CHAPTER I

INTRODUCTION

TANGERINE

Citrus fruits can be divided into two groups. The first group is sour citrus fruits, which include limes and lemons. The other group is sweet citrus fruits such as grapefruits and oranges. Mandarin or commonly known as Tangerine is a group name for a class of oranges with thin loose peel. These are treated as members of a distinct species, *Citrus reticulata,* Blanco and belongs to the family *Rutaceae* and the subfamily *Aurantioideae* (Considine and Considine,1982).

Tangerine, which is known as Som Kheaw Wan in Thai, may have come from China long time ago. The tangerine is flat or oblate to pear-shaped of medium size (5.4-7.5 cm. wide and 4-5.4 cm. high), bright-orange or red orange peel when ripe, smooth, glossy, loose, separating easily from the segments. Seeds are small, pointed at one end and green inside (Morton *et al.,* 1987). In Thailand, the commercial size grader is used to separate tangerines into 6 sizes by diameter at 5.0, 5.5, 6.0, 7.0, 7.5, and more than 7.5 centimeter with no. 3, 2, 1, 0, 00, and 000 respectively.

Total production of tangerines in Thailand was around 600,000 metric tons in 1996 (DOAE, 1996). Although most of tangerines is consumed and exported in fresh form, excess production as well as large quantities of rejected fruit has led to efforts to explore new means of utilization such as jam and fruit juice. High demand for juice extracted from tangerines has added value to the crop which ultimately increase the

income of farmers and increase the export value of the country. With the growing awareness of nutrition value from fruit and vegetable juice, the potential for fruit juice market is great and worth exporting (Table 1). Tangerine juice is preferable by consumers not only for its taste but also its high nutritive value. Table 1 shows the quantity and value of fruits export.

Table 1 Quantity and value of citrus fruits and juice export

Items	Quantity (metric tons) / Value (million bahts)		
	1995	1996	1997
Tangerines	434/ 4.2	612/ 6.1	574/ 6.8
Mandarine	3 / 0.04	2 / 0.1	24 / 0.7
Neck orange	-	-	12 / 0.3
Pomeloes	4,776 / 56.1	6,182 / 66.9	3,247 / 44.4
Other citrus	5 / 0.1	9 / 0.2	1,282 / 6.5
Orange juice	2,342 / 40.0	1,943 / 34.3	5,233 / 100.6

Source: Department of customs

PHYSICAL STRUCTURE

The structure in all citrus fruits are very similar except for their size and shape (Albrigo and Carter, 1977). The cross-section diagram of typical citrus fruit is shown in Figure 1.

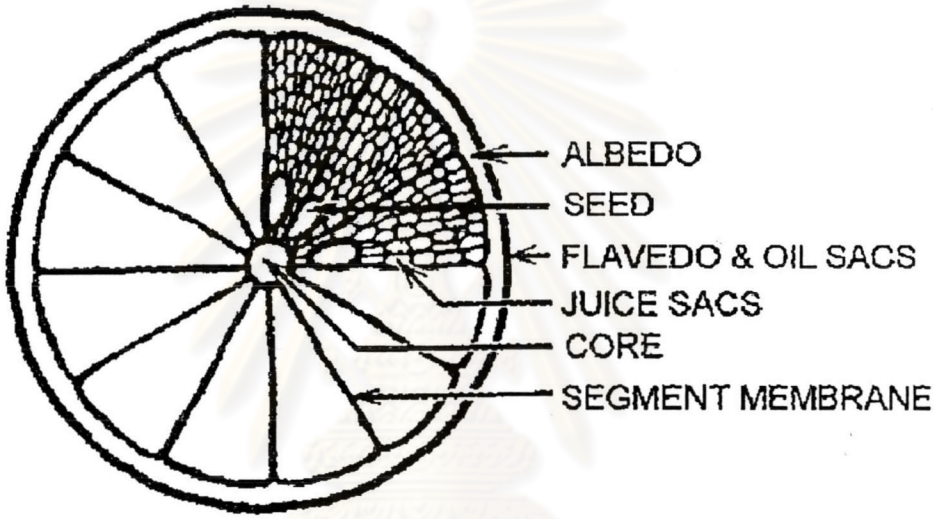


Figure 1 Cross-section of citrus fruit (Considine and Considine, 1982)

Epidermis is an epicuticular wax of platelets. The wax layer is composed of amorphous and soft material, which is deposited slowly as fruit develops. The next inner layer is flavedo, which is either green, yellow or orange in color. Numerous fragile oil glands also scatter throughout the flavedo. Albedo, which consisted of cells arranged in a network with lots of air space is the next inner layer from flavedo. This layer is

composed of pectic substances and hemicelluloses. The next layer is an edible part of fruit called carpel segment which is consisted of 10-14 segments. In each segment there are plenty of juice vesicles connected with segment membranes. The core of the fruit is quite similar to albedo (Ting and Attaway, 1971a).

CHEMICAL COMPOSITION

The principal chemical components in citrus juice are pigments (carotenoids), nitrogeneous compounds (amino acids, amines, peptides and proteins), carbohydrate (sugar, polysaccharides), organic acids (citric, tartaric, benzoic and succinic acids), lipids (oleic, linolenic, palmitic and steric acids), volatile compounds, miscellaneous lipid-solvent soluble compounds, vitamins, inorganic constituents and flavonoids and limonoids

1. Pigments

Chlorophyll and xanthophyll are mostly found in both citrus fruit and juice. When citrus fruit is immature, the dominant color is green. During ripening, chlorophyll a and b break down, and the yellow or orange pigments in the peel begin to increase (Ting and Attaway, 1971b cited Miller *et al.*, 1940b).

2. Nitrogenous compounds

Nitrogenous compunds in citrus fruits consist of amino acids, for example alanine, asparagine, aspartic acid, glutamic acid, proline and serine (Ting and Attaway, 1971c cited Underwood and Rocklands, 1953), amines, peptides and proteins.

The amount of the nitrogenous compounds distributes in various parts of citrus fruit. Bain (1958) observed that in the early stage of development of the fruit, protein nitrogen is predominant (Ting and Attaway, 1971d cited Bain, 1958). As fruit continues to mature, soluble nitrogen which is mostly found in the juice increases and is almost equal to protein nitrogen at the early stage of development.

3. Carbohydrate

The principal carbohydrates in both citrus fruit and juice are sugars and polysaccharides, 75-85% of the total solids of orange juice are sugars, (Ting and Attaway, 1971e cited Bartholomew and Scinclair, 1943). Curl and Veldhuis (1948) concluded that the main sugars in Florida valencia oranges were sucrose, glucose and fructose, which occurred in approximate ratio of 2:1:1. As the early and mid season oranges and tangerines riped on the tree, the total sugars in the juice increase rapidly due to an accumulation of sucrose (Ting and Attaway, 1971f cited Curl and Veldhuis, 1948).

During the course of development of fruits, starch is found in all components of the fruit including the juice vesicles (Webber and Bachelor, 1943). It is especially abundant in the albedo, but is also found in flavedo. The other principal polysaccharide found in citrus fruits is pectic substances which are mostly distributed in albedo and citrus tissue. The pectic substances are important to the processing industry because of their function as a cloud stabilizer in the juice.

4. Organic acids

While, the main acids of the citrus peel are oxalic, malic and malonic acid, citric acid is the principal acid of the endocarp of all citrus. However, tartaric, benzoic and succinic acid have also been reported in the citrus peel (Braverman, 1949). In oranges and tangerines, Sinchair and Ramsey (1944) found that the free acid per fruit increased in early growth and then declined. Decrease in the concentration of acid with the gradual increase in total sugars during development results in an increase in the ratio of total soluble solids to acidity, which is the basis for determining the maturity of the fruit as well as their palatability. Due to the concentration of acid in the citrus juice, the pH of citrus fruit generally varies from about 2 for lemons and about 5 for over-mature tangerines or oranges (Ting and Attaway, 1971g cited Sinchair and Ramsey, 1944).

