APPENDIX C
VALIDATION OF HPLC METHOD

Linearity

The three concentration levels of each standard were plotted against peak area (n=3) for a linear calibration curve which its correlation coefficient (r^2), regression equation and range were show below (Figures 1 - 6 and Tables 1 - 6).

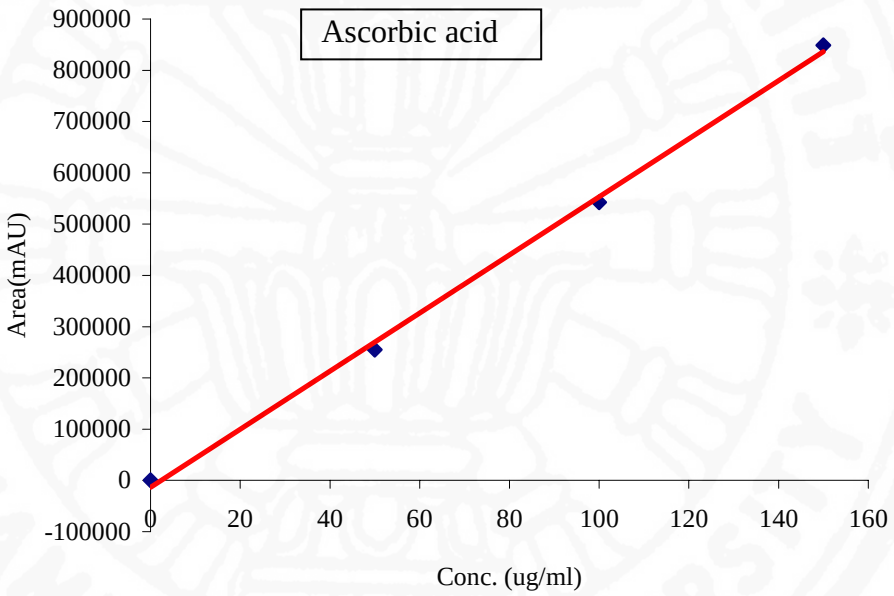

Figure 1 The calibration curve of ascorbic acid

Table 1 The regression data of ascorbic acid

Validation	Ascorbic acid
Regression equation	y = 5667.4x - 13427
Correlation coefficient (r^2)	0.9984
Range (μg/ml)	50-150
Retention time (min) (9 replicates, mean ± SD)	4.70 ± 0.11

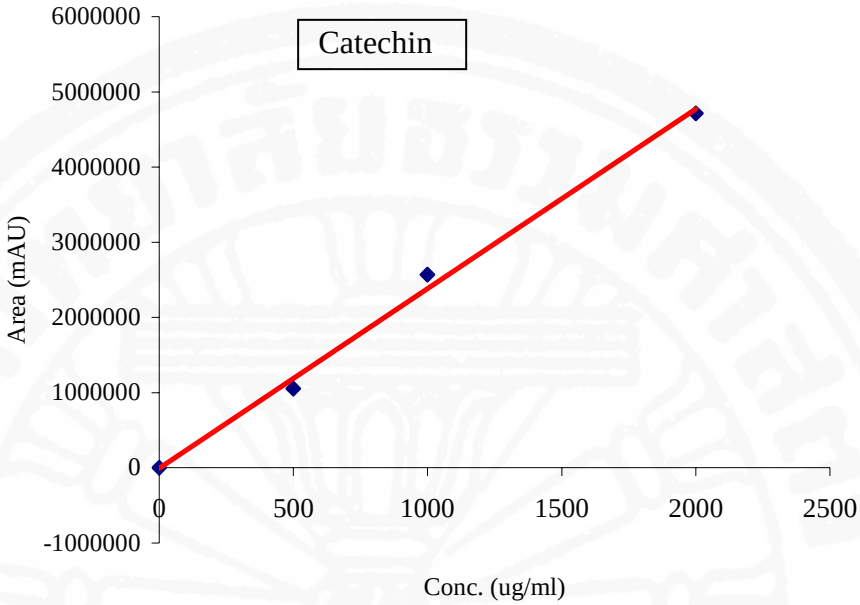

Figure 2 The calibration curve of catechin

Table 2 The regression data of catechin

Validation	Catechin
Regression equation	y = 2390.4x - 6721
Correlation coefficient (r^2)	0.9954
Range (μg/ml)	500-2000
Retention time (min) (9 replicates, mean ± SD)	13.4 ± 0.33

