CHAPTER 6

Extraction and purification of isoflavone aglycones

6.1 Introduction

The solid-liquid extraction with solvent is a common method utilized in many industrial processes. This method often uses organic solvents such as methanol, ethanol and acetonitrile. In case of isoflavones, the results from many researches had shown that solvent type, solvent concentration and extract condition could affect isoflavones extraction. The optimum solvent for isoflavones extraction was polar solvents (Hutabarat *et al.*, 2001). The 58% acetonitrile was used to effectively extract isoflavones from soy meal and ground manokin soybeans at room temperature for 2h (Lee *et al.*, 2004; Lin and Giusti, 2005). Griffith and Collison (2001) had found that 80% acetonitrile could extracted isoflavones more efficient than 80% methanol. Rostagno *et al.* (2004) also compared different amount of ethanol and methanol mixtures in water and found that ethanol in water was the optimum extraction solvent. Nonetheless, Genovese and Lajolo (2001) had showed that the best solvent for isoflavones extraction was 80% methanol when compared with 70% methanol, 60% and 80% acetonitrile with HCl 0.1N and water. Thus, due the contradicting results, the suitable solvent for isoflavones extraction is still inconclusive and required further investigation.

Solid phase extraction using column chromatography is one of most widely used method for purifying functional materials; such as anthocyanins (Sandhu and Gu, 2013), rebaudioside A (Liu *et al.*, 2011), resveratrol (Xiong *et al.*, 2014), puerarin (Guo *et al.*, 2015) and flavonoids (Cao *et al.*, 2004; Fu *et al.*, 2005; Zhang *et al.*, 2007). The method uses adsorbent to adsorb compound which is desorbed with gradient of solvent elution resulting in separation of the compound. Likewise, isoflavone aglycones can be purified using the method. Adsorbents for separate isoflavones are resins including Diaion HP-20,

Amberlite XAD-2, Amberlite XAD-4 and Amberlite XAD-16HP resins (Chang *et al.*, 2004; Cho *et al.*, 2009; Sevillano *et al.*, 2014; Wu and Lai, 2007)

Hence, the objective of this chapter was to find the optimum condition to extract and purify isoflavone aglycones from fermented soygerm.

6.2 Methods

6.2.1 Fermented soygerm and inoculum preparation

Soygerm from soybean variety SJ2 was used to prepare fermented soygerm. Soygerm was mixed with water (1:2 w/w) and autoclaved at 121°C for 15 min. Added with 15% (v/w) of *Bacillus coagulans* PR03 and fermented at 30-35°C for 96 h with 6 L/min air feeding. Inoculum of *B. coagulans* PR03 was prepared in nutrient broth (NB) by transferring cells of *B. coagulans* PR03 to 100 ml NB and incubating at 120 rpm for 18 h before used.

6.2.2 The optimum solvent for isoflavone aglycones extraction

The fermented soygerm was extracted with various solvents and concentrations; ethanol, methanol, acetonitrile and acetone. The varied concentrations of each solvent were 40%, 60%, 80% and 100%.

Twenty grams of fermented soygerm were mixed with 100 ml of solvent and homogenized in homogenizer for 5 min. Mixed solution was swirled at 200 rpm for 2 h and centrifuged at 5,000 rpm for 5 min. The solvent was evaporated from extracted solution by rotary evaporator. The crude extract was filtered with 0.45 µm nylon filter and analyzed for isoflavone aglycones (see Appendix A). The optimal extract solution was selected based on isoflavone aglycones content.

6.2.3 The optimum extraction process for isoflavone aglycones

The extraction machine with 46 L capacity was used in this study. This machine was constructed and developed by Traditional Food Research and Development Unit, Science and Technology Research Institute, Chiang Mai University, Chiang Mai, Thailand. The accessories involved in this study were rotor blade and inverter controller. The configuration of extraction machine is shown in Figure 6.1. The extraction speed (rpm) was controlled by inverter frequency (Hz) and can be calculated back to extraction speed by the following equation.

$$s = [f \times 121] \div 2$$

When s = extraction speed (rpm) and f = inverter frequency (Hz)

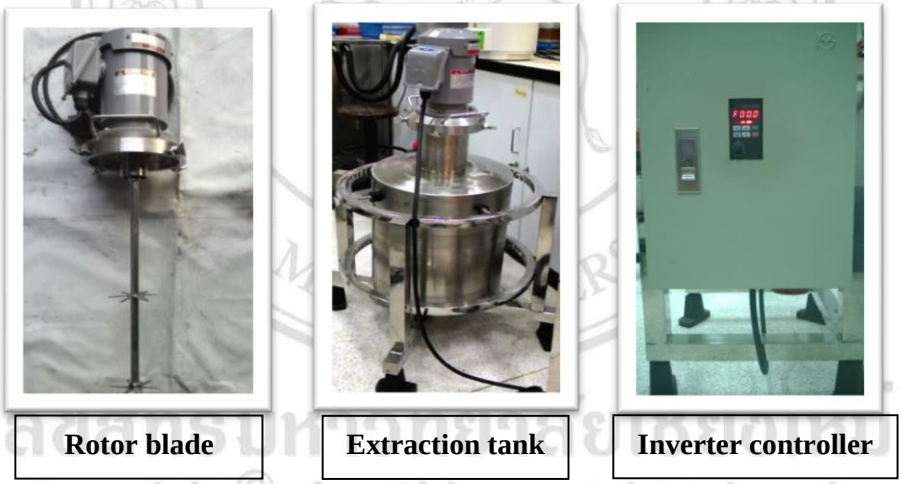

Figure 6.1 Configuration of 46 L extraction machine

The optimum inverter frequency and extraction time was studied using 2^2 factorial experiments in central composite design. Inverter frequency of extraction machine was varied in the range of 45-75 Hz and extraction time was varied in the range of 11-139 min. The experimental design is shown in Table 6.1.

