylated according to the suppliers). Because all solutions except the controls showed a decrease in pH initially to values of 3.0 - 3.4, this decrease can best be ascribed to traces of acidic compounds from the heat-catalysed degradation of sucrose. The fall in the pH of solutions containing pectin but no sugar could be due to pectin hydrolysis, while the fall in the pH of the control solutions suggests the formation of acidic material from limonin itself. Since limonin is recovered unchanged after hydrolysis by dilute alkali (being degraded only by strong acids and alkalis), it can be assumed that this acidic material is the hydroxyacid form of limonin produced by heat-catalysed neutral hydrolysis of one or both of the lactone groups.

A comparison of the effect of the various solutes on TEL content shows that, while all the solutes significantly increased TEL content, 10% sucrose had the greatest effect (mean values of 182.5 ppm compared to control values of 112 ppm). The effect of the other solutes was not significantly different from each other (mean of 0.10% pectin 133 ppm; mean of 10% sugars 143 ppm, and mean of 10% sucrose plus 0.10% pectin 142.5 ppm). These solutes could influence TEL contents in two ways, both of which could operate together in the present systems.

Firstly, the difference between sucrose, glucose and fructose in their effect on the solubility of organic compounds has been reported by others. For example, Nango *et al.* (1980) observed both 'sugaring out' (decreased solubility) and 'sugaring in' (increased solubility) phenomena in studies on the solubility of aromatic hydrocarbons in aqueous solutions of sugars. Thus the addition of sugars to water decreased the solubility of benzene, the decrease in solubility following the order: glucose < fructose < sucrose < water. On the other hand, the addition of sugars to water increased the solubility of phenanthrene, the increase in solubility following the order: sucrose > fructose > glucose > water. It was concluded that the solubility of aromatic hydrocarbons in sugar solutions depended not only on the sugar used but also on the size of the aromatic hydrocarbon. While Nango *et al.* provided no explanation for the effect of the different sugars on aromatic hydrocarbon solubility (or for that matter on TEL solubility), their results provide a precedent for the differing effects of sugars on TEL solubility as shown in Table XXIV.

Secondly, the various solutes could affect TEL contents differently by affecting not only the rates of conversion of limonin into the hydroxyacid but also the composition of the equilibrium mixture. The effect of hydroxylic components on the hydrolysis of esters in aqueous systems is particularly well recognised and, although the studies have been made principally with simple alcohols as the solute, the results can be extrapolated to hydroxylic sugars; lactones could be expected to behave as internal esters in this respect. However, no particular results are available to which the present results can be specifically compared and the order of TEL solubilities in the presence of the various solutes must remain unexplained. With respect to the solutions containing pectin, reference may be made to the effect of solution structure on reaction rates and equilibrium composition.

The results in Table XXIV differ from those of Chandler (1971b) who reported that the presence of 10% sucrose and

0.10% pectin had a greater effect on the solubility of limonin in aqueous solutions refluxed for 3 h than either of the solutes separately. However, the shorter reflux times used by Chandler mean that the results cannot be directly compared, it being apparent that differing reflux times will result in differing equilibria in the solutions.

Even more interesting effects resulting from the various solutes are evident in Table XXIV. In pure water, there was a rapid decline in TEL concentration to about 5.5 ppm in the first week. However, in solutions containing 10% sucrose, the decline in TEL concentration was much less rapid, the TEL concentrations after 65 days being 36 ± 5 ppm in the two experiments. A similar trend was observed in those solutions containing the sugar mixture, although in one experiment the TEL concentration was 73 ppm after 65 days. This difference in the rate at which limonin came out of apparently identical solutions was also observed by Chandler (*ibid.*). In the solutions containing 0.10% pectin, TEL concentrations fell more rapidly, reaching about 17 ppm after 65 days, while there was a wide discrepancy of 47 ppm in the TEL concentrations of the two solutions containing both 10% sucrose and 0.10% pectin after 65 days.

As discussed above in connection with their effect on TEL contents in the refluxed solutions, the various solutes can be expected to affect (both directly and indirectly) the TEL contents in the cooled solutions, and especially the rates at which equilibrium conditions are reached. Indeed, since equilibrium involves lactonization of the hydroxyacid by internal esterification (which requires the relevant hydroxyl and carboxylic acid groups to come into close proximity), the varying effect of solutes and particularly solution structures would be even more marked than was the case with hydrolysis of the lactone ring during the solubilization process.

From these results it can be concluded that pectin, sucrose, and sucrose, glucose and fructose in combination all increase the solubility of TEL in aqueous solutions and all hold TEL in solution to a greater extent than when it is present alone

in solution. There is a suggestion that the more complex solutions (the mixed sugar and the sucrose-pectin solution) could under certain circumstances hold TEL in solution for longer periods than solutions containing only pectin. Far longer storage periods than 65 days would be necessary to determine just how stable these solutions of high TEL content were.

4.4. SOLUBILITY OF LIMONIN IN MODEL SOLUTIONS PREPARED FROM HOT REFLUXED LIMONIN SOLUTIONS

4.4.1. Experimental

Two 1 L round-bottomed flasks, each containing water (500 mL) and powdered limonin (0.10 g), were heated under reflux for 52 h. While the solutions were still hot (95 C), they were mixed together and immediately used to prepare eight solutions, two containing 10% (w/w) sucrose, two 0.10% pectin, two 10% sucrose and 0.10% pectin, and two with no additional solutes. The pH was adjusted to 3.2 by the addition of 5 N HCl, using Oxyphen narrow range (pH 2.7 - 4.2) indicating paper. After cooling the solutions and adding sodium metabisulphite (100 mg), TEL analyses were made. The flasks were then stored in the dark at room temperature (20 ± 4 C), and the TEL concentrations determined periodically.

4.4.2. Results

These are summarized in Table XXV, results from duplicated experiments being reported separately.

4.4.3. Discussion

Firstly, in solutions without sucrose or pectin, equilibrium solubility was reached much more quickly in the previous experiment (Section 4.3.); after 7 days the mean TEL concentration was 5.7 ± 1.6 ppm compared with 30.6 ± 1.2 ppm after 11 days in the present experiment. A possible explanation lies in the method of preparing the solutions for storage. In the previous experiment, the solutions were stored in the flasks in which they were refluxed and these

Table XXV Total extractable limonin concentration (ppm) in model solutions prepared from hot refluxed limonin solutions

Composition of solution	Days in storage							
	0		11		24		59	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Water	112.0	112.0	31.7	29.4	6.1	6.8	n.d.	n.d.
10% sucrose	101.0[a]	101.0[a]	29.2	34.7	13.2	12.1	9.4	n.d.
0.10% pectin	112.0	112.0	58.5	48.0	10.4	10.1	8.6	n.d.
10% sucrose plus 0.10% pectin	101.1[a]	101.0[a]	38.5	40.5	6.6	7.1	n.d.	n.d.

a = by calculation.

would have contained undissolved limonin. Therefore, there would have been more centres for crystal formation than were present in the solutions in the present experiment, since these were transferred from the refluxed flasks into the storage flasks.

Secondly, in the present experiment (Table XXV) in marked contrast to the previous experiment (Table XXIV), the rates of fall in TEL concentration were very similar in all solutions. The falls were initially somewhat slower in solutions containing pectin than in those without pectin (mean values after 11 days 46.3 ± 9.0 and 31.4 ± 2.7 ppm respectively) but in all cases reached values below 10 ppm after 59 days storage. Moreover, the rates of fall were much greater than in the comparable solutions in the previous experiment. The differences were especially marked when it is considered (as mentioned above) that undissolved limonin crystals would be more plentiful in the solutions in the previous experiment.

The faster decline in TEL contents shown in Table XXV compared with that shown in Table XXIV (pure aqueous solutions excepted) is almost certainly due to the adjustment of the pH of these solutions to 3.2 with acid before storage. The general similarities in the rates of falls in TEL contents among the solutions in Table XXV (compared with those in Table XXIV) indicate a dominant effect of added acid in the rapid establishment of equilibrium, presumably by acid-catalysed lactonization of the hydroxyacid in the stored solutions. This conclusion is justified despite the fact that only small amounts of acid were added and the pH of the resultant solutions were not markedly different from those in Table XXIV to which no acid had been added.

4.5. CRYSTALLIZATION OF LIMONIN FROM SUPERSATURATED SOLUTIONS CONTAINING SUCROSE AND PECTIN

4.5.1. Experimental

Supersaturated aqueous solutions of limonin were prepared by heating under reflux four flasks each containing distilled water (250 mL) and powdered limonin (25 mg) at 86 C for four days. The solutions were allowed to cool to room temperature before mixing and filtering through Whatman No. 540 filter paper to remove any undissolved limonin. The bulk solutions were used to prepare eight solutions, two containing 10% (w/w) sucrose, two 0.10% pectin, two 10% sucrose and 0.10% pectin, and two without additional solutions. After adding sodium metabisulphite (50 mg) and adjusting the pH to 3.2 with 10% w/v citric acid, samples were taken to determine the TEL concentrations. The solutions were stored in the dark at room temperature (20 ± 4 C) and TEL concentrations were determined periodically.

4.5.2. Results

These are summarized in Table XXVI.

4.5.3. Discussion

The first point to note is the difference between the initial TEL concentrations of the various model solutions. The solutions were sampled 1 h after they had been prepared and it is obvious that even in this short time there had been a significant decrease in the TEL concentration of certain of the solutions. Although it was not possible to determine the initial TEL contents, they must all have been the same and in excess of 45.7 ppm. Even if some decrease had occurred in the TEL contents in the 0.10% pectin solution, the decrease was much greater in all other model systems. Allowing for the fact that the solutions containing sucrose would have had an initial TEL concentration of at least 40.5 ppm (since the solutions were prepared on a w/w basis) they had still undergone a decrease in TEL concentration of at least 23% in the case of those containing only sucrose and at least 19% in those containing sucrose and pectin within 1 h of being prepared.

Table XXVI Total extractable limonin concentration (ppm) in model solutions prepared from cold supersaturated solutions of limonin

Composition of solution	Days in storage									
	0		7		17		39		88	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Water	26.1	28.3	11.1	13.8	13.0	8.5	9.1	7.0	11.3	9.6
10% sucrose	29.8	32.5	12.2	10.4	10.8	11.0	7.8	7.3	9.9	9.4
0.10% pectin	43.0	45.7	30.2	30.4	28.3	32.6	23.9	21.3	26.9	30.5
10% sucrose plus 0.10% pectin	33.8	32.1	25.3	26.1	16.9	24.9	21.7	27.5	20.5	15.9

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The present results for solutions acidified after cooling (Table XXVI) can be compared with those in Table XXV for solutions acidified before cooling. Even allowing for the different initial TEL contents, solutions acidified after cooling, with the notable exception of those containing pectin, reached about the same TEL content (approximately 10 ppm) within 20 days storage as those acidified before cooling.

That it was the pectin which delayed the decline in TEL concentration in the solutions is evident from the values obtained at each of the various storage periods. Those solutions containing only sucrose behaved in an almost identical manner to those containing only water. On the other hand, the presence of sucrose and pectin together considerably reduced the effectiveness which pectin by itself exhibited in keeping the TEL in solution.

The origin of this effect of pectin, which was not observable in solutions acidified before cooling, would appear to lie in its inhibition of lactonization of the hydroxyacid by reason of the structure it imposes on the solution. As discussed before, this structure could inhibit lactonization by preventing the hydroxyl group coming close enough to the carboxylic acid group for reaction to occur between them.

In the case of pectin solutions acidified before cooling, the lactonization process had already occurred to a considerable extent under the influence of residual heat (and hydrogen ions) in the cooling solution when the pectin structure had not been fully established. As the limonin passed out of solution, the hydroxyacid - lactone equilibrium readily shifted in compensation towards the lactone.

In the solutions cooled before acidification, lactonization occurred less spontaneously in the cooling solutions since acid catalysis would be negligible. When the residual hydroxyacid is placed in a structured pectin environment, even one containing acid, lactonization is still limited from the steric consideration mentioned above.

It is noteworthy that although unacidified sucrose solutions retained very high TEL contents on storage (Table XXIV), a comparatively rapid fall in TEL content occurred in acidified sucrose solutions, irrespective of when the acidification occurred. Apparently the effect of sugar in promoting the retention of high TEL contents is nullified by the presence of acid.

