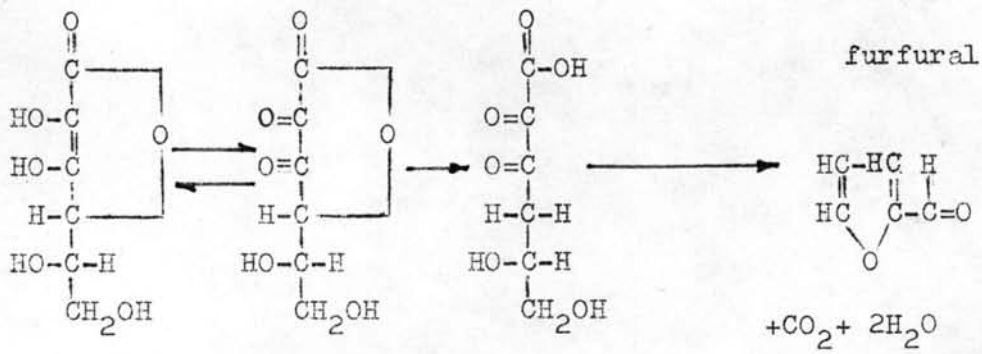
CHAPTER II

LITERATURE REVIEW

During storage and processing of citrus juice, many physical and chemical changes as well as microbial deterioration such as loss of cloudy appearance, changes of color and flavor, and fermentation caused by micro-organisms always occur. Freshly extracted citrus juices are generally opaque due to the presence of a heterogeneous mixture of cellular materials and perhaps emulsoids held in suspension by pectin. Hydrolysis of pectin by enzyme pectin-esterase will cause a phenomena of cloud loss and gelation occurs in unheated citrus juices. To prevent the hydrolysis of pectins, the pectin enzymes should be destroyed by inactivating them through heating the juice for a short time to a minimum temperature of 85°C. But heat treatment and also high storage temperature will result in cooked taste which might be a result of loss and/or chemical alteration of the volatile substances, as well as through the formation of new odorous materials. Generally, all of these changes are undesirable and can be kept to a minimum. Deaeration, absence of excess essential oils, short-term processing, rapid cooling after pasteurization and low temperature storage condition are some of the precaution to be taken to avoid undesirable change.

2.1 Non-Enzymic Browning

The main color problem facing the citrus industry is the non-enzymic browning occuring during storage. Three main theories have been put forward to explain non-enzymic browning (Stadtman, 1948). One pathway is the formation of sugar-amine condensation products which, after undergoing Amadori rearrangement and a variety of secondary reactions, give rise ultimately to dark colored melanoidin' compounds. The Amadori rearrangement requires a near neutral, or slightly alkaline medium for optimal efficiency of reaction, therefore it is unlikely that this mechanism is the major contributor to the browning of a highly acid product such as lemon juice at pH 2.5. A secondary theory postulates that browning involves the decomposition of sugars and sugar acids to furfuraldehydes, or similar compounds characterised by having an active carbonyl group. These products then condense with nitrogeneous compounds and/or polymerise to form brown resinous materials. The third theory is based on oxidation, yielding reactive products which similarly may polymerise or react with nitrogeneous constituents, but the precursors are specified as ascorbic acid or related compounds.



This third theory seems the most likely to apply to the conditions pertaining to an acidic product such as lemon juice; the concentration of ascorbic acid is relatively high and free amino acids are present to combine with the reactive products resulting from the oxidation of the ascorbic acid lead to the formation of brown pigments. Browning of lemon juice in model systems was proportional to the level of ascorbic acid; the presence of amino acid in model systems increased the intensity of browning (Clegg, 1964). In model systems, citric acid is one of the reactants leading directly to the formation of brown polymers and does not have the role of catalyst or source of carbonyl compounds (Clegg, 1966).

