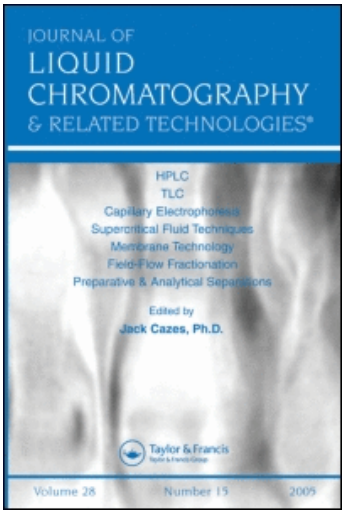

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Preparative Isolation of Isoflavones from Defatted Soy Flakes

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Abstract: Defatted soy flakes (DSF) were used as the starting material for the preparative isolation of isoflavones in this study. First, the crude DSF extracts were prepared using an extractor with solvent refluxing, operated under optimal extraction conditions (using 500 mL 80% ethanol aqueous for 3 h per 100 g DSF). The extraction yield was about 10% and the purity of isoflavones was about 2.0–2.5 wt%. Before the isolation operations, the extracts were dissolved in deionized water. The isolation procedures included the method of liquid–liquid extraction and the method of column chromatography. For the method of liquid–liquid extraction using a mixed solvent of 10% n-butanol and 90% ethyl acetate operated under the optimal extraction conditions, the purity and yield of isoflavones were ~35% and ~70%. For the method of column chromatography, XAD-7HP and XAD-4 adsorbents with different polarities were used as the packing materials. For the XAD-4 column, a part of non-polar impurities was efficiently separated with the majority of isoflavones by a proper step gradient elution, which resulted in an efficient isolation: the purity and yield of isoflavones were ~58% and ~89%. In comparison, the method of column chromatography using XAD-4 adsorbents achieved both the highest purity and yield, and was found to be the best isolation method in the current isolation stage.

Keywords: Defatted soy flakes, Isoflavones, Preparative isolation, Liquid–liquid extraction, Column chromatography

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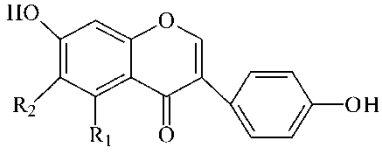
INTRODUCTION

Isoflavones are considered to be important fractions in soybeans. They possess the functions of preventing cancer and osteoporosis, and reducing the cholesterol levels in the human body, so they can be used as nutrition foods and medicines. However, isoflavones are found in very low abundance (0.1–0.3%) in soy foods. Isoflavones can be categorized into two groups: glucosides and aglycones. Approximately 97 to 98% of isoflavones are glucosides, but aglycones may have more biological activity than their respective glucosides. Figure 1(a) shows the chemical structures of the three isoflavone-aglycones including Daidzein (De), Genistein (Ge), and Glycitein (Glye). Figure 1(b) shows the chemical structures of the nine isoflavone-glucosides including Daidzin (D), Genistin (G), Glycitin (Gly), 6″ -O- acetyldaidzin, 6″ -O- acetylgenistin, 6″ -O- acetylglycitin, 6″ -O- malonyldaidzin, 6″ -O- malonylgenistin, and 6″ -O- malonylglycitin.[1–6]

Current procedures for the isolation and purification of the various isoflavones mainly include liquid–liquid extraction and column chromatography. In the method of liquid–liquid extraction, the organic solvents used include ethyl acetate, n-butanol, isobutanol, and isoamyl alcohol;[1,4,7,8] in the method of column chromatography, the adsorbents used include polystyrene-divinylbenzene, such as Amberlite XAD-4, XAD-1180, and XAD-16HP resins,[4,5,9,10] polylmethacrylate, such as TossHaas CG-71 resins,[11] acrylic ester, such as Amberlite XAD-7HP resins,[9] cation exchangers, such as DOWEX MSC-1 resins,[12] and anion exchangers, such as Amberlite IRA 410 resins[13] with either non-polar, intermediate polar, or polar adsorption characteristics. However, most of the processes involve multiple steps and various solvents, which are tedious, time consuming, and not easily scaled up. Therefore, there exists a need for a process, which is efficient, relatively inexpensive, and easy to scale-up into industrialized usage.

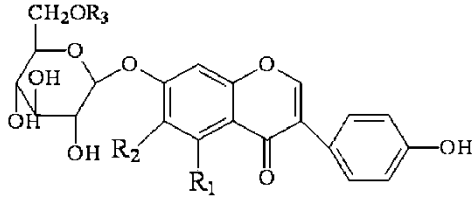
Defatted soy flakes (DSF), the by-product from the production of soybean oil, were used as the starting material for the production of isoflavones, from which their added values can be increased. This study is aimed at developing an efficient and economic technique for the preparative isolation of isoflavones with high purity, high yield, and high production rate. The entire isolation procedure for isoflavones from defatted soy flakes is represented in the flow diagram depicted in Figure 2. The isolation procedures investigated in this study included the method of liquid–liquid extraction and the method of column chromatography. In the method of liquid–liquid extraction, ethyl acetate and n-butanol were chosen as the organic solvents and the effects of mixed solvent composition, volume ratio of aqueous phase to organic phase, temperature, and duration time on the extraction performance were investigated. In the method of column chromatography, two different packing materials, i.e., XAD-7HP and XAD-4 adsorbents, with different polarities were used. The column operation included the adsorption, washing, elution, and regeneration steps in sequence. The effects of the

(a) Isoflavone-aglycones



Compounds	Molecular formula	R_1	R_2	Molecular weight
Daidzein (De)	$C_{15}H_{10}O_4$	H	H	254
Genistein (Ge)	$C_{15}H_{10}O_5$	OH	H	270
Glycitein (Glye)	$C_{16}H_{12}O_5$	H	OCH_3	284

(b) Isoflavone-glucosides



Compounds	Molecular formula	R_1	R_2	R_3	Molecular weight
Daidzin (D)	$C_{21}H_{20}O_9$	H	H	H	416
Genistin (G)	$C_{21}H_{20}O_{10}$	OH	H	H	458
Glycitin (Gly)	$C_{21}H_{22}O_{10}$	H	OCH_3	H	446
6″-O-Acetyldaidzin	$C_{23}H_{22}O_{10}$	H	H	$COCH_3$	458
6″-O-Acetylgenistin	$C_{23}H_{22}O_{11}$	OH	H	$COCH_3$	474
6″-O-Acetylglycitin	$C_{23}H_{24}O_{11}$	H	OCH_3	$COCH_3$	476
6″-O-Malonyldaidzin	$C_{24}H_{23}O_{12}$	H	H	$COCH_2COOH$	502
6″-O-Malonylgenistin	$C_{24}H_{22}O_{13}$	OH	H	$COCH_2COOH$	518
6″-O-Malonylglycitin	$C_{24}H_{24}O_{13}$	H	OCH_3	$COCH_2COOH$	520

Figure 1. Chemical structures of isoflavones: (a) isoflavone-aglycones (b) isoflavone-glucosides.

packing material and corresponding column operating conditions on the column performance were investigated. Finally, the isolation performances, in terms of purity, concentration ratio, and yield of isoflavones between these two methods were compared.

