CHAPTER IV
DISCUSSION

Tangerines are called "Som Khew Wan" in Thai. The production of the tangerines in Thailand was around 60,000 metric tons in 1996. Because of its desirable taste, tangerine are modified to many products such as chilled single strength juice, canned single strength juice, frozen concentrated juice and so on. However, in the case of mass production, resulting the delayed bitterness of citrus juice has been a long-standing problem affecting consumer acceptance. There are many attempts to eliminate the bitter compounds eg., limonin and naringin from the products. Many types of polymer such as β-CD and XAD-16 resin were used in the debittering process. Using adsorbent technique is available for commercial practice at present. Reports for debittering citrus juices with β-CD are, however, quite limited and mainly by few groups of investigator. This might be due to the regulatory policies on the use of cyclodextrin in food industries by certain countries, especially the United States of America. With the current status of β-CD safety, approval for β-CD used are increasing worldwide. In U.S.A., β-CD is allowed for used as a processing aid (www.betacyclodextrin.com).

The aim of this study was to examine the reduction of limonin content in Thai tangerine juice using β-CD polymer. There are two main parts of studies reported in this work. First is the development of method for naringin and limonin determination. Second is the investigation of batch and column debittering processes by β-CD polymer,

including comparison of the use of XAD-16 column for debittering. An assessment of the production cost and potential of β-CD polymer for commercial debittering used was also reported.

Naringin and limonin are two major bitter compounds generally found in citrus fruits. Naringin is a bitter flavonoid existing in fresh juice. On the other hand, limonin is formed from a non-bitter precursor present in the juice sacs: limonoate A-ring lactone when the fruits are extracted and allowed to stand or are heated during processing (Shaw *et al.*, 1999). Unlike limonin, which is found in all citrus species, naringin does not occur in a number of citrus species such as sweet orange (*Citrus sinesis)*, lemon (*Citrus limon)*, lime (*Citrus aurantifolia)* and tangerine (*Citrus reticulata)(*Nagy *et al.*, 1977).

There are several reports concerning naringin determination by Davis test, a color development of the reaction between flavonoids and alkaline solution detecting by means of spectrophotometry, (Peaschan, 1975; Show and Wilson, 1985; Shaw and Buslig, 1986). Since the Davis test is positive for all bitter and non-bitter flavonoids, it can not indicate with Davis test that naringin actually presents in Thai tangerine juice. Thus, in this work, after fractionation by Extra Sep C-18 column suggested by Rouseff *et al.* (1987), the naringin content in Thai tangerine juice was determined by reverse phase HPLC, which was recommended by Fisher and Weaton (1976) because the method can differentiate between naringin and its tasteless isomers. The accuracy of the method for naringin determination was 81% with the resonable precision (±5.30 %

C.V.) (Table A 1). This study suggested that naringin in Thai tangerine juice was not detectable (Figure 21). This finding is supported by the study of Nagy and co-workers (1977) which reported that naringin did not occur in some citrus species such as sweet orange (*Citrus sinensis*), lemon (*Citrus limon)* and tangerine *(Citrus reticulata)*. It could be stated here that limonin is the major bitter compound in Thai tangerine existing in the range of 1-4 ppm in fresh juice from fruits harvested during 8-12 months of age (Jungsakulrujirek, 1997).

Various methods for quatitative determination of limonin in citrus juice have been developed for many years. TLC (thin layer chromatography), an immunoassay method, and HPLC techniques were reported by Tatum and Berry (1973); Weiler and Mansell (1980), and Rouseff and Fisher (1980) respectively. At present reverse phase HPLC becomes the most popular method because it is accurate and take short time of analysis. The HPLC resutls could be improved by solvent extraction step before analysis. However, for reliable data, the partition solvent extraction is not recommended because of numerous interfering peaks (Figure 25). Alternatively, the use of rapid solid phase extraction (SPE) is more proper for the limonin separation (Figure 24). In this study, Extra Sep C-18 column (C-18 octadecyl silane chemically bond to< 5μm microsilica packing) was used for limonin separation. Basically, SPE using Extra Sep C-18 column can be explained in term of reverse phase adsorption chromatography. The limonin in sample juice was slowly passed through the Extra Sep C-18 column and retained in the column before slowly flushed down by moderate polar solvent.