5. Lipids

There are many lipids distribute in all part of citrus. In the lipid fraction of the rind, Matalack (1929) found oleic, linoleic, palmitic, steric acids, glycerol, phytoseterolin and cerol alcohol. The pulp contained, in addition to those fatty acids and alcohol found in the peel, cerotic acid, phytosterol and penthcosane (Matalack, 1940). The mixture of glycerines and fatty acids is also found in seeds and small amount in juice (Ting and Attaway, 1971h; i cited Matalack, 1929; 1940).

6. Volatile compounds

The most important volatile materials of citrus fruit are those associated with flavor and aroma. These include terpene hydrocarbon ((+)-limonene, α-pinene,

β-pinene, myrcene, sabinene.,etc), carbonyl components (n-octanal, n-heptanal, methyl heptenone, citrnellaln-nonanal, n-decanal and carvone), alcohols (linalool, octanol, α-terpineol, methanol, ethanol, n-propanol, n-heptanol, citronellol, carveol, nerol, geraniol.,etc), esters (ethyl formate, ethyl caproate, ethyl acetate, linalyl acetate, ethyl butyrate.,etc) and volatile organic acids (acetic, propionic, butyric, caproic and capric), which are generally found in peel oil in the flavedo and in oil sacs embedded in the juice vesicles (Ting and Attaway, 1971j cited Davis,1932).

7. Miscellaneous compounds

These compounds are found in the residue after distillation of citrus oil and that they are all solute in lipid solvents. Among these compounds are waxes, coumarins, triterpenoids and steroids.

8. Vitamins

Vitamin C, or ascorbic acid, is by far the most abundant vitamin in citrus fruits, which are important sources of this vitamin. On a whole fruit basis, the juice contained about 25 % of the total vitamin C of the oranges (Ting and Attaway, 1971l cited Atkins, 1945). Tangerines generally contain from 20 to 50 mg of ascorbic acid /100ml of juice. Harding reported that ascorbic acid was usually high in immature oranges, but as fruit ripen and increase in size, the concentration generally decreased. Besides vitamin C, citrus juice also consists of others important vitamins such as inositol, tocopherol, vitamin A, thiamin, niacin and riboflavin (Ting and Attaway, 1971 m; n cited Harding *et al.*, 1940; Harding and Fisher, 1945).

9. Inorganic constituents

The inorganic substances of the citrus fruit are all found in the ash which is found in citrus juice approximately 0.4% by weight. Harding *et al* (1940) found that the ash content was generally the highest in immature fruit and gradually decrease as the fruit maturity progressed. Potassium is by far the most abundant element in citrus juice. Furthermore, iron, manganese, copper, zinc, boron, strontium, barium, aluminium, titanium, lead, tin, nickel, silver, chromium and some anions such as phosphates, sulphates and chlorides have also been found in citrus juice as well (Ting and Attaway, 1971o; p cited Rakienten *et al.,* 1952; Stevens, 1954).

10. Flavonoids and Limonoids

Flavonoids comprised a class of chemical compounds widely distributed in citrus fruits. The principal citrus flavonoids are anthocyanins, flavones, flavonols or flavanones. The last-mentioned group contains hesperidin and naringin, which are the main flavonoids of oranges and grapefruits respectively. These two flavonoids are important as quality indicators for citrus industry. For example, the cloudiness of marmalade made from sweet oranges is due to precipitation of hesperidin, which is less soluble than naringin (Ting and Attaway, 1971q cited Smith, 1953). In addition, high concentration of naringin also reduces juice quality because of its bitter taste, which can be detected at a dilution of 1:50,000 (Ting and Attaway, 1971r cited Zoller, 1918).

Limonoids are chemicals that, like the flavonoids, widely distributed in all citrus species. The most important limonoid compound, limonin, is an intensely bitter

compound that is of commercial significance in the citrus industry as even low concentration of this compound (e.g. 5 ppm) may cause significant reduction in juice quality (Mozaffar *et al.*, 2000).

The two major chemical compounds of citrus, which cause bitterness, are limonoids and flavonoids. Naringin and limonin, the main chemical components causing bitterness in citrus juice, are widely distributed throughout the fruits (Rouseff and Fisher, 1980). Naringin is generally found in grapefruits but does not occur in a number of citrus species such as sweet orange (*Citrus sinensis)*, lemon (*Citrus limon),* lime (*Citrus aurantifolia)* and tangerine *(Citrus reticulata)*(Nagy *et al.,* 1977). In contrast, limonin appears to all citrus species. Moreover, limonin is widely distributed throughout the Rutaceae species (MacIntoch and Rouseff, 1982). The bitter compound of Thai tangerine (*Citrus reticulata* Blanco) is also limonin (Jungsakulrujirek, 1997).

NARINGIN IN CITRUS FRUITS

Naringin is a flavanone glucoside with IUPAC name, 4',5,7-trihydroxy flavanone-7-rhamnoglycosidase or naringenin-7-rhamnoglucoside. The chemical formula of naringin is $C_{27}H_{32}O_{14}$ with molecular weight of approximately 580. Naringin is composed of one flavonoid group attached to a disaccharide. The structure of naringin is shown in Figure 2. If the rhamnose attached at C-7 position of the flavonoid, the compound is bitter however if it attached to the C-2 position of the flavonoid, the compound is tasteless. Naringin is only slight soluble in water, it is soluble in acetone, alcohol and warm acetic acid (Mozaffar *et al.*, 2000). The taste threshold level of naringin issue by the State of Florida, Department of citrus (1982) was 600 ppm measured by Davis test.

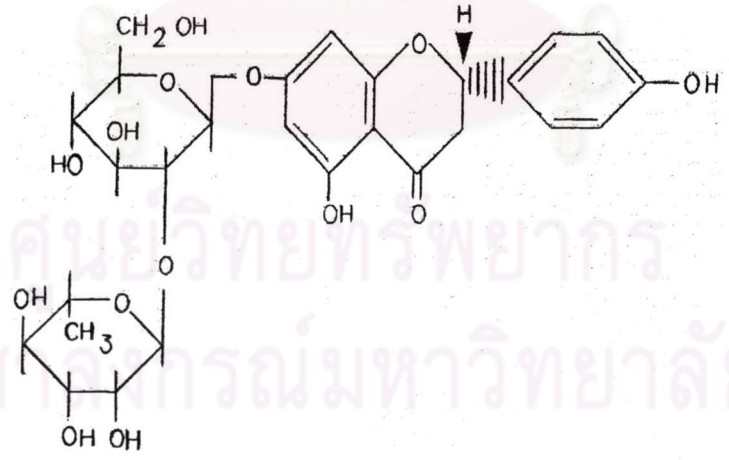


Figure 2 Chemical structure of naringin (Mozaffar *et al.*, 2000)

LIMONIN IN CITRUS FRUITS

Limonin is a highly oxygenated triterpene dilactone with the IUPAC name, Limonoic acid 3,19:16, 17-dilactone; 8-(3-furyl) decahydro –2,2, 4a, 8a-tetramethyl-11H,13H-oxireno-[d]pyrano[4',3':3,3a] isobenofuro [5,4f][2] benzopyran-4,6,-13 (2H,5aH)-trione. The chemical formula of limonin is $C_{26}H_{30}O_8$ and the molecular weight approximately 470, and a volume of approximately 402 cubic angstorms. The melting point of limonin is 298^{0}C and the size is 13.3 A*7.6 A*3.4 A (Norman, 1989). As shown in Figure 3, limonin includes an epoxidic, two lactone rings, a five-membered ether ring, and a furan ring. Limonin is slightly soluble in water and alcohol, although its solubility is increased in the presence of sugar and pectin. It is soluble in acetonitrile, chloroform and glacial acetic acid (Windholz *et al*, 1975).