EXPERIMENTAL

Materials

The defatted soy flakes (DSF) were obtained from Central Union Oil Company, (UCOC, Taichung, Taiwan). Isoflavones standards including

Figure 2. Flow diagram of the entire isolation procedure for isoflavones from defatted soy flakes.

isoflavone-aglycones daidzein and genistein (De and Ge, 98% purity) and fluorescein (F, 98% purity), used as an internal standard for HPLC, were purchased from Sigma (St. Louis, MO, USA) and isoflavone-glucosides daidzin, genistin, and glycitin (D, G, and Gly, 95% purity) were prepared in our laboratory.[14] Purified water was obtained using a Milli-Q purifier (Millipore, USA). Acetonitrile, ethanol, and n-butanol were bought from J. T. Baker (Pillipsburg, NJ, USA), methanol was from Mallinckrodt (St. Louis, Missouri, USA), ethyl acetate was from TEDIA (Fairfield, OH, USA), and acetic acid was from Merck (St. Frankfurter, Darmstadt, Germany). All the organic solvents used were of HPLC grade and were ultrasonically degassed before use.

Amberlite XAD-7HP polymeric adsorbents with the specific surface area of 450 m^2/g were from Supelco Inc. (USA), and XAD-4 polymeric adsorbents with the specific surface area of 725 m^2/g were from Rohm and Haas company (Philadelphia, PA, USA), each with the particle size of 250–840 μm (20-60 mesh). Based on the "like attracts like" principle,[15] XAD-7HP adsorbent, possessing intermediate polarity due to its both aliphatic and acrylic structure,[16] is effective for adsorbing non-polar compounds from polar solvents and polar compounds from non-polar solvents; XAD-4 adsorbents, possessing non-polarity due to their aromatic structure,[17] is effective for adsorbing non-polar compounds from polar solvents.

Preparation of the Crude Defatted Soy Flakes Extracts

A batch of 100 g DSF and 500 mL of 80% aqueous ethanol was placed in a 1000 mL conical flask, and the solution was refluxed on a hot plate for 3 h. The concentrated extracts of the first extract solution (Solution A) were then dissolved in a certain amount of deionized water to form the second extract solution (Solution B). Some water insoluble lipophilic components were precipitated and separated. Solution B was used as the aqueous phase solution for the liquid–liquid extraction and the feed solution for the adsorption separation using column chromatography.

The amount and mass fraction of isoflavones in the concentrates of Solution A and Solution B were analyzed by HPLC in the next section. The loss and concentration ratio of isoflavones of the extracts from Solution A (80% ethanol aqueous solution) to Solution B (deionized water) were calculated.

Quantitative Analysis of the Active Ingredients

High Performance Liquid Chromatography (HPLC) System

The high performance liquid chromatography system included a Jasco Model PU-980 solvent metering pump, a Jasco Model UV-970 UV detector (Tokyo,

Japan), a Rheodyne Model 7125 6-way syringe loading valve fitted with a 20 μL sample loop (Cotati, CA, USA), and a Sunway Model 940-CO column oven (Taipei, Taiwan). The HPLC column was Hypersil-100 C_{18}, 250 × 4.6 mm I.D., 5 μm (Hypersil, UK). All analyses were carried out at 30°C.

Quantitative Analysis of Isoflavones

In this study, the contents of D, G, Gly, De, and Ge were used as the quantitative indices of isoflavones. To analyze the isoflavones in the crude DSF extracts, the extracted solution (dissolved in methanol) was directly analyzed by the reversed-phase HPLC. Elution was carried out at a flow rate of 1.0 mL/min with the following gradient cycle: A = acetonitrile, B = acetic acid/water (1/99 v/v); 14% A (v/v) to 15% A over 10 min, 15% A to 20% A over 0.1 min, 20% A to 40% A over 50 min, 40% A to 14% A over 1 min, and 14% A for 5 min. The peaks were monitored with the UV detector at 262 nm.

The internal standard fluorescein (F) was employed to quantify the contents of D, G, Gly, De, and Ge.[18] Figure 3 shows the corresponding chromatogram of one particular example where a successful separation of D, G, Gly, De, Ge, and F was obtained. This gradient elution system was proven

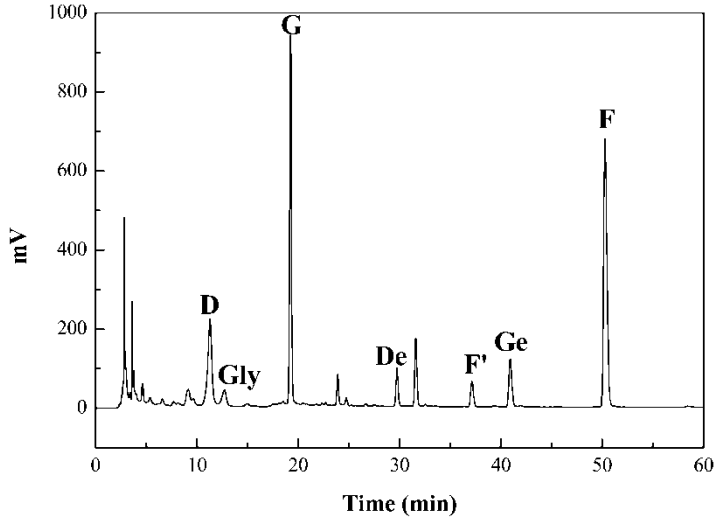


Figure 3. Typical HPLC analysis results of the crude DSF extracts. Column: Hypersil RP-C_{18} (250 mm × 4.6 mm i.d., 5 μm); injection volume: 20 μL; flow rate: 1 mL/min; monitor: UV detector with wave length of 262 nm; gradient elution mobile phase: acetonitrile/H_2O (1% acetic acid) = 14:86 (v/v) to 15:85 over 10 min, 15:85 to 20:80 over 0.1 min, 20:80 to 40:60 over 50 min, 40:60 to 14:86 over 1 min, and 14:86 for 5 min; concentration of internal standard (F): 0.6 mg/mL; oven temperature: 30°C.

