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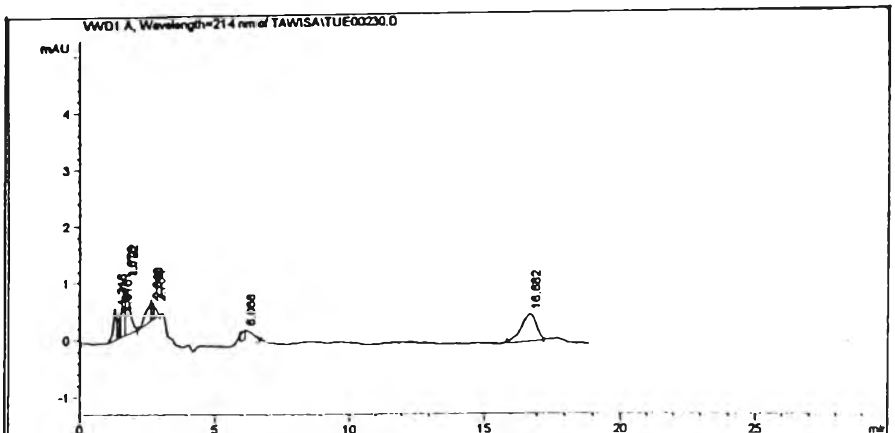
APPENDICES

Appendix A

1. Chromatogram and standard curve of Limonin



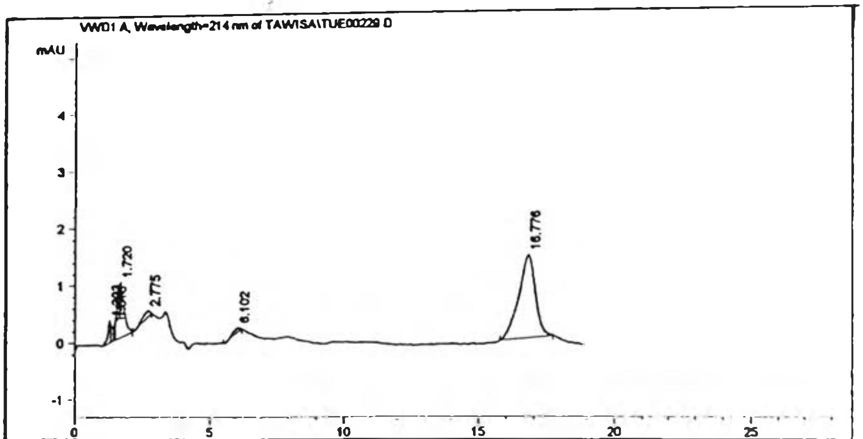
Area Percent Report

Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000

Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.316	PV	0.105	4.54269	5.59821e-1	8.3301
2	1.447	VV	0.054	1.59232	3.80153e-1	2.9199
3	1.516	VV	0.040	1.17215	4.11536e-1	2.1494
4	1.676	VV	0.088	6.64687	9.55352e-1	12.1886
5	1.722	VV	0.137	10.98958	9.80970e-1	20.1519
6	2.640	BV	0.181	4.89459	3.25604e-1	8.9754
7	2.666	VV	0.070	1.76007	3.22515e-1	3.2275
8	2.764	VB	0.122	1.71808	2.35612e-1	3.1505
9	6.068	VV	0.127	1.79980	1.68439e-1	3.3003
10	16.682	VV	0.501	19.41747	4.83679e-1	35.6064
Totals :				54.53360	4.82368	