Five kilograms of fermented soygerm was mixed with 25 L of optimal extract solution (from section 6.2.1) and extracted with extraction machine. After that, mixed solution was filtered with filter cloth and filter paper (Whatman® No.1) until a clear filtrate was obtained. The solvent was evaporated from extracted solution by rotary evaporator. The crude extract was filtered again with 0.45 μm nylon filter and analyzed for isoflavone aglycones (see appendix A). Raw data were analyzed by Design-Expert©. The inverter frequency and extraction time was optimized.

Table 6.1 2^2 Factorial experiments with central composite design

Treatments	**Code**	**Frequency of inverter (Hertz)**	**Time(minute)**
1	(1)	-1	-1
2	a	1	-1
3	b	-1	1
4	ab	1	1
5	-α a	-1.414	0
6	±α a	1.414	0
7	-α b	0	-1.414
8	±α b	0	1.414
9	cp1	0	0
10	cp2	0	0
Level of code	-1.414	45.86	11.36
	-1	50.00	30.00
	0	60.00	75.00
	1	70.00	120.00
	1.414	74.14	138.64

6.2.4 The optimum resin for isoflavone aglycones purification

The extract solution (from section 6.2.3) was removed ethanol by rotary evaporator. The crude extract was purified with different resins using column chromatography. The resins included Diaion HP-20, Amberlite XAD-4, Amberlite XAD-7HP and AmberliteXAD-16HP. The resin was prepared before studying by soaking 50 grams of each resin in methanol for 24 h to eliminated impure substance. After that, the resin was packed in glass column (Ø 2.8 cm) and washed with 1 L of deionized water.

The process of purification was modified method of Wu and Lai (2007) that divided into 6 steps (all steps using a flow rate of 1.5 ml/min).

Step 1: crude extract was mixed with 80% ethanol and deionized water in ratio of 1: 2: 4 (w/v/v). Mixed solution was purified by passing through column which isoflavones were absorbed by the resin. The resulting eluent was collected and called fraction 1.

Step 2: column was washed with 400 ml of deionized water to eliminate dissolved solid in water exterior of the resin. The resulting eluent was called fraction 2.

Step 3: column was washed with 20% ethanol and the eluent was collected; fraction 3.

Step 4: column was washed with 40% ethanol and the solution that passed through the column was collected and called fraction 4.

Step 5: column was washed again with 60% ethanol. The resulting eluent; fraction 5, was collected.

Step 6: column was washed with 80% ethanol and the final eluent (fraction 6) was collected.

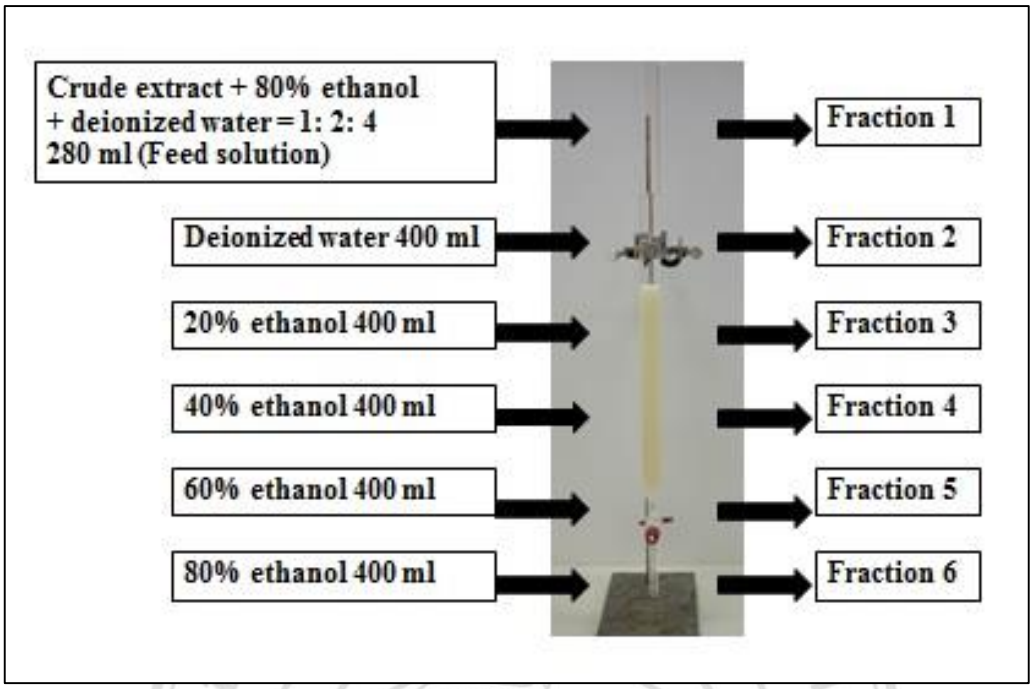

Figure 6.2 The process of isoflavone aglycones purification.