Much work has been carried out on the factor affecting the development of brown color in various food. Curl (1947) found that browning increased during storage at room temperature. Meanwhile, Pederson et al (1947) studied in some detail the darkening or browning in some juices during storage at various temperatures. They found that a soluble brown compound was formed in all samples stored at various temperatures. Seaver and Kertesz (1946) reported that d-galacturonic acids formed on heating colored compounds at a rate exceeding that found with common sugars. Pederson et al (1947) found that browning developed during storage at most temperatures. McCready and Owens (1950), Underwood and Rockland (1953) in their works on citrus fruits mentioned that evidence had indicated that amino acid-sugar interactions may be

important in the darkening and development of off-flavor in citrus products. Later on Jones (1959) concluded that glucose was the limiting factor in the browning in various food.

According to the result of fresh lime juice by the use of chemical additives at the Department of Chemical Technology, Chulalongkorn University (Sinchumpasak, 1976). It was found that the lime juice treated with single chemical additive, 200 or 300 ppm of potassium metabisulfite or potassium sorbate had the same acceptable qualities at the end of 4 months when stored at refrigerator temperature, while the acceptability of the juice treated similarly stored at room temperature was only at the end of 2 months. The combined effect of both additives,200 ppm of potassium metabisulfite and 300 ppm of potassium sorbate resulted in a good quality of fresh lime juice at the end of the 4 months at refrigerator temperature, while at room temperature it was still acceptable only at the end of 2 months. The potassium metabisulfite could protect the color change better than potassium sorbate.

2.2 Ascorbic Acid

The principle value of the citrus fruits in nutrition is a source of ascorbic acid. As for the effect of ascorbic acid on the development of browning, Koppanyi et al (1945) reported that dehydroascorbic acid but not ascorbic acid, reacted in solution at 98.8°C with amino acid to form strongly colored compounds. Tressler and Joslyn (1954) reported that in single-strength juice, and

especially in concentrates, much more ascorbic acid disappearance on room temperature storage could not be accounted for by the oxygen originally present. This appearance of ascorbic acid was considered to be among the important factors in forming the colored compounds in such juices.

Much works concerned with the preservation of citrus juice especially lime juice have been done. Moored et al (1944) found that the retention of ascorbic acid of citrus juices stored in tin had a higher retention (3-8%) than the corresponding pack in glass. Eveden and Marsh (1948) found that sulfur dioxide lowered the loss of ascorbic acid in citrus juices. Heikal et al (1967) reported that concentrations varying from 100 to 200 ppm sulfur dioxide, are the most suitable for such preservation. It was reported that the color of the processed lemon juice was affected by processing technique, storage temperature and storage periods, and that sulfur dioxide was effective in preserving the color and flavor of the processed lemon juices, and there was no appreciable change in pH and total acidity during storage of the processed lemon juice. (Heikal et al., 1964, 1967)

According to the results from the Department of Science, Ministry of Industry. (Chumwatana et al., 1966) It was found that single strength lime juice with 1000 ppm of potassium metabisulfite as preservative could be kept at 24°C for one year without much change in qualities except for the decrease in ascorbic acid content to zero at the end of second month, but it was still acceptable. When 500 ppm of sodium sorbate or sodium benzoate were used, the

juice could be kept only for half a month and its color turned brown.

Oxygen can react with ascorbic acid in the presence of trace amounts of copper to give dehydroascorbic acid and hydrogen peroxide. The hydrogen peroxide formed during this reaction can lead to further oxidation of vitamin C either directly or indirectly, by breaking down to water and oxygen, both reactions being catalysed by cupric ion (Weissberger and Luvalle, 1944). Dehydroascorbic acid retains full vitamin C activity but is more thermolabile than ascorbic acid and would therefore be easily destroyed during heat processing. It is essential for vitamin C retention that citrus juice should be kept in a de-aerated condition. Oxygen in the head space of the can has been shown to have a detrimental effect on the ascorbic acid concentration in de-aerated sweetened orange juice (Boyd and Peterson, 1945)

