CHAPTER IV
RESULTS

4.1 Limonin extraction by solid phase extraction (SPE) and determination by reverse phase HPLC

4.1.1 Extraction of limonin by solid phase extraction (SPE)

Limonin was extracted from tangerine juice and determined by HPLC using the condition described in Section 3.1.1 and 3.1.2 (Chapter III). Figure 4.1 shows the elution profile of limonin extraction. It was found that limonin could not be extracted in the water fraction, the extraction of limonin was carried out with the total volume of 1.5 ml acetonitrile at 3.7, 4.2 and 4.7 ml of eluted volume. These 1.5 ml fractions were collected to determine limonin content by HPLC.

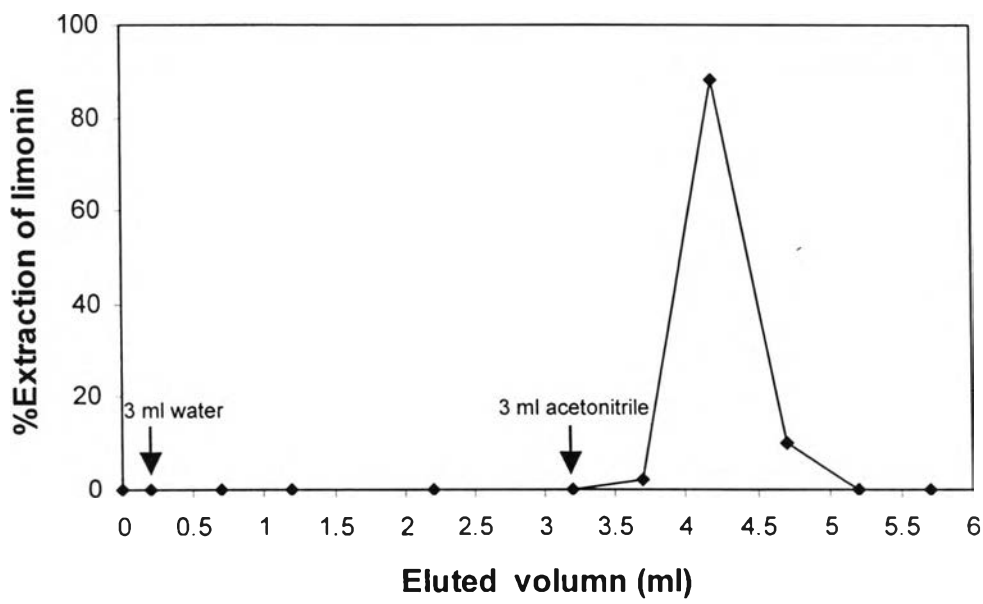


Figure 4.1 Chromatogram of limonin extraction by 3 ml acetonitrile with initial 0.2 ml standard limonin, 0.5 ml fraction was collected for limonin determination.

4.1.2 Determination of limonin content by reverse phase HPLC

For determination of limonin content by HPLC, the standard limonin chromatogram was shown in Figure 4.2. It was found that the retention time of limonin was around 16 min. The peak of limonin was further confirmed by comparing the chromatograms of limonin sample and the spiked limonin sample (Figure 4.3 and 4.4).

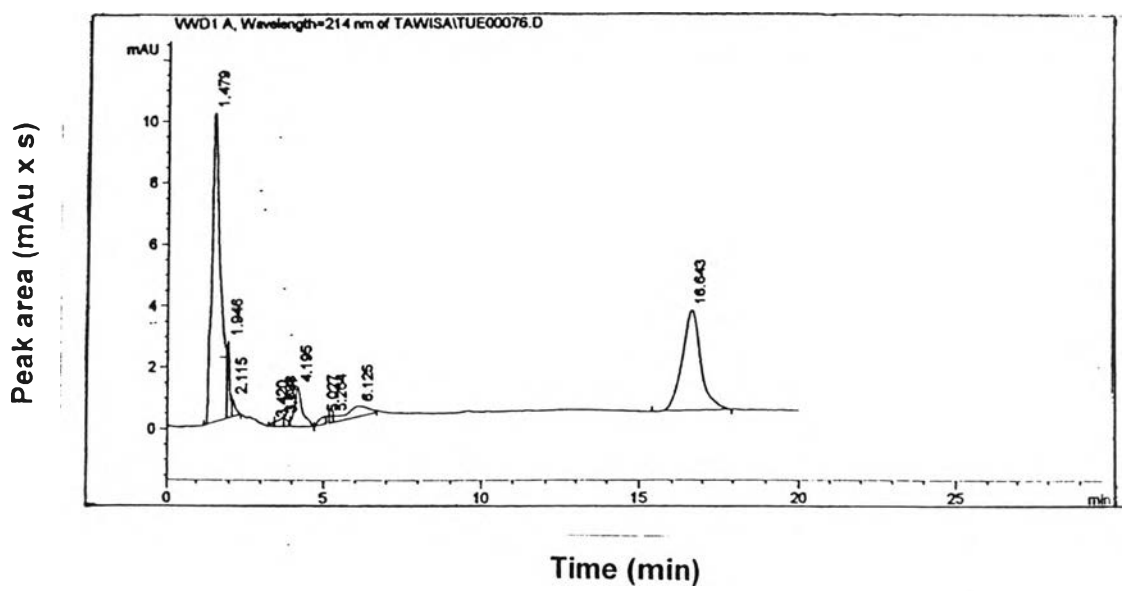


Figure 4.2 Chromatogram of standard limonin (20 µl sample injection containing 0.35 µg standard, C-18 column, acetonitrile:water (37:63 v/v), flow rate 1.0 ml/min, 214 nm)

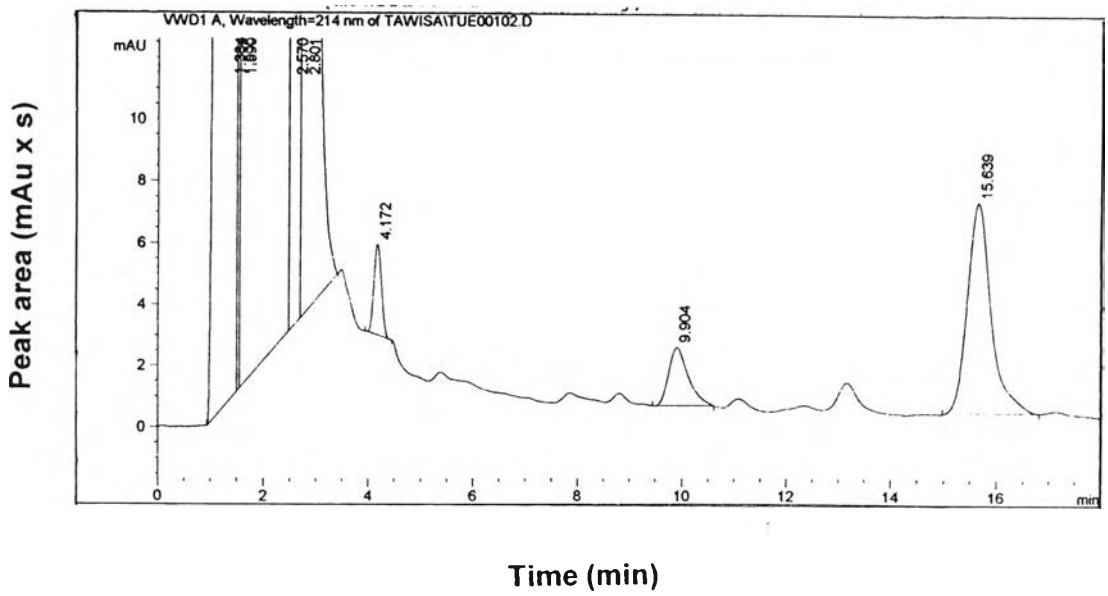


Figure 4.3 Chromatogram of limonin sample extracted by SPE method (20 µl sample injection, C-18 column, acetonitrile:water (37:63 v/v), flow rate 1.0 ml/min, 214 nm)

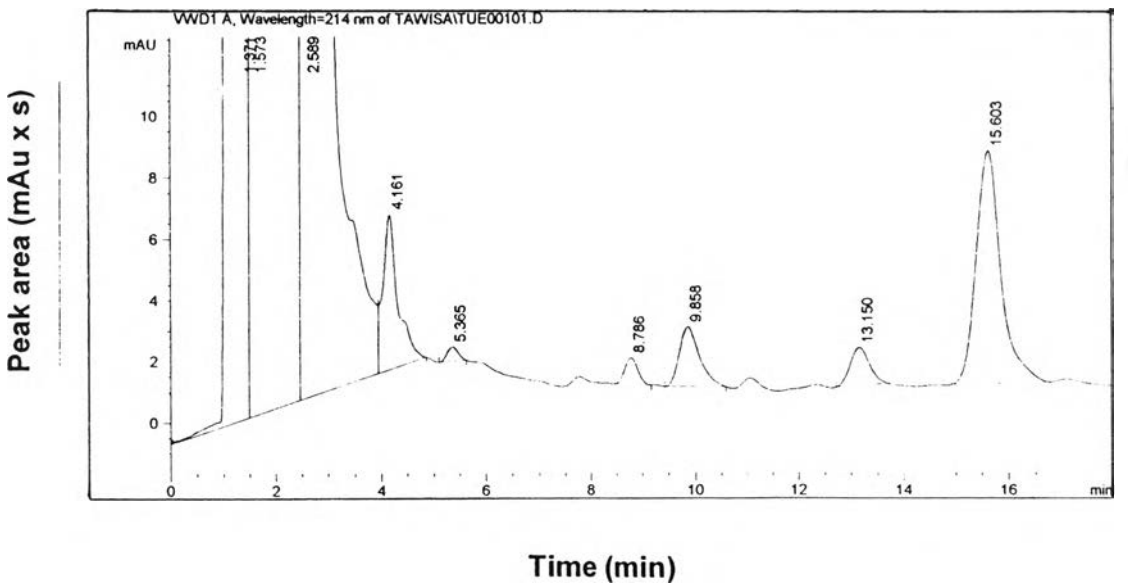


Figure 4.4 Chromatogram of spiked limonin sample prepared by SPE method (20 µl sample injection containing 0.075 µg standard limonin, C-18 column, acetonitrile:water (37:63 v/v), flow rate 1.0 ml/min, 214 nm)

