CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

1. Analytical sieve 60, 80, 120 and 170 mesh and receiver: Retsch, F.Kurt Restch GmbH & CoKG, Germany.
2. C-18 column (250 mm x 4.6 mm) Model LUNA 5µm i.d., Phenomenex, U.S.A.
3. Extra-Sep HL C-18, 500 mg, 50 µm: Lida Manufacturing Crop. U.S.A.
4. Fraction collector Model 2211 Pharmacia LKB, Sweden
5. HPLC Model Hewlett Packard Series 1050, Japan
6. Laboratory juice extractor
7. Magnetic stirrer: model MR3003 Heidolph, Germany
8. Membrane filter: Nylon membrane filter 0.45 micron, 1.3 mm i.d., Sartorious, Germany
9. Peristaltic pump: Pharmacia LKBpump P-1, Pharmacia Biotech. Sweden
10. Refrigerated centrifuge: model J-21C, Beckman Instrument Inc.,U.S.A.
11. Spectrophotometer : Jenway UV/VIS 6400, England
12. Vacuum manifold: Lida® 12 samples, U.S.A.
13. Vortex: model K-550GE, Scientific Industries, U.S.A.

2.2 Chemicals

1. Acetonitrile (HPLC grade): Scharlau, Scharlau Chemie S.A., Spain.
2. Amberlite XAD-16 resin: Rohm and Hass, Philadephia, U.S.A
3. β–Cyclodextrin epichlorohydrin copolymer gift from : Cerestar U.S.A., Inc., Indiana, U.S.A.
4. Hydrochloric acid: Merck, Germany.
5. Methanol (HPLC grade):- Scharlau, Scharlau Chemie S.A., Spain.
6. Sodium Hydroxide pellets: Merck, Germany.
7. Standard Limonin (HPLC grade): Sigma, U.S.A.
8. Standard Naringin (95%): Sigma, U.S.A.

2.3 Sample preparation

No.3 commercial size of Thai tangerines, harvested from an orchard at Rangsit, Pathumthani province, were purchased from a local market in April – June, 2000. The fruits were washed and cut in half, then extracted with laboratory juice extractor. The tangerine juice was kept at –20 ^{0}C and thawed before used. All sample were centrifuged to remove pulps before further processed.

2.4 Determination of naringin and limonin content in Thai tangerine juice

2.4.1 Determination of naringin content by HPLC

The naringin content was determined by reverse-phase HPLC method using analytical C-18 column (octadecyl silane chemically bonded to < 10 μm porous microsilica packing 250 mm x 4.6 mm i.d.) as recommended by Fisher and Wheaton (1976). HPLC analyses were performed with a Hewlett Packard (HP) Series 1050 4 pumps (shown in Figure16). The chromatographic condition was 40^{0} C column temperature, pressure 120 bar, flow rate of 1.0 ml/min using 20 % acetonitrile as mobile and measured at 280 nm.

2.4.1.1 Standard curve of naringin

The naringin standard curve was established. The standard naringin solutions were prepared at 100, 200, 300, 400, 500 and 600 ppm in acetonitrile (HPLC grade). Twenty μl of each concentration was injected onto C-18 column and run at the condition described in 2.4.1 Appendix A1-7 shows the chromatograms of standard naringin at 100 – 600 ppm.

The naringin standard curve, shown in Appendix A 7, was plotted between standard naringin concentrations and peak areas, representing by axial X and Y respectively. The equation derived for the naringin standard curve was shown in Appendix A (Figure A 7).

2.4.1.2 Preparation of naringin extract by solid phase extraction (SPE) technique

The narinign extract was prepared by means of SPE using Extra Sep C-18 column recommended by Rouseff *et al* (1987). The Extra Sep C-18 column was pre-conditioned with 4 ml of methanol (HPLC grade) followed by 8 ml of water. Three ml of clarified fresh tangerine juice was slowly (1drop/20sec) passed through the column. The sugar was removed by washing the column with 3 ml of 10% methanol. Finally, the flavanone glycosides including naringin were slowly (1 drop/20 sec) flushed by 3 ml of methanol (HPLC grade). The naringin extract was filtered through 0.45 μm nylon filter membrane before determination by HPLC.

2.4.2 Determination of limonin content by HPLC

The limonin content was determined by reverse-phase HPLC method, using analytical C-18 column (octadecyl silane chemically bonded to < 10 μm porous microsilica packing, 250 mm x 4.6mm i.d.) as recommended by Mozaffar *et al* (2000). HPLC analyses were performed with HP series 1050 4 pumps (shown in Figure 16). The chromatographic conditions: 40°C column

temperature, pressure 120 bar, and flow rate of 1.0 ml/min using 37% acetonitrile as mobile phase. Detection was done at 214 nm.

Figure 16 HPLC (Hewlett Packard Series 1050 with 4 pumps)

2.4.2.1 Standard curve of limonin

To prepare 25 ppm of limonin standard solution, 1.25 g of limonin crystal (Sigma) was dissolved in 50 ml of acetonitrile (HPLC grade). The standard limonin was diluted with 37% aqueous acetonitrile aqueous 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 using the condition described in 2.4.2. The chromatograms of all standard limonin solutions were shown in Appendix A 8-13.