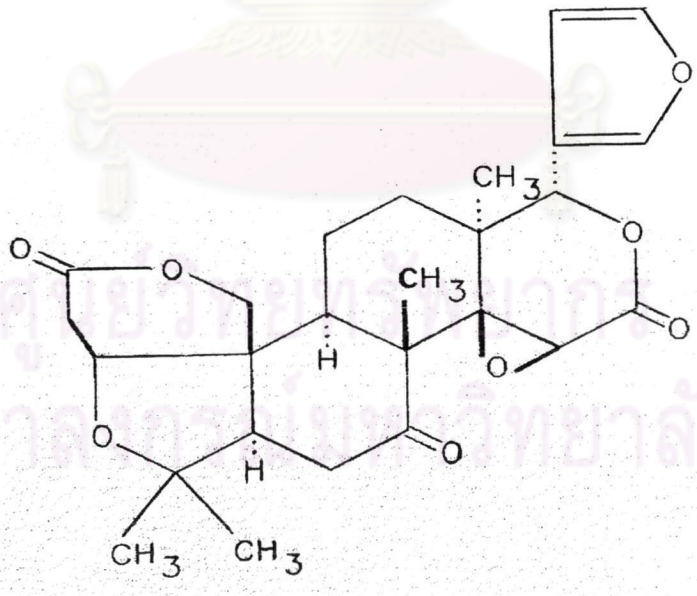
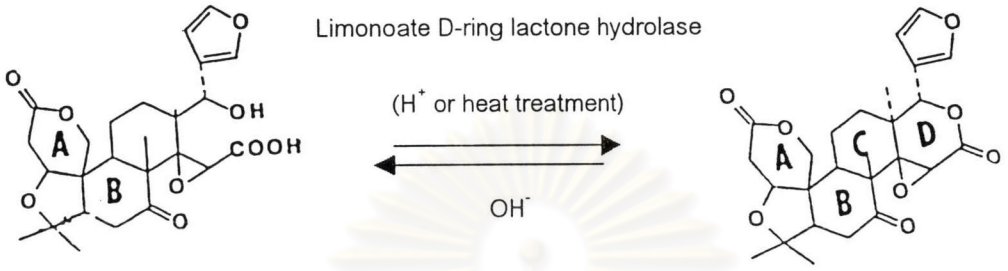


Figure 3 Chemical structure of limonin (Windholz *et al.*, 1975)

Limonin appears to be ubiquitous to all citrus species (Maier *et al.*,1977). In general, most citrus fruits are not bitter tasting if eaten fresh or if freshly-squeezed juice is consumed. However, after pasteurization or a few hours after extraction even kept at low temperature, the juice becomes bitter. This phenomenon is known as "delayed bitterness". The intact fruit barely contained limonin; however, its nonbitter precursor, limonoate-A-ring lactone (LARL), is found to be endogenously present in membrane sacs which is most probably at a neutral to slightly alkali pH. When these sacs are ruptured during juice processing, the LARL encounters the net acidic pH of the juice, which gradually catalyzes closure of the ring to form limonin (Kimball, 1991). In addition, this conversion is accelerated by the action of an endogenous enzyme named limonin D-ring lactone hydrolase as illustrated in Figure 4.



Limonoate A-ring lactone

(non-bitter)

Limonin

(bitterness)

Figure 4 Conversion of LARL (a non-bitter precursor) to limonin (a bitter end product) (Puri *et al.*, 1996)

Microorganisms such as *Arthrobacter globiformis* and *Corynebacterium fascians* isolated from soil, which produce limonoate dehydrogenase and limonol dehydrogenase which convert LARL and limonin to nonbitter 17-dehydroxy-limonoate A ring lactone and limonol respectively (Hasegawa *et al.*, 1972; Hasegawa *et al.*, 1983; Hasegawa, 1985). Figure 5 shows the catabolic pathways for the conversion of limonin to its nonbitter metabolites.

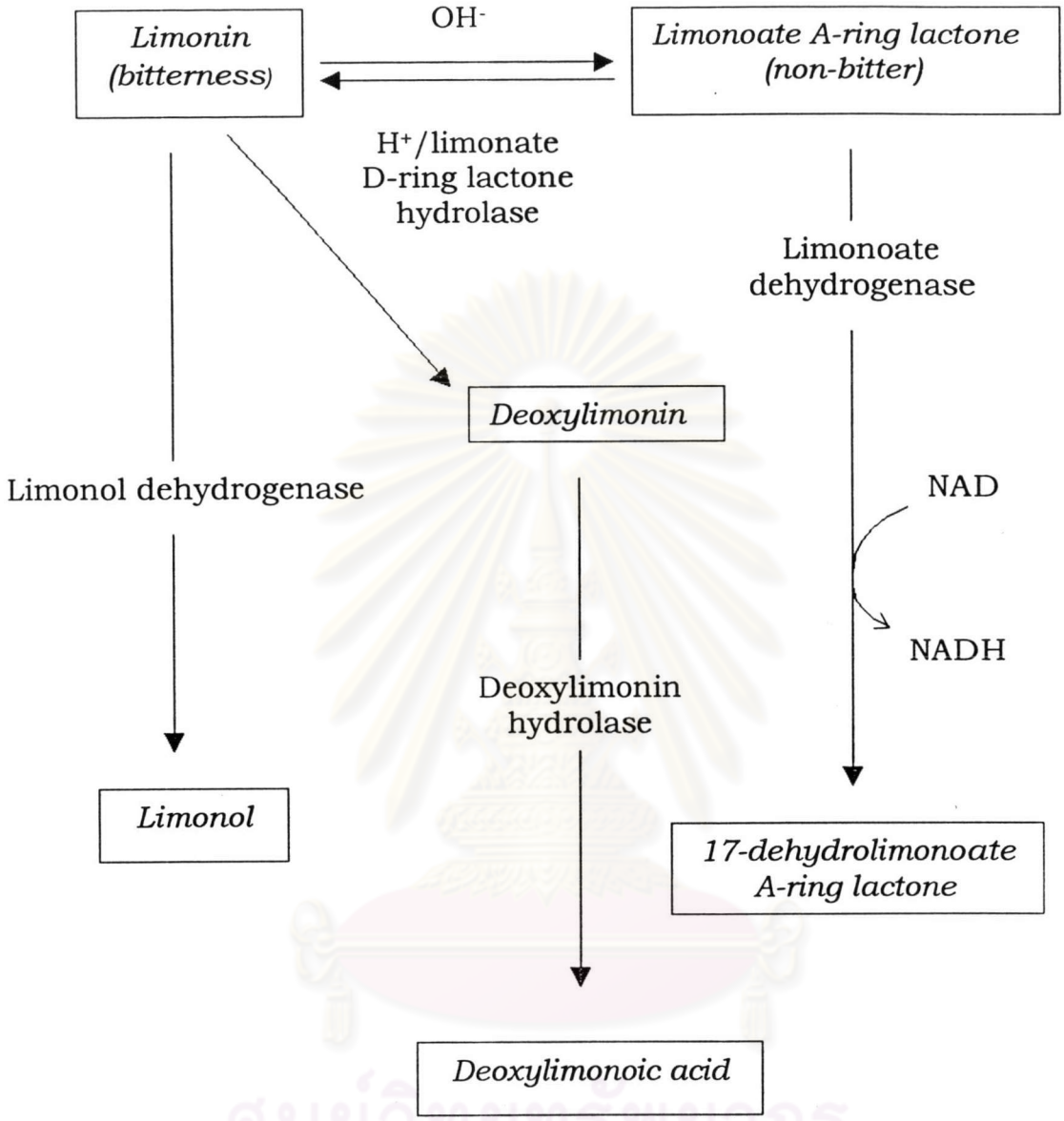


Figure 5 Catabolic pathways for the conversion of limonin to its nonbitter metabolites (Puri *et al.*, 1996)

ANALYSIS OF LIMONIN

The quantification of limonin in juice has been continuously developed using several methods. The first method for the quantitative analysis of limonin, developed by Chandler and Kefford (1966), involved extraction with chloroform, conversion to a dinitrophynylhydrazone derivative, separation by TLC, elution and measurement of the ultraviolet absorption. This method required more time (2 days) and had low sensitivity.