workable, and accordingly adopted in the rest of this work. The purity of each of the five compounds in the sample was then determined by:

$$\text{Purity of compound j}(\%) = W_{in} \times (A_j/A_{in}) \times RR_j/W_{sam} \times 100\%$$

where W_{sam} is the weight of the sample, W_{in} and A_{in} is the weight and the peak area of the internal standard F, respectively, W_j and A_j is the weight and the peak area of compound j, respectively, and RR_j is the relative response of compound j, which can be obtained from the slope of the calibration curve, i.e., $RR_j = (W_j/W_{in})/(A_j/A_{in})$.

Isolation of Isoflavones Using Liquid–Liquid Extraction

Liquid–Liquid Extraction System

Solution B was used as the aqueous phase solution. The extraction was done by placing the mixture in a 250 mL conical flask and shaking it in a thermostat water bath. Upon extraction, the organic and aqueous phases were separated and collected by a 500 mL separatory funnel. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness under reduced pressure. The aqueous layer was dried overnight in a vacuum oven. The residues from both layers were dissolved in methanol and kept for HPLC analysis.

Evaluation of the Extraction Performance

The residues of the organic layer and the aqueous layer were analyzed by HPLC. The extraction performance indices of the organic layer, including purity, concentration ratio, and loss of isoflavones, are defined as follows:

Purity (%) = mass fraction of isoflavones in the organic layer

Concentration ratio = purity of isoflavones in the organic layer/purity of isoflavones in Solution B

Loss (%) = [1 − (mass of isoflavones in the organic layer/mass of isoflavones in Solution B)] × 100%

Meanwhile, the mass of the residual and the mass of isoflavones in the aqueous layer were also calculated to examine the mass balances of the residue and isoflavones.

Experimental Design and Analysis

First, ethyl acetate was chosen as the extraction solvent because it has a relatively low cost, exhibits low toxicity, and has good biodegradability. The three independent variables included volume ratio of aqueous phase to organic phase or water-oil ratio in abbreviation (factor A), extraction temperature (factor B), and

extraction time (factor C). A 2^3 factorial design with center points was used and the experimental design matrix consisting of a set of 11 experiments is shown in Table 1. The purity and loss of isoflavones of the organic layer were selected as the two response functions. Using Design-Ease software, the effects of the three factors on the two responses were analyzed, respectively, by the analysis of variance (ANOVA) and the corresponding regression models were established.[19,20] Based on the above analysis, the extraction conditions were selected.

Then, n-butanol was added into ethyl acetate with different percentages (0%, 10%, 20%, 40%, 60%, 80%, and 100%, v/v) to form a mixed solvent. Based on the chosen extraction conditions, the effects of the new added solvent on the extraction performances were examined.

Isolation of Isoflavones Using Column Chromatography

Medium Pressure Liquid Chromatograph (MPLC) System

The medium pressure liquid chromatograph system included a Lab Alliance Model Series II medium pressure pump (Lemont, PA, USA), a ISCO Model 2360 gradient programmer, and a ISCO Model Retriever® 500 fraction collector (Lincoln, NE, USA). Each of the two packing materials,

Table 1. Arrangement of the operating conditions of each run in the 2^3 factorial design with center points

Levels	−1	0	+1
Factors			
A:water-oil ratio (mL/mL)	1:1	1:2	1:3
B:temperature (°C)	45	55	65
C:time (hr)	1	2	3
Runs	**Factor A**	**Factor B**	**Factor C**
1	−1	−1	−1
2	+1	−1	−1
3	−1	+1	−1
4	+1	+1	−1
5	−1	−1	+1
6	+1	−1	+1
7	−1	+1	+1
8	+1	+1	+1
9	0	0	0
10	0	0	0
11	0	0	0

Note: 1:2 water-oil ratio (mL/mL) stands for 40 mL extract solution B:80 mL ethyl acetate.

i.e., XAD-7HP and XAD-4, was dry packed in a glass column. The column was located in a water bath, where the temperature was controlled at 30°C.

Adsorption Separation Process

It is noted that the triangular relationship among the adsorbate, the adsorbent, and the eluent is formed based on the "like attracts like" principle,[15] i.e., whether the adsorbate will stay in the adsorbent or in the eluent depends on the relative strength of attraction between them. Applying the principle to the current system, as intermediate polar and non-polar adsorbents, all the two packing materials can adsorb intermediate polar isoflavones from aqueous solutions; on the contrary, using an eluent at a higher percentage of ethanol is favorable for the desorption of isoflavones.

The whole adsorption separation process, thus, included four steps in sequence: conditioning, adsorption, washing, and elution steps. First, the column was conditioned with deionized water. Then, the feed solution (Solution B) was loaded onto the column. At the end of adsorption, the column was washed with a certain volume of deionized water in order to remove the impurities remaining in the pore of the adsorbents and the packing space. Finally, the packed column was eluted with a certain volume of the ethanol aqueous solution varying step gradiently from 20% to 100%.

Evaluation of the Column Performance

The effluent for each step in the adsorption separation process was collected for compositional analysis. Each effluent was concentrated and each concentrate was weighed. Based on the compositions of isoflavones in all effluents, the following column performance was evaluated. First, the dynamic adsorption curve (breakthrough curve) of isoflavones was established to evaluate the suitable loading volume for the adsorption step. Then, the dynamic elution curve of isoflavones was established to find the proper step gradient elution program for achieving an efficient isolation in the elution step. The column performance indices, including purity, concentration ratio, and yield of isoflavones, are defined as follows:

Purity (%) = mass fraction of isoflavones in the concentrated elution effluent fraction × 100

Concentration ratio = purity of isoflavones in the concentrated elution effluent fraction/purity of isoflavones in Solution B

Recovery (%) = mass of isoflavones in the concentrated elution effluent fraction/sum of the masses of isoflavones in Solution B × 100

Finally, the column performances among the two different packing materials were compared.