Figure A 1 Chromatogram of standard limonin at 2.5 ppm



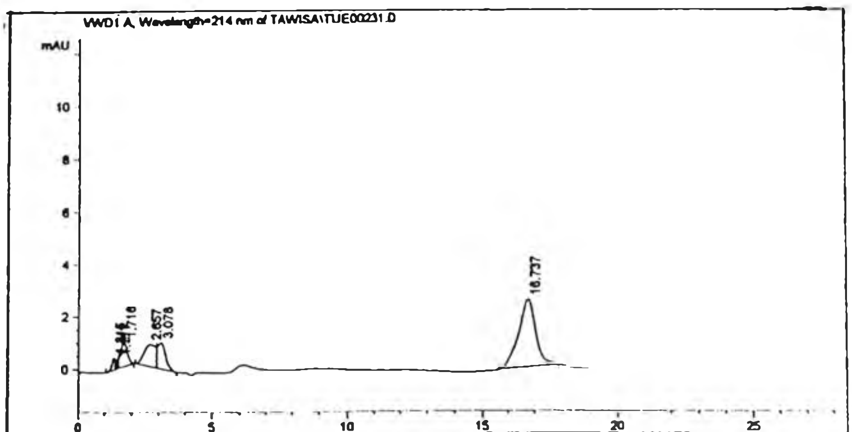
Area Percent Report

Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000

Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.303	BV	0.086	2.87760	4.08149e-1	3.2304
2	1.441	VV	0.082	1.74274	2.72847e-1	1.9564
3	1.518	VV	0.044	1.00263	3.34670e-1	1.1256
4	1.720	VB	0.219	18.09196	9.87734e-1	20.3103
5	2.775	BB	0.246	2.31412	1.22720e-1	2.5979
6	6.102	BB	0.247	1.37916	8.05776e-2	1.5483
7	16.776	BB	0.513	61.66935	1.45473	69.2311
Totals :				89.07756	3.66143	

Figure A 2 Chromatogram of standard limonin at 7.5 ppm



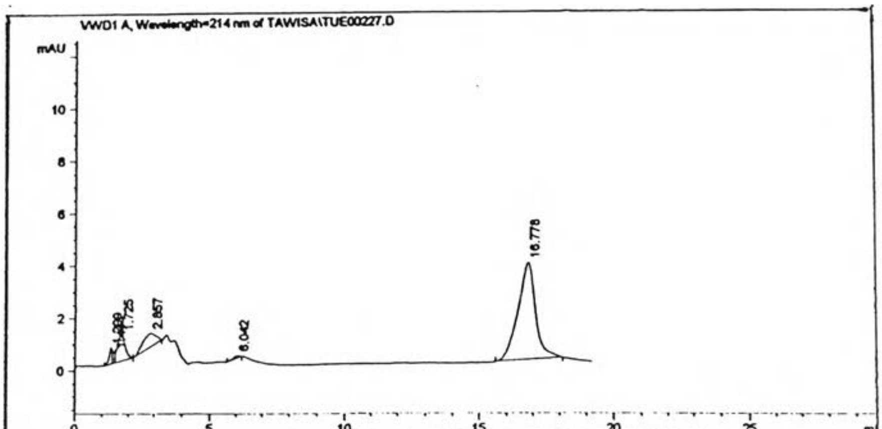
Area Percent Report

Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000

Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.315	BV	0.087	3.22284	4.89159e-1	1.7555
2	1.441	VV	0.059	1.45695	3.32836e-1	0.7936
3	1.517	VV	0.047	1.09348	3.90309e-1	0.5956
4	1.716	VV	0.210	16.45072	9.62871e-1	8.9608
5	2.657	BV	0.382	24.95917	8.52654e-1	13.5954
6	3.078	VB	0.262	20.86027	1.00910	11.3627
7	16.737	BB	0.540	115.54169	2.57175	62.9363
Totals :				183.58511	6.60868	

Figure A 3 Chromatogram of standard limonin at 12.5 ppm



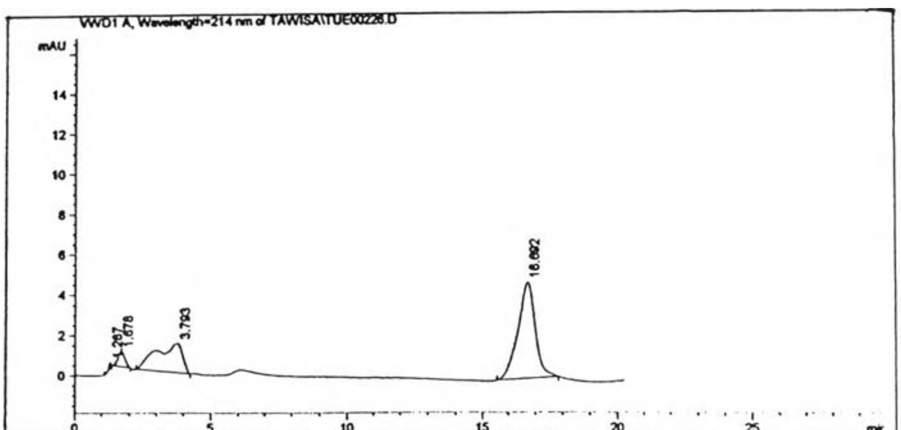
Area Percent Report

Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000

Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.299	BV	0.096	4.75334	6.42361e-1	2.1825
2	1.444	VV	0.076	2.58861	4.33771e-1	1.1885
3	1.725	VV	0.251	21.00517	1.01923	9.6444
4	2.857	PB	0.430	18.85574	5.28300e-1	8.6575
5	6.042	BB	0.240	1.88865	1.05101e-1	0.8672
6	16.778	BB	0.561	168.70444	3.67219	77.4599
Totals :				217.79594	6.40095	