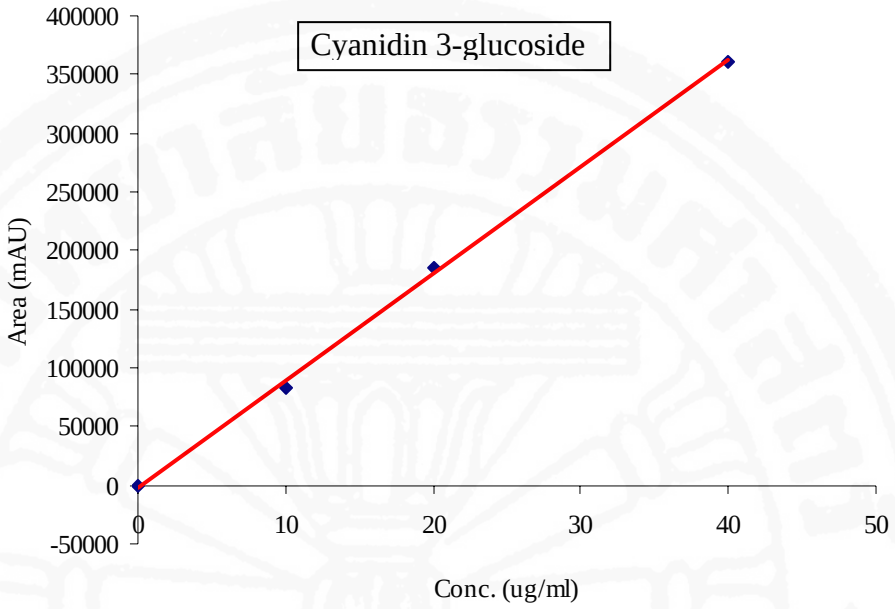

Figure 3 The calibration curve of cyanidin 3-glucoside

Table 3 The regression data of cyanidin 3-glucoside

Validation	Cyanidin 3-glucoside
Regression equation	y = 9092.4x - 1994.6
Correlation coefficient (r^2)	0.9991
Range (μg/ml)	10-40
Retention time (min) (9 replicates, mean ± SD)	22.8 ± 0.24

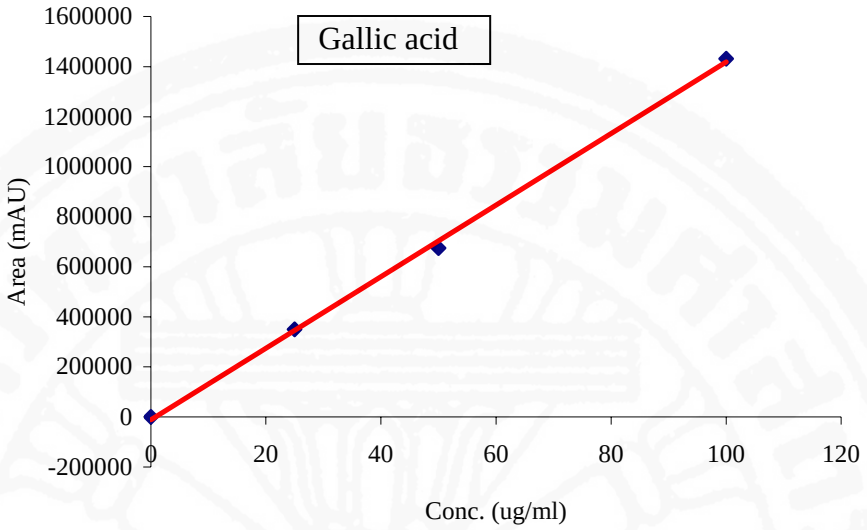

Figure 4 The calibration curve of gallic acid

Table 4 The regression data of gallic acid

Validation	Gallic acid
Regression equation	y = 14301x - 11316
Correlation coefficient (r^2)	0.9990
Range (μg/ml)	10-100
Retention time (min) (9 replicates, mean ± SD)	9.3 ± 0.15

