CHAPTER III
MATERIALS AND METHODS

Materials

1. HyperSEP C-18 cartridge, 500 mg, 6 ml: ThermoHypersil-Keystone Inc., UK.
2. Bitter tangerine juice, 40 L:Tipco Foods Thailand Public Co., Ltd. Thailand.

Equipments

1. Analytical sieve 60, 80, 120 and 170 mesh and reciever: Retsch, F.Kurt Retsch GmbH and CoKG, Germany.
2. Gas chromatography: series US2202831H and HP-5 capillary column (30 m x 0.32 mm), coated with 5% phenylsiloxane, J&W Scientific, Agilent Technologies Inc., U.S.A.
3. Hand refractometer: model MNL-1125, Atago Co., Ltd. Japan.
4. HPLC: series 1050, Hewlett Packard, Agilent Technologies Inc., Japan ; C-18 column (150 mm x 4.6 mm i.d.), Inertsil ODS-3, 5 μm i.d., GL Sciences Inc., Japan.
5. Laboratory juice extractor
6. Magnetic stirrer: model MR3003, Heidolph Instrument Co., Ltd. Germany.
7. Membrane filter: Nylon membrane filter 0.45 μm, 13 and 25 mm i.d., Sartorious AG, Göttingen, Germany.
8. Minoltachroma meter: series CR-300, Minolta camera Co., Ltd. Japan.

9. Refrigerated centrifuge: model J-21C, Beckman Instrument Inc., U.S.A.
10. Scanning microscope: model JSM-6400, JEOL Ltd., Japan.
11. Water pump: Lifetech® AP2500, Electric Appliance Co., Ltd. China.

Chemicals

1. Absolute ethanol: Scharlau & Cia Ltda. Spain.
2. Acetonitrile (HPLC grade): Lab-Scan Ltd. Ireland.
3. Ascorbic acid: Farmatalia Carlo Erba Ltd., Italy.
4. β-Cyclodextrin epichlorohydrin polymer Gift: Cerestar U.S.A. Inc., Indiana, U.S.A.
5. β-Cyclodextrin hydrate, 99%: Acros Organics, Fisher Scientific Chemical Co., Ltd. U.S.A.
6. Carbontetrachloride: Farmatalia Carlo Erba Ltd., Italy.
7. Epichlorohydrin: Acros Organics, Fisher Scientific Chemical Co., Ltd. U.S.A.
8. Glacial acetic acid: Farmatalia Carlo Erba Ltd., Italy.
9. Limonin (HPLC grade): Sigma-Aldrich Corp., U.S.A.
10. Metaphosphoric acid: Farmatalia Carlo Erba Ltd., Italy.
11. Sodium bicarbonate: BDH Chemicals Co., Ltd. England.
12. Sodium borohydride: Fisher Scientific CHEMICAL Co., Ltd. U.S.A.
13. Sodium 2,6-dichloroindophenol: Aldrich Chemical Co., Ltd. Germany.
14. Sodium hydroxide pellets: Merck KGaA, Germany.

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

Sample preparation

Forty litres of tangerine juice from Tipco Foods Thailand Public Co., Ltd. The juice was preheated at 70 °C for 15 min, cooled down to 4 °C and centrifuged at 12,000 rpm for 15 min to remove pulps. The sample of clarified juice was divided into 10 groups, kept at -20 °C and thaw before using.

3.1.1 Extraction of limonin by solid phase extraction (SPE) (Shaw and Wilson, 1984)

A HyperSEP C-18 cartridge (ThermoHypersil-Keystone, UK.), as shown in Figure 3.1, was activated consecutively with 2 ml acetonitrile (HPLC grade) and 5 ml water. Three millilitres of the clarified tangerine juice was then passed through the SPE column by gravity flow and followed by 3 ml of water to remove sugar. Eventually, it was flushed with 3 ml of acetonitrile. Before loading, 0.5 ml fraction was collected and filtered through a 0.45 μm nylon filter membrane (Sartorious, Germany). The limonin content in each fraction was determined by reverse phase HPLC. The elution profile of limonin extraction was developed by plotting the eluted volume (X-axial) and the % extraction of limonin (Y-axial).