Figure A 4 Chromatogram of standard limonin at 17.5 ppm



Area Percent Report

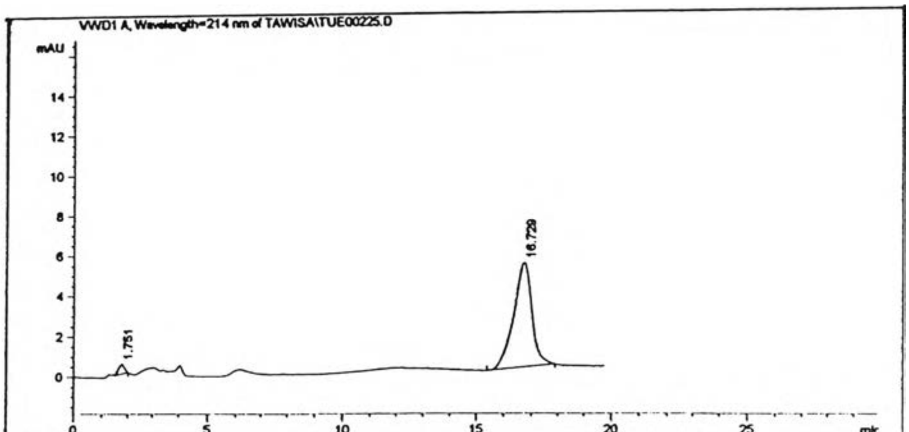
Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000

Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.287	BB	0.058	1.22371	3.07409e-1	0.3789
2	1.678	VB	0.214	13.42850	7.84763e-1	4.1584
3	3.793	BV	0.783	96.71919	1.46322	29.9512
4	16.692	BB	0.535	211.55150	4.75914	65.5115

Totals : 322.92291 7.31453

<u>Figure A 5</u> Chromatogram of standard limonin at 22.5 ppm



Area Percent Report

Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000

Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.751	BB	0.185	8.01883	5.28654e-1	3.2892
2	16.729	BB	0.544	235.77185	5.16708	96.7108
Totals :				243.79068	5.69573	