RESULTS AND DISCUSSION

Preparation of the Crude Isoflavone Extracts

The crude extraction results of two batches of Solution A were as follows: the masses of the concentrated extracts were 10.70 and 10.68 g and the masses of total isoflavones were 234.8 and 266.1 mg, respectively. The results showed that the yield of the crude extracts (mass of the concentrated extracts/mass of DSF used) ranged from 10 to 12% (w/w) and the mass fraction (or purity) of isoflavones in the concentrated extracts ranged from 2.2 to 2.5% (w/w).

The feed solution for the following isolation processes was prepared by collecting the crude extract solution (Solution A), concentrating it, dissolving the concentrates in deionized water by the ratio of 1.2 g extract:100 mL water, centrifuging the solution, and then filtrating it to get the filtrate (Solution B). Averaging the results of two batches of Solution B obtained from the above two batches of Solution A, the mass of the concentrated extracts were 9.67 and 9.30 g, the mass of isoflavones were 220.2 and 185.7 mg, and the mass fraction (or purity) of isoflavones were 2.3 and 2.0% (w/w), respectively, which was slightly less than that in the concentrates of Solution A. From Solution A (80% ethanol aqueous solution) to Solution B (deionized water), the loss of the concentrated extracts ranged from 5 to 10% and the loss of isoflavones of the extracts ranged from 5 to 30%. Finally, the concentration of isoflavones of the feed solution (Solution B) ranged from 0.2 to 0.3 mg/mL.

Isolation of Isoflavones Using Liquid–Liquid Extraction

According to the arrangement of Table 1, the liquid–liquid extraction results of 11 batches of Solution B are shown in Table 2. For each batch, the extraction was done two times and the averaged value was taken. It is noted that the sums of the masses of the residues and the masses of isoflavones in both layers were very close to their corresponding amounts in Solution B, and the masses of the residues and isoflavones were proven to be conserved.

The statistical analysis results for the two response functions, i.e., the purity and loss of isoflavones of the organic layer, are listed in Tables 3 and 4, respectively. Based on the established regression models, the contour and response surface plots for the two response functions are shown in Figures 4 and 5, respectively. Here, the Z axis represents the response function and X and Y axes represent the two independent variables, while keeping the other one variable constant. It was found that the purity of isoflavones increased, but the loss of isoflavones also increased with the decrease of the three operating variables. For compromising the two responses, the extraction conditions were then selected as those of the center point, i.e., water-oil ratio = 1:2, temp. = 55°C, and extraction time = 2 hr, at which the purity and loss (or yield) of isoflavones were 40∼45% and ∼40% (or ∼60%).

Table 2. Liquid-liquid extraction results of each run in the 2^3 factorial design with center points

Run no.	Mass of concentrate in the aqueous phase (g)	Mass of concentrate in the organic phase (g)	Mass of isoflavones in the aqueous phase (mg)	Mass of isoflavones in the organic phase (mg)	Purity of isoflavones in the aqueous phase (%)	Purity of isoflavones in the organic phase (%)	Loss of isoflavones in the organic phase (%)	Concentration ratio
1	0.4138	0.0068	6.15	3.24	1.37	47.6	62.30	24.0
2	0.4470	0.0111	3.36	4.64	0.70	42.7	46.04	21.5
3	0.4168	0.0082	6.09	3.90	1.35	47.6	54.68	23.9
4	0.4391	0.0124	3.08	4.87	0.66	39.2	43.40	19.7
5	0.4105	0.0089	6.30	3.31	1.42	37.2	61.53	18.7
6	0.4358	0.0106	3.34	4.51	0.71	42.5	47.58	21.4
7	0.4163	0.0080	7.09	2.90	1.55	36.2	66.28	18.2
8	0.4354	0.0163	4.43	5.38	0.92	33.0	37.45	16.6
9	0.4149	0.0121	4.48	5.38	0.99	44.5	37.38	22.4
10	0.4372	0.0122	4.81	4.89	1.01	40.2	43.09	20.2
11	0.4259	0.0131	4.86	5.07	1.05	38.7	40.97	19.5

Note: Original aqueous phase solution (Solution B): volume = 40 mL, mass of concentrate = 0.433 g, mass of isoflavones = 8.60 mg, purity of isoflavones = 1.99%.

Table 3. The results of analysis of variance and the regression model for the case of selecting the purity of isoflavones as the response function

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value[a]
ANOVA table					
A	15.68	1	15.68	1.73	0.3190 (NS)
B	24.50	1	24.50	2.70	0.2419 (NS)
C	99.40	1	99.40	10.97	0.0803 (S)
AB	18.00	1	18.00	1.99	0.2941 (NS)
AC	29.64	1	29.64	3.27	0.2122 (NS)
BC	6.13	1	6.13	0.68	0.4974 (NS)
ABC	3.13	1	3.13	0.34	0.6165 (NS)
Quadratic effect (Curvature)	0.32	1	0.32	0.035	0.8682 (NS)
Pure error	18.13	2	9.06		
Total	214.93	10			

Regression model:[b]
Purity (%) $= +40.75 - 1.40 \times A - 1.75 \times B - 3.52 \times C - 1.50 \times A \times B + 1.92 \times A \times C - 0.88 \times B \times C - 0.63 \times A \times B \times C$; $R^2 = 0.9155$.

Note: [a]NS: non-significant ($P > 0.1$); S: significant ($0.01 < P < 0.1$).
[b]1st order regression model for the 2^3 factorial design w/o center points.

Table 4. The results of analysis of variance and the regression model for the case of selecting the loss of isoflavones as the response function

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value[a]
ANOVA table					
A	618.11	1	618.11	74.19	0.0132 (S)
B	30.58	1	30.58	3.67	0.1955 (NS)
C	5.15	1	5.15	0.62	0.5140 (NS)
AB	12.25	1	12.25	1.47	0.3491 (NS)
AC	29.03	1	29.03	3.48	0.2029 (NS)
BC	2.98	1	2.98	0.36	0.6107 (NS)
ABC	49.3	1	49.3	5.92	0.1355 (NS)
Quadratic effect (Curvature)	310.4	1	310.4	37.26	0.0258 (S)
Pure error	16.66	2	8.33		
Total	1074.46	10			

Regression model:[b]
Loss (%) $= +52.41 - 8.79 \times A - 1.95 \times B + 0.80 \times C - 1.24 \times A \times B - 1.91 \times A \times C + 0.61 \times B \times C - 2.48 \times A \times B \times C$; $R^2 = 0.9782$.