Figure 3.1 Limonin extraction using HyperSEP C-18 cartridge

3.1.2 Determination of limonin content by reverse phase HPLC

The limonin content was determined by reverse-phase HPLC method, using analytical C-18 column (octadecyl silane chemically bonded to 5 μm porous microsilica packing, 150 mm x 4.6 mm i.d.) as recommended by Mozaffar *et al* (2000). HPLC analyses were performed with HP 1050 (Hewlett Packard, Japan.) as shown in Figure 3.2. The chromatographic condition was 40 °C column temperature, maximum pressure 120 bar, and flow rate of 1.0 ml/min using 37% acetonitrile as mobile phase and detection was done at 214 nm (Piriya Rodart, 2001).

Figure 3.2 HPLC series 1050 (Hewlett Packard, Japan)

3.1.3 Standard curve of limonin

To prepare 25 ppm of limonin standard solutions, 1.25 mg of limonin crystal (Sigma, USA.) was dissolved in 50 ml of acetonitrile (HPLC grade). The initial standard solution was further diluted with acetonitrile to various concentrations (2.5, 7.5, 12.5, 17.5, 22.5 and 25 ppm).

Twenty μl of each concentration of standard limonin solution was injected onto the C-18 column and analyzed according to the condition described in 3.1.2. The chromatograms of all standard limonin solutions were shown in Appendix A (Figure A1-A6)

The limonin standard curve was plotted between standard limonin concentrations and peak areas, which were represented by axial X and Y respectively (Figure A7). The equation derived for the limonin standard curve which was used to estimate the limonin content in juice sample was shown in Appendix A.

3.2 Evaluation of reliability of the method for limonin determination

3.2.1 Efficiency of HyperSEP C-18 cartridge in limonin separation

Two hundred μl of limonin standard solution (25 ppm) was passed through the SPE column which was consecutively activated by 2 ml of acetonitrile (HPLC grade) and 5 ml of water. The SPE column was then washed with 3 ml of water to remove sugar. Limonin was extracted by 3 ml of acetonitrile running through the column by gravity flow. The fraction was analyzed by reverse phase HPLC using condition described in 3.1.2 to estimate the limonin content. The same SPE column was used five times to evaluate the effectiveness of reused column. The experiment was designed as completely randomized design (CRD). Before reused, the HyperSEP C-18 cartridge was regenerated by washing with 5 volumes of 2 ml of acetonitrile and air dried.

3.2.2 Sensitivity and reliability of the method for limonin determination

To estimate the sensitivity of the method, the standard limonin was prepared at the concentrations of 2.2, 2.3, 2.4, 2.5, 5.0, 7.5 and 10.0 ppm. Two hundred μl of each standard was passed through the preconditioned HyperSEP

C-18 cartridge by gravity flow, then washed with water and acetonitrile respectively as described in 3.1.1. The limonin extracted from the column was analyzed by HPLC.

For assessment of the precision of the method, the experiment was designed as completely randomized design (CRD). Five replications were assayed and reported as % C.V. that should be less than 10. Accuracy of the method was represented by % recovery of limonin in the eluant.

3.3 Reduction of limonin by fluidized β-CD polymer process

Activation of β-CD polymer

Twenty-five grams of β-CD polymer, obtained as a gift from Cerestar company, Indiana, U.S.A., were washed with acetone. The acetone concentration was decreased from 100 to 0 % with water by 10 % decrements. The β-CD polymer was then continuously washed with 10 x 100 ml of water until pH of the solution was around 7, followed by 2 x 100 ml of ethanol and dried (Piriya Rodart, 2001).