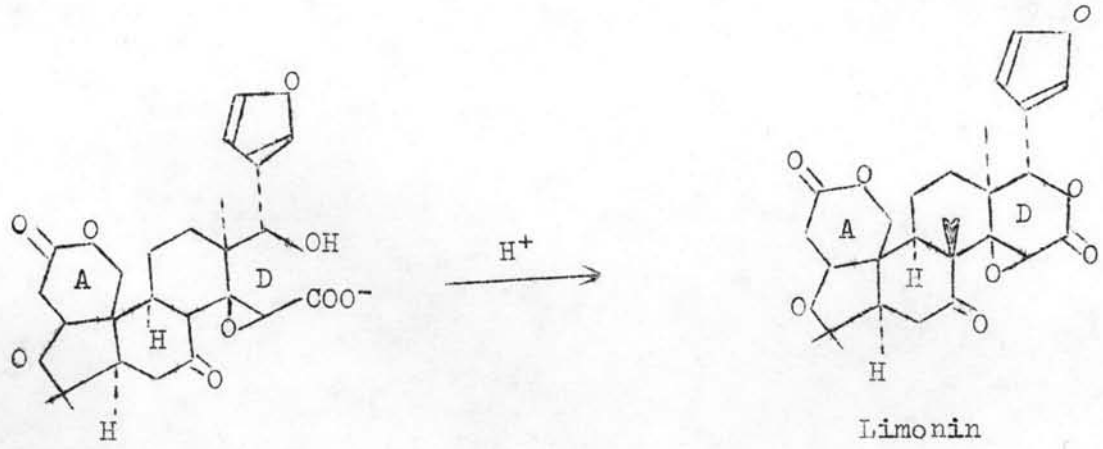
2.3 Bitter Principle-Limonin

There appears to be four main groups of chemical constituents contributing to the taste and flavor of citrus products. These are organic acids of which citric acid is the major representative, sugars, bitter principle and volatile constituents, mainly terpenes and carbonyls.

A serious problem in the production of juice from certain citrus fruit is the bitterness in the juice after extraction from the fruit. The component responsible for the bitter taste of the juice from fruits exhibiting delayed bitterness is the triterpenoid,

limonin (Figure I)

Limonin was first isolated from Washington Navel Orange juice by Higby (1938). It was shown to be the sole bitter limonoid in Navel Orange juice by Emerson (1949), and its complete structure was reported by Arigoni et al (1960). Maier et al (1965) isolated small amounts from grapefruit juice. The bitterness due to limonin develops gradually in the juice after extraction from certain verieties of oranges, lemons, and grapefruit. The intact fruits do not normally contain limonin but rather a nonbitter precursor limonoate A-ring lactone (Maier and Beverly 1968, Maier and Margileth, 1969). This nonbitter precursor is converted to limonin under acidic conditions (Maier and Beverly, 1968) and the conversion is also accelerated by the action of limonoate D-ring lactone hydrolase which has been shown to be present in citrus fruit (Maier et al.,1969).



Limonoate A-ring lactone
(nonbitter)

Limonin
(bitter)

Figure I Conversion of limonoate A-ring lactone to limonin

Delayed bitterness, numerous attempts have been made to determine the cause of delayed bitterness. Two theories have been proposed for describing the delayed bitterness phenomena. One is the precursor theory which is proposed by Higby in 1938 and the other one is diffusion theory which is proposed by Kefford (1959) and Joslyn et al.,(1961).

The precursor theory proposed that the fruit tissues contain a nonbitter, water-soluble substance limonoate A-ring lactone which after disruption of the fruit tissues in the juice manufacture, enters the juice where it is slowly converted into limonin. When juice is extracted from citrus fruits, the acidic environment of juice results in the eventual conversion of limonoate A-ring lactone to limonin, the juice becomes bitter.