Note: [a]NS: non-significant ($P > 0.1$); S: significant ($0.01 < P < 0.1$).
[b]1st order regression model for the 2^3 factorial design w/o center points.

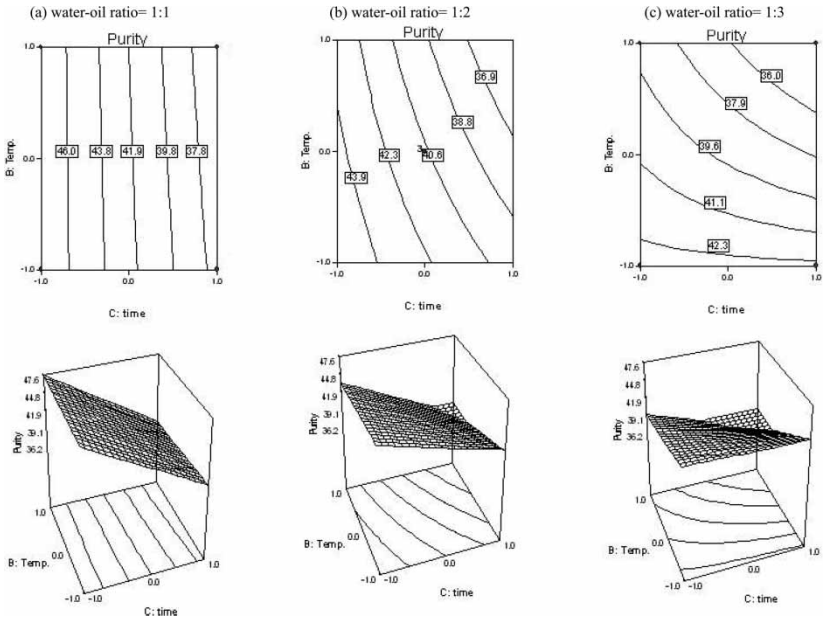


Figure 4. Contour and response surface plots of the purity of isoflavones.

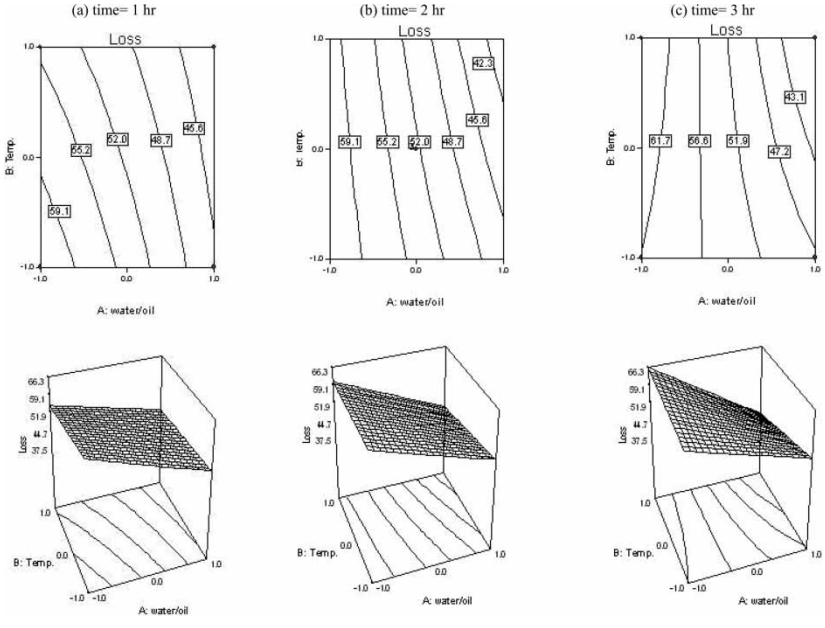


Figure 5. Contour and response surface plots of the loss of isoflavones.

In order to further improve the extraction efficiency, the polarity of the extraction solvent was increased by adding different percentages (0%, 10%, 20%, 40%, 60%, 80%, and 100%, v/v) of n-butanol into ethyl acetate. The extraction results of the mixed solvent system are shown in Table 5. It can be seen that the loss of isoflavones decreased (or the yield of isoflavones increased) at the expense of decreased purity of isoflavones when the percentage of n-butanol in the mixed solvent system was increased. This was due to the simultaneous increase in the amounts of isoflavones and other impurities when the polarity of the mixed solvent was increased by adding n-butanol. Again, for compromising the two responses, the extraction conditions were then selected as: n-butanol-ethyl acetate ratio in the mixed solvent = 10%:90%, water-oil ratio= 1:2, temp.= 55°C, and extraction time = 2 hr, at which the purity and loss (or yield) of isoflavones were ~35% and ~30% (or ~70%).

Isolation of Isoflavones Using Column Chromatography

The bed lengths of the two glass columns (I.D. = 15 mm), packed with approximately 6.34 g and 5.5 g of XAD-7HP and XAD-4 adsorbents, were 90 mm and 102 mm, respectively.

Dynamic Adsorption Performance

The dynamic adsorption experiment was carried out on each of the two columns at a certain flow rate and feed concentration (C_{in}). For each column, the effluent fractions of the adsorption step were collected in several intervals, e.g., 20–30 mL for each interval, for compositional analysis. The dynamic adsorption curves of isoflavones are shown in Figures 6 and 7 for the XAD-7HP and XAD-4 columns, respectively. To avoid the loss of isoflavones from the adsorption stage, the break point was set at the point as the exit concentration of isoflavones approached 5% of the feed concentration. The loading volume of the adsorption process was then selected according to the elution volume at the break point.