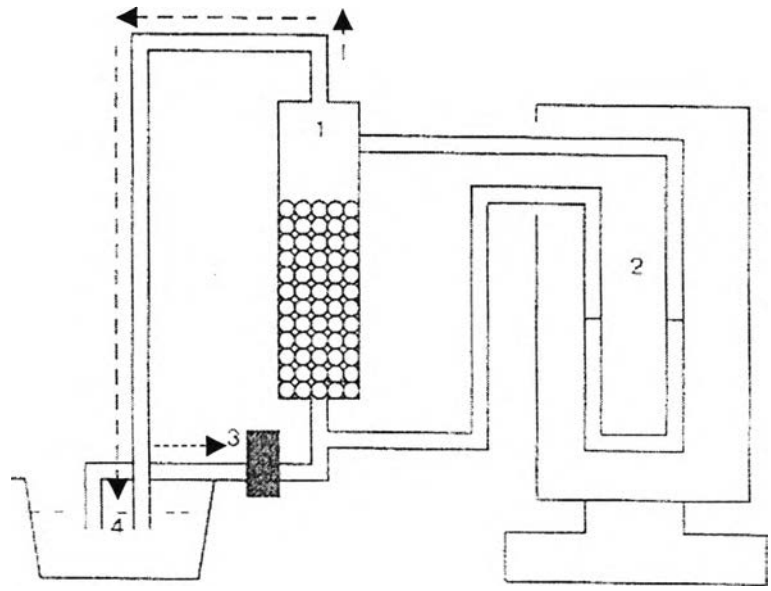
3.3.1 Optimization of fluidized process

3.3.1.1 Size of fluidized column

Size of the β-CD polymer used was 60-80 mesh, the Reynold number of β-CD polymer was below 50. The minimum fluidization velocity (U_{mf}) was calculated from Equation 2.7 (Chapter II). Then, U_{mf}

could be calculated to the height and diameter of fluidized column, the calculation was shown in Appendix B.

The height of the fluidized column was 50 cm with the inside diameter of either 5 cm or 3 cm. The bottom of each column was equipped with a distributor (200 mesh) and manometer containing carbontetrachloride to measure pressure drop (ΔP) when juice fluid was counter flow through the column. The schematic flow of fluidized debittering column was shown in Figure 3.3A. The experimental equipment, debittering fluidized column was shown in Figure 3.3B.



A) The schematic flow of fluidized debittering column

1 = fluidized column, 2 = manometer, 3 = pump, 4 = tangerine juice tank

B) Experimental equipment

Figure 3.3 The experimental equipment, debittering fluidized column

3.3.1.2 Minimum fluidization velocity

Fifteen grams of β-CD polymer (60-80 mesh) from 3.3.1 were swelled in water overnight and packed into both fluidized columns (50 cm x 5 cm i.d. and 50 cm x 3 cm i.d.). At each fluidized column, the flow rate of water was controlled by AP2500 Lifetech aquarium pump to15 ml/min at the beginning and increased regularly each 15 ml until the pressure drop (ΔP) at the monometer was stable. ΔP was recorded at every increased flow rate. Fluidization occurred when β-CD polymer was expanded upward in the column until the bed was orderly rearranged and the pressure drop was stable (Figure 3.4). The relationship of velocity (X- axial) and pressure drop (Y- axial) was plotted to estimate the minimum fluidization velocity.