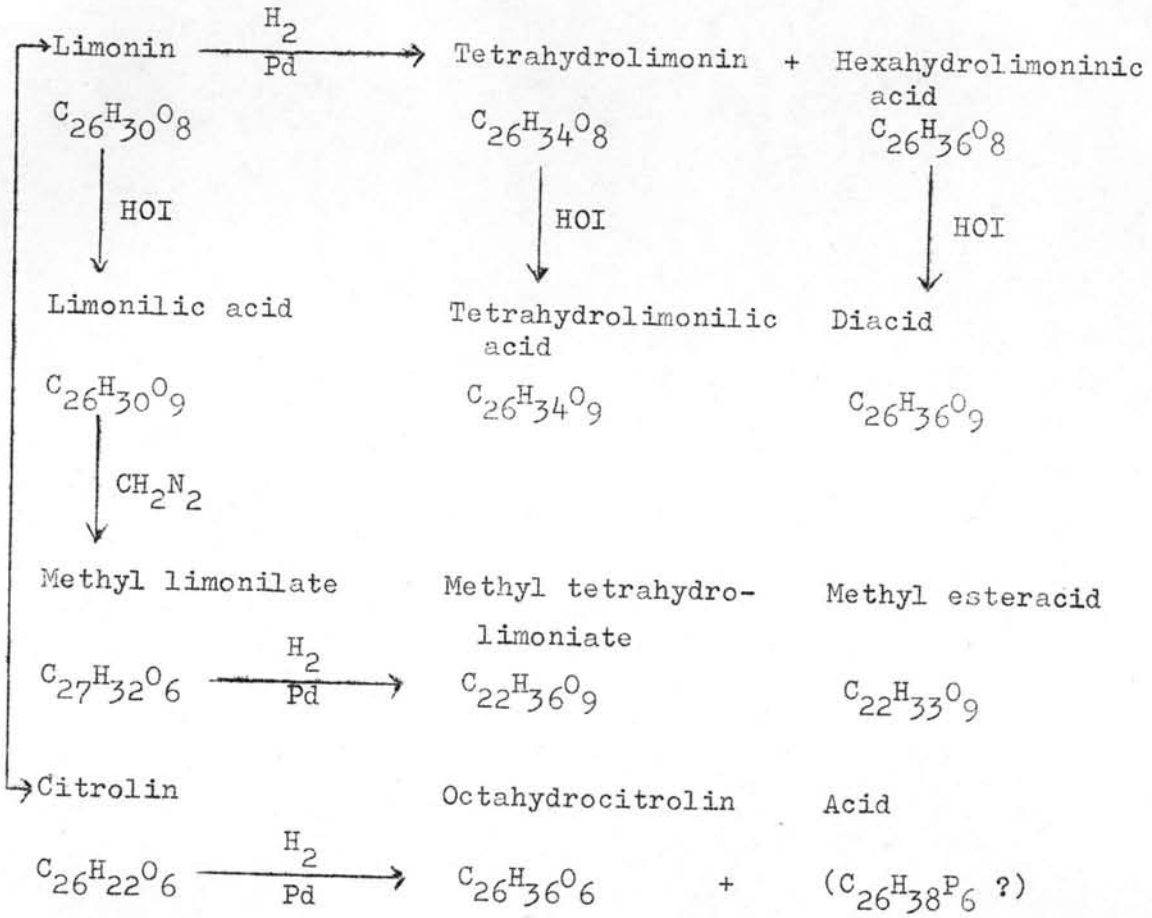
The diffusion theory, proposed by Kefford (1959) explains that limonin itself is present in the fruit tissues but because of its low solubility, it takes an appreciable time to diffuse from the tissue fragments of the juice into solution and to reach a concentration sufficient to impart a bitter taste.

Limonin, a limonoid bitter principle in citrus juice, the amount required in a juice before bitterness becomes detectable varies with the sweetness and the acidity of the juice as well as the sensitivity of the factor. As a general rule a juice containing less than 6 ppm of the limonin is unlikely to taste bitter, but a juice with more than 9 ppm will seem bitter to most tasters. Limonin contents higher than 30 ppm are comparatively rare.