The limonin standard curve was plotted between standard limonin concentrations and peak areas, which were represented by axial X and Y respectively. The equation derived for the limonin standard curve which was used to estimate the limonin content in juice sample was shown in Appendix A (Figure A 14).

2.4.2.2 Preparation of limonin extract

Limonin in tangerine juice was extracted by two principles, namely adsorption (solid phase extraction) and partition (solvent extraction).

2.4.2.2.1 Limonin extraction by solid phase extraction (SPE) (Shaw and Wilson, 1984)

An Extra Sep C-18 column (Lida®) was fitted at one end of the column with an adapter and was connected to a vacuum manifold as illustrated in Figure 17. The column was activated

consecutively with 2 ml acetonitrile (HPLC grade) and 5 ml water. Three ml of the clarified tangerine juice was then slowly passed through the Extra Sep C-18 column (0.35 ml/min) and followed by 3 ml of water to remove sugar. Eventually, it was slowly flushed with 3 ml of acetonitrile (0.35ml/min) to collect the limonin fraction. The acetonitrile wash was filtered through a 0.45 μm nylon filter membrane (Sartorious, Germany) before determination of limonin content by HPLC.

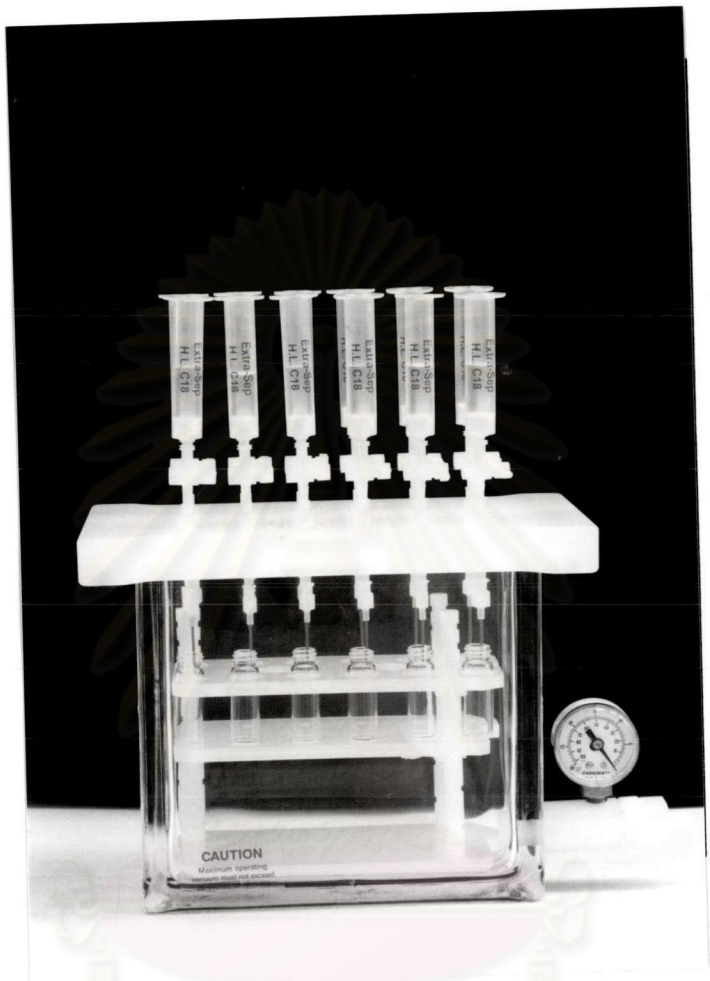

Figure 17 Limonin separation using Extra Sep C-18 and vacuum manifold (12 ports)

2.4.2.2.2 Limonin extraction by solvent extraction (SE) (Mozaffar *et al.*, 2000)

Mozaffar *et al* (2000) recommended to heat the juice in 40% aqueous acetonitrile at 90°C for 5 min (1:1 dilution), in order to dissolve any precipitated limonin and convert all limonin precursors to limonin before HPLC analysis.

2.5 Evaluation of reliability of the method

2.5.1 Efficiency of Extra Sep C-18 column in limonin separation

Two hundred and fifty µl of limonin standard solution (100 ppm) were passed through the column which was activated by 2 ml of acetonitrile (HPLC grade) and 5 ml of water consecutively. The column was then washed with 3 ml of water to remove sugar. Limonin was collected by 2.5 ml of acetonitrile wash at the flow rate of 0.35 ml/min. The fraction was analyzed by HPLC using the condition described in 2.4.2 to estimate the limonin content. The same column would be thrice to evaluate the efficacy of reused column. Before reused, the Extra Sep C-18 column was regenerated by washing with 5 volumes of 2 ml of acetonitrile (HPLC grade) and air dried.

2.5.2 Reliability of the method for limonin determination

To estimate the sensitivity of the method, the standard limonin was prepared at the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 ppm. One ml of each standard was passed through the preconditioned Extra Sep C-18 column at the flow rate of 0.35 ml/min, then washed with water and acetonitrile (HPLC

grade) respectively as described previously in the section of 2.4.2.2.1. The limonin extract from the column was analyzed by HPLC.