Wilson and Crutchfield (1968) reported a procedure in which limonin was isolated by extracting with dichloromethane, treating with alumina, and partitioning between petroleum ether and acetonitrile. The limonin was then converted to a hydroxamic acid derivative, treated with ferric ion, and absorbance of the color complex was measured at 510 nm. The success of the method depended upon the isolation procedure because the colorimetric determination was specific for lactones and esters. If these compounds were not separated from limonin, they would interfere with the measurement. Because of its limitation, the method was only suitable for orange juice.

Kruger and Colter (1972) developed a gas chromatographic method for determination of limonin. After extraction with chloroform and partitioning between hexane and acetonitrile, the sample was injected onto SE-30 column at 300^{0}C. The height of peak was used as a measure of its concentration. Since the detection system was nonspecific, any compound which overlapped the limonin peak would interfere.

Fluorometric determination of limonin has been reported by Fisher (1973). Grapefruit juice and orange were extracted with chloroform and then chromatograph on an alumina column. The limonin concentration was determined by treatment with sulfuric acid and measurement of the fluorescence produced. Its lower limit was 0.5 ppm and six samples per day could be analyzed.

Tatum and Berry (1973) developed a procedure based on TLC, which did not include the extraction step. Juice samples were spotted directly on the TLC plates and were detected with 2 % sulfuric acid. Limonin concentrations were estimated by visual comparison with the standards. This method was faster than the previous ones (24 samples per 3 hours). It was applicable to orange and grapefruit juice (Ting and Attaway, 1971a).

An improved high-performance liquid chromatography (HPLC) method using normal-phase has been developed for determination of limonin concentration in citrus products (Rouseff and Fisher, 1980). This normal-phase is accurate but requires lengthy chloroform extraction. A rapid method with enzyme-linked immunoassay has also been developed and made into a commercially available kit (Jourdan *et al.*, 1984). The method has the advantage of short analyzing time and the equipment cost is less than HPLC. However, a collaborative study done by Widmer and Rouseff (1991) using the commercial kit indicated some problems with reproducibility of results.

Several reverse-phase HPLC methods were also developed (FMC Corporation, 1983; Shaw and Wilson, 1984; Shaw, 1986; Van Beek and Blaakmeer, 1989). At present,

this method is considered to be the best method for limonin determination. Either a C-8 or C-18 column was used for analysis with rapid solid phase extraction (SPE) to separate limonin from interfering components. Many solvent combinations used as a mobile phase were investigated.

Normal and reverse phase HPLC are used for adsorption and many bond phase separation. Normal phase means that the polarity of stationary phase is higher than of the mobile phase, while reverse phase means that the polarity of stationary phase is less than that of the mobile phase. With both techniques, normal phase retain polar compounds from non-polar matrices and conditioning is done firstly with a non-polar solvent followed by eluting with more polar solvent. In the case of reverse phase, non-polar to moderately polar compounds in polar matrix are retained. These sorbents require prior conditioning with an organic solvent then eluting with less polar solvent.

TASTE THRESHOLD LEVELS

There are many reports concerning the threshold point of limonin in citrus juice presented by several researchers. Barmore *et al* (1986) reported that the threshold point for sensory detection of limonin in distilled water was at 1 ppm while Guadagni *et al* (1973) stated that only 17 % of panels could detect limonin in orange juice at 1 ppm and 75 % of tasters at 5-6 ppm.

The upper limits of limonin and naringin in grapefruit juice issued by the State of Florida, Department of Citrus (1982) were 5 ppm (HPLC) and 600 ppm (Davis test) respectively.

FACTORS AFFECTING BITTERNESS IN CITRUS JUICE

There are several factors, which influence bitterness in citrus fruit, for example, climatic condition, maturity of fruits at harvesting time, and horticultural practices. Besides, many processes in juice production such as extraction and pasteurization also affected its quality.

DEBITTERING OF CITRUS JUICE

Due to the bitterness in citrus has been a long-standing problem affects consumer acceptance especially in juice product. There have been many attempts through the years to eliminate or control levels of bitterness in citrus juice products. The methods are described below.

1. Controlling harvesting time and processing parameters

Because of higher level of limonin in early season fruits than others, one of the method to control the bitterness in citrus is by controlling the harvesting time. Only mid to late season fruits is advised to be processed. In addition, the regulation of processing parameters such as pressure in juice extraction and the immediate separation of pulp from the juice after extraction were recommended as a method for controlling bitterness in juice (Rangana *et al.*, 1983).

2. Chemical methods

Earlier methods of debittering citrus juice used lengthy extraction techniques and involved pH adjustment of the juice after extraction and immediately before consumption (Pritchet, 1957; Swisher, 1958).

Treatments with ethylene (20μg/ml) for 3 hrs to accelerate ripening in navel orange, lemon, and grapefruit with a concomitant reduction in bitterness have been reported by Maier *et al* (1973). Kimball (1987) proposed that citrus juice treatment using carbon dioxide at pressures of 21 to 41 MPA at 30°C to 60°C for 1 hr resulted in average removal of 25% of the limonin from navel orange juice. By extending the treatment to 4 hrs, 60% of the limonin was removed.

Although using chemical treatment seems to be effective in bitterness reduction it was difficult to control and might cause environmental problem (Puri *et al.*, 1996).

3. Enzyme techniques

3.1 Immobilized microbial mass for debittering

Limonoid catabolic pathways in microorgamisms have been studied. An isolate of *Anthrobacter globiformis* which grew on sodium-limonate-rich medium secreted intracellular limonoate dehydrogenase. It catalyzed reversible conversion of LARL to 17-dehydrolimonoate A-ring

lactone in the presence of NAD as shown in Figure 5 (Hasegawa *et al.*, 1972).

To reduce limonin in grapefruit juice, the application of two other bacterial isolates, *Pseudomonas* 321-18 and *Bacterium* 342-152-1 have also been investigated (Hasagawa *et al.*, 1972; Hasegawa and Kim, 1975).

Vaks and Lifshitz (1981) purified *Acinetobacter* sp. from soil and entrapped the bacteria in a dialysis sac. This bacterium could use limonin as a sole carbon source and convert limonin to deoxylimonic acid, a nonbitter compound.

In 1983, Hasegawa and coworkers proposed that packing *Pseudomonas* sp. and *Arthrobacter globiformis* II isolated from soil in column could reduce limonin in citrus juice by metabolizing the limonin to nonbitter metabolites named 17-dehydroxy-limonoate A ring lactone and limonol respectively. Immobilizing *Corynebacterium fascians* cells which produce limonol dehydrogenase for the metabolism of limonin to limonol in acrylamide gel was also investigated by Hasegawa and King (1983) and Hasegawa *et al* (1985).

3.2 Immobilized enzyme for debittering

Immobilization of naringinase from *Aspergillus niger* on various kinds of polymer such as copolymer styrene, maleic anhydride, tannin

amino hexylcellulose and chitin with glutaraldehyde and sodium borohydride to hydrolyze naringin in grapefruit juice have been reported (Goldstein *et al.*, 1971; Ono *et al.*, 1978; Tsen, 1984).

Tsen *et al* (1987) stated that the operational stability of naringinase from *Penicillium* sp. was better than that from *Aspergillus niger* for debittering process in citrus juice. Naringin in grapefruit (*Citrus paradisi* Macf) juice was removed with naringinase from *Penicillium* sp. entrapped in cellulose triacetate fiber, while limonin was being absorbed into the fiber. The immobilized enzyme column could remove both bitter compounds simultaneously without affecting to levels of total organic acids and pulp contents (Tsen and Yu, 1991)

However, it is difficult to control optimum conditions for enzyme activity because of the high variability of factors involved and the biological nature of enzymes. Moreover, it is very difficult to separate the enzymes used to remove bitter compounds since the enzymes are dissolved in citrus juice and this may be affect juice flavor. Hasegawa *et al* (1985) cautioned that the use of immobilized bacteria or enzymes for removing limonin and naringin was difficult to scale up to commercial level.