Shaw and Wilson (1984) recommended to use this technique for limonin separation before HPLC analysis. They also reported that, besides high effective interfering removal, the preliminary separation by SPE could be used on all samples to protect the column and guard column from possible contamination by solid juice particles. In this work, the limonin peak was at 22 minutes.

The limonin standard curve was established by using six standards at levels of 2.5 – 25 ppm in 5 ppm increments at 20 μl injection volume. The standard curve showed a reliable linear response ($R^2 = 0.9997$).

The % recovery of limonin by first use of Extra Sep C-18 was around 83 % (at the started limonin concentration of 10 ppm) with very good precision (% C.V. of 0.94). From Figure 28, it can be seen that the % limonin recoveries decreased when the Extra Sep C-18 was reused (83.17±0.78, 78.91±1.23 and 66.86±2.17 respectively). It might be because the Extra Sep C-18 lost its limonin adsorption ability. For this reason, the researcher recommended to use the Extra Sep C-18 column only once for the best separation.

The sensitivity of the method for limonin determination was observed at the lower limit of 0.3 ppm (Table 2). The lower limit of the method in this study is lower than the method reported by Shaw and Wilson (1984) which was at 1 ppm. That means the method used in this study was more sensitive. It may be due to different condition during SPE extraction such as flow rate, composition of solvent or type of HPLC column. In addition, the % recoveries of Extra Sep-C-18 column were decreasing at the lower

limitation 0.3 ppm compared to 10 ppm load. It was demonstrated here that the % recovery of limonin was decreasing from around 83 to 66 when initial limonin concentration was varied from 10 to 0.3 ppm (Table 2 and 3). The flow rate was also affected the accuracy of this method which gave higher accuracy when low flow rate used(data not shown). Hence, both initial limonin concentration and flow rate and number of reused might affect the accuracy of the method. Although, there was limonin loss during separation by Extra Sep C-18 column, the precision of the determination represented by % C.V. at 3.70 was still good for 3 ppm level.

In this study, the limonin content in fresh, chilled (24 hours) and pasteurized juice, extracted from Thai tangerine harvested in August, 2000, were explored. From Figure 29, it was found that the limonin contents obtained from fresh, chilled and pasteurized juice were 3.97±0.02, 4.07±0.06 and 4.60±0.09 ppm. The pasteurized juice showed the highest of limonin content. It can be explained in term of delayed bitterness in which a non-bitter precursor was converted to bitter compound due to the process of juice extraction or heat treatment and upon prolonged standing. The non-bitter precursor, limonoate-A-ring lactone (LARL), is found to be endogenously present in membrane sacs which is 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 which works well under heat treatment and in the acidic

condition. Such observation was supported by the finding of Moshonas and Shaw (1995) who concluded from the results of sensory evaluations comparing fresh and pasteurized orange juice that fresh juice had significantly higher hedonic rating than the pasteurized juice. The reason was that higher limonin increment during pasteurization effecting on consumers' acceptance. Likewise, other reports supported the notion that pasteurization is a cause of limonin increment of the juice until it was higher than the taste threshold level (Shaw and Wilson, 1983; Shaw *et al.*,1984; Shaw and Wilson,1985; Couture and Rouseff,1992).

Since the level of limonin in thai tangerine juice was too small for debittering study, it was necessary to increase its level by heating. The results from Figure 30 and 31 showed that heating at 70 ^{0}C for 10 minutes was adequate for complete limonin conversion. Therefore, sample juice for debittering process would be preheated at this condition. Furthermore, the pasteurized juice was centrifuged before debittering to prevent the column plugging.