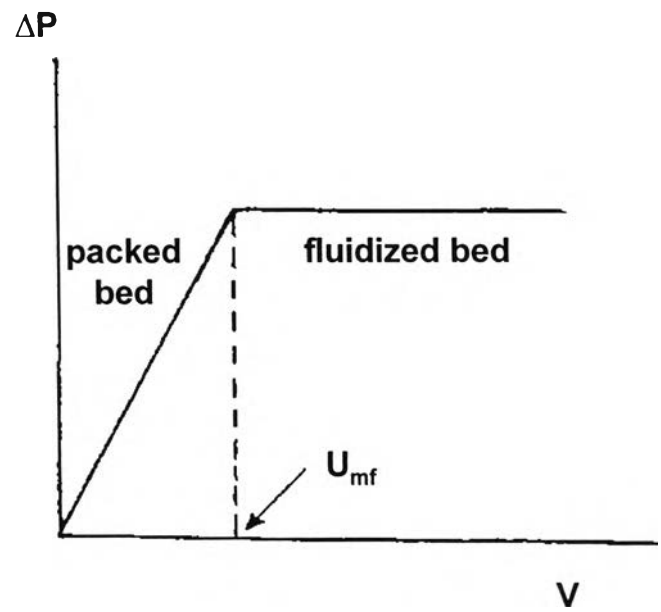


Figure 3.4 Experimental determination of U_{mf}

3.3.1.3 Flow rate of tangerine juice

According to the determination of minimum fluidization velocity, 50 cm x 3 cm i.d. fluidized column was applied to scale-up debittering process. Consequently, the appropriate flow rate for debittering tangerine juice was evaluated. Fifteen grams of β-CD polymer was fluidized by using the flow rate of 75, 100 and 120 ml/min for 30 min. At intervals, 100 ml juice was collected for determination of limonin content. Limonin was extracted and quantitated as described in 3.1.1 and 3.1.2.

3.3.1.4 Amount of β-CD polymer

To estimate the efficiency of adsorption, the juice was flowed through the fluidized column with different amount of the slurry of β-CD polymer (15, 20 and 25 g) by the appropriate flow rate from 3.4.2.3 at processing time of 30 minutes. At intervals, 100 ml juice was collected for determination of limonin content. Then the juice was passed through HyperSEP C-18 cartridge in order to separate limonin from other components. The experiment was designed as completely randomized design (CRD). After limonin extraction as described in the Section 3.1.1, the sample was injected onto HPLC as mentioned in the Section of 3.1.2, to determine the limonin content.

For regeneration, the β-CD polymer was washed with water, followed by 2% NaOH at room temperature until the alkali washed

fraction was colorless (determined spectrophotometrically at 275 and 360 nm). Finally, the β-CD polymer was washed with absolute ethanol and air dried before reused (Shaw and Wilson, 1983).

The suitable amount of regenerated β-CD polymer has been investigated before estimating the adsorption capability of fluidized column. The slurry of 8, 11 and 15 g of β-CD polymer was packed into a 50 cm x 3 cm i.d. fluidized column. The sample juice was counter-flowed by pump into the column at the proper flow rate from 3.4.2.3 for 30 min. At intervals, 100 ml juice was collected for determination of limonin content. Then the limonin was separated using HyperSEP C-18 cartridge (3.1.1) and determined by HPLC (3.1.2).

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

The practical maximum load of β-CD polymer fluidized column was studied for debittering process. The β-CD polymer was packed into the column at the optimum quantity as mentioned in the Section 3.3.2.3 and 3.3.2.4. Various amount of sample juice was loaded into the bottom of column and analyzed for the debittering profile. Estimation of the practical maximum load was on the basis that the acceptable amount of the limonin in eluant should not exceed 6 ppm which is the threshold level of limonin.

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

The β-CD polymer was regenerated as the procedure described previously in 3.3.1.4. The clarified juice was passed through 15 g regenerated β-CD polymer column at the same condition with 15 g β-CD polymer fluidized column. At intervals, 100 ml fraction of debittered juice was collected and subjected for determination of limonin content using the same procedures described in 3.1.1 and 3.1.2.

3.4 Reduction of limonin by fluidized XAD-16 resin process

3.4.1 Activation of XAD-16 resin

XAD-16, a neutral, hydrophobic, cross-linked polystyrene adsorbent available in commercial debittering process was used to compare its efficiency with that of β-CD polymer at the same of debittering condition.

XAD-16 resin was activated before using by the method described by Mozaffar *et al* (2000). Eleven grams of XAD-16 resin (60-80 mesh particle size) were swelled in excess water overnight, washing with 100 ml of 95 % ethanol before placing into the fluidized column (50 cm x 3 cm i.d.). At the batch activation, the XAD–16 resin was treated with 100 ml of distilled water followed by 100 ml 0.5 N hydrochloric acid, 50 ml distilled water, 100 ml 0.5 N sodium hydroxide and 100 ml distilled water respectively.

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

The sample juice was preheated and clarified by centrifugation before passing through XAD-16 resin fluidized column at the same condition used for β-CD polymer fluidized column. At intervals, 100 ml fraction of the debittered juice was collected and subjected for determination of limonin content using the same procedures described in 3.1.1 and 3.1.2.