4.2 Evaluation of reliability of the method for limonin determination

4.2.1 Efficiency of HyperSEP C-18 cartridge in limonin separation

Because of high cost of HyperSEP C-18 cartridge, the efficiency of HyperSEP C-18 cartridge for limonin extraction was evaluated the effectiveness of reused column. Two hundred microlitres of 25 ppm standard limonin was loaded onto the HyperSEP C-18 cartridge as the procedure described previously in Section 3.1.1, Chapter III. Then the limonin content in samples eluted from the HyperSEP C-18 cartridge was determined by HPLC as described in Section 3.1.2, Chapter III.

Figure 4.5 shows that the % recovery of limonin using HyperSEP C-18 cartridge at each cycle. The percentage of limonin recoveries decreased when the HyperSEP C-18 cartridge was reused (83.95$\pm$2.6%, 80.29$\pm$0.26%, 76.89$\pm$1.07%, 63.37$\pm$0.19% and 63.99$\pm$0.43% respectively). It was found that the accuracy of the method for limonin determination was 84%. The accuracy decreased gradually from 84 to 80 to 77% for the second and third time of reuse, but decreased considerably for the forth and fifth time of reuse (84% to 64%) and there was significant difference at the confidential level of 0.05 between each reuse. Therefore, the HyperSEP C-18 cartridge was recommended to be reused for only 3 times (~80% limonin recovery), although there was a significant difference of limonin contents between the initial, second used and third used of HyperSEP C-18 cartridge at the confidential level of 95%.

Furthermore, the difference of % recovery in three reused is acceptable when compare with the cost of HyperSEP C-18 cartridge. The precision of the data was confirmed by % C.V. of 0.3 – 2.8 as shown in Table A 1. In term of % C.V., it could be stated that the data in the experiment were reliable.

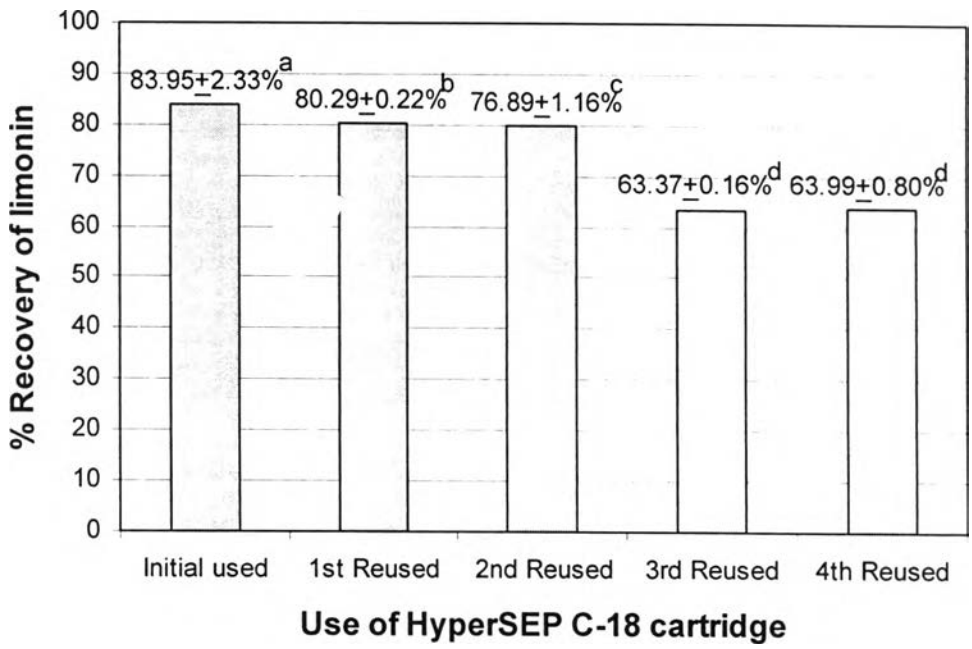


Figure 4.5 % Recovery of limonin of initial and reused HyperSEP C18 column (a, b, c and d means with the same letter were significantly different at P>0.05 by Duncan's multiple range test with three replications)

4.2.2 Sensitivity and reliability of the method for limonin determination

The sensitivity of the technique using HyperSEP C-18 cartridgeand HPLC was also investigated as described in the Section 3.1.1 and 3.1.2, Chapter III.

Peak area of each concentration of standard limonin before and after passing through the HyperSEP C18column and % recoveries of limonin were shown in Table 4.1. It can be seen that the lowest concentration of standard limonin which could be detected by this method was at 2.4 ppm.

In order to evaluate the reliability of the method, five replications of 2.4 ppm standard limonin were assayed at the same condition. The results were shown in Table 4.2. The precision of the method was acceptable with error of $\pm$ 0.01 and %C.V. 3.24.

Table 4.1 Limitation of limonin determination using HyperSEP C-18 cartridge and HPLC

200 µl std.limonin (ppm)	limonin (µg)		% recovery
	Before passing through HyperSEP C18 column	After passing through HyperSEP C18 column	
10	2.00	1.74	87.0
7.5	1.50	1.33	88.7
5	1.00	0.88	88.0
2.5	0.50	0.43	86.0
2.4	0.48	0.41	85.4
2.3	0.46	UD	-
2.2	0.44	UD	-

Note : UD = Undetectable

Table 4.2 Peak areas of 2.4 ppm (0.48 µg) standard limonin for 5 replications

Replications	limonin (µg)	%Recovery
1	0.41	85.41
2	0.39	81.49
3	0.40	82.92
4	0.42	86.57
5	0.39	82.05
Average±SE	0.40±0.01	83.69±2.20
%C.V.	3.24	2.63

n=5

4.3 Reduction of limonin by fluidized β-CD polymer process

The aimed of this experiment was to scale-up and using fluidization process to increase production rate of debittered tangerine juice.

4.3.1 Optimization of fluidized process

4.3.1.1 Size of fluidized column

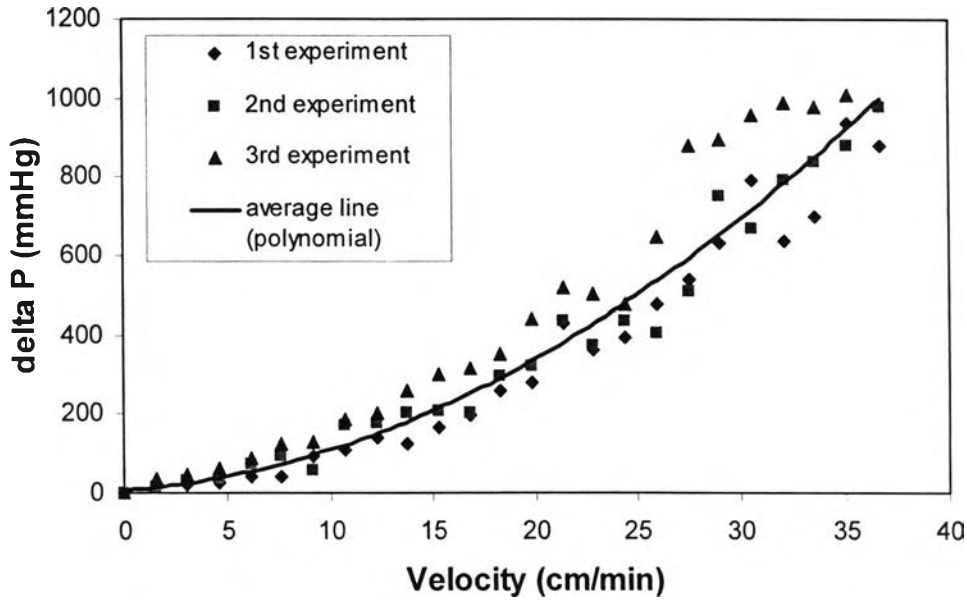
The size of fluidized column for scale-up debittering process was designed as describe in the 3.3.1.1 (Chapter III). The appropriate fluidized column for this experiment was chosen from the determination of minimum fluidization velocity.