It can be seen, from each figure, that the break point took place earlier as the flow rate and/or the feed concentration increased. The earlier breakthrough at higher flow rate is believed to be caused by the increasing turbulent mixing in the axial dispersion.[21] On the other hand, due to the loading amount (=loading volume × feed concentration) is about fixed for a certain column operated at a certain flow rate, the increase in the feed concentration lead to the decrease in the loading volume. Based on the dynamic adsorption behaviors of Figures 6 and 7, and in order to have an efficient column operation in terms of the loading volume and the operating time, for the XAD-7HP column, the flow rate of 2 mL/min was selected and the loading volume was estimated to be 400 mL at the feed concentration of 0.2–0.3 mg/mL; for the XAD-4 column, the flow rate of 1 mL/min was

Table 5. Liquid-liquid extraction[a,b] results of the mixed solvent system

	Percentage of n-butanol (v/v) added with ethyl acetate (%)						
	0	10	20	40	60	80	100
Mass of concentrate (mg)							
Organic phase	16.4	24.8	39.9	65.5	86.4	128.5	132.8
Aqueous phase	426.0	425.0	403.4	393.0	365.0	328.4	281.8
Mass of isoflavones (mg)							
Organic phase	7.02	8.69	9.16	10.89	10.49	11.83	11.80
Aqueous phase	4.72	3.07	2.82	1.73	1.46	0.79	0.60
Purity of isoflavones (%)							
Organic phase	42.59	35.04	22.97	16.63	12.14	9.21	8.92
Aqueous phase	1.11	0.72	0.70	0.44	0.40	0.24	0.21
Loss of isoflavones (%)							
Organic phase	41.50	27.60	23.70	9.30	12.70	1.50	1.75
Concentration ratio							
Organic phase	20.3	15.0	9.8	7.1	5.2	3.9	3.8

Note: [a]Extraction conditions:water-oil ratio = 1:2 (40 mL Solution B:80 mL mixed solvent), 55°C, 2 hr.
[b]Contents of the 40 mL Solution B: mass of concentrate = 514 mg, mass of isoflavones = 12.01 mg, purity of isoflavones = 2.34%.

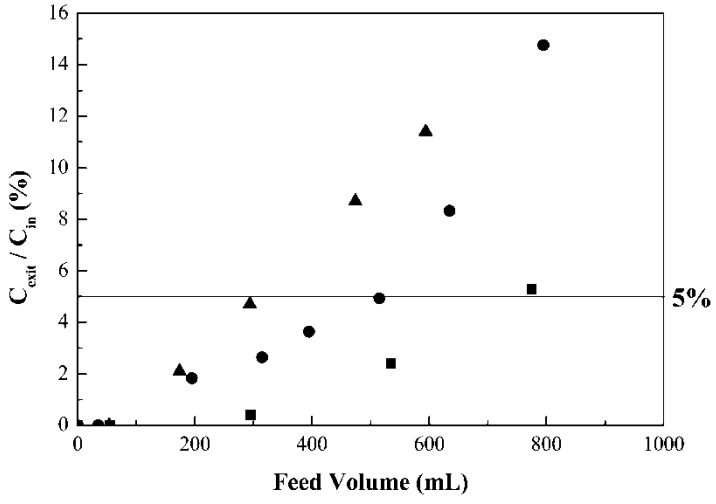


Figure 6. The dynamic adsorption curves of isoflavones for the XAD-7HP column. Column size: 90 × 15 mm i.d., 250-840 μm; temperature: 30°C; (■) flow rate: 1 mL/min, C_{in}: 0.2256 mg/mL; (●) flow rate: 2 mL/min, C_{in}: 0.2789 mg/mL; (▲) flow rate: 3 mL/min, C_{in}: 0.2256 mg/mL.

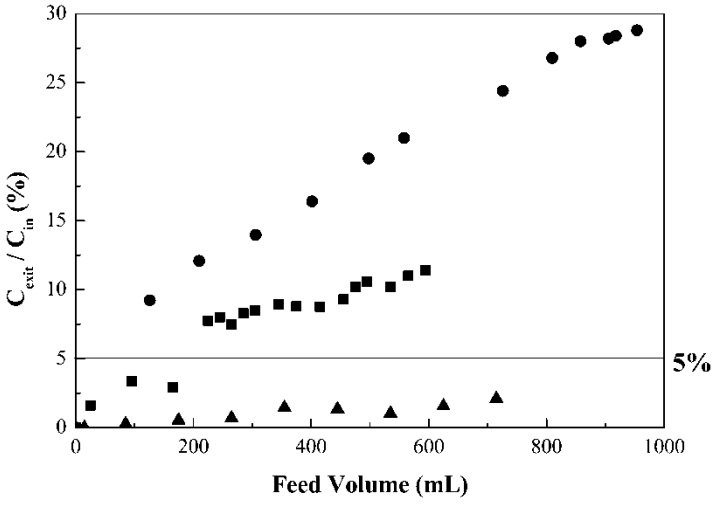


Figure 7. The dynamic adsorption curves of isoflavones for the XAD-4 column. Column size: 102 × 15 mm i.d., 250-840 μm; temperature: 30°C; (▲) flow rate: 1 mL/min, C_{in}: 0.2629 mg/mL; (■) flow rate; 2 mL/min, C_{in}: 0.2786 mg/mL; (●) flow rate: 3 mL/min, C_{in}: 0.2786 mg/mL.

selected and the loading volume was estimated to be 700 mL at the feed concentration of 0.2–0.3 mg/mL. The loading volume has to be carefully adjusted when the flow rate or the feed concentration changes.

Dynamic Elution Performance

A dynamic elution experiment was then carried out on each of the two columns. Each column was eluted with a certain volume of the ethanol aqueous solution varying step gradiently from 0∼100% at a flow rate 2.0 mL/min, following the adsorption step of a suitable loading volume of the feed solution, and the washing step of 150–200 mL deionized water. The effluent fractions of the elution step were collected in several intervals, e.g. 10∼20 mL for each interval, for compositional analysis. Their dynamic elution curves are shown in Figures 8 and 9 for the XAD-7HP and XAD-4 columns, respectively. There are three parts in each figure: the mass of isoflavones, the mass of concentrate, and the purity of isoflavones versus the elution volume shown in parts (a)–(c), respectively.

The results of part (a) show that the proper eluent composition for an efficient elution of the majority of isoflavones ranged from 20 to 40% ethanol for both the XAD-7HP and XAD-4 columns. The results of parts (b) and (c) show that some more polar impurities were eluted separately by 0% ethanol, which contributed to an effect for the concentration of isoflavones. However, some impurities with similar polarity as isoflavones were eluted together with isoflavones by 20 to 40% ethanol, which limited the purity of isoflavones collected at this range. The only difference between these two columns was, for the XAD-4 column, a part of the nonpolar impurities (eluted by 60 to 80% ethanol) was efficiently separated with the majority of isoflavones (eluted by 20 to 40% ethanol), which resulted in the higher purity of isoflavones collected at 20 to 40% ethanol fractions, compared with the corresponding fractions collected from the XAD-7HP column.