Figure A 6 Chromatogram of standard limonin at 25.0 ppm

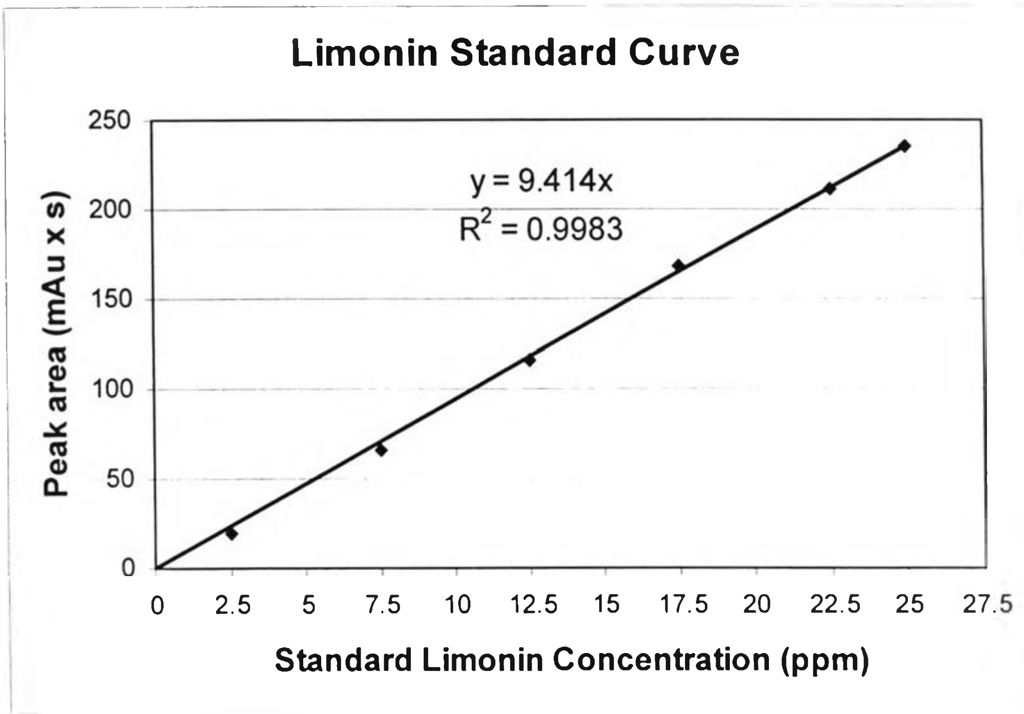


Figure A 7 Standard curve of limonin

Table A 1 % Recoveries of limonin of initial and reused HyperSEP C18 column

Replications	%Recoveries				
	Initial C-18	1^{st} Reused	2^{nd}Reused	3^{rd} Reused	4^{th} Reused
1	86.55	80.15	77.06	63.56	64.91
2	83.24	80.55	75.66	63.28	63.56
3	82.06	80.18	77.96	63.28	63.50
Average	83.95 ± 2.33^{a}	80.29 ± 0.22^{b}	76.89 ± 1.16^{c}	63.37 ± 0.16^{d}	63.99 ± 0.80^{d}
%C.V.	2.77	0.28	1.51	0.26	1.24

Note: 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.

Table A 2 Color values (L, a, b) of each sample juice

Sample	L	a	b	Average / %C.V		
				L	a	b
Fresh juice	102.46 101.17 101.77	-11.32 -10.92 -10.86	+33.50 +31.83 +31.66	101.80±0.64[A] / 0.63	-11.03±0.25[A] / 2.26	+32.33±1.01[A]/ 3.14
Clarified juice	92.23 92.34 92.72	-6.11 -6.10 -6.33	+9.90 +10.39 +10.54	92.43±0.2 [B] / 0.28	-6.18±0.13 [B] / 2.10	+10.28±0.33 [B] / 2.60
Debittered juice by β-CD polymer	92.54 92.87 91.98	-6.54 -6.42 -6.21	+10.34 +10.23 +10.11	92.46±0.44 [B] / 0.49	-6.39±0.17 [B] / 2.61	+10.23±0.12 [B] / 4.54
Debiitered juice by XAD-16 resin	92.05 92.89 92.56	-6.86 -6.15 -5.97	+10.15 +10.17 +10.28	92.5±0.42 [B] / 0.46	-6.33±0.47 [B] / 7.43	+10.18±0.04 [B] / 0.69
Debittered juice by prepared β-CD polymer	92.19 92.43 92.54	-6.38 -6.19 -6.22	+10.14 +9.98 +10.37	92.39±0.18 [B] / 0.36	-6.26±0.10 [B] / 1.63	+10.16±0.20 [B] / 1.43

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

A and B means with the same letter were significant different at P>0.05 by Duncan's multiple range test with three replications.

<u>Table A 3</u> Total soluble solids of each sample juice

Juice	Total soluble solids			Average*NS	%C.V
Fresh juice	11.6	11.7	11.6	11.63±0.06	0.50
Clarified juice	11.6	11.5	11.6	11.55±0.07	0.50
Debittered juice by β-CD polymer	11.6	11.5	11.5	11.54±0.05	0.50
Debiitered juice by XAD-16 resin	11.7	11.6	11.6	11.63±0.06	0.50
Debittered juice by prepared β-CD polymer	11.5	11.6	11.7	11.6±0.10	0.86

Note: *NS= not significantly different at $P \leq 0.05$ by Duncan's multiple range test with three replications.

Table A 4 Vitamin C content of each sample juice

Juice	Vitamin C content (mg/100 ml juice)			Average*NS	%C.V
Clarified juice	5.67	5.80	5.67	5.71±0.08	1.31
Debittered juice by β-CD polymer	5.80	5.80	5.94	5.85±0.08	1.37
Debiitered juice by XAD-16 resin	5.80	5.67	5.80	5.76±0.08	1.30
Debittered juice by prepared β-CD polymer	5.94	5.67	5.80	5.80±0.14	2.33

Note: *NS= not significantly different at $P \leq 0.05$ by Duncan's multiple range test with three replications.