4.3.1.2 Minimum fluidization velocity

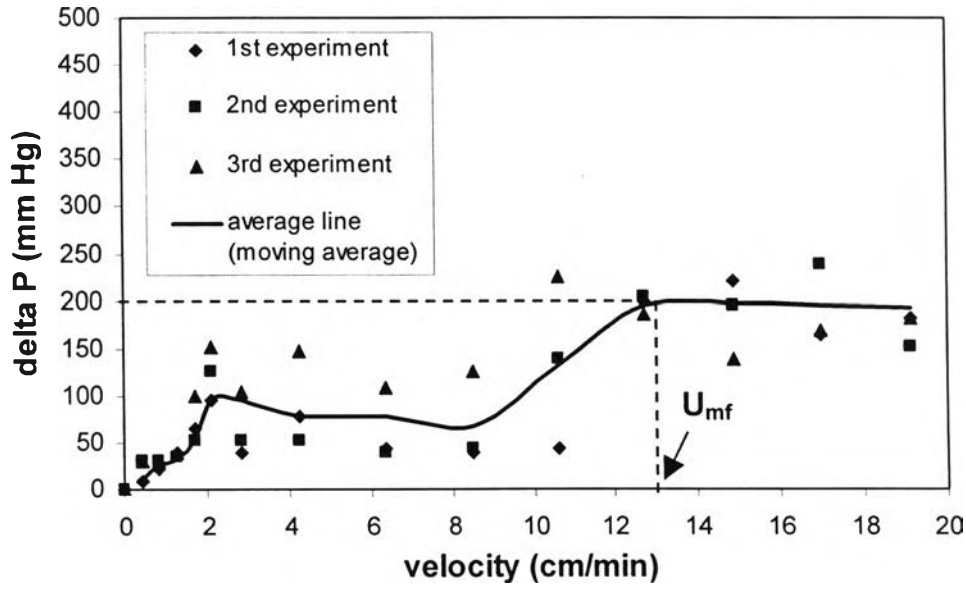
The suitable fluidized column for debittering process was estimated from investigating the flow rate which the fluidization of β-CD polymer was onset. Fifteen grams of β-CD polymer were suspended in water overnight and packed in both columns (50 cm x 5 cm i.d. and 50 cm x 3 cm i.d.). The minimum fluidization velocity was examined by the procedure described previously in Section 3.3.1.2, Chapter III.

Figure 4.6A shows that the fluidizing point in 50 cm x 5 cm i.d. column could not be determined because the pressure drop was not stable eventhough the velocity increased more than 35 cm/min. In the case of the 50 cm x 3 cm i.d. column, the minimum velocity of fluidization (13 cm^2/min or 90 ml/min) could be determined as shown in Figure 4.6B.

Therefore, the 50 cm x 3 cm i.d. fluidized column was selected for the appropriate fluidized column.



A) 50 cm x 5 cm i.d. fluidized column



B) 50 cm x 3 cm i.d. fluidized column

Figure 4.6 Experimental determination of minimum fluidization velocity (U_{mf})

4.3.1.3 Flow rate of tangerine juice

The effect of flow rate on residual limonin in the debittering process was explored. At the chosen condition, the flow rate of clarified juice was varied at 75,100 and 120 ml/min as mentioned Section 3.3.1.3 (Chapter III). The residual limonin at each flow rate was displayed in Figure 4.8. It can be seen that the flow rate below and above the minimum fluidizing velocity, 75 and 120 ml/min respectively, resulted in less debitter efficiency and were unsuitable. At the flow rate of 75 ml/min of feed rate, complete fluidization may not yet reached as can be seen from the fluctuated level of residual limonin. At too high flow rate (120 ml/min), the juice was passed through the fluidized bed column rapidly, the contact between limonin in the juice and the β-CD polymer decreased, thus reducing debittering efficiency. Consequently, the researcher decided to choose the debittering process at the flow rate 100 ml/min.

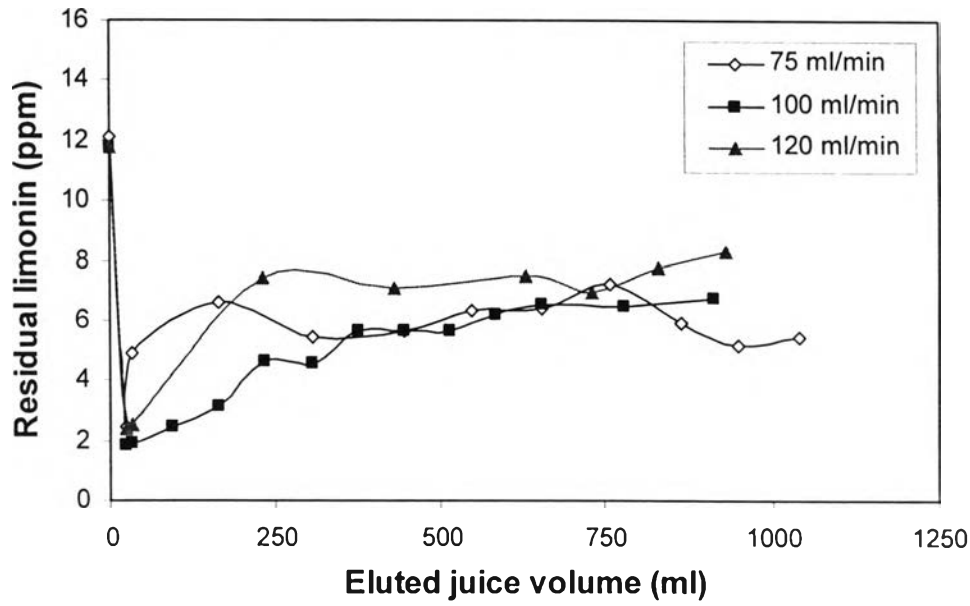


Figure 4.7 **Residual limonin in debittered juice with the flow rate of 75, 100 and 120 ml/min, 15 g β-CD polymer, ~ 12 ppm initial limonin content. At intervals, 100 ml juice was collected for limonin determination.**

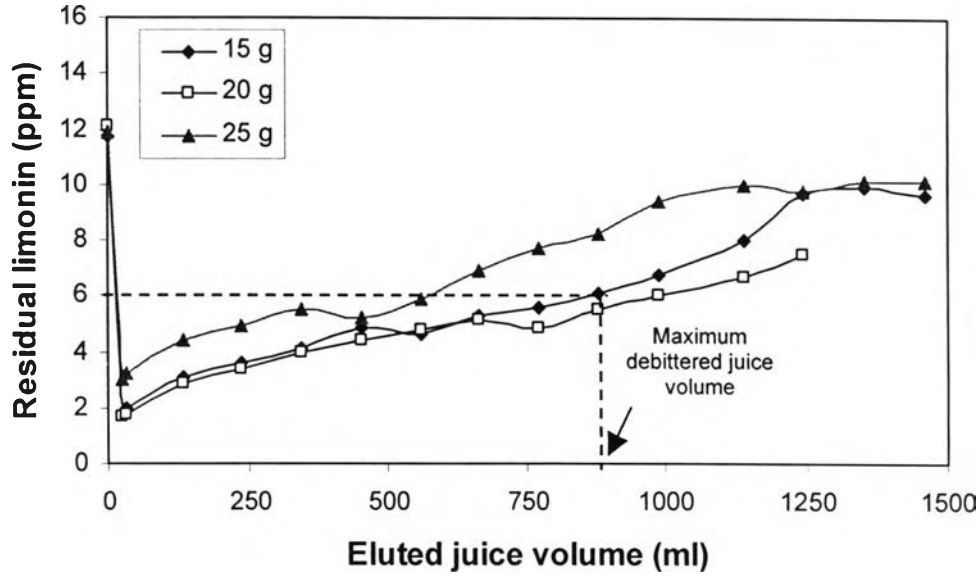
4.3.1.4 Amount of β-CD polymer

The amount of β-CD polymer for limonin reduction in the 50 cm x 3 cm i.d. column was varied at 15, 20 and 25 g. Clarified juice was flowed at 100 ml/min for 30 min. Then, the limonin in each fraction of juice was separated by SPE column and analyzed by HPLC as the method described in Section 3.1.1 and 3.1.2 (ChapterIII).

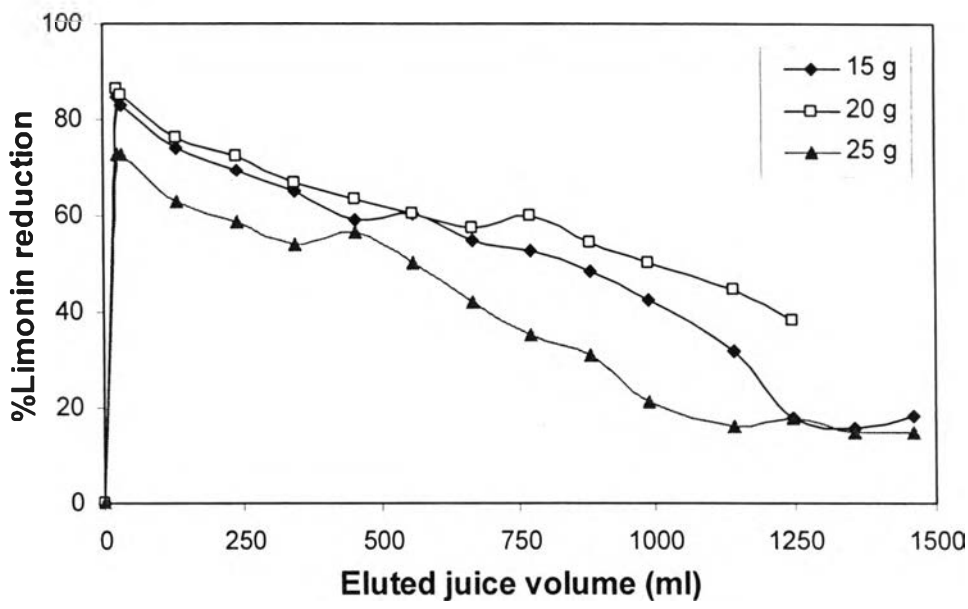
From Figure 4.8B, all treatments showed very rapid limonin reduction (~80%) in the first 25 ml fraction and the efficiency gradually decreased. Increasing the amount of β-CD polymer did not increase the capacity and efficiency of the process. Considering the capacity of polymer, 25 g of polymer gave the least favorable result, both efficiency and maximum practical load wise. Figure 4.8A shows the slurry of 15-20 g β-CD polymer significantly produced better debittering results than 25 g and both treatments gave the similar result. Considering processing cost, the amount of 15 g should be appropriate. It is possible that maximum complexation of limonin in the sample juice was already attained at this bed concentration and lower amout of polymer could be used.