All six fractions were analyzed isoflavone aglycones (see appendix A) and solid mass by drying at 105°C. The column performance indices, including yield and purity of isoflavone aglycones, were calculated. Finally, suitable resin for isoflavone aglycones purification was selected.

6.3 Results and discussion

6.3.1 Optimum extracts solutions for isoflavone aglycones extraction

The results in Table 6.2 demonstrate that different extracting solvent significantly affects daidzein, genistein, glycitein and total aglycones content ($P<0.05$). The extraction ability depended on concentration and type of the solvent. Increasing solvent concentration resulted in increasing efficiency of isoflavone aglycones extraction. Ethanol, methanol, acetonitrile and acetone either at 80% or 100% showed the highest total aglycones contents which were 253.42, 257.61, 264.92 and 265.75 mg/100 g of fermented soygerm, respectively. Acetonitrile and acetone were the superior solvent for extracting isoflavone aglycones from fermented soygerm. Methanol was less efficient isoflavone aglycones extraction solvent and ethanol was the least efficient solvent for the extraction. The optimum solvent for isoflavone extraction was relevant to the study reported by Murphy *et al.* (2002) which stated that acetonitrile was superior solvent than acetone, ethanol and methanol in soy isoflavone extraction from soy foods. However, the objective of this research was to produce isoflavone aglycones as a food supplement. So, the extraction solvent should be non-toxic and easily removed. As a result, ethanol was the optimum extraction solvent in this study. In general, ethanol is considered as a good solvent for extracting bioactive compound in food and pharmaceutical industry. Moreover, ethanol was effectively used to extract isoflavones from various sources such as soybean, soy foods, defatted soybean flakes, soybean sprout cotyledon and okara (by-product of soymilk and tofu production) (Achouri *et al.*, 2005; Chang and Chang, 2007; Cho *et al.*, 2009; Jankowiak *et al.*, 2014; Rostagno *et al.*, 2009). Nonetheless, since isoflavone aglycone contents extracted from 80% and 100% ethanol were not statistically different ($P>0.05$), to reduce the usage of solvent, 80% ethanol was used as extracting solvent for isoflavone aglycones from soygerm.

Table 6.2 Isoflavone contents in crude extracts from each solvent at various concentrations

Treatment	Isoflavone aglycones (mg/100 g of FSG)			
	Diadzein	**Genistein**	**Glycitein**	**Total aglycones**
FSG	251.80±4.40^{a}	45.70±0.80^{a}	74.42±1.30^{a}	371.92±6.49^{a}
EtOH 100%	171.99±1.89^{b}	30.94±0.39^{b}	50.49±0.70^{b}	253.42±2.99^{b}
EtOH 80%	169.85±3.34^{bc}	30.50±0.69^{b}	49.24±0.96^{bc}	249.59±4.99^{bc}
EtOH 60%	163.02±1.42^{c}	28.98±0.09^{c}	47.70±0.34^{c}	239.71±3.88^{c}
EtOH 40%	139.94±4.40^{d}	22.01±0.80^{d}	41.73±1.30^{d}	203.68±6.40^{d}
MeOH 100%	174.56±0.78^{b}	31.42±0.47^{b}	51.63±0.54^{b}	257.61±0.74^{b}
MeOH 80%	174.65±0.01^{b}	31.57±0.21^{b}	50.05±0.24^{b}	256.27±0.69^{b}
MeOH 60%	164.29±0.98^{c}	28.71±0.11^{bc}	46.92±0.84^{c}	239.92±0.27^{c}
MeOH 40%	139.80±0.58^{d}	22.10±0.68^{c}	42.01±0.46^{d}	203.91±0.85^{d}
ACN 100%	176.90±0.13^{b}	30.47±0.15^{b}	54.64±0.47^{b}	262.01±1.12^{b}
ACN 80%	177.52±0.45^{b}	32.23±0.23^{b}	55.17±0.54^{b}	264.92±1.15^{b}
ACN60%	164.53±1.12^{c}	28.46±1.23^{c}	47.08±0.17^{d}	240.08±1.18^{c}
ACN 40%	140.51±0.95^{d}	24.51±1.12^{d}	44.71±0.14^{d}	209.73±1.16^{d}
ACT 100%	175.19±1.48^{b}	30.04±1.45^{b}	53.20±0.19^{b}	258.43±0.15^{c}
ACT 80%	178.97±0.32^{b}	34.25±0.24^{b}	52.54±1.75^{b}	265.75±0.24^{b}
ACT 60%	166.25±0.79^{c}	27.97±0.69^{c}	47.20±1.46^{c}	241.42±1.65^{d}
ACT 40%	142.51±1.24^{d}	25.19±0.79^{c}	47.31±0.33^{c}	215.01±2.36^{e}

Note: Means within same column and solvent with different superscripts are significantly different ($P \leq 0.05$) (compared with FSG); FSG = fermented soygerm; EtOH = ethanol; MeOH = methanol; ACN = Acetonitrile; ACT = acetone

6.3.2 Optimum extraction process for isoflavone aglycones

The optimum inverter frequency and extraction time was studied. The fermented soygerm was extracted with 80% ethanol. Inverter frequency of extraction machine was varied in the range of 45-75 Hz and extraction time was varied in the range of 11-139 min. The results are shown in Table 6.3.