Table A 5 Operating cost for β-CD polymer fluidized column (Laboratory scale)

Items	Unit	Quantity	Cost/unit (Bahts/unit)	Cost (Bahts)	(%)
Tangerine fruit	kg	10	7	70	1.56
β-CD polymer	G	11	400	4,400	98.36
NaOH	G	12	0.26	3.12	0.07
Electricity for pump	kw*h	0.004	4	0.016	0.0004
Water	m^3	0.02	10	0.2	0.0045

Total cost (Bahts/column) =4,473.336

Practical maximum capacity of column (mg limonin/g β-CD polymer) = 0.47

Productivity (ml juice/column/hour) = 6,000

Appendix B

1. Calculation of limonin content in tangerine juice

Standard limonin equation : $Y = 9.414 X$

Slope (S) = 9.414

When Y represents to peak area (mAu x s) and

X represents to limonin concentration (ppm)

From HPLC analysis

Peak area of the limonin peak at 16 min = P

Thus limonin concentration of the extract = P/S ppm or μg/ml

The 1.5 ml of extract was prepared from 6 ml of sample juice

The efficiency of HyperSEP C18 column = 80%

Limonin content in the 6 ml sample juice = 1.5 x (P/S) x100/80 μg

= 1.875(P/S) μg

Finally, the limonin concentration in the sample juice = 1.875(P/S) μg/6 ml

= 0.3215(P/S) ppm

2. Calculation of size of fluidized column

The equation of minimum fluidization velocity, for small particles and Re<50:

$$U_{mf} = \frac{688\, d_p^{1.82} (\rho_s - \rho_f)^{0.94}}{\mu^{0.88}}$$

where d_p = 0.015 in, ρ_s = 67.8 lbm/ft^3, ρ_f = 64.74 lbm/ft^3, μ =1.2 centipoise

Thus, U_{mf} = 0.805 ft/hr

= 0.409 cm/min

The equation of cross-section area of column was

$Q = U_{mf} \cdot A$ cm^3/min

If Q = 5 cm^3/min

Thus, A = 12.22 cm^2

The diameter of column was

$A = (\pi/4)\, D^2$ cm^2

Thus, D = 3.94 cm

The bed height was

$U_{mf} = s/t$ cm/min

If t = 30 min

s = 12.27 cm

From this calculation, the fluidized column was approximately designed as the height of column was 50 cm and the diameter of column was between 3 to 5 cm.

Appendix C

Statistical Analysis

Table C 1 ANOVA for the study of regeneration of HyperSEP C18 column for limonin determination

SOURCE	DF	SUM OF SQUARE	MEAN SQUARE	F VALUE	Pr>F
Model	4	1078.9682	269.7420	180.48	0.0001
Error	10	14.9458	1.4946		
Corrected total	14	1093.9140			

Duncan's Multiple Range Test

Duncan Grouping	Mean	N	Between group
A	83.9500	3	1
B	80.2933	3	2
C	76.8933	3	3
D	63.9900	3	5
D			
D	63.3733	3	4

R-square	0.9863	C.V.	1.6588
Root MSE	1.2225	Data Mean	73.7000

Table C 2 ANOVA for the study of color value (L) in sample juice

SOURCE	DF	SUM OF SQUARE	MEAN SQUARE	F VALUE	Pr>F
Model	4	205.4938	51.3734	262.37	0.0001
Error	10	1.9581	0.1958		
Corrected total	14	207.4519			

Duncan's Multiple Range Test

Duncan Grouping	Mean	N	Between group
A	101.8000	3	1
B	92.8200	3	5
B			
B	92.5000	3	4
B			
B	92.4633	3	3
B			
B	92.4300	3	2

R-square	0.9906	C.V.	0.4687
Root MSE	0.4425	Data Mean	94.4027

Table C 3 ANOVA for the study of color value (a) in sample juice

SOURCE	DF	SUM OF SQUARE	MEAN SQUARE	F VALUE	Pr>F
Model	4	54.0706	13.5176	199.26	0.0001
Error	10	0.6784	0.0678		
Corrected total	14	54.7490			

Duncan's Multiple Range Test

Duncan Grouping	Mean	N	Between group
A	-6.1800	3	2
A			
A	-6.2633	3	5
A			
A	-6.3267	3	4
A			
A	-6.3900	3	3
B	-11.0333	3	1

R-square	0.9876	C.V.	3.5982
Root MSE	0.2605	Data Mean	7.2387

Table C 4 ANOVA for the study of color value (b) in sample juice

SOURCE	DF	SUM OF SQUARE	MEAN SQUARE	F VALUE	Pr>F
Model	4	1165.9718	291.4930	1039.68	0.0001
Error	10	2.8037	0.2804		
Corrected total	14	1168.7755			

Duncan's Multiple Range Test

Duncan Grouping	Mean	N	Between group
A	32.3300	3	1
B	10.5600	3	3
B			
B	10.2767	3	2
B			
B	10.2000	3	4
B			
B	10.1300	3	5