The maximum debittered juice volume which limonin content still below 6 ppm was about 900 ml for these columns (50-60 % reduction). Fifteen grams of β-CD polymer supported 900 ml of maximum volume of tangerine juice or approximately 1.67g% (w/v) β-CD polymer. According to Piriya Rodart (2001), 1.25g% (w/v) β-CD polymer was used in the optimum condition for packed bed

column debittering. Therefore using 15 g of β-CD polymer might be excess to reduce limonin in tangerine juice.



A) Residual limonin in debittered juice



B) %Limonin reduction of debittering process

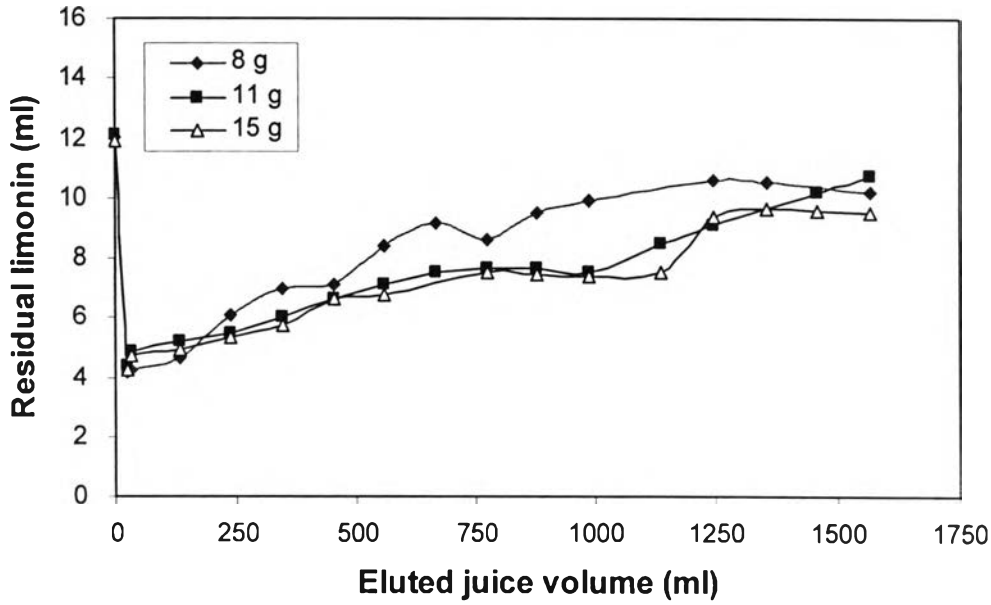
Figure 4.8 Debittering of tangerince juice by 15, 20 and 25 g of β-CD polymer, at the flow rate of 100 ml/min, ~ 12 ppm initial limonin content. At intervals, 100 ml juice was collected for limonin determination.

The optimum amount of β-CD polymer to reduce limonin by fluidization process was determined. The same amount of β-CD polymer with Piriya Rodart (2001), 1.25g% (w/v) β-CD polymer was used to study. Since the new β-CD polymer was not enough, the regenerated β-CD polymer was used instead for debittering.

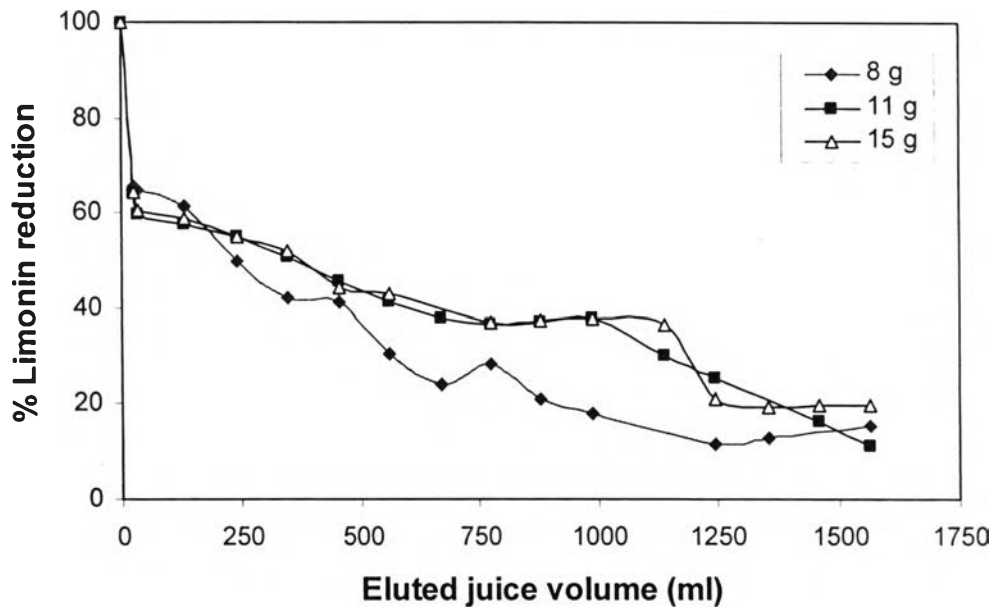
The slurry of 8, 11 and 15 g regenerated β-CD polymer (0.89, 1.25,1.67 g%, w/v) was packed into a 50 cm x 3 cm i.d. fluidized column. The sample juice was counter-flowed at the flow rate of 100 ml/min. At intervals, 100 ml juice was collected for determination of limonin content. Then, the limonin was separated and determined by HPLC (3.1.1 and 3.1.2, ChapterIII).

Figure 4.9A shows the residual limonin in debittered tangerine juice. The trend of the line of using 11 g regenerated β-CD polymer was similar to 15 g, the residual limonin was suddenly decreased and gradually increased. From Figure 4.9B, it can be seen that all concentrations of the regenerated bed showed very rapid limonin reduction (~60%) in the first 25 ml product and the debittering efficiency was gradually decreased. However, the use of 8 g regenerated β-CD polymer gave the least efficiency for debittering process.

In the above condition, the researcher decided to operate the fluidized column for debittering process at the condition of 50 cm x 3 cm i.d. column, 11 g β-CD polymer (1.25 g%,w/v) and 100 ml/min of flow rate at room temperature (27 ^{o}C) in order to give residual limonin in tangerine juice was less than 6 ppm.



A) Residual limonin in debittered juice



B) %Limonin reduction of debittering process

Figure 4.9 Debittering of tangerine juice by 8, 11 and 15 g of regenerated β-CD polymer, at the flow rate of 100 ml/min, ~12 ppm initial limonin content. At intervals, 100 ml juice was collected for limonin determination.

4.3.2 Practical maximum load of β-CD polymer fluidized column for debittering process

This result could be used to indicate the maximum debittered juice volume when using both new β-CD polymer and regenerated β-CD polymer to produce debittered juice with the limonin content still less than 6 ppm (threshold level of limonin).

From Figure 4.10A, the maximum debittered juice volume by 15 g new β-CD polymer and regenerated β-CD polymer fluidized column under the same condition was about 900 and 350 ml with the initial limonin of tangerine juice was 11.76 and 11.89 respectively. In term of adsorption capacity, it was 0.14 mg limonin/g regenerated β-CD polymer and 0.35 mg limonin/g β-CD polymer at the maximum debittered juice volume (Appendix D). The adsorption capacity of regeneration of β-CD polymer was decreased around 60 %.

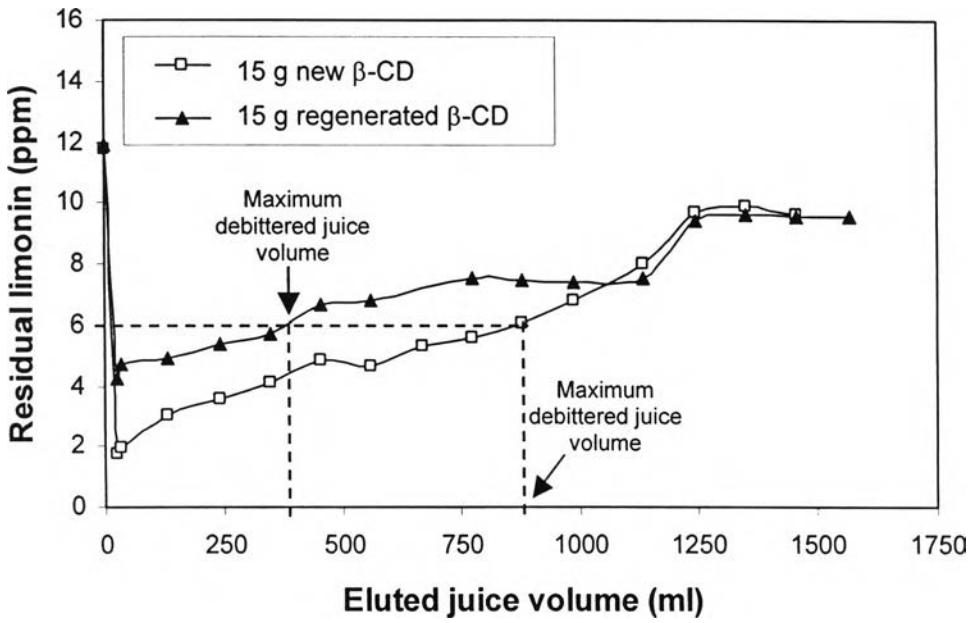


Figure 4.10 Residual limonin in debittered juice by 15 g of β-CD polymer and regenerated β-CD polymer fluidized column. The initial limonin was 12 ppm. At intervals, 100 ml of juice was collected.