Cyclodextrin, with its hydrophobic cavities, had been demonstrated that it could form inclusion complexes with naringin and limonin (Konno *et al.,* 1982). In case of the molecular study, NMR spectra have been recognized as direct methods of verification of the formation of inclusion complexes. The formation of naringin and β-CD inclusion complex has been reported by Konno *et al.,* (1982). From NMR spectra, the chemical shifts of the β-CD protons definitively established that the phenyl ring and not the rhamnose moiety, of naringin is positioned within the β-CD cavity. In case of limonin,

examination of ^{1}H NMR spectra was impossible because of its low solubility. However, Konno and co-workers (1982) also assumed from the chemical structure of limonin, similar to naringin, the limonin can forms inclusion complex with β-CD at the position of its furan ring.

Not only the formation of inclusion complexes between bitter compound and β-CD monomer has been reported, but the inclusion of naringin by β-cyclodextrin ephichlorohydrin polymer (β-CD polymer) was also discussed. Su and Yang (1990) reported that using insoluble polymeric gel (ECH: β-CD >15) gave higher capacity to form complex with naringin than using soluble polymeric gel (ECH: β-CD <15). They belief that, the inclusion of naringin is due not only to the cavities in the β-CD structure itself but also to some form of interaction between naringin and the cross-linked ECH structure of the gel. For the debittering process, the β-CD polymer was preferred over β-CD monomer because of its specific characteristics. Since the β-CD polymer is insoluble material and has larger size than the monomer, the β-CD polymer can be easily excluded from the juice after debittering process. β-CD monomer, on the other hand which has slight solubility and difficult to be separated from the juice and some of the monomer may contaminate to the juice product, which is undesirable due to legal regulation by many countries. Besides, the adsorption ability of the β-CD polymer was higher than monomer because of its porosity, particle size and surface area.

The β-CD polymer in this study obtain as a gift from Cerestar Inc., U.S.A. is an epichlorohydrin polymer. It is insoluble, bright yellow puffy bead that can swell in water

(~0.3g/ml). However it needed to be cleaned before applying to the debittering process. In this work, the unwashed β-CD polymer was compared with the washed β-CD polymer (Figure 32). It can be seen that after treating with acetone and water, the β-CD polymer was brighter in their color and more uniform.

The use of β-CD polymer as an adsorbent in the batch debittering process was studied. It was observed that limonin reduction varied with amount of debittering agent, time and temperature (Table 5). This is consistent with the results of previous works (Shaw *et al.,* 1984; Shaw and Wilson, 1985). The best condition for maximum limonin reduction in batch process was at cold room temperature (~6^{0}C), 5g% β-CD polymer and 60 minutes processing time. The limonin reduction was around 81% as shown in Table 5. Nevertheless, in practice, not only limonin reduction efficiency was considered but also other factors such as production cost, processing time, nutrition loss during process and limonin content in acceptable level. Basing on the standard of acceptable limonin level in citrus juice product issued by the State of Florida, Department of Citrus, U.S.A., the final limonin, found in debittered Thai tangerine juice should not be over 5 ppm. The researcher decided to operate the batch debittering process at the condition of: room temperature (~30 ^{0}C), 3g% of β-CD polymer and 30 minutes, which gave around 68.0 % limonin reduction and 2.80 ppm limonin left in final juice product as shown in Table 6. The reason why the researcher chose room temperature (~30^{0}C) as the suitable condition was due to lower energy consumption and processing cost (dose not need a cold room or refrigerator). Three g% of β-CD polymer was selected instead

of 5g% because it still gave reasonable % limonin reduction (~68.0) with acceptable limonin level in juice products (< 5 ppm). In term of processing time, 90 minutes was not appropriate for this process because comparing with 60 minutes, there was no significant difference in % limonin reduction at confidence level of 0.05. Although statistical analysis showed the difference in % limonin reduction between 30 and 60 minutes as 68.05 and 71.83 respectively, the increased debittering was very slight. In consideration of possible nutrition loss, production cost and energy consumption cost that would increase with longer process time, the appropriate processing time was selected at 30 minutes. At the selected condition, the limonin content in the final juice product was 2.8 ppm and the limonin adsorption capacity was 0.17 mg limonin/g β-CD polymer (Table 5 and A 6). The data obtained from this experiment was used for planning of column process development.