Table 6.3 Effect of inverter frequency and extraction time on isoflavone aglycones extraction

Treatment	Frequency of inverter (Hertz)	Time (min)	Isoflavone aglycones (mg/100g of fermented soygerm)			
			Daidzein	Genistein	Glycitein	Total Aglycones
1	50.00	30.00	99.53±0.12	18.06±0.81	69.27±0.19	186.85±0.73
2	70.00	30.00	115.06±0.22	22.56±0.87	80.06±0.15	217.68±0.50
3	50.00	120.00	121.14±0.21	24.72±0.14	83.68±0.14	229.54±0.48
4	70.00	120.00	146.81±0.06	26.06±0.46	101.32±.012	274.18±0.64
5	45.86	75.00	104.41±0.03	20.02±0.60	72.17±0.07	196.59±0.50
6	74.14	75.00	143.83±0.13	28.20±0.22	100.07±0.07	272.10±0.28
7	60.00	11.36	83.72±0.02	15.23±0.04	58.26±0.03	157.20±0.09
8	60.00	138.64	155.43±0.02	31.17±0.08	106.85±0.02	293.45±0.08
9	60.00	75.00	155.98±0.12	28.12±0.81	107.17±0.19	291.27±1.89
10	60.00	75.00	142.18±0.22	25.80±0.87	98.95±0.15	266.94±1.24

From Table 6.3, it was found that inverter frequency and extraction time were significantly affected daidzein, glycitein and total aglycones (P<0.05). The stepwise regression models were obtained as the following:

Dein $= -457.92 + 16.55(F) + 1.55(T) - 0.13(F)^2 - 7.43 \times 10^{-3} (T)^2$ $R^2 = 0.9252$

Glein $= -310.45 + 11.24(F) + 1.06(T) - 0.09(F)^2 - 5.16 \times 10^{-3} (T)^2$ $R^2 = 0.9274$

Tagy $= -825.60 + 29.96(F) + 2.86(T) - 0.23(F)^2 - 1.36 \times 10^{-2} (T)^2$ $R^2 = 0.9234$

When Dein = dainzein; Glein = glycitein; Tagy = total aglycones
F = frequency of inverter (Hz); T = extraction time (min)

The stepwise regression models show that increasing inverter frequency and extraction time positively affect daidzein, glycitein and total aglycones at P<0.05. The respond surface plot of relationship between inverter frequency and extraction time of daidzein, glycitein and total aglycones are shown in Figure 6.3, 6.4 and 6.5.

The extraction process was optimized using numerical method by Design-Expert© (7.1.0). The optimization criteria are shown in Table 6.4. Consequently, the optimum inverter frequency and extraction time were 65.90 Hz (equal to 3,986.95 rpm) and 90.31 min. The resulting daidzein, glycitein and total aglycones were predicted as 156.62, 108.29 and 293.73 mg/100 g fermented soygerm (Figure 6.6), respectively. Moreover, the overlay plot of optimum point by graphical method is shown in Figure 6.7.

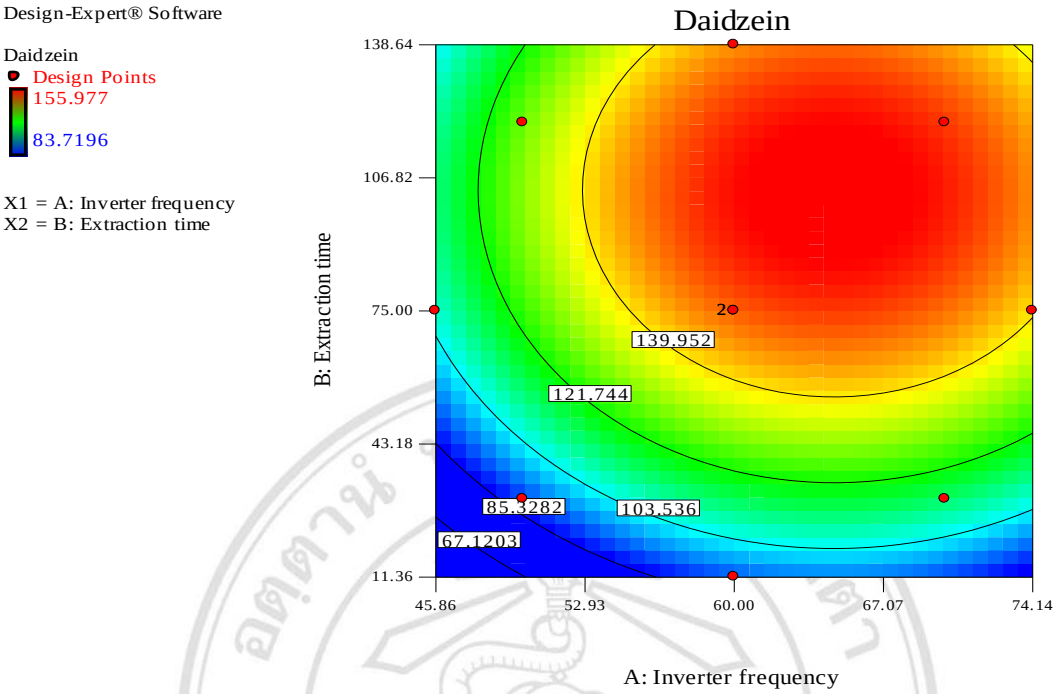

Figure 6.3 Respond surface plot of daidzein.