2.4 Chemistry of Limonin

The constitution of limonin. Limonin ($C_{26}H_{30}O_8$) is a tetracyclic triterpenoid dilactone with a furan ring side chain and the A-, and D-rings are δ lactones which can be reversibly opened with dilute alkali. (Arigoni et al.,1960 and Barton et al., 1961). The dihydroxy diacid derived from limonin by complete hydrolysis of the lactones, the limonoic acid can lead to two monolactone-forms; limonoic acid A-ring lactone and limonoic acid D-ring lactone. The limonoic acid A-ring lactone is the naturally occuring monolactone in tissues of citrus fruit.

Reaction of Limonin. The hydrogenation of limonin $C_{26}H_{30}O_8$ gives a mixture of tetrahydrolimonin $C_{26}H_{34}O_8$ and hexahydrolimoninic acid $C_{26}H_{36}O_8$. The oxidation of limonin with alkaline hypoiodite gives limonilic acid $C_{26}H_{30}O_9$ (Emerson, 1952). Tetrahydrolimonin and hexahydrolimonin and hexahydrolimoninic acid behave similarly. The reaction apparently involves the openning of a lactone ring and the formation of a new carbocyclic ring. One lactone ring is opened by hydrogenolysis and the other by oxidation. Treatment of limonin with hydriodic acid gives citrolin, which appears to be tricarbocyclic, and has four ethylenic links, one of which is conjugated with the carbonyl group and one with a lactone group.



Formation of dinitrophenylhydrazone. The saponified limonin is allowed to react with 2,4-dinitrophenylhydrazine under standardised conditions of time and temperature (16 hours at 25°)(Chandler and Kefford, 1966). Recrystallisation of the crude product from butanol gave orange needles, melting point 305° (uncorr.), $C_{32}H_{34}O_{11}N_4$.

Hydroxamation and ferric complex formation. Neutral methanolic hydroxylamine was unreactive toward limonin at room temperature and at reflux. An alkaline hydroxamation reagent is required to react with the stable lactone rings of limonin (Wilson and Crutchfield, 1968).

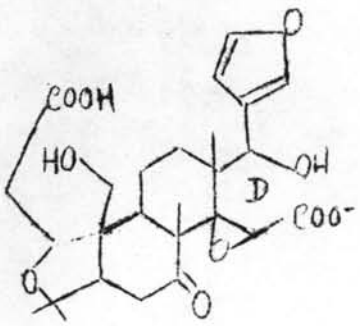
Limonin is sensitive to air oxidation, it can be controlled by addition of antioxidants to the juice extract to prevent serious losses during limonin isolation procedure (Chandler and Kefford, 1966). It appears to involve aerial oxidation of limonin either to limonexic acid or to limonin disophenol. Three antioxidants are butylated-hydroxytoluene, butylatedhydroxyanisole, propyl gallate.

2.5 Bitterness Prevention in Citrus Juice

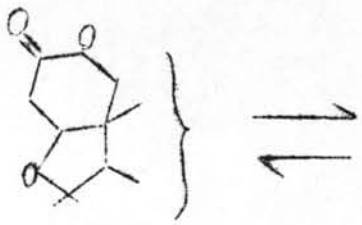
Limonin bitterness in citrus juice continues to be an important economic problem for the citrus industry. Recently, three methods were reported which reduced limonin-caused bitterness in citrus products. One is the metabolic debittering method (Maier et al., 1973) which reduces the amount of limonin precussor in the intact fruit. Maier et al found that a three hour treatment of citrus fruits (navel oranges, lemons, grapefruit) with 20 ppm of ethylene accelerated limonoate A-ring lactone metabolism. Accelerated metabolism continues after ethylene exposure ceases and results in substantial loss of limonoate A-ring lactone. The juice from the treated fruit has 30 to 50 percent lower limonin concentration than the juice from untreated fruit. It is less bitter, and is more prefered by judge than juice from untreated fruit. Longer exposure to ethylene has no greater effect on limonoate A-ring lactone metabolism than the 3 hour treatment, but it can be detrimental to juice quality.