These results confirm the conclusions of Chandler (1971b) who suggested that the function of pectin was to hold the limonin in solution in citrus juice. However, as the results in Section 3.2. showed, degradation of juice pectin *in situ* does not cause the limonin to pass out of solution any more rapidly than in juices where the native pectin remains undegraded. This suggests that the molecular species of pectin and limonin present in the model solutions are different from those present in citrus juices, even though the analytical procedures used do not distinguish between them. Thus the above results have only a partial relationship to events occurring in natural juice systems.

Previous results have shown that high concentrations of limonin cannot be achieved in model aqueous solutions without the use of heat even in the presence of pectin, sugar and acid. Thus the possibility exists that some other juice component associates with the limonin in solution, leading to concentrations far in excess of the equilibrium concentration (5 ppm at 25 C).

Two juice constituents which could associate with limonin are the lipid and protein; both could interact with the hydrophobic backbone of the limonin molecule to form a weak but effective association. Therefore, experiments were undertaken to investigate this possibility.

4.6. THE SOLUBILITY OF LIMONIN IN LIPID-CONTAINING SOLUTIONS

4.6.1. Experimental

Initially an attempt was made to extract the lipids from New Zealand grapefruit segments using the method of Shomer *et al.* (1975). This involved extracting segments in a Soxhlet apparatus with solvent, using diethyl ether which removed only lipids, instead of chloroform which removed both lipids and carotenoids. However, yields were extremely low, and because fresh grapefruit were in short supply (it was the end of the season) and the extraction process was time-consuming, an alternative approach was adopted.

This approach consisted of finding an oil which had a fatty acid composition similar (if not identical) to that found in citrus lipids. According to Nagy (1977), the major fatty acids in grapefruit juice are palmitic (23%), oleic (24%) and linoleic (35%). After reference to a standard text on lipids (Hilditch and Williams 1964), a sample of purified corn oil was used in the subsequent experiments as a substitute for citrus lipids, since it had a similar lipid composition. The fatty acids in the corn oil were determined as their methyl esters by GLC using a Hewlett Packard gas chromatogram fitted with a hydrogen flame ionization detector and a DEGS polyester column. (This analysis was kindly performed by Mr. G.A. Ramsay of the Biotechnology Department, Massey University).

After a preliminary trial, an aqueous solution containing lipid was prepared by homogenizing distilled water (2 L) and corn oil (2 mL) at 34,500 kPa (5000 psig) for 5 min in an homogenizer (Alfa Laval Pilot model). The resulting solution was stable when centrifuged at 2300 x *g* for 20 min and no separation occurred after storage in a measuring cylinder at room temperature for 6 weeks. Microscopic examination revealed that the diameter of the oil particles ranged from 1 - 15 micron, the majority of particles having a diameter of 5 micron. A fresh solution was prepared and used for the experiments below on the next day.

The lipid-containing aqueous solution (100 mL) was placed together with citric acid (1 g) in a 250 mL conical flask and the pH of the solution was adjusted to 3.2 with 4 N NaOH. Sodium metabisulphite (20 mg) and powdered limonin (10 mg) were added and the flask was shaken in a reciprocating water bath at 25 C for 5 days. A 20 mL sample was withdrawn for limonin analysis 2 h after shaking had ceased. The experiment was carried out in triplicate.

4.6.2. Results

The fatty acid composition of the corn oil is presented in Table XXVII.

Table XXVII Fatty acid compositions of corn oil and grapefruit juice lipids

Fatty acid	Corn oil	Grapefruit juice lipids[a]
Palmitic (16:0)	11.1%	22.7%
Palmitoleic (16:1)	0.1	3.7
Stearic (18:0)	2.8	0.0
Oleic (18:1)	28.6	23.9
Linoleic (18:2)	55.6	34.5
Linolenic (18:3)	1.1	8.8

a = after Nagy (1977).

The limonin concentration in the lipid-containing aqueous solutions after 5 days shaking was 5.1 ppm with a standard error of ± 0.3 ppm.

4.6.3. Discussion

The fatty acid composition of the corn oil used in this experiment was similar to that of the lipids in grapefruit juice, the major differences being that the corn oil had a lower level of palmitic and linolenic acids but a higher level of linoleic acid. These differences were unlikely to have significantly affected the results obtained in this experiment.

The concentration of limonin in the lipid-containing solutions after 5 days was not measurably different from that obtained when limonin and water are shaken together. Thus, as the lipid has not increased the limonin solubility, it can be assumed that the limonin and the lipid molecules have not associated to the extent that above normal concentrations of limonin would be obtained in the cold. It is therefore concluded that citrus juice lipids are not responsible for the comparatively high concentrations of limonin found in citrus juices.

4.7 THE SOLUBILITY OF THE PROTEINS IN NEW ZEALAND GRAPEFRUIT JUICES

4.7.1. Background

According to Smith (1925), under ordinary conditions citrus protein is insoluble within the juice sacs since the isoelectric point of the protein is 4.7 compared to the juice pH of around 3.2. If this is correct, i.e. if the protein present in citrus juices is in an insoluble form, then any association between limonin and protein would lead to measurable limonin concentrations in citrus juice cloud. In contrast, earlier trials (Section 3.1.) showed that the limonin concentration of the clear supernatant was (within experimental limits) not measurably different from that in cloudy juice. However, to resolve completely the question of a possible association between limonin and citrus protein, it was necessary to establish whether there was indeed any soluble protein in citrus juices with which limonin could be associated to increase its solubility in the juice.

4.7.2. Experimental

Two cans of New Zealand grapefruit juice which had been extracted using a Kenwood extractor, processed as described in Section 2.1.4. and then stored at 2 C for five months were opened and their contents mixed. The usual parameters used to characterize citrus juices were measured. Juice (200 mL) was placed in each of two 250 mL conical flasks, together with sodium metabisulphite (40 mg). The pectolytic

enzyme Rohament P (Rohm GmbH, Darmstadlt) was added (0.10% w/w) to one of the flasks. The flasks were placed in a 30 C incubator for 48 h and then at room temperature (20 ± 4 C) for a further 48 h.

The supernatants and shaken juices from both samples, as well as the sediment from the enzyme-treated sample were analysed for protein content using both a modified Folin-Ciocalteau method (Potty 1969) and an automated micro-Kjeldahl digestion procedure; this latter analysis was kindly performed by the Analytical Section, New Zealand Dairy Research Institute, Palmerston North. In addition, the juice turbidity (A_{660c}) and the limonin concentrations were measured.

4.7.3. Results

The canned juice had a pH of 3.9; a soluble solids content of 10.5^{O}B; a titratable acidity of 0.74 g citric acid/100 mL; a Brix:acid ratio of 14.2:1; and a pulp content of 7%.

The protein contents of the juices are shown in Table XXVIII, together with the juice turbidity measurements.

The protein content of the sediment from the juice treated with pectic enzyme was 910 mg/100 mL of sediment as determined by the Kjeldahl method, while the protein content of a 1% w/v solution of Rohament P was 76 mg/100 mL as determined by the Potty method and 110 mg/100 mL as determined by the Kjeldahl method.

The limonin concentrations in the juice samples were 23.1 ppm with a standard error of ± 0.9 ppm.

4.7.4. Discussion

The results in Table XXVIII indicate that approximately 90% of the protein as determined by either the Potty or the Kjeldahl method was present in a soluble form in New Zealand grapefruit juice. Also the Kjeldahl protein contents for the supernatants from untreated and enzyme-treated juices were not significantly different, even though the supernatant of the enzyme-treated juice was virtually water-clear, all

Table XXVIII Protein content and juice turbidity of New Zealand grapefruit juice

Sample	Protein content (mg/100 mL juice)		Juice turbidity
	Potty method	Kjeldahl method[a]	(A_{660c})
Control juice (no enzyme treatment):			
Supernatant	313	490	0.848
Whole juice	333	530	0.612
Treated juice (added pectic enzyme):			
Supernatant	220	490	0.063
Whole juice	285	560	0.050

a = protein calculated from total nitrogen x 6.38.

the suspended cloud material having settled out. The soluble protein was therefore not precipitated when the cloud suspension was disrupted by enzyme action. The discrepancy between the corresponding figures using the Potty assay could be explained by the precipitation with the cloud of water-soluble compounds interfering with this analytical method. The lower value of the enzyme-treated juice compared with its untreated counterpart suggests some other kind of interference in the Potty method. Because the actual reaction on which the Potty method is based is unknown, the nature or effect of the interfering compounds is uncertain. Although Potty demonstrated that his procedure overcame interference from phenols and pectins, he provided no comparative protein values obtained using a Kjeldahl digestion. The present results suggest that the Potty method is subject to considerable interference from water-soluble juice components which (unlike Kjeldahl-detectable nitrogen compounds) are precipitated when the cloud suspension is disrupted.

The above discussion has taken into account the presence of added protein in those samples treated with the enzyme. The protein analysis of Rohament P (76 and 110 mg/100 mL by the Potty and Kjeldahl methods respectively) indicates that this enzyme preparation is only 11% protein, the remainder presumably being an inert carrier such as diatomaceous earth. On this basis, the protein content of the enzyme-treated juice would be expected to be 11 mg higher than that for the untreated juice.

4.7.5. Conclusion

This experiment has demonstrated that 90% of the proteins present in both cloudy and clarified New Zealand grapefruit juices are in a soluble form. Such information does not appear to have been reported previously and in fact disagrees with the statement of Smith (1925) concerning the insoluble nature of citrus juice proteins. Since 90% of the protein in New Zealand grapefruit juice is in the soluble form, a limonin-solubilizing interaction between limonin and protein could account for the high limonin solubility in citrus juices. Therefore, this possibility was investigated.

4.8. THE SOLUBILITY OF LIMONIN IN PROTEIN-CONTAINING SOLUTIONS

4.8.1. Experimental

Juice (10 L) was extracted from mature New Zealand grapefruit (20 kg) using a Kenwood extractor and screened by passing through a pulper/finisher fitted with a 100 mesh screen. Protein was extracted from the juice using the procedure described by Townsley *et al.* (1953). Briefly, the juice was centrifuged at 2300 x *g* for 30 min and the precipitate (520 mL) containing the chromatophores was retained. An equal volume of 95% ethanol containing 1% NaOH was added and the precipitated pectin was removed by filtration through a double layer of cheese cloth. On lowering the pH of the clear, dark orange solution from 12.2 to 4.7 with glacial acetic acid, the protein precipitated out; it was recovered by filtering through Whatman No. 541 filter paper. Approximately 50 mL of an orange-yellow material was obtained which could not be dispersed in water. On refluxing this material with acetone in a Soxhlet extractor (five cycles), substantially all the colour was removed, leaving a pale cream powder (7.55 g wet wt.). The protein content of the powder was determined by the method of Potty (1969).

A problem arose when an attempt was made to disperse the protein powder in an aqueous solution. However, when the protein was dispersed in an aqueous NaOH solution at pH 7.9, experiments showed that the pH could not be lowered to 3.2 without the protein precipitating out, no matter how rapidly the pH was lowered. It was therefore decided to attempt to reproduce a citrus juice system by the reverse process, i.e. by dispersing the protein in 1% citric acid (pH 2.23) and then raising the pH to 3.2 with 4 N NaOH. Provided that mixing took place in an ultrasonic water bath for 3 min, effectively all the protein was dissolved by this procedure. The protein content of the solution was determined using the method of Potty (*ibid.*).

A solubility trial was set up in triplicate, in which three 250 mL conical flasks containing the protein solution (100 mL),

sodium metabisulphite (20 mg) and powdered limonin (10 mg) were shaken at 25 C for 5 days. Although after this time it was found that some of the protein had precipitated out, it could be almost completely redispersed by gentle swirling of the flasks. Nevertheless, it was difficult to withdraw a sample for limonin analysis without possibly sucking some undissolved limonin crystals into the pipette, since the sample could not be sucked through Whatman No. 541 filter paper as was possible in the limonin analysis of model solutions. However, by leaving the flasks undisturbed for several hours and carefully placing the tip of the pipette only a few millimetres below the surface of the solution, it was possible to withdraw a sample for limonin analysis.

4.8.2. Results

The protein content of the powder was 50% and the protein content of solutions prepared from it were 335 mg/100 mL.

The limonin concentration of the solutions was 5.2 ppm with a standard error of ± 0.3 ppm. As well, some of the solution was filtered through Whatman No. 541 filter paper in a Buchner funnel and then analysed for limonin, when a concentration of 4.9 ppm with a standard error of ± 0.3 ppm was obtained.