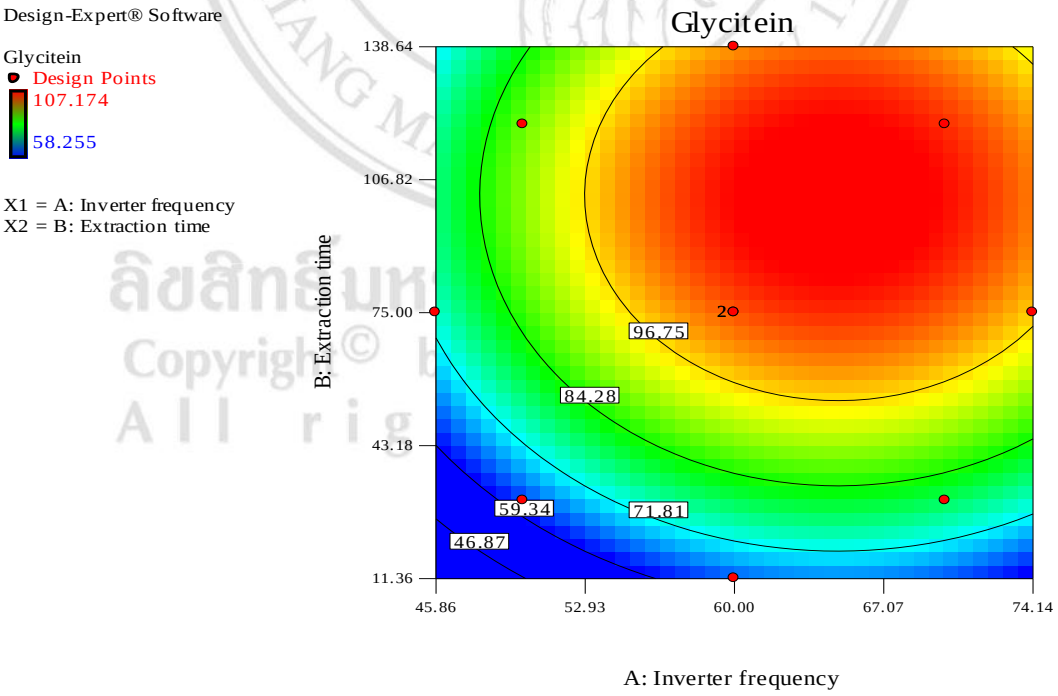

Figure 6.4 Respond surface plot of glycitein.

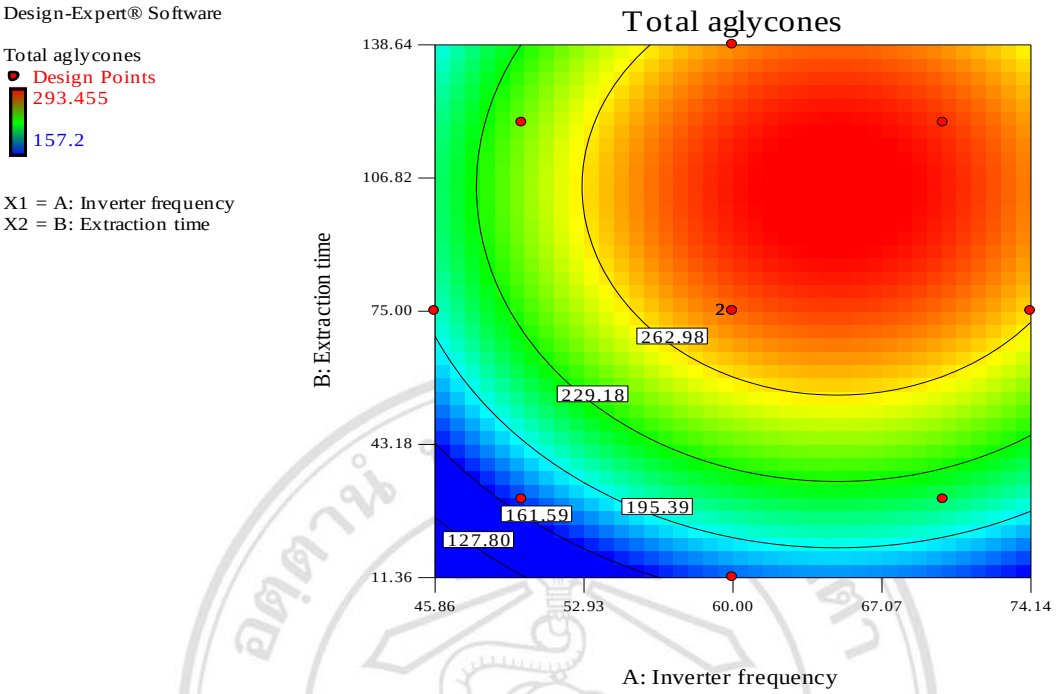

Figure 6.5 Respond surface plot of total aglycones.