4.3.3 Comparison of limonin reduction efficiency using new β-CD polymer with regenerated β-CD polymer fluidized column

Regeneration of the polymer for reused was done by means of Shaw and Wilson (1985). The β-CD polymer was washed with excessive amount of water, 2% NaOH and finally by absolute ethanol as mentioned in Section 3.3.1.4 (ChapterIII).

Figure 4.11 shows that the %limonin reduction was decreased when debittered juice by regenerated β-CD polymer fluidized column (~60% reduction) whereas new β-CD polymer showed very rapid limonin reduction (~80% reduction) in the first 25 ml product. The % limonin reduction of debittering process by regenerated β-CD polymer was 20% lower than new β-CD polymer.

In addition, the surface area of new β-CD polymer and regenerated β-CD polymer was investigated by scanning electron microscope. From Figure 4.12, it can be seen that the regenerated β-CD polymer bead was raptured than new β-CD polymer. This might the reason that the efficiency of limonin adsorption was decreased.

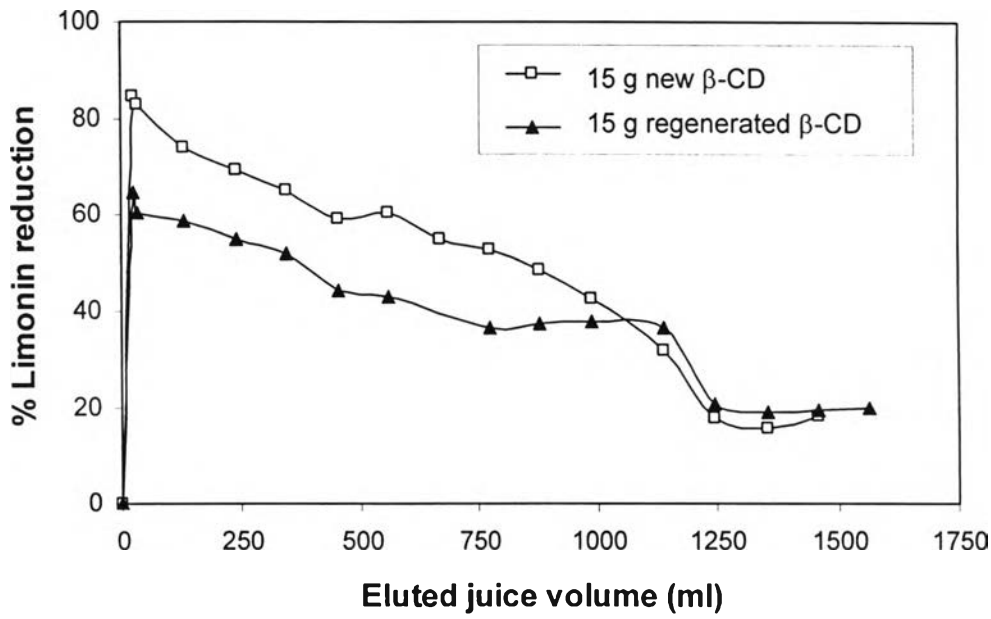
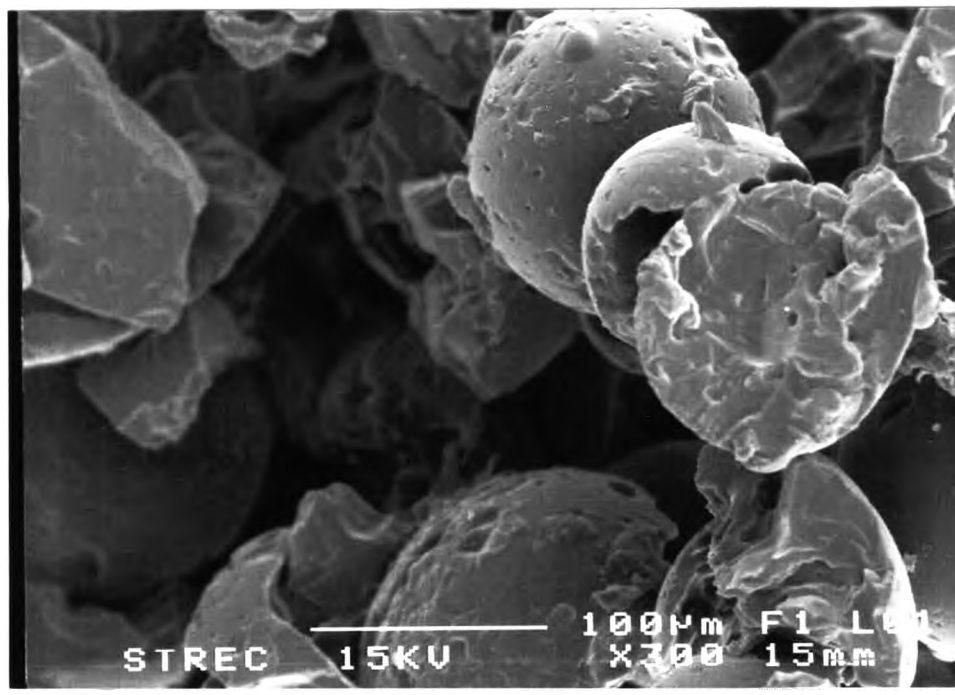
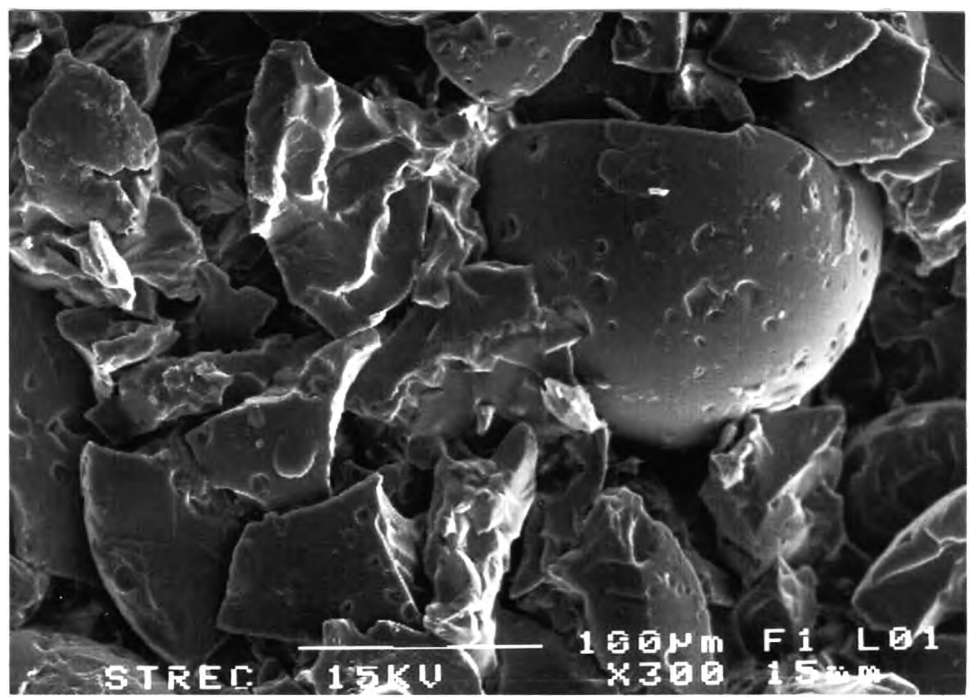


Figure 4.11 Debittering of tangerine juice by 15 g of β-CD polymer and regenerated β-CD polymer fluidized column. The initial limonin was 12 ppm. At intervals, 100 ml of juice was collected.



A) new β-CD polymer



B) regenerated β-CD polymer

Figure 4.12 Scanning electron micrograph of new β-CD polymer and regenerated β-CD polymer

4.4 Reduction of limonin by fluidized XAD-16 resin process

4.4.1 Scanning electron microscope of XAD-16 resin

The scanning electron micrograph of XAD-16 resin was shown in Figure 4.13. It can be seen here that the XAD-16 resin has more surface area and porosity more than β-CD polymer (Figure 4.12A).