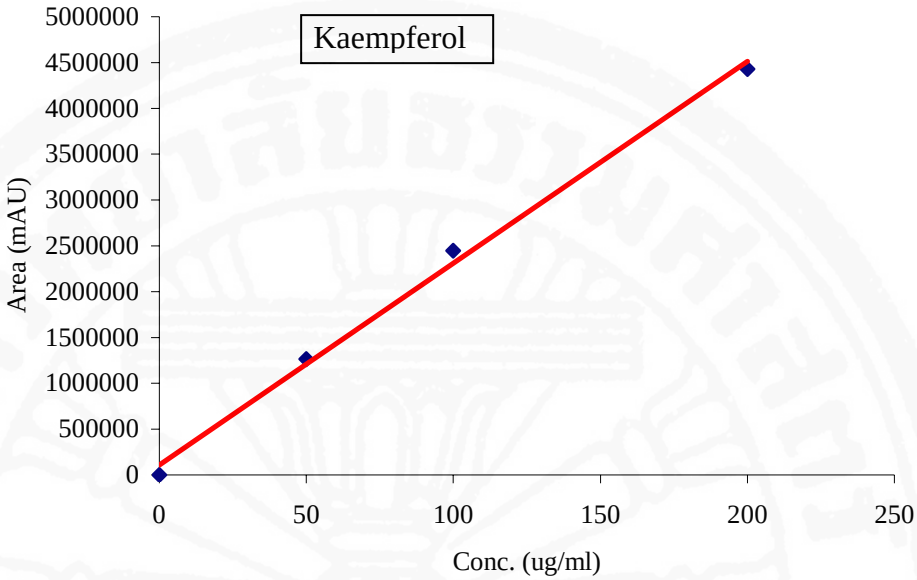

Figure 5 The calibration curve of kaempferol

Table 5 The regression data of kaempferol

Validation	Kaempferol
Regression equation	y = 22012x + 109266
Correlation coefficient (r^2)	0.9962
Range (μg/ml)	50-200
Retention time (min) (9 replicates, mean ± SD)	83.4 ± 0.26

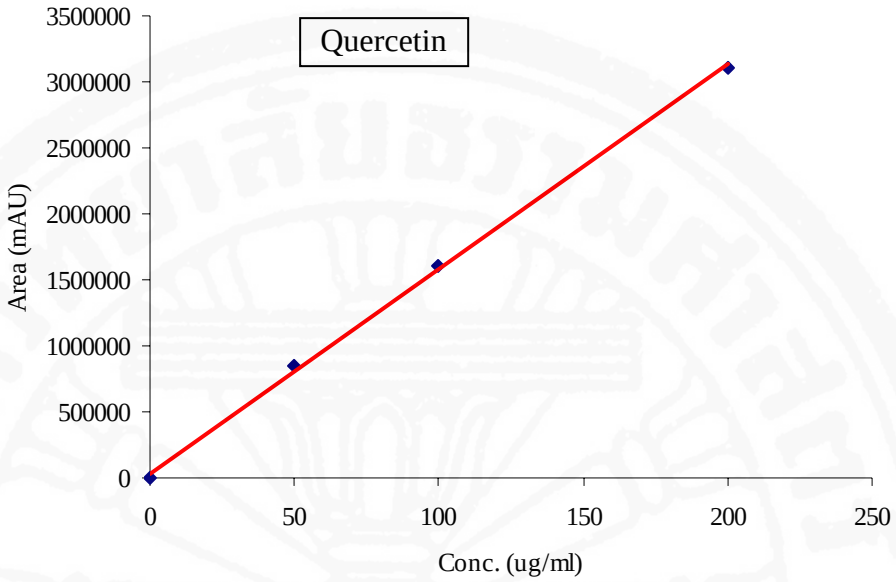

Figure 6 The calibration curve of quercetin

Table 6 The regression data of quercetin

Validation	Quercetin
Regression equation	y = 15470x + 35565
Correlation coefficient (r^2)	0.9994
Range (μg/ml)	50-200
Retention time (min) (9 replicates, mean ± SD)	80.4 ± 0.39

Precision

Repeatability (intra-day) was evaluated by assaying each standard during the same day. The % RSD of repeatability of ascorbic acid, catechin, cyanidin 3-glucoside, gallic acid, kaempferol and quercetin were 0.83, 0.70, 0.20, 1.52, 1.81 and 4.6, respectively (Tables 7 - 12). The RSD indicated that the method is precise.

Table 7 The repeatability (intra-day) precision of ascorbic acid by HPLC (6 replicates, n = 1, mean ± SD)

Concentration	Inject No.	Peak area
100 µg/ml	1	549771
	2	538995
	3	539570
	4	537568
	5	541356
	6	538671
	Average	540988.5
	SD	4479.38
	%RSD	0.83

Table 8 The repeatability (intra-day) precision of catechin by HPLC (6 replicates, n = 1, mean ± SD)

Concentration	Inject No.	Peak area
1000 µg/ml	1	2681795
	2	2731920
	3	2722164
	4	2721320
	5	2702329
	6	2695535
	Average	2709177
	SD	19058.5
	%RSD	0.70

Table 9 The repeatability (intra-day) precision of cyanidin 3-glucoside by HPLC (6 replicates, n = 1, mean ± SD)

Concentration	Inject No.	Peak area
20 µg/ml	1	185666
	2	184697
	3	184951
	4	185162
	5	185214
	6	185589
	Average	185213
	SD	369.89
	%RSD	0.20