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

3.5.1 Preparation of β-CD polymer

A mixture of 25 g of β-CD monomer (2.2 mmol) (Acros Organics, U.S.A.) and 11 ml of water in a 500 ml three-necked, round-bottomed flask fitted with a mechanical stirrer, reflux condenser, and dropping funnel was shown in Figure 3.5. All processes were operated at 80 °C then a mixture was stirred with a freshly solution of 13.5 g of sodium hydroxide in 13.5 ml water. Then, 50 mg of sodium borohydride was added and the mixture was stirred for 2 min. A 25 ml portion of epichlorohydrin (33 mmol) was rapidly dropped in the reaction mixture at 2 min interval. The resulting pasty mixture underwent a rapid, exothermic reaction in 10-20 min with brief, spontaneous refluxing, and a frothy, hard, glassy reaction product was formed. The mixture was allowed to stand at room temperature overnight and then heated to 50 °C for 5 h (Shaw *et at.*, 1984; Shaw

and Buslig, 1986). The glassy polymeric reaction product was easily broken up and was activated by procedure described in 3.3.1.

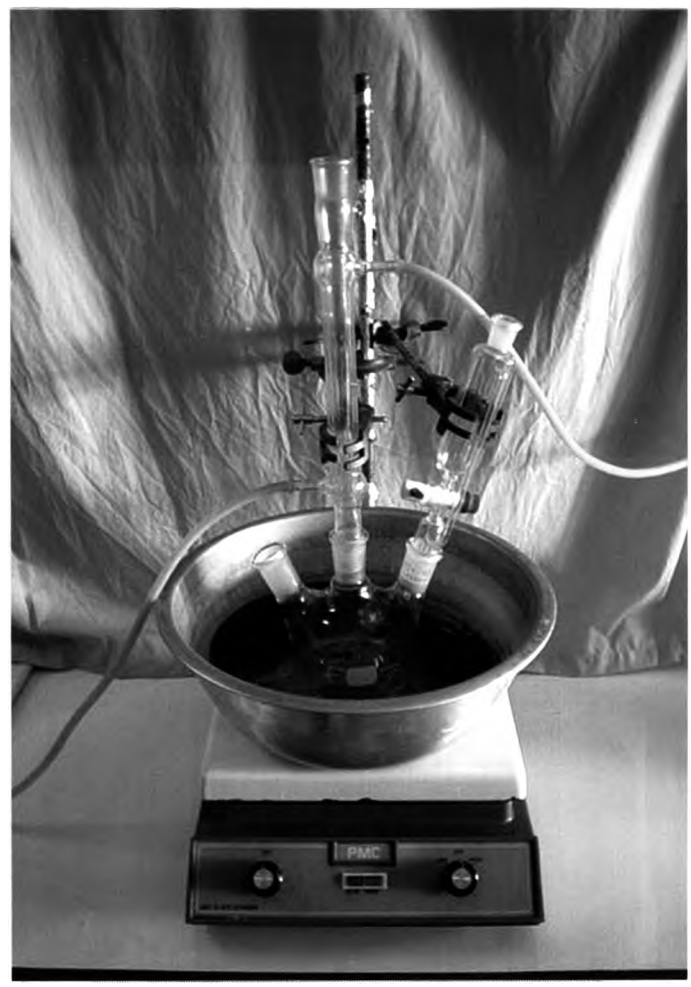


Figure 3.5 **β-cyclodextrin polymer preparation using round-bottomed flask fitted with a mechanical stirrer, reflux condenser, and dropping funnel**

3.5.2 Determination of residual epichlorohydrin

Residual epichlorohydrin in the washed polymer was determined by injection of 1 μl of ethanol wash solution into a gas chromatograph equipped with a 0.32 mm i.d. x 30 m fused silica capillary column coated with 5% phenylmethylsiloxane (HP-5). Temperature programming was 40 °C for 0.5 min, then increased to 60 °C at 20 °C/min, held for 1.5 min, and increased at 4 °C/min to 100 °C. The carrier gas (He) flow was 25 cm/s, the flame ionization detector was at 350 °C, and an injection port spiltter at 250 °C was used with a split ratio of 20:1. If epichlorohydrin was still present, the polymer was washed further with ethanol until no epichlorohydrin was detected (< 0.5 ppm).