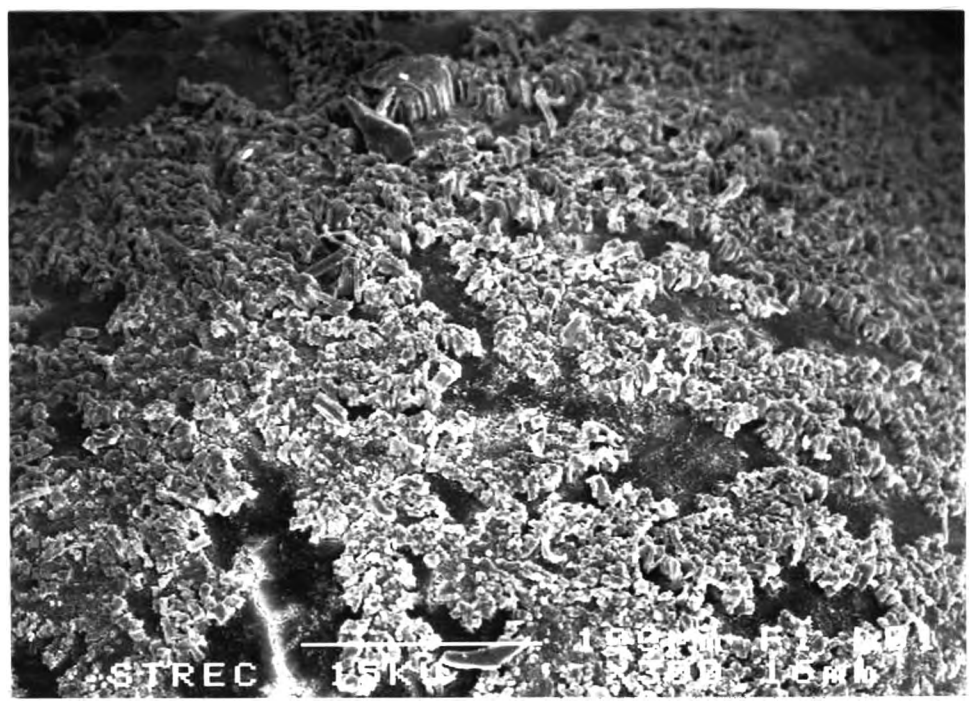
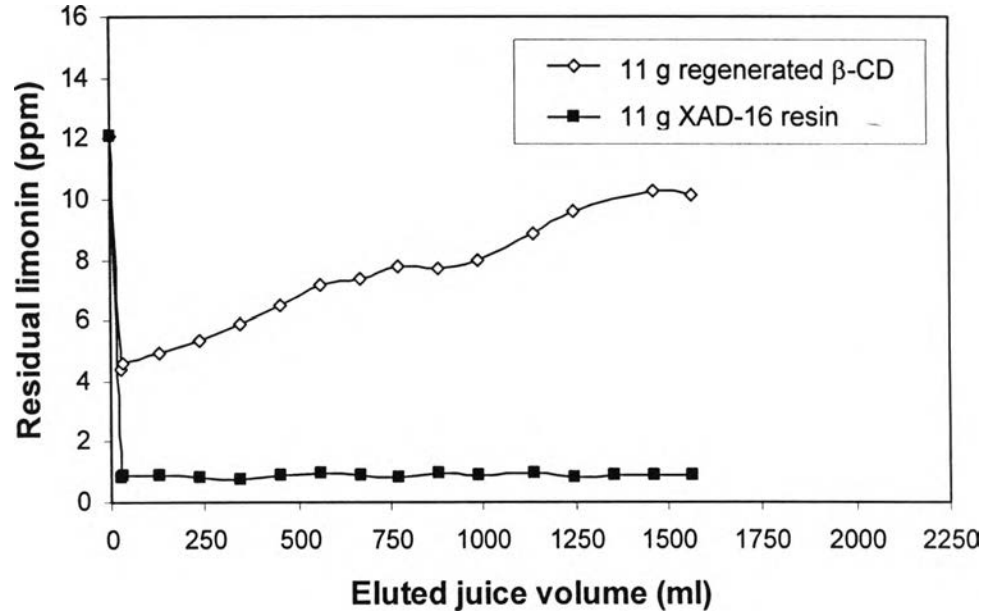


Figure 4.13 Scanning electron micrograph of XAD-16 resin

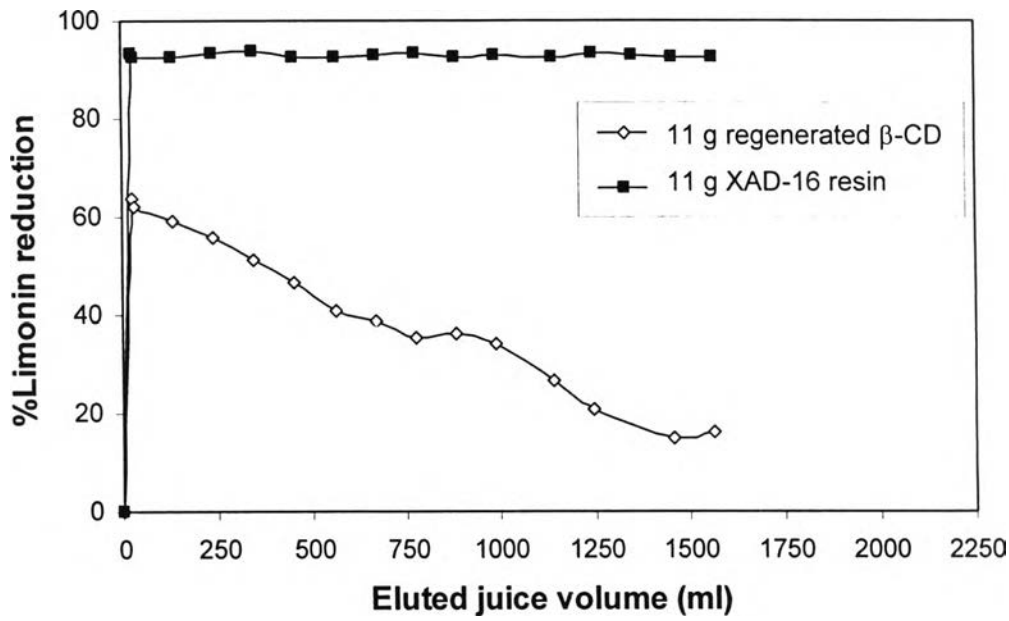
4.4.2 Comparison of limonin reduction efficiency using β-CD polymer fluidized column with XAD-16 resin fluidized column

The XAD-16 resin was used for fluidized debittering process under the same condition as the β-CD polymer column (11 g of absorbent, 100 ml/min of clarified juice, at room temperature, 30 min processing time). The initial limonin content in clarified juice was about 12 ppm.

From Figure 4.14A, it was found that the residual limonin in every debittered juice fraction by XAD-16 resin fluidized column was very low. Greater %limonin reduction (~90%) was shown in Figure 4.14B. When debittering 1,600 ml of juice, the limonin content of the juice was still below threshold level of limonin (6 ppm). The capacity in limonin reduction of this XAD-16 resin fluidized column at the chosen condition was 1.58 mg limonin / g XAD-16 resin.



A) Residual limonin in debittered juice



B) %Limonin reduction of debittering process

Figure 4.14 Debittering of tangerine juice by 11 g of the regenerated β-CD polymer and XAD-16 resin fluidized column. The initial limonin was 12 ppm. At intervals, 100 ml of juice was collected.

4.5 Reduction of limonin with self-prepared β-CD polymer by fluidized process

4.5.1 Scanning electron microscope of β-CD polymer preparation

Because of the high cost of commercial β-CD polymer, the researcher had prepared the β-CD polymer by the method of Shaw *et al.* (1984). The β-CD polymer was prepared with the molar ratio of epichlorohydrin:β-CD = 15:1 as described in 3.5.1 (Chapter III).

Characteristic and surface area of prepared β-CD polymer was investigated using scanning electron microscope. From Figure 4.15, it can be seen that the surface area and porosity of prepared β-CD polymer did not see obviously.

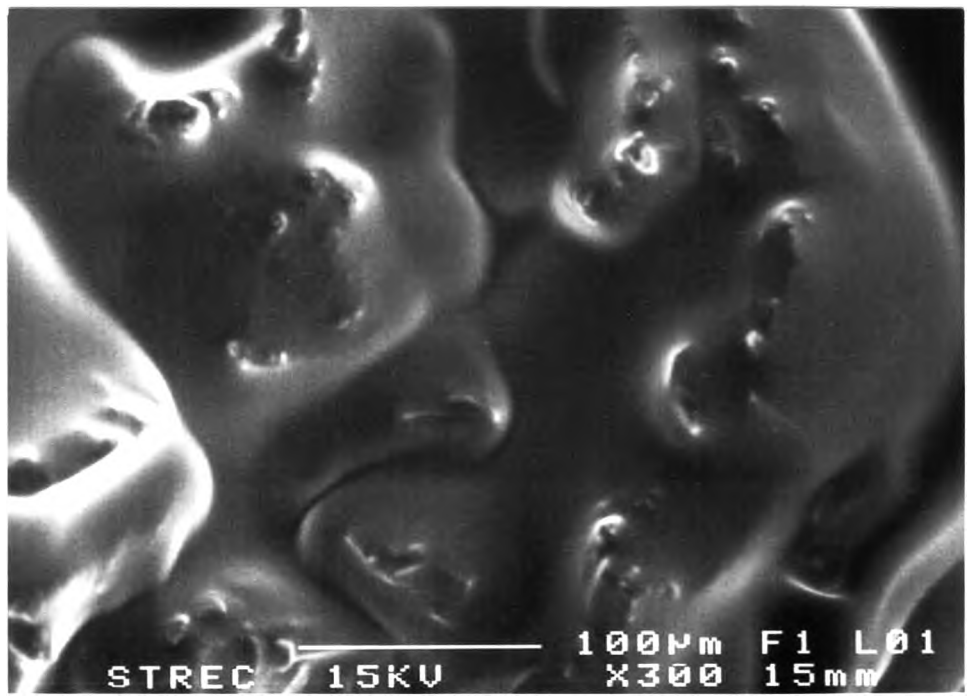


Figure 4.15 Scanning electron micrograph of self-prepared β-CD polymer

4.5.2 Determination of residual epichlorohydrin

The removal of epichlorohydrin, residual epichlorohydrin in the washed polymer was determined by injection of 1 μl of ethanol wash solution into a gas chromatography as the mention described in Section 3.5.2 (Chapter III).