4.8.3. Discussion

This trial has demonstrated the unlikelihood of any association between limonin and soluble protein that would account for the comparatively high concentration of limonin in grapefruit juice. However, since isolation procedures may have affected some change in the protein structure, the possibility of such an association cannot be entirely ruled out. From the discussion in the previous section it is unlikely that there is any association between protein (soluble or insoluble) and limonin leading to the incorporation of limonin into the cloud fraction by formation of an insoluble limonin-protein complex.

4.9. GENERAL DISCUSSION

Experiments with model solutions have shown that it is only possible to achieve high concentrations of limonin by the application of heat. The equilibria operating in heated solutions leading to the establishment of such high concentrations apparently involves the hydroxyacid form of limonin. The high concentrations that have been reported in citrus juices cannot be explained satisfactorily by the presence of pectin, sugars, protein and lipids. Therefore the precursor of limonin must be involved.

Since a hydroxyacid form of limonin has been postulated to be the naturally occurring precursor of limonin in processed orange juice, it was appropriate to undertake a detailed study of the changes occurring in the limonin and total extractable limonin (TEL) contents of citrus juice. The studies already discussed with model solutions of limonin and hydroxyacid should help to interpret these changes, so leading to a better understanding of the phenomenon of delayed bitterness. As the literature review indicated, neither the precursor theory nor the diffusion theory adequately explains all the observations concerning this phenomenon.

CHAPTER FIVE

THE SOLUBILIZATION OF LIMONIN IN CITRUS JUICES AND FACTORS CONTROLLING ITS CONCENTRATION

5.1. FORMS OF LIMONIN PRESENT IN HEATED SOLUTIONS

5.1.1. Background

After the experiments described in Sections 4.1. - 4.5. inclusive had been completed, Chandler and Nicol (personal communication) provided details of a procedure which they had only recently developed for the examination of the limonin and limonin precursor contents of parts of citrus fruits. The procedure involved extraction of the sample at two different pH levels, with and without the application of heat.

For acidic samples (such as orange juice) an aliquot was adjusted to pH 5.6 before extraction with the organic solvent, which would then remove only limonin, since the acidic limonin precursor would be fully ionized and so would remain preferentially in the aqueous phase. A second aliquot of the acidic sample was extracted without pH adjustment, so giving the TEL content (limonin plus precursor) of the sample.

For nearly neutral samples (such as orange albedo which has a natural pH about 5.6), extraction was made of an aliquot without pH adjustment, only limonin then being removed, and a second aliquot was adjusted to pH 3.2 and heated (5 min at 90 C) before being extracted to give the total potential limonin content.

In both cases the difference between the extractions at the pH levels provided a measure of the precursor content.

This procedure depends on the extraction of acidic precursor at pH 3.2 and its conversion to limonin during the analytical procedure, in the same way as the hydroxyacid was extracted from model solutions examined in Sections 4.1. - 4.5. and analysed as limonin.

The results obtained by Chandler and Nicol (personal communication) could be best interpreted in this way since no additional limonoid spots showed on TLC of samples with

pH adjusted to 5.6 to match the increased limonin spot in the samples extracted at acidic pH. Although it would be impossible to prove this conversion without isolation of the precursor, the basic assumption regarding the non-extractability at pH 5.6 of the hydroxyacids derived from limonin and presumably therefore the acidic limonin precursor has been confirmed by Johnson and Chandler (personal communication).

5.1.2. Experimental

Powdered limonin (20 mg) was added to two 250 mL round-bottomed flasks, one containing distilled water (100 mL) at pH 6.3, and the other 1% w/v citric acid (100 mL) adjusted to pH 3.2 with 0.1 N NaOH. Each flask was then refluxed for 96 h, and one hour after boiling had ceased the pH of the solutions was measured and samples removed for limonin analysis, the extracting solvent being chloroform. The pH of the solution in distilled water was then adjusted to pH 3.2 by the addition of 10 N HCl, and it was refluxed for 5 min and sampled 1 h later for limonin content as described above. The pH of the citric acid solutions was adjusted to pH 5.6 with 0.1 N NaOH and also sampled for limonin. The experiment was performed in triplicate.

5.1.3. Results

These are summarized in Table XXIX.

5.1.4. Discussion

As before (Section 4.1.), the changes in the pH of limonin solutions in distilled water on refluxing and cooling can be explained in terms of the opening and closing of one or more of the two lactone rings in the dissolved limonin and the subsequent ionization of the hydroxyacid(s). The pH of the limonin solutions in aqueous citric acid remained constant because of the buffering effect of sodium and citrate.

Although chloroform was used as the extractant in this experiment instead of chloroform:ethanol normally used, the TEL contents recorded for heated aqueous solutions (107.0 and 114.0 ppm) were of the same order as those reported earlier

Table XXIX Results of analyses[a] of refluxed limonin solutions

	Distilled water	Aqueous 1% citric acid[b]
pH values:		
Initial	6.3 ± 0.0	3.2 ± 0.0
1 h after end of reflux (53 C)	5.1 ± 0.2	3.2 ± 0.1
4 h after end of reflux (25 C)	6.8 ± 0.1	3.3 ± 0.0
Total extractable limonin (ppm):		
Extracted at pH 3.2	107.3 ± 1.5[c]	107.0 ± 1.7
Extracted at pH 5.6	114.0 ± 4.4	23.0 ± 1.7

a = presented as means with standard errors.
b = adjusted to pH 3.2 with 0.1 N NaOH.
c = solution refluxed 5 min after acidification with 10 N HCl.

5.1.4. Discussion (continued)
(Section 4.1.2.) using mixed solvents.

The major difference appears in the TEL content of the acidic solution when it is extracted at pH 5.6 and can be explained in terms of hydrolysis of limonin to its hydroxyacid, as discussed earlier (Section 4.1.3.). That is, from these results the "true" limonin solubility in refluxed acidic aqueous solutions is 23.0 ppm; this may be compared with the value for "true" limonin solubility estimated from the solubility curve (Fig. 4) as 8.5 ppm. The discrepancy here may be due to errors arising from extrapolation of this curve or to the fact that some of the hydroxyacid (possibly the unionized portion) had been extracted at pH 5.6.

As before, the apparent anomaly in the similarity of the limonin solubilities in aqueous solutions at pH 3.2 and 5.6 is eliminated when it is considered that the solution at

pH 5.6 was not buffered and extraction with solvent would effectively shift any ionized hydroxyacid toward the unionized state (Fig. 5).

Therefore this procedure for limonoid analysis involving extraction at two pH levels was used in subsequent studies on the solubilization of limonin in citrus juices.

5.2. EFFECT OF PASTEURIZATION ON LIMONOID CONTENTS OF ORANGE JUICES AND SERA

5.2.1. Experimental

Washington Navel oranges, which had been picked at Gosford, Australia, were fumigated with ethylene dibromide and air-freighted to Massey University, Palmerston North. Although the following experiments were commenced within three days of picking, they were not completed for up to two weeks, during which time the fruit was stored in a chill room at 5 C. No other material was in the chill room and it was assumed that no significant changes in limonoid content occurred during chill storage, since no such changes have been observed in freshly picked Navel oranges similarly stored in Sydney, Australia (Nicol, personal communication).

Juice was extracted from the oranges using a Kenwood extractor and passed through a pulper/finisher fitted with a 100 mesh screen. One batch of the juice was left at the natural juice pH of 3.2 and the other was adjusted to pH 5.6 with 1 N NaOH. Samples of the two juices were subjected to various treatments as recorded in Table XXX, and the resultant products were stored at room temperature, together with the sera prepared from them by centrifugation at 800 x *g* for 10 min.

Limonoid analyses were carried out on the samples after various periods of storage. To facilitate comparisons between samples with and without pH adjustment, water was added to the samples with natural pH in volume equivalent to that needed for the adjustment of pH; consequently no allowance was made for dilution of the pH-adjusted samples when limonoid

analyses are reported.

5.2.2. Results

The changes in limonoid content of the Navel orange juices subjected to the various treatments are presented in Table XXX.

5.2.3. Discussion

For the purposes of this discussion an initial assumption will be made that there was no difference between the results obtained using whole juice and serum. In fact, as Table XXX shows, this assumption is not true in all 38 cases (there were five exceptions) but these exceptions will be discussed later.

In control juice and serum (A) allowed to stand without any treatment, TEL appeared in solution according to the relationship:

$$TEL = A - B \log_e t$$

where A is the TEL content when $t = 0$; B is a "rate of change" constant; and t is the holding period in hours. Although this relationship was statistically significant at the 99.9% level, it has no physico-chemical meaning and a more meaningful relationship was therefore sought.

This was done by assuming that the samples initially contained 14 ppm of a non-extractable limonin precursor which disappeared (mole for mole) from solution as TEL was formed. Using the mean values from the control juice and serum, the data can be fitted (significant at 99.9% level) to the relationship:

$$\log_e \frac{a}{a - x} = kt$$

where a is the precursor limonin content (ppm); x is the TEL content when $t = 0$; t is the holding period in hours; and k is the specific reaction rate constant.

The above relationship describes the kinetics of a first order irreversible reaction in which a non-extractable limonin precursor, hereafter called protolimonin A, is

Table XXX Limonin analyses of Navel orange juices and sera[a] subjected to various treatments

Treatment before storage	Period after extraction (hours)				
	1	2	8	17	41
With no pH adjustment before pasteurization:					
A: control; no treatment	1.8	3.6	8.4	11.6	13.0(9.7)
B: pasteurized[b]	14.1	13.7	14.0	13.5	15.6
C: pasteurized[c]	9.3	9.2	9.0	8.7	10.8(13.7)
D: pasteurized[b]; pH adjusted[d] to 5.6	6.6	7.3	7.4	7.9	7.4
With pH adjustment to 5.6 before pasteurization:					
E: control; no treatment	<1	<1	2.3(<1)	4.0(<1)	4.5(1.7)
F: pasteurized[b]	n.d.	3.1	3.4	4.0	4.5
G: pH adjusted[d] to 3.2; pasteurized[c]	8.3	9.0	8.7	11.3	14.3
H: pasteurized[b]; pH adjusted[d] to 3.2; repasteurized	n.d.	11.4	13.2	14.6	13.6

a = sera were prepared from unpasteurized juices within 20 min of extraction and from pasteurized juices within 40 min of extraction; limonin analyses for sera were not significantly different from those for juices except where two results are given, that in brackets being the serum result.

b = pasteurized within 20 min of extraction. c = pasteurized within 40 min of extraction.

d = pH raised with 1 N NaOH; pH lowered with 10 N HCl.

converted into TEL. The low TEL value in the serum after 41 h compared with the juice could be due to incomplete leaching of precursor from albedo before centrifugation.

Highly significant differences in TEL content were obtained depending on whether or not the samples were pasteurized 20 (B) or 40 (C) min after juice extraction, the former giving higher total limonin contents than the latter. These differences could have been due to a limonin precursor degrading (LPD) enzyme present in the albedo particles (Nicol and Chandler 1978) being sufficiently active at pH 3.2 in the additional 20 min to degrade 4.5 - 5.0 ppm of the limonin precursor in juice held longer before pasteurization. This is a high level of activity for this enzyme, which shows optimum activity at pH 5.6, but not enough is known about variations in its activity (except that it is most active over a limited period of the year) for this suggestion to be supported by previous reports. The high limonin analyses recorded for the late (i.e. 40 min) pasteurized serum after 41 h compared with the juice also suggests LPD activity in the juice sample before pasteurization. This difference might have been expected to show up at earlier withdrawals but with albedo particles in the juice being both the source of the limonin precursor and the LPD enzyme, the system is probably too complex to give such a direct result.

The final value recorded for the limonin concentration in the unpasteurized juice (A) was intermediate between the values recorded for the two pasteurized juices. In the unpasteurized juice, activity of the LPD enzyme would have continued until it was stopped by hydrogen ion inactivation or product inhibition. It is therefore to be expected that the final recorded value in the unpasteurized juice would be lower than that of the pasteurized juices. This is the case with the juice pasteurized 20 min after extraction (B) but not with the juice pasteurized 40 min after extraction. The fact that this latter juice had a lower value than the control juice suggests that the conversion of protolimonin A into extractable limonin is more efficient (though less rapid) in juice allowed to stand at room temperature than in

processed juice where side reactions may reduce the efficiency of the conversion.

Over the 40 h period there was only a slight increase in TEL in both pasteurized juices, suggesting that very little precursor remained after pasteurization, some being converted to TEL with the remainder otherwise degraded.