Table 6.4 The criteria for optimization by Design-Expert© (version 7.1.0)

Variable	Criteria	Lower limit	Upper limit	Unit
Inverter frequency	is in range	45.86	74.14	Hertz
Extraction time	is in range	11.36	138.64	minute
Daidzein	maximum	-	155.98	mg/100g of fermented soygerm
Glycitein	maximum	-	107.17	mg/100g of fermented soygerm
Total aglycones	maximum	-	293.45	mg/100g of fermented soygerm

Criteria | Solutions | Graphs

Solutions 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Solutions

Number	Inverter freque	Extraction time	Daidzein	Glycitein	Total aglycones	Desirability	
1	66.80	108.73	157.453	108.74	295.78	1.000	
2	64.79	104.96	158.158	109.20	296.79	1.000	
3	63.14	99.14	157.669	108.87	295.62	1.000	
4	65.99	105.87	157.937	109.08	296.52	1.000	
5	66.39	106.76	157.756	108.96	296.25	1.000	
6	62.92	108.97	157.552	108.70	295.61	1.000	
7	61.48	103.04	156.805	108.21	294.00	1.000	
8	67.36	107.76	157.177	108.58	295.31	1.000	
9	67.05	107.88	157.365	108.70	295.63	1.000	
10	65.59	105.53	158.054	109.15	296.69	1.000	
11	61.61	100.84	156.841	108.25	294.02	1.000	
12	66.87	100.35	157.49	108.84	295.66	1.000	
13	67.43	106.27	157.2	108.61	295.33	1.000	
14	65.62	109.09	157.869	108.99	296.43	1.000	
15	65.90	90.31	156.617	108.29	293.73	1.000	Selected
16	66.65	109.72	157.448	108.73	295.78	1.000	

Figure 6.6 The optimum point of inverter frequency and extraction time.

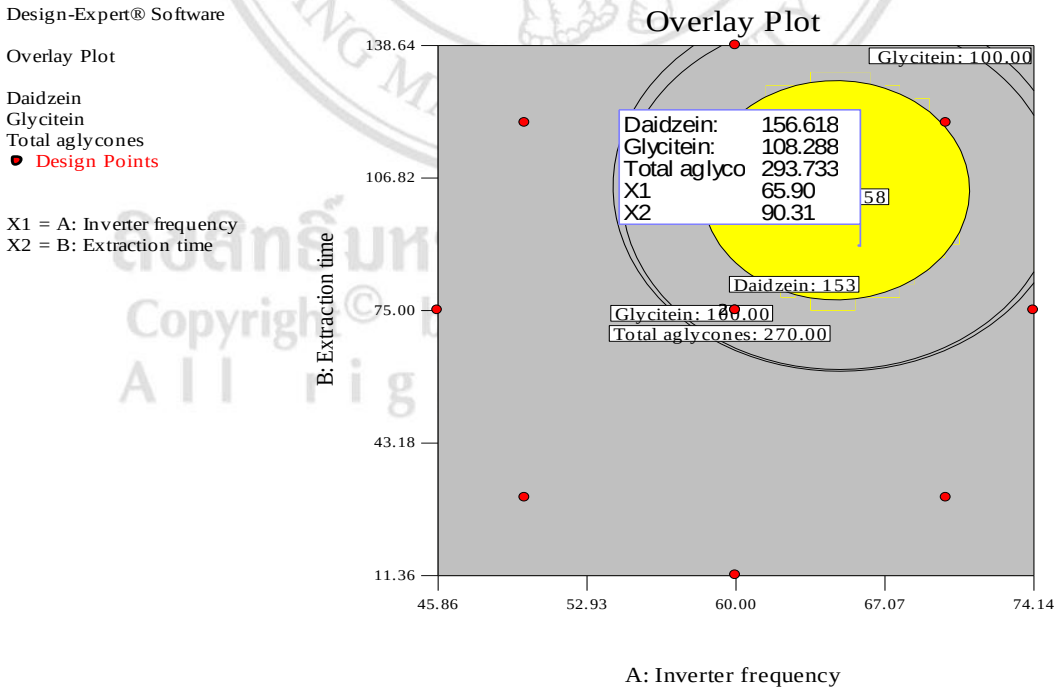

Figure 6.7 The overlay plot of optimum point.

6.3.3 Optimum resin for isoflavone aglycones purification

The optimum resin for isoflavone aglycones purification was studied. The fermented soygerm was extracted in extraction machine with 80% ethanol at 66 Hz inverter frequency for 90 min. After that, the ethanol was evaporated from extracted solution by rotary evaporator. The crude extract was purified by solid phase extraction with adsorbtion column chromatography with different resins; Diaion HP-20, Amberlite XAD-4, Amberlite XAD-7HP and Amberlite XAD-16HP. The purification results are shown in Table 6.5-6.8. It was found that, during adsorption and washing step, the losses of isoflavone aglycones by Diaion HP-20, Amberlite XAD-4, Amberlite XAD-7HP and Amberlite XAD-16HP columns were 2.81, 4.06, 4.28 and 1.82%, respectively. In other word, less than 5% of isoflavone aglyecones were lost when 280 ml of feed volume was loaded for all resins. Since 72.86, 81.02, 77.17 and 73.30% of the total mass of concentrates were removed without significant loss in isoflavone aglycones in the adsorption and washing step by Diaion HP-20, Amberlite XAD-4, Amberlite XAD-7HP and Amberlite XAD-16HP columns, respectively, the active ingredient contents were further isolated by an efficient separation from other impurities in the gradient elution step. In the gradient elution step, all the effluent fractions were collected. The sums of concentrates were 16,802, 16,289, 15,951 and 16,444 mg and the sums of isoflavone aglycones were 1,748.34, 1,706.98, 1,684.36 and 1,661.52 mg for the Diaion HP-20, Amberlite XAD-4, Amberlite XAD-7HP and Amberlite XAD-16HP column, respectively.