4. Adsorptive debittering

The use of polyamides to selectively adsorb significant quantities of limonin from Washington navel orange juice has been explored successfully (Griffith, 1969). Likewise, the uses of variety of adsorbents such as cellulose acetate, nylon-based matrices, porous polymers, and ion exchangers have been investigated to reduce bitterness and acidity in grapefruit juice (Johnson and Chandler, 1988). Treatment of grapefruit juice with activated magnesium silicate (Florisil) in a batch mode significantly reduced limonin, naringin, narirutin, and total acid without adversely affecting its nutritional quality (Vitamin C, sugars) and the flavor of the Florisil-treated juice was improved significantly (Barmore *et al.*, 1986).

Other methods to debitter citrus juice have included the use of ultrafiltration and adsorption which remove limonin from grapefruit juice at a pilot plant scale (Fernandez *et al.*, 1992). Puri (1984) reported that the use of cross-linked divinylbenzene-styrene resin could reduce naringin and limonin content in grapefruit juice by 80-90 % respectively. Laboratory studies on the effect of mean pore diameter, percent cross-linkage and specific area of polystyrene-divinylbenzene resins for the adsorption of limonin and naringin from grapefruit juice have also been reported (Manlan *et al.*, 1990).

Debittering with neutral XAD-16 resin, the polystyrene-divinylbenzene, and deacidifying with weak base anionic exchange (IRA-93) for sour orange

juice in a series of four glass columns (30cm x 15 mm i.d) were studied by Couter and Rouseff (1992). Limonin reduction by XAD-16 and IRA93 were about 100% and 24-30% respectively.

However, despite the large number of methods studied to remove bitterness from citrus juice, most of the previously reported methods have serious limitations. For example, carbon adsorbents are non-specific and consequently remove other components present in the juice (Berry, 1981). The use of polyamide has a major drawback in that it results in the substantial loss of ascorbic acid from orange juice. In addition, the use of polyamides requires a two-stage treatment, due to the preferential adsorption of phenolic compounds such as polyamines. Thus, this method is not commercially viable.

5. Debittering by cyclodextrin

Konno *et at* (1981) reported that the use of soluble β-CD effectively reduced the bitterness from grapefruit juice, lyo orange, and *C. natsudaidai* due to its ability to form inclusion complexes with bitter substances. This information led to utilization of insoluble β-CD polymer in a batch/continuous column mode to remove limonin and naringin from their aqueous solutions in orange and grapefruit juices (Shaw and Wilson, 1983). It was notable that using the β-CD polymer to remove bitterness has no effect on the amount of total solid, acidity and ascorbic acid as well. The ability to regenerate of the β-CD polymer by extraction with ethanol enhanced its use for scale-up trials.

Significant quantities of limonin, nomilin, and naringin from grapefruit juice as well as limonin and nomilin from navel orange juice were removed in a continuous flow fluid column (Shaw *et al.*,1984). A taster's panel indicated more consumer preference for such debittered juice compared with the control. This led to a scale-up application of the β-CD polymer on a pilot-scale fluidized bed column, enhancing the possibilities of using such a system for commercial operations (Wagner *et al.*, 1988).

CURRENT COMMERCIAL DEBITTERING PROCESS

The first commercial debittering unit was installed in a plant in Australia in the late 1970's based on the research of Johnson and Chandler (1981). The debittering units consisted of 12 long cylindrical column packed with cellulose acetate which was one of the polymer approved for food use at that time. The juice was passed through the column in series. However there was a continuing problem of column plugging. During the same time period, Maeda and co-workers (1984) studied 33 types of neutral and ion exchange resins for their capacities to deacidify and debitter juice from Hassaku mandarin grown in Japan. They found styrene-divinylbenzene and acrylonitrile-divinylbenzene to be the most effective resins for reduction of naringin and limonin (Shaw, 1999a cited Maeda *et al.*, 1984).

In 1988 the first debittering unit used in the United States was installed in California using a styrene-divinylchloride polymer that was approved for food use at that

time (Shaw, 1999b cited Kimball and Norman, 1990). That unit, which is still in use in 1999, consists of two stages, centrifugation of the juice to remove most of the pulp and passage of the clarified juice through one or two column packed with a hydrophilic styrene-divinylbenzene adsorbent. After the first unit was installed, the second unit, which was designed to use ultrafiltration for pulp removal in conjunction with the use of neutral styrene-divinylbenzene as adsorbent, was installed in another plant in California. The schematic processes involving combined technology of ultrafiltration and adsorption are illustrated in Figures 6 and 7. The styrene-divinylbenzene resin is popularly used in current debittering unit and they remain to be a suitable adsorbent for debittering unit as long as they satisfied the U.S. Food and Drug Administration requirements for food contact use.

The use of β-CD polymer in food industries is also interesting because it is natural material which can form inclusion complex with suitable guest molecules. In addition, in form of insoluble materials, the β-CD polymer is easily removed from the food processes. Thus, in this work the researcher proposed to study the efficacy and feasibility of using the β-CD polymer as adsorbent for debittering process. The β-CD monomer and polymer are described below.

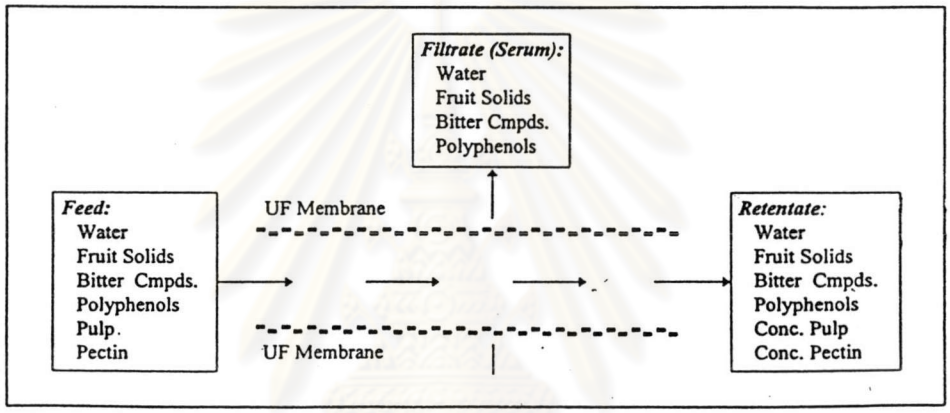


Figure 6 Ultrafiltration (UF) membrane performance in citrus processing (Milnes, 1995)