When pasteurized juice was adjusted to and held at pH 5.6 so that no acidic-type limonoids were extracted by the solvent (D), the actual limonin analyses soon levelled out at 7.5 ± 0.3 ppm. This result suggests that about 85% of the TEL in the normal pasteurized sample was actually limonin. On the other hand, when the juice was adjusted to a pH of 5.6 without pasteurization (E), the actual limonin contents were low, increasing from 1 ppm to 4.5 ppm after 40 h. The conversion of precursor to extractable limonin would appear to be more rapid at natural juice pH than at pH 5.6.

In samples pasteurized at pH 5.6 (F), there was an apparent slight increase in actual limonin from 3.1 ppm at 2 h to 4.5 ppm at 41 h, suggesting that conversion of precursor limonin was not complete under these conditions. In juices adjusted to pH 5.6 (E and F) there was no difference in the final limonin contents, whether the juices had been pasteurized or not.

When samples pasteurized at pH 5.6 were readjusted to pH 3.2 and repasteurized (H), high TEL contents were again recorded, though not quite as high as in juices pasteurized directly at pH 3.2, possibly because side reactions undergone by the precursors at pH 5.6 reduced the overall conversion to limonin. In these samples also there was evidence for a more significant increase in TEL contents during storage than in samples pasteurized directly at pH 3.2 (B and C). Although the evidence is scanty, it is possible that treatment at pH 5.6 converted the precursor to a product which was less readily converted to TEL at pH 3.2 than the precursor itself. That this conversion of precursor at pH 5.6 did not require heat is indicated by the fact that the increase in TEL contents

in the stored juice was even greater when the juice (G) was not pasteurized at pH 5.6 before acidification and pasteurization at pH 3.2.

However, comparisons of the samples pasteurized (H) and unpasteurized (G) at pH 5.6 before adjustment to pH 3.2 and pasteurization confirm the conversion of precursor to TEL at pH 5.6, previously demonstrated by comparison of juices (F and E) stored with and without pasteurization at pH 5.6.

This work demonstrates that the solubilization of limonin in citrus juice is a more complex process than had previously been suggested. During the period over which bitterness slowly develops in the freshly extracted juice, or during the short pasteurization treatment, considerable changes occur qualitatively and quantitatively in the limonoid composition of the juice. Subsequent experiments sought a better understanding of the processes involved.

5.3. EFFECT OF HOLDING TIMES ON LIMONOID CONTENTS OF UNPASTEURIZED JUICES AND SERA

5.3.1. Experimental

Carter Navel oranges picked from the Massey University orchard were used for the following experiments. The fruit not used immediately was stored for a maximum of 2 days in a chill room at 5 C before being used.

Juice was extracted from the fruit using a Kenwood extractor and passed through a pulper/finisher fitted with a 100 mesh screen before being subjected to the following treatments:

(a) held at room temperature and sampled periodically for analysis as whole juice;
(b) centrifuged immediately and the serum held at room temperature and sampled periodically;
(c) held at room temperature for 1 h before being centrifuged: serum held at room temperature and sampled periodically;
(d) held at room temperature for 4 h before being centrifuged: serum held at room temperature and sampled periodically.

At each withdrawal, three samples of juice or serum were taken and the limonin content determined in each as follows: the first sample was extracted without further treatment; the second sample was adjusted to pH 5.6 prior to extraction, while the third sample was heated at 90 C for 5 min and rapidly cooled before commencing the limonin determination.

5.3.2. Results

These are summarized in Tables XXXI to XXXIV.

5.3.3. Discussion

The most striking feature of these results is the evidence they provide for the presence of two limonin precursors in orange juice. On examination of Table XXXI, it is seen that both TEL extractable at pH 5.6 (which represents dilactone limonin) and TEL directly extractable at pH 3.2 (which has been assumed to measure limonin and its acidic precursor) increase slowly on standing. Therefore there must be a second limonin precursor, not extractable at either pH 3.2 or 5.6, to account for these increases.

The existence of this second non-extractable precursor is further demonstrated by the results of TEL analyses made after heating the juice at pH 3.2, this treatment converting the second precursor to TEL. Whereas the TEL content determined after heating reaches a maximum after about 24 h, neither the TEL extractable at pH 5.6, nor that extractable at pH 3.2, reach a maximum, the latter always being slightly but significantly higher than the former. In subsequent discussions, these two precursors will be referred to as protolimonin A and protolimonin B.

Protolimonin A is the non-extractable precursor, presumably a much more water-soluble compound than either limonin or protolimonin B; it could be a strong acid and/or a compound with several hydrophilic groups. It is converted to limonin or protolimonin B by heat treatment or by allowing the juice to stand at room temperature. Protolimonin B is a weakly acidic compound, extractable by organic solvents and readily converted to limonin during the analytical procedure.

Table XXXI Changes in the limonoid content[b] of Carter Navel orange juice

Time after extraction (h)	Limonoid content (ppm)						
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin	Increase in TEL[d] after heating[c]
0	<1	<1	13.6	>17.8	0	<1	>12.6
½	<1	<1	12.2	>17.8	0	<1	>11.2
1	<1	<1	13.2	>17.8	0	<1	>12.2
2	1.7	<1	13.2	17.1	>0.7	<1	11.5
4	3.4	2.4	17.6	15.4	1.0	2.4	14.2
8	7.1	4.7	17.2	11.7	2.4	4.7	10.1
24	15.0	13.4	19.1	3.8	1.6	13.4	4.1
48	18.1	15.5	18.5	0.7	2.6	15.5	0.4

a = see footnotes on next page; the maximum TEL content in the juice after heating was taken as the mean of the last two withdrawals (18.8 ppm).

Footnotes for Table XXXI:

b = limonin was measured by extraction after pH adjustment to 5.6; protolimonin A was estimated as the difference between the maximum TEL content (limonin and proto-limonins) determined after heating[c], and the TEL content determined at pH 3.2 at that withdrawal; protolimonin B was estimated as the difference between TEL analyses at pH 3.2 and 5.6.

c = 5 min at 90 C.

d = the amount of limonin formed from the protolimonins on heating[c] is represented by the difference between TEL at pH 3.2 in heated and unheated samples.

Table XXXII[a] Changes in the limonoid content[b] of Carter Navel orange serum prepared immediately after juice extraction

Time after extraction	Limonoid content (ppm)						
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin	Increase in TEL[d] after heating[c]
0	<1	<1	n.d.	>23.9	0	<1	-
20 min	<1	<1	12.6	>23.9	0	<1	>11.6
40 min	<1	<1	n.d.	>23.9	0	<1	-
1 h	<1	<1	11.9	>23.9	0	<1	>10.9
2	1.7	<1	n.d.	23.2	>0.7	<1	-
4	3.4	2.5	15.0	21.5	0.9	2.5	11.6
8	9.0	7.0	15.3	15.9	2.0	7.0	6.3
24	11.8	7.9	n.d.	13.1	3.9	7.9	-
48	23.4	14.8	24.9	1.5	8.6	14.8	1.5

a = see footnotes for Table XXXI; the maximum TEL content in the serum after heating was taken as 24.9 ppm.

Table XXXIII[a] Changes in the limonoid content[b] of Carter Navel orange serum prepared 1 h after juice extraction

Time after extraction (h)	Limonoid content (ppm)						
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin	Increase in TEL[d] after heating[c]
1	2.4	1.9	9.0	12.6	0.5	1.9	6.6
2	3.6	1.9	10.3	11.4	1.7	1.9	6.7
3	3.9	3.5	10.3	11.1	0.4	3.5	6.4
7	5.2	4.8	10.2	9.8	0.4	4.8	5.0
24	14.2	9.8	15.0	0.8	4.4	9.8	0.8

a = see footnotes for Table XXXI; in this case the maximum TEL content after heating was taken as 15.0 ppm.

Table XXXIV[a] Changes in the limonoid content[b] of Carter Navel orange serum prepared 4 h after juice extraction

Time after extraction (h)	Limonoid content (ppm)					
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin B	Limonin	Increase in TEL[d] after heating[c]
4	4.5	1.0	12.2	3.5	1.0	7.7
5	3.4	1.6	11.5	1.8	1.6	8.1
6	7.8	2.0	12.4	5.8	2.0	4.6
24	8.2	7.2	14.9	1.0	7.2	6.7

a = see footnotes for Table XXXI; calculations of protolimonin A content have not been made because considerable amounts of protolimonin A were still present after 24 h, as shown by the difference between TEL determined before and after heating at pH 3.2. This means that the amount of TEL formed on heating after 24 h would not provide a realistic measurement of the maximum TEL content.

An estimate of protolimonin content at each withdrawal is provided by the difference between the maximum TEL content extractable at pH 3.2 after heating and the TEL content extractable at pH 3.2 at that withdrawal. This may provide an underestimate since the heat-catalysed conversion of protolimonin A into TEL (shown by the increase in TEL after heating) is only about 60 - 70% efficient. An estimate of protolimonin B content is provided by the difference between TEL extracted at pH 3.2 and 5.6. As previously, TEL extractable at pH 5.6 provides a measure of the dilactone limonin content. Tables XXXI to XXXIV show values for protolimonin A, protolimonin B and limonin calculated in this way, together with the amount of protolimonin A converted into TEL on heating.

From Table XXXI, it appears that the dilactone limonin content of the juice increases on standing while the protolimonin A content decreases. Little change occurs within the first 2 h and it is only after this period that appreciable amounts of protolimonin B accumulate, reaching a comparatively constant level after 8 h.

The serum from the juice immediately after extraction (Table XXXII), although showing the same general pattern in the changes in its limonoid content as the juice, showed some significant differences. Firstly, the final TEL extractable at pH 3.2 either with or without heating was considerably (30%) higher than the comparable value for the juice, indicating that the albedo-associated LPD enzyme had been active in the juice but not in the serum. Secondly, the protolimonin B content increased to a considerably higher level in the serum than in the juice, suggesting that it was the substrate for the LPD enzyme. A lower efficiency in the heat-catalysed conversion of protolimonin A to limonin can be noted in the serum but as yet no explanation can be offered for this.

Sera centrifuged 1 and 4 h after extraction (Tables XXXIII and XXXIV) showed no consistent significant differences in limonoid content from the serum prepared immediately (Table

XXXII). Limonin contents rose to a mean value of 8.3 ± 1.2 ppm after 24 h. Some differences showed up between the sera, most notably the almost complete disappearance after 24 h of protolimonin B from the serum prepared 1 h after extraction compared with its high concentration in the serum prepared later. However, there are no consistent effects that could be ascribed to the delay in centrifugation affecting either the extent of LPD enzyme activity or the diffusion of limonoids from the albedo particles. Instead, comparison with results from the juice itself suggest that the limonoids immediately passed into solution in the extracted juice, since they apparently reached a higher concentration in the serum prepared immediately than in the juice.

However, since albedo particles are the source of both limonoids and the LPD enzymes, it is possible that freshly extracted juice initially had a higher total limonoid content than recorded, and that degradation by the LPD enzyme was taking place contemporaneously with the diffusion of limonoids from the albedo. Such a dynamic situation could account for the fact that very little appears to have happened in either juice or sera during the first few hours after extraction. It could also account for the discrepancies that appear in the results recorded for sera. For example, at 24 h the TEL content (after heating) in the sera prepared 1 and 4 h after extraction (15.0 and 14.9 ppm) was lower than that in the juice (18.5 ppm) when it would be expected to be higher from comparison of the results from juice and serum prepared immediately.

5.4. EFFECT OF HEAT TREATMENTS ON LIMONOID CONTENTS OF JUICES AND SERA

5.4.1. Experimental

Carter Navel oranges were picked from the Massey University orchard and within one hour the juice was extracted from the fruit using a Kenwood extractor. After passing through a pulper/finisher fitted with a 100 mesh screen, the juice was divided into two parts: to one part was added sufficient 1 N NaOH (approximately 11% v/v) to raise the pH from 3.4 to 5.6. No pH adjustment or water addition was made to the other part, but to simplify comparisons, analyses reported take into account these dilutions.

The two juices were placed in conical flasks (500 mL) and heated rapidly to 90 C over a bunsen before placing in an oil bath maintained at 90 ± 1 C. The juices were sampled for limonin content periodically as follows. For the juice held at pH 3.4, two aliquots were taken at each sampling and prior to the limonin determination, one aliquot was adjusted to pH 5.6 with 4 N NaOH, the other aliquot being analysed directly. For the juice held at pH 5.6, three aliquots were taken at each sampling and prior to the limonin determination, one aliquot was adjusted to pH 3.2 with 10 N HCl and analysed without heating, one was adjusted to pH 3.2, heated to and held at 95 C for 5 min and then cooled rapidly to room temperature before analysis, and the other was analysed at pH 5.6.