The second method to reduce the bitterness of juice (Hasegawa et al., 1973) is an enzymatic juice treatment which converts the limonin precursor to a monbitter product. The enzyme limonoate dehydrogenase

Limonoate dehydrogenase

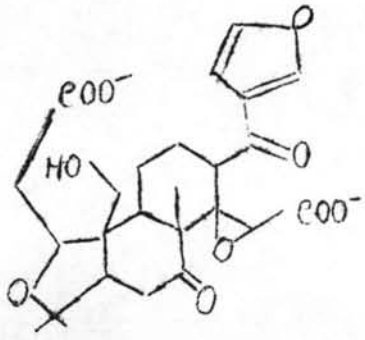


or

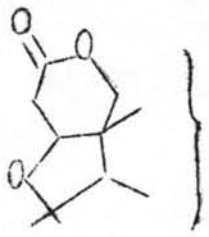


Limonoate

Limonoate A-ring lactone

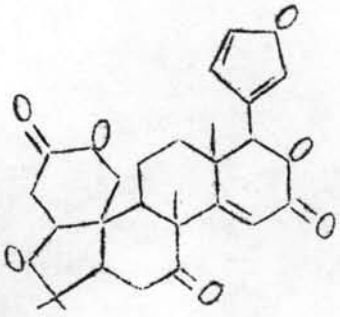


or

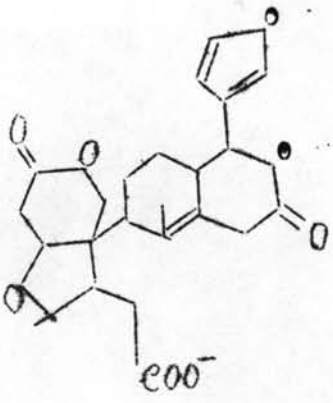


17 Dehydrolimonoate

17-Dehydrolimonoate A-ring lactor



Deoxylimonin



Deoxylimonoate

of Arthrobacter globiformis (LD-Ag) is used to convert the limonin precursor present in the juice limonoate A-ring lactone (LARL) to nonbitter 17-dehydrolimonoate A-ring lactone (17-DLARL) and thereby prevent the formation of bitter limonin (Hasegawa et al.,1972 b). Another enzyme limonoate dehydrogenase, from Pseudomonas sp.321-18 (LD-Ps)(Hasegawa et al., 1974 c) also catalyzes the conversion of LARL to 17-DLARL and the other via deoxylimonin and requires Zn ions and sulfhydryl groups for its activity. LD-Ps differs markedly from LD-Ag in three major characteristics.

LD-Ag	LD-As
NAD as a cofactor Activity of A.globiformis was optimal at pH 9.00	NAD and NADP as cofactor Activity of Pseudomonas-sp.321-18 was optimal at pH 8.0 more stable at low pH

The third method is the removal of limonin by selective sorbent. Polyamide powders will sorb limonin from bitter Navel orange juice serum (Chandler, B.V. et al., 1968), but their use requires a preliminary separation of cloud from the juice, treatment of the serum with the sorbent polyamide powder, and recombination of the debittered serum with the cloud. Moreover polyamide also sorbs other juice constituents, the polyphenolic flavonoids much more strongly than they sorb limonin and for an effective process two treatments with polyamide have to be given, the second removing two to three times more limonin than the first. Ascorbic acid, is partially lost in the process. Cellulose acetate is found to be more specific for limonin than the polyamides (Chandler, 1977); it has much greater capacity than the polyamides for limonin, removes negligible amounts of ascorbic acid, and cheaper than poly amides.