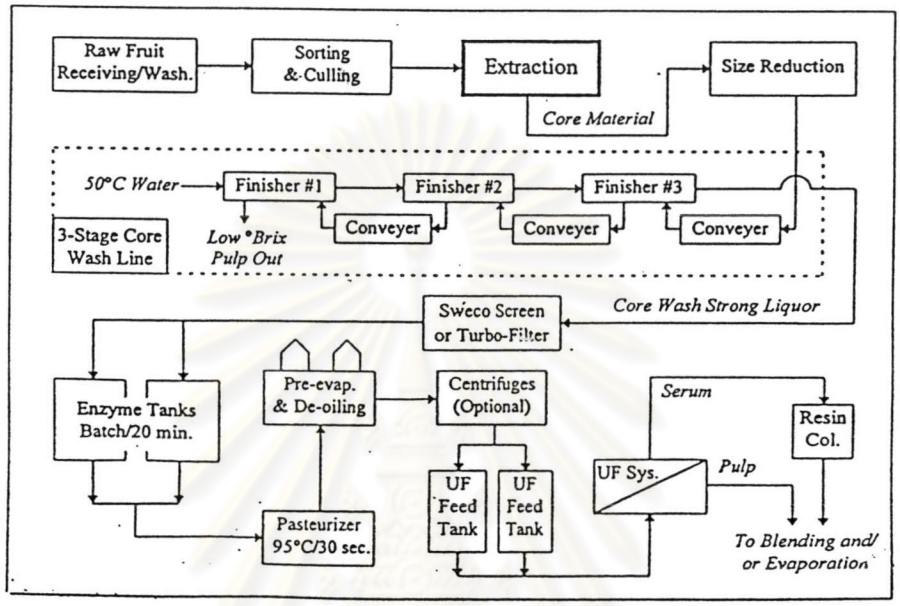
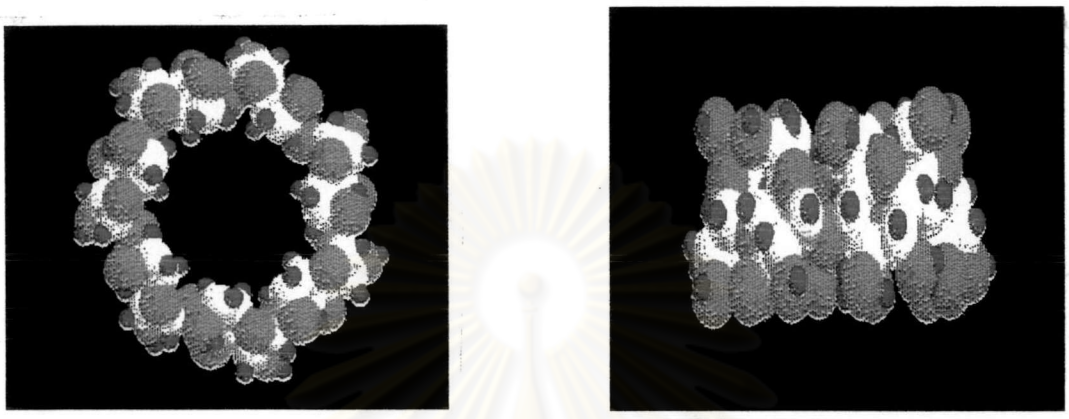


Figure 7 **Schematic flow of commercial debittering process (Milnes, 1995)**

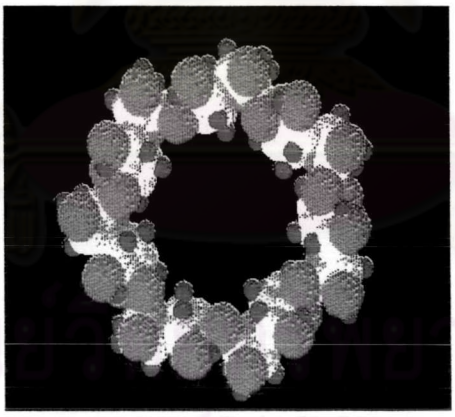
CYCLODEXTRINS

Cyclodextrins are cyclic oligosacchrides shaped like a truncated cone which has a hydrophobic cavity. Cyclodextrins are composed of six or more D-glucose residues, and are named α-CD (six glucose residues), β-CD (seven glucose residues), and γ-CD (eight glucose residues) according to the number of glucose residues, respectively. Primary hydroxy groups are located on the narrower rim of the cyclodextrin cavity, while the secondary hydroxy groups are on the wider rim of the cyclodextrin cavity. The numbers of the primary and secondary hydroxy groups are one and two, respectively, per a single glucose unit in cyclodextrin. Because of the presence of the hydrophilic hydroxy groups existing at the two ends of the cavity, cyclodextrins are soluble in water. Due to the hydrophobicity of the cavity interior, however, hydrophobic molecules can be bound into the cavity when the molecules have dimensions suitable with the cavity size. Figures 8 – 10 show the α, β and γ- CD structures and their physical properties are shown in Table 2.



a) Top view of β-CD

b) Side view of β-CD



c) Bottom view of β-CD

Figure 8 **Molecular model of β- CD (white, red and blue balls represent C, O and H atoms) (http://www.akita-u.ac.jp/~hamai/bCD_fig1e.htm)**

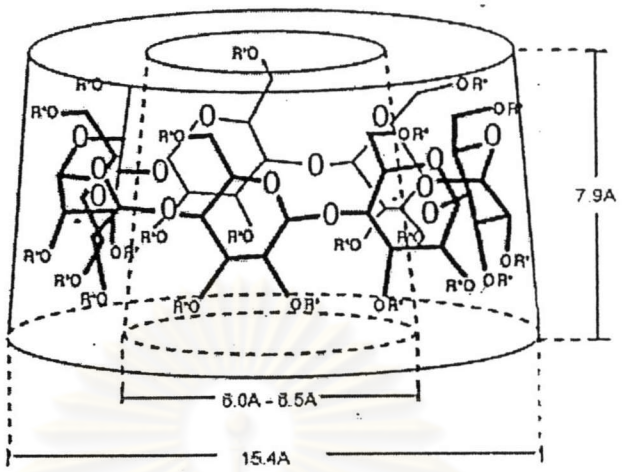


Figure 9 Structure of β-CD (Szejtli, 1998)

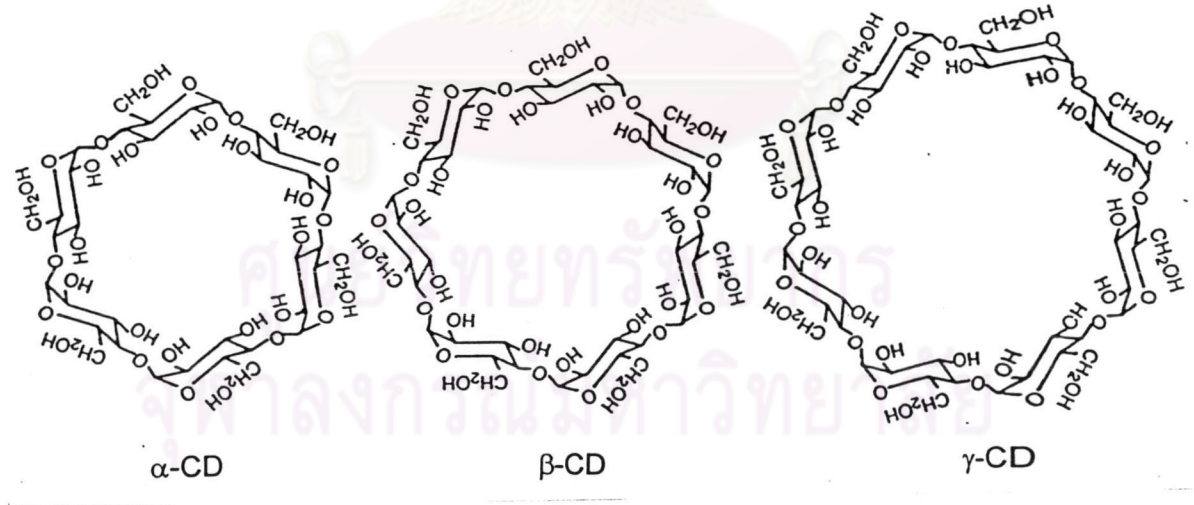


Figure 10 Chemical structure of α, β, and γ- CD (Szejtli, 1998)

Table 2 **Physical properties of α, β, and γ- CD (Szejtli, 1998)**

	α	β	γ
Molecular weight	972	1135	1297
Glucose monomers	6	7	8
Internal cavity diameter (angstroms)	5	6	8
Water solubility (g/100mL: 25 ^{o}C)	14.2	1.85	23.2
Surface tension (mN/m)	71	71	71
Melting range (^{o}C)	255-260	255-265	240-245
Water of crystallization	10.2	13-15	8-18
Water molecules in cavity	6	11	17

CYCLODEXTRINS AND INCLUSION COMPLEX

In liquid and solid phases, organic and inorganic molecules of appropriate size can be incorporated into the cyclodextrin cavity to form inclusion complexes. The incorporated compounds and cyclodextrins are referred to as guests and hosts, respectively. On the basis of the hydrophobic, van der Waals, and hydrogen bonding interactions, a host-guest inclusion complexation is believed to occur. α-CD, which has the smallest cavity among cyclodextrins, best accommodates a benzene nucleus while β-CD best accommodates a naphthalene nucleus. Usually, a single guest molecule is encapsulated into the cavity of a single cyclodextrin molecule to form a 1:1 inclusion

complex. However, two small guest molecules are often incorporated into a single cyclodextrin cavity, resulting in the formation of a 1:2 host-guest inclusion complex. In some cases, one guest molecule, which is bulky or long, is encapsulated by two cyclodextrin molecules, leading to the formation of a 2:1 host-guest inclusion complex. In addition, the self-association of 1:1 inclusion complexes takes place to form a 2:2 inclusion complex. More generally, the association of different two kinds of 1:1 inclusion complex occurs to generate a 2:1:1 host-guest 1-guest 2 inclusion complex.