Fruit picked at the same time as that used for the above experiment was held in a chill room at 5 C for 5 days before the juice was extracted and screened as described above and then centrifuged at 800 x *g* for 10 min to separate the insoluble solids. The serum was then treated in an identical manner to that described above for the juice.

5.4.2. Results

These are summarized in Tables XXXV to XXXVIII.

Table XXXV[a] Change in the limonoid content[b] of Carter Navel orange juice held at 90 C

Time after extraction (h)	Limonoid content (ppm)				
	TEL at pH 3.4	TEL at pH 5.6	Protolimonin A	Protolimonin B	Limonin
0	1.5	1.4	23.9	0.1	1.4
½	20.5	19.8	4.9	0.7	19.8
1	22.7	21.1	2.7	1.6	21.1
4	21.2	23.1	4.2	-	23.1
8	26.3	19.3	-	7.0	19.3
24	25.3	18.6	0.1	6.7	18.6
48	25.0	18.6	0.4	6.4	18.6
120	25.1	24.8	0.3	0.3	24.8

a = see footnotes for Table XXXI; calculation of protolimonin A content based on assumption that maximum TEL content at pH 3.4 (mean value at last four withdrawals 25.4 ppm) would not be different from maximum TEL content after heating at pH 3.4.

Table XXXVI[a] Changes in the limonoid content[b] of Carter Navel orange juice held at 90 C after adjustment of pH to 5.6

Time after extraction (h)	Limonoid content (ppm)					
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin
0	3.1	<1	23.2	19.1	>2.1	<1
½	15.8	12.7	22.2	6.4	3.1	12.7
1	20.7	15.8	22.2	1.5	4.9	15.8
4	18.9	17.4	22.1	3.3	1.5	17.4
8	22.1	17.1	21.2	0.1	5.0	17.1
24	20.9	20.0	21.7	1.3	0.9	20.0
48	21.2	18.3	21.2	1.0	2.9	18.3
120	19.5	17.6	21.7	2.7	1.9	17.6

a = see footnotes for Table XXXI; the maximum TEL content after heating was taken as the mean of all analyses (22.2 ppm).

Table XXXVII[a] Changes in the limonoid content[b] of Carter Navel orange serum prepared immediately after juice extraction and held at 90 C

Time after extraction (h)	Limonoid content (ppm)				
	TEL at pH 3.4	TEL at pH 5.6	Protolimonin A	Protolimonin B	Limonin
0	4.3	1.6	25.2	2.7	1.6
1	23.7	17.3	5.8	6.4	17.3
4	25.2	17.5	4.3	7.7	17.5
8	20.9	19.3	8.6	1.6	19.3
24	22.3	23.2	7.2	-	23.2
48	29.3	23.9	0.2	5.4	23.9
120	29.7	29.6	-	0.1	29.6

a = see footnotes for Table XXXI; calculation of protolimonin A content based on assumption that maximum TEL content at pH 3.4 (mean value at last two withdrawals 29.5 ppm) would not be different from maximum TEL content after heating at pH 3.4.

Table XXXVIII[a] Changes in the limonoid content[b] of Carter Navel orange serum prepared immediately after extraction and held at 90 C after adjustment to pH 5.6

Time after extraction (h)	Limonoid content (ppm)					
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin
0	6.3	<1	18.4	26.2	>5.3	<1
1	22.2	17.9	33.3	10.3	4.3	17.9
4	22.0	21.7	34.0	10.5	0.3	21.7
8	20.9	19.3	32.0	11.6	1.6	19.3
24	19.9	16.3	31.9	12.6	3.6	16.3
48	35.3	23.5	n.d.	-	11.8	23.5
96	29.8	20.2	31.1	2.7	9.6	20.2

a = see footnotes for Table XXXI; the maximum TEL content after heating was taken as the mean of the last five withdrawals (32.5 ppm).

5.4.3. Discussion

Before discussing the above results, reference must be made to the difficulties in establishing a true zero time for the systems under study. The very nature of the juice extraction process, especially from large batches of fruit, and the period required for centrifugation and pH adjustment, impose a barrier to ensuring the degree of uniformity that is possible with simple model systems. Consequently it is believed that the differences in the analytical results recorded for the various treatments at zero time are related not to the systems themselves but to unavoidable differences in the time at which the various operations were completed. Because of the hitherto unrecognized complexity of the processes involved in limonin solubilization and in the development of bitterness in citrus juices, even slight differences in timing of the operations could have a significant effect on the analytical values recorded.

Furthermore, it is noteworthy that some of the biggest differences occurred between juice and serum samples, most notably the TEL contents of the samples at pH 5.6. This difference did not, as might be expected, lie in a greater TEL content in the juice as a result of opportunity for more complete diffusion of limonoids from the albedo particles. Instead, it was the serum sample which had the greater TEL content. This difference could be ascribed to the activity of an LPD enzyme known to be associated with the albedo particles (Nicol and Chandler 1978), which was most active at pH 5.6 and would lead to higher TEL contents in serum despite the above mentioned diffusion process leading to higher TEL contents in the juice. However, these differences were recorded in samples which were rapidly heated to and held at 90 C. Since it is unlikely that the LPD enzyme could have remained active during this period, it would appear that the differences developed during unavoidable delays in manipulation of the samples. Indeed, it is impossible to develop a rational interpretation of the results without assuming such delays and associated variations in zero time.

The serum maintained at pH 3.4 showed a gradual increase in

dilactone limonin content (extractable at pH 5.6) over the 120 h period, after a rapid increase in the first hour. On the other hand, the TEL content (extractable at pH 3.4) rose, fell and then rose again, before becoming stable at 29.5 ± 0.2 ppm after 48 h. At the final sampling, the similarity in the values for TEL and limonin contents indicated that conversion of the protolimonins to limonin had been completed during storage.

The decline in protolimonin A was very rapid, being 80% complete in the first hour, while protolimonin B contents fluctuated widely as might be expected if it were an intermediate in the conversion of protolimonin A to limonin. Also in this system, evidence would be expected of the hydrolysis of limonin to hydroxyacid, such as occurs in heated model solutions (Section 4.1.). The hydroxyacid so formed is extractable at pH 3.4 but not at 5.6, and one would expect it to show up as an increasing difference in the two TEL analyses. The lactone-hydroxyacid equilibrium was established within 72 h at 100 C in solutions containing limonin and 1% citric acid, and while there is some evidence for the presence of such an equilibrium at 48 h, such evidence disappears at 120 h. It is difficult to see why the behaviour of the model solution has not been reproduced in the natural system.

In the juice maintained at natural pH, the changes in limonoid content generally followed the same pattern as those in the comparable serum. There was a major difference in the final limonin contents (about 20% higher in the serum) which could be accounted for by LPD enzyme activity in the juice. In the serum, the evidence for an hydroxyacid-lactone equilibrium was even stronger than in the juice over the period 8 - 48 h after extraction, with very high differences between the TEL extractable at pH 3.4 and 5.6, but again this difference disappeared after 120 h.

In the samples adjusted to pH 5.6, there was greater fluctuation in the analyses, apart from the remarkably constant values recorded for the TEL contents of juices heated at

pH 3.2 before analysis. Some of these fluctuations would suggest degradation of limonin on heating at pH 5.6, but since limonin is known to be completely stable under these conditions, apart from simple hydrolysis of the lactone rings (Emerson 1952), the fluctuations must be ascribed to analytical difficulties. Nevertheless, the general pattern of changes in the limonoid content of the samples at pH 5.6 is similar to that reported above for samples at pH 3.4. Limonin contents increased and protolimonin A contents decreased, both less rapidly than in the samples at pH 3.4.

It is noteworthy that the lowest TEL content was recorded for the juice sample at pH 5.6, possibly because the albedo-associated LPD enzyme (present in juice but not in serum) is more active at pH 5.6 than at pH 3.2. In contrast, the highest TEL contents were recorded for the serum sample at pH 5.6. This would suggest that heat treatment of limonin precursors at pH 5.6 followed by further heating at pH 3.2 is the most efficient method for their conversion to limonin. It was previously reported (Section 5.2.3.) that such pre-treatment had the reverse effect on final TEL contents, but the juices used in the two experiments were derived from different cultivars and had different limonoid contents. It is not suggested that processes occurring in these two juices are qualitativley different, but simply that they may be sufficiently different quantitatively for such apparent differences to appear in the analysis of the complicated equilibria involved.

Finally, the serum (but not the juice) at pH 5.6 showed definite evidence for the lactone-hydroxyacid equilibrium in the high values recorded for protolimonin B (about 30% of total limonoids) toward the end of the trial. Such a result is to be expected in samples naturally buffered at this high pH; in fact, the lack of evidence for the presence of hydroxyacid in the juice sample at pH 5.6 is surprising, and (as in the case of both samples at pH 3.2), no explanation can be offered for it.

5.5. CHANGES IN THE LIMONOID CONTENT OF NEW ZEALAND GRAPEFRUIT JUICE SERUM AND INSOLUBLE SOLIDS AT ROOM TEMPERATURE

5.5.1. Experimental

New Zealand grapefruit were picked from the Massey University orchard and held in a chill room at 5 C before being used for the following experiments. Fruit was stored for a maximum of 7 days after picking.

Juice was extracted from a random selection of the fruit using a Kenwood extractor and immediately centrifuged at 2000 x *g* for 4 min to separate the insoluble solids, the extraction and centrifugation steps being completed in 7 min. The serum was held at room temperature and sampled for limonoid content as follows. Three aliquots were taken at each sampling: one was analysed without any further treatment; one was analysed after raising the pH to 5.6 with 4 N NaOH, and the third was analysed after heating to and holding at 95 C for 5 min and then rapidly cooling to room temperature. To ensure that the extraction and centrifugation steps were carried out rapidly, only small quantities of fruit were juiced at any one time.

Ths insoluble solids were suspended in model solutions containing sucrose (10% w/v) and citric acid (1% w/v) at levels equivalent to that found in the extracted juice (15% v/v). The model solutions (pH 2.5) were held at room temperature and sampled periodically for limonoid content as described above for the sera.

The above studies for sera and model solutions containing insoluble solids were repeated with the inclusion of an additional step; this involved rapidly heating the samples immediately after preparation to 95 C, holding them at that temperature for 1 min and then quickly cooling to room temperature. This treatment was to inactivate any enzymes present.

5.5.2. Results

These are presented in Tables XXXIX to XLII.

Table XXXIX[a] Changes in the limonoid content[b] of New Zealand grapefruit juice serum prepared immediately after extraction and held at room temperature

Time after extraction[f] (h)	Limonoid content (ppm)					
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin
0	1.5	1.5	19.4	24.9	0	1.5
1	2.3	1.7	22.3	24.1	0.6	1.7
4	5.8	5.0	28.0	20.6	0.8	5.0
8	17.5	11.2	21.3	8.9	6.3	11.2
24	22.2	16.0	26.3	4.2	6.2	16.0
48	19.2	15.6	17.7	7.2	3.6	15.6
96	26.3	18.9	26.4	0.1	7.4	18.9

a = see footnotes for Table XXXI; the maximum TEL content after heating was taken as 26.4 ppm.
f = samples for analysis were taken from the same batch of juice at the storage periods linked by vertical lines.

Table XL[a] Changes in the limonoid content[b] of model solutions[e] containing suspended insoluble solids from New Zealand grapefruit juice and held at room temperature

Time after extraction[f] (h)	Limonoid content (ppm)					
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin
0	<1	<1	<1	>2.7	0	<1
1	<1	<1	<1	>2.7	0	<1
4	1.6	1.1	1.0	2.1	0.5	1.1
8	2.0	1.5	1.3	1.7	0.5	1.5
24	3.0	2.4	3.4	0.7	0.6	2.4
48	2.1	2.1	2.0	1.6	0	2.1
96	2.8	2.0	3.7	0.9	0.8	2.0

a = see footnotes for Table XXXI; the maximum TEL content after heating was taken as 3.7 ppm.

e = aqueous solutions containing 10% sucrose, 1% citric acid and 15% insoluble solids separated centrifugally from freshly extracted juice.

f = samples for analysis were taken from the same batch of juice at the storage periods linked by vertical lines.