Table 6.5 Column performances of the Diaion HP-20 column

Step of column operation Diaion HP-20	Fraction	Operating volume (ml)	Mass of concentrate(mg)	Mass ratio of concentrate (%)	Mass of aglycones (mg)	Yield of aglycones (%)	Purity of aglycones (%)
Adsorption	1	280.00	10,680.00	63.56	44.72	2.56	0.42
Washing (DI water)	2	400.00	1,562.00	9.30	4.44	0.25	0.28
Gradient elution step							
20% Ethanol	3	400.00	237.00	1.41	3.96	0.23	1.67
40% Ethanol	4	400.00	1,088.00	6.48	542.90	31.05	49.90
60% Ethanol	5	400.00	1,981.00	11.79	1030.20	58.92	52.00
80% Ethanol	6	400.00	1,254.00	7.46	122.12	6.98	9.74
Total			16,802.00	100.00	1,748.34	100.00	10.41

Note: Crude extract: mass of concentrate = 418.48 mg/ml; mass of aglycones = 43.05 mg/ml; purity of aglycones = 10.29%.

Adsorption = feed of isoflavone aglycones solution (crude extract of fermented soygerm : 80% ethanol : DI water = 1:2:4);

Operating volume (ml) = fractional volume of solution for eluted column;

Mass of concentrate (mg) = solid mass in each fraction; Mass ratio of concentrate (%) = (fractional mass of concentrate; mg) / total mass of concentrate) x 100;

Mass of aglycones (mg) = total aglycone content in each fraction; Yield of aglycones (%) = (fractional mass of aglycones; mg) / total mass of aglycones) x 100;

Purity of aglycones (%) = (fractional mass of aglycones; mg / fractional mass of concentrate; mg) x 100.

Table 6.6 Column performances of the Amberlite XAD-4 column

Step of column operation XAD-4	Fraction	Operating volume(ml)	Mass of concentrate (mg)	Mass ratio of concentrate (%)	Mass of aglycones (mg)	Yield of aglycones (%)	Purity of aglycones (%)
Adsorption	1	280.00	12,130.00	74.47	63.22	3.70	0.52
Washing (DI water)	2	400.00	1,068.00	6.56	6.12	0.36	0.57
Gradient elution step							
20% Ethanol	3	400.00	161.00	0.99	4.82	0.28	2.99
40% Ethanol	4	400.00	702.00	4.31	467.60	27.39	66.61
60% Ethanol	5	400.00	1,088.00	6.68	819.74	48.02	75.34
80% Ethanol	6	400.00	1,140.00	7.00	345.48	20.24	30.31
Total			16,289.00	100.00	1,706.98	100.00	10.48

Note: Crude extract: mass of concentrate = 418.48 mg/ml; mass of aglycones = 43.05 mg/ml; purity of aglycones = 10.29%.

Adsorption = feed of isoflavone aglycones solution (crude extract of fermented soygerm : 80% ethanol : DI water = 1:2:4);

Operating volume (ml) = fractional volume of solution for eluted column;

Mass of concentrate (mg) = solid mass in each fraction; Mass ratio of concentrate (%) = (fractional mass of concentrate; mg) /total mass of concentrate) x 100;

Mass of aglycones (mg) = total aglycone content in each fraction; Yield of aglycones (%) = (fractional mass of aglycones; mg) / total mass of aglycones) x 100;

Purity of aglycones (%) = (fractional mass of aglycones; mg / fractional mass of concentrate; mg) x 100.

Table 6.7 Column performances of the Amberlite XAD-7HP column

Step of column operation XAD-7HP	Fraction	Operating volume (ml)	Mass of concentrate (mg)	Mass ratio of concentrate (%)	Mass of aglycones (mg)	Yield of aglycones (%)	Purity of aglycones (%)
Adsorption	1	280.00	11,160.00	69.96	67.60	4.01	0.61
Washing (DI water)	2	400.00	1,149.00	7.20	4.56	0.27	0.40
Gradient elution step							
20% Ethanol	3	400.00	181.00	1.13	11.86	0.70	6.55
40% Ethanol	4	400.00	774.00	4.85	481.74	28.60	62.24
60% Ethanol	5	400.00	1,515.00	9.50	892.82	53.01	58.93
80% Ethanol	6	400.00	1,172.00	7.35	225.78	13.40	19.26
Total			15,951.00	100.00	1,684.36	100.00	10.56

Note: Crude extract: mass of concentrate = 418.48 mg/ml; mass of aglycones = 43.05 mg/ml; purity of aglycones = 10.29%.

Adsorption = feed of isoflavone aglycones solution (crude extract of fermented soygerm : 80% ethanol : DI water = 1:2:4);

Operating volume (ml) = fractional volume of solution for eluted column;

Mass of concentrate (mg) = solid mass in each fraction; Mass ratio of concentrate (%) = (fractional mass of concentrate; mg) /total mass of concentrate) x 100;

Mass of aglycones (mg) = total aglycone content in each fraction; Yield of aglycones (%) = (fractional mass of aglycones; mg) / total mass of aglycones) x 100;

Purity of aglycones (%) = (fractional mass of aglycones; mg / fractional mass of concentrate; mg) x 100.