Figure 11 show the complexation process. The circles represent water molecules. The outer surface of the β-CD is hydrated, but the water molecules in the ring cavity are in the energetically unfavorable position due to its non-polar surface cavity. The potential guest molecule repulses the water molecules. The result of the complex formation is that the non-polar the guest molecule penetrates into the β-CD cavity (Pagington, 1985). The inclusion complex formation of β-CD and pyretroid is also illustrated in Figure 12.

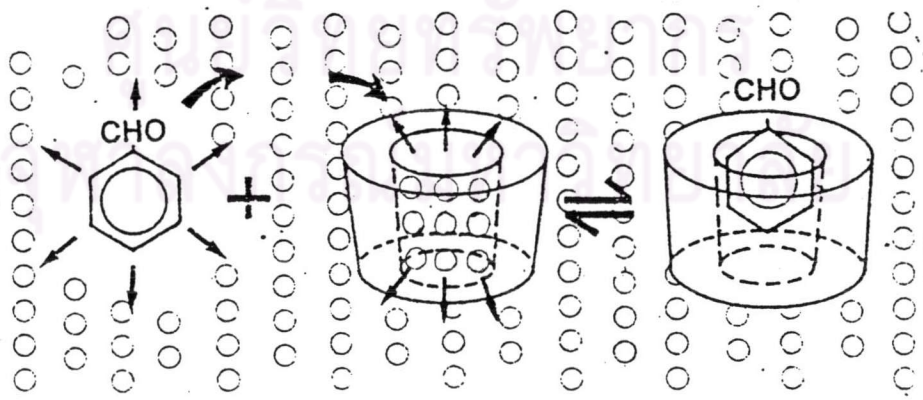


Figure 11 The complexation process, the circle present water molecules (Pagington, 1985)

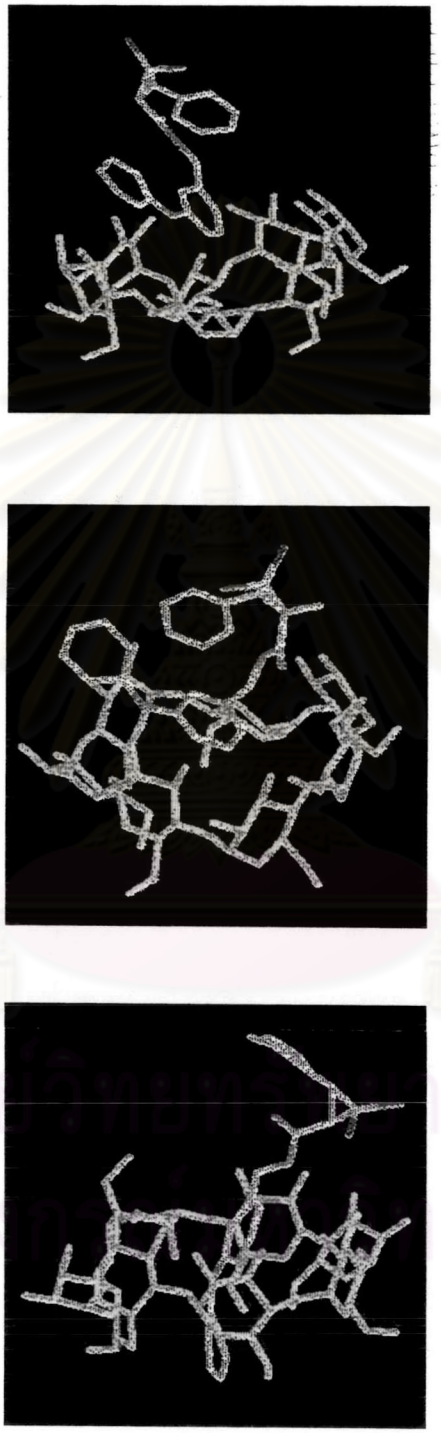


Figure 12 **Snapshots of the equilibrium configuration for each of β-CD-pyretroid complex (http://antas.agraria.uniss.it)**

APPLICATIONS OF CYCLODEXTRINS

Cyclodextrins are widely used in many industries, particularly foods, pharmaceuticals, cosmetics and agricultural industries, in order to increase the solubility of guest molecules and to protect the guest molecules from degradation, oxidation, volatility and sublimation. Moreover, cyclodextrins are used in the isolation of desirable and undesirable compound processes.

In food industries, cyclodextrins are used in food formulations for flavor protection or flavor delivery by means of encapsulation technology. Cyclodextrins are used as process aids for example, to remove cholesterol from products such as milk, butter and eggs.

In pharmaceutical industries, most of pharmaceutical active agents do not sufficient solubility in water although they should have a certain level of water solubility to be readily delivered to the cellular membrane. Cyclodextrins are added into drugs so they can overcome the problem by improving its solubility. They also offer distinct advantages such as the stabilization of active compounds, reduction on volatility of drug molecules, and masking malodors and bitter taste without side-effected.

In cosmetic industries, the major benefits of cyclodextrins in this sector are stabilization, odor control, and process improvement upon conversion of a liquid ingredient to a solid form. Applications include toothpaste, skin cream, liquid and solid fabric softeners, paper towels, tissues and underarm shields.

In agricultural industries, cyclodextrins are used to stabilize and increase solubility of agricultural chemicals including herbicides, insecticides, fungicides, repellents, pheromones, and growth regulator.

In chemical industries, cyclodextrins are widely used to separate isomers and enantiomers, to catalyze reaction, to aid in various processes, and to remove or detoxify waste materials.

Application of cyclodextrins in various sections could be represented by Figure 13.

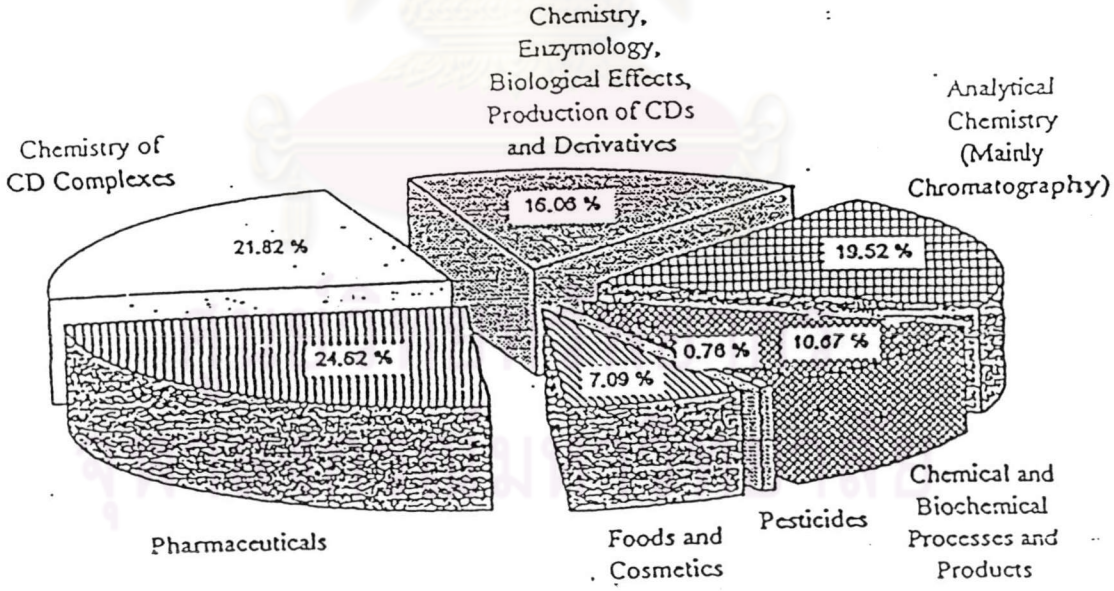


Figure 13 Distribution of the 1706 CD relevant abstract published in 1996 by Cyclodextrin News (Szeijtli,1998)