3.5.3 Comparison of limonin reduction efficiency using β-CD polymer with prepared β-CD polymer fluidized column

The clarified juice was passed through 11 g prepared β-CD polymer column at the same condition with β-CD polymer fluidized column. At intervals, 100 ml fraction of debittered juice was collected and subjected for determination of limonin content using the same procedures described in 3.1.1 and 3.1.2.

3.6 Analysis of Thai tangerine juice compositions

3.6.1 Measurement of color

The color value of fresh juice, clarified juice and debittered juice by β-CD polymer, XAD-16 resin and prepared β-CD polymer was measured by Minoltachroma meter: series CR-300 (Minolta camera Co., Ltd. Japan.) using D_{65}

light source as shown in Figure 3.6. The experiment was designed as completely randomized design (CRD).

Figure 3.6 Minoltachroma meter

3.6.2 Determination of total soluble solids

The total soluble solids (°Brix) of the fresh juice, clarified juice and debittered juice by β-CD polymer, XAD-16 resin and prepared β-CD polymer was determined using of Hand refractometer (Model MNL-1125, Japan.) as shown in Figure 3.7. The experiment was designed as completely randomized design (CRD).

Figure 3.7 Hand refractometer

3.6.3 Determination of vitamin C content (AOAC, 1995)

Vitamin C or ascorbic acid reduces oxidation-reduction indicator dye, 2,6-di-chloroindophenol, to colorless solution. At the end point, excess unreduced dye is rose pink in acid solution. Vitamin is extracted and titration performed in the presence of HPO_3-CH_3COOH or HPO_3-CH_3COOH-H_2SO_4 solution to maintain proper acidity for the reaction and to avoid auto-oxidation of ascorbic acid at high pH.

3.6.3.1 Reagent preparation

Dye solution: 0.25 g sodium 2,6-dichlorophenolindophenol was dissolved in 500 ml water (60 °C) before adding 100 mg sodium

bicarbonate. The resulting solution was filtered through filter paper into a glass- bottle. Dye solution was kept out of direct sunlight in refrigerator.

Acid solution: 15 g meta-phosphoric acid (HPO_3) pellets was dissolved in 20 ml water (60 °C). After cooling, 40 ml acetic acid was added and the solution filtered rapidly through filter paper into a glass-bottle. The solution was transferred to 500 ml volumetric flask and adjusted to the volume with distilled water (HPO_3 slowly changes to H_3PO_4, but if stored in refrigerator, solution would remains stable for 7-10 days).

Ascorbic acid standard solution : 0.1 g ascorbic acid (Carlo Erba, Italy) was dissolved in 10 ml acid solution, adjusted volume to 100 ml in volumetric flask, the concentration of this solution was 1 mg/ml.

3.6.3.2 Standardization of dye solution

Ten millilitres ascorbic acid standard solution and 10 ml acid solution was transferred into 50 ml Erlenmeyer flask and titrated rapidly with dye solution until the solution was rose pink for at least 5 second. The concentration of dye solution was calculated as mg ascorbic acid equivalent to 1.0 ml reagent.

3.6.3.3 Determination of vitamin C

Ten millilitres of each sample juice (fresh juice, clarified juice and debittered juice by β-CD polymer, XAD-16 resin and prepared β-CD polymer) and 10 ml acid solution was transferred into 50 ml Erlenmeyer

flask. The solution was titrated immediately with standardized dye solution until the solution was rose pink for at least 5 second. Vitamin C content was calculated as ascorbic acid content from this equation.

$$\text{mg of Ascorbic acid / 100 ml} = \frac{\text{(dye conc.)(ml of titrated) (100 ml of juice)}}{\text{(10 ml of sample)}}$$

3.7 Statistical analysis

For statistical analysis, The SAS (stat analysis system) program V 6.12 was used. Coefficient of variation (C.V.) is a parameter indicating the level of reliability of the data.