For assessment of the precision of the method, 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.

2.6 Optimization of juice preparation for the study of limonin reduction

2.6.1 Limonin content in juice kept at consumption conditions

Before exploring the optimum condition of juice preparation for the limonin reduction study, the limonin contents in juice treated at the general consumption conditions e.g., fresh juice, chilled juice (at 6^{0}C for 24 hours) and pasteurized juice (70^{0}C for 15 minutes) were explored. These samples were derived from the same pool of fresh tangerine juice but were treated at different conditions. Limonin was quantitated as described in 2.4.2 and 2.4.2.2.1.

2.6.2 Effect of temperature on limonin content in Thai tangerine juice

To study the appropriate temperature for treating juice before debittering, the juice was heated at 60, 70 and 80 ^{0}C for 15 min and centrifuged at 4^{0} C, 10,000 rpm for 10 min. Then the juice was passed through Extra-Sep C-18 column in order to separate limonin from other components. After limonin separation as a procedure described in the section 2.4.2.2.1, the sample was injected onto HPLC as mentioned in the section of 2.4.2 for determining limonin content.

2.6.3 Effect of heating time on limonin content in Thai tangerine juice

To estimate the effect of heating time on limonin content in tangerine juice, the juice was heated at the appropriate temperature from 2.5.2 for 5, 10 and 15 min respectively. Then the limonin was separated using Extra Sep C-18 column and determined by HPLC (2.4.2).

For economical reasons the optimum condition for juice preparation should be at the lowest temperature and heating time, which could completely converts limonin precursors to limonin.

2.7 Reduction of limonin by β-CD polymer

2.7.1 Preparation 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 75 ml of water until pH of the solution was around 7, followed by 2 X75 ml of ethanol and dried. This method was recommended by Alan Hedge, the director of Cerestar U.S.A., Inc.

2.7.2 Procedures for debittering

Two debittering processes were employed in this study.

2.7.2.1 Batch process

For the batch process, 0.25, 0.75 or 1.25 g of β-CD polymer was added to 25 ml of preheated clarified juice sample (1, 3 and 5g%β-CD polymer). The resulting mixture was magnetically stirred at cold room

temperature (6 ^{0}C) and room temperature (~30^{0}C) for 0.5, 1.0, and 1.5 hour respectively. The experiment was designed as 3x3x2x3 factorial design. At designated time, the polymer was removed by filtration through 170 mesh screen. After that the treated juice was passed through Extra Sep C-18 column in order to separate limonin before determination by HPLC as mentioned in 2.4.2.2.1 and 2.4.2 respectively. Figure 18 A shows the batch debittering process.

After determining the optimum condition of the batch debittering process, the effect of mixing speed was also investigated by varying into two speeds No. 3 and 5 (Magnetic stirrer model MR3003 Heidolph, Germany) using the conditions chosen from the above studies.

2.7.2.2 Column process

The process was conducted at room temperature (~30^{0}C). Three grams of β-CD polymer (0.03 g β-CD polymer/ml bed volume) from 2.7.1 were suspended in water for 30 minutes and were packed in glass column (12 cm x 1.2 cm i.d.) at the flow rate 0.35 ml/min monitored by a peristaltic pump . The β-CD polymer column was equilibrated with water for 1 hour before loading 100 ml of sample juice at the same flow rate. Five ml fraction of debittered juice fraction was collected and subjected to the determination of limonin content. The column process was illustrated in Figure 18 B.

A) Debittering by batch process

B) Debittering by column process

Figure 18 Batch and column processes

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

To study the practical maximum load of β-CD polymer column for debittering process, the β-CD polymer was packed into the column as mentioned in section 2.7.2.2. Various amount of sample juice was loaded onto the column and analyzed for the debittering profile. Estimation of the practical was based on the criterion that the acceptable amount of the limonin in eluant should not exceed 5 ppm.

2.7.4 Regeneration of β-CD polymer

For reused, the column was treated with 30 ml (3 Bed volume) of water, followed by 2 % NaOH until the alkali washed fraction was limpid (determined spectrophotometrically at 275 and 360 nm). Finally the β-CD polymer was washed with absolute ethanol and dried in air before reused (Shaw and Wilson, 1983).

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

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

2.8.1 Resin preparation

XAD-16 resin was prepared before used by Mozaffar *et al* (2000) method. Five point six grams of XAD-16 resin (80-120 mesh particle size) were swelled in excess water for 30 min. (with 0.56 g resin/ml)

followed by 50 ml of 95 % ethanol before placing into the glass column (20 cm x 1.2 cm i.d.). At the flow rate of 0.35 ml/min, the XAD–16 column was treated with 50 ml of distilled water followed by 50 ml 0.5 N HCl, 50 ml distilled water, 50 ml 0.5 N NaOH and 50 ml distilled water respectively. The sample juice was preheated and clarified by centrifugation before it was passed through the column at the flow rate of 0.35 ml/min. Five ml fraction of debittered juice was collected and subjected for determination of limonin content using the same procedures described in 2.4.2.2.1 and 2.4.2.

2.9. 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.