2.6 Detection of Limonin

Since limonin has an effect on quality of citrus juice, a study of the limonin content of lime juice products is of much interest to citrus processors. Limonin is the serious problem in the production of juice from citrus fruits. There are many methods for determining limonin content in citrus juices. The extraction of limonin from citrus juice was described by Chandler and Kefford (1966), Maier and Grant (1970), Kruger and Colter (1972), using chloroform as solvent and by Wilson and Crutchfield (1968), using dichloromethane.

Limonin has been quantitatively analyzed by many methods, each of which suffers to some degrees in length of analysis, sensitivity, and reproducibility. It was found that limonin can be determined mainly by spectrophotometric analysis, chromatography and others.

Spectrophotometric Analysis

After the extraction of limonin from the juice, limonin can be converted into 2,4-dinitrophenylhydrozone by reacting with 2,4-dinitrophenylhydrazine (Chandler and Kefford, 1966). The product is subjected to thin-layer chromatography to single out the limonin derivative whose concentration was determined spectrophotometrically as the absorbance of the eluted limonindinitrophenylhydrazone spot. It was found that Ehrlich's reagent (0.5% of p-N-dimethylaminobenzaldehyde in methanol) can detect limonin (Maier and Grant, 1970) by using thin layer chromatography and treatment with gaseous hydrogen chloride to reveal spot with quantitation by visual or spectrodensitometric comparison with spots of known limonin content. This method suffers from the lack of sensitivity and specificity of the reagent, which is used in thin layer chromatography for the detection of a variety of natural products. Later, Chandler (1971) found a rapid assay for limonin by using Tollen's reagent to detect the complex mixture obtained from the reaction with bromine under controlled condition. This method is sensitive, detecting five limonoids in orange juice extracts. Tatum et al (1973) developed a simplified thin layer chromatography method for limonin estimation which required

no extraction or preparation. The limonin concentration was estimated by observation of either visible color or of fluorescence, compared with the limonin standard. The method based on the formation of the colored ferric complex of the hydroxamic acid derivative of limonin was developed by Wilson and Crutchfield (1968). An alkaline hydroxamation method (Gutmikov and Schenk, 1962) (Wilson and Crutchfield, 1968) proved satisfactory for the conversion of limonin to the hydroxamic acid. The limonin content was determined spectrophotometrically.

Fisher (1973) converted crude limonin into water-extractable sodium limonoate which was reconverted to chloroform extractable limonin and measured the limonin content fluorometrically in concentrated sulfuric acid.

Chromatographic Method

Kruger and Colter (1972) developed a procedure based on gas chromatography. After having concentrated the extracted limonin it was injected into a 5% SE-30 column and determine quantitatively. This method is quite specific for limonin, with a sensitivity of 0.25 ppm. High pressure liquid chromatography was used by Fisher (1975) to determine limonin content and his latest method (Fisher 1978) was improved from the previous one by using micro CN column and eluting with a water methyl alcohol system. The limonin was detected at 210 nm

Paper Electrophoresis (PE)

The method used by Maier et al., (1968) was proved to be an effective method of separating limonin disalt, limonin monolactone,

and limonin because of charge differences exhibited by these compounds at pH 5.5. All forms of limonin were stable during PE and were readily detected with Ehrlich's reagent.

Advantage and Disadvantage of the Method of Limonin Assay

Method	Advantage	Disadvantage
1. TLC+Ehrlichs' reagent (Maier and Grant, 1970)	Simple and specific for limonin, suitable for industrial quality control purpose, and research studies	Total time for analysis of 4 samples is about 4 hours. The color intensities of the spots are so difficult to reproduce.
2. TLC+Tollen's reagent (Chandler, 1971)	More selective and sensitive than other methods, detecting five limonoid in orange juice extracts	The identification of these compounds are not known.
3. TLC of phenylhydrazine + spectrophotometer (Chandler and Kefford, 1966)	Selectivity, reliability, accuracy, straight forward if time is allowed	Rate of formation of the dinitrophenylhydrozone is very slow.
4. TLC+UV (Tatum and Berry, 1973)	No extraction or preparation, less time consuming, determine six juices in 1.5 hour	-
5. Colorimetric determination (Numura and Santo, 1965)	-	Non reproducible, and non specific because colors are produced from non-bitter juice.