Comparison of the Column Performance

The dynamic elution curves of Figures 8 and 9 were then referred and their step gradient elution programs were modified in order to enhance their isolation efficiencies. Using the modified step gradient elution programs, the column performances, in terms of purity, concentration ratio, and yield of isoflavones, between the two different packing materials were compared, as presented in Tables 6 and 7 for the XAD-7HP and XAD-4 columns, respectively.

It can be seen, from each table that, in the adsorption step, the loss of isoflavones was less than 10% when the selected volume of feed solution was loaded. Since nearly 80–90% of the total mass of concentrates was

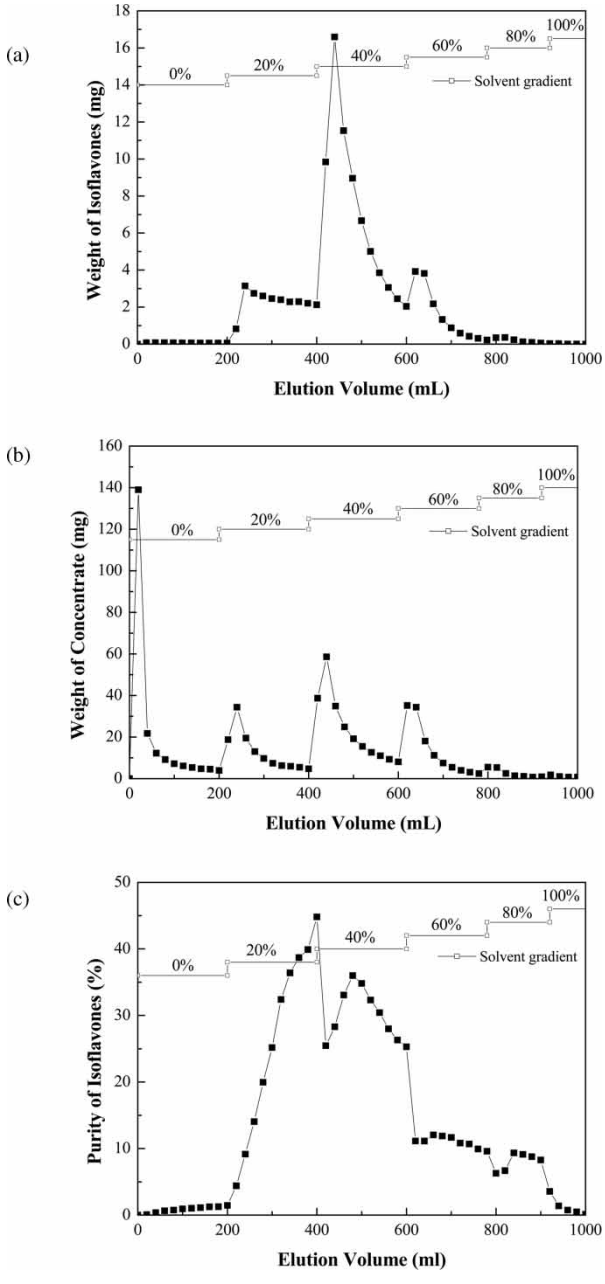


Figure 8. The dynamic elution curves for the XAD-7HP column: (a) mass of isoflavones, (b) mass of concentrate and (c) purity of isoflavones. Column size: same as Figure 6; feed solution: 400 mL Solution B; feed concentration: 0.266 mg/mL; feed flow rate: 2 mL/min; elution solution: 0, 20, 40, 60, 80, 100% aqueous ethanol; elution flow rate: 2 mL/min; temperature: 30°C.

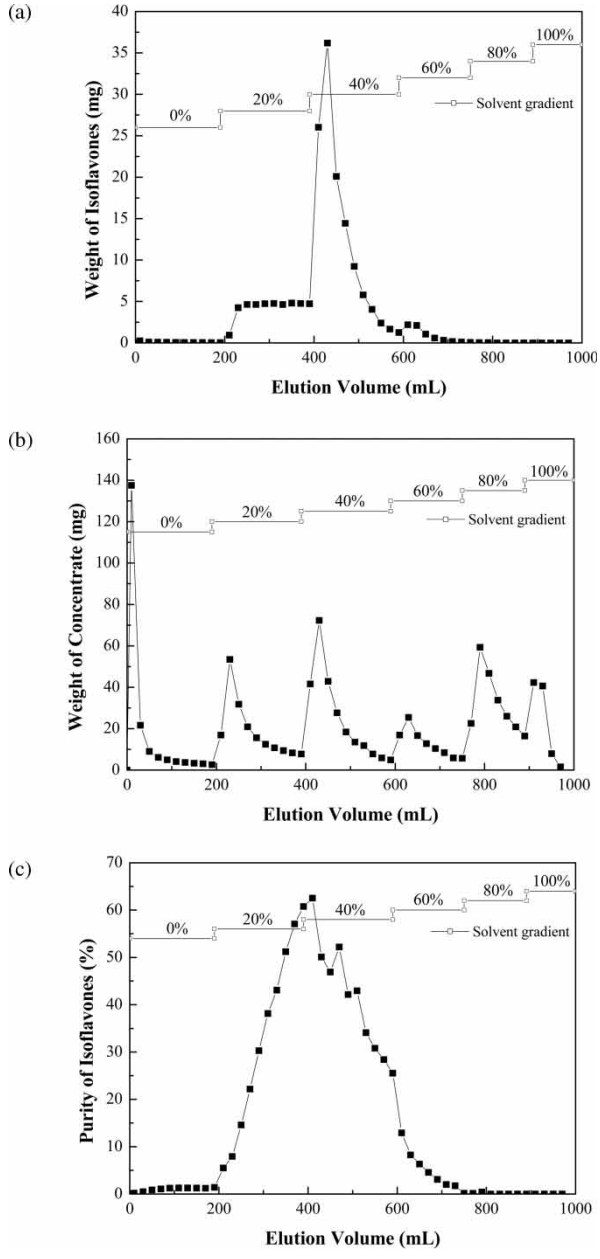


Figure 9. The dynamic elution curves for the XAD-4 column: (a) mass of isoflavones, (b) mass of concentrate and (c) purity of isoflavones. Column size: same as Figure 7; feed solution: 700 mL Solution B; feed concentration: 0.254 mg/mL; feed flow rate: 1 mL/min; elution solution: 0, 20, 40, 60, 80, 100% aqueous ethanol; elution flow rate: 2 mL/min; temperature: 30°C.