Table 6.8 Column performances of the Amberlite XAD-16HP column

Step of column operation XAD-16HP	Fraction	Operating volume(ml)	Mass of concentrate (mg)	Mass ratio of concentrate (%)	Mass of aglycones (mg)	Yield of aglycones (%)	Purity of aglycones (%)
Adsorption	1	280.00	10,660.00	64.83	26.64	1.60	0.25
Washing (DI water)	2	400.00	1394.00	8.48	3.68	0.22	0.26
Gradient elution step							
20% Ethanol	3	400.00	157.00	0.95	3.58	0.22	2.28
40% Ethanol	4	400.00	896.00	5.45	527.12	31.73	58.83
60% Ethanol	5	400.00	1,884.00	11.46	802.30	48.29	42.58
80% Ethanol	6	400.00	1,453.00	8.84	298.20	17.95	20.52
Total			16,444.00	100.00	1,661.52	100.00	10.10

Note: Crude extract: mass of concentrate = 418.48 mg/ml; mass of aglycones = 43.05 mg/ml; purity of aglycones = 10.29%.

Adsorption = feed of isoflavone aglycones solution (crude extract of fermented soygerm : 80% ethanol : DI water = 1:2:4);

Operating volume (ml) = fractional volume of solution for eluted column;

Mass of concentrate (mg) = solid mass in each fraction; Mass ratio of concentrate (%) = (fractional mass of concentrate; mg) /total mass of concentrate) x 100;

Mass of aglycones (mg) = total aglycone content in each fraction; Yield of aglycones (%) = (fractional mass of aglycones; mg) / total mass of aglycones) x 100;

Purity of aglycones (%) = (fractional mass of aglycones; mg / fractional mass of concentrate; mg) x 100.

Based on isoflavone aglycones yield and purity, the fractions with 40% and 60% ethanol elution were chosen and used as the purified product for each of the four columns. The yield and purity of isoflavone aglycones in each column were calculated as column performance and summarized in Table 6.9.

Table 6.9 The yield and purity of isoflavone in 40-60% ethanol elutions

Type of column resin	Yield of aglycones (%)	Purity of aglycones (%)	Column performance (%)
Diaion HP-20	89.98	51.26	56.97
Amberlite XAD-4	75.42	71.92	95.36
Amberlite XAD-7HP	81.61	60.05	73.58
Amberlite XAD-16HP	80.01	47.82	59.77

Note: column performance (%) = (purity of aglycones / yield of aglycones) x 100

The results in Table 6.9 shows that the highest yields of isoflavone aglycones was achieved with Diaion HP-20 column (89.98%) while Amberlite XAD-4 column resulted in the lowest yield (75.42%). The hightest purity of isoflavone aglycones was achieved by using Amberlite XAD-4 column (71.92%) while Amberlite XAD-16HD column resulted in the lowest purity (47.82%). However, eluents from all resins had purity of isoflavone aglycones more than the crude extract, which was 10.29%. In comparison, Amberlite XAD-4 column had the best performance with column performance of 95.36% while Diaion HP-20 performed the least with only 56.97% performance score. As a result, Amberlite XAD-4 was optimum absorbent. The superior performance of Amberlite XAD-4 might come from its structure; macroporous styrene-divinylbenzenecopolymer, which is the best resin for eliminated phenolic compounds from water soluble substances (Li *et al.*, 2001). This finding corresponded with the report from Wu and Lai (2007), which concluded that isoflavones from defatted soy flakes were best purified by Amberlite XAD-4 column. They found that purity and yield of isoflavones in purified solution were approximately 58% and 89%, respectively.

Isoflavone aglycones from our research had higher purity because fermented soygerm were used to produce crude extract resulting in high purity isoflavones. Therefore, Amberlite XAD-4 was optimum resin for isofalvone aglycones purification.

6.5 Conclusion

The optimum solvent for isoflavone aglycones extraction by solid-liquid extraction method was 80% ethanol. For the extraction process of isoflavone aglycones with extraction machine, the optimum inverter frequency and extraction time were 65.90 Hz (equal to 3,986.95 rpm) and 90.31 min, respectively. The resulting crude extract contained total isoflavone aglycones in an amount 293.73 mg/100 g fermented soygerm, respectively.

For the method of isoflavone aglycones purification by solid-liquid phase column chromatography, the majority of isoflavone aglycones in all resins were found in the portions eluted by 40% and 60% ethanol. The yield and purity of isoflavone aglycones were 89.98% and 51.26% for the Diaion HP-20 column, 75.42% and 71.92% for the Amberlite XAD-4 column, 81.61% and 60.05% for Amberlite XAD-7HP column and 80.01% and 47.82% for Amberlite XAD-16HP columns. In comparison, the order of isoflavone aglycones yield was Diaion HP-20 column > Amberlite XAD-7HP column > Amberlite XAD-16HP column > Amberlite XAD-4 columns. The order of isoflavone aglycones purity was Amberlite XAD-4 column > Amberlite XAD-7HP column > Diaion HP-20 > Amberlite XAD-16HP column. And, the order of column performance was Amberlite XAD-4 column > Amberlite XAD-7HP column > Amberlite XAD-16HP column > Diaion HP-20. The Amberlite XAD-4 column achieved the highest purity and column performance. Therefore, Amberlite XAD-4 was optimum resin for isoflavone aglycones purification.