Table 10 The repeatability (intra-day) precision of gallic acid by HPLC (6 replicates, n = 1, mean ± SD)

Concentration	Inject No.	Peak area
50 µg/ml	1	672908
	2	661593
	3	691241
	4	685214
	5	679562
	6	681050
	Average	678595
	SD	10316.1
	%RSD	1.52

Table 11 The repeatability (intra-day) precision of kaempferol by HPLC (6 replicates, n = 1, mean ± SD)

Concentration	Inject No.	Peak area
100 µg/ml	1	2503939
	2	2392041
	3	2453122
	4	2415870
	5	2418656
	6	2489007
	Average	2445439.2
	SD	44322.6
	%RSD	1.81

Table 12 The repeatability (intra-day) precision of quercetin by HPLC (6 replicates, n = 1, mean ± SD)

Concentration	Inject No.	Peak area
100 µg/ml	1	1661709
	2	1548327
	3	1502548
	4	1675624
	5	1539549
	6	1641085
	Average	1594807
	SD	73313.7
	%RSD	4.60

Accuracy

The recovery of each standard from the extract was performed on samples spiked with each standard. The accuracy of ascorbic acid, catechin, cyanidin 3-glucoside, gallic acid, kaempferol and quercetin were determined and the average of percent recovery was found to be 100.23 ± 1.23, 100.55 ± 0.60, 96.8 ± 2.70, 96.03 ± 0.99, 102.27 ± 1.21 and 85.61 ± 1.92, respectively (Tables 12 - 17).

Table 13 Recovery study of ascorbic acid by HPLC (3 replicates, n = 1, mean ± SD)

Amount of ascorbic acid added (µg)	Amount of ascorbic acid (µg)			Recovery (%)
	In mixture	In sample	Mixture- Sample	
100	111.34	11.49	99.85	99.85
100	110.56	11.33	99.23	99.23
100	112.15	10.55	101.60	101.60
Average				100.23
SD				1.23

Table 14 Recovery study of catechin by HPLC (3 replicates, n = 1, mean ± SD)

Amount of catechin added (µg)	Amount of catechin (µg)			Recovery (%)
	In mixture	In sample	Mixture- Sample	
1000	1023.25	11.97	1011.28	101.13
1000	1012.2	12.88	999.32	99.93
1000	1019.8	13.87	1005.93	100.59
Average				100.55
SD				0.60

Table 15 Recovery study of cyanidin 3-glucoside by HPLC (3 replicates, n = 1, mean ± SD)

Amount of cyanidin 3-glucoside added (µg)	Amount of cyanidin 3-glucoside (µg)			Recovery (%)
	In mixture	In sample	Mixture- Sample	
20	47.35	28.17	19.18	95.89
20	50.51	31.58	18.93	94.67
20	51.26	31.29	19.97	99.83
Average				96.8
SD				2.70

Table 16 Recovery study of gallic acid by HPLC (3 replicates, n = 1, mean ± SD)

Amount of gallic acid added (µg)	Amount of gallic acid (µg)			Recovery (%)
	In mixture	In sample	Mixture- Sample	
50	73.26	24.88	48.38	96.76
50	72.54	25.09	47.45	94.91
50	74.13	25.91	48.22	96.43
Average				96.03
SD				0.99

Table 17 Recovery study of kaempferol by HPLC (3 replicates, n = 1, mean ± SD)

Amount of kaempferol added (µg)	Amount of kaempferol (µg)			Recovery (%)
	In mixture	In sample	Mixture- Sample	
100	103.87	0.94	102.93	102.93
100	102.60	1.73	100.87	100.87
100	104.28	1.28	103.00	103.00
Average				102.27
SD				1.21

Table 18 Recovery study of quercetin by HPLC (3 replicates, n = 1, mean ± SD)

Amount of quercetin added (µg)	**Amount of quercetin (µg)**			**Recovery (%)**
	In mixture	**In sample**	**Mixture- Sample**	
100	138.25	53.98	84.27	84.27
100	140.09	52.28	87.81	87.81
100	137.12	52.37	84.75	84.75
Average				85.61
SD				1.92

Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection and limit of quantitation of ascorbic acid, catechin, cyanidin 3-glucoside, gallic acid, kaempferol and quercetin were shown in Table 19.

Table 19 LOD and LOQ of each standard by HPLC (10 replicates, n = 1)

Compound	Limit of detection (µg/ml)	Limit of quantitation (µg/ml)
Ascorbic acid	3.2	10.0
Catechin	9	30
Cyanidin 3-glucoside	0.12	0.40
Gallic acid	1.5	5.0
Kaempferol	0.5	1.8
Quercetin	2.0	7.0