Regarding of the mixing speed in batch system, it can be seen that there was no significant difference in % limonin reduction between using rotor speed No.3 and 5 at confidence level of 0.05. However, from Table 6, it was observed that the use of higher speed for mixing β-CD polymer and sample juice gave a little lower % limonin removal than that of lower speed. It might be because of there were detriment of the beads and more diffusion between limonin and β-CD polymer occurred when higher speed was used. Both phenomenon may effect the ability to form inclusion complex between limonin and β-CD polymer.

In conclusion, the optimum condition for batch debittering process was; room temperature (~30 ^{0}C), 3g% β-CD polymer, 30 minutes and mixing speed No.3 (Heidolph MR3003 magnetic stirrer).

Shaw and Wilson (1983) and Shaw *et al* (1984) reported that using 0.2-1.8 g% β- CD polymer, 30 minutes in batch process gave around 50 % limonin reduciton.

Column process was developed from the data of the batch process with four times scaled-up. In this work, the column process showed better % limonin reduction than in the batch process (94% and 68% respectively). From Table 10, due to its high effectiveness in limonin removal, it was observed that the limonin absorption capacity of column process at the chosen condition was at 0.27 mg limonin/g β-CD polymer. It was about 1.6 folds higher than the data obtained from the batch process (0.17 mg limonin/g β-CD polymer. This finding is consistent with the report of Shaw and Wilson (1983) who reported that the % limonin reduction from batch and debittering process was around 50 and 90% repectively at the condition of 1.2-1.8 g% β-CD polymer, room temperature, 60 minutes flow rate 3-5 ml/min. The better efficiency probably because, in the column process, the limonin was constantly in contact with β-CD polymer and the concentration of β-CD polymer per unit volume was higher. Moreover, there was less diffusion occurred during the process run. For these reasons the limonin can be easily absorbed with minimum disturbances.

Besides giving better result, was found that operating with column process gave more effective for debittering and regeneration because unlike batch process there was

no need to have a filtration unit. Regeneration process is also easy. In addition, in column process, it is easier to develop to large scale than the batch process. The batch process is usually used for first trial studies to obtain preliminary data. Jungsakulrujirek (1997) studied and compared of batch, column and fluidized bed debittering processes using XAD-16 resin. She reported that using cloud juice sample, the efficiency of batch was better than the column (operated under gravity flow) (0.011 and 0.001 mg limonin/g XAD-16resin) because there was no clogging occurred. However, using fluidized bed gave the most effective limonin removal for cloud juice (0.023 mg limonin/g XAD-16 resin) compared to batch and column processed because there was no resin destruction and clogging found under this operation. It should be noted that the capacity of this process was very low. To prevent this clogging, the sample juice was recommended to be clarified by filtration or centrifugation before loading. Some other debittering techniques were also suggested e.g., fluidized bed system and resin in cage system. (Shaw *et al.*, 1984; Shaw and Wilson, 1985; Shaw and Buslig, 1986). In commercial debittering unit, more than one adsorption resin columns were installed to provide high productivity and to insure that the process could be carried on continuously while the other columns were regenerated.

When the practical maximum load of the β-CD polymer column was investigated, using 3 g % of β-CD polymer at the same running condition, it was found that starting with juice containing ~10 ppm limonin, the maximum load for sample juice of the column

was 240 ml and the practical maximum limonin absorption capacity of the column was 0.81 mg limonin/g β-CD polymer.

In practice, prior to debittering, the pulp was separated from the juice. It was then washed by water to remove some bitter compounds existing in the pulp. Such pulp would be stored and later reblend with the debittered juice. From Figure 35, it can be seen that the color and appearance of the clarified debittered juice was not different from the clarified pasteurized juice. In addition, after adding the pulp into the clarified debittered juice, the color of the juice was similar to fresh juice by visual observation. Therefore, the process did not cause apparent change in the debittered juice.