R-square	0.9976	C.V.	3.6021
Root MSE	0.5295	Data Mean	14.6993

Table C 5 ANOVA for the study of total soluble solids in sample juice

SOURCE	DF	SUM OF SQUARE	MEAN SQUARE	F VALUE	Pr>F
Model	4	0.0227	0.0057	1.21	0.3639
Error	10	0.0467	0.0047		
Corrected total	14	0.0693			

Duncan's Multiple Range Test

Duncan Grouping	Mean	N	Between group
A	11.6333	3	1
A			
A	11.6333	3	4
A			
A	11.6000	3	5
A			
A	11.5667	3	2
A			
A	11.5333	3	3

R-square	0.3269	C.V.	0.5892
Root MSE	0.0683	Data Mean	11.5933

Table C 6 ANOVA for the study of vitamin C content in sample juice

SOURCE	DF	SUM OF SQUARE	MEAN SQUARE	F VALUE	Pr>F
Model	3	0.0535	0.0178	1.98	0.1957
Error	8	0.0721	0.0090		
Corrected total	11	0.1256			

Duncan's Multiple Range Test

Duncan Grouping	Mean	N	Between group
A	5.8933	3	2
A			
A	5.8033	3	4
A			
A	5.7567	3	3
A			
A	5.7133	3	1

R-square	0.4261	C.V.	1.6388
Root MSE	0.0949	Data Mean	5.7917

Appendix D

1. Capacity of β-CD polymer column in limonin adsorption at maximum juice volume

Productivity of the laboratory scale

At maximum load, it produced 900 ml of debittered juice

Flow rate	=	100 ml/minute
Process time for 900 ml juice	=	9 minutes or 0.15 hours / column
Productivity of laboratory scale (P)	=	900 ml debittered juice / column / 0.15 hour
	=	6000 ml juice/column/hour

Limonin adsorption capacity of the laboratory scale

When used 15 g β-CD polymer

= (11.76-6) (μg/ml juice)*900 ml juice/ 15 g β-CD polymer

= 345.6 μg limonin /g β-CD polymer

≃ 0.35 mg limonin /g β-CD polymer

When used 15 g regenerated β-CD polymer

= (11.89-6) (μg/ml juice)*350 ml juice/ 15 g β-CD polymer

= 137.4 μg limonin /g β-CD polymer

≃ 0.14 mg limonin /g β-CD polymer

When used 11 g β-CD polymer

= (11.76-6) (μg/ml juice)*900 ml juice/ 11 g β-CD polymer

= 471.3 μg limonin /g β-CD polymer

≃ 0.47 mg limonin /g β-CD polymer

2. Capacity of XAD-16 in limonin adsorption at the optimum condition

Initial limonin content in juice load = 12.07 ppm

Juice load = 1600 ml

Amount of XAD-16 resin used = 11 g

Total limonin in 100 ml juice = 19312 μg

% limonin reduction ~ 90%

Capacity of XAD-16 resin in limonin reduction = (19312 x 0.9)/11

= 1580.07 μg limonin/ g XAD-16 resin

= 1.58 mg limonin/ g XAD-16 resin

3. Capacity of prepared β-CD polymer in limonin adsorption at the maximum juice volume

Initial limonin content in juice load = 12.15 ppm

Maximum juice volume = 25 ml

Amount of prepared β-CD polymer used = 11 g

Total limonin in 100 ml juice = 303.75 μg

Capacity of β-CD polymer in limonin reduction = 303.75/11

= 27.614 μg limonin/ g β-CD polymer

= 0.028 mg limonin/ g β-CD polymer

BIOGRAPHY

Miss Tawisa Pipatthitikorn was born on May 11, 1982 in Nakhonpathom, Thailand. She graduated her Bachelor degree of Biochemistry, Faculty of Science from Chulalongkorn University in 2001 and then enrolled in the graduate program for Master of Science in Biotechnology at Chulalongkorn University in 2002. She has publication:

1. Tawisa Pipatthitikorn, Lersuang Mekasut, Peerada Mongkolkul and Romanee Sa-nguandeekul. 2004. Scale-up of Debittering Tangerine *Citrus reticulata* Blanco Juice by Fluidized β-Cyclodextrin Polymer Process. *In* The 16th Annual Meeting of the Thai Society for Biotechnology, Pittsanulok, Thailand during December 12-15, 2004 p.109.