Method	Advantage	Disadvantage
6. Alkaline hydroxamation and colorimetric determination (Wilson and Crutchfield, 1968)	Less time consuming, it is useful for determination of limonin in the 5 to 40 ppm range	Non specific because all compounds that form hydroxamates such as esters, lactones, coumarin are interfering substances.
7. Fluorometer determination (Fisher, 1973)	Procedure can detect 0.5 ppm of limonin	
8. High pressure liquid chromatography (Fisher, 1975)	Six complete analysis takes about 4 hr, simple.	

2.7 Additives

The role of chemical additives is used to prevent chemical and microbial deterioration, many food additives are introduced into citrus juice for extending shelf-life for example; sodium benzoate, sorbic acid and its salts, sulfite salts, ascorbic acid, citric acid, stannous chloride.

Metabisulfite

Application of sulfiting to food products can prolong shelf-life because of antimicrobial activity. Sulfurous acid inhibits yeasts, molds and bacteria. Sulfur dioxide is very effective against molds because the oxygen present oxidizes some of the preservative and is thereby eliminated from the juice and the headspace. Besides

antimicrobial activity, sulfiting can prevent enzymatic and nonenzymatic discoloration of food and helps in reducing loss of ascorbic acid. It is used to prevent the oxidation of essential oils and carotenoids and consequent development of off-flavor and loss of color in citrus juices. It has no effects on total acidity analysed as citric acid (Vandercook and Guerrero, 1968) as well as no effect on pectic enzymes which are responsible for the breakdown of tissue or which cause loss of cloud in citrus juices. The amount of sulfur dioxide required depends on the pH of the juice and the amount of suspended pulp. The use of sulfites is limited by the fact that at residual levels above 500 ppm, the taste begins to be noticeable. The sulfite salts and sulfur dioxide are generally recognized as safe (GRAS) for use in food by the U.S. Food and Drug Addministration. Potassium metabisulfite ($K_2S_2O_5$) is a white crystal or powder having odor of sulfur dioxide that is freely soluble in water. Under humid conditions, the metabisulfites are more stable than the sulfites. Sulfur dioxide is the only chemical preservative which can be removed from the juice by applying heat or vacuum.

Sorbic acid

Sorbic acid and its salts have a broad spectrum of activity against yeast and molds, but are less active against bacteria. Sorbic acid and potassium sorbate are generally recognized as safe (GRAS) for use in foods under regulation of the U.S. Food and Drug Administration. Potassium sorbate can be applied to fruit juice at 0.025 to 0.10% or they can be used together with other preservatives, each at a lower level.

Stannous Chloride

A number of tin salts can serve as source of stannous ions for preservation such as stannous chloride, stannous sulfate and stannous tartrate, which are water-soluble; stannous oxalate which is soluble in hydrochloric acid. Stannous chloride is preferred since it is readily available as a reagent grade chemical of known purity and therefore is suitable as such for use in foods. It was found that the addition of small amounts of stannous ions to citrus juice products, such as single strength lemon, orange and grapefruit juices, concentrated lemon, orange and grapefruit juices and concentrates for orangeade and lemonade, retards or inhibits browning of such products (Higby, W.K. and Pritchett, D.E., 1965). The addition of stannous ion as low as 25 ppm retards browning moderately. Stannous chloride is generally recognized as safe (GRAS) for use in food under regulation of the U.S. Food and Drug Administration. Stannous chloride can be applied to food up to 0.0015% calculated as tin.