The standard epichlorohydrin chromatogram was shown in Figure 4.16. It was found that the retention time of limonin was around 3.6 min. Figures 4.17, 4.18 showed the chromatograms of residual epichlorohydrin in the second and third cycle of ethanol wash solution of β-CD polymer preparation by the method of Shaw *et al* (1984). The peak of epichlorohydrin was absent in the chromatogram of the third cycle of ethanol wash solution. Base on this experiment, it was concluded that the prepared β-CD polymer shoukd be washed for 3 times.

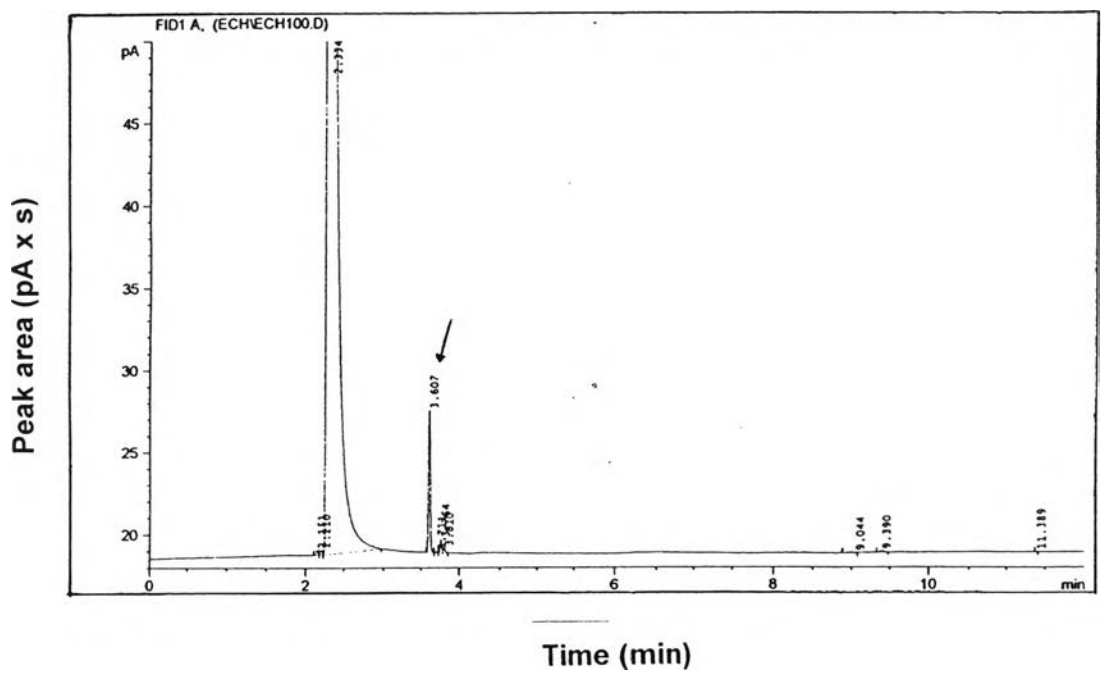


Figure 4.16 **Chromatogram of 100 ppm standard epichlorohydrin (1 μl sample injection, fused silica capillary column coated with 5% phenylmethylsiloxane (HP-5), carrier gas (He) flow was 25 cm/s, the flame ionization detector was at 350 ^{o}C, with a split ratio of 20:1)**

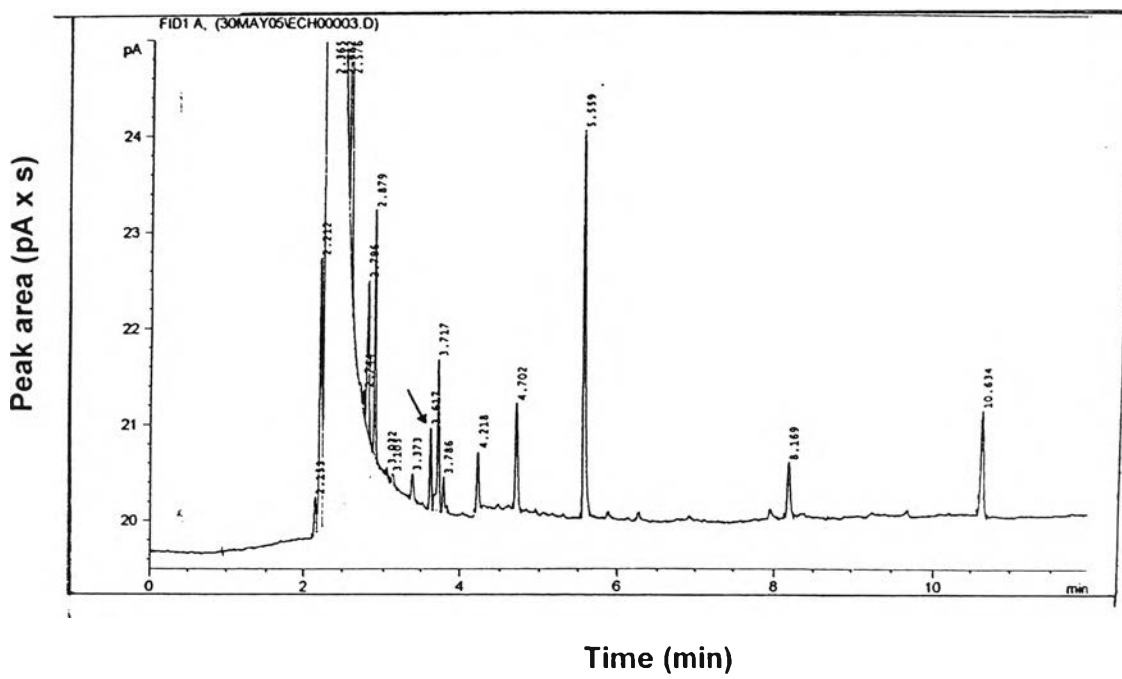


Figure 4.17 **Chromatogram of the second cycle of ethanol wash solution (1 μl sample injection, fused silica capillary column coated with 5% phenylmethylsiloxane (HP-5), carrier gas (He) flow was 25 cm/s, the flame ionization detector was at 350 °C, with a split ratio of 20:1)**

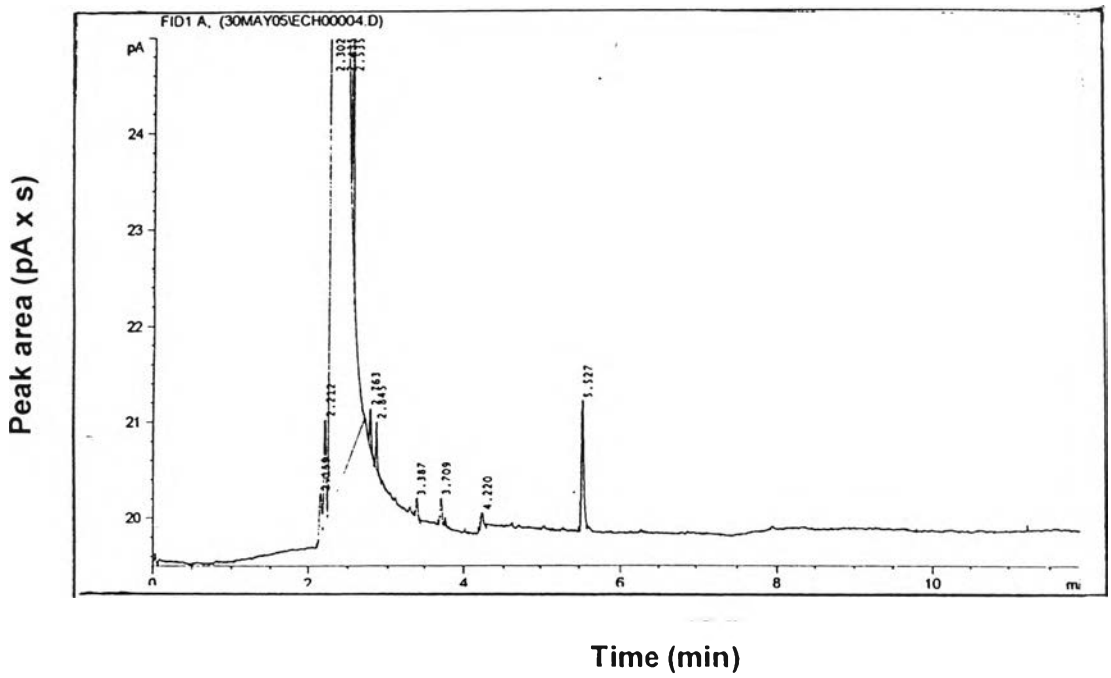
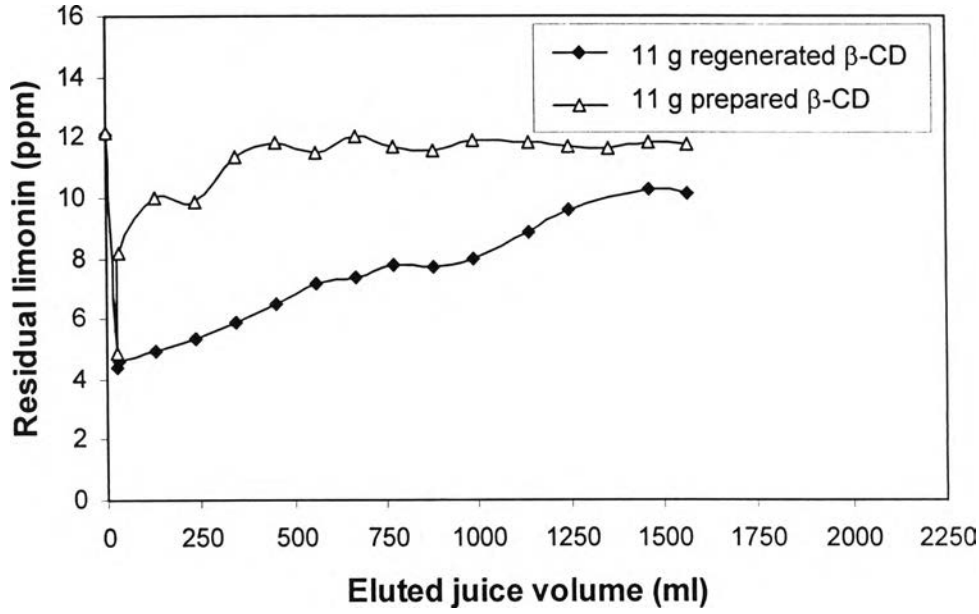