Table XLI[a] Changes in the limonoid content[b] of New Zealand grapefruit juice serum held at room temperature after heat treatment (1 min at 95 C)

Time after extraction[f] (h)	Limonoid content (ppm)					
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin
0	6.8	6.0	16.3	13.3	0.8	6.0
1	7.6	5.8	15.9	12.5	1.8	5.8
4	8.2	5.4	16.4	11.9	2.8	5.4
8	10.4	7.6	16.5	9.7	2.8	7.6
24	15.3	7.7	15.1	4.8	7.6	7.7
48	20.6	20.0	19.7	-	0.6	20.0
96	17.8	18.3	20.1	2.3	-	18.3

a = see footnotes for Table XXXI; the maximum TEL content after heating was taken as 20.1 ppm.
f = samples for analysis were taken from the same batch of juice at the storage periods linked by vertical lines.

Table XLII[a] Changes in the limonoid content[b] of model solutions[e] containing suspended insoluble solids from New Zealand grapefruit juice and held at room temperature after heat treatment (1 min at 95 C)

Time after extraction[f] (h)	Limonoid content (ppm)					
	TEL at pH 3.2	TEL at pH 5.6	TEL after heating[c] at pH 3.2	Protolimonin A	Protolimonin B	Limonin
0	1.9	1.4	2.2	0.6	0.5	1.4
1	1.5	1.5	1.6	1.0	0	1.5
4	1.2	1.2	1.6	1.3	0	1.2
8	2.0	2.0	3.0	0.5	0	2.0
24	2.2	2.1	2.2	0.3	0.1	2.1
48	3.1	2.5	2.6	-	0.6	2.5
96	4.0	2.4	2.2	-	1.6	2.4

a = see footnotes for Table XXXI; the maximum TEL content after heating was taken as the mean of the last four withdrawals (2.5 ppm).

e = aqueous solutions containing 10% sucrose, 1% citric acid and 15% insoluble solids separated centrifugally from freshly extracted juice.

f = samples for analysis were taken from the same batch of juice at the storage periods linked by vertical lines.

5.5.3. Discussion

Before proceeding with the discussion, the point must again be made that the limonoid system in freshly extracted citrus juices is very labile, making it difficult to avoid variations arising from delays in manipulation. For example, in the results in Table XXXIX, the fluctuations in TEL contents could have arisen from such difficulties in manipulation. Two apparently anomalous results for TEL contents (after 8 and 48 h) can be explained by a lower limonoid content in the fruit used in this particular extraction, since all other values for TEL form part of a definite trend. In discussing the results of these experiments, therefore, emphasis will be placed on broad trends.

The results in Table XXXIX show a very similar trend to those presented in Table XXXVII (Carter Navel orange serum prepared immediately after juice extraction). In other words, the limonin and protolimonin B concentrations increased, while the protolimonin A concentration declined to zero. The TEL content after heating at pH 3.2 increased on storage to reach a maximum at 4 h which was significantly higher than the maximum limonin concentration at 96 h, indicating that conversion of protolimonin to limonin had not been completed.

The results in Table XL indicate that there were very small but measurable quantities of limonoids present in the insoluble solids of freshly extracted grapefruit juice, i.e. all the protolimonins and limonin did not pass into solution from the albedo immediately on juice extraction. As in the serum itself, the limonin content increased and the protolimonin A content decreased, but the conversion of protolimonin A to limonin was incomplete at the end of the trial.

The effect of a heat treatment (1 min at 95 C) on the changes in limonoid content of grapefruit juice serum can be observed by comparing the results in Table XXXIX (no heat treatment) with those in Table XLI. The TEL content did not increase to as great a concentration as in the comparable unheated serum. In the short heat treatment used, this result is unlikely to be due to heat-catalysed degradation of protolimonin A to a

product which did not behave as limonin during the analytical procedure. Instead, it suggests the presence of an enzyme or enzymes capable of converting protolimonin to limonin which were inactivated by the heat treatment. As a consequence of this inactivation, the dilactone limonin concentration increased at a slower rate in the serum which had been heated, even though it eventually reached a value similar to the maximum TEL content. In the absence of the enzyme, the conversion of protolimonin to limonin was accompanied by simultaneous degradation of some protolimonin to other products, thus reducing the potential limonin content by about 20%.

In the heated serum, conversion of protolimonins A and B to limonin was virtually complete after storage at room temperature for 48 h, whereas the conversion of protolimonin B to limonin was still incomplete after this time in the unheated serum. This result is easiest to interpret if protolimonin B is an intermediate in the conversion of protolimonin A to limonin. The sudden drop in protolimonin B content in the heated serum at 48 h could have resulted from the fact that by this time the conversion of protolimonin A to protolimonin B was complete and no further protolimonin B was being introduced into the system to match that lost by its conversion to limonin.

Similarly, in the heated model systems containing suspended insoluble solids (Table XLII), conversion of protolimonin B to limonin was complete within 24 h. Comparison of these results with those for comparable unheated samples (Table XL) failed to reveal any evidence for the action of albedo-associated enzymes.

5.6. GENERAL DISCUSSION

When juices were held at room temperature, both dilactone limonin and TEL concentrations eventually reached the same values, whereas when similar juice was heated, even just for 1 min at 95 C, the maximum TEL content was lowered, with the maximum limonin content being lowered still further. This result could be explained if an enzyme was destroyed at 90 C which at room temperature catalysed the conversion of some precursor to dilactone limonin. In the absence of the enzyme, conversion of precursor to limonin would still occur but at greatly reduced rates; the action of heat could catalyse the conversion, but at considerably reduced yields.

When freshly prepared serum was held at room temperature, the TEL content rose to a much lower level than that in the similarly treated juice. On the other hand, the limonin concentration rose to approximately the same value (lower than both TEL contents) in either serum or juice. This suggests the presence of an albedo-associated enzyme which was absent from the serum but which in the juice inhibited conversion of precursor limonin to limonin; the most likely activity of such an enzyme would be degradation of the precursor to some other product.

When freshly prepared serum was held at 90 C, limonin and TEL concentrations rose to approximately the same value; so did limonin and TEL contents in the juice, but the value was about 20% lower. These results can only be satisfactorily explained by the action of an LPD enzyme in the juice before the heat treatment could affect enzyme activity.

In freshly extracted juice, most but not all of the limonoids diffused into the juice during the extraction process. The amount still remaining in the albedo particles (about 3 ppm), though small, would just be sufficient to affect the bitterness in the final product as it slowly passed into solution on standing or more rapidly on heat treatment.

However, it is clear that the small amount of dilactone

limonin, either in solution or in albedo particles, was not the main origin of bitterness in the juices examined in this study. Development of high bitterness levels depended on the conversion into limonin of two limonin precursors, the water-soluble protolimonin A and the readily extractable and very labile protolimonin B. There was evidence that protolimonin B was not present as such in the fresh juice but was an intermediate in the conversion of protolimonin A to limonin. There was also evidence that the latter conversion was enzyme catalysed, although it could occur in the absence of the enzyme, and rapidly but less efficiently by heat catalysis.

Also affecting the limonoid composition of the juice was an LPD enzyme associated with the albedo particles. This enzyme appeared to act on protolimonin B to convert it to a compound which, in contrast to protolimonin B, was not extracted and analysed as limonin during the analytical procedure.

Heat treatment of juices, as in normal pasteurization, could actually reduce the potential limonin content of the juice or increase it, according to whether the limonin-promoting enzyme or the limonin-degrading enzyme was more active in the fresh juice. Similarly, the dominant effect of centrifugation was not to remove albedo particles early enough to prevent diffusion of limonoids into the juice so decreasing bitterness, but to remove the albedo-associated LPD enzyme from contact with the aqueous phase, so increasing bitterness.

On pasteurization of juices, protolimonin A was converted via protolimonin B into limonin, but this conversion was by no means complete, and indeed was not completed for many hours even when the juice was held at 90 C. This heat-catalysed conversion of protolimonin to limonin was less efficient (though more rapid) than the enzyme-catalysed conversion, and this is another reason why the dilactone limonin content of pasteurized juice fell a good deal below its potential value.

A number of factors are therefore involved in bitterness development in citrus juices. In addition to the limonoid

composition of the albedo and the albedo content of the juice, there are enzymes which promote and enzymes which prevent the conversion of limonin precursors into limonin. Variations in the final bitterness in the juice will occur according to the delay before removal of pulp from the juice, the delay before the application of any heat treatment, and the extent of the heat treatment. It is understandable under these circumstances why the literature abounds with so many conflicting reports on this subject, particularly in respect to the effect of certain processing variables.

Adding to this complication are the analytical difficulties involved in following the phenomenon of delayed bitterness, most notably the difficulty in establishing a realistic zero time for comparable experiments. Also, prior studies have been complicated by the failure to recognise that proto-limonin B behaves as limonin during the analytical procedures used in the past. Additional complications could arise by the establishment of the hydroxyacid-lactone equilibrium which developed in heated model solutions of limonin. Fortunately, for reasons yet unknown, this equilibrium does not appear to operate in citrus juices.

CHAPTER SIX

CONCLUSIONS

The work reported in this thesis has shown that the solubilization of limonin and the phenomenon of delayed bitterness in citrus juices are very complicated processes. There are two aspects of the bitterness problem: the processes whereby limonin is formed from its non-bitter precursors, and the conditions under which high limonin concentrations are achieved and maintained in citrus juices.

Much more work is required before the question of how high concentrations of limonin are maintained in citrus juices is resolved. Work reported in this thesis demonstrated that high limonin concentrations are possible in heated model solutions, but such high concentrations are only stable in cases where cooling occurs in the absence of hydrogen ions. Such a situation would not be found in citrus juices. Moreover, even in model solutions where high concentrations of limonin existed for longer than two months, there was evidence that limonin concentrations were still falling.

It was shown that simple hydroxyacid derivatives of limonin were involved in the establishment of high limonin concentrations in the model solutions. Although earlier workers had produced evidence that the limonin precursor is a simple hydroxyacid derivative of limonin, such a precursor is unlikely to be involved in the establishment of stable high limonin concentrations in citrus juices. This conclusion is reached because when acidified refluxed solutions containing hydroxyacids were cooled, limonin concentrations rapidly fell to a low level equivalent to the true solubility of limonin in aqueous solutions due to lactonization of hydroxyacid to limonin which then passed out of solution.

Studies using New Zealand grapefruit and Navel orange juices have shown that there are actually two precursors of limonin, and studies on the phenomenon of delayed bitterness development must recognise the complicated enzyme-catalysed and chemical-catalysed changes to these precursors that can occur during extraction, processing and storage of juice.

The above considerations require reassessment of previous

work on this subject. Clearly all the previous work has been done without adequate controls over what is an extremely complex process. Furthermore, what results have been reported have been obtained using experimental designs which must now be considered unacceptable, and analytical methods which did not correctly distinguish limonin from its precursors.

The classic papers from which the currently accepted precursor theory developed (Maier and Beverly 1968; Maier and Margileth 1969) can be criticised for their complete lack of quantitative measurements. For instance, the statement made in the second paper that the first paper showed that the monolactone in the albedo was readily converted to limonin on preparation of the juice is simply not true. The presence of the monolactone was demonstrated in albedo extracts and the presence of limonin was demonstrated in juice extracts, but there was no attempt to follow quantitatively the changes in the limonoid components of freshly extracted juice with time, and the presence of the monolactone in juice was never demonstrated. Furthermore, where a rough estimate was made of the free limonin contents (based on organoleptic assessment or visual comparison of chromatographic or electrophoretic spots), the samples had been filtered using celite, a good adsorbent for limonin, as a filter aid. Consequently, the statement in the second paper that limonin was shown to be an insignificant constituent of the albedo samples examined is also subject to considerable doubt.

The first paper contained further conflicting evidence. Organoleptic assessments were carried out by individuals who could detect limonin bitterness at 2 to 7 ppm and who judged the monolactones and disalt to be nonbitter. When these individuals tasted a homogenate of the carpellary membranes after it had been filtered through celite, they found it to be "just perceptibly bitter". However, after paper electrophoresis and treatment with Ehrlich's reagent, no disalt or limonin was detected in the homogenate. Similarly, when albedo was treated in the same manner as the carpellary membrane fraction, it was described as tasting "very slightly bitter" yet only monolactone was shown to be present. The

reason why the samples tasted bitter in the absence of limonin was not discussed, and the possibility that some other bitter-tasting compound was present was not raised. It is obvious that this work should be repeated under closely controlled experimental conditions which take into account the various factors involved in citrus juice bitterness.