Table 6. Column performances of the XAD-7HP column

Step of column operation	Operating volume (mL)	Mass of concentrate (mg)	Mass ratio of concentrate (%)[b]	Mass of isoflavones (mg)	Yield of isoflavones (%)[c]	Purity of isoflavones (%)[d]
Adsorption[a]	400	4575	87.10	1.06	0.94	~0
Washing (0% ethanol)	150	190	3.60	0.40	0.36	0.21
Step-gradient elution						
10% ethanol	100	57	1.10	2.69	2.39	4.72
20% ethanol (a)	50	28	0.50	4.80	4.27	17.14
20% ethanol (b)[e]	250	64	1.20	26.80	23.80	41.87
40% ethanol[e]	300	242	4.60	68.90	61.30	28.47
60% ethanol	150	88	1.70	7.20	6.41	8.18
80% + 100% ethanol	200	11	0.20	0.50	0.44	4.54
Total	—	5255	100.00	112.35	100.00	—

Note: [a]Feed solution (Solution B): concentration of concentrate = 12.66 mg/mL; concentration of isoflavones = 0.248 mg/mL; purity of isoflavones = 1.96%.

[b]Mass ratio of concentrate (%) = (fractional mass of concentrate/total mass of concentrate) × 100%.

[c]Yield of isoflavones (%) = (fractional mass of isoflavones/total mass of isoflavones) × 100%.

[d]Purity of isoflavones (%) = (fractional mass of isoflavones/fractional mass of concentrate) × 100%.

[e]The fraction was collected as a part of the concentrated product.

Table 7. Column performances of the XAD-4 column

Step of column operation	Operating volume (mL)	Mass of concentrate (mg)	Mass ratio of concentrate (%)[b]	Mass of isoflavones (mg)	Yield of isoflavones (%)[c]	Purity of isoflavones (%)[d]
Adsorption[a]	700	8100	91.10	2.35	1.24	0.03
Washing (0% ethanol)	150	186	2.09	0.24	0.12	0.13
Step-gradient elution						
10% ethanol	100	63	0.71	1.85	0.97	2.93
20% ethanol (a)	50	34	0.38	5.66	2.97	16.64
20% ethanol (b)[e]	300	104	1.17	48.05	25.28	46.20
40% ethanol[e]	200	189	2.13	121.00	63.65	64.02
60% ethanol	100	67	0.75	10.08	5.30	15.04
80% + 100% ethanol	200	143	1.61	0.89	0.47	0.62
Total	—	8886	100.00	190.10	100.00	—

Note: [a]Feed solution (Solution B): concentration of concentrate = 12.76 mg/mL; concentration of isoflavones = 0.274 mg/mL; purity of isoflavones = 2.15%.

[b]Mass ratio of concentrate (%) = (fractional mass of concentrate/total mass of concentrate) × 100%.

[c]Yield of isoflavones (%) = (fractional mass of isoflavones/total mass of isoflavones) × 100%.

[d]Purity of isoflavones (%) = (fractional mass of isoflavones/fractional mass of concentrate) × 100%.

[e]The fraction was collected as a part of the concentrated product.

removed without too much loss of isoflavones in the adsorption step, the active ingredient contents were first isolated more than 4.0 times (concentration ratio >4.0) after the adsorption step and were further isolated by an efficient separation from the other impurities in the elution step. In the elution step, for all the effluent fractions collected, the sum of concentrates weighed around 5255 mg and 8886 mg and the sum of isoflavones weighed around 112 mg and 190 mg for the XAD-7HP and XAD-4 columns, respectively. When comparing the collected amounts with their loading amounts, the errors in mass balances of the concentrate and isoflavones were about $<2\%$ and $<10\%$, respectively. The mass conservation was satisfactory and each column was proven to be sufficiently regenerated.

The fractions of the second part of 20% ethanol elution and 40% ethanol elution were collected as the isolation product for each of the two columns. The purity and yield of isoflavones were $\sim 31\%$ and $\sim 85\%$ for the XAD-7HP column and $\sim 58\%$ and $\sim 89\%$ for the XAD-4 column. In comparison, the purity and yield were both in the same order: XAD-4 column $>$XAD-7HP column.

CONCLUSIONS

The preparation of the crude DSF extracts, using an extractor with solvent refluxing operated at 500 mL of 80% ethanol aqueous solution and 3 h for a batch of 100 g DSF, gave a satisfactory result. The yield of the crude extracts was about 10% (w/w) and the purity of isoflavones in the concentrated extracts ranged from 2.0 to 2.5 wt%. In order to be effectively processed in the isolation processes, the crude extracts were dissolved in deionized water. This process led to a 5–10% loss for the concentrated extracts, a 5–30% loss for isoflavones, and a slightly lower purity of isoflavones ranged from 2.0 to 2.3 wt%.

For the method of liquid–liquid extraction, increasing the polarity of the mixed solvent by adding n-butanol into ethyl acetate led to an increase in the yield of isoflavones, but at the expense of decreased purity of isoflavones. For compromising the two responses, the extraction conditions were selected as: n-butanol-ethyl acetate ratio in the mixed solvent = 10%:90%, water-oil ratio = 1:2, temp. = 55°C, and extraction time = 2 hr, at which the purity and yield of isoflavones were $\sim 35\%$ and $\sim 70\%$.

For the method of column chromatography, in the adsorption step, the XAD-4 column had a higher loading capacity than that of the XAD-7HP column. In the elution step, the majority of isoflavones were eluted by 20 to 40% ethanol for both the XAD-7HP and XAD-4 columns. Since a part of non-polar impurities was efficiently separated with the majority of isoflavones at higher percentages (60 to 80%) of ethanol for the XAD-4 column, the purity of isoflavones collected at 20 to 40% ethanol fractions was much higher than those collected from the XAD-7HP column. The purity and yield of

isoflavones were ~31% and ~85% for the XAD-7HP column and ~58% and ~89% for the XAD-4 column.

In comparison, the order of purity was found to be XAD-4 column >liquid–liquid extraction >XAD-7HP column, and the order of yield was found to be XAD-4 column >XAD-7HP column >liquid–liquid extraction. The XAD-4 column achieved both the highest purity and yield. Therefore, the method of column chromatography using XAD-4 adsorbents was found to be the best choice in the current isolation stage.

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