Figure 4.18 Chromatogram of the third cycle of ethanol wash solution (1 μl sample injection, fused silica capillary column coated with 5% phenylmethylsiloxane (HP-5), carrier gas (He) flow was 25 cm/s, the flame ionization detector was at 350 ^{o}C, with a split ratio of 20:1)

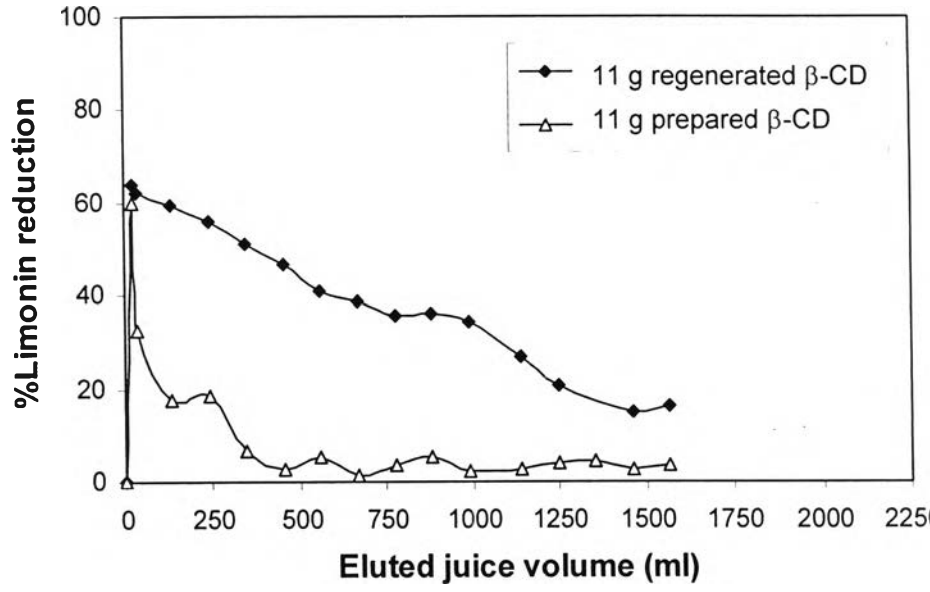
4.5.3 Comparison of limonin reduction efficiency between β-CD polymer and prepared β-CD polymer fluidized column

The efficiency of limonin reduction by prepared β-CD polymer was evaluated, 11 g prepared β-CD polymer was used in fluidized bed as the optimum condition of debittering tangerine juice as described in 4.3.3.

Figure 4.19 shows the residual limonin and % limonin reduction of debitered juice with prepared β-CD polymer. The efficiency of the prepared β-CD polymer for fluidized debittering process was very lower. The limonin level initially decreased but rapidly increased later.



A) Residual limonin in debittered juice



B) %Limonin reduction of debittering process

Figure 4.19 Debittering of tangerine juice by 11 g of the regenerated β-CD polymer and prepared β-CD polymer fluidized column. The initial limonin was 12 ppm. At intervals, 100 ml of juice was collected.

4.5.4 Comparison of limonin reduction efficiency among new β-CD polymer, regenerated β-CD polymer, XAD-16 resin and self-prepared β-CD polymer

Comparison of using β-CD polymer, regenerated β-CD polymer, XAD-16 resin and prepared β-CD polymer for debittering processes was demonstrated in Table 9. It clearly indicated that the capacity of limonin absorption in XAD-16 resin fluidized column was higher than β-CD polymer, regenerated β-CD polymer and self-prepared β-CD polymer respectively.

Table 4.3 Comparison of limonin reduction efficiency between β-CD polymer, XAD-16 resin and prepared β-CD polymer

Items	new β-CD polymer	regenerated β-CD polymer	XAD-16 resin	self-prepared β-CD polymer
% g β-CD polymer (g/100ml juice)	1.67/1.25*	1.67/1.25	1.25	1.25
Process Temperature (^{0}C)	27	27	27	27
Contact time (minutes)	30	30	30	30
Initial limonin content in sample juice (ppm)	11.76	11.89	12.07	12.15
% Limonin reduction in first 25 ml product	80	60	90	60
Capacity for limonin absorption (mg limonin/g polymer)	0.35/0.47	0.14/0.19	1.58	0.028

Note: *1.25g% of new β-CD polymer was the estimated results from optimization of fluidized debittering process.

4.6 Analysis of Thai tangerine juice compositions

The objective of this experiment was to evaluate the debittered juice compositions which is the important factor in citrus juice products affecting consumer's acceptance. The color, total soluble solids and vitamin C before and after debittering were analysed.

4.6.1 Measurement of color

The clarified tangerine juice was passed through the debittering fluidized columns using new β-CD polymer, XAD-16 resin and prepared β-CD polymer as an absorbent. The color of the fresh juice and the clarified juice before and after debittering process were measured by Minoltachroma meter as shown in Table 4.4. It was found that there was no significantly difference in the color value of the fresh juice, clarified juice and debittered juice at confidential level of 95%.

Table 4.4 Color values (L, a, b) of fresh juice, clarified juice and debittered juice

Juice	L	a	b
Fresh juice	101.80 ± 0.64^{A}	-11.03 ± 0.25^{A}	$+32.33\pm1.01^{A}$
Clarified juice	92.43 ± 0.25^{B}	-6.18 ± 0.13^{B}	$+10.28\pm0.33^{B}$
Debittered juice by β-CD polymer	92.46 ± 0.44^{B}	-6.39 ± 0.17^{B}	$+10.23\pm0.12^{B}$
Debittered juice by XAD-16 resin	92.50 ± 0.42^{B}	-6.33 ± 0.47^{B}	$+10.18\pm0.04^{B}$
Debittered juice by prepared β-CD polymer	92.39 ± 0.18^{B}	-6.26 ± 0.10^{B}	$+10.16\pm0.20^{B}$

Note: L = Lightness, a and b = color coefficient

A and B means with the same letter in the column were significantly different at P>0.05 by Duncan's multiple range test with three replication.

4.6.2 Determination of total soluble solids

Total soluble solids, which include carbohydrates, organic acids, proteins, fats and various minerals, comprise from 10-20% of the juice. In tangerine juice, total soluble solids were refered as sweetness because 75-85% of total soluble solids were sugars. From Table 4.5, the total soluble solids of juice which was measured as °brix by Hand refractometer, was compared between fresh juice, clarified juice and debittered juice. It was found that the total soluble solids content in all of juice was rather unchanged. Therefore, The debittering process has no effect on total soluble solids content.

Table 4.5 Total soluble solids of fresh juice, clarified juice and debittered juice

Juice	Total soluble solid (°brix)^{ns}
Fresh juice	11.63±0.06
Clarified juice	11.55±0.07
Debittered juice by β-CD polymer	11.54±0.05
Debiitered juice by XAD-16 resin	11.63±0.06
Debittered juice by prepared β-CD polymer	11.60±0.10

ns: not significantly different at P≤0.05 by Duncan's multiple range test with three replications.

4.6.3 Determination of vitamin C content

To study the effect of debittering process on vitamin C content in tangerine juice, the vitamin C content of the juice was determined according to the AOAC method described in Section 3.6.3 (Chapter III). Vitamin C levels in clarified juice and debittered juice were expressed as mg/100 ml juice, was shown in Table 4.6. It could be seen that there was no significant difference of vitamin C content between clarified juice and debittered juice at the confidential level of 0.05. In other words, vitamin C was not affected by the polymer treatment

From the above data, the results consistently showed that the color and nutritional value of tangerine juice in terms of total soluble solids and vitamin C remained unchanged when the tangerine juice was passed through fluidized debittering process.

Table 4.6 Vitamin C content of clarified juice and debittered juice

Juice	Vitamin C content (mg/100 ml juice)^{ns}
Clarified juice	5.71±0.08
Debittered juice by β-CD polymer	5.85±0.08
Debiitered juice by XAD-16 resin	5.76±0.08
Debittered juice by prepared β-CD polymer	5.80±0.14

ns: not significantly different at P≤0.05 by Duncan's multiple range test with three replications.

4.7 Evaluation of the debittering cost

Evaluation of debittering cost using β-CD polymer was described in Table A 5. The debittering cost was around 4,500 bahts/column and gave the productivity of 6 L juice/column/hour. It was noticed that the 99 % of the operating cost was due to the β-CD polymer.