The conclusions from this thesis also cast doubt on the previously reported studies related to more applied aspects of the bitterness problem. For example, the work of Guadagni *et al.* (1973) on taste thresholds for limonin in distilled water and the effect of some citrus juice constituents on these thresholds will need to be repeated. They assumed (in the absence of any analytical checks) that heating limonin and distilled water in a boiling water bath with occasional shaking until the limonin dissolved would result in solutions containing known amounts of dilactone limonin. However, the results reported in this thesis on the solubility of limonin in aqueous solutions demonstrate that, during the solubilization of limonin at high temperatures, hydrolysis of the lactone rings occurs, resulting in the presence of dilactone limonin, hydroxyacid and ionized hydroxyacid in solution. Since it has been reported (Maier and Beverly 1968) that the hydroxyacids are not bitter, the determination of taste thresholds based on the assumption that all the dilactone limonin had dissolved unchanged in aqueous solution is now seen to be erroneous.

As a further example of the implications of the present results, reference can be made to the method patented by Sperti (1964) for the production of acceptable juice from orange varieties susceptible to bitterness development. His procedure involved immediate separation of pulp from the juice, boiling of the pulp in water, and addition of the treated pulp back to the serum. Results in this thesis demonstrate that centrifugation immediately after juice extraction does not prevent bitterness development in the serum. Therefore, even if boiling of the pulp with water reduced its bitter content, recombined juice would taste bitter. The improbability if not absolute impossibility of this patented procedure being

successful is confirmed by the fact that it has not apparently been adopted by citrus juice processors (Berry and Veldhuis 1977).

If a commercial process is ever to be achieved for the prevention of bitterness development in citrus juices, further work is essential, taking into consideration the results obtained in this thesis.

Let the thick curtain fall; I better know than all
How little I have gained, how vast the unattained.

Others shall sing the song, others shall right the wrong,
Finish what I begin, and all I fail of win.

What matter I or they? Mine or another's day,
So the right word be said, and life the sweeter made?

Whittier

BIBLIOGRAPHY

ALMADINGER, V.V., DILLMAN, C.A., and BEISEL, C.B. 1954. Determination of free galacturonic acid in citrus products Food Technol. *8*: 86 - 88.

ANDERSON, D.M.W. 1959. Studies on materials containing uronic acid. 1. An apparatus for routine semi-micro estimations of uronic acid content. Talanta *2*: 73 - 78.

ARIGONI, D., BARTON, D.H.R., COREY, E.J., JEGER, O., CAGLIOTO, L., DEV, S., FERRINI, P.G., GLAZIER, E.R., MELERA, A., PRADHAN, S.K., SCHAFFNER, K., STERNHILL, S., TEMPLETON, J.F., and TOBINAGA, S. 1960. Constitution of limonin. Experientia *16*: 41 - 49.

ARNOTT, S., DAVIE, A.W., ROBERTSON, J.M., SIM, G.A., and WATSON, D.G. 1960. The structure of limonin. Experientia *16*: 49 - 52.

BAKER, R.A., and BRUEMMER, J.H. 1969. Cloud stability in the absence of various orange juice soluble components. Proc. Fla. State Hortic. Soc. *82*: 215 - 220.

BAKER, R.A., and BRUEMMER, J.H. 1972. Pectinase stabilization of orange juice cloud. J. Agric. Food Chem. *20*: 1169 - 1173.

BARNETT, D. 1975. Electrodes for estimating sulphur dioxide. CSIRO Food Res. Q. *35*: 68 - 69.

BARTON, D.H.R., PRADHAN, S.K., STRENHILL, S., and TEMPLETON, J.F. 1961. Triterpenoids. XXV. The constitutions of limonin and related bitter principles. J. Chem. Soc. Part I, p. 255 - 275.

BASKER, H.B., BEN-SHALOM, N., KATZ, M., and SKLAN, D. 1973. Gas chromatographic detection of limonin. Lebensm.-Wiss. Technol. *6*: 34 - 35.

BATH, I.H. 1958. The ultraviolet spectrophotometric determination of sugars and uronic acids. Analyst (London) *83*: 451 - 455.

BERLEPSCH, K. VON. 1969. Two automated procedures for determination of uronic acids in acid glycosaminoglycans. Anal. Biochem. *27*: 424 - 432.

BERRY, R.E., and VELDHUIS, M.K. 1977. Processing of oranges, grapefruit and tangerines. In : Citrus Science and Technology. S. Nagy, P.E. Shaw and M.K. Veldhuis (Editors), Vol. 2. p. 177 - 252. Avi Publishing Co. Inc., Westport, Connecticut, USA.

BISSET, O.W., VELDHUIS, M.K. and RUSHING, N.B. 1953. Effect of heat treatment temperature on the storage life of Valencia orange concentrates. Food Technol. *7*: 258 - 260.

BITTER, T., and MUIR, H.M. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. *4*: 330 - 334.

BLUMENKRANTZ, N., and ASBOE-HANSEN, G. 1973. New method for quantitative determination of uronic acids. Anal. Biochem. *54*: 484 - 489.

BREWSTER, L.C., HASEGAWA, S., and MAIER, V.P. 1976. Bitterness prevention in citrus juices. Comparative acitivities and stabilities of the limonoate dehydrogenases from *Pseudomonas* and *Arthrobacter*. J. Agric. Food Chem. *24*: 21 - 24.

BUSLIG, B.S., and CARTER, R.D. 1974. Particle size distribution in orange juices. Proc. Fla. State Hortic. Soc. *87*: 302 - 305.

CASTAGNE, A.E., and SIDDIQUI, I.R. 1975. Uronic acid determination. Carbohy. Res. *42*: 382 - 386.

CHANDLER, B.V. 1958. The chemistry of the bitter principle of oranges. PhD Thesis, University of Sydney Library,

Sydney, NSW, Australia.

CHANDLER, B.V. 1971a. A limonin-degrading enzyme in citrus albedo. J. Sci. Food Agric. *22*: 634 - 637.

CHANDLER, B.V. 1971b. Some solubility relationships of limonin and their importance in orange juice bitterness. CSIRO Food Res. Q. *31*: 36 - 40.

CHANDLER, B.V. 1971c. Rapid assay for limonin using a new selective detecting system for limonoids. J. Food Agric. *22*: 473 - 476.

CHANDLER, B.V., and JOHNSON, R.L. 1979. New sorbent gel forms of cellulose esters for debittering citrus juices. J. Sci. Food Agric. *30*: 825 - 832.

CHANDLER, B.V., and KEFFORD, J.F. 1966. The chemical assay of limonin, the bitter principle of oranges. J. Sci. Food Agric. *17*: 193 - 197.

CHANDLER, B.V., and NICOL, K.J. 1975. Debittering citrus products with enzymes. CSIRO Food Res. Q. *35*: 79 - 88.

CHANDLER, B.V., NICOL, K.J., and VON BIEDERMANN, C. 1976. Factors controlling the accumulation of limonin and soluble constituents within orange fruits. J. Sci. Food Agric. *27*: 866 - 876.

CONNOLLY, J.D., OVERTON, K.H., and POLONSKY, J. 1970. The chemistry and biochemistry of the limonoids and quassinoids. In : Progress in Phytochemistry. L. Reinhold and Y. Liwschitz (Editors), Vol. 2. p. 385 - 455. John Wiley and Sons, New York.

DEKKER, R.F.H., and RICHARDS, G.N. 1972 Determination of pectic substances in plant material. J. Sci. Food Agric. *23*: 475 - 483.

DIETZ, J.H., and ROUSE, A.H. 1953. A rapid method for

estimating pectic substances in citrus juices. Food Res. *18*: 169 - 177.

DI GIACOMO, A., CALVARANO, I., CALVARANO, M., and BOVALO, F. 1976. Limonin content of orange juice. III. Role of processing technology. Essen. Deriv. Agrom. *46*: 247 - 263.

DISCHE, Z. 1947a. A new specific colour reaction to hexuronic acids. J. Biol. Chem. *167*: 189 - 198.

DISCHE, Z. 1947b. A specific colour reaction for glucuronic acid. J. Biol. Chem. *171*: 725 - 730.

DISCHE, Z. 1948. A new specific colour reaction of galact-uronic acid. Arch. Biochem. *16*: 409 - 414.

DOESBURG, J.J. 1957 Relation between the solubilization of pectin and the fate of organic acids during maturation of apples. J. Sci. Food Agric. *8*: 206 - 216.

DREYER, D.L. 1965. Citrus bitter principles. III. Isolation of deacetylnomilin and deoxylimonin. J. Org. Chem. *30*: 749 - 751.

DREYER, D.L. 1966. Citrus bitter principles. V. Botanical distribution and chemotaxonomy in the *Rutaceae*. Phyto-chemistry *5*: 367 - 378.

DREYER, D.L. 1968. Limonoid bitter principles. Fortschr. Chem. Org. Naturst. *26*: 190 - 244.

EAGERMAN, B.A., and ROUSE, A.H. 1976. Heat inactivation temperature-time relationships for pectinesterase inactivation in citrus juices. J. Food Sci. *41*: 1396-1397.

EDWARDS, J.W., and JOSLYN, M.A. 1952. Inhibition and activation of orange pectinesterase. Food Res. *17*: 51 - 55.

EMERSON, O.H. 1948. The bitter principles of citrus fruit. I. Isolation of nomilin, a new bitter principle from the

seeds of oranges and lemons. J. Am. Chem. Soc. *70*: 545 - 549.

EMERSON, O.H. 1949. The bitter principle of Navel oranges. Food Technol. *3*: 248 - 250.

EMERSON, O.H. 1952. Bitter principles in citrus. III. Some reactions of limonin. J. Am. Chem. Soc. *74*: 688 - 693.

FISHER, J.F. 1973. Fluorimetric determination of limonin in grapefruit and orange juice. J. Agric. Food Chem. *21*: 1109 - 1110.

FISHER, J.F. 1975. Quantitative determination of limonin in grapefruit juice by high-pressure liquid chromatography. J. Agric. Food Chem. *23*: 1199 - 1201.

FLAVIAN, S., and LEVI, A. 1970. Natural disappearance of the limonin monolactone in the peel of Shamouti oranges. J. Food Technol. *5*: 193 - 195.

GALAMBOS, J. 1967. The reaction of carbazole with carbohydrates. 1. Effect of borate and sulphamate on the carbazole colours of sugars. Anal. Biochem. *19*: 119 - 132.

GEE, M., McCOMB, E.A., and McCREADY, R.M. 1958. A method for the characterization of pectic substances in some fruit and sugar-beet marcs. Food Res. *23*: 72 - 75.

GLASSTONE, S., and LEWIS, D. 1965. Elements of physical chemistry. 2nd edition. Macmillan and Co. Ltd., London.

GOODWIN, T.W., and GOAD, L.J. 1970. Carotenoids and triterpenoids. In: The Biochemistry of Fruits and Their Products. A.C. Hulme (Editor), Vol. 1, p. 305 - 368. Academic Press, New York and London.

GUADAGNI, D.G., MAIER, V.P., and TURNBAUGH, J.G. 1973. Effect of some citrus juice constituents on taste thresholds

for limonin and naringin bitterness. J. Sci. Food Agric. *24*: 1277 - 1288.

HANSON, J. 1968. Navel orange juice and process for its preparation. British Patents 1, 134, 279 and 1, 134, 280.

HASEGAWA, S., and MAIER, V.P. 1979. Approaches to the limonin bitterness problem in citrus juice. In: Developments in Food Science 2. Proceedings of the Fifth International Congress of Food Science and Technology. Chiba, H., Fujimaki, M., Iwai, K., Mitsuda, H., and Morita, Y. (Editors). Kodansha: Tokyo and Elsevier: Amsterdam.

HASEGAWA, S., BENNETT, R.D., and MAIER, V.P. 1972. Limonoate dehydrogenase from *Arthrobacter globiformis*. J. Agric. Food Chem. *20*: 1031 - 1034.

HASEGAWA, S., BREWSTER, L.C., and MAIER, V.P. 1973. Use of limonoate dehydrogenase of *Arthrobacter globiformis* for the prevention or removal of limonin bitterness in citrus products. J. Food Sci. *38*: 1153 - 1155.

HASEGAWA, S., MAIER, V.P., and KING, A.D. 1974. Isolation of new limonoate dehydrogenase from *Pseudomonas*. J. Agric. Food Chem. *22*: 523 - 526.

HELBERT, J.R., and BROWN, K.D. 1956; 1957. Colour reaction of hexuronic acids with anthrone. Anal. Chem. *28*: 1098 - 1100; *29*: 1464 - 1466.

HIGBY, R.H. 1938. The bitter constituents of Navel and Valencia oranges. J. Amer. Chem. Soc. *60*: 3013 - 3018.

HIGBY, R.H. 1941. Canning Navel orange juice. Calif. Citrograph *26*: 360 - 361.