β-CYCLODEXTRIN SAFETY STATUS

It has taken many years to dispel the notion of β-CD toxicity created by some early works using adulterated material. The acceptances of using cyclodextrin in food industry as food additive or food processing aid in many countries are different. The β-CD is permitted in the Australia Food Standards Code for extraction of cholesterol from eggs, but not as a flavor carrier. In Europe, β-CD is used as processing aid and permitted to use in foodstuffs in tablet and coated tablet. The use of β-CD as solvent carrier at limit if 1 g/kg body weight is expected that the EU Commission will soon amend. Similar to Korea, β-CD is included in the " List of Existing Food Additives" published by the Ministry of Health and Welfare in Japan. The joint Expert Committee on Food Additives (JECFA) considers β-CD as approved food additive with an ADI (acceptable dairy intake) of 0-5 mg/kg body weight.

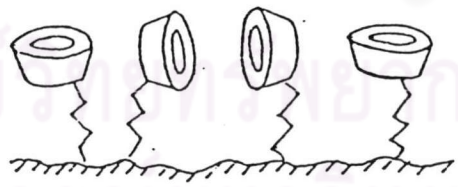
Recently, used of β-CD as a food additive is approved in United States by Generally Recognized as Safe (GRAS) in many food categories with different limitations. As flavour carrier and protectant, the β-CD is allowed to be used in chewing gum, compressed candies and breakfast cereal at the maximum level of 2%. In processes of chess products, gelatins & pudding, baked goods and beverage prepared from dry mixes and flavored coffee & tea products, it is used at maximum level of 1% (www.betacyclodextrin.com)

CYCLODEXTRIN POLYMERS

Cyclodextrin polymers are prepared by crosslinking the cyclodextrin rings, by polymerising bifunctional substituent containing cyclodextrin-derivatives, or binding cyclodextrin to other polymers. Many appropriate crosslinking agents using in preparation process are di-or polyfunctional compounds, such as aldehydes, ketones, allyl halides, isocyanate, epoxides etc., epichlorohydrin, and ethyleneglycol diepoxypropyl. The cyclodextrin polymers may be water soluble, insoluble strongly, swelling, moderately swelling, amorphous powders, beads, foils, solidified forms. Figure 14 shows the structure of many kinds of CD polymer.

a) Chain CD-polymer

b) Network CD- polymer



c) Immobilized CD-polymer

Figure 14 The predicted structure of many kinds of cyclodextrin polymers (Szejtli and Pagington, 1987)

β-CYCLODEXTRIN EPICHLOROHYDRIN POLYMER (β-CD polymer)

The β-cyclodextrin polymers (β-CD polymer) have been used in several fields such as analytical chemistry, thermochemistry, catalysis, waste water treatment, pharmaceutical and food industries. It is prepared by cross-linking β-CD with epichlorohydrin. Reacting one or more of 21 hydroxyl groups with epichlorohydrin can polymerize the β-CD generally. This cross-linking agent, which cointains two reactive functional groups can react with β-CD molecules in cross- linking step and/ or itself in polymerization step. Therefore, the structure of β-cyclodextrin epichlorohydrin polymer (β-CD polymer) is very complicated. It is evident that in this material, two kinds of structure could exist:β-cyclodextrin cross-linked by epichlorohydrin and polymerized epichlorohydrin. These solids contain only glucose units and hydroxyalkyl groups (Crini *et al.*,1999).

Figure 15 shows the reaction scheme of polycondensation of the β-CD with epichlorohydrin.

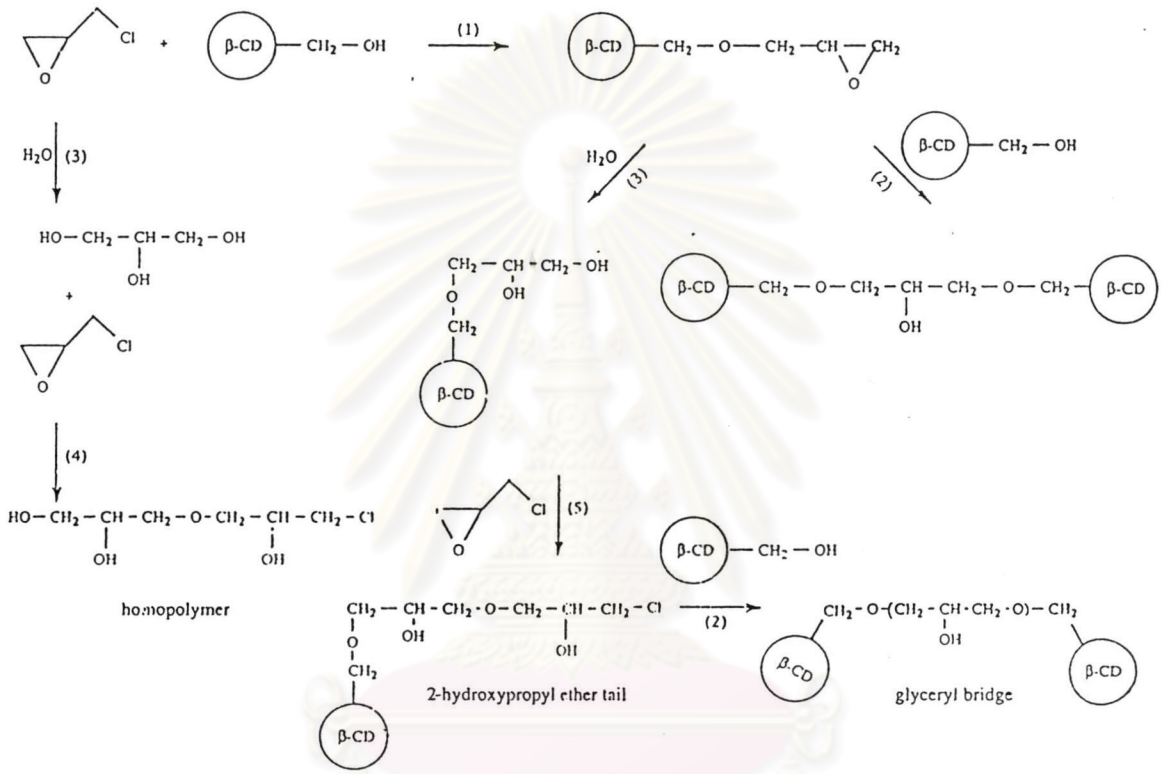


Figure 15 Reaction scheme of the polycondensation of β-CD with epichlorohydrin (Renard *et al.*, 1997)

THE OBJECTIVES OF THIS STUDY

1. To develop the method of determination of limonin from tangerine juice using EXTRA SEP C-18 column and reverse phase HPLC.
2. To investigate the optimum condition for a batch debittering process using β-CD polymer as an adsorbent.
3. To compare the efficiency of β-CD polymer in the batch and column debittering process.
4. To compare the % limonin reduction efficiency of β-CD polymer column and XAD-16 resin column.