The commercial adsorbent XAD-16, the neutral resin cross-linked polystyrene adsorbent was tested for column debittering process using the same condition of β-CD polymer column. It needed to be pointed out that, due to its less swelling property, high amount of XAD-16 resin was needed to provide the same 10 ml bed volume. The chromatographic condition was 5.6 g% XAD-16 resin, flow rate 0.35 ml/min, contact time 30 minutes and at room temperature. It was found that the limonin reduction obtained from the XAD-16 resin was complete (undetectable limonin in eluant) column. However, the limonin adsorption capacity was only 0.18 mg limonin/g XAD-16 which is lower than the β-CD polymer (0.27 mg limonin /g β-CD polymer). However, It would be more justified if the optimum of XAD-16 column debittering process is investigated and compare with that of the β-CD polymer column.

The debittering cost of β-CD polymer column was estimated at the practical maximum load. The estimation cost of this process included the expense on fruits, debittering column, energy consumption and regeneration process was around 1,200 bahts/column (details in Table A 6). Ninety-nine percent of total debittering cost was due to the price of β-CD polymer. The productivity of the β-CD polymer column was 21 ml/hour with ~0.5 ppm limonin in final juice product. Although productivity of the column was low and was not practical, it could be improved by increasing the flow rate to the point where limonin content in the fruit juice is just below 5 ppm. Using the flow rate in this study, the debittered fruit juice contained only ~0.5 ppm limonin. Although this study did not examine the efficiency of the regenerated β-CD polymer, some previous works had reported that the β-CD polymer can be effectively used for column debittering process around 19-21 times of regeneration (Shaw and Wilson,1985; Shaw *et al.*,1988). Su and Yang (1991) in their studies reported 7 cycles of regenerations. This is advantage for reducing the operating cost.

In commercial debittering process, the establishment of XAD-16 debittering unit is very costly because it is patented. Moreover, the process for XAD-16 resin preparation was more complicated than that of the β-CD polymer (section 2.8.1). On the other hand, use of β-CD polymer in food processing has the GRAS status in most countries and the market price of this kind of polymer is gradually decreasing. Therefore, β-CD polymer has a good potential for being the absorbent for debittering process if further developed both in equipment designs and process.

Although study about consumers' acceptance does not include in this work, the researcher would like to refer this from other previous reports. As regards to the quality of debittered juice, there are a number of reports concerning the nutritional value of the juice in terms of both quantitatively and qualitatively reports. Vitamin C, the main vitamin tangerine juice and total soluble solids, which was refered as sweetness found in citrus fruits, were determined from the juice after passed through the β-CD polymer treatment by Shaw and Wilson (1983) and Shaw *et al* (1984). The results consistently showed that both the ascorbic acid contents (Vitamin C) and total soluble solids of debittered juice were unchanged whereas the reduction of oil level was about 40 %. In case of sensory evaluations, there are a number of studies. Konno *et al* (1982); Shaw *et al* (1984); and Wagner *et al* (1988), which have similar conclusion reported that the flavor evaluations on debittered juice sample and their original samples showed a significant preference for the debittered juice at a confidence level of 95 % or greater.

From this preliminary study, the researcher recommended that, the column process can be developed more in the further studies, for example increase the flow rate of juice or decrease contact time might be studied in the future. There are some of recommendations, which the reseracher would like to suggest for the further studies in term of process development as described below. For enhancing knowledge of the debittering of Thai tangerine juice by β-CD, additional studies on the following topics should be achieved.

1. Development of rapid limonin determination method in order to rapidly use for routine work.
2. Modification of the batch process using resin in cage technique in order to overcome the occurrence of the beads' breakdown.
3. Improvement of the column design and scale-up the process.
4. Sensory and color evaluation of the final juice products are needed for evaluating consumer acceptance.
5. Development of the production of β-CD and β-CD polymer using the local raw materials to reduce cost.

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