HILDEBRAND, J.H. and SCOTT, R.L. 1970. Regular and Related Solutions; The Solubility of Gases, Liquids, and Solids. Van Nostrand Reinhold Co., New York.

HOLLAND, R.R., REEDER, S.K., and PRITCHETT, D.E. 1976. An accelerated test for residual cloud reducing enzyme activity in citrus juices. J. Food Sci. *41*: 812 - 815.

HOLT, R. 1954. Volumetric determination of pectin as calcium pectate. Analyst (London) *79*: 623 - 627.

HULTIN, H.O., SUN, B., and BULGER, J. 1966. Pectin methyl esterase of the banana. Purification and properties. J. Food Sci. *31*: 320 - 327.

JOSLYN, M.A. and CHEN, T.S. 1967. Determination of pectic substances in the presence of dextrins. J. Agric. Food Chem. *15*: 398 - 402.

KATZ, S., and TACKER, L.H. 1972. A new sensitive ultra-violet detection system for carbohydrates eluted during column chromatography. J. Chromatogr. *64*: 247 - 252.

KEFFORD, J.F. 1959. The chemical constituents of citrus fruits. In : Advances in Food Research. C.O. Chichester, E.M. Mrak, and G.F. Stewart (Editors), Vol. 9. p. 285 - 372. Academic Press, New York and London.

KEFFORD, J.F., and CHANDLER, B.V. 1970. The chemical constituents of citrus fruits. In : Advances in Food Research, Supplement 2. C.O. Chichester, E.M. Mrak, and G.F. Stewart (Editors), Academic Press, New York and London.

KEFFORD, J.F., and CHANDLER, B.V. 1977. Citrus processing around the world Part 3. Australia. In : Citrus Science and Technology. S. Nagy, P.E. Shaw, and M.K. Veldhuis (Editors), Vol. 2. p. 609 - 616. Avi Publishing Co. Inc., Westport, Connecticut, USA.

KEW, T.J., and VELDHUIS, M.K. 1960. Citrus pectinesterase inhibitor in grape leaf extract. Proc. Fla. State Hort. Soc. *73*: 293 - 297.

KEW, T.J., and VELDHUIS, M.K. 1961. Cloud stabilization in citrus juice. USA Patent 2,995,448.

KROP, J.J.P. 1974. The mechanism of cloud loss phenomena in orange juice. Agric. Res. Rep. (Versl. Landb. Onderz.) 830, Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands.

KROP, J.J.P., and PILNIK, W. 1974a. Effect of pectic acid and bivalent cations on cloud loss of citrus juices. Lebensm.-Wiss. u. Technol. *7*: 62 - 63.

KROP, J.J.P., and PILNIK, W. 1974b. Cloud loss studies in citrus juices: cloud stabilization by a yeast polygalacturonase. Lebensm.-Wiss. u. Technol. *7*: 121 - 124.

KROP, J.J.P., and PILNIK, W. 1974c. Effect of commercial protease preparations on cloud loss of citrus juice. Lebensm.-Wiss. u. Technol. *7*: 125 - 126.

KRUGER, A.J., and COLTER, C.E. 1972. Gas chromatographic identification of limonin in citrus juice. Proc. Fla. State Hortic. Soc. *85*: 206 - 210.

KULP, K. 1975. Carbohydrases. In : Enzymes in Food Processing. G. Reed (Editor), 2nd edition p. 107 - 122. Academic Press, New York and London.

LANKVELD, J.M.G. 1970. Adsorption of polyvinylalcohol on paraffin-water interfaces and the properties of paraffin-in-water emulsions stabilized by polyvinlyalcohol. Meded. Landb. Hogesch. 70 - 21, Wageningen, The Netherlands.

LANKVELD, J.M.G. 1973. Fundamental aspects concerning the cloud stability of soft drinks. Lecture held at the International Symposium 'Emulsions and Foams in Food Technology', Ebeltoft, Denmark, 3 - 5 April.

LEE, M., and MACMILLAN, J.D. 1968. Mode of action of pectic enzymes. I. Purification and certain properties of tomato

pectinesterase. Biochem. *7*: 4005 - 4010.

LEVI, A., FLAVIAN, S., HAREL, S., BEN-GURA, I., STERN, F., and BERKOVITZ, S. 1974a. The bitter principle and the prevention of bitterness in Shamouti orange juice products. Special Publication No. 29, Agricultural Research Organization, The Volcani Centre, Bet Dagan, Israel.

LEVI, A., FLAVIAN, S., HAREL, S., STERN, F., and BERKOWITZ, S. 1974b. The bitter principle in Shamouti orange juice. I. Seasonal changes and distribution in different parts of the fruit. Lebensm.-Wiss. Technol. *7*: 234 - 235.

LINEWEAVER, H., and BALLOU, G.A. 1945. The effect of cations on the activity of alfalfa pectinesterase. Arch. Biochem. *6*: 373 - 387.

McCOLLOCH, R.J. 1950. Preliminary studies on debittering Navel orange products. Calif. Citrogr. *35*: 290, 292.

McCOMB, E.A., and McCREADY, R.M. 1952. Colorimetric determination of pectic substances. Anal. Chem. *24*: 1630 - 1632.

McCREADY, R.M., and McCOMB, E.A. 1952. Extraction and determination of total pectic materials in fruits. Anal. Chem. *24*: 1986 - 1988.

MABEY, W., and MILL, T. 1978. Critical review of the hydrolysis of organic compounds in water under environmental conditions. J. Phys. Chem. Ref. Data *7*(*2*): 383 - 415.

MAIER, V.P., and BEVERLY, G.O. 1968. Limonin monolactone, the non-bitter precursor responsible for delayed bitterness in certain citrus juices. J. Food Sci. *33*: 488 - 492.

MAIER, V.P., and GRANT, E.R. 1970. Specific thin-layer chromatographic assay of limonin, a citrus bitter principle. J. Agric. Food Chem. *18*: 250 - 252.

MAIER, V.P., and MARGILETH, D.A. 1969. Limonoic acid A-ring lactone, a new limonin derivative in citrus. Phytochemistry *8*: 243 - 248.

MAIER, V.P., BENNETT, R.D., and HASEGAWA, S. 1977. Limonin and other limonoids. In : Citrus Science and Technology. S. Nagy, P.E. Shaw and M.K. Veldhuis (Editors), Vol. 1. p. 355 - 396. Avi Publishing Co. Inc., Westport, Connecticut, USA.

MAIER, V.P., HASEGAWA, S., and HERA, E. 1969. Limonin D-ring lactone hydrolase. A new enzyme from citrus seeds. Phytochemistry *8*: 405 - 407.

MARKH, A.T. 1953. Biochemical alterations of citrus juices Tr. Odessk. Tekhnol. Inst. Pishchevoi i Kholodil'n. Prom. *5*(*2*): 80 - 89; Chem. Abstr. *49*: 6499i (1955).

MARKH, A.T., and FEL'DMAN, A.L. 1949. Removing the bitter taste from citrus products. USSR Patent 77, 160; Chem. Abstr. *47*: 10150i (1953).

MARKH, A.T., and FEL'DMAN, A.L. 1950. Biochemical transformations of flavanone glucosides of citrus fruits. Biokhimiya *15*: 230 - 235; Chem. Abstr. *44*: 9582i (1950).

NAGY, S. 1977. Lipids: identification, distribution, and

NANGO, M., YAMAMOTO, H., JOUKOU, K, UEDA, M, KATAYAMA, A. and KUROKI, N. 1980. Solubility of aromatic hydrocarbons in water and aqueous solutions of sugars. J.C.S. Chem. Comm. 104.
Enzymes in Food Processing. G. Reed (Editor), 2nd edition p. 397 - 442. Academic Press, New York and London.

NICOL, K.J., and CHANDLER, B.V. 1978. The extraction of the enzyme degrading the limonin precursor in citrus albedo. J. Sci. Food Agric. *29*: 795 - 802.

NOMURA, D., and SANTO, T. 1965. A new method for quantitative

determination of limonoid in Citrus natsudaidai juice. Nippon Shokuhin Kogyo Gakkaishi *12*: 100 - 101; Chem. Abstr. *64*: 18316e (1966).

PILNIK, W., and VORAGEN, A.G.J. 1970. Pectic substances and other uronides. In : The Biochemistry of Fruits and Their Products. A.C. Hulme (Editor), Vol. 1. p. 63 - 87. Academic Press, New York and London.

POTTY, V.H. 1969. Determination of proteins in the presence of phenols and pectins. Anal. Biochem. *29*: 535 - 539.

ROBERTSON, G.L. 1976. Pectinesterase in New Zealand grapefruit juice. J. Sci. Food Agric. *27*: 261 - 265.

ROBERTSON, G.L. 1978. Time-temperature for PE inactivation questioned. J. Food Sci. *43*(*6*): vi - vii.

ROTHSCHILD, G., VAN VLIET, C., and KARSENTY, A. 1975. Pasteurization conditions for juice and comminuted products of Israeli citrus fruits. J. Food Technol. *10*: 29 - 38.

ROUSE, A.H., and ATKINS, C.D. 1955. Pectinesterase and pectin in commercial citrus juices as determined by methods used at the Citrus Experiment Station. Technical Bulletin 570, Univ. Florida Agric. Exp. Stns., Gainesville, Fla.

SAMISCH, Z., and GANZ, D. 1950. Bitterness in Shamouti oranges. Canner *110*(*23*): 7 - 9; *110*(*24*): 36 - 37; *110*(*25*): 22, 24.

SARRIS, J., VIJAYALAKSHMI, M.A., and LATTRASSE, A. 1975. Automatic determination of galacturonic acid: application to the study of pectins. Ind. Aliment. Agric. *92*: 1265 - 1269.

SCOTT, W.C., KEW, T.J., and VELDHUIS, M.K. 1965. Composition of orange juice cloud. J. Food Sci. *30*: 833 - 837.

SMITH, A.H. 1925. A protein in the edible portion of orange. J. Biol. Chem. *63*: 71 - 73.

SPERTI, G.S. 1964. Orange juice process. U.S. Patent 3, 385, 711.

SPIRO, M. 1977. Uronic acid analysis by automated anion exchange chromatography. Anal. Biochem. *82*: 348 - 352.

TARIQ, A.M., and CHAUDREY, M.S. 1974. Effect of processing and storage on the development of bitterness in the orange juice. Pakistan J. Sci. Ind. Res. *17*: 27 - 28.

TATUM, J.H., and BERRY, R.E. 1973. Method for estimating limonin content of citrus juices. J. Food Sci. *38*: 1244 - 1246.

TERMOTE, F., ROMBOUTS, F.M., and PILNIK, W. 1977. Stabilization of cloud in pectinesterase active orange juice by pectic acid hydrolysates. J. Food Biochem. *1*: 15 - 34.

TOWNSLEY, P.M., JOSLYN, M.A., and SMIT, C.J.B. 1953. The amino acids in various tissues of citrus fruits and orange protein. J. Food Res. *18*: 522 - 531.

VAN DE HULST, H.C. 1957. Light Scattering by Small Particles. John Wiley and Sons, New York.

VAS, K., NEDBOLEK, M., SCHEFFER, H., and KOVACS-PROSZT, G. 1967. Methodological investigations on the determination of some pectic enzymes. Fruchtsaft-Industrie *12*: 164 - 184.

WALSTRA, P. 1964. Approximation formulae for the light scattering coefficient of dielectric spheres. Br. J. Appl. Phys. *15*: 1545 - 1552.

WARDI, A.H., ALLEN, W.S., and VARMA, R. 1974. A simple method for the detection and quantitative determination of hexuronic acids and pentoses. Anal. Biochem. *57*: 268 - 273.

SHOMER, I., FAHN, A. and BEN-GARA, I. 1975. Epicuticular wax on the juice sacs of citrus fruits - a possible adhesive in the fruit segments. J. Food Sci. *40*, 925-930.

WARREN, D.S., and WOODHAM, J.S. 1973. Distribution of cell wall components in potato tubers: a new titrimetric procedure for the estimation of total polyuronide (pectic substances) and its degree of esterification. J. Sci. Food Agric. *24*: 769 - 777.

WHYTE, J.N.C., and ENGLAR, J.R. 1974. Determination of uronic acid content of saccharides by acidic decarboxylation: a simplified approach. Anal. Biochem. *59*: 426 - 435.

WILSON, K.W., and CRUTCHFIELD, C.A. 1968. Spectrophotometric determination of limonin in orange juice. J. Agric. Food Chem. *